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Development of Loop-mediated Isothermal Amplification (LAMP) for rapid detection of Methicillin-resistance *Staphylococcus aureus* (MRSA) from dairy cattle

Laohasatian, T., Eardmusic, S. and Seritrakul, P.*

Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Samphraya, Cha Am district, Phetchaburi 76120, Thailand.

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Abstract Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of mastitis in dairy cattle, resulting in the loss of economic value in livestock. The gene *mecA* encodes penicillin-binding protein 2A (PB2A), which only binds weakly to β-lactam antibiotics such as penicillin and methicillin, thus conferring the antibiotic resistant property in MRSA. Currently, detection of MRSA in laboratory setting is commonly performed using bacterial culture or PCR assay targeting *mecA* gene, which is time-consuming, labor-intensive, and requires specialized personnel and equipment. Therefore, a more rapid detection of MRSA is needed to effectively prevent the spread of MRSA. In this study, a novel Loop-mediated Isothermal Amplification (LAMP) assay that can detect MRSA more conveniently, quickly, and accurately was developed. Bacterial culture were isolated from milk samples from dairy cattle with mastitis and PCR primers were designed to target *femA*, *blaZ*, and *mecA* gene to detect *Staphylococcus aureus* (*S. aureus*), penicillin-resistant *S. aureus*, and methicillin-resistance *S. aureus*, respectively. We found that all isolates possessed *femA* and *blaZ* genes, and 30% of the isolates possessed *mecA* gene. For LAMP assay development, primers were designed to target the coding region of *mecA* gene, and LAMP reactions were monitored in real-time using the fluorescence labelling of the amplified products. We were able to detect the presence of MRSA quickly, and the results were consistent with those obtained by PCR and by bacterial culture. These results indicated that the LAMP assay successfully detected *mecA* gene in MRSA strains isolated from dairy cattle milk, and this assay could be further developed into a test kit to be used in the field.

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, Loop-mediated isothermal amplification, Mastitis

Introduction

Mastitis is an infectious disease that causes inflammatory of the udder tissue in bovine, which affects dairy cattle and causes considerable economic

* **Corresponding Author**: Seritrakul, P.; **Email:** seritrakul_p@silpakorn.edu

losses in global dairy industry, resulting in decreased milk production, reproductive complications, high costs associated with veterinary care, replacing tainted milk, and infectious animal culling losses (Hogeveen, 2005; Hogeveen *et al.,* 2011; Deb *et al.,* 2013; Gomes and Henriques, 2016; Ashraf and Imran, 2018). The cause of mastitis involves infectious pathogenic bacterium in the udder such as *Streptococcus agalactiae* (*S. agalactiae*), *Staphylococcus aureus* (*S. aureus), Escherichia coli* (*E. coli*), and *Klebsiella pneumoniae* (*K. pneumoniae*) (Zadoks *et al.,* 2011). The main focus of mastitis detection and treatment is aimed at the disease caused by *S. agalactiae* and *S. aureus* (Ruegg, 2017). Two classifications of mastitis includes clinical stage and subclinical stage of the disease (Pinzón-Sánchez and Ruegg, 2011). For clinical stage, the clinical signs include symptoms in the udder such as heating, swelling, redness, and pain, high fever, reduced milk yield, and abnormalities in milk such as watery appearance, clots, flakes, and pus. For subclinical stage the animal does not show signs of illness or abnormalities but may yield deterioration in milk quality (Sandra Björk, 2013).

