Salmonella detection in powdered dairy products using a novel molecular tool

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ABSTRACT

In this study, we developed a rapid, specific, and sensitive loop-mediated isothermal amplification technique combined with a lateral flow dipstick (LAMP-LFD) method to detect *Salmonella* targeting the *stx*A gene in powdered infant formula (PIF). The specificity of the detection method (LAMP-LFD) approached 100% using 21 *Salmonella* and 31 non-*Salmonella* bacterial strains. This detection method exhibited high sensitivity limits for pure cultures at 3.7 cfu/mL and in PIF at 2.2 cfu/g without enrichment. To evaluate the applicability of the LAMP-LFD method, we detected 60 positive PIF samples and 20 negative PIF samples. The results showed that the method of LAMP-LFD had a high diagnostic specificity of 100% for detection of *Salmonella* in PIF. To reduce incidence of LAMP contamination, we applied propidium monoazide (PMA) to eliminate carryover contamination of LAMP. At the same time, we found that PMA does not affect observation of LFD for measurement of LAMP signal. The results verified that the method of LAMP-LFD targeting the *stx*A gene is rapid, accurate, and sensitive for *Salmonella* detection in PIF, and that PMA shows great potential to be widely used to eliminate the amplicon contamination risk generated by the highly sensitive LAMP reaction in the detection process.

Key words: loop-mediated isothermal amplification, lateral flow dipstick, *Salmonella*, powdered infant formula

INTRODUCTION

Salmonella is one of the most common foodborne pathogenic bacteria worldwide, infecting humans and a variety of other animals via their respective habitats, feeding activities, or reservoirs (Zhao et al., 2003; Alves et al., 2015). Thus, *Salmonella* can infect humans by

improper cooking and processing of foods of animal origin such as raw milk, meat, and eggs (Moussa et al., 2010). If ingested, *Salmonella* may cause diseases such as typhoid fever and salmonellosis (Salzman et al., 2003; Zhang et al., 2003). Previous studies have reported that powdered infant formula (PIF) is a newly identified source of *Salmonella* infection in infants, leaving the dairy industry with a high priority to develop innovative means to detect *Salmonella* in PIF (Cahill et al., 2008).

Conventional culture-based methods for detection of *Salmonella* require 2 to 3 d (Salam et al., 2013) and are not suitable for rapid detection of *Salmonella*. In recent years, many methods have been developed to rapidly detect nucleic acid targets of *Salmonella*, including PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP; Zhao et al., 2010; Maciel et al., 2011). The methods of PCR and real-time PCR have many disadvantages, including a relatively high cost for equipment and consumables, and the need for trained personnel. However, LAMP is based on a nucleic acid amplification approach developed by Notomi et al. (2000). It may generate up to 10⁹ copies of the target DNA within 1 h using a specific stem-loop structure based on auto-cycling strand displacement and isothermal conditions (60 to 65°C; Notomi et al., 2000). Therefore, LAMP is a fast, isothermal process (requiring only a heat block) and is robust, offering greater sensitivity than other comparable methods (Mori and Notomi, 2009). Thus, it is a method with great promise for food safety applications, or clinical diagnostics, especially for resource-poor laboratories (Dhama et al., 2014; Kokkinos et al., 2014). Many different monitoring methods are available for results of LAMP, including agarose gel electrophoresis (AGE), real-time turbidity measurement (Wang et al., 2012), electrochemical methods (Safavieh et al., 2012), lateral flow dipstick (LFD; Jung et al., 2015), or ELISA-based quantification (Ravan and Yazdanparast, 2012). Previous studies have reported that the visual detection method of LFD have many advantages such as rapid speed, long-term stability, accuracy, user-friendly format, and not requiring expensive laboratory equipment (Peng et al., 2014).

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Thus, LFD is the preferred method and has attracted significant attention (Rigano et al., 2014).

