Original Article

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Microbiology Section

Evaluation of LAMP Assay Using Phenotypic Tests and Conventional PCR for Detection of *nuc* and *mecA* genes Among Clinical Isolates of *Staphylococcus* spp

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ABSTRACT

Introduction: The purpose of this study is to develop a *nuc* and *mecA* gene specific Loop-mediated isothermal Amplification (LAMP) assay for rapid identification and detection of methicillin resistant *Staphylococcus aureus* among clinical isolates.

Materials and Methods: A total of 100 (70 from pus and 30 from blood), clinical isolates of *Staphylococcus* spp were screened for the *nuc* gene to differentiate between *S.aureus* and Coagulase negative *Staphylococci* (CONS) by a *nuc* gene specific LAMP assay. The isolates were also screened for the presence of the *mec A*gene by the *mecA* specific LAMP assay. The results were compared with the phenotypic identification and methicillin resistance by Vitek-2 system (*bioMérieux, Marcy l'Etoile, France*) and conventional PCR.

Results: Among 100 *Staphylococcus* isolates, there were 82 (82%) *Staphylococcus aureus* isolates and 18 (18%) coagulase negative *Staphylococcus* as detected by the Vitek 2, conventional PCR and the LAMP assay using the *nuc* gene. The *mecA* gene was detected by the LAMP assay in 56(56%) isolates (44 Methicillin resistant *Staphylococcus aureus* (MRSA) and 12 Methicillin resistant coagulase negative *Staphylococcus* (MRCONS), which were also identified by the Vitek 2 and conventional PCR as methicillin resistant. The results of the LAMP assay were available within 90min as compared to the Vitek 2 results (18- 24hours) and conventional PCR (3-4 hours).

Conclusion: The present study proved that LAMP assay can be used for the simultaneous differentiation of *Staphylococcal spp* and detection of methicillin resistance.

Keywords: Benchside assay, Methicillin resistance, Turnaround time, Vitek 2

INTRODUCTION

Staphylococcus aureus (S.aureus) is one of the major pathogens causing community and health care associated infections [1,2]. The clinical manifestations of *S.aureus* ranges from local infections to severe life threatening systemic manifestations such as bacteremia, toxic shock syndrome [3].

MRSA strains started emerging one year after the introduction of methicillin in clinical settings in 1960s [4]. The *mecA* gene, which encodes the low-affinity penicillin-binding protein 2a causes methicillin resistance in *Staphylococci* [5]. In recent times MRSA has emerged as an important pathogen of public health importance causing significant morbidity. Most of them are multidrug resistant. Rapid detection of MRSA is essential for early treatment of the patients and implementation of infection control policies to prevent its spread and outbreaks. The *nuc* gene is specific for *S.aureus* and molecular detection of this gene helps in the rapid identification of *S.aureus* from clinical specimens.

There are several phenotypic methods for the detection of methicillin resistance in *Staphylococci* which includes manual methods and automated systems such as Vitek 2C [6]. The disadvantage of the phenotypic methods are that they are time consuming and have a turnaround time of 18-24 hours.

Molecular methods like Polymerase chain reaction (PCR) and real-time PCR have been used for rapid identification of *S. aureus,* particularly for MRSA [7,8]. Loop-mediated isothermal amplification (LAMP) assay is a novel method for amplifying DNA and RNA with high specificity, sensitivity, and simplicity. This newly developed tool has enlightened the world of today's diagnosis and changing the way infectious diseases are monitored in developing countries. Loop mediated isothermal amplification, as the name implies, the reaction takes place at an isothermal temperature, which is the greatest credit to the test. Essentially, the assay consists of incubating a mixture of the target gene along with six sets of target specific primers (2 inner, 2 outer, 2 loop), Bst DNA polymerase and the substrates at an optimal temperature (63–65°C). The inner primers introduce self-complementarity into the amplification product, causing loops to form, while extension of the outer primers causes displacement of the extension products of the internal primers [9].

