Multiplex loop-mediated isothermal amplification-based lateral flow dipstick for simultaneous detection of 3 food-borne pathogens in powdered infant formula

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ABSTRACT

In this study, we established a rapid, simple, and sensitive method for visual and point-of-care detection of *Salmonella* spp., *Cronobacter* spp., and *Staphylococcus aureus* in powdered infant formula (PIF) based on multiplex loop-mediated isothermal amplification (mLAMP) combined with lateral flow dipstick (LFD). Three different species-specific target genes, *stx*A of *Salmonella* spp., internal transcribed space (*ITS*) of *Cronobacter* spp., and *nuc* of *Staph. aureus*, were applied in the mLAMP with biotin-, digoxin-, and Texas Red-modified forward inner primers and fluorescein isothiocyanate (FITC)-modified backward inner primers. After mLAMP, a large number of modified amplicons were detected with LFD; one end of the amplicon was conjugated to the anti-FITC antibody on gold nanoparticles and the other end to streptavidin (anti-digoxin or anti-Texas Red antibody) on test lines. Visual inspection of the device relies on the presence of a red band formed by accumulation of sandwich composites. The detection limits of this mLAMP-LFD assay for *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* in PIF without enrichment were 4.2, 2.6, and 3.4 cfu/g, respectively. The whole method can be completed in less than 1 h. Thus, mLAMP-LFD is a rapid and efficient method for simultaneously detecting *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* in PIF.

Key words: multiplex loop-mediated isothermal amplification, lateral flow dipstick, food-borne pathogen, powdered infant formula

INTRODUCTION

As a significant burden on public health, food-borne pathogens cause large numbers of outbreaks worldwide. Food-borne illnesses are frequently reported to be caused by *Salmonella* spp. (Angulo et al., 2008; Hyeon et al., 2010), *Cronobacter* spp. (Drudy et al., 2006), and *Staphylococcus aureus* (Wang et al., 2012b). These pathogens infect newborns and infants via intrinsic or extrinsic contamination of reconstituted powdered infant formula (PIF), which represents a significant hazard to the health of infants. Illnesses include diarrhea (Hyeon et al., 2010), neonatal meningitis (Liu et al., 2006b), and food poisoning (Wang et al., 2012b). Hence, there is an urgent need to develop a highly sensitive platform for simultaneous detection of these 3 pathogens to monitor PIF and prevent associated infections.

Conventional detection methods to identify food-borne pathogens such as *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* typically rely on culture-based assays, which require 5 to 7 d for the processes of pre-enrichment, growth on selective media, and biochemical confirmation (*Salmonella* spp. additionally needs serological testing; Law et al., 2015). These detection methods are both time consuming and laborious (Shao et al., 2011). Although more recent methods using PCR or real-time PCR have been developed, they often require long detection times and expensive instruments, which limit their application in the field of rapid detection. Loop-mediated isothermal amplification (LAMP) is based on nucleic acid amplification and was developed by Notomi et al. (2000). The target fragments can be amplified by LAMP with high efficiency and satisfactory specificity, only using a heating block or heating water bath. Therefore, it is suitable for food safety testing or clinical diagnostics, especially in resource-poor environments or settings (Dhama et al., 2014; Kokkinos et al., 2014).

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Recently, the multiplex loop-mediated isothermal amplification (**mLAMP**) assay, which mixes several sets of primers in a single reaction system, has attracted attention. Unlike a single LAMP, mLAMP produces a more complex ladder-like pattern of amplicons, which cannot be analyzed by agarose gel electrophoresis (**AGE**). Several methods have been reported to detect and differentiate mLAMP products, such as real-time turbidity measurement (Wang et al., 2012a; Nagamine et al., 2014), restriction patterns based on an endonuclease recognition site in the LAMP inner primers (Shao et al., 2011), fluorophore-labeled primers combined with an intercalator dye (Tanner et al., 2012), and sequence-based barcodes coupled with nicking endonuclease-mediated pyrosequencing (Liang et al., 2012). However, these methods are limited by the use of precision instruments, making them unsuitable for point-of-care testing. As a rapid, visual, and user-friendly detection method, the lateral flow dipstick (**LFD**) presents great potential for point-of-care testing (Liu et al., 2006a; Jung et al., 2015). Because the LFD method can discriminate a mixture of mLAMP amplicons, it has been used for the detection of toxins (Chen et al., 2016), pathogenic *Leptospira* (Nurul Najian et al., 2016), and *Babesia* (Yang et al., 2016). To the best of our knowledge, there are no reports of 3 pathogenic species being simultaneously detected by the mLAMP-LFD method in a single reaction.

