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Comparison of Loop mediated isothermal amplification with polymerase chain reaction for detection of methicillin resistant *Staphylococcus aureus* in chevon

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Abstract
Methicillin-Resistant *Staphylococcus aureus* has been recognized as a major nosocomial pathogen and has also been widely associated with foodborne illnesses. The main aim of the study was to compare Loop Mediated Isothermal Amplification assay and Polymerase Chain Reaction technique based on the basis of sensitivity and specificity for detection of Methicillin-Resistant *Staphylococcus aureus*. Total 26 *Staphylococcus aureus* isolates recovered from 150 raw chevon samples were used in this study. These isolates were subjected to both the techniques for detection of Methicillin-Resistant *Staphylococcus aureus*. In results, both the techniques could detect 2 (1.33%) isolates. The sensitivity (detection limit) of the Loop Mediated Isothermal Amplification assay was noted to be 10-fold higher than that of Polymerase Chain Reaction whereas the specificity of both was found to be similar (100%).

Keywords: Methicillin-resistant *Staphylococcus aureus*, loop mediated isothermal amplification, polymerase chain reaction

Introduction
Staphylococcus aureus (*S. aureus*) is an important foodborne pathogen which is known to cause outbreaks many times. They contaminate various food products which causes food poisoning due to the ingestion of preformed Staphylococcal Enterotoxins. It acts as one of the most important economic illness and is a major issue for the worldwide public health program [1]. From last few decades antibiotic resistance in the bacteria is of great concern all around the world. Methicillin-Resistant *Staphylococcus aureus* (MRSA) is one of the highest ranking pathogen all around the world and has a significant public health concern. It is resistant to methicillin and many other antimicrobials of β lactam group and also resistant to macrolides and aminoglycosides. MRSA has been recognized as a major nosocomial pathogen and has also been widely associated with foodborne illnesses [2]. It is mediated by the *mecA* gene, which encodes penicillin binding protein 2a (PBP2a), with a low affinity for β lactam antibiotics. The *mecA* gene is part of a large mobile genetic element called Staphylococcal Cassete Chromosome *mec* (SCC*mec*) [3]. MRSA strains are one of the biggest public health concerns because the treatment of infection is more difficult and complicated when resistance is encounterd and considered as one of the most important agents of food poisoning around the world [4].

Detection of MRSA is based on cefoxitin and oxacillin disc diffusion method and detection of *mecA* gene by Polymerase Chain Reaction (PCR). PCR is one of the most widely used methods in diagnostic applications because it allows sensitive and rapid diagnosis. However, this technique is not suitable for usual food safety testing as it requires expensive thermal cycler, complex DNA amplification operations and post amplification protocol such as electrophoresis. To overcome such limitations, several nucleic acid amplification methods have been developed in which thermal cycling is not required and the operation is simple [5].

Notomi *et al*. (2000) [6], developed Loop Mediated Isothermal Amplification assay (LAMP) which can amplify the target gene under isothermal conditions (60–65°C) with high efficiency, specificity and sensitivity. This novel method can amplify a few copies of DNA to 10 copies in less than an hour.

It serves as a useful tool to quickly detect and identify foodborne pathogens [7]. This method is based on the auto cycling strand displacement nature of *Bst* DNA polymerase using a set of two specially designed inner and two outer primers. As it is conducted under isothermal conditions and findings can be visually interpreted, it is well suited for adoption as a field level diagnostic in developing countries and poorly equipped laboratories [8].

Hence, looking towards the scanty work in India regarding LAMP based diagnosis of MRSA from chevon, this study was planned with objectives to detect MRSA by PCR and LAMP technique and comparison of both techniques based on sensitivity and specificity.

Materials and Methods

Sample collection

Total 26 *S. aureus* isolates recovered from 150 raw chevon samples were used in this study. Raw chevon samples were collected aseptically from different retail meat shops in Anand district of Gujarat. These 26 isolates were identified by cultural methods and biochemical tests and further confirmed by PCR.

Molecular characterization of MRSA by PCR

DNA extraction

The DNA from isolates was extracted by boiling method. A loopful of pure culture was suspended in 100 µl nuclease free water in a sterilized microcentrifuge tube. The suspension was vortexed and then heated at 95˚C for 10 mins in thermal cycler. This was then centrifuged at 10000 rpm for 6 mins so that the cell debris settle down. The upper aqueous phase was used as a DNA template for PCR.