Compared to PCR, LAMP has the advantages of reaction simplicity and rapidity.

This study was conceptualized with an aim to develop and evaluate the LAMP assay for detection of *Staphylococcal* spp from cultures using *nuc* (for identification of *S. aureus*) and *mecA* (for detection of methicillin resistance) gene specific LAMP assays.

MATERIALS AND METHODS

This study was done at Nizam's Institute of Medical Sciences, Hyderabad from June 2013 to May 2014. The standard control strains used in this study were American Type Culture Collection (ATCC) of 25923 methicillin Sensitive *Staphylococcus aureus* (positive control for Methicillin Sensitive *SA*), ATCC 43300 MRSA (positive control for MRSA), ATCC 25922 *Escherichia coli* (negative control for *mecA* and *nuc* genes) (commercially procured from microbiologics, USA). The LAMP assays for *mecA* and *nuc* genes were optimized using these ATCC strains, which were later applied to screen the clinical isolates of *Staphylococci.*

Optimization of the LAMP Assays for *mecA* and the *nuc* genes:

Genomic DNA Extraction

Genomic DNA was extracted from overnight growth cultures of all the ATCC strains on 5% sheep blood agar (*bioMérieux, Marcy l'Etoile, France*) by boiling method, as per the Centers for Disease Control and Prevention (CDC) protocol [10].

Sequence of DNA Target Selection and Primer Designing

mecA and *nuc* specific gene sequence of different *Staphylococcus* strains were taken from NCBI site (ncbi.nlm.nih.gov.in) and the sequences were aligned by using clustal W software. After alignment LAMP primers were designed by using highly conserved regions of *mecA* and *nuc* genes of *Staphylococcal* strains with accession no X52593.1 and BA000018.3 respectively, by using Primer Explorer V4 software (http://primerexplorer.jp:81/lam/).

The LAMP primers included two outer primers F3 and B3, two inner primers FIP and BIP, two loop primers (forward loop primer (FLP) and backward loop primer (BLP) (Notomi et al.,). The list of all the LAMP primer sequences selected and used in this study is shown in [Table/Fig-1].

Genes	Primer sequences
	PCR
*nuc*FP	CAAAGCATCAAAAAGGTGTAGAGA
*nuc*RP	TTCAATTTTCTTTGCATTTTCTACCA
*mecA*FP	GGCAATATTACCGCACCTCA
*mecA*RP	GTCTGCCACTTTCTCCTTGT
	LAMP assay
nuc-F3	TCGCTTGCTATGATTGTGG
nuc-B3	ACATACGCCAATGTTCTACC
nuc-FIP	GTACAGTTTCATGATTCGTCCCGCCATCATTATTGTAGGTGT
nuc- BIP	TGTTCAAAGAGTTGTGGATGGTGTACAGGCGTATTCGGTT
nuc-FLP	TTGAAAGGACCCGTATGATTCA
nuc-BLP	GATACGCCAGAAACGGTGA
mecA-F3	GGTACAAGATGATACCTTCGTT
mecA-B3	ATAGCAGTACCTGAGCCAT
mecA-FIP	TCTTCAGAGTTAATGGGACCAAACAGAAAGTCGTAACTATCCTC
mecA-BIP	AAGCTCCAACATGAAGATGGCTTGTATGTGCGATTGTATTGC
mecA-FLP	ACCTAATAGATGTGAAGTCGCT
mecA-BLP	CGTGTCACAATCGTTGACG

[Table/Fig-1]: Primer sequences for *nuc* and *mecA* genes for PCR and LAMP assay
FP-Forward primer
RP-Reverse primer
F3-Forward outer primer, B3-Backward outer primer
FIP-Forward inner primer, BIP-Backward inner primer
FLP-forward loop primer, BLP-backward loop primers

All the LAMP primers were synthesized commercially (Bioserve, Hyderabad).