In this study, we developed a method based on mLAMP-LFD for the simultaneous detection of *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* in PIF, providing a simple, rapid, sensitive, and low-cost detection system for point-of-care testing in the dairy industry.

MATERIALS AND METHODS

Reagents

Bst 2.0 WarmStart DNA polymerase and MgSO_4 were purchased from New England BioLabs (Ipswich, MA). Betaine was obtained from Sigma-Aldrich (St. Louis, MO). Deoxynucleoside triphosphates (dNTP) were purchased from TianGen Co. Ltd. (Beijing, China). The nitrocellulose membrane was obtained from Sartorius (Göttingen, Germany). The plastic backing, sample pad, conjugate pad, and absorption pad were purchased from Ahlstrom Co. Ltd. (Helsinki, Finland). The mouse anti-fluorescein isothiocyanate (**FITC**) monoclonal antibody was obtained from Santa Cruz Biotechnology Co. Ltd. (Shanghai, China). The rabbit anti-Texas Red polyclonal antibody was purchased from Thermo Fisher Scientific (San Jose, CA). The streptavidin, mouse anti-digoxin monoclonal antibody

and gold chloride trihydrate were obtained from Sigma (St. Louis, MO). Goat anti-mouse secondary polyclonal antibody (IgG) was purchased from Bioss Co. Ltd. (Beijing, China). Sucrose and BSA were acquired from Sangon Biotech Co. Ltd. (Shanghai, China). All other reagents were of analytical grade. The LFD sprayer and automatic programmable cutter were acquired from Biodot (Irvine, CA) and Autokun Co. Ltd. (Hangzhou, China), respectively.

Bacterial Strains and DNA Extraction

A total of 42 bacterial strains, including *Salmonella* spp. ($n = 13$), *Cronobacter* spp. ($n = 8$), *Staph. aureus* ($n = 6$), and other nontarget bacteria ($n = 15$) were used for specificity testing of the mLAMP-LFD assay and are listed in Table 1. All bacteria were cultured overnight in Luria-Bertani (**LB**) broth at 37°C with shaking at 150 rpm. *Salmonella* Typhimurium ATCC 14028, *Cronobacter sakazakii* ATCC 29544, and *Staph. aureus* ATCC 13565 were used as positive controls, and nuclease-free water was used as the negative control. Genomic DNA samples were extracted from 1 mL of enrichment broth by using the Gen Elute Bacterial Genomic DNA Kit (Tiange, Beijing, China) and suspended in 100 μL of Tris-EDTA buffer. Genomic DNA of bacteria was stored at -20°C until use.

Design of LAMP Primers

According to previous studies (Ruan et al., 2013; Wang et al., 2017; Zhao et al., 2017), 3 conserved fragments from the *siiA* gene of *Salmonella* spp., the internal transcribed spacer (*ITS*) sequences of *Cronobacter* spp., and the *nuc* gene of *Staph. aureus* were selected as target sequences to design LAMP primers using Primer Explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>). Primers are shown in Table 2 and were synthesized by Invitrogen (Shanghai, China). The forward inner primers (**FIP**) targeting the *siiA*, *ITS*, and *nuc* genes were labeled with biotin, digoxin, and Texas Red at the 5' end, and the backward inner primers (**BIP**) of the 3 sets were labeled with FITC at the 5' end.

mLAMP Reaction

The multiplex LAMP reaction was performed in a 25- μL volume containing the following components: 0.1 μM *siiA*-F3 and *siiA*-B3 primers, 0.8 μM *siiA*-FIP and *siiA*-BIP primers, 0.1 μM *ITS*-F3 and *ITS*-B3 primers, 0.7 μM *ITS*-FIP and *ITS*-BIP primers, 0.1 μM *nuc*-F3 and *nuc*-B3 primers, 0.9 μM *nuc*-FIP and *nuc*-BIP

Table 1. Strains used for the multiplex loop-mediated isothermal amplification (mLAMP) specificity test¹