Many target genes have been used to detect *Salmonella* by different methods. Some, such as *iroB*, *hilA*, *rfb*, *opmC*, and *invA* are occasionally detected in some non-*Salmonella* bacteria (Luk et al., 1993; Bäumler et al., 1997; Ziemer and Steadham, 2003; De Clercq et al., 2007; Chen et al., 2011). Conversely, genes such as *fimA*, *iroB*, *pipA*, and *stn* may not be detected in all *Salmonella* strains (Bäumler et al., 1997; Khoo et al., 2009; Smith et al., 2010; Wang et al., 2013). It is worth mentioning here that *invA* encodes a *Salmonella* invasion protein and is thus considered a virulence gene located on *Salmonella* pathogenicity island (SPI) 1, so it is frequently used to detect *Salmonella* (Chen et al., 2011). However, several studies have shown that some *Salmonella* strains including the important strain of *Salmonella* Kentucky lack this gene (Turki et al., 2012). Therefore, a more specific and conserved gene is needed as an ideal target for accurate detection of *Salmonella* in food. The *Salmonella* SPI4 has an important role in intra-macrophage survival and cooperation with the SPI for invasion (Gerlach et al., 2008). The SPI4 region includes genes from *siiA* to *siiF* that are important for adhesion to polarized epithelial cells (Kiss et al., 2007). Wille et al. (2014) reported that *siiA* and *siiB* encode novel type I secretion system subunits that have important roles in controlling the SPI4-mediated adhesion of *Salmonella enterica*. Also, the *siiA*-regulatory protein is essential for SPI4-encoded type I secretion system function and can affect the expression of *Salmonella* virulence genes (Gerlach et al., 2007; Wille et al., 2014). Therefore, *siiA* has an extremely important role in the field of *Salmonella* pathogenesis. Hassena et al. (2015) used the novel gene of *siiA* to detect *Salmonella* by the conventional method of real-time PCR (Hassena et al., 2015). To the best of our knowledge, this is the first report using the LAMP and LAMP-LFD methods to specifically target the *siiA* gene for detection of *Salmonella* in a dairy food safety application.

It is well known that after repeated experiments, spurious aerosolized LAMP amplicons of various lengths may be present in pipettes, equipment, reaction reagents, the working environment, and even experimenters' skin (He and Xu, 2011; Kil et al., 2015). Amplicon amounts less than 10 ag have been successfully used to simulate the level of aerosol droplets, confirming contamination (Hsieh et al., 2014). Therefore, the high sensitivity of LAMP might also become its largest potential disadvantage (Ou et al., 2014). Also, it is of great significance to eliminate any contamination from previous LAMP reactions. Until now, some methods to eliminate carryover contamination have been established, such as the use of separate rooms for each step

of LAMP, 5% sodium hypochlorite solution to wipe the surface of the entire experimental environment, UV light (Ou et al., 2014), uracil-DNA-glycosylase (Hsieh et al., 2014), a closed tube with an agar dye capsule (Karthik et al., 2014), and overlaying the top of the LAMP reaction mixture with sterile mineral oil (Wu et al., 2014). Although the results of these methods are credible and effective, it could be time-consuming, labor-intensive, occasionally inhibitory to LAMP amplification, and unable to eliminate the contaminants from the environment. It is well known that propidium monoazide (PMA) has been applied in selective detection of viable bacteria and removal of exogenous DNA signal in quantitative PCR experiments (Elizaquível et al., 2012; Schnetzinger et al., 2013). Therefore, PMA appears to be useful for the elimination of LAMP carryover contamination.

In this study, we used the *siiA* gene to develop a rapid, specific, and sensitive LAMP-LFD method for the detection of *Salmonella* in PIF and applied PMA to eliminate the LAMP carryover contamination. Thus, the aim of this study was to provide an accurate, fast, sensitive, and convenient detection system for the detection of *Salmonella* in the dairy industry.

MATERIALS AND METHODS

Bacterial Strains and DNA Extraction

A total of 50 bacterial strains including 21 *Salmonella* strains and 31 non-*Salmonella* strains were used to evaluate the specificity of the LAMP method (Tables 1 and 2). All *Salmonella* strains were grown in 20 mL of NB medium at 37°C shaking at 5 × g for 8 h to achieve late-log phase (10⁸ cfu/mL). Non-*Salmonella* strains were cultured in 20 mL of TSB medium under similar conditions.

In this study, *Salmonella* Typhimurium ATCC 14028 was used as the positive control and the template DNA was extracted from 1 mL of bacterial culture by the Gen Elute Bacterial Genomic DNA Kit (TIANGE, Beijing, China) following the manufacturer's instructions (<http://www.tiange.com/?productShow/t1/1/id/18.html>) and suspended in 100 µL of Tris-EDTA buffer. Templates were stored at -20°C until further use.