PCR protocol

All the isolates were screened for the presence of *mecA* gene by PCR as per the protocol described by Lee (2003) [3]. The details of oligonucleotide primers for *mecA* gene is given in Table 1. The reaction mixture for PCR was prepared in 200 µl PCR tubes on ice to a final volume of 25 µL and the amplification to screen the *mecA* gene was done by using Thermocycler PCR machine (Eppendorf Mastercycler gradient, Germany). The reaction mixture contained 12.5 µL PCR master mix (2X), 1 µL each of forward and reverse primer (10pmol), 5.5 µL nuclease free water and 5 µL template. The details of thermal profiling of PCR are mentioned in Table 2. The final amplified product was analysed by agarose gel electrophoresis on 1% agarose gel and visualized under gel documentation system.

Table 1: Description of primer used for detection of MRSA

Sr. No.	Target- Gene	Primer sequence (5' — 3')	Product Size (base pairs)	Reference
1.	*mecA*	F : AAA ATC GAT GGT AAA GGT TGG C R : AGT TCT GCA GTA CCG GAT TTG C	533 bp	Lee (2003) [3]

Table 2: PCR conditions for detection of *mecA* gene

Cycling Conditions		Temperature	Time
Initial Denaturation		94ºC	4 min
35 cycles	Denaturation	94ºC	30 sec
	Annealing	55ºC	30 sec
	Extension	72ºC	60 sec
Final Extension		72ºC	5 min

Molecular Characterization of MRSA by LAMP

The DNA of MRSA isolates was extracted by boiling method as described previously.

LAMP assay using primers for *mecA* was performed. The details of the primers are mentioned in Table 3. Total 25 µL of LAMP reaction mixture was prepared, the composition and concentration of which is given in Table 4. The reaction mixture was prepared in 200 µL PCR tubes and then it was incubated in water bath for isothermal amplification at 65ºC for 45 mins and further heated to 80°C for 2 min to terminate the reaction. The LAMP products were visualised either by visual detection after addition of dyes like SYBR green or by agarose gel electrophoresis in which a ladder like pattern is seen. After the amplification of DNA, 1 µl of SYBR Green (1:100) was added to each LAMP reaction tube in a closed environment for the visual detection of amplified product. In positive reaction fluorescence and colour change were visualized under U. V. transilluminator of gel documentation system (Biovis, India). After completion of LAMP reaction, amplified DNA were analyzed on 2% agarose gel by electrophoresis at 100 V for 45 mins and then observed under U. V. transilluminator of gel documentation system (Biovis, India).

Table 3: Description of primers used for detection of MRSA by LAMP

Sr. No.	Target Gene	Primer sequence (5' — 3')	Reference
1.	*mecA*	F3:CGT ATA TTA AAC AAC AAG CTG AAC A B3:GCT TTT TGC TTT AAT TCT TCT GAG FIP:TTC AAC AAA ACG CTT TGT GTC TTG AGG ATT GGG TTA AAG ATG ATA CAT TC BIP:CAT CTC ACA ACA CAA GAA ACA GAA AAG GGCCAACATAACCAAG LF:TTT TGA ACA GTC TTG AGA GGG AC LB:GTC GAC AGT ATC CGC TTG AAG	Lin *et al.* (2017) [9]

Table 4: Reaction mixture of LAMP for detection of MRSA

Sr. No.	LAMP reagents	Quantity (µL)	Concentration
1.	10X Isothermal buffer	2.50	1.50
2.	Mg_2SO_4 (100mM)	1.50	1.50
3.	Dntp	3.50	1.6 mM
4.	Primer *mecA* FIP	1.8	1.6 µM
5.	Primer *mecA* BIP	1.8	1.6 µM
6.	Primer *mecA* F3	2	0.2 µM
7.	Primer *mecA* B3	2	0.20 µM
8.	Primer *mecA* LF	0.5	0.80 µM
9.	Primer *mecA* BF	0.5	0.80 µM
10.	*Bst* DNA polymerase	1.00	8 units/ µL
11.	DNA template	3.00	2.00
12.	Nuclease free water	4.90	5.50
Total		25.00	

Detection of specificity and sensitivity of LAMP assay and PCR

Specificity

For checking the specificity of LAMP and PCR, DNA was extracted from MRSA isolates and some other bacterial strains like *E. coli*, *Salmonella* spp. *Bacillus cereus* and *Klebsiella* spp. MRSA specific LAMP and PCR reaction was performed for all these bacteria according to the above mentioned procedures and then the results were compared.