Conventional PCR Assay

A 210 bp region of *mecA* and 90 bpregion of *nuc* genes were tested by conventional PCR assay. Published primers [11] used in the study were shown in [Table/Fig-1] (Bioserve Technologies, Hyderabad). The bacterial genomic DNA extracted was used as template for amplification. PCR was done on GeneAmp PCR system 2400 (Applied Biosystems, USA) with quick load master mix (New England Biolabs, UK). PCR conditions were maintained at 95^{0}C for 10 minutes, initial denaturation followed by 40 cycles of 95 ^{0}C 15 seconds, 58^{0}C for 30 seconds and 70^{0}C for 30 seconds and final extension 70^{0}C for 2 min thermal cycler (Perkin Elmer, USA). The PCR products were tested for *nuc* and *mecA* genes by running agarose gel electrophoresis. The bands were visualized by the Gel documentation system, (Syngene, UK).

LAMP Reactions

LAMP reactions were carried out as per manufacturer's instructions (DNA amplification kit; Eiken Chemical Co. Japan). The reaction was standardized at 60^{o}C for 60 min and inactivated at 80^{o}C for 5 min in a water bath.

Detection of LAMP products was by: 1) visual fluorescence by adding 0.2µl of 1/10000 DMSO SYBR Green I (Sigma Aldrich, USA) to 25 µl of LAMP product; 2) A 10µl of the LAMP product was electrophoresed on 2% agarose gel and documented using a gel documentation system (Syngene, UK).

Clinical Isolates

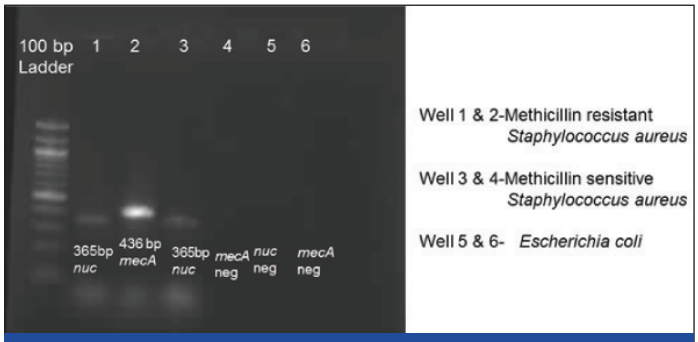
A total of 100 (70 from purulent aspirates of deep seated abscesses and postoperative wounds and 30 from blood cultures), clinically significant, non-duplicated *Staphylococcal* isolates were included in this study. Phenotypic identification and antimicrobial susceptibility of the isolates were carried out with the Vitek 2 system, using the ID GP and the P628 AST panels (*bioMerieux, Marcy l'Etoile, France).* Vitek 2 Advanced Expert System (AES) analysis findings were recorded.

The optimized LAMP assays and conventional PCR for the 2 genes were applied to screen these 100 clinical isolates of *Staphylococci.*

The phenotypic, conventional PCR and LAMP assays were compared for the identification and detection of clinical isolates of MRSA. The turnaround times (TAT) and cost per test were calculated for these methods.

RESULTS

The specificity of the LAMP F3 and B3 primers were confirmed by PCR on the ATCC strains (positive and negative controls). [Table/Fig-2] shows the agarose gel image of the PCR result of the *nuc* and *mecA* genes, found only in the ATCC standard for MRSA and the *nuc* gene alone in the ATCC MSSA.



[Table/Fig-2]: *nuc* and *mecA* conventional PCR agarose gel image by using LAMP outer (F3 B3) primers

The specificity of the LAMP assays for the 2 genes were 100% on the ATCC strains.

