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A novel, sensitive, accurate multiplex loop-mediated isothermal amplification method for detection of *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* in food

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Abstract

For fast and efficient reasons, a multiplex loop-mediated isothermal amplification (mLAMP) to detect foodborne pathogens was studied. Three sets of LAMP primers for 3 kinds of pathogens were designed from nuc gene of *S. aureus*, ipaH gene of *Shigella* spp. and invA gene of *Salmonella* spp., respectively. The DNA(s) were specifically amplified in the presence of the templates from three foodborne pathogens in a single tube. The sensitivities of the mLAMP method were shown 1000 times higher in average on the detection for the genes than those of the classical PCR methods. The sequences of mLAMP assay-positive pathogen products were 99% fidelity with the individual pathogen DNA sequence by sequencing. The large number of tests with field samples showed that the specificity of the LAMP assay was high, and no cross-reaction of the primers and the templates occurred in the same tube. The mLAMP method is further improved and simplified in the detection of the three foodborne pathogens with great specificity and sensitivity.

Key words: Foodborne pathogen, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, multiplex (m)LAMP, food safety.

Introduction

Foodborne pathogens continue to be a common and serious threat to public health all over the world and are major causes of morbidity. Both industrialised and developing countries suffered large number of illnesses and the incidence, on a global basis, appears to be increasing. Most foodborne illnesses are mild, and associated with acute gastrointestinal symptoms such as diarrhoea and vomiting. However, sometimes foodborne diseases are much more serious and life-threatening, particularly in children in developing countries, and infection can also be followed by chronic sequelae or disability.

Foodborne diseases in China are increasing due to rapid development of market economy and lack of legistive control in recent years. Accurate yearly incidences are difficult and sometimes impossible, partly depending on the reporting systems in different regions, but very much depend on techniques and facilities. In a study of diarrhoeal disease in south-eastern China between 1986–1987, the overall incidence of diarrhoeal illness was 730 episodes per 1000 population caused by low hygiene standards, particularly in rural areas, and weak quality control [1]. As matter of fact the outbreaks of food poisoning caused by *Salmonella* more often to come to light because of more and more commercial food products, but with poor management [2-5]. However, the traditional morphological observations for the detection of pathogens are being replaced with ELISA and molecular biology method, especially nucleic acid probing or PCR detection.

Staphylococcus aureus is another one of the most common causes of foodborne disease worldwide. Staphylococcal food poisoning (SFP) is caused by the ingestion of food contaminated with the enterotoxin produced by enterotoxigenic *Staphylococcus aureus*. If foods containing enterotoxigenic *Staphylococcus aureus* are stored under inappropriate temperature conditions, sufficient bacterial growth to produce toxic levels of staphylococcal enterotoxins (SEs) can occur. Because SEs are heat stable, heat treatment such as normal cooking and pasteurization cannot totally inactivate them. Indeed, it is common that the enterotoxin in food has caused outbreaks where the incriminated food had already undergone heat treatment [6]. *Shigella* spp., which is often found in tins or other sealed food products, can cause neurotoxicity, even though the techniques improvement, but because of increasing production and international trade it is also an important criteria in food safety worldwide [7, 8].

Loop mediated isothermal amplification (LAMP) is a single tube technique for the amplification of DNA, which uses a single temperature incubation thereby obviating the need for expensive thermal cyclers [9]. The amplified products can be detected by photometry for turbidity caused by increasing quantity of magnesium pyrophosphate in solution, a colour change observed without equipment, or with addition of SYBR green, which can be visible under UV light. Also in-tube detection of DNA amplification is possible using manganese (Mn) loaded calcein, which starts fluorescing upon complexation of Mn by pyrophosphate during DNA synthesis *in vitro* [10, 11].

In China, almost all food must be detected for *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* to ensure biological safety of food by law since 1999 [12]. The conventional procedure is mainly based on microbiology and immunology assay, even though large number of molecular biological methods has been frequently used to analyze foodborne pathogens and detect very low concentration of nucleic acids. Traditional microbiological methods for detection of foodborne pathogens, like *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* are time-consuming and laborious, and the criteria are frequently subject to constrains by the inspection of different professional personnel's experience and other individual factors, which are prone to give false reports. The ordinary PCR has been widely used in today's pathogen analysis, which needs a standard cycler [13, 14]. Thus, further simplization and accuracy of the analysis of LAMP is needed and we here report a new innovative method, multiplex loop-mediated isothermal amplification (mLAMP). So far no more than 2 sets of primers for 2 pathogens in one reaction tube have been reported [15, 16]. Here we report the detection of 3 kinds of foodborne pathogens at one tube simultaneously for first time. The method has been proved to reduce the workload and improve the quality of reports in food safety analysis.