Staphylococcus aureus (*S. aureus*) is an important pathogenic bacterium affecting bovine and other livestock worldwide (Botaro *et al.,* 2015). *S. aureus* is a gram-positive cocci, immobile, and non-spore forming bacterium (Toltzis, 2018). Bacteria can be found in the forms of one cell, two cells, or resembling bunches of grapes (Licitra, 2013). *S. aureus* is a facultative anaerobic bacterium but grows better in the presence of oxygen than in anaerobic conditions (Genigeorgis, 1989). *S. aureus* can produce catalase enzyme for metabolizing hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) (Das and Bishayi, 2010), produce coagulase for altering the fibrinogen to fibrin, resulting in plasma clotting, and produce β-hemolysin to metabolize red blood cells (Harris *et al.,* 2002; John and Harvin, 2007; Johannessen *et al.,* 2012). Some *S. aureus* strains can produce β-lactamase, causing β-lactam antibiotic resistance (Lowy, 2000). The pathogenesis of *S. aureus* involves many factors such as enterotoxin, hyaluronidase, hemolysin, leucocidin, coagulase, and biofilm (Tam and Torres, 2016). However, important factors involved in the pathogenic process are controlled by genes such as synthesis of extracellular thermostable nuclease (thermonuclease: TNase) encoded by *nuc* gene, adhesion protein formation encoded by *cna* and *fnbA* genes (Brakstad *et al.,* 1992; Gillaspy *et al.,* 1997; Shinji *et al.,* 2011). *S. aureus* detection is commonly done by detecting the presence of *femA* gene, which is found in all strains of *S. aureus* genome and encodes the essential factors for resistance to methicillin (Maidhof *et al.,* 1991; Mehrotra *et al.,* 2000). *S. aureus* can be killed by β-lactam antibiotics such as ampicillin, penicillin, methicillin, and oxacillin that structurally contain a β-lactam ring. β-lactam antibiotics bind to the active site

of penicillin-binding protein (PBP), which cross-link with and destroy bacterial peptidoglycan formation, resulting in the inhibition of bacteria cell wall formation (Rayner and Munckhof, 2005).

Over the past several decades, *S. aureus* has evolved to become more resistant to a diverse type of antibiotics. After 1941, *S. aureus* became resistant to penicillin by producing β-lactamase, encoded by *blaZ* gene on Tn552-like transposon (Rowland and Dyke, 1989), to break down the bond within the β–lactams ring of penicillin (Corrigan *et al.,* 2009). Methicillin-resistance *Staphylococcus aureus* (MRSA) is a multiplex of *S. aureus* resistant to antibiotics such as aminoglycosides, fluoroquinolones, chloramphenicol, trimethoprim/sulfamethoxazole, clindamycin, and penicillin (Taj *et al.,* 2010). Mechanistically, MRSA functions by altering the PBP to PBP2a, resulting in the β-lactam inability to bind to PBP2a active site and loss of antibiotics affinity (Lee, 2003; Cuny *et al.,* 2015). PBP2a formation is encoded by *mecA* gene residing on Staphylococcal cassette chromosome *mec* (SCC*mec*) (Itou *et al.,* 2000).

Loop-mediated isothermal amplification (LAMP) is a novel molecular technique developed in 2000 by Notomi *et al*. (2000). The LAMP assay works by amplifying specific sequences of nucleic acid under a constant temperature, allowing a simple and rapid detection of target DNA sequence with minimal laboratory equipment. In addition, it has higher efficiency, accuracy, and specificity than conventional PCR (Notomi *et al.,* 2000). In LAMP method, four or six sequence-specific primers are required: for four primers, inner primers consist of forward inner primer (FIP) and backward inner primer (BIP), outer primers consist of F3 and B3; for six primers, loop primers consist of loop forward and loop backward are added to the existing four primers. The primers bind to specific regions in the target gene (Wong *et al.*, 2018). Currently, LAMP is commonly used in diagnosis of diseases in humans and animals and suitable for applications in biotechnology (Sahoo *et al.,* 2016). LAMP products are easy to detect by various methods, and results can be interpreted readily without sophisticated instrument. LAMP-amplified products can be detected by fluorescent assays in real-time fluorescent detector instrument, visualized by fluorescence under UV light, detected by turbidity in spectrophotometer, or run on gel electrophoresis. LAMP can also be coupled with detection on lateral flow device (LFD) for test kit development (Li *et al.,* 2017; Wong *et al.,* 2018). Additionally, LAMP reactions can amplify minimal amount of nucleic acid molecules under constant temperature (60 - 72 ℃) without the use of a thermocycler, making it a sensitive and convenient assay to use in the field.

