EVALUATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD FOR DETECTING ENTEROTOXIN A GENE OF *STAPHYLOCOCCUS AUREUS* IN PORK

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Abstract. *Staphylococcus aureus* is an important food-borne pathogen of humans due to ingestion of food containing enterotoxigenic strains. We evaluated the detection of *S. aureus* enterotoxin A gene (*sea*) using a loop-mediated isothermal DNA amplification (LAMP) method. Specificity of LAMP method was comparable to that of conventional PCR. Both methods correctly identified *sea* and did not detect 14 other non-*Staphylococcus* strains. Limit of detections of LAMP assay for direct detection of *S. aureus* was 10^4 cfu/ml while that of PCR was 10^3 cfu/ml. Both methods were employed for the detection of *S. aureus* spiked pork samples to assess their sensitivity. LAMP method was able to detect a minimum of 10^3 cfu/g while PCR 10^4 cfu/g of pork sample following incubation in tryptic soy broth for 6 hours. The LAMP assay allows a one-step identification of gene of interest without any specialized equipment and requires less time than conventional PCR, thus suggesting that the LAMP technique might be an appropriate alternative diagnostic method for detecting enterotoxin A gene of *S. aureus* in food sample.

Keywords: *Staphylococcus aureus*, loop-mediated isothermal amplification (LAMP), *sea* gene, pork sample

INTRODUCTION

*Staphylococcus aureu*s is often found closely associated with the human body, but may also be found in many parts of our environment, including dust, water, air and feces and on clothing (Bergdoll, 1983). Many healthy people carry *S. aureus* as part of the normal microflora of micro-organisms associated with nose, throat and skin, with the nasal passage reported to harbor *S. aureus* in 10-50% of the healthy population (Genigeorgis, 1989).

S. aureus also causes infection that compromises food safety. It produces a wide variety of toxic proteins such as toxic shock syndrome toxin 1, exfoliative toxins, and enterotoxins (SEs), which

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can cause staphylococcal food poisoning (SFP) (Becker *et al*, 2003). SFP is a typical intoxication due to ingestion of food contaminated with enterotoxigenic strains, even with small amounts of SEs. SEs are difficult to destroy by heating, so the usual heat treatments used to cook food and destroy bacterial cells cannot inactivate those toxins (Hilker *et al*, 1968). About 100-200 ng of SEs produced by $> 10^5$ cfu/g of *S. aureus* can cause SFP in healthy adults (Evenson *et al*, 1988). Among SEs, enterotoxin A (SEA) is the most common toxin implicated in SFP (Balaban and Rasooly, 2000), and has been identified as the causative agent in numerous outbreaks of food poisoning (Asao *et al*, 2003). As meat and meat products present an ideal substrate supporting their growth, the presence of spoilage and pathogenic bacteria in raw meat is the result of contamination from skin of animals, equipments, employees and environment (Mataragas *et al*, 2008).

Various rapid detection methods have been developed for detection of enterotoxins and their genes from *S. aureus*. Polymerase chain reaction (PCR) was proved to be a useful tool for rapid and reliable detection of such genes (Tamaparu *et al*, 2001; Ercolini *et al*, 2004). However, this assay requires electrophoresis to detect amplified DNA products, which is a time-consuming protocol. Although real-time PCR assay has been applied to rapidly detect enterotoxigenic *S. aureus* this method requires expensive equipment (Klotz *et al*, 2003).

Notomi *et al* (2000) have developed a loop-mediated isothermal amplification (LAMP) method, which is capable of amplifying DNA under isothermal conditions (60ºC - 65ºC) with high specificity, efficiency and speed. This method depends on an autocycling strand displacement DNA synthesis performed by *Bst* DNA polymerase large fragment and requires a set of 4 oligonucleotide primers recognizing 6 regions. The LAMP reaction can be monitored in real-time through measurement of turbidity, which is correlated with the production of magnesium pyrophosphate, by means of an inexpensive photometer (Mori *et al*, 2001). The amplification efficiency of LAMP assay is extremely high because there is no time loss through thermal changes, as in PCR due to its isothermal reaction. Moreover, Nagamine *et al* (2002) had advanced the method by including forward loop primers (LF and LB) that accelerate the LAMP reaction.