Materials and Methods

Bacterial strains: The different strains of *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus* and other non-target strains were originally acquired from the American Type Culture Collection (ATCC, Maryland, USA), China Center of Industrial Culture Collection (CICC, Beijing, China) and National Center for Medical Culture Collections (CMCC, Beijing, China) (Table 1).

Table 1. Target and non-target strains used in mLAMP.

No.	Name of strains	Source	Medium for enrichment
1.	*Salmonella typhimurium*	ATCC 14028	Nutrient Broth
2.	*Salmonella cholerae-suis*	CICC 21493	Nutrient Broth
3.	*Salmonella arizona*	CICC 21506	Nutrient Broth
4.	*Salmonella paratyphi-A*	CICC 21501	Nutrient Broth
5.	*Salmonella paratyphi-B*	CICC 21495	Nutrient Broth
6.	*Salmonella paratyphi-C*	CICC 21512	Nutrient Broth
7.	*Shigella flexneri*	ATCC 12022	Nutrient Broth
8.	*Shigella dysenteriae*	ATCC 51252	Nutrient Broth
9.	*Shigella sonnei*	ATCC 25931	Nutrient Broth
10.	*Shigella bogdii*	ATCC 9207	Nutrient Broth
11.	*Staphylococcus aureus*	ATCC 6538	Nutrient Broth
12.	*Staphylococcus aureus*	ATCC 25923	Nutrient Broth
13.	*Staphylococcus aureus*	ATCC 27217	Nutrient Broth
14.	*Staphylococcus aureus*	CICC 23699	Nutrient Broth
15.	*Listeria monocytogenes*	ATCC 19114	Brain Heart Infusion Broth
16.	*Listeria monocytogenes*	ATCC 19115	Brain Heart Infusion Broth
17.	*Listeria innocua*	ATCC 33090	Brain Heart Infusion Broth
18.	*Listeria welshimeri*	CICC 21672	Brain Heart Infusion Broth
19.	*Listeria seeligeri*	CICC 21671	Brain Heart Infusion Broth
20.	*Listeria ivanovii*	CICC 21663	Brain Heart Infusion Broth
21.	*Listeria grayi*	CICC 21670	Brain Heart Infusion Broth
22.	*Staphylococcus epidermidis*	ATCC 12228	Nutrient Broth
23.	*Staphylococcus saprophyticus*	ATCC 49453	Nutrient Broth
24.	*Enterococcus faecalis*	CICC 23536	Nutrient Broth
25.	*Enterococcus faecium*	CICC 21605	Nutrient Broth
26.	*Vibrio parahaemolyticus*	CICC 21528	3% NaCl Alkaline Peptone Water
27.	*Pseudomonas aeruginosa*	ATCC 15442	Nutrient Broth
28.	*Escherichia coli*	ATCC 25922	Nutrient Broth
29.	*Enterobacter sakazakii*	ATCC 51329	Nutrient Broth
30.	*Proteus mirabilis*	CICC 23676	Nutrient Broth
31.	*Citrobacter youngae*	CICC 21596	Nutrient Broth
32.	*Bacillus cereus*	ATCC 11778	Brain Heart Infusion Broth
33.	*Streptococcus hemolytic-β*	CMCC 32210	Brain Heart Infusion Broth

DNA extraction: The bacteria were incubated in a variety of medium (Table 1) at 36ºC for 24 hours, and 1.5 ml enrichment broth of each bacteria were collected for DNA extraction with Qiagen QIAamp DNA Mini Kit (QIAGEN, Hidlen, Germany) to obtain the DNA templates for mLAMP detection. The OD260/OD280 ratio of DNA template solutions was among 1.8 to 1.9, and the concentrations were 50-100 ng DNA μl^{-1}.