No.	Bacterial species	Source ²	Result ³	
			mLAMP-AGE	mLAMP-LFD
1	<i>Salmonella</i> Typhimurium	ATCC 14028	+	+
2	<i>Salmonella</i> Typhimurium	ATCC 23564	+	+
3	<i>Salmonella</i> Dublin	CMCC 50042	+	+
4	<i>Salmonella</i> Arizona	CMCC(B) 47001	+	+
5	<i>Salmonella enterica</i> ssp. <i>Salamae</i>	ATCC 43972	+	+
6	<i>Salmonella</i> Choleraesuis	CMCC 50018	+	+
7	<i>Salmonella</i> Pullorum	ATCC 9120	+	+
8	<i>Salmonella</i> Enteritidis	ATCC 13076	+	+
9	<i>Salmonella enterica</i> Paratyphi B	CMCC(B) 50094	+	+
10	<i>Salmonella</i> Typhi	CMCC(B)50071	+	+
11	<i>Salmonella</i> Typhi	ATCC 19430	+	+
12	<i>Salmonella</i> Paratyphi A	CMCC(B)50093	+	+
13	<i>Salmonella</i> Thompson	CMCC 50023	+	+
14	<i>Cronobacter sakazakii</i>	ATCC 29544	+	+
15	<i>Cronobacter sakazakii</i>	ATCC 12868	+	+
16	<i>Cronobacter malonaticus</i>	DSM 18702	+	+
17	<i>Cronobacter turicensis</i>	DSM 18703	+	+
18	<i>Cronobacter universalis</i>	NCTC 9529	+	+
19	<i>Cronobacter dublinensis</i>	CICC 18705	+	+
20	<i>Cronobacter muytjensii</i>	ATCC 51329	+	+
21	<i>Cronobacter condimenti</i>	LMG 26250	+	+
22	<i>Staphylococcus aureus</i>	CMCC 26075	+	+
23	<i>Staphylococcus aureus</i>	ATCC 25923	+	+
24	<i>Staphylococcus aureus</i>	ATCC 29213	+	+
25	<i>Staphylococcus aureus</i>	ATCC 12600	+	+
26	<i>Staphylococcus aureus</i>	ATCC 13565	+	+
27	<i>Staphylococcus aureus</i>	ATCC 12598	+	+
28	<i>Bacillus cereus</i>	ATCC 14579	—	—
29	<i>Shigella flexneri</i>	CMCC 51572	—	—
30	<i>Staphylococcus epidermidis</i>	ATCC 12228	—	—
31	<i>Listeria monocytogenes</i>	CMCC 54006	—	—
32	<i>Micrococcus luteus</i>	Laboratory	—	—
33	<i>Enterococcus faecalis</i>	Laboratory	—	—
34	<i>Shigella flexneri</i>	CICC 21678	—	—
35	<i>Bacillus subtilis</i>	Laboratory	—	—
36	<i>Escherichia coli</i> O157:H7	CICC 21530	—	—
37	<i>Listeria monocytogenes</i>	ATCC 19114	—	—
38	<i>Listeria monocytogenes</i>	ATCC 19116	—	—
39	<i>Listeria welshimeri</i>	ATCC 43550	—	—
40	<i>Listeria welshimeri</i>	ATCC 43548	—	—
41	<i>Vibrio parahaemolyticus</i>	ATCC 33847	—	—
42	<i>Vibrio parahaemolyticus</i>	ATCC 17802	—	—

¹mLAMP-AGE = detection by mLAMP combined with agarose gel electrophoresis (AGE); mLAMP-LFD = detection by mLAMP combined with lateral flow dipstick (LFD).

²ATCC = American Type Culture Collection (Manassas, VA); CMCC = China Medical Culture Collection Center, Ministry of Public Health (Beijing, China); DSM = DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); NCTC = National Collection of Type Cultures, Central Public Laboratory Service (London, UK); CICC = China Center of Industrial Culture Collection, China National Research Institute of Food and Fermentation Industries (Beijing, China); LMG = Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University (Ghent, Belgium).

³+, positive result; —, negative result.

primers, 1.6 mM dNTP, 4 mM MgSO₄, 2.5 µL of 10× ThermoPol Buffer (New England BioLabs), 0.9 µL of 8 U/µL *Bst* 2.0 WarmStart DNA polymerase (New England BioLabs), and 2 µL of template DNA (68.5 ng/µL) of each target bacteria. Then, amplification was carried out in a conventional water bath and incubated at 64°C for 45 min followed by 80°C for 2 min to terminate the reactions.