LAMP method has been used to detect 4 classical enterotoxins genes of *S. aureus* strains isolated from food and clinical samples (Goto *et al*, 2007). The LAMP assay has the same sensitivity but is more time-efficient than the PCR assay (at 35 cycles). However the study did not examine sensitivity using food samples. Thus, in the present study we compared the sensitivity of the LAMP assay with that of conventional PCR using spiked pork samples for possible application in food industry.

MATERIALS AND METHODS

Bacterial strains and DNA preparation

A total of 17 bacterial strains were acquired either from Department of Microbiology, Faculty of Science, Chulalongkorn University; Department of Microbiology, Faculty of Public Health, Mahidol University; or The National Institute of Health, Thailand (Table 1). All bacterial strains were cultured on tryptic soy broth (TSB) medium (Difco, Becton Dickinson, Franklin, NJ) at 37ºC for 18-24 hours. *S. aureus* ATCC 13565 carrying enterotoxin A gene (*sea*) was used as positive control.

Table 1
Bacterial species and strains used in the study.

Bacterial strain	Source
Staphylococcus aureus ATCC 13565 (*sea*) *Staphylococcus aureus* ATCC 14458 *Staphylococcus aureus* ATCC 25923 *Staphylococcus epidermidis* ATCC12228	The National Institute of Health, Thailand
Streptococcus agalactiae DMST 16992 *Listeria monocytogenes* *Enterococcus faecalis* *Escherichia coli* ATCC 25922 *Enterobacter aerogenes* ATCC 13048 Enterotoxigenic *E. coli* *Vibrio parahaemolyticus*	Department of Microbiology, Faculty of Public Health, Mahidol University
Micrococcus luteus MSCU0350 *Yersinia enterocolitica* ATCC27729 *Klebsiella pneumoniae* *Salmonella* Typhimurium ATCC 13311 *Pseudomonas aeruginosa* MSCU0359 *Serratia marcescens*	Department of Microbiology, Faculty of Science, Chulalongkorn University

A single colony of a bacterial strain was cultured for 18-24 hours in 5 ml of TSB, and then 1 ml aliquot was centrifuged at 13,200*g* for 10 minutes at 4ºC. Bacterial cell pellet was suspended in 100 l of Tris-EDTA (TE) and heated at 95ºC for 15 minutes. Following centrifugation at 13,200*g* for 10 minutes, the supernatant was used as source of DNA template for LAMP and PCR assays (Reischl *et al*, 2008).

LAMP assay

LAMP assay for the detection of *sea* was performed using a set of five primers, FIP, BIP, F3, B3 and LF, as previously described (Goto *et al*, 2007) (Table 2). LAMP reaction was carried out in 25 l reaction mixture containing 1.6 M each of inner primers (FIP and BIP), 0.2 M/l each of outer primers (F3 and B3), 0.8 M of loop primer (LB), 1.4 mM of dNTPs, 0.5 M betain (Sigma, St Louis, MO), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.2% Tween 20, 8 U *Bst* polymerase large fragment (New England Biolabs, Ipswich, MA), and 1 l of sample DNA. Negative control contained no added DNA, and positive control contained DNA of *S. aureus* ATCC 13565 containing *sea*. The reaction mixture was incubated at 60ºC for 60 minutes and reaction terminated at 80ºC for 2 minutes. Positive and negative results were distinguished by observing white turbidity of the reaction mixture or the LAMP products were subjected to electrophoresis in 2.0% agarose gel, stained with ethidium bromide and visualized under UV light.

PCR

Primers used for PCR amplification of *sea* were as previously described (Johnson *et al*, 1991) (Table 2). PCR amplification was performed in a 50 l reaction mixture

Table 2
Base sequences of primers used for staphylococcal toxin A-specific detection by LAMP and PCR.

	Primer sequence (5′-3′)
Primer (LAMP)	
FIP	5′-GAT CCA ACT CCT GAA CAG TTA CAA TAC AGT ACC TTT GGA AAC G-3′
BIP	5′-CTG ATG TTT TTG ATG GGA AGG TTC CCG AAG GTT CTG TAG AAG T-3′
F3	5′-TCA ATT TAT GGC TAG ACG GT-3′
B3	5′-CTT GAG CAC CAA ATA AAT CG-3′
LB	5′- AGA GGG GAT TAA TCG TGT TTC A -3′
Primer (PCR)	
SEA-1F	5′-TTGGAAACGGTTAAAACGAA-3′
SEA-2R	5′-GAACCTTCCCATCAAAAACA-3′

Table 3
Cell concentration of *S. aureus* (cfu/g) used in spiking pork samples.

