A multiplex loop-mediated isothermal amplification assay for rapid detection of *Bacillus cereus* and *Staphylococcus aureus*

Yanglong Deng[1], Yanquan Liu[2], Zubin Jiang[3], Jingxin Wang[4], Qing Zhang[1], Yeqian Qian[1], Yuan Yuan[1], Xiangyu Zhou[1], Guiling Fan[1], Yufeng Li[1,*]

[1] *School of Food and Bioengineering, Xihua University, Chengdu, Sichuan, China;*
[2] *College of Life and Geographic Sciences, Kashi University, Kashi, Xinjiang, China;*
[3] *Culinary school, Sichuan Tourism University, Chengdu, Sichuan, China;*
[4] *School of Materials and Environmental Protection, Chengdu Textile College, Chengdu, Sichuan, China.*

Summary   ***Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) are major human food-borne pathogens that may produce a variety of toxins and cause diarrhea, food poisoning, and even death. In order to monitor and prevent the spread of these pathogens, a multiplex loop-mediated isothermal amplification (multi-LAMP) assay was developed to simultaneously and rapidly detect *B. cereus* and *S. aureus*. The sensitivity and specificity of the loop-mediated isothermal amplification (LAMP) reactions were determined *via* electrophoresis. The multi-LAMP showed 100% inclusivity and exclusivity, the sensitivity was 10 fg/μL and was 10 times more sensitive than that of polymerase chain reaction (PCR), the results were consistent with those of conventional PCR assay, and the entire assay should be finished within 40 min. This multi-LAMP assay was confirmed as a rapid and reliable diagnostic technique upon application for clinical samples and food samples. To our knowledge, this is the first study to report the application of multi-LAMP to detect *B. cereus* and *S. aureus*.**

Keywords: Multi-LAMP, *Bacillus cereus*, *Staphylococcus aureus*, pathogen, rapid detection

1. Introduction

Bacillus cereus (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) are the most common causes of foodborne pathogens in developed and developing countries. In the United States, during 2009-2015, Foodborne Disease Outbreak Surveillance System (FDOSS) received reports of 5,760 outbreaks that resulted in 100,939 illnesses, 5,699 hospitalizations, and 145 deaths (*1*). 1,229 food-borne outbreaks caused by *B. cereus* and *S. aureus* were reported; 39% were reported with a confirmed etiology. Vomiting was commonly reported in *B. cereus* (median, 75% of cases) and *S. aureus* outbreaks (median, 87%) (*2*). At the same time, China, Japan, South Korea and other

countries had also reported the outbreak of foodborne epidemics (*3,4*). Rice or soy dishes were commonly implicated in *B. cereus* (50%) outbreaks, and meat or poultry dishes were commonly implicated in *S. aureus* (55%) outbreaks (*5*). These pathogens cause illness through preformed toxin production in improperly handled foods or in vivo toxin production within the gastrointestinal tract after consumption of a contaminated food (*6,7*). Therefore, the development of a rapid and ready-to-use method for simultaneous detection of these pathogens is of great importance to improve food safety and protect human health.

Many methods have been developed to detect *B. cereus* and *S. aureus*, including convention culture-based, immunology-based, and molecular methods (*8,9*). Conventional culture-based methods are safe and simple and Cost efficiency, but time-consuming. Immunology-based assay, including enzyme-linked immunosorbent and immunofluorescence, are fast but not very effective in terms of sensitivity (*10*). Molecular methods such as PCR and nucleic acid probe technology, Which are rapidity, sensitivity and

specificity, however, the instruments used in these methods are expensive and profession (*11,12*).