LAMP primers: The LAMP method requires a set of four primers that recognize a total of six distinct sequences in the target DNA and two loop primers that increase the amplication efficiency. One set of four primers include two outer primers (forward outer primer F3 and backward outer primer B3) and two inner primers (the forward inner primer [FIP] and the backward inner primer [BIP]) [9]. The primers used for mLAMP and sequencing were designed against the sequence of the *nuc* gene of *Staphylococcus aureus* (Genebank NC_002952.2), *ipaH* gene of *Shigella* spp. (Genebank AY206449.1) and *invA* of *Salmonella* spp. (Genebank NC_003198.1) genes (Table 1) by using Primer Explorer (version 4) software (Fujitsu, Tokyo, Japan), and synthesized by Invitrogen (Shanghai, China). The location and the sequence of each primer are shown in Table 2.

LAMP reaction: The mLAMP reactions were carried out in a thermostat-controlled water bath (LAUDA, Mannheim, Germany) with the following cycle: 61ºC for 1 h, followed by 80ºC for 4 min to terminate the reaction. The mLAMP reactions were performed as the system described in Table 3.

Validation by sequencing: To further confirm the homogeneity and specificity of mLAMP, especially when there were multiple targeted bacteria DNA templates in the system, a mLAMP-PCR-sequencing strategy was applied to analyze the mLAMP products. The mLAMP product of multiple target bacteria DNA (*Staphylococcus aureus* + *Shigella* spp. + *Salmonella* spp.) was verified by electrophoresis, and the ladders between 100 bps and 1000 bps were extracted and purified by using QIAquick Gel Extraction Kit (QIAGEN, Hidlen, Germany) to obtain the template for PCR. Three sets of primers (Table 2) were used, and the PCR reactions were performed as described in Table 4, respectively, in a Veriti™ PCR system (Applied Biosystems, Foster City, CA, USA). The reaction was performed with initial denaturing at 95ºC for 5 min followed by 35 cycles of 30 s at 95ºC, 30 s at 56ºC and 30 s at 72ºC, plus a final extension at 72ºC for 5 min. The PCR products of each reaction was verified by electrophoresis, and then extracted and purified by using QIAquick Gel Extraction Kit (QIAGEN) again to obtain the template for sequencing. Sequencing was performed using the same primers for PCR and BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instruction. The POP-7™ Polymer (Applied Biosystems) was used for electrophoresis using an ABI Prism 3730 DNA sequencer (Applied Biosystems). The sequence data were analyzed by SeqScape software (version 2.5) and compared with the target gene sequences in GenBank database.

Table 2. Primers used in mLAMP and sequencing.

Objective	Type	Sequence (5' to 3')	Loci
nuc of *Staphylococcus aureus*	nuc-F3	GCTCAGCAAATGCATCACAAA	(167-187)
	nuc-B3	TACGCTAAGCCACGTCCAT	(509-491)
	nuc-FIP	GTTGAAGTTGCACTATATACTGTTGG CGTAAATA GAAGTGGTTCT	(225-248)+(216-196)
	nuc-BIP	GTTGATACACCTGAAACAAAGCATCA TTTTTTTCGTAAATGCACTTGC	(352-376)+(431-409)
	nuc-sF	GGCGTAAATAGAAGTGGTTCT	(196-216)
	nuc-sF	TTTTTTTCGTAAATGCACTTGC	(431-409)
ipaH of *Shigella* spp.	ipaH-F3	CCTTTTCCGCGTTCCTTGAC	(97-116)
	ipaH-B3	GGAACATTTCCCTGCCCA	(375-358)
	ipaH-FIP	GCAGCGACCTGTTCACGGATTGCCTT TCCGATACCGTCTCT	(175-157)+(118-137)
	ipaH-BIP	ATCTCCGGAAAACCCTCCTGGTAGC GCCGGTATCATTATCGA	(283-304)+(347-328)
	ipaH-sF	GCCTTTCCGATACCGTCTCT	(118-137)
	ipaH-sB	AGCGCCGGTATCATTATCGA	(347-328)
invA of *Salmonella* spp.	invA-F3	ATTGGTGTTTATGGGGTCGTTCT	(147-169)
	invA-B3	ACCACGGTGACAATAGAGAAGACA	(371-348)
	invA-FIP	CTGGTACTGATCGATAATGCCAAGTT TTTCAACGTTTCCTGCGG	(257-236)+(187-208)
	invA-BIP	TGCCGGTGAAATTATCGCCACACAA AACCCACCGCCAGGCTA	(282-302)+(347-327)
	invA-sF	AAGTTTTTCAACGTTTCCTGCGG	(187-208)
	invA-sB	ACAAAACCCACCGCCAGGCTA	(327-347)

Table 3. The composition of mLAMP reaction mixture.