LAMP Target Sequence and Primer Design

The LAMP primers were designed based on the conserved sequence between the *Salmonella* *siiA* gene (STM4257, GenBank accession number: NC_003197) and the 7 different strains of *Salmonella* Pathogenicity Island 4 genomic sequence (GenBank accession numbers: AJ576316, KP234069.1, KP234070, KP234071,

KP234072, KP234073, and KP234074.1). A set of 4 primers including 2 outer primers (**F3** and **B3**) and 2 inner primers (**FIP** and **BIP**) were designed by the PrimerExplorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>). Then the specificity of the amplified fragment and all primers was assessed by searching the basic local alignment search tool (BLAST) in the National Center for Biotechnology and Information. The 2 inner primers (FIP and BIP) were labeled with fluorescein isothiocyanate (**FITC**) and biotin at the 5'-end, respectively. The primer sequences were shown in Table 3 and Figure 1. All the primers were synthesized commercially by Invitrogen (Shanghai, China).

LAMP Assay

Each LAMP reaction mixture included 0.2 μ M F3 and B3, 1.6 μ M FIP and BIP primers, 2.5 μ L of 10 $\times$ ThermoPol Buffer (New England BioLabs, Ipswich, MA), 1.8 mM each deoxynucleoside triphosphate, 4 mM MgSO_4 , 0.9 μ L of 8 U/ μ L *Bst* 2.0 WarmStart DNA polymerase (New England BioLabs), 2 μ L of the target DNA (68.5 ng/ μ L), and nuclease-free water up to 25 μ L. A conventional water bath was used to incubate the LAMP assay reactions. Then, the reagent mixture was incubated at 65°C for 45 min in a water bath, followed by 80°C for 2 min to terminate each reaction.

Lateral Flow Dipstick Setup and Assay

Reagents and Instrumentation. Bovine serum albumin and sucrose were obtained from Sangon Biotech (Shanghai, China). The anti-FITC were acquired from Santa Cruz Biotechnology (Shanghai, China). The streptavidin was purchased from Sigma (St. Louis, MO) and anti-mouse secondary antibody was obtained from Bioss (Beijing, China). The nitrocellulose (**NC**) membranes were purchased from Sartorius (Göttingen, Germany). The conjugate pad, sample pad, absorption pad, and the plastic backing were acquired from Ahlstrom (Helsinki, Finland). The LFD cutter and sprayer were obtained by Autokun (Hangzhou, China) and Biodot (Irvine, CA), respectively.

Synthesis of Gold Nanoparticles Conjugated with Antibody. The gold nanoparticles (**AuNP**, 30 nm) were obtained by the YiRui (Shenzhen, China). The conjugations were produced as follows: 3 μ L of the 0.2 M K_2CO_3 was added into 1-mL AuNP solutions and sufficiently mixed to regulate the pH of AuNP, then 4.5 μ L of the anti-FITC (1 mg/mL) was added and incubated at room temperature for 15 min, after which 100 μ L of 10% BSA was mixed and incubated at room temperature for 15 min. Each solution was centrifuged at 12,000 $\times g$ for 15 min at 4°C. Subsequently, the dark red sediments (anti-FITC-AuNP conjugates) were

Table 1. Strains of *Salmonella* used for this study and the results for the loop-mediated isothermal amplification (LAMP) detection methods