The objective of this study is to develop a prototype of LAMP assay to detect MRSA from milk samples from dairy cattle with mastitis, in comparison

to the conventional PCR assay. MRSA strains can be detected by both PCR and LAMP assays. Similarly to PCR assay, LAMP assay was able to quickly detect the presence of *mecA* gene, which confers antibiotic resistance. It showed that LAMP assay can effectively detects *mecA* gene in MRSA strains isolated from dairy cattle milk.

Materials and methods

Sample collection

Milk samples were collected at Cha-am/Huai-sai dairy cooperative, Phetchaburi province. All animal procedures were done in accordance with the protocol reviewed and approved by Silpakorn University Animal Care and Use Committee (SUACU, Project ID: 07/2562). Dairy farms were randomly selected and 55 farms were categorized by size into 25 small farms (no more than 20 cows), 28 medium farms (21-100 cows), and 2 large farms (more than 100 cows). Milk samples were collected from each udder of cows after thoroughly cleaning and drying the udder and teats. California Mastitis Test (C.M.T.) was used to preliminarily detect mastitis and identify the udder with positive C.M.T. results. Then the udder was cleaned and treated again with 70% ethanol before milking into sterile 5-10 ml bottles. The collected milk samples were placed on ice and immediately transferred to the laboratory at Faculty of Animal Sciences and Agricultural Technology, Silpakorn University within 6-12 hours.

Bacterial isolates

Milk samples were spreaded onto Mannitol salt agar (MSA) plates and incubated at 37 ℃ overnight. Single colonies were picked up and streaked onto Nutrient Agar (NA) plates and incubated at 37 ℃ overnight. The *S. aureus* isolates were identified by gram staining, coagulase test, and catalase test. Gram stain method was used to distinguish between gram-positive and gram-negative bacteria by staining the cells with crystal violet (violet) or safanin (red). Gram-positive bacteria cells are positively-stained by crystal violet and gram-negative bacteria cells are positively-stained by safanin (Smith and Hussey, 2005). Coagulase and catalase tests were used to detect *S. aureus,* which can produce both of the enzymes. Coagulase test detects the coagulase enzyme from *S. aureus* which converts fibrinogen to fibrin. Positive coagulase test results in a plasma clotting and negative coagulase results in no plasma clotting (SenGupta and Sengupta, 2016). Catalase test detects the catalase

enzyme produced by *S. aureus* for metabolizing H_2O_2 to H_2O and O_2. A positive catalase test results in a vigorous bubbling when cells are mixed with H_2O_2, and a negative catalase test results in the absence of bubbling (Reiner, 2013).

Bacteria DNA Extraction

S. aureus isolates in NA were transferred into Nutrient broth (NB) and incubated at 37 ℃ overnight. Before DNA extraction, *S. aureus* isolates in NB were transferred to 2 ml microtube and centrifuged at 16,000 rpm for 1 minute, then the supernatant was discarded. DNA extraction from *S. aureus* pellet was performed using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) according to manufacturer recommendation. DNA samples were eluted into 100 µl of the elution buffer and kept at –20 ℃ until use.

PCR assay

S. aureus isolates were confirmed using PCR assay by the detection of *femA*, *blaZ*, and *mecA* genes. Primers for PCR assays specifically targeting each gene were picked from previously published works and verified using Geneious Prime program (Biomatters, USA) (Table 1). The 25 µl PCR mixture contains 1 µl DNA template from *S. aureus*, 0.5 µl forward primer, 0.5 µl reverse primer, 12.5 µl OneTaq Hot Start master mix buffer (New England Biolabs, USA), and 10.5 µl ddH_2O. The PCR condition is as follows: 94 ℃ for 10 min of initial denaturation; 34 cycles of 94 ℃ 30 seconds for denaturation, 60 ℃ 30 seconds for annealing, and 68 ℃ 60 seconds for extension; and a final extension at 68 ℃ 10 minutes. PCR products were separated by running on 1.0% agarose gel electrophoresis and visualized under UV light in UV transilluminator.