Composition	Volume (µl)
primer nuc-F3 and nuc-B3 (0.5 µmol l^{-1})	0.2 for each
primer nuc-FIP and nuc-BIP (5 µmol l^{-1})	3 for each
primer ipaH-F3 and ipaH-B3 (0.5 µmol l^{-1})	0. 2 for each
primer ipaH-FIP and ipaH-BIP (5 µmol l^{-1})	3 for each
primer invA-F3 and invA-B3 (0.5 µmol l^{-1})	0. 2 for each
primer invA-FIP and invA-BIP (5 µmol l^{-1})	3 for each
Template DNA	1 for each
*Bst*DNA Polymerase (8 U μl^{-1}, NEB)	1
10×*Bst*Buffer (NEB)	2.5
Mg^{2+} (100 mmol l^{-1}, Promega)	1.2
dNTP mix (10 mmol l^{-1}, Promega)	2
Betaine (8 mol l^{-1}, Sigma)	2
Sterile ultrapure water	up to 25

Table 4. The composition of PCR mixture.

Composition	Volume (µl)
Premix Ex Taq (Lodding dye mix) (TAKARA, Kyoto, Japan)	12.5
primer nuc-sF and primer nuc-sB (50 µmol l^{-1}) or primer ipaH-sF and primer ipaH-sB (50 µmol l^{-1}) or primer invA-sF and primer invA-sB (50 µmol l^{-1})	0.2 for each
DNA template	1
Sterile ultrapure water	up to 25

Determination of mLAMP sensitivity compared with conventional PCR: The template DNA of each target bacteria was diluted by 10-fold-series in order to check the approximate detection sensitivity of the mLAMP and conventional PCR. Reaction of mLAMP and PCR were carried out as described above (LAMP primers, LAMP reaction and Validation by sequencing).

mLAMP detection of bulk samples: In order to evaluate the feasibility of mLAMP assay in food sample, 164 raw milk, 76 raw pork, 34 raw eggs and 24 raw chickens were tested, 25 g of each sample was inoculated into nutrient broth and 7.5% sodium chloride broth, respectively, and incubated overnight at 37ºC. Following inoculation, the cultures were subjected to DNA extraction, and the DNA extracted from nutrient broth and 7.5% sodium chloride broth was 2:1 mixed by volume. The mixed DNA was applied to perform the mLAMP detection. The positive PCR(s) were performed to prove foodborne pathogen DNA(s) involved in template DNA solution (Table 4). Meanwhile, each sample from the three pathogens by different culture methods, including the steps of enrichment, direct plating, biochemical identification and serological identification were also tested in order to validate mLAMP results from different conditions.

Results

mLAMP with DNA of target and non target strains: With specifically amplification, turbid tubes could be observed in target strains and no turbid tubes in non-target strains (data not shown), and the result of DNA gel electrophoresis (Fig. 1) fits precisely with expected result.

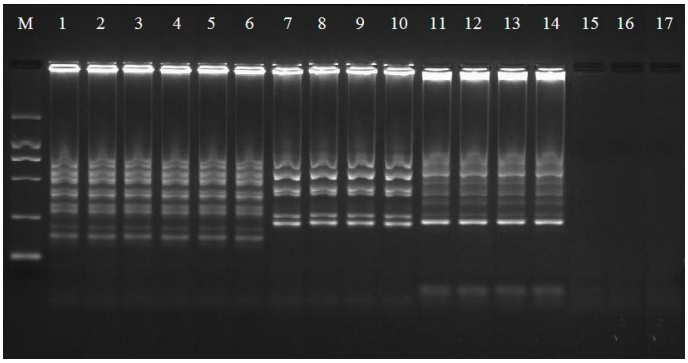

Figure 1. Result of mLAMP with target and non-target standard foodborne pathogen strain DNAs.
M: DNA Marker, the size of bands were 2000, 1000, 750, 500, 250 and 100 bp, respectively, from top to bottom; Lane 1-4, 4 *Staphylococcus aureus* stains; Lane 5-10, 6 *Salmonella* spp. stains; Lane 11-14, 4 *Shigella* spp. strains; Lane 15-17, the non-target strains (*Staphylococcus epidermidis*, *Escherichia coli* and *Enterobacter sakazakii*).

mLAMP with different combination of the target DNA templates: For efficient assay, the mLAMP was performed with the combination of 2 or 3 target strains, which give similar specific results (Fig. 2).