Establishment of the LFD

Preparation of Gold Nanoparticles and Functionalization with Antibody. Gold nanoparticles (AuNP), with a diameter of 15 ± 3.5 nm, were prepared based on a previously reported method (He et al., 2011; Chen et al., 2016). Three microliters of 0.2 M K₂CO₃ was added to 1 mL of AuNPs to adjust them to a suit-

Table 2. Sequences of multiplex loop-mediated isothermal amplification (mLAMP) primers

Primer	Sequence (5'→3')	Label
nuc-F3	CGATTGATGGTGATACGGTTA	5'-modified Texas Red 5'-modified FITC ¹
nuc-B3	CCACGTCCATATTTATCAGTTC	
nuc-FIP	GGATGCTTTGTTTCAGGTGTATCATGTACAAAGGTCAACCAATG	
nuc-BIP	AGAGAAATATGGTCCTGAAGCAAGCCTTTGTCAAACCTCGACTTC	
ITS-F3	AAATGCGCGGTGTGTGTCAG	5'-modified digoxin 5'-modified FITC
ITS-B3	GGTTTCCCCATTTCGGACAT	
ITS-FIP	ACCGTGTACGCTTGTTCGCTTTTCTCTCAAACCTCGCAGCAC	
ITS-BIP	GGCAGTCAGAGGCGATGCGCCGGTTATAACGGTTCA	
siiA-F3	AGAAGCTTTTCAGAAGAGGA	5'-modified biotin 5'-modified FITC
siiA-B3	GATAATAGCTTTACTCGTTGTACT	
siiA-FIP	CTGCCTGAGGAATAACCATTTCTATAATAAAGGCTTGGTTAGAAGACA	
siiA-BIP	TACGACTGGGATATGAACGGGACTCATGTCAATAACTACATCAG	

¹Fluorescein isothiocyanate.

able pH. Since AuNPs would damage the electrode of the pH meter, 0.2 M K₂CO₃ was added to the solution as a pH indicator. Then, 3 µL of 1 mg/mL anti-FITC antibody was added to the solution and incubated at room temperature for 15 min. Then, 10% BSA (wt/vol) was added and mixed thoroughly, blocking the residual sites on the surface of AuNPs. After incubation for 15 min, the mixture was centrifuged at $9,175 \times g$ at 4°C for 15 min. Subsequently, the supernatant was removed and the crimson precipitate was resuspended in 100 µL of dilution buffer (wt/vol) containing 100 mM Tris (pH 9), 4% trehalose (wt/vol), and 0.2% BSA and stored in 4°C until further use.

Assembly of the LFD. The LFD was assembled from 4 components (sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad), which were pasted on the polyvinyl chloride (PVC) baseplate. The sample and conjugate pads were saturated with running buffer [100 mM Tris (pH 9), 0.05% casein (wt/vol), 0.5% Tween 20, 4% trehalose (wt/vol), 0.002% trisodium citrate (wt/vol), 1% polyvinylpyrrolidone (PVP; wt/vol), 0.2% BSA (wt/vol), and 0.05% sodium azide (wt/vol)] and then dried at 45°C for 2 h. Then, the conjugate pad was treated with anti-FITC–AuNP conjugates and dried at 37°C for 2 h. Streptavidin (0.8 mg/mL), anti-digoxin antibody (0.8 mg/mL), anti-Texas Red antibody (0.8 mg/mL), and goat anti-mouse IgG (1 mg/mL) were applied, in turn, to the test lines (T1 to T3) and control line (line C) on the nitrocellulose membrane. The test strip was assembled as shown in Figure 1 and stored in bags with a silica-gel desiccant at 4°C.

mLAMP-LFD Assay. The 25-µL mLAMP amplicon reaction was mixed with 75 µL of running buffer and incubated for 5 min. The mixture was then added dropwise to the sample pad of the LFD. Results were then observed by the naked eye after the device was left to stand for 5 min.