Incubation time (h)	*S. aureus* cell concentration (cfu/g)					
	10^6	10^5	10^4	10^3	10^2	0
0	6.11	5.48	4.56	3.60	2.15	0
2	6.63	5.89	4.62	3.77	2.38	0
4	7.43	7.04	6.26	4.52	3.55	0
6	9.08	8.04	7.27	6.48	4.00	0

containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM of each primer, 0.2 mM dNTPs, 2.5 U TaKaRa *Taq* DNA polymerase (Takara Bio, Shiga, Japan) and 1 l of template DNA. Thermocycling conditions in a thermal cycler (Applied Biosystem, Carlsbad, CA) were as follows : 94°C for 2 minutes; 35 cycles of 94°C for 2 minutes, 55°C for 2 minutes and 72°C for 1 minute; with a final step of 72°C for 7 minutes. PCR amplicons were analyzed by 2% agarose gel-electrophoresis, staining with ethidium bromide and observation under UV light.

Determination of detection limit of LAMP and PCR assays using bacterial culture

S. aureus ATCC 13565 was cultured in TSB at 37°C for 18 hours and a series of 10-fold dilution was conducted in normal saline solution. Cell suspensions were centrifuged at 13,200*g* for 10 minutes. Cell pellets were resuspended in 100 l of Tris-EDTA and subjected to DNA extraction for LAMP and PCR assays as described above. To confirm the inoculum size, 0.1 ml aliquot of each dilution of cell culture was plated onto Baird-Parker agar (BPA) in duplicate. After incubation at 37°C

