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Comparative evaluation of loop mediated isothermal amplification assay for rapid detection of *Staphylococcus aureus*

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ABSTRACT

A loop mediated isothermal amplification (LAMP) method for rapid detection of *Staphylococcus aureus* from milk using standard strain ATCC 33591 was optimized targeting thermonuclease (*nuc*) gene. LAMP protocol was optimized for detection of *S. aureus* at 62°C for 50 min in water bath. The analytical sensitivity of LAMP and qPCR was found to be equal, *ca.*10^2 CFU/ml, both in broth and spiked milk by commercial kit and phenol-chloroform-isoamyl method of DNA extraction, while the sensitivity of conventional PCR was low (*ca.* 10^3 and 10^4 CFU/ml in broth and spiked milk samples, respectively), using kit method of DNA extraction. LAMP was 100% specific and in complete agreement with real time and conventional PCR. LAMP protocol optimized in the study was rapid and sensitive in detection of *S. aureus* in comparison to qPCR and PCR when kit method of DNA extraction was used. Application of LAMP for rapid detection of *S. aureus* on 126 field milk samples detected 93 milk samples positive out of 97 positive by culture without enrichment, hence giving sensitivity of 95.87%. Whereas, LAMP detected all 29 milk samples negative by culture as negative, thereby giving detection specificity of 100%. However, on enrichment of LAMP negative samples for four hours, LAMP could detect them positive for *S. aureus*. Enrichment of the milk sample for four hours enhanced the sensitivity of detection to 100% and detection limit to < 10^2 CFU/ml.

Key words: DNA extraction, LAMP, PCR, qPCR, *Staphylococcus aureus*

One of the major global concerns in the field of food safety and security remains the food-borne infections and intoxications. It is estimated that every year 241,000 illnesses in United States are reported due to *Staphylococcus aureus* (Scallan *et al.* 2011). Food-borne *S. aureus* illness is present in India, but owing to lack of available database its extent of severity is not known.

S. aureus is highly adaptive bacteria as it can grow in a wide range of temperature (7–48.5°C) (Schmitt *et al.* 1990), pH 4.2-9.3 (Bergdoll 1989) and sodium concentration (up to 15%) hence these characteristics enable it to thrive in a wide variety of foods. *S. aureus* occurs both as commensals on skin, mucous membranes and as environmental contaminants. Milk provides a good media for thriving of bacteria and for production of enterotoxins. *S. aureus* infective dose of *ca.* 10^6 to 10^8 CFU/g or ml of food is enough to produce food poisoning (Johnson *et al*. 1990).

Early and rapid detection of this pathogen in food is important for preventing food related illnesses. Culture-based conventional procedures for the detection of *S. aureus* are laborious and require approximately seven days to identify positive samples. This paved the way for rapid molecular based detection methods such as PCR and qPCR. However, the requirement of trained personnel, operating space, expensive equipment and reagents poses an obstacle for their broad application.Therefore, quest for novel rapid method with less turnaround time further gained importance and a newer method known as loop-mediated isothermal amplification (LAMP) was developed by Notomi *et al.* (2000) which relies on auto cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi *et al.* 2000, Mori *et al.* 2001) using 4 or 6 primers for the target gene amplification. This procedure uses isothermal conditions (60–65°C) for amplification and give results within 1 h. Hence LAMP can also be used for rapid diagnosis of foodborne pathogens in food safety laboratories. The current study was done to optimise the LAMP protocol for *S. aureus* detection in milk targeting thermonuclease (*nuc*) gene and compare it with other rapid detection methods such as PCR and qPCR. Various researchers have proved in their studies that *nuc* gene has potential for the rapid diagnosis of *S. aureus* (Brakstad *et al*. 1992, Martineau *et al.* 1998).

MATERIALS AND METHODS

Bacterial strains: S. aureus strain ATCC 33591 was used for optimization of LAMP. Gram positive strains, viz. *Staphylococcus epidermidis* ATCC 49134, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC

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14506 and Gram negative strains, viz. *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis ATCC 13076 and *Escherichia coli* ATCC 10536 were used as negative control in LAMP, qPCR and PCR for evaluation of its analytical specificity.