The LAMP technique is a novel constant-temperature nucleic acid amplification technique invented by Japanese scholars in 2000 with high sensitivity, specificity, and rapidity for the low-cost detection of pathogens (*13-17*). Song found that a loop-mediated isothermal amplification (LAMP) method for detecting *Shigella* and enteroinvasive *Escherichia coli*, the LAMP method efficiently detected the gene within 2 h at a minimal amount of bacteria (8 CFU) per reaction (*18*). Wan selected the heat-labile enterotoxin gene to design LAMP primers, optimized the reaction conditions of LAMP, and examined the specificity and sensitivity of the method (*19*). All the related reports did not study the *B. cereus* and *S. aureus*, and the LAMP assay can only detect a gene in a single reaction. In the present study, a multi-LAMP assay was developed to simultaneously detect *B. cereus* and *S. aureus*. The aim of this study is to establish a rapid and low-cost method for detecting *B. cereus* and *S. aureus*, and getting a better understanding of the distinguishing epidemiologic and clinical characteristics of outbreaks caused by these pathogens, it will help investigators determine which one pathogen was the likely cause.

2. Materials and Methods

2.1. Bacterial strains

11 bacterial strains were used in the present study (Table 1). They were cultured for 24-48 h at 37°C in nutrient broth. *B. cereus* and *S. aureus* were used as standard strains to develop the multi-LAMP assay.

2.2. DNA extraction

DNA from 11 compared strains used in this study was extracted according to DNA purification Kit (Shanghai Weijie Biological Engineering Co., Ltd., Shanghai, China). The DNA extracted was used as template in the later assay determining the optimum reaction conditions and analyzing the specificity of multi-LAMP in detecting two goal strains.

Use the bacterial genomic DNA extraction kit, follow the instructions in the instructions: 1.5 mL of the bacterial solution, centrifuge (Tabletop refrigerated centrifuge, Thermo Fisher Scientific, Chengdu, Sichuan Province, China), add buffer GA suspension, 37°C for 30 min, add 20 μL of proteinase K and mix well into 220 μL buffer GB, mix well, Warm bath at 70°C for 30 min. 220 μg of absolute ethanol was added, mix well, transfer to the adsorption column CB3, centrifuge, add 500 μL buffer GD and centrifuged at 10,000g for 2 min. 600 μL of the rinse solution PW was added to the adsorption column, centrifuged, and finally 75 μL of the elution buffer TE was added to the collection tube to extract

Table 1. Bacterial strains used in the present study and their sources

No	Bacterial species	Source
1	*Listeria monocytogenes:1.4255*	CDCP
2	*Escherichia coli:1.2574*	CDCP
3	*Salmonella enteritidis:50040*	CGMCC
4	*Listeria ivanovii1:1527*	CDCP
5	*Lactobacillus delbrueckii subsp:50040*	CDCP
6	*Saccharomyces cerevisiae:1.10599*	CDCP
7	*Enteroinvasive E.coli:ATCC44338*	CGMCC
8	*Shigella flexneri1:1.1059*	CDCP
9	*Enterotoxingenic E.coli:44274*	CGMCC
10	*Bacillus cereus:NC7401*	CGMCC
11	*Staphylococcus aureus: 1.6739*	CDCP

CDCP, Sichuan Center for Disease Control and Prevention, CGMCC, China General Microbiological Culture Collection Center.

the genomic DNA of the bacteria. The DNA of the extracted strain was detected by electrophoresis on a 0.7% agarosegel (DYY-8B type steady current electrophoresis instrument, Liuyi Instrument Factory, Beijing, China).

2.3. LAMP primer design

The result of comparison of similar strain sequences in the Gene Bank database showed that the *nhe* and *nuc* genes are well conserved. Then the LAMP primers (Shanghai Shenggong Company, Shanghai, China) were designed on the website (*http://primerexplorer.jp/e/*). According to the characteristics of LAMP primer design, two groups of LAMP primers were selected, including external primers F3 and B3, internal primers FIP and BIP, as shown in Table 2.

2.4. Optimization of LAMP reaction system

The reaction system of LAMP was optimized. First determine the composition of the 25 μL reaction system: 10 × Thermopol Buffer 2.5 μL Betaine (4 mol/L) 5 μL; four primers each (F3:B3:FIP:BIP = 5:5:40:40, μmol/L) 2 μL; Bst DNA polymerase large fragment (8 U) 1 μL; dNTPs (10 mmol/L) 4 μL; MgSO4 (100 mmol/L) 1.5 μL; template 1 μL; sterile deionization 2 μL of water.