Principle of the mLAMP-LFD Detection

The protocol outlined in this study combines the rapid detection of mLAMP with visual assessment of the LFD. The principle of visual detection of mLAMP products on an LFD is shown in Figure 1. In the presence of target bacteria, large amounts of biotin- and FITC-, digoxin- and FITC-, or Texas Red- and FITC-labeled amplicons are produced under the trigger of specific labeled primers by mLAMP for the target genes. After mixing with the running buffer, the amplicons are added dropwise to the sample pad of the LFD. The mixture moves through the LFD by capillary action and combines with anti-FITC antibody–AuNPs on the conjugate pad to form an amplicon–FITC–anti-FITC antibody–AuNP complex. Then, the complexes continue to migrate along the LFD and are sequentially captured by the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3), which is visualized as a red line because of the aggregation of AuNPs. Then, excessive anti-FITC antibody–AuNP conjugates continue migrating and are captured to form the other red line (line C) by nonspecific reactions between IgG and anti-FITC antibody on the AuNP surface. When target bacteria are not present in the sample, only line C is red and no red lines appear on the test lines. For all test results, line C must be red to demonstrate the validity of LFD detection.

Sensitivity of mLAMP-LFD

To determine the sensitivity of the LAMP-LFD assays, fresh overnight cultures in LB of *Salmonella* Typhimurium ATCC 14028, *C. sakazakii* ATCC 29544, and *Staph. aureus* ATCC 13565 were 10-fold serially diluted with normal saline. One milliliter of each dilution was plated on LB agar for enumeration, and 1 mL

from the same dilution was used to extract genomic DNA as described above. Then, 2 μ L of each genomic DNA sample was used to determine the sensitivity of the mLAMP-LFD and mLAMP-AGE methods. Image analysis software (Quantity One, Bio-Rad Inc., Hercules, CA) was used to estimate the intensity of line signals on the LFD by converting the red bands to gray values (Lee et al., 2015). All reactions were conducted in triplicate for each concentration of bacteria.

Specificity of mLAMP-LFD

The specificity of the mLAMP-AGE and mLAMP-LFD detection methods was validated by testing extracted genomic DNA from all 42 bacterial strains (Table 1); each strain was tested in triplicate. At the same time, to verify that the 3 sets of primers did not cross-react with each other (resulting in false-positive results), different types of target genomic DNA were added to the double or triple system (containing 2 or 3 sets of primers) for amplification. All mLAMP products were analyzed using the multiplex LFD assay.

Stability of the LFD

As an important indicator for evaluating the mLAMP-LFD detection method, stability was determined using an accelerated aging test. Using a modified version of the method reported by Peng et al. (2014), LFD were stored in a closed container with a silica gel desiccant at 45°C, and mLAMP products were detected using these LFD at 0, 10, 20, and 30 d after storage.

Application in Contaminated PIF Samples

Commercial PIF samples were obtained from local stores in Harbin, China, and determined to be free of *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* by standard culture methods (ISO, 2017a,b, 1999). Artificially contaminated PIF samples were then pre-treated by using the procedure reported by Pan et al. (2018). Briefly, 10 g of PIF was reconstituted with 90 mL of buffer peptone water that was preheated to 44°C, and *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* were added at concentrations ranging from 10^9 to 10^{-1} cfu/mL. To avoid matrix effects, samples were

Figure 1. Schematic diagram for the visual detection of multiplex loop-mediated isothermal amplification (mLAMP) products on a lateral flow dipstick (LFD). PVC = polyvinyl chloride; FITC = fluorescein isothiocyanate; AuNP = gold nanoparticle. T1, T2, and T3 are the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3); C is the control line.

centrifuged for 5 min at $2,300 \times g$ and 4°C to remove the upper layer of whey and fat. Then, the sediment was resuspended in an equal volume of normal saline. Subsequently, 1 mL of solution was used for extraction of genomic DNA and the mLAMP-LFD assay was conducted as described above. In this paper, detection was assessed by the naked eye, so we did not perform statistical analysis on the results.

RESULTS

Optimization of the mLAMP-LFD Assay

To achieve the best detection efficiency and sensitivity, we optimized several key parameters of the mLAMP assay individually, including the concentrations of primers and reagents and reaction temperature and time. Optimal mLAMP conditions were determined from the strongest ladder-like pattern following 2% AGE. The optimal concentration ratios of F3/B3 to FIP/BIP (*siiA*, *ITS*, *nuc*) were 1:8, 1:7, and 1:9, respectively (Supplemental File S1 and Figure S1; <https://doi.org/10.3168/jds.2019-17538>). The optimal mLAMP reaction was obtained with 0.9 μL of *Bst* 2.0 WarmStart DNA polymerase, 4 mM MgSO_4 , and 1.6 mM dNTP (Supplemental Figure S2; <https://doi.org/10.3168/jds.2019-17538>) at 64°C for 45 min (Supplemental Figure S3; <https://doi.org/10.3168/jds.2019-17538>). Based on our previous study (Zhao et al., 2017), we further opti-