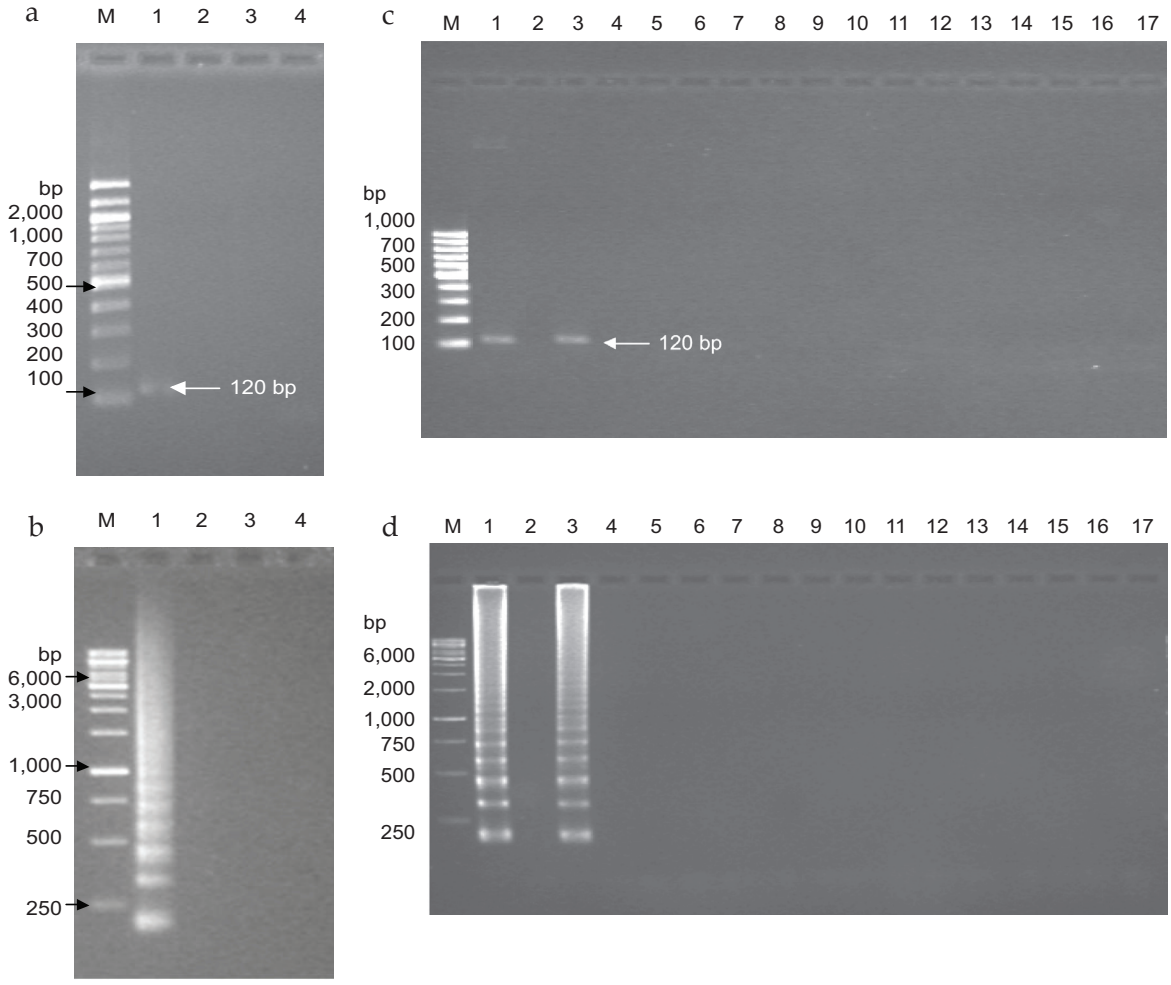

Fig1–Agarose gel-electrophoresis of PCR and LAMP products of *S. aureus* . Primer used are listed in Table 2. (a) PCR amplicons of *sea*. Lane M, 100 bp DNA ladder; lane 1, *S. aureus* ATCC 13565 ; lane 2, *S. aureus* ATCC 14458; lane 3, *S aureus* ATCC 25923; lane 4, negative control. (b) LAMP products of *sea*. Lane M, 1 Kb DNA ladder; lane 1, *S. aureus* ATCC 13565 ; lane 2, *S. aureus* ATCC 14458; lane 3, *S. aureus* ATCC 25923; lane 4, negative control. (c) PCR amplicons of *sea*. Lane M, 100 bp DNA ladder; lane 1, positive control; lane 2, negative control; lane 3, *S. aureus* ATCC 13565; lane 4, *S. epidermidis* ATCC 12228; lane 5, *S. agalactiae* DMST 16992; lane 6, *L. monocytogenes*; lane 7, *E. faecalis*; lane 8, *E. coli* ATCC 25922; lane 9, *E. aerogenes* ATCC 13048 ; lane 10, enterotoxigenic *E.coli*; lane 11, *V. parahaemolyticus*; lane 12, *M. luteus* MSCU 0350; lane 13, *Y. enterocolitica* ATCC 27729; lane 14, *K. pneumoniae* ; lane 15, *S.* Typhimurium ATCC13311; lane 16, *P. aeruginosa* MSCU 0359 ; lane 17, *S. marcescens*; (d) LAMP products of *sea*. Lane M, 1 Kb DNA ladder; lane 1, positive control; lane 2, negative control; lane 3, *S. aureus* ATCC 13565; lane 4, *S. epidermidis* ATCC 12228; lane 5, *S. agalactiae* DMST 16992; lane 6, *L. monocytogenes*; lane 7, *E. faecalis* ; lane 8, *E. coli* ATCC 25922; lane 9, *E. aerogenes* ATCC 13048 ; lane 10, enterotoxigenic *E.coli*; lane 11,*V. parahaemolyticus*; lane 12, *M. luteus* MSCU 0350; lane 13, *Y. enterocolitica* ATCC 27729; lane 14, *K. pneumoniae* ; lane 15, *S.* Typhimurium ATCC 13311; lane 16, *P. aeruginosa* MSCU 0359 ; lane 17, *S. marcescens.*

for 24 hours, the numbers of black shiny colonies with clear zones around the colonies were counted.