The following changes were attempted in the reaction to optimize the clearness of strips. (*i*) Optimization of amount of Mg^{2+} addition amounts were increased progressively from 1.3 μL to 1.7 μL *i.e.*, 1.3, 1.5, 1.6, 1.7 μL; (*ii*) the temperatures were increased from 58°C to 64°C, *i.e.* 58, 60, 62, 64°C, (*iii*) the concentrations of primer were increased from1.8 μL to 2.4 μL, *i.e.*, 1.8, 2.0, 2.2, 2.4 μL. According to the principle of the same amplification efficiency of the two LAMP systems, the 25 μL multiple LAMP reaction conditions were finally determined.

2.5. Specificity of multi-LAMP assay

All 9 bacterial strains in Table 1 were used as templates

Table 2. LAMP primers used in this study to detect *B. cereus* and *S. aureus*

Target genes	Primer name	Sequence(5'-3')
B. cereus	CES-F3	AACAGTATATAGTGCAACTTCAA
	CES-B3	CTTTGTCAAACTCGACTTCAA
	CES-FIP	TGTCATTGGTTGACCTTTGTACATT-AAAATTACATAAAGAACCTGCGA
	CES-BIP	GTTGATACACCTGAAACAAAGCATC-ATTTTTTTCGTAAATGCACTTGC
S. aureus	SEA-F3	CGATTGATGGTGCGGTTA
	SEA-B3	CAGTTCTTTGACCTTTGTCA
	SEA-FIP	TGCTTTGTTTCAGGTGTATCAACCA
		TTAATGTACAAAGGTCAACC
	SEA-BIP	AAGGTGTAGAGAAATATGGTACTGAT
		CGACTTCAATTTTCTTTGCA

Table 3. PCR primer for detecting *B. cereus* and *S. aureu*

Primer name	Sequence(5'-3')	*Ref.*
CES-F	CGCCGAAAGTGATTATACCAA	(*23*)
CES-R	TATGCCCCGTTCTCAAACTG	(*23*)
CES-P	GGGAAAATAACGAGAAATGCA	(*23*)
SEA-F	AAAATACAGTACCTTTGGAAACGGTT	(*23*)
SEA-R	TTTCCTGTAAATAACGTCTTGCTTGA	(*23*)
SEA-P	AACGAATAAGAAAAATGTAACTG	(*23*)
	TTCAGGAGTTGGATC	

to determine the specificity of multiplex LAMP reaction. Two sets of primers were added to the optimized reaction system (25 μL), and the LAMP detection was carried out by using the genomic DNAs of the two strains and the non-target strains as templates. The results were detected by agarose gel electrophoresis, to verify the accuracy and specificity of the method for detecting B. cereus and *S. aureus*.

2.6. Sensitivity of multi-LAMP assay

The DNA template (100 ng/μL) of the pathogenic bacteria *B. cereus* and *S. aureus* was diluted 10-fold to $100-10^{-7}$ times to 1 fg/μL, and multiplex LAMP amplification was performed to detect the reaction. The multiple LAMP experiment was repeated 2 times, LAMP products were subjected to electrophoresis on 2.0% agarose gel.

2.7. PCR assay

A PCR assay was performed to compare its sensitivity and the clinical detection rates with those of the LAMP assay. Each plasmid sample was amplified in 20 μL reaction mixtures containing 10 μL PCR Master Mix (Tiangen Biotech Co., Ltd., Beijing, People's Republic of China), 400 pM primers (Table 3), and 1 μL DNA template. The cycling conditions were as follows: 3 mins at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; 5mins at 72°C. The PCR products were analyzed electro-phonetically on a 2% agarose gel (*13*).