LAMP assay

S. aureus genomic DNA was extracted as a template for the development of LAMP assay using the LavaLAMP kit (Lucigen®, USA) to detect the *mecA* gene. Primers for the LAMP assays (F3, B3, FIP, BIP), that specifically target the *mecA* gene were designed using PrimerExplorer V4 software (https://primerexplorer.jp/e/index.html) (Table 2). The sequence encoding *S. aureus mecA* gene was downloaded from NCBI Genbank with accession number KC243783.1. A total of 25 µl for LAMP reaction consists of: 1 µl DNA template from *S. aureus*, 2.5 µl target-specific primer (2 mM F3 and B3, 16 mM FIP and BIP), 1 µl green fluorescent dye, 12.5 µl LavaLAMP™ DNA

master mix, and 8 μl ddH_2O. The LAMP condition is as follows: 90 ℃ 3 minutes for preheat, 72 ℃ 30-60 minutes for amplification, and hold at 4 ℃ until detection. LAMP products were detected by fluorescent assays using the FAM channel to detect amplification in real-time by fluorescent detector instruments. The end product of LAMP was then visualized under UV light from a UV lamp in a dark chamber. Additionally, turbidity of the LAMP product was detected by visual inspection of the final reaction products. Finally, the LAMP products were confirmed by running on 1.0% agarose gel electrophoresis and visualized under UV light in UV transilluminator.

Table 1. Primers used for PCR of *femA*, *blaZ*, and *mecA* genes

Gene		Primer sequence	Product size (bp)	Ref.
femA	F	5'-CGATCCATATTTACCATATCA-3'	450	(Davoodi *et al.*, 2015)
	R	5'-ATCACGCTCTTCGTTTAGTT-3'		
blaZ	F	5'-TACAACTGTAATATCGGAGGG-3'	774	(Wilailuckana *et al.*, 2005)
	R	5'-AGGTTCAGATTGGCCCTTAGG-3'		
mecA	F	5'-TGTCCGTAACCTGAATCAGC-3'	519	(Wilailuckana et al., 2005)
	R	5'-TGCTATCCACCCTCAAACAG-3'		

Table 2. Primers used for LAMP of *mecA* gene

Primer name	Primer sequence
F3	5'-GCGACTTCACATCTATTAGGTT-3'
B3	5'-GCCATCTTTTTTCTTTTTCTCT-3'
FIP	5'-GTCCCTTTTTACCAATAACTGCATCATGTTGGTCCCATTAACTCTG-3'
BIP	5'-AAGCTCCAACATGAAGATGGCCGATTGTATTGCTATTATCGTCAA-3'

Results

Bacterial isolates

A total of 145 raw milk samples were cultured on MSA plates and 153 bacterial colonies were isolated and identified by gram staining, coagulase test, and catalase assay. Of these, 10 isolates (6.54%) showed characteristics of *S. aureus*: positively-stained with crystal violet in gram staining test, produced oxygen bubbles in catalase test, and caused plasma clotting in coagulase test.

Additionally, the *S. aureus* isolates were able to grow in MSA, which is a selective growth medium for *Staphylococcus*. Of the remaining 143 colonies, 17 isolates (11.11%) were Coagulase Negative Staphylococcus (CNS), and 126 isolates (82.35%) did not show characteristic of *S. aureus*. This indicates that in raw milk samples, the majority of bacteria were other species and only a small fraction were *S. aureus*. However, CNS and other species such as *S. agalactiae*, *E. coli*, and *K. pneumoniae* are still important pathogenic bacterial of mastitis in cattle. The 10 isolates (I1-I10) of *S. aureus* were used for subsequent molecular analyses (PCR and LAMP).

PCR assay

DNA samples were extracted from Reference MRSA strain (RI) and the 10 isolates of *S. aureus* (I1-I10) and used as templates for PCR assay to detect *femA*, *blaZ*, and *mecA* genes with primers specific to each target gene. Analysis of the PCR products on 1.0% agarose gel showed successful amplification of 450 bp for *femA*, 774 bp for *blaZ*, and 519 bp for *mecA* (Figure 1). All isolates were positive for *femA* and *blaZ* genes, and *mecA* gene was detected in four isolates consisting of RI, I2, I8 and, I9 (Table 3). The result indicates that all bacterial isolates were penicillin-resistant *S. aureus*, while isolate RI, I2, I8, and I9 were MRSA.