Determination of detection limit of LAMP and PCR assays using *S. aureus*-spiked pork samples

Pork was purchased from a local supermarket. The sample was determined to be negative for *S. aureus* by microbiological examination in BPA. The pork sample then was cut into small pieces (25 g) using sterile technique. Serial 10-fold dilution of *S. aureus* ATCC 13565 cells were prepared ranging from 10^9 cfu/ml to 10^5 cfu/ml in normal saline solutions. A 100 l aliquot of various cell suspensions were spiked into the pork samples. A 225 ml aliquot of TSB was added to 25 g of pork sample, which was homogenized using a stomacher (IUL Instruments, Barcelona, Spain) for 2 minutes and then incubated at 37°C for 0, 2, 4 and 6 hours. Then 1 ml of the cultured sample was removed for DNA extraction for LAMP and PCR assays as described above. To confirm the inoculum size of the spiking cell samples, 0.1 ml aliquot of each cell suspension was plated onto BPA, incubated at 37°C for 24 hours in duplicate and colonies identified as described above.

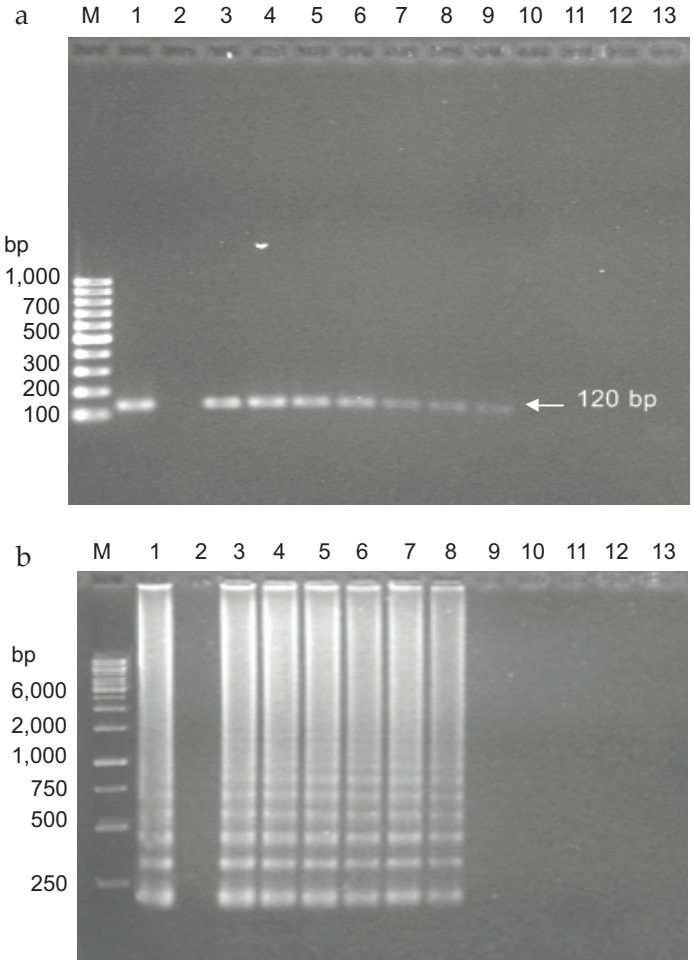

Fig 2–Agarose gel-eletrophoresis of PCR (a) and LAMP (b) of *sea* products from various concentrations of *S. aureus* ATCC 13565. Lane M, 100 bp DNA ladder; lane 1, positive control; lane 2, negative control; lane 3, 10^9 cfu/ml; lane 4, 10^8 cfu/ml; lane 5, 10^7 cfu/ml; lane 6, 10^6 cfu/ml; lane 7, 10^5 cfu/ml; lane 8, 10^4 cfu/ml; lane 9, 10^3 cfu/ml; lane 10, 10^2 cfu/ml; lane 11, 10 cfu/ml; lane 12, 1 cfu/ml; and lane 13, 0 cfu/ml).