3. Results

3.1. Optimization of LAMP reaction system

The three reaction conditions of Mg^{2+} concentration, temperature and primer concentration were optimized. The results of LAMP reaction of *B. cereus* were detected by 0.5% agarose gel electrophoresis (Figure 1). It can be known from the specific ladder strips in the group diagram of Figure 1 that the LAMP reaction was ideal, and the designed primer for the *nhe* gene can specifically and accurately identify the *B. cereus*. From Figure 1A, 25 μL of LAMP reaction system can be obtained, and the optimum amount of $MgSO_4$ is 1.5 μL. From Figure 1B, the optimum temperature was 60°C, and the optimal concentration of primers is 2.0 μL from Figure 1C. The LAMP reaction system was optimized based on the LAMP amplification assay of *B. cereus*, and the determination of amplification conditions laid the foundation for further identification of two pathogenic bacteria by multi-LAMP.

3.2. Specificity of multi-LAMP

The two pathogenic bacteria *B. cereus* and *S. aureus* were specifically detected by multiple LAMP, and the results are shown in Figure 2. It can be seen from Figure 2 that the pathogenic bacteria *B. cereus* and *S. aureus* bands in the LAMP amplification result was positive. The other 9 strains (1 to 9 in Figure 2) was negative. Comparing the blanks in 0, the positive results were accurate. It was indicated that the *B. cereus* and *S. aureus*, which were detected by multi-LAMP, were successful, and the reaction was rapid, simple and specific, which provided a basis for rapid detection of food pathogenic bacteria.

3.3. Sensitivity of multiple LAMP and PCR assays

The template concentration of the pathogens were 10-fold gradient dilution from 10 ng/μL DNA to 10^{-7} times (1 fg/μL), and the electrophoresis results were shown in Figure 3. It can be seen from Figure 3 that the multiple
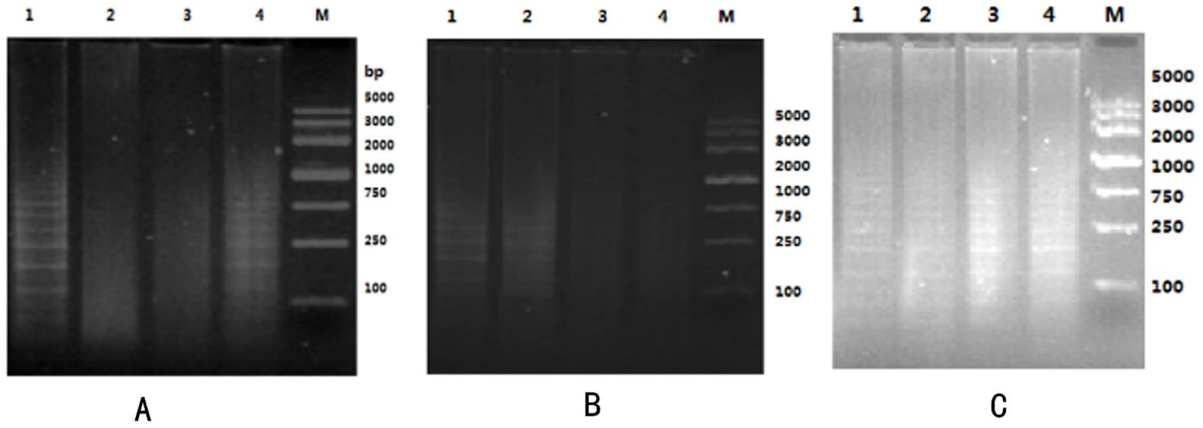
Figure 1. Electrophoresis of LAMP products. M indicates DL 2000 DNA Marker, 1~4 in A indicates 1.3, 1.5, 1.6, 1.7 μL of MgSO₄, 1~4 amplification temperature in B is 58°C, 60°C, 62°C, 64°C, 1~4 primer in C Concentrations 1.8, 2.0, 2.2, 2.4 μL.
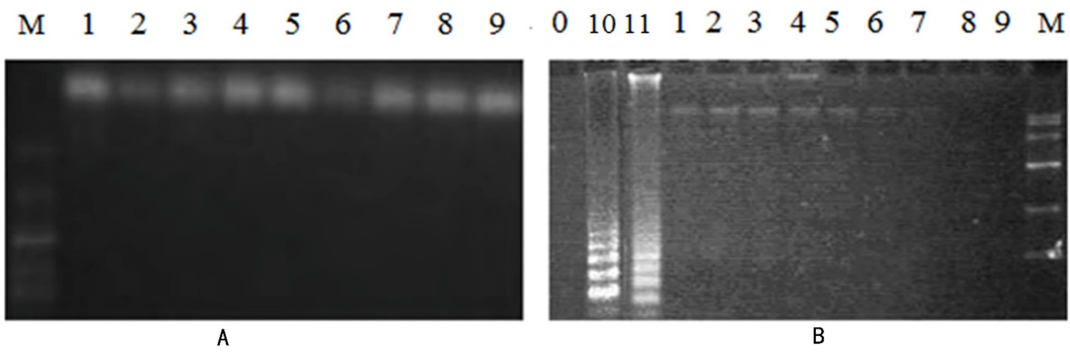