Table 3. *S. aureus* isolate analysed for PCR assay to detect *femA*, *blaZ*, and *mecA* genes

***S. aureus* isolates**	**PCR assay**		
	femA	*blaZ*	*mecA*
RI	+	+	+
I1	+	+	-
I2	+	+	+
I3	+	+	-
I4	+	+	-
I5	+	+	-
I6	+	+	-
I7	+	+	-
I8	+	+	+
I9	+	+	+
I10	+	+	-

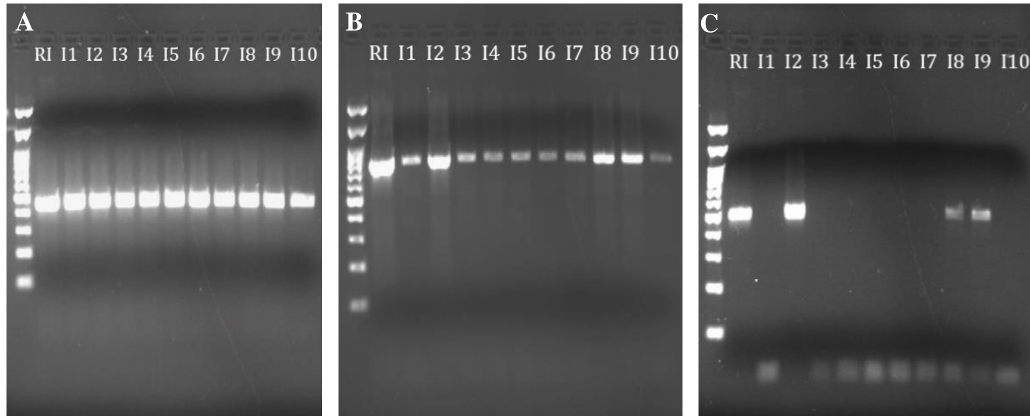

Figure 1. 1.0% agarose gel electrophoresis showed PCR amplification of *femA*, *blaZ*, and *mecA* gene from bacterial cultures isolated from raw milk samples: (A) 450 bp product for *femA*, (B) 774 bp product for *blaZ*, (C) 519 bp product for *mecA*. Lane 1: 100 bp DNA ladder, lane 2: MRSA reference strain (RI), lanes 3-12: samples isolated from raw milk (isolates I1-I10)

LAMP assay

S. aureus isolates (I2, I8, I9, and RI) DNA samples were detected by LAMP assay for *mecA* gene with positive control (control DNA and primers) and negative control (no-template control). Primers for LAMP assays were designed to specifically target the *mecA* gene (Figure 2). All LAMP reactions were done in microcentrifuge tubes containing fluorescent dye (FAM) for detection of amplification reaction. Analysis of real-time fluorescent detection in FAM channel showed a detectable increase in signal corresponding to positive amplification of *mecA* gene (I2 and RI), while the lack of signal represented negative amplification of *mecA* gene (I8 and I9) (Figure 3). Analysis of LAMP product on a 1.0% agarose gel showed successful amplification of *mecA* gene, which showed a robust and characteristic ladder-like pattern due to the various product sizes of folded DNA amplicons from LAMP reaction (Figure 4). This indicates that the LAMP assay successfully detected *mecA* gene in MRSA strains isolated from dairy cattle milk.

Figure 2. LAMP primer design for *mecA* gene (KC243783.1) in *S. aureus* consisted of inner primer: forward inner primer (FIP; F1c and F2), backward inner primer (BIP; B1c and B2) and outer primers: F3, B3