All mentioned reference strains were procured from Hi Media Labs Mumbai and were revived as per recommended protocol and stored at –80ºC.

Genomic DNA extraction: Extraction of genomic DNA from *S. aureus* reference strains in BHI broth was done using commercial kit QIAamp DNA mini extraction kit (Qiagen). DNA extraction from *S. aureus* reference strain in milk samples was done by using kit (PowerFood™ Microbial DNA Isolation Kit, MoBIO Laboratories) following manufacturer's guidelines. Conventional or phenol-chloroform-isoamyl alcohol DNA extraction method was followed as per Sambrook and Russel (2000).

In heat shock DNA extraction method, 1 ml of broth or spiked milk from respective dilutions was taken in 2 ml microcentrifuge tube which was centrifuged at 15,000×g for 15 min. Supernatant was discarded and pellet was washed twice in nuclease free water (NFW) by centrifuging at 15,000×g for 2 min. Resuspended pellet in one ml of NFW was boiled in a beaker on hot plate for 10 min and then cooled suddenly on ice for 10 min. After heat-cold treatment, mixture was centrifuged at 15,000×g for 10 min. Supernatant with DNA was collected and stored at 4°C for a week or at –20°C indefinitely.

Optimization of LAMP assay: LAMP protocol was optimised by targeting thermonuclease gene (*nuc*) of the standard strain *S. aureus* ATCC 33591. Three sets of primers (Table 1) were used as described by Wang *et al.* (2015). The LAMP reaction was carried out in a total reaction volume of 25 µl containing 2.5 µl of 10× ThermoPol Reaction Buffer (New England Biolabs, UK), 1 µl of *Bst* DNA polymerase (8 U/µl) (New England Biolabs, UK), 3.5 µl of 10 mM dNTP Mix (Fermentas, Thermo Scientific, Mumbai), 5 µl of 5 M Betaine (Sigma, USA), 1.5 µl of 100 mM $MgSO_4$ (New England Biolabs, UK), 10 pmol forward outer primer (F3) and backward outer primer (B3), 40 pmol forward inner primer (FIP) and backward inner primer (BIP), 20 pmol forward loop primer (LF) and backward loop primer (LB), 1 µl of template DNA and rest nuclease free water to make up the volume.

Table 1. Primers sequences for LAMP, qPCR and PCR

LAMP primer	Sequence	Product Size/ Visualization
nuc-F3	5'-AACAGTATATAGTGCAACTTCAA-3'	Ladder
nuc-B3	5'-CTTTGTCAAACTCGACTTCAA-3'	Pattern
nuc-FIP	5'ATGTCATTGGTTGACCTTTGTACAT-AAATTACATAAAGAACCTGCGA-3'	
nuc-BIP	5'-GTTGATACACCTGAAACAAAGCATC-ATTTTTTTCGTAAATGCACTTGC-3'	
nuc-LF	5'-GTATCACCATCAATCGCTTT-3'	
nuc-LB	5'-GGTGTAGAGAAATATGGTCC-3'	
qPCR primers		
*nuc*F	5'-GCGATTGATGGTGATACGGTT-3'	279 bp
*nuc*R	5'- AGCCAAGCCTTGACGAACTAAAGC-3'	
PCR primers		
*nuc*F	5'-GCGATTGATGGTGATACGGTT-3'	279 bp
*nuc*R	5'-AGCCAAGCCTTGACGAACTAAAGC-3'	

For optimization of time-temperature combination for LAMP, the reaction mixture was incubated for 20, 30, 45 and 60 min in a water bath (Sub Aqua Dual Plus, Grant, UK) at each temperature of 60, 61, 62, 63, 64 and 65°C. Reaction was terminated by heating the reaction mixture to 80°C for 5 min. Positive and negative controls were included in each run, and three-room policy, i.e. one room for extraction of DNA, second for preparation of reaction mixture and third for incubation was followed to prevent cross-contamination.