Sensitivity comparison of mLAMP and conventional PCR: To ascertain the sensitivity of mLAMP assay, 10-fold serial dilution of the extracted DNA of *Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923) was subjected to the mLAMP assay. The sensitivity of mLAMP assay was found to be 10 fg DNA in tube for *Salmonella* spp. or *Shigella* spp., respectively, and 1 fg DNA in tube for *Staphylococcus aureus*. Comparatively, conventional PCR present the sensitivity of 100 pg DNA in tube for *Salmonella* spp., 1 pg DNA in tube for *Shigella* spp. and 10 pg DNA in tube for *Staphylococcus aureus.* The sensitivity of mLAMP was at least 100 times higher than that of conventional PCR (Fig. 3).

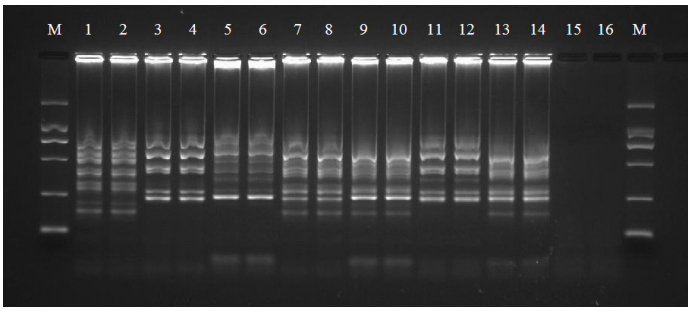

Figure 2. Result of mLAMP with different combination of the target DNA templates. M: DNA Marker, the size of bands were 2000, 1000, 750, 500, 250 and 100bp, respectively, from top to bottom; Lane1-2, *Salmonella typhimurium* (ATCC 14028) only; Lane 3-4, *Shigella flexneri* (ATCC 12022) only; Lane 5-6, *Staphylococcus aureus* (ATCC 25923) only; Lane 7-8, *Salmonella typhimurium.* (ATCC 14028) and *Shigella flexneri* (ATCC 12022); Lane 9-10, *Salmonella typhimurium* (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923); Lane 11-12, *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923); Lane 13-14, *Salmonella typhimurium.* (ATCC 14028), *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923); Lane 15-16, non-target strains (*Staphylococcus epidermidis* and *Escherichia coli*).

Validation of the specificity of LAMP by DNA sequencing: To further confirm the specificity of mLAMP, we established a mLAMP-PCR-sequencing strategy to analyze the mLAMP products. The sequences of PCR amplicons which amplified from mLAMP products were 99% match with the expected sequence (data not shown).

Detection result of bulk samples: We tested 164 raw milk, 76 raw pork, 34 raw eggs and 24 raw chickens. mLAMP assay screen out 5 positive samples, and PCR further confirmed that 4 raw milk samples were contaminated by *Staphylococcus aureus* and 1 raw pork sample was contaminated by *Salmonella* spp., while these three foodborne pathogens were not found in raw chicken and raw eggs. The results of mLAMP assay were in accordance with the results of culture methods.

Discussion

The conventional procedure for pathogenic microorganism requires an accurately weighed sample (usually 25 g) to be homogenized in a primary enrichment broth and incubated for a stated time at a known temperature. In some cases, a sample of the primary enrichment may require transfer to a secondary enrichment broth and further incubation. The final enrichment is usually streaked out onto a selective agar plate that allows the growth of the organisms under test. The long enrichment procedure is used because the sample may contain very low levels of the test organism in the presence of high number of background microorganisms. In recent years, besides the detection of foodborne pathogens [17-19], LAMP technology is also widely used to detect other targets, including viruses [20], parasites [21] and genetic modified organisms [22]. LAMP is a novel technique for amplification of specific DNA sequences, and has several advantages over PCR. First, the specificity of LAMP is high because the LAMP method uses multiple primers, recognizing six distinct sequences in the target DNA. Second, the method is both rapid and simple; only 45 min are needed to amplify the target sequences. Third, the cost of the equipment is low compared with PCR, which is one of the major reasons why PCR diagnostics have not been more widely utilized. With these advantages, LAMP has the potential to become adopted for widespread use in hospitals, laboratories, and out-stations and food industry.