No.	Bacterial species	Source ¹	Result ²	
			LAMP-AGE	LAMP-LFD
1	<i>Salmonella</i> Typhimurium	ATCC 14028	+	+
2	<i>Salmonella</i> Typhimurium	ATCC 23564	+	+
3	<i>Salmonella</i> Typhimurium	Laboratory	+	+
4	<i>Salmonella</i> Typhimurium	Laboratory	+	+
5	<i>Salmonella</i> Dublin	CMCC 50042	+	+
6	<i>Salmonella</i> Arizona	CMCC(B) 47001	+	+
7	<i>Salmonella enterica</i> ssp. <i>salamae</i>	ATCC 43972	+	+
8	<i>Salmonella</i> Choleraesuis	CMCC 50018	+	+
9	<i>Salmonella</i> Pullorum	ATCC 9120	+	+
10	<i>Salmonella</i> Pullorum	Laboratory	+	+
11	<i>Salmonella</i> Pullorum	Laboratory	+	+
12	<i>Salmonella</i> Pullorum	Laboratory	+	+
13	<i>Salmonella</i> Pullorum	Laboratory	+	+
14	<i>Salmonella</i> Enteritidis	ATCC 13076	+	+
15	<i>Salmonella</i> Enteritidis	Laboratory	+	+
16	<i>Salmonella</i> Enteritidis	Laboratory	+	+
17	<i>Salmonella</i> Enterica paratyphi B	CMCC(B) 50094	+	+
18	<i>Salmonella</i> Typhi	CMCC(B) 50071	+	+
19	<i>Salmonella</i> Typhi	ATCC 19430	+	+
20	<i>Salmonella</i> Paratyphi A	CMCC(B) 50093	+	+
21	<i>Salmonella</i> Thompson	CMCC 50023	+	+

¹ATCC = American Type Culture Collection; CMCC = National Center for Medical Culture Collections.

²LAMP-AGE = the detection method of loop-mediated isothermal amplification-agarose gel electrophoresis. LAMP-LFD = the detection method of loop-mediated isothermal amplification-lateral flow dipstick.

³+ = positive result.

Table 2. Strains of non-*Salmonella* used for this study and the results of the loop-mediated isothermal amplification (LAMP) detection methods

No.	Bacterial species	Source ¹	Result ²	
			LAMP-AGE	LAMP-LFD
1	<i>Staphylococcus aureus</i>	CMCC 26074	— ³	—
2	<i>Staph. aureus</i>	CMCC 26075	—	—
3	<i>Staph. aureus</i>	ATCC 25923	—	—
4	<i>Staph. aureus</i>	ATCC 29213	—	—
5	<i>Staph. aureus</i>	ATCC 12600	—	—
6	<i>Staph. aureus</i>	ATCC 13565	—	—
7	<i>Staph. aureus</i>	ATCC 12598	—	—
8	<i>Bacillus cereus</i>	CMCC 63303	—	—
9	<i>B. cereus</i>	ATCC 14579	—	—
10	<i>Cronobacter sakazakii</i>	ATCC 29544	—	—
11	<i>C. sakazakii</i>	ATCC 51329	—	—
12	<i>Escherichia coli</i>	ATCC 25922	—	—
13	<i>Shigella flexneri</i>	CMCC 51572	—	—
14	<i>Listeria innocua</i>	ATCC 33090	—	—
15	<i>Staphylococcus epidermidis</i>	ATCC 12228	—	—
16	<i>Listeria monocytogenes</i>	CMCC 54004	—	—
17	<i>L. monocytogenes</i>	CMCC 54005	—	—
18	<i>L. monocytogenes</i>	CMCC 54006	—	—
19	<i>Micrococcus luteus</i>	Laboratory	—	—
20	<i>Enterococcus faecalis</i>	Laboratory	—	—
21	<i>Shigella flexneri</i>	CICC 21678	—	—
22	<i>Bacillus subtilis</i>	Laboratory	—	—
23	<i>E. coli</i> O157:H7	CICC 21530	—	—
24	<i>L. monocytogenes</i>	ATCC 19114	—	—
25	<i>L. monocytogenes</i>	ATCC 19116	—	—
26	<i>L. monocytogenes</i>	ATCC 19112	—	—
27	<i>Listeria welshimeri</i>	ATCC 43550	—	—
28	<i>L. welshimeri</i>	ATCC 43548	—	—
29	<i>Vibrio parahaemolyticus</i>	ATCC 33847	—	—
30	<i>V. parahaemolyticus</i>	ATCC 17802	—	—
31	<i>Staphylococcus xylosum</i>	ATCC 29971	—	—

¹ATCC = American Type Culture Collection; CMCC = National Center for Medical Culture Collections.²AGE = agarose gel electrophoresis; LFD = lateral flow dipstick.³— = negative result.

suspended in 100 μ L of buffer [containing 100 mM Tris pH 8.0, 0.2% BSA (wt/vol), and 4% trehalose (wt/vol)] and stored at 4°C before being used.