LAMP amplification was visualized by addition of 1 µl of SYBR green I dye (1:1000) (Invitrogen, USA) to finished LAMP reaction tube and then determined through both visual observations for colour change and an UV florescence assay in gel documentation system (Syngene, USA).The amplified LAMP product was also separated on 1.5% agarose gel in 1× TBE buffer. Positive reaction showed ladder pattern bands and negative reactions had no such pattern.

Optimization of qPCR: For optimization of qPCR, primers (Table 1) targeting *nuc* gene as described by Brakstad (1992) were used. The qPCR amplification was carried out in LightCycler®96 Roche (Germany). A total reaction volume of 25 µl containing 12.5 µl LightCycler® 480 SYBR Green I master mix (Roche, Germany), 1 µl each of 10 pmol/µl of primer set containing forward and reverse primers, 5 µl template and sterilized nuclease free water to make up the reaction volume. The cycling conditions for qPCR included an initial denaturation of DNA at 95°C for 7 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, followed by one cycle of thermal profile/melt curve analysis of qPCR products at 95ºC for 1 min, 55ºC for 30 sec and 95ºC for 30 sec. The result of each reaction was expressed in threshold cycles (Ct). The qPCR software was used for data analyses. Samples which showed amplification before cycle threshold (Ct) of 30 were considered as positive.

Optimization of conventional PCR: All DNA amplification reactions in conventional PCR were performed in Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a pre-heated lid as per protocol optimized in department (Zehra *et al.* 2017).

Comparative sensitivity evaluation of LAMP, qPCR and PCR assays

Evaluation of sensitivity in broth: Freshly grown standard strain culture was diluted 10-fold serially in BHI broth (containing *S. aureus ca* 10^0–10^8 CFU/ml), additionally viable plate count was also done. Genomic DNA was extracted from these dilutions by commercial kit, phenol-chloroform-isoamyl alcohol and heat shock method for

amplification in LAMP, qPCR and PCR.

Evaluation of sensitivity in milk: Retail pasteurized milk samples were collected and re-heated to 63ºC for 30 min. Heated sample was checked for sterility by plating on Baird-Parker Egg yolk Tellurite agar for *Staphylococcus* species and negative milk samples were used for sensitivity study. Freshly grown standard strain culture of *S. aureus* was diluted 10-fold serially in milk for spiking. From each dilution of spiked milk sample, 1 ml from each dilution was used for isolation of DNA by commercial kit, conventional and heat shock method.

Comparative specificity evaluation of LAMP, qPCR and PCR assays

Evaluation of specificity in broth: Different strains of Gram positive and Gram-negative bacteria were revived. A dilution tube containing *ca.* 10^4 CFU/ml dilution was subjected to genomic DNA extraction by kit method and LAMP, qPCR and PCR were done to analyse analytical specificity.

Evaluation of specificity in milk: Genomic DNA was extracted from milk samples spiked with *ca.* 10^5 CFU/ml of standard strains of Gram positive and Gram-negative bacteria in individual experiment for each standard strain. Extracted DNA from spiked milk samples was subjected to all 3 assays to analyse specificity.

Application of optimized LAMP protocol for rapid detection of S. aureus *in field milk samples*

Approximately 126 raw milk samples were collected from dairy farms, retail market and clinical cases reported in Teaching Veterinary Clinical Services Complex, Guru Angad Dev Veterinary and Animal Sciences University. About 20 ml of each milk sample was collected aseptically in a sterile container and transported on ice to the laboratory for immediate processing or stored at 4ºC; and were processed within 2 to 3 h of collection. Collected milk samples were processed for isolation of *S. aureus* by conventional isolation procedures and were subjected to species identification by conventional PCR. Same milk samples which were used for isolation were also processed for DNA extraction by using kit and extracted DNA was subjected to LAMP.