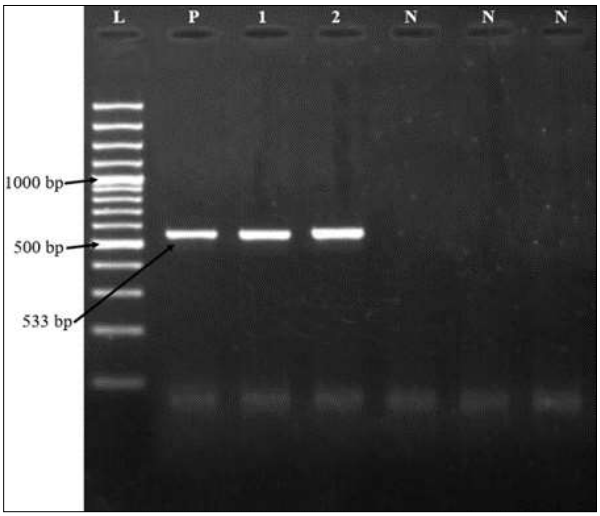
Sensitivity

Sensitivity was assessed by diluting the template DNA followed by LAMP and PCR. The DNA was extracted and then serially diluted to get concentrations 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg. Then 3 µL of DNA was taken from each dilution and *S. aureus* and MRSA specific LAMP and PCR was performed making the resultant concentrations of 300 ng/tube, 30 ng/tube, 3 ng/tube, 300 pg/tube, 30 pg/tube and 3 pg/tube DNA. Finally, the results of both the techniques were compared.

Results and Discussion

Polymerase chain reaction

Out of the total isolates, PCR technique could detect 2 samples as MRSA by targeting *mecA* gene, shown in Fig 1. So the prevalence of MRSA in the present study was 1.33% (2/150).



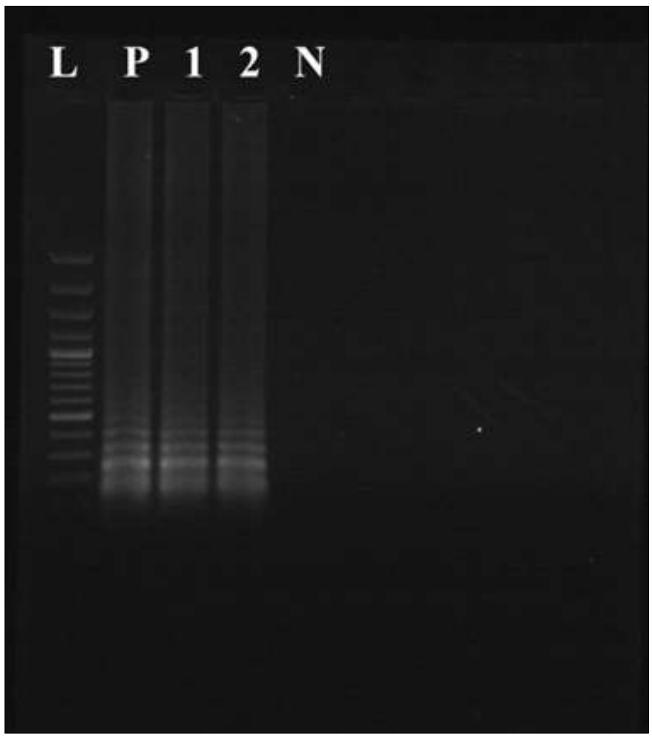
L – 100bp DNA ladder**,** P- Positive control**,** Lane 1 & 2 – Positive samples**,** N – Negative control

Fig 1: Agarose gel showing amplification product of *mecA* gene (Approxi.533 bp)

Sergelidis *et al*. (2011) [10] reported 3.40% prevalence of *mecA* gene from chevon which is slightly higher than the finding in the present study. A very high prevalence of 20.40% of *mecA* gene in chevon was reported by Hasanpour *et al.* (2017) [11]. Zehra *et al.* (2019) [12] also studied the prevalence of MRSA in various meat species but none of the *S. aureus* isolates from chevon samples showed the presence of *mecA* gene.