Figure 2. Detection specificity of LAMP reaction for strain. M in A indicates DL 2000 DNA Marker, 1~9 corresponds to the positive plasmid of 9 strains in sequence; 0 in B is blank control, 10 indicates *B. cereus,* 11 indicates *S. aureus*, 1~9 indicates 9 strains, M indicates DL 2000 DNA Marker.
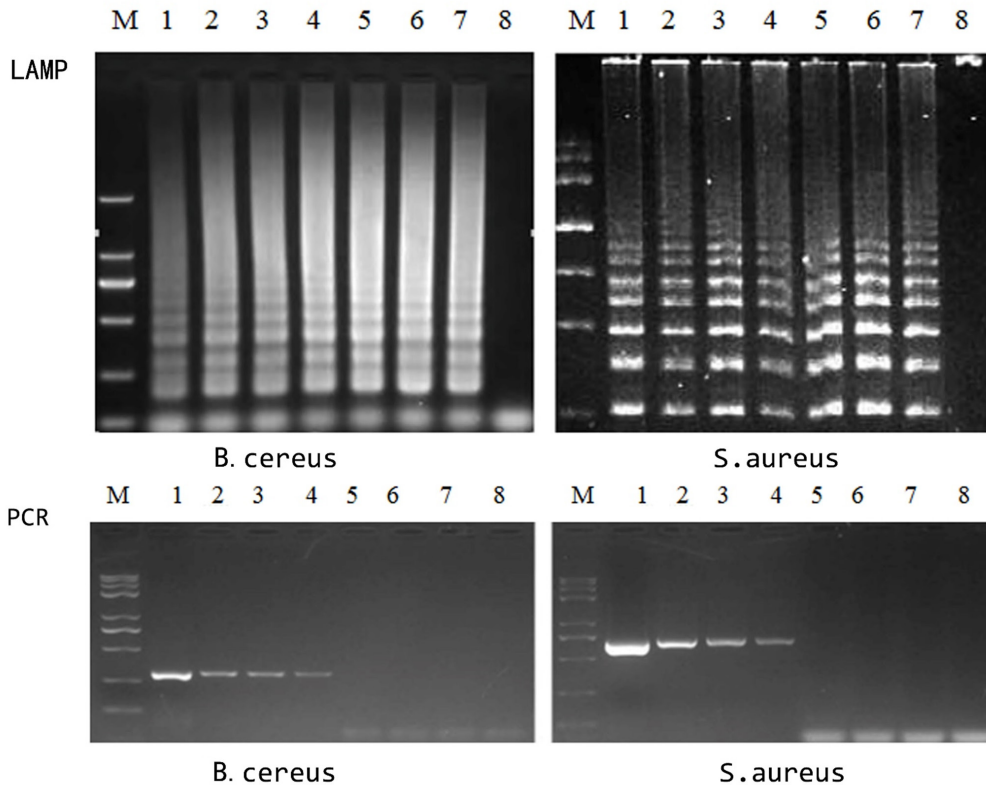

Figure 3. The sensitivity of LAMP and PCR assays. M indicates DNA Marker, 1~8 is the DNA template mass concentration 10, 1 ng/μL100; 10, 1 pg/μL; 100, 10, 1 fg/μL.