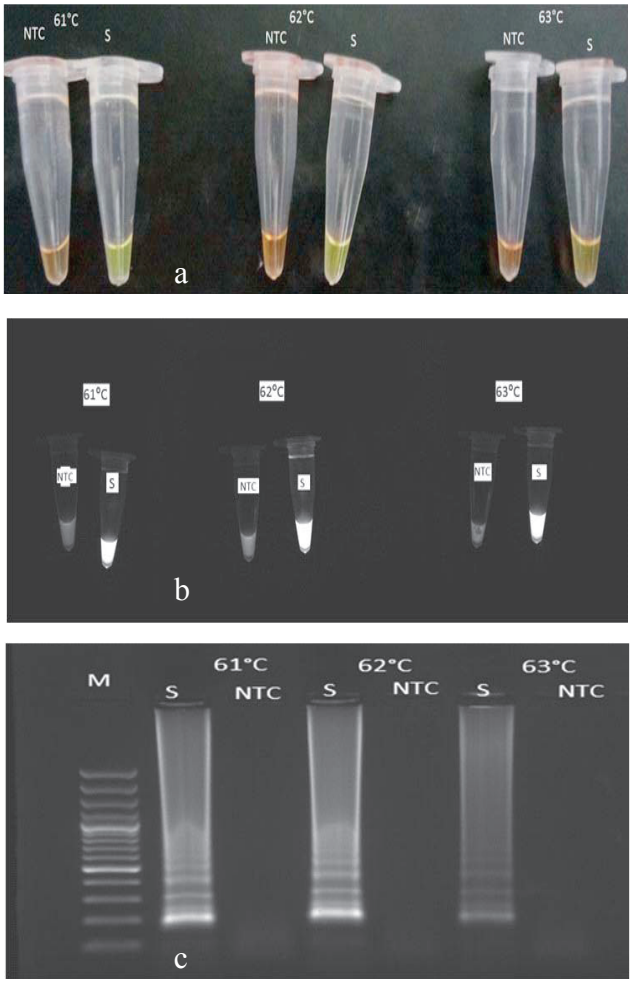

Fig. 1(A-C). **A**. Colour change following addition of 1 µl of SYBR green I dye to the tube. **B.** Fluorescence under UV illuminator. **C**. Gel electrophoresis of optimized LAMP. NTC, No template control; S, standard.

The milk samples which showed positive results by culture and negative results by LAMP were subjected to enrichment. The enriched samples were again subjected to LAMP. Comparison of detection sensitivity and specificity between culture method and LAMP was done.

Table 2. Comparative detection sensitivity of LAMP, qPCR and PCR assay in broth

DNA extraction method	Detection limit (*ca.* CFU/ml)			Detection time (excluding DNA extraction method)			Time for DNA extraction	Total detection time[1]		
	LAMP	qPCR	PCR	LAMP	qPCR	PCR		LAMP	qPCR	PCR
Kit method (QIAmp DNA mini extraction kit, Qiagen)	10^2	10^2	10^3				1 h 20 min	2 h 10 min	3 h 30 min	3 h 20 min
Conventional method (Phenol-chloform-isoamyl alcohol method)	10^2	10^2	10^3	50 min	2 h 10 min	2 h	15 h	15 h 50 min	17 h 10 min	17 h
Heat shock method	10^3	10^3	10^4				30 min	1 h 20 min	2 h 40 min	2 h 30 min

[1]Detection time including time for DNA extraction

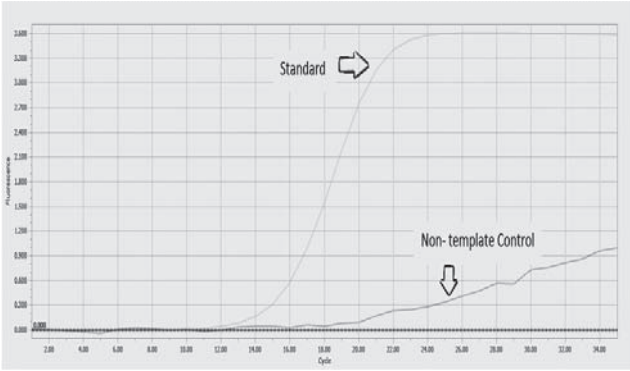

Fig. 2. Real time optimization- standard curve.