mized the pH value and NaCl concentration of the running buffer to achieve sensitive and accurate detection. As shown in Supplemental Figure S4 (<https://doi.org/10.3168/jds.2019-17538>), detection signals on test lines were enhanced at pH 9 compared with that pH 7.5 and pH 8. However, more false-positive results occurred at pH 9. To solve this problem, the NaCl concentration of the running buffer was optimized (Supplemental Figure S4). The color of the test lines decreased with increasing NaCl concentration. When the NaCl concentration was 50 mM, the false-positive signal was completely eliminated without affecting detection of positive samples. Thus, the best formula for running buffer was 50 mM NaCl at pH 9.

As described above, we established the mLAMP-LFD detection method and used mLAMP-AGE and mLAMP-LFD to detect different target strains; the results are shown in Figure 2.

Sensitivity of mLAMP-LFD

To determine the sensitivity, genomic DNA preparations of *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* ranging from 10^8 to 10^{-2} cfu/mL were detected by mLAMP-LFD and mLAMP-AGE. As shown in Figure 3, weak bands were observed in lane 10 with detection by AGE and LFD. Thus, the limits of detection (LOD) of LFD and AGE are the same; both

Figure 2. The multiplex loop-mediated isothermal amplification (mLAMP) method with detection of target strains by agarose gel electrophoresis (mLAMP-AGE; A) and by lateral flow dipstick (mLAMP-LFD; B). M = 2,000-bp DNA marker; lane 1 = negative control; lane 2 = genomic DNA of *Salmonella* Typhimurium ATCC 14028; lane 3 = genomic DNA of *Cronobacter sakazakii* ATCC 29544; lane 4 = genomic DNA of *Staphylococcus aureus* ATCC 13565; lane 5 = genomic DNA of all 3 bacteria. T1, T2, and T3 are the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3); C is the control line.

could detect 7, 2.1, and 4.5 cfu/mL of *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus*, respectively.

For artificially contaminated PIF samples with varying concentrations of 3 target bacteria, the LOD of mLAMP-LFD were consistent with the LOD mLAMP-AGE, which were 4.2, 2.6, and 3.4 cfu/g for *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus*, respectively (Figure 4). However, in Figure 4, only the LFD method

Figure 3. Sensitivity of the multiplex loop-mediated isothermal amplification (mLAMP) methods for pure bacterial culture. (A) Detection of mLAMP products by agarose gel electrophoresis; (B) detection of mLAMP products by lateral flow dipstick (LFD); (C) analysis of gray values of lines on LFD. DPI = dots per inch. M = 2,000-bp DNA marker; lane 1 = negative control; lanes 2 to 12 = genomic DNA of serial concentrations of the *Salmonella* Typhimurium ATCC 14028, *Cronobacter sakazakii* ATCC 29544, and *Staphylococcus aureus* ATCC 13565, ranging from 3.7×10^8 to 3.7×10^{-2} cfu/mL, 2.1×10^8 to 2.1×10^{-2} cfu/mL, and 4.5×10^8 to 4.5×10^{-2} cfu/mL, respectively. T1, T2, and T3 are the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3); C is the control line.

Figure 4. Sensitivity of the multiplex loop-mediated isothermal amplification (mLAMP) methods for powdered infant formula. (A) Detection of mLAMP products by agarose gel electrophoresis; (B) detection of mLAMP products by lateral flow dipstick (LFD); (C) analysis of gray values of lines on LFD. DPI = dots per inch. M = 2,000-bp DNA marker; lane 1 = negative control; lanes 2 to 12 = genomic DNA of serial concentrations of the *Salmonella* Typhimurium ATCC 14028, *Cronobacter sakazakii* ATCC 29544, and *Staphylococcus aureus* ATCC 13565, ranging from 4.2×10^8 to 4.2×10^{-2} cfu/g, 2.6×10^8 to 2.6×10^{-2} cfu/g, and 3.4×10^8 to 3.4×10^{-2} cfu/g, respectively. T1, T2, and T3 are the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3); C is the control line.

could clearly distinguish between different species when using multiplex LAMP reactions to produce amplicons.