We here described the successful development of an mLAMP method for the simultaneous detection of respective genes of foodborne *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* using thermostat-controlled water bath. The three foodborne bacteria could be detected within one tube at a constant temperature. Previous study show that *nuc* gene, *ipa*H gene and *invA* gene were the specific virulence factor genes for 3 pathogens, the sequences of which were highly conserved [23-25]. Three sets of primers involved in the mLAMP were designed based on these three evolution remoted genes so that the mLAMP had good specificity. Recently, several LAMP methods combining the technique of restriction enzyme cleavage, such as restriction fragment length polymorphism (RFLP) have been constructed using an original cleavage site within the amplified DNA products rather than within the designed primers [20, 26]. The strategy has been used to confirm whether or not the amplified DNAs are derived from the target genes. In this study, we provided a mLAMP-PCR-sequencing strategy to validate the sequence of mLAMP product in order to further confirm the specificity of the mLAMP. The primers for PCR and sequencing were located on the F2 and B2 region of the inner primer FIP and BIP for each pathogen, respectively, in order not to miss any non-specific amplification products from previous step (mLAMP). The obtained sequence data further confirmed the specificity of this mLAMP method (100% match of the reference sequences). The comparison of mLAMP assay with different culture conditions also validated the accuracy of mLAMP primers and the methods.

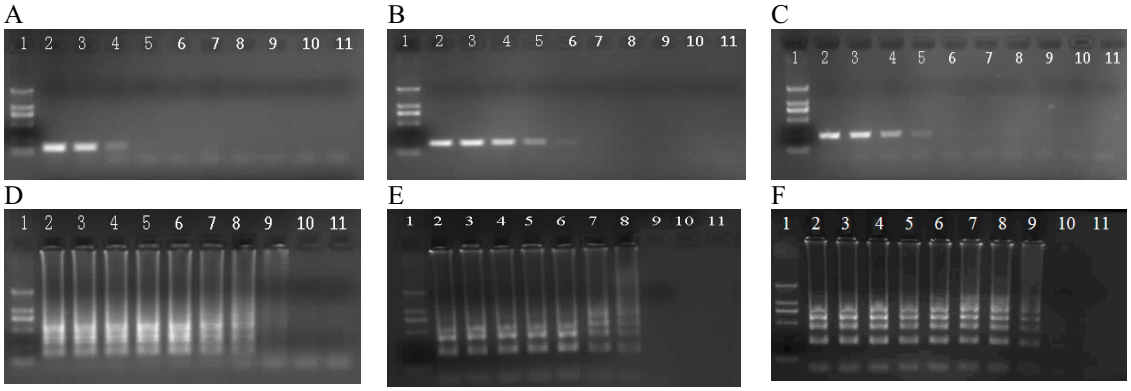

Figure 3. Sensitive comparison of the mLAMP and conventional PCR.
Panel A and D, PCR and mLAMP with *Salmonella typhimurium* (ATCC 14028) DNA; Panel B and E, PCR and mLAMP with *Shigella flexneri* (ATCC 12022) DNA; Panel C and F, PCR and mLAMP with *Staphylococcus aureus* (ATCC 25923) DNA; Lane 1 in each panel was DNA Marker; Lane 11 was negative control; For Lane 2-10, the template DNA concentration were 10 ng μl^{-1}, 1 ng μl^{-1}, 100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1}, 0.1 fg μl^{-1} for each panels.

The established mLAMP method also showed high sensitivity. *Salmonella* spp. or *Shigella* spp. could be detected with the template DNA of 10 fg in tube, respectively, while *Staphylococcus aureus* with 1 fg in tube. Comparatively, the concentration of target DNA template for conventional PCR needs 100 times higher at least. However, the current mLAMP methods still need an effective validation, such as DNA sequencing for each set-up even though not necessary for each run. The pattern of LAMP products of the three kinds of foodborne pathogens had a tiny difference between each other; however, electrophoresis gels do not distinguish in which foodborne pathogen DNA(s) was/were involved in template DNA solution. Observing the result with fluorescence intensity or turbidity was less likely to distinguish between the three foodborne pathogens. However, for food, especially processed foods, the results of the three foodborne pathogens were often negative, so the mLAMP method is still a good choice for rapid screening. If positive results occur, other molecular biology methods or conventional culture method could be used for further confirmation.

Conclusions

The established mLAMP method has been proved to be a new, convenient and powerful tool for simultaneous screening of the three foodborne pathogens

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