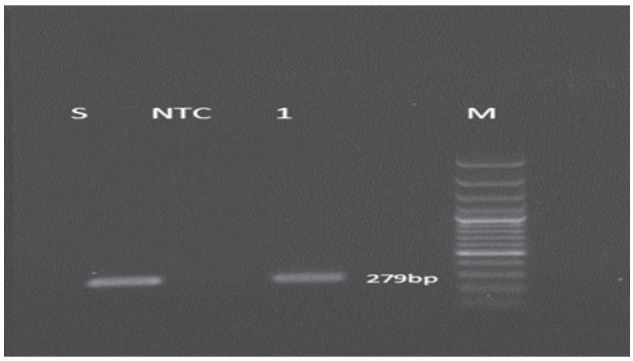

Fig. 3. Optimization of conventional PCR. Lane M, gene ruler; lanes S, 1, standard strain *S. aureus;* NTC, no template control.

RESULTS AND DISCUSSION

Optimization of LAMP reaction conditions and analysis: For optimizing LAMP protocol, reaction mixture was subjected to temperatures ranging from 60 to 65ºC with 1ºC increment. Reaction at each temperature was also subjected to amplification for different time durations such as 20, 30, 45 and 60 min each. Isothermal amplification was carried out in water bath. Optimal amplification time-temperature combination was found to be 62ºC for 45 min (Figs 1A, 1B, 1C).

Wang (2015) optimized LAMP for rapid detection of *S. aureus* by targeting *nuc* gene at time-temperature combination of 62ºC for 60 min. Difference in isothermal amplification temperature and amplification time in the studies could be due to difference in instrument's efficiency and lab to lab variations.

Optimization of qPCR: Quantitative PCR was optimized using SYBR Green qPCR.This optimized protocol showed real time amplification in the form of amplification curve (C_t) (Fig. 2). After the end of the amplification cycle, amplified product was subjected to melt curve analysis which showed melting at 82ºC. For further confirmation, product was also separated on 1.5% agarose gel for 45 min and an amplicon of 279 bp was observed.

Optimization of conventional PCR: Optimized conventional PCR yielded amplicon of 279 bp (Fig. 3).

Comparative sensitivity evaluation of LAMP, qPCR and PCR assay from broth and spiked milk samples: Effect of different DNA extraction methods on analytical sensitivity of LAMP, qPCR and PCR was also studied. The results of comparative sensitivity evaluation of LAMP, qPCR and PCR assay from broth and milk are given in Tables 2 and 3, respectively.

As per the results, LAMP and qPCR were equally sensitive with detection limit of *ca.* 10^2 CFU/ml from broth when kit and conventional methods of DNA extraction were used as compared to a detection limit of *ca.* 10^3 CFU/ml in broth with conventional PCR using same DNA extraction methods (Table 2) (Fig. 4). When heat shock method of DNA extraction was used, sensitivity of LAMP, qPCR and PCR reduced further with a detection limit of *ca.* 10^3 CFU/ml from broth for LAMP and qPCR and *ca.* 10^4 CFU/ml for conventional PCR (Fig. 4). Taking into consideration time for detection of *S. aureus* from broth using all 3 methods, LAMP was quicker with detection time of 50 min excluding time for DNA extraction, whereas qPCR and PCR took 2 h or more for detection.

Application of LAMP, qPCR and PCR for rapid detection of *S. aureus* in spiked milk sample also showed LAMP to be sensitive method of detection with a detection limit of *ca.* 10^2, 10^3 and 10^4 CFU/ml using kit, conventional and heat shock methods of DNA extraction, respectively (Fig. 5). Here also LAMP performed equally well in comparison to qPCR (Table 3). When detection limit was

Table 3. Comparative detection sensitivity of LAMP, qPCR and PCR assay in spiked milk sample

DNA extraction method	Detection limit (*ca.* CFU/ml)			Detection time (excluding DNA extraction time)			Time for DNA extraction	Total detection time[1]		
	LAMP	qPCR	PCR	LAMP	qPCR	PCR		LAMP	qPCR	PCR
Kit method (PowerFood™ Microbial DNA Isolation Kit method)	10^2	10^2	10^4				45 min	1 h 35 min	2 h 55 min	2 h 45 min
Conventional method (Phenol-chloform-isoamyl alcohol method)	10^3	10^3	10^5	50 min	2 h 10 min	2 h	15 h	15 h 50 min	17 h 10 min	17 h
Heat shock method	10^4	10^4	10^6				30 min	1 h 20 min	2 h 40 min	2 h 30 min