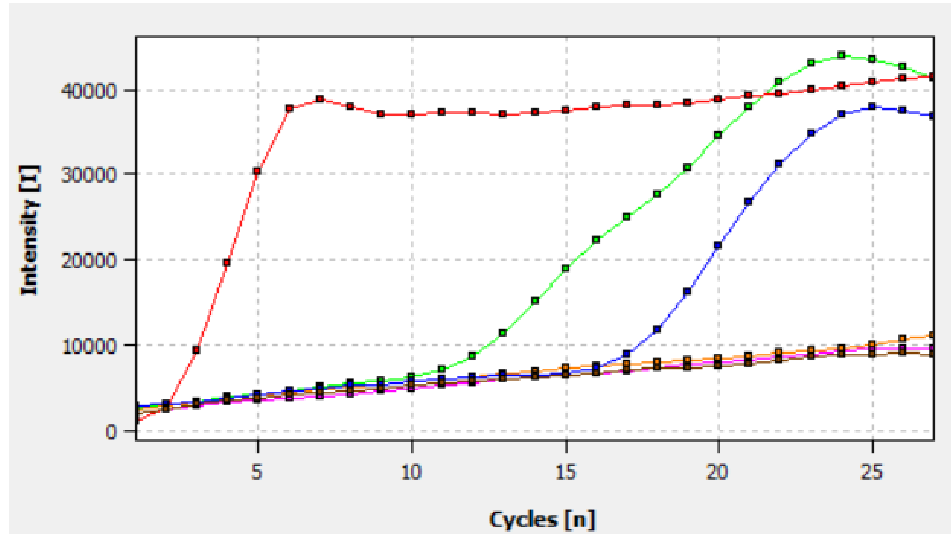

Figure 3. Analysis of LAMP reaction by fluorescent reaction using a real-time fluorescent detector in the FAM channel. Red line: positive control, green line: I2, blue line: RI, pink line: I8, orange line: I9, and brown line: no-template control

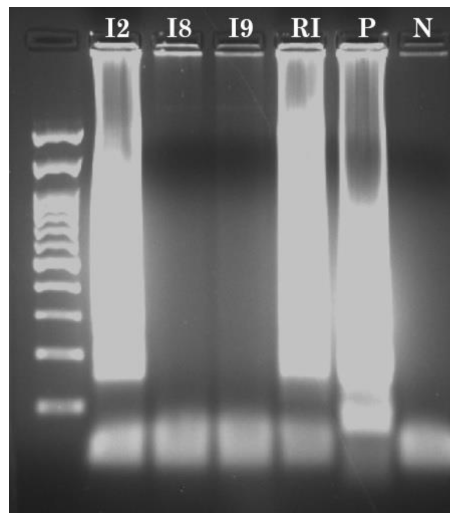

Figure 4. 1.0% agarose gel electrophoresis showing characteristics laddering and smearing pattern of LAMP amplification product of *mecA* gene. Lanes were loaded with ladder, sample I2, I8, I9, RI, positive control, and no-template control, from left to right, respectively

Discussion

Mastitis is an important infectious disease in bovine (Gomes and Henriques, 2016). The cause of the disease involves infectious pathogenic bacterium species that can be categorized into two main types: 1) Contagious pathogens including *S. aureus* and *S. agalactiae*, that are responsible for transmission of infection from cow to cow, 2) Opportunistic pathogens originating from the environment such as Coagulase Negative Staphylococcus (CNS) and *E. coli* (Ruegg, 2017). In the recent history, mastitis pathogenic bacteria have evolved to become antibiotic resistant, necessitating animal husbandry practices that minimize unnecessary usage of antibiotics. Treatments that involve antibiotic uses often result in high cost of treatment and poor efficacy in antibiotic resistant bacteria in the long run (Deb *et al.,* 2013).

In this study, the results of CMT test showed that 145 raw milk samples were positive in the CMT test (CMT score +1, +2, and +3). Subsequently, CMT-positive raw milk samples were investigated by microbiology techniques including differential medium, gram staining, coagulase test, and catalase test, which showed that 6.54% of the isolates were *S. aureus*, these results are consistent with previous studies in mastitis cow in Nepal and Ethiopia, which showed percentage of *S. aureus* from total bacterial isolates at 15.2% and 17.2% respectively. In addition, this study showed 11.1% rate of CNS, that is slightly lower than in the previous studies done in Nepal and Ethiopia at 15.7% and 39.1% respectively (Balemi *et al.,* 2021; Shrestha *et al.,* 2021). The lower percentage of *S. aureus* and CNS could be an indication of good husbandry and health management of dairy cow in Cha-am-Huai Sai, Phetchaburi province, Thailand.