LAMP sensitivity detection is ideal, and the 10-fold gradient dilution of the two bacterial DNA templates to 10^{-6} times (10 fg/µL) can still enlarge the clear ladder-like strips, while the dilution is not detected at a low concentration of 10^{-7} times (1 fg/µL). Simultaneously, the bacterial samples were also subjected to traditional PCR. It was found that the sensitivity of the multiple LAMP amplification detection of pathogens in the experiment was 10 times higher than that of PCR.

4. Discussion

The multiple LAMP reaction was used to detect the two pathogenic bacteria. In the experiment, two sets of primers were able to specifically amplify, indicated that the primers were specific. The sensitivity of the multiple LAMP reaction to identify pathogenic bacteria was also high, (reaching 10 fg/µL), which is higher than the sensitivity of 0.2 ng/L in the pistachio DNA detected by Liu and Huang (*20*). Simultaneously, we compared the sensitivity of LAMP with that of traditional PCR analysis, reporting that the detection limit of the LAMP assay was 10-fold that of conventional PCR analysis, and the results of LAMP being consistent with those of conventional PCR analyses (*21*). It shows that this method can identify food samples contaminating pathogens quickly, conveniently and accurately. Compared with traditional PCR, the present method exhibited the following advantages: *1*) high specificity and sensitivity and ease of operation; *2*) multiplex detections of genes using the same detection system, thus reducing manual operation; *3*) a total operating time of < 40 min, as opposed to 90 min to detect via conventional PCR analysis; *4*) greater user-friendliness than conventional PCR analysis, with no requirement of specialized instruments and complicated operations.

This experiment only uses a simple agarose gel electrophoresis test to obtain an ideal positive test result. However, the LAMP reaction only has negative and positive results. It is prone to false positives during the experiment, and the detection sensitivity is too high and it is easy to cause pollution, which has a great impact on the results. Therefore, it is necessary to pay attention to the addition of multiple LAMP reagents of different species in different rooms and places, personnel exchange to avoid contamination and false positives, and the length of the reaction target sequence is controlled below 300 bp, once non-specific amplification occurs, not easy to identify (*22*). If these deficiencies of LAMP are further improved and improved, the potential of multiple LAMP in food and sanitation identification of pathogenic bacteria can be made even greater.