Specificity of mLAMP-LFD

The mLAMP primers are the main factors affecting the specificity of mLAMP-LFD. Thus, to validate the specificity of the mLAMP-LFD method described here, we considered 2 criteria. First, we validated the

specificity of the mLAMP method by testing 42 different bacterial strains. The results showed that mLAMP-AGE and mLAMP-LFD could detect 13 *Salmonella* spp., 8 *Cronobacter* spp., and 6 *Staph. aureus* strains but did not detect 15 nontarget bacterial strains (Table 1). These results confirmed that the mLAMP-LFD method established here had high specificity for target bacteria. In addition, cross-reactivity was validated by testing the 3 target bacteria with all LAMP primers and without target-specific primers, respectively. The mLAMP reaction without target-specific primers did not detect the corresponding target-specific DNA. Conversely, when all 3 sets of mLAMP primers were included, successful detection of the target-specific DNA was achieved, with no nonspecific detection observed

(Figure 5). These results indicate that the mLAMP-LFD method showed no cross-reactivity among primers. Thus, the mLAMP-LFD method can detect and differentiate the 3 target bacteria with high specificity.

Stability of the LFD

We verified the stability of the prepared LFD by using an accelerated aging test. As shown in Figure 6, for LFD stored at 45°C for 30 d, no false-positive results were observed for negative samples and the brightness of the 3 test lines did not change significantly for positive samples. Therefore, storage for 1 mo at 45°C did not affect detection performance of LFD, demonstrating stability of the LFD.

Figure 5. Specificity of the multiplex loop-mediated isothermal amplification (mLAMP)-lateral flow dipstick (LFD) method. Lanes 1, 2 = genomic DNA of *Salmonella* Typhimurium ATCC 14028 amplified by mLAMP without and with *Salmonella* target-specific primers, respectively; lanes 3, 4 = genomic DNA of *Cronobacter sakazakii* ATCC 29544 amplified by mLAMP without and with *C. sakazakii* target-specific primers, respectively; lanes 5, 6 = genomic DNA of *Staphylococcus aureus* ATCC 13565 amplified by mLAMP without and with *Staph. aureus* target-specific primers, respectively; lanes 7, 8: negative control and template DNA from the 3 target strains detected by mLAMP-LFD. + (-): component present (absent) in mLAMP reaction. T1, T2, and T3 are the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3); C is the control line.

Table 3. Detection of real samples (artificially contaminated with a certain pathogen) to evaluate the performance of the multiplex loop-mediated isothermal amplification (mLAMP) method in the artificially contaminated powdered infant formula (PIF) samples (values 20 and 40 indicate the number of test samples)

Method ¹	PIF contamination				Specificity (%)
	<i>Salmonella</i>	<i>Cronobacter</i>	<i>Staphylococcus aureus</i>	Negative	
mLAMP-AGE	20	20	20	40	100
mLAMP-LFD	20	20	20	40	100

¹mLAMP-AGE = detection by mLAMP combined with agarose gel electrophoresis (AGE); mLAMP-LFD = detection by mLAMP combined with lateral flow dipstick (LFD).

Application of mLAMP-LFD Assay in PIF

To determine the feasibility of the mLAMP-LFD method in PIF samples, we tested 60 positive samples that were artificially contaminated with 1 of 3 different species (20 each for *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus*) and 40 negative samples using the mLAMP-LFD method. All positive samples produced the corresponding test line along with the control line on the LFD. All negative samples only produce the control line of the LFD (Table 3 and Supplemental Figure S5, <https://doi.org/10.3168/jds.2019-17538>), verifying that the mLAMP-LFD method can be effectively applied to detect 3 different bacterial species in PIF samples.

DISCUSSION

Salmonella spp., *Cronobacter* spp., and *Staph. aureus* are highly prevalent food-borne pathogens globally that cause infant health problems by contaminating PIF

(Drudy et al., 2006; Moussa et al., 2010; Wang et al., 2012b). Because of the low efficiency and high cost of single-pathogen detection, the development of multiple detection systems has great application prospects (Li and Macdonald, 2016). The LAMP technology has been developed and applied to diverse pathogens because of its high sensitivity and specificity and outstanding efficiency. To date, several target genes have been used in different molecular biology assays for the detection of *Salmonella*. Among these genes, the *siuA* gene located on *Salmonella* pathogenicity island 4 (SPI 4), which plays a crucial role in the survival of macrophages and invasion of polarized cells, has excellent specificity (Gerlach et al., 2008). The internal transcribed spacers (ITS) of 16S-23S rDNA are the most commonly used target sequence in bacterial taxonomy, and the length and sequence vary with different bacterial species. Therefore, the ITS sequence of *Cronobacter* can be used to design specific primers for the whole genus (Ruan et al., 2013). The *nuc* gene, which encodes an extracellular enzyme unique to *Staph. aureus*, was selected because it has been widely used in the molecular detection of the species (Wang et al., 2017).