Among the 100 *Staphylococcus* isolates, the Vitek 2 identified 82(82%) isolates as *S.aureus* and 18(18%) isolates as CONS and detected methicillin resistance in 56 isolates (44 MRSA and 12 MRCONS)

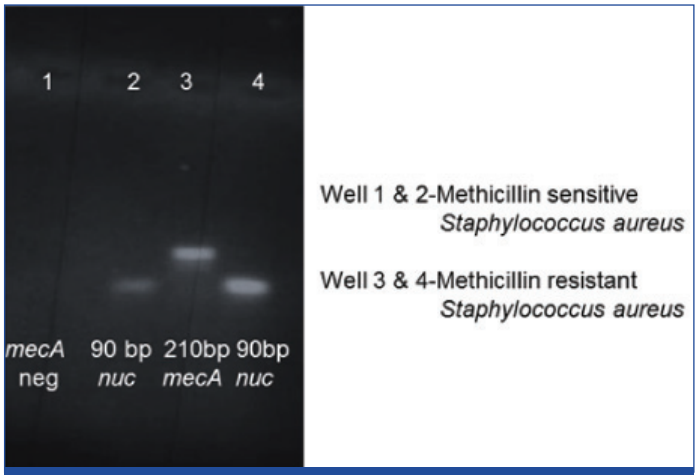
The conventional PCR and LAMP assays also showed identical results. However, the results of the LAMP assay were rapid and were available within 90 minutes as compared to the Vitek 2 results (18-24 hours)

The gel images of conventional PCR using published forward and reverse primers are shown in [Table/Fig-3]. The visual fluorescence of the LAMP products and the gel images are shown in [Table/Fig-4,5]. The amplified target sequences of LAMP assays can be visualized as ladder like bands in the gel image due to the formation of stem loop structures of amplified DNAs of various stem lengths.

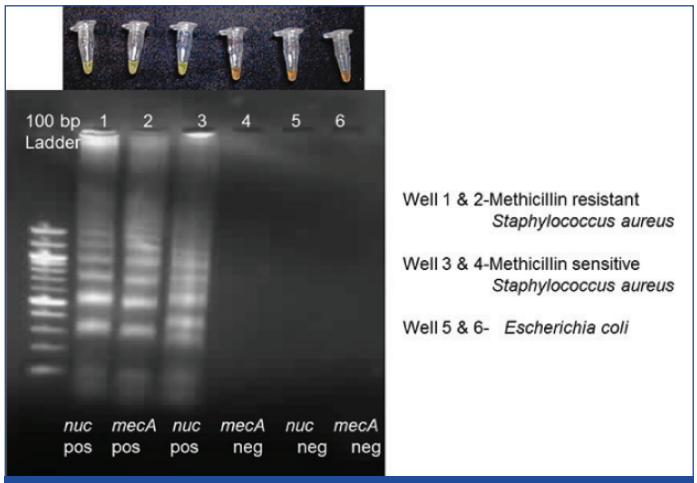
The comparison of TATs and cost per test of phenotypic, conventional PCR and LAMP assays are shown in [Table/Fig-6].

DISCUSSION

S. aureus, including MRSA, is one of the most important bacteria causing a wide spectrum of human infections [12]. Timely detection



[Table/Fig-3]: *nuc* and *mecA* conventional PCR agarose gel image by using published primers



[Table/Fig-4]: Visual fluorescence and gel images of LAMP products of MRSA and MSSA

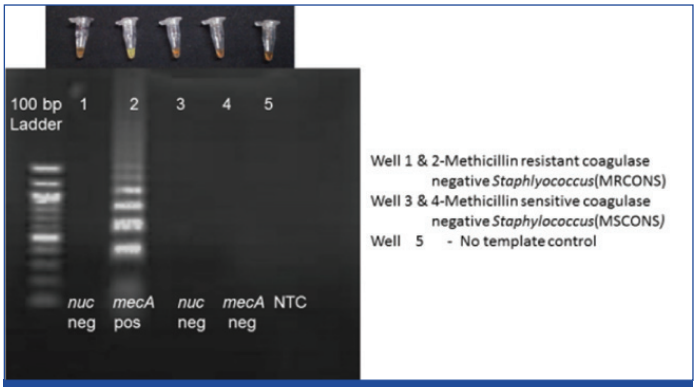
and confirmation of MRSA is important for adequate and appropriate treatment, surveillance and infection control.