[1]Detection time including time for DNA extraction

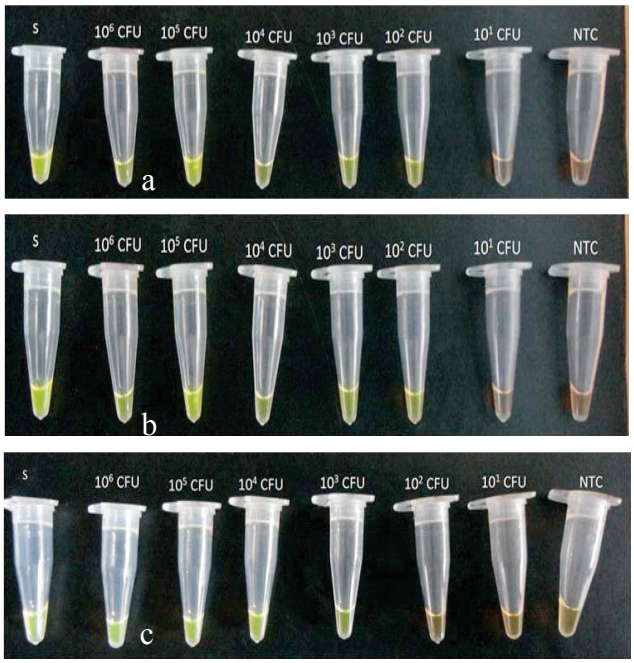

Fig. 4. Sensitivity evaluation of LAMP from broth by kit (**A**), conventional method/phenol-chloform-isoamyl alcohol (**B**), heat shock method (**C**) of DNA extraction. NTC, No template control; S, standard.

combined with time for DNA extraction, LAMP was able to detect *S. aureus* rapidly from milk in 1 h 35 min and 1 h 20 min using kit and heat shock methods of DNA extraction, respectively.

If we normalize amount of eluted DNA aliquot used for LAMP, qPCR and PCR to 1µl taking into consideration the amount (1 ml) of broth and spiked milk sample used for DNA extraction, LAMP and qPCR were equally sensitive in detecting *S. aureus* both in broth and spiked milk with a detection limit of *ca.* 0.1 CFU/ml (Table 4). Whereas, conventional PCR could detect one and 10 CFU/ml from broth and spiked milk, respectively. Based on detection time, LAMP was rapid in detecting *S. aureus* from broth and milk samples with detection time of 50 min (Tables 2, 3).

Comparing our results with the studies published earlier regarding rapid detection of *S. aureus* using LAMP and PCR, there also LAMP had been reported to be sensitive method of detection (Xu *et al.* 2012, Lim *et al.* 2013, Zhao

Table 4. Comparative sensitivity evaluation of LAMP, qPCR and PCR assay from broth and spiked milk samples

DNA extraction method	Detection limit (*ca.* CFU/ml)*					
	Broth			Spiked milk		
	LAMP	qPCR	PCR	LAMP	qPCR	PCR
Kit method	0.1	0.1	1.0	0.1	0.1	10.0
Conventional method (Phenol- chloform-isoamyl alcohol method)	0.1	0.1	1.0	1.0	1.0	100.0
Heat shock method	1.0	1.0	10.0	10.0	10.0	1000.0

*Detection limit based on if amount of eluted DNA aliquot used for LAMP, qPCR and PCR is normalized to 1 µl.