The development of multiple LAMP-LFD assays is not a simple process and is more challenging than designing single assays. First, to avoid false-negative results, mLAMP must be optimized extensively, including the appropriate quantity of reagents and the concentration ratio for each primer. To determine the optimal primer concentrations, it is necessary to determine the ratio of the outer primer to the inner primer in a single LAMP assay, and then halve it to optimize the double and triple LAMP primers (Shao et al., 2011). Second, the composition of the running buffer affects the accuracy, sensitivity, specificity, and stability of the LFD assay. Currently, PBS (Nurul Najian et al., 2016), Tris (Rivas et al., 2015), saline sodium citrate (Chen et al., 2016), sodium carbonate (Jung et al., 2015), and borate buffer (Peng et al., 2014) have been used as LFD running buffers. Notably, the Tris buffer selected in our study has unique advantages for preventing DNA degradation and dissolution (Rivas et al., 2015). Sucrose and

Figure 6. Stability of multiplex loop-mediated isothermal amplification (mLAMP)-lateral flow dipstick (LFD) method. Lanes 1, 3, 5, and 7 = LFD stored at 45°C for 0, 10, 20, and 30 d and then used to detect mLAMP products of negative control; lanes 2, 4, 6, and 8 = LFD stored at 45°C for 0, 10, 20, and 30 d and used to detect mLAMP products of the 3 target strains. T1, T2, and T3 are the test lines coated with streptavidin (T1), anti-digoxin antibody (T2), and anti-Texas Red antibody (T3); C is the control line.

trehalose have been used to stabilize AuNPs labeled with antibody conjugates. However, Wang et al. (2013) indicated that trehalose can better maintain conjugate stability than sucrose during storage. Therefore, trehalose was selected in this study to maintain the stability of LFD. Surfactant (Tween-20) has an important role in alleviating nonspecific reactions in the detection zone, inducing better flow of AuNPs on the membrane, and promoting the release of colloidal gold from conjugated mats (Kalogianni et al., 2009; Mao et al., 2009; Hua et al., 2012). Based on these factors, we optimized the composition of the LFD running buffer.

Using the mLAMP-LFD method, the total time for simultaneous detection of *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* was only 57 min. Furthermore, the results can be monitored clearly and easily in just 10 min (the time to detect the mLAMP products by LFD, including 5 min for incubating products with running buffer and 5 min for LFD standing and reading, which is included in the 57 min). Jiang et al. (2012) constructed mLAMP detection systems for simultaneous detection of *Shigella*, *Salmonella*, and *Staph. aureus* in food using AGE to visualize results. However, the AGE method used in that study is time consuming and cannot distinguish the types of amplicons; moreover, the ethidium bromide reagent required in the AGE method is carcinogenic to humans.

During the transportation and storage of LFD, the activity of antigens and antibodies can be affected by environmental factors such as temperature and humidity, resulting in a rapid decrease in the stability of LFD (Peng et al., 2014). In the development process, it is impractical to place the LFD at room temperature for 1 to 2 yr to evaluate its stability. Consequently, to evaluate their stability, we placed the test strips in a high temperature environment for an accelerated aging test. According to the Arrhenius equation (Wang et al., 2009), we estimated that LFD can be stored for more than 1 yr at room temperature or at 4°C for more than 2 yr. Thus, the LAMP-LFD method herein developed is rapid, reliable, practical, and stable.

CONCLUSIONS

The mLAMP-LFD method described here is suitable for rapid, simple, convenient, and sensitive simultaneous detection of *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* in PIF and dairy food systems that are crucial to the economy of China and many other countries. Compared with conventional mLAMP, our detection method is simple and convenient. At the same time, the method shows high specificity (100%) with a detection limit of $<10^1$ cfu/mL and can be read within 57 min. This method provides a rapid, sensitive, simple,

and effective detection system for *Salmonella* spp., *Cronobacter* spp., and *Staph. aureus* in PIF samples and be suitable for point-of-care testing applications in dairy foods.

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