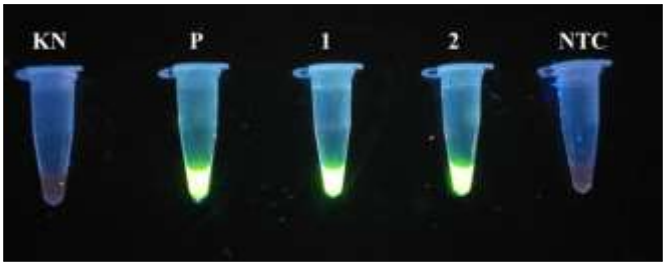
Loop mediated isothermal amplification

After subjecting the samples to LAMP assay it was found that 2 samples (1.33%) were found positive using LAMP technique. The result of LAMP after gel electrophoresis are shown in Fig 2a and results after addition of SYBR Green are shown in Fig 2b. The detection rate for both PCR and LAMP in this study were similar which was 7.69% (2/150). Higher detection rate of MRSA of 94.30%, 44% and 71.09% was reported by Xu *et al.* (2012) [13], Sudhaharan *et al.* (2015) [14] and Chen *et al*. (2017) [15] respectively.



L: 100 bp DNA ladder, P: Positive control, Lane 1 & 2 : Ladder like pattern of LAMP products of MRSA, N: Negative control

Fig 2a: Ladder like pattern of LAMP products on 2% agarose gel for MRSA

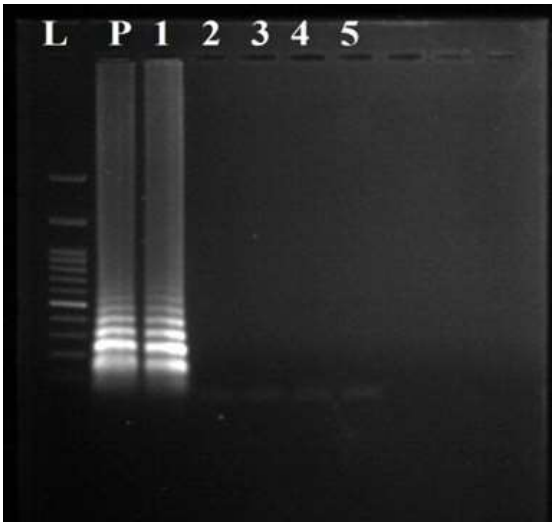


KN: Known negative, P : Positive control, 1 & 2 : Positive samples, NTC: No Template Control

Fig 2b: Visualization of LAMP products under UV light for fluorescence for MRSA

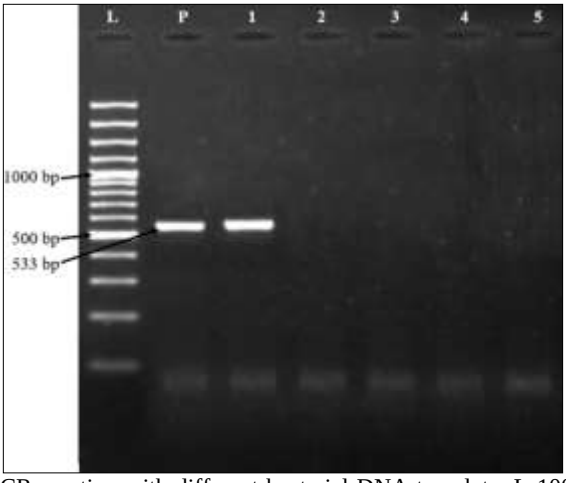
Comparison

It was observed that both PCR and LAMP assay successfully gave positive result only for DNA isolates of standard MRSA, while it did not amplify any non MRSA organisms. The specificity of both PCR and LAMP assay was found to be 100% (Fig 3a & 3b).