Acknowledgements

References

1. Dewey-Mattia D, Manikonda K, Hall AJ, Wise ME, Crowe SJ. Surveillance for Foodborne Disease Outbreaks – United States, 2009-2015. MMWR Surveill Summ. 2018; 67:1-11.
2. Bennett SD, Walsh KA, Gould LH. Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* – United States. 1998-2008. Clin Infect Dis. 2013; 57:425-433.
3. Wu G, Yuan Q, Wang L, Zhao J, Chu Z, Zhuang M, Zhang Y, Wang K, Xiao P, Liu Y, Du Z. Epidemiology of foodborne disease outbreaks from 2011 to 2016 in Shandong Province, China. Medicine. Medicine (Baltimore). 2018; 97:e13142.
4. Lee MJ, Song KY, Chang LW, Cheon JH, Sook KH, Jeong J, BAE D, Seo GH. Epidemiological aspects of pathogenic microbial foodborne disease outbreaks in Korea and Japan from 2011 to 2015. J Prev Vet Med. 2019; 43:62-67.
5. Ke BX, He DM, Tan HL, Zeng HH, Yang T, Li BS, Liang Y H, Lu LL, Liang JH, Huang Q, Ke CW. Active etiological surveillance for foodborne diseases in Guangdong province, 2013-2014. Zhonghua Liu Xing Bing Xue Za Zhi. 2016; 37:1373-1378.
6. Stefano Z, Marina B. Growth of *Staphylococcus aureus* and enterotoxin production in fresh egg pasta. J Food Process Preserv. 2018; 42:37-53.
7. Pexara A, Govaris A. *Bacillus cereus*: an important foodborne pathogen. J Hellenic Vet Med Soc. 2018; 61:127-133.
8. Law JW, Ab Mutalib NS, Chan KG, Lee LH. Rapid methods for the detection of foodborne bacterial pathogens: Principles, applications, advantages and limitations. Front Microbiol. 2014; 5:1-19.
9. Zhao X, Lin CW, Wang J, Oh DH. Advances in rapid detection methods for foodborne pathogens. J Microbiol Biotechnol. 2014; 24:297-312.
10. Kim HJ, Cho JC. Rapid and sensitive detection of *Listeria monocytogenes* using a PCR-Enzymelinked immunosorbent assay. J Microbiol Biotechnol. 2008; 18:1858-1861.
11. Arunrut N, Kiatpathomchai W, Ananchaipattana C. Multiplex PCR assay and lyophilization for detection of *Salmonella* spp. *Staphylococcus aureus* and *Bacillus cereus* in pork products. Food Sci Biotechnol. 2018; 27:867-875.
12. Chiang YC, Tsen HY, Chen HY, Chang YH, Lin CK, Chen CY, Pai WY. Multiplex PCR and a chromogenic DNA macroarray for the detection of *Listeria monocytogens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterobacter sakazakii*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Salmonella* spp. and *Pseudomonas fluoresc*. J Microbiol Methods. 2012; 88:110-116.
13. Zhong LL, Zhou Q, Tan CY, Roberts AP, El-Sayed Ahmed MAE, Chen G, Dai M, Yang F, Xia Y, Liao K, Liang Y, Yang Y, Feng S, Zheng X, Tian GB. Multiplex loop-mediated isothermal amplification (multi-LAMP) assay for rapid detection of *mcr-1* to mcr-5 in colistin-

resistant bacteria. Infect Drug Resist.2019; 12:1877-1887.

14. Niessen L, Luo J, Denschlag C, Vogel RF. The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. Food Microbiol. 2013; 36:191-206.

15. Sul SY, Kim MJ, Kim HY. Development of a direct loop-mediated isothermal amplification (LAMP) assay for rapid and simple on-site detection of chicken in processed meat products. Food Control. 2018; 98:194-199.

16. Wachiralurpan S, Sriyapai T, Areekit S, Kaewphinit T, Sriyapai P, Santiwatanakul S, Chansiri K. Development of a rapid screening test for *Listeria monocytogenes* in raw chicken meat using loop-mediated isothermal amplification (LAMP) and lateral flow dipstick (LFD). Food Anal Methods. 2017; 10:3763-3772.

17. Lim KT, Teh CSJ, Thong KL. Loop-mediated isothermal amplification assay for the rapid detection of *Staphylococcus aureus*. BioMed Res Int. 2013; 2013:895816.

18. Song T, Toma C, Nakasone N, Iwanaga M. Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. FEMS Microbiol Lett. 2010; 243:259-263.

19. Wan HM, Li YF, Li S, Huang TR, Zheng CL. Optimization of reaction conditions for rapid detection of heat-labile enterotoxin *Escherichia coli* by loop-mediated isothermal gene amplification. Mod Prev Med. 2011; 38:321-323.

20. Liu H, Huang WS, Deng TT, Fu K, Ju XR, Chen Y. Detection of pistachio allergens in food by LAMP method. Food Sci. 2013; 34:128-132.

21. Wei S, Daliri EBM, Chelliah R, Park BJ, Lim JS, Baek MA, Nam YS, Seo KH, Jin YG, Oh DH. Development of a multiplex real – time PCR for simultaneous detection of *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* in food samples. J Food Saf. 2019; 39:1-7.

22. Sowmya N, Thakur MS, Manonmani HK. Rapid and simple DNA extraction method for the detection of enterotoxigenic *Staphylococcus aureus* directly from food samples: comparison of PCR and LAMP methods. J Appl Microbiol. 2012; 113:106-113.

23. Rebelo AR, Bortolaia V, Kjeldgaard JS, *et al.* Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. Euro Surveill. 2018; 23:29-39.