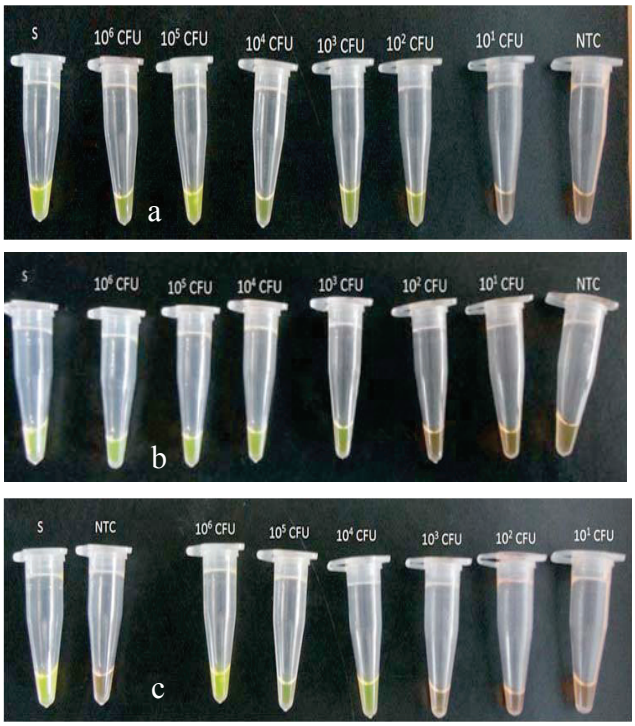

Fig. 5. Sensitivity evaluation of LAMP from milk by kit (**A**), conventional method/phenol-chloform-isoamyl alcohol (**B**), heat shock method (**C**) of DNA extraction method. NTC, No template control; S, standard.

et al. 2013). Lim *et al.* (2013) reported detection limit of *S. aureus* as 10^2 and 10^3 CFU/ml for LAMP and conventional PCR, respectively. Xu *et al.* (2012) in their study reported a detection limit of 10^1 and 10^3 CFU/amplification reaction for LAMP and PCR, respectively. Application of LAMP for detection of enterotoxigenic *S. aureus* in raw milk, *burfi* and *khoa* samples recorded sensitivity of 10^2 CFU/ml or g of food sample taking into consideration amount of food sample processed for DNA extraction (Sowmya *et al.* 2012). Detection limit of qPCR had also been reported in the range of 10^1–10^2 CFU/ml in various studies (Hein *et al.* 2001). Quigley *et al.* (2012) reported that DNA extraction done by PowerFood™ Microbial DNA Isolation Kit, MoBIO Laboratories had a detection sensitivity of 10^1 CFU/ml using qPCR.

Comparative specificity evaluation of LAMP, qPCR and PCR assays from broth and spiked milk sample: Detection by LAMP, qPCR and PCR assay both in broth and spiked milk sample with standard strains of Gram positive and Gram negative organisms for rapid detection of *S. aureus* showed all three detection methods to be 100% analytically specific with no false positive result (Figs 6A-C). Thermonuclease gene (*nuc*) was highly specific hence, can be used for rapid detection purposes and results were in concordance with previous scientific findings (Brakstad *et al*. 1992, Martineau *et al.* 1998). Detection specificity results were in agreement with findings published in scientific literature (Xu *et al.* 2012, Lim *et al.* 2013, Zhao *et al.* 2013).

Application of optimized LAMP protocol for rapid detection of S. aureus *in field milk samples:* Out of 126