RESULTS

A total of 17 bacterial strains were included in the evaluation of the specificity of LAMP and PCR assays to detect enterotoxin A gene (*sea*) of *S. aureus*. In LAMP assay, positive and negative results were distinguished by observing the white turbidity of the reaction mixture. LAMP products formed a ladder-like pattern

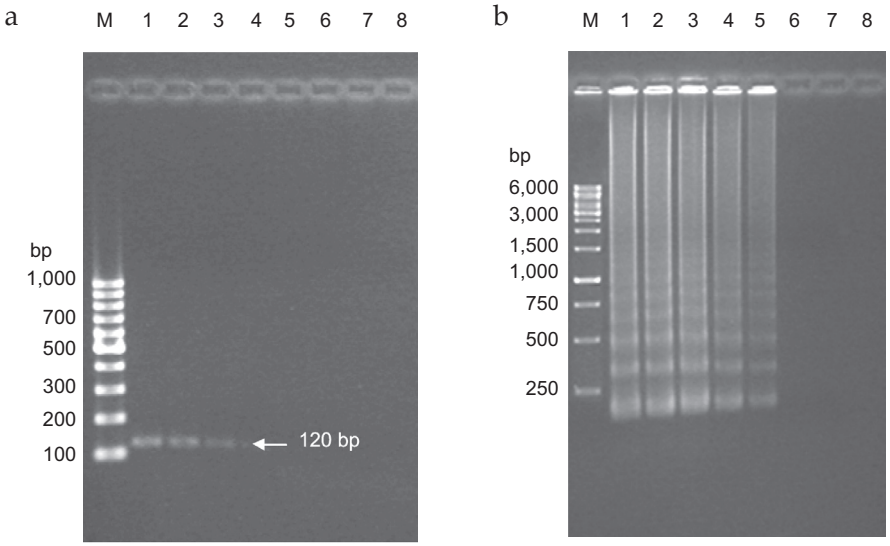

Fig 3–Agarose gel-eletrophoresis of PCR (a) and LAMP (b) of *sea* products in pork samples spiked with *S. aureus* ATCC 13565.Cells were incubated in TSB broth for 6 hours. Primers used are listed in Table 2. Lane M (a), 100 bp DNA ladder; lane M (b), 1 kb DNA ladder; lane 1, positive control [*S. aureus* (*sea*) ATCC 13565 10^6 cfu/g]; lane 2,10^6 cfu/g; lane 3, 10^5 cfu/g; lane 4, 10^4 -cfu/g; lane 5, 10^3 cfu/g; lane 6, 10^2 cfu/g; lane 7, 10 cfu/g; and lane 8, negative control.

upon electrophoresis. Both LAMP and PCR method correctly identified *sea* of *S. aureus* ATCC 13565 and did not detect the other 14 non-*Staphylococcus* strains. Results from gel-electrophoresis analyses are shown in Fig1. Sensitivity of LAMP assay for *sea* detection was 10^4 cfu/ml, while that of PCR was 10^3 cfu/ml (Fig 2). For detection of *S. aureus* in spiked pork samples, LAMP was able to detect 10^3 cfu/g of pork sample while PCR could detect only 10^4 cfu/g of pork sample after incubation in TSB for 6 hours (Fig 3).

DISCUSSION

Sensitivity of the LAMP assay in this study was approximately 10-fold lower than in their original study (Goto *et al*, 2007). It may be due to our simpler DNA preparation method that excluded the incubation step with achromopeptidase. LAMP method was 10-fold more sensitive than PCR for the detection of *sea* in *S. aureus* spiked pork sample. The less sensivity of PCR than the LAMP method may be due to the presence of PCR inhibitors in the food samples, such as lipid and protein components. Kaneko *et al* (2007), Karanis *et al* (2007) and Okada *et al* (2010) have reported that PCR is less sensitive than LAMP method owing to the presence of inhibitors in water samples and clinical specimens, such as rectal swab.

A short incubation of the enrichment culture usually ensures detection in the case of nonuniform distribution of pathogens in food products or of a low microbial load. However, the increase in levels of *S. aureus* after 6 hour incubation was not significantly changed (Table 4). In a real situation, the target is sometimes killed or damaged by cooking and food processing, but the enterotoxin produced

by the *S. aureus* still retains biological activity, which is a hazard for consumers. In such cases, we cannot expect to increase the number of target by enrichment culture. Thus, LAMP method is potentially useful for detection of *S. aureus* carrying *sea* in food samples in the food industry and outbreak investigation.

This is the first report of LAMP assay applied for detection of entertoxin A gene of *S. aureus* in spiked raw meat. Further studies are needed to determine the usefulness of LAMP assay for detection of enterotoxigenic A gene of *S. aureus* in actual food samples.

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