There are very few studies using LAMP assays for the detection of *Staphylococcal* isolates, which include *femA* and *mecA* genes from food isolates [13], *femA* from clinical and food samples [14], *spa* and *arc C* genes from spiked blood specimens [7], *spa* and *mecA* gene from positive blood culture bottles [15], *orfX* (a highly conserved open reading frame of *S.aureus*) from clinical isolates [16].

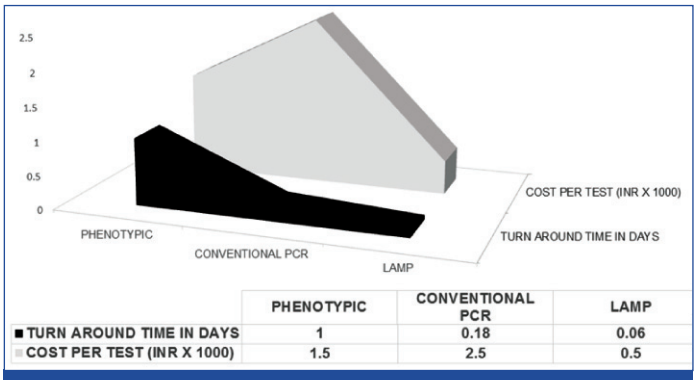
In this study, we differentiated *S.aureus* from CONS using the *nuc*gene. *S. aureus* strains produce an extracellular thermostable nuclease (thermonuclease {TNase}), which is an endonuclease that degrades both DNA and RNA characterized by its gene *nuc* specific for *S.aureus* [17,18].

Methicillin resistance was detected by *mecA* gene in both *S.aureus* and CONS. *mecA* gene, specific for Methicillin resistance, is carried by a mobile genetic element *Staphylococcal* cassette chromosome *mec* (SCC*mec*) is characterized by the presence *mecA* gene in *mec* gene complex,*ccr*genes*(ccrA* and *ccrB) in ccr*gene complex, and the presence of flanking direct repeat sequences containing the integration site sequence (ISS) [19,20].

In comparison to phenotypic methods, which are time consuming and PCR, which requires thermal cycler and requires technical expertise, LAMP uses a simple water bath and allows visual detection of amplified product by turbidity or fluorescence. The LAMP assay is a more rapid and simpler method than PCR. The total amplification time for LAMP is 60 minutes, as compared to PCR, which is about 3-4 hours. It is based on autocycling strand displacement DNA synthesis using the *Bst* DNA polymerase enzyme. It uses six primers recognizing 6–8 distinct regions of the target gene facilitating the



[Table/Fig-5]: Visual fluorescence and gel images of LAMP products of MRCONS and MSCONS



	PHENOTYPIC	CONVENTIONAL PCR	LAMP
TURN AROUND TIME IN DAYS	1	0.18	0.06
COST PER TEST (INR X 1000)	1.5	2.5	0.5

[Table/Fig-6]: Comparison of Turnaround time and cost per test for phenotypic, conventional PCR and LAMP Assay

detection of very minute quantities of target DNA. The amplification occurs under isothermal conditions between 63°C [9,14] and time loss due to thermal changes is prevented [7]. In addition the reaction is not inhibited by the inhibitors in the sample [21]. The amplified gene product can be visualized by the unaided eye, either as turbidity in the form of a white precipitate or through a colour change employing a fluorescent intercalating dye (SYBR Green I) [9]. The results of LAMP assay was comparable with phenotypic and conventional PCR with the advantage that the LAMP assay is rapid with a turnaround time of 90 minutes following the isolation of the organism from clinical specimen and is also cost effective.

CONCLUSION

The present study proved that LAMP assay can be used as a rapid molecular diagnostic assay for the simultaneous differentiation of *Staphylococcal spp* and detection of methicillin resistance. It can be easily adapted in any microbiology laboratory as a rapid molecular bench side assay due to its simplicity and ease of performance without any sophisticated instrumentation.

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