LAMP reaction with different bacterial DNA template; L-100 bp DNA ladder, P-Positive control, 1- MRSA, 2-*Salmonella* spp., 3-*Bacillus cereus*, 4-*Klebsiella* spp., 5-*Escherichia coli*

Fig 3a: LAMP assay specificity confirmation for MRSA by electrophoresis

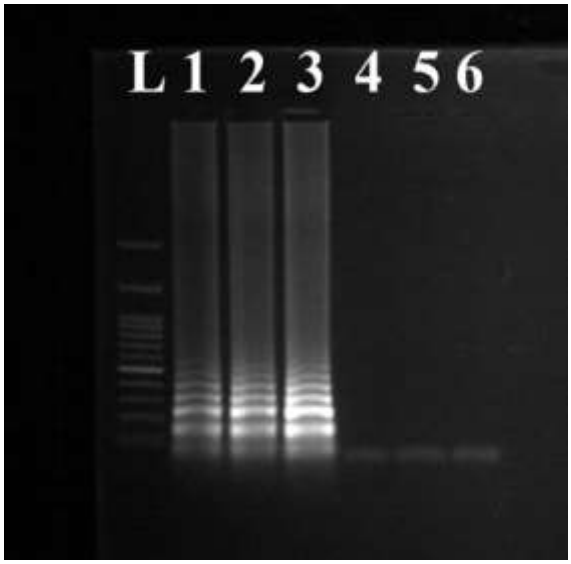


PCR reaction with different bacterial DNA template; L-100 bp DNA ladder, P-Positive control, 1- MRSA, 2-*Salmonella* spp., 3-*Bacillus cereus*, 4-*Klebsiella* spp., 5-*Escherichia coli*

Fig 3b: PCR assay specificity confirmation for MRSA by electrophoresis

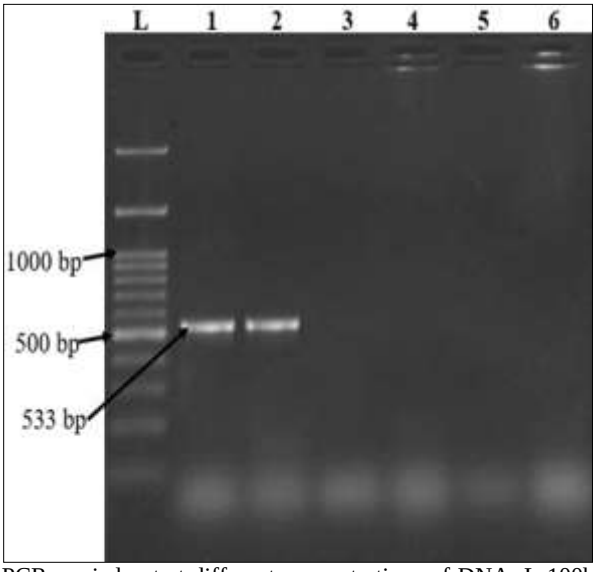
The specificity results (100%) observed in present study are in accordance with Xu *et al.* (2012) [13] and Sudhaharan *et al.* (2015) [14] who reported 100% specificity of both LAMP and PCR.

The current study showed that LAMP could detect upto 3 ng/tube concentration of DNA but further failed to detect 300 pg/tube concentration of DNA for MRSA. However PCR could detect the DNA upto 30 ng/tube of DNA and failed to detect any further dilutions. Thus the sensitivity of the LAMP assay was found 10 folds greater than that of PCR. The findings in the present study are similar to those of Xu *et al.* (2012) [13].



LAMP carried out at different concentrations of DNA; L-100bp DNA Ladder, 1-300 ng/tube, 2-30 ng/tube, 3-3 ng/tube, 4-300 pg/tube, 5-30 pg/tube, 6-3 pg/tube

Fig 4a: LAMP assay sensitivity confirmation for MRSA by electrophoresis



PCR carried out at different concentrations of DNA; L-100bp DNA Ladder, 1-300 ng/tube, 2-30 ng/tube, 3-3 ng/tube, 4-300 pg/tube, 5-30 pg/tube, 6-3 pg/tube

Fig 4b: PCR assay sensitivity confirmation for MRSA by electrophoresis

Conclusion

On screening 26 *S. aureus* isolates by PCR and LAMP for MRSA, 2 isolates showed positive results. The specificity of

LAMP and PCR assay was found to be 100%. The sensitivity (detection limit) of the LAMP assay was noted to be 10 fold greater than that of PCR. Thus, both LAMP assay and PCR are convenient testing method for detection of MRSA with reliable sensitivity and specificity.

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