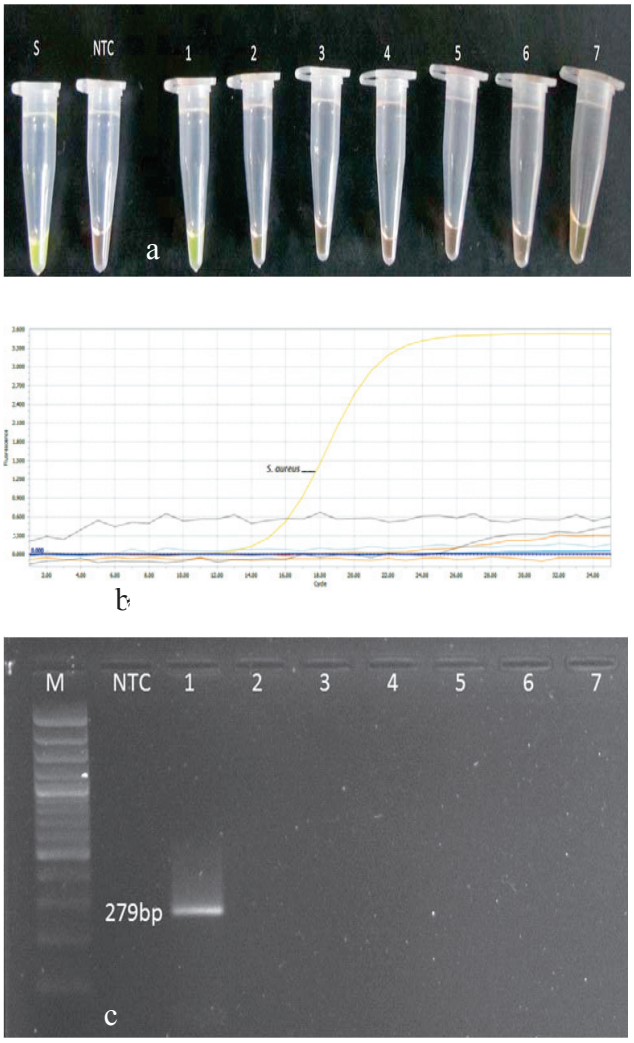

Fig. 6(A-C). **A**. Specificity evaluation of LAMP from broth. **B**. Comparative specificity evaluation of LAMP, qPCR and PCR assays from broth. **C**. Gel electrophoresis of PCR of *nuc* gene. S, Standard strain *S. aureus*; NTC, No template control; 1, *S. aureus*; 2, *Staphylococcus epidermidis;* 3, *Enterococcus faecalis*; 4, *Listeria monocytogenes*; 5, *Salmonella* Typhimurium; 6, *Salmonella* Enteritidis; 7, *Escherichia coli.*

samples, 97 were positive for *S. aureus* and 29 negative by conventional culture method. When these positive samples were subjected to detection of *S. aureus* by optimized LAMP protocol, LAMP detected 93 milk samples positive for *S. aureus,* hence giving detection sensitivity of 95.87% in comparison to gold standard culture method (Table 5). Four samples, which were negative by LAMP directly returned positive after enrichment, hence giving 100% detection sensitivity when coupled with 4 h enrichment step. All the 29 milk samples negative by culture method were also negative by LAMP, thereby giving detection specificity of 100%. Zhao *et al.* (2013) in their study reported sensitivity of LAMP assay to be 98.4% for the detection of *S. aureus* from clinical and food samples. Similarly, Xu *et al.* (2012) reported detection sensitivity of LAMP assay ranging from 100% to 98.5% depending upon genes of *S. aureus* targeted.The lower detection sensitivity observed in this

Table 5. Comparison of *S. aureus* isolation results and LAMP results

Test status		True status	
		S. aureus positive	*S. aureus* negative
Before enrichment	LAMP positive	93	0
	LAMP negative	4	29
Total		97	29
After enrichment	LAMP positive	97	0
	LAMP negative	0	29
Total		97	29

study was due to the presence of *S. aureus* in milk below the detection limit of 10^2 CFU/ml for LAMP as counts in those four milk samples ranged from 10 to 80 CFU/ml.

LAMP assay was found to be analytically sensitive, specific and rapid for detecting *nuc* gene as compared to quantitative and conventional PCR assays. It can be carried out in less sophisticated laboratories. Therefore, it can be a promising alternative method for the rapid identifiction of *S. aureus* and could be used in resource-limited laboratories and field conditions.

A loop mediated isothermal amplification (LAMP) method for rapid detection of *S. aureus* from milk using standard strain ATCC 33591 was optimized at 62°C for 50 min in water bath targeting thermonuclease (*nuc*) gene. The analytical sensitivity of LAMP and qPCR was found to be equal (*ca.*10^2 CFU/ml) both in broth and spiked milk samples, while sensitivity of conventional PCR was low (*ca.* 10^3 and 10^4 CFU/ml in broth and spiked milk samples, respectively) using kit method of DNA extraction. LAMP was 100% specific and in complete agreement with real time and conventional PCR. Application of LAMP for rapid detection of *S. aureus* on 126 field milk samples gave sensitivity of 95.87% and specificity of 100%. Enrichment of the milk sample for four hours enhanced the sensitivity of detection to 100% and detection limit to < 10^2 CFU/ml.

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