Aside from the microbiological test results, this study describes the development of a novel LAMP assay to detect MRSA strains isolated from dairy cattle milk, in comparison to the conventional PCR assay. In PCR assay, results were consistent with the microbiological techniques. All isolates were positive for *femA* genes, indicating that all bacterial isolates were *S. aureus*. This is because *femA* gene is generally found in practically all strains of *S. aureus* genome (Maidhof *et al.,* 1991; Mehrotra *et al.*, 2000). In addition, all isolates were also positive for *blaZ* gene that is involved in the production of penicillinase and causes penicillin resistance (Takayama *et al.,* 2018), which indicates that all bacteria isolates tested were also penicillin resistant. The *mecA* gene showed positive results at 30% (3 out of 10 *S. aureus* isolates), indicating that only a small fraction of total *S. aureus* carried *mecA* gene.

Running the *mecA* PCR products on an agarose gel electrophoresis revealed a bright band at the correct product size for I2 isolate, while I8 and I9

isolates showed slightly fainter band but were still at the correct size for *mecA* gene. Subsequently, antimicrobial susceptibility tests were performed to check for antibiotic resistance using 1 μg oxacillin and 30 μg cefoxitin. The results showed that I8 and I9 isolates were susceptible to both of the antibiotics, contradicting the results of the PCR test. This is likely because *mecA* resides on the mobile bacterial chromosome SCCmec, and the two isolates (I8 and I9) may contain few copies of the SCCmec chromosome than I2, thus conferring low level of antibiotic resistance. Alternatively, this could be due to gene silencing in bacteria gene, which has been shown before in antibiotic resistance gene silencing in *E. coli*. In some bacteria samples isolated from the environment and clinical samples, antibiotic resistant genes were detected in the genome of the bacteria, although the genes were not expressed and presumed to be silenced by an unknown mechanism (Enne *et al.,* 2006). Taken together, these results indicated that PCR assay has a higher sensitivity and accuracy for detection of resistant genes than antimicrobial susceptibility test.

Currently, *femA*, *blaZ*, and *mecA* genes are widely used for molecular detection of antibiotic-resistant *S. aureus* in many host species in various settings (Mehrotra *et al.,* 2000; Davoodi *et al.,* 2015; Wang *et al.,* 2018; Badua *et al.*, 2020). However, because *femA* and *blaZ* genes were found in virtually all *S. aureus* isolates, we reasoned that it was not necessary to detect these genes for MRSA using LAMP assay. Therefore, we primarily focused only on *mecA* gene, which correlates with the MRSA phenotype, for the development of LAMP assay.

For the LAMP results, visualization of turbidity of the LAMP products were shown in all tube, as well as visualization of end-point products of LAMP under UV light, that were likely caused by a long reaction time (data not shown). Detection of fluorescent products from the reactions by real-time fluorescent detector and analysis of LAMP product on 1.0% agarose gel showed an obvious positive amplification of *mecA* gene in RI and I2, consistent with the PCR assay. However, LAMP fluorescent and gel electrophoresis showed no amplification in I8 and I9, which is not consistent with PCR assay but consistent with the antimicrobial susceptibility test. Again, this may indicate that I8 and I9 possessed few copies of SCCmec below the level of detection by LAMP assay. The results indicated that, in this case, the sensitivity of PCR assay is higher than LAMP assay, which can detect very few copies of SCCmec in *S. aureus* that were insufficient for rendering methicillin resistance in antimicrobial susceptibility test.

Finally, we conclude that the LAMP assay can be used for amplification of *mecA* gene in MRSA strains isolated from dairy cattle milk, but the samples must contain a relatively high copies of SCCmec for accurate detection. This

assay can be further developed into a prototype of rapid test kit that will facilitate usage in the diagnosis in the field. Additionally, LAMP reaction is highly sensitive and users must be stringent about preventions of contamination which can cause a false positive result.

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