Assembly of the LFD. The schematic diagram of the LFD is illustrated in Figure 2. The LFD consists of 4 components: sample pad, conjugate pad, detection region with control line and test line, and absorbent pad. The sample and conjugate pad were made from glass fiber and saturated with the running buffer containing 100 mM Tris (pH 8.0), 0.2% BSA (wt/vol), 0.05%

casein (wt/vol), 4% trehalose (wt/vol), 0.5% Tween 20, and 0.05% sodium azide (wt/vol). Then the sample and conjugate pad were dried at 45°C for 2 h. After that the conjugate pad was sprayed with the anti-FITC-AuNP conjugate and dried at 37°C for 2 h. Streptavidin (5 mg/mL, dissolved in 100 mM Tris, pH 7.5) and anti-mouse secondary antibody (1 mg/mL) were sprayed with 1.0 μ L/cm onto the NC membrane to form the test line and the control line on the detection region, respectively. Each distance between 2 adjacent detec-

Table 3. The sequences of loop-mediated isothermal amplification (LAMP) primers for the *siiA* gene of *Salmonella*

Primer ¹	Sequence (5'–3')	Item
siiA-F3	AGAAGCTTTTCAGAAGAGGA	
siiA-B3	GATAATAGCTTACTCGTTGTTACT	
siiA-FIP	CTGCCTGAGGAATAACCATTTCTATAATAAAGGCTTGTTAGAAAGACA	5' modified biotin
siiA-BIP	TACGACTGGGATATGAACGGGACTCATGTCAATAACTACATCAG	5' modified FITC ²

¹A set of 4 primers including 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP).²FITC = fluorescein isothiocyanate.

Figure 1. The target sequences for loop-mediated isothermal amplification (LAMP). The boxes and arrows show the sequences and direction of the LAMP primers. Color version available online.

tion lines was 1 cm and the NC membrane was then air-dried at room temperature. To assemble the test strip, the NC membrane was pasted on the center of the plastic backing and covered 2 mm with the absorbent pad and the conjugate pad on each side. The absorbent pad was on one end of the test strip. The other side of conjugate pad was covered 2 mm with the sample pad to the other end of the test strip. Then the LFD were cut to a width of 3 mm and stored at 4°C in a desiccated container.

The LAMP-LFD detection method was prepared by mixing 10 µL of LAMP amplicons and 90 µL of running buffer, then immersing the LFD into the mixture. The result was visualized within 15 min. If the detection region presents 2 red lines (test line and control line), the result was confirmed to be positive. Only the control line appears as red in a negative result.

PCR Assay

The F3 and B3 of the LAMP outer primers (Table 3) were used as the PCR primers to detect *Salmonella* with an amplicon length of 240 bp. The composition of the PCR reaction mixture included 12.5 µL of 2× Taq DNA polymerase buffer (TIANGE), 0.4 µM of the outer primers F3 and B3, 2 µL of the prepared template DNA (68.5 ng/µL), and nuclease-free water up to a final volume of 25 µL. The PCR reactions were performed in the Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA) with the following steps:

an initial denaturation at 94°C for 3 min, each of the 30 cycles containing a denaturation at 94°C for 30 s, an annealing at 58°C for 45 s, and an elongation at 72°C for 1 min, with a final elongation at 72°C for 5 min. The results were observed by the 1% AGE.

Specificity of LAMP-LFD

Primer specificity was tested by amplifying 52 bacterial strains under optimum reaction conditions and observed by the LAMP-AGE and LAMP-LFD detection methods. All experiments were repeated 3 times.

Assay Sensitivity for Pure Bacterial Culture and Powdered Infant Formula

The *Salmonella* Typhimurium ATCC 14028 was aerobically cultured 8 h at 37°C (5 × g) to achieve late-log phase. Then, sterilized 0.85% normal saline was used to 10-fold serially dilute the *Salmonella* Typhimurium ATCC 14028 to achieve concentrations ranging from 3.7×10^8 to 3.7×10^{-2} cfu/mL and the density was evaluated by the standard plate counting method. Afterward, the different concentrations of genomic DNA were extracted by the method earlier described. Aliquots of the 2-µL template DNA were used to detect the sensitivity of LAMP-LFD and PCR methods.

The PIF samples were purchased from a local retailer in Harbin, