



Rapid Detection of MRSA by Loop Mediated Isothermal Amplification in Bovine Milk Samples

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Authors' contributions

This work was carried out in collaboration between all authors. Author GS designed the study, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Authors GS, EH and KCD managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To develop a simple and rapid detection method for diagnosis of MRSA in bovine milk samples suspected for mastitis.

Methodology: The laboratory sensitivity and specificity of LAMP assay was carried out using available laboratory strains. Milk samples were collected from Thiruvallur and Kanchipuram districts of Tamilnadu, India. DNA was directly isolated from the milk samples and *mecA* gene was screened by both PCR and LAMP methods.

Results: The LAMP assay successfully amplified the *mecA* gene under isothermal conditions at 64°C within 60 min. LAMP assay was able to detect 10 pg of DNA and did not amplify *mecA* gene from bacterial DNA of other species. The screening of milk samples for MRSA showed 47 Positive out of 77 Samples by PCR and 43 positive out of 77 Samples by LAMP.

Conclusion: Application of LAMP assay enabled rapid and easy detection of MRSA in milk samples suspected for bovine mastitis.

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1. INTRODUCTION

In Dairy farming and production, mastitis treatment is a challenge due the antibiotic resistance. *Staphylococcus aureus* is one of the major causative agent of mastitis and a primary reason for antibiotic use on dairy farms [1,2]. There has been increasing reports from all over the world about the prevalence of Methicillin resistance among cattle. Da silva et al. [3] reported the presence of MRSA in 11% of milk samples in dairy farms of Brazil compared to 29% in India [4]. Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for those with zoonotic potential [5]. Methicillin resistance in *S. aureus* is primarily mediated by the *mecA* gene carried on a mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec), and at least five types of SCC mec elements have been reported [6]. *mecA* gene codes for the modified penicillin-binding protein 2a (PBP 2a or PBP 2'). PBP2a is located in the bacterial cell wall and has a low binding affinity for β -lactams. The role of inappropriate antibiotic usage, under dosage and inappropriate administration are also considerable in emergence of drug resistant zoonotic pathogen in the community, since MRSA of Human and bovine origin are epidemiologically related, it can be understood that the transmission of MRSA from cow to human is possible. Hence it is essential to diagnose antibiotic resistance in bovine mastitis and detect MRSA in milk before it becomes a major public threat [7,8].

Bacterial culturing, ELISA and PCR are some of the Diagnostic methods used to detect MRSA in bovine mastitis. These techniques are often time-consuming and require specialized equipments, which may not be available. Loop-mediated isothermal amplification (LAMP) is a simple and rapid technique, in which DNA can be isothermally amplified using only one enzyme [7]. Since LAMP can amplify genes isothermally, the amplification can be carried out with a simple heater, water bath, thermal block making LAMP assay, the optimal method of choice for diagnosis of methicillin resistance in milk samples. Although LAMP technique was previously developed for rapid detection of *Staphylococcus aureus* including MRSA which targeting *arcC* gene [9], here we are targeting *mecA* gene for specifically MRSA as it reduces the time for differentiation of MRSA from MSSA.

In Bovine mastitis causative factors MRSA is difficult one in treatment comparing to MSSA. Hence this study aimed at developing LAMP assay for detection of MRSA in milk samples suspected for bovine mastitis and its usefulness in field conditions.

2. MATERIALS AND METHODS

2.1 Lamp Assay

For LAMP assay, four sets of primers (B3, F3, BIP, and FIP) recognizing a total of 6 distinct sequences (B1-B3 and F1-F3) on the *mecA* gene of MRSA, were used as described by Koide et al. [10]. The LAMP assay was performed in a 25 μ l reaction mixture and was incubated at 64°C for 60 min and terminated by heating for 10 min at 80°C [10]. It was then observed under UV Light followed by Agarose gel electrophoresis.

Total DNA from *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 700603) were used to check the specificity of LAMP primers. Positive and negative samples for LAMP reaction were tested thrice to check the reproducibility of the assay. The diagnostic sensitivity of LAMP assay was determined by serially diluting Positive control MRSA DNA (TN/TVR/BM/SA1/14) (GenBank Accession No KP336393) (977 ng/ μ l) [8] to 10 fold.

2.2 Sample Collection

The milk samples were collected from 77 cows with clinical mastitis (hard swollen udder with change in milk consistency) attended to the veterinary hospitals of Tiruvallur and Kanchipuram district between April 2015 and March 2016. Aseptic centrifuge tubes were used for milk collection and the udders were wiped using cotton swab during collection. The consistency and colour of the milk were noted. The collected samples were transported to laboratory in ice and processed immediately.

2.3 Bacterial Identification and Microscopic Inhibitory Concentration Test (MIC)

The milk samples were initially inoculated in buffered peptone water. The growth suspension was placed into blood agar plates. The hemolytic colonies were grown into Mannitol salt agar for

S. aureus. Coagulase test was also done for further confirmation. The Methicilin resistance was identified using Oxacilin disc (5 µg) in colony growth, which occurred in Mueller Hinton Agar as per [11].

2.4 DNA Isolation

DNA was isolated directly from the milk samples as reported by [12] with modifications. Briefly 1:1 dilution of milk samples and saline was centrifuged for 20 min at 1008 x g. The supernatant was discarded. The pellet was then incubated with Lysis buffer [3 M guanidine thiocyanate, 20 mM EDTA, 10 mM Tris-HCl (pH 6.8), 40 mg/mL Triton X- 100, 10 mg/mL DL-dithiothreitol] and binding buffer [40 mg/mL silica] for 10 minutes and centrifuged at 448 x g for 1 min. To the pellet, 300 µl of lysis buffer was added and centrifuged at 448 x g for 1 min. This step was again repeated and the pellet was precipitated with absolute Ethanol and Isopropanol. Air dried pellet was resuspended in Nuclease Free Water and stored at -20°C for LAMP assay. The DNA purity and concentration was determined using spectrophotometer.

2.5 Screening of DNA Samples

The DNA samples were screened using LAMP primers by incubation at 64°C for 60 min and termination by heating for 10 min at 80°C. PCR targeting *mecA* gene (FP 5' GGCCAATACAGGAACAGCAT3' RP 5'TCACCTTGTCGGTAACCTGA3') [8] was also used to screen the DNA samples for comparing the results of LAMP.

3. RESULTS AND DISCUSSION

The need for rapid and reliable identification of mastitis causing organisms and investigation of local antibiotic resistant pattern of the isolates has become more important in the recent decade [13] Effective infection control efforts obviously depend on the performance of the laboratory to detect emerging resistant pathogens accurately and confirm resistance patterns by additional molecular techniques [14]. LAMP assay can be performed in isothermal conditions 60-65°C, does not require sophisticated instruments like thermocycler which is not pertinent in most of the dairy farm sector. It does not require trained personnel and the notable feature of LAMP is that the results can be visualised by naked eye as a white precipitate at the end of the reaction. In the present study, LAMP primers did not

amplify *mecA* gene from *E.coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) strain and *K. pneumoniae* (ATCC 700603) DNA indicating the assay to be specific only for *mecA* gene of MRSA (Fig. 1). When positive control MRSA DNA (TN/TVR/BM/SA1/14) (GenBank Accession No KP336393) (977 ng/µl) was serially diluted 10-fold and subjected to LAMP assay detected as little as pico gram amount of DNA (upto 0.097 ng/µl) (Fig. 2).

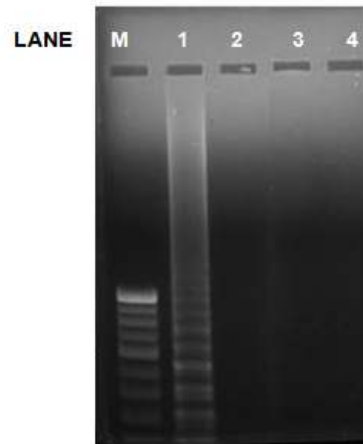


Fig. 1. Specificity of LAMP assay

Lane M: 100 bp DNA Ladder
 Lane 1: Positive Control MRSA DNA (TN/TVR/BM/SA1/14)
 Lane 2: *E. coli* DNA (ATCC 25922)
 Lane 3: *Staphylococcus aureus* ATCC 25923 DNA
 Lane 4: *K. pneumoniae* DNA (ATCC 700603)

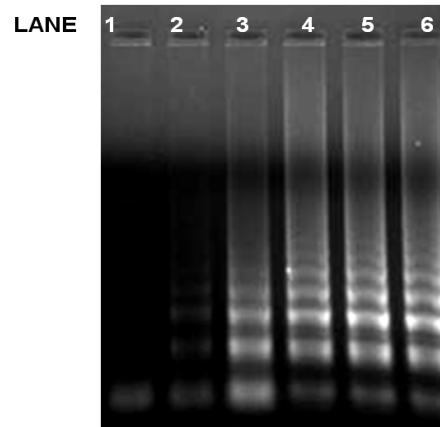


Fig. 2. Diagnostic sensitivity of LAMP assay

Lane 1-6: 0.0097, 0.097, 0.97, 9.7, 97.7, 977 ng/µl of Positive control MRSA DNA (TN/TVR/BM/SA1/14) for *mecA* gene amplification

DNA can be directly isolated from the milk samples rapidly and easily by using silica resin to

bind DNA in the presence of high concentrations of guanidine thiocyanate as described previously by Cremonesi et al. [12], which guarantees excellent disruption of bacterial cells. Forty-two samples showed resistance to Oxacillin by MIC test, forty seven samples showed positive by PCR and forty three samples showed positive for LAMP (Fig. 3). Forty three samples were positive by both PCR and LAMP assay. Hence LAMP assay can also be used for detection of MRSA in bovine milk samples.

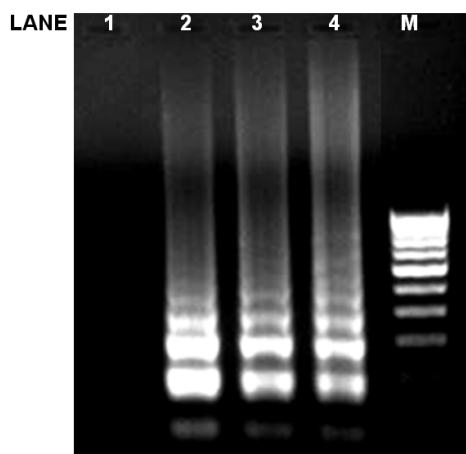


Fig. 3. Screening of MRSA suspected milk samples by LAMP

Lane 1: Negative Control (*E. coli* DNA (ATCC 25922))

Lane 2: Positive Control MRSA DNA (TN/TVR/BM/SA1/14)

Lane 3-4: *mecA* Gene LAMP Products

Lane M: 100 bp DNA Ladder

From this study, it can be concluded that LAMP assay is a potential method for rapid large scale screening of MRSA in bovine mastitis in endemic areas.

4. CONCLUSION

The LAMP assay successfully amplified *mecA* gene of MRSA in DNA samples directly isolated from milk samples. It can be concluded that LAMP assay can be employed for simple and rapid detection of MRSA in bovine mastitis suspected milk samples but further studies should focus on the performance analysis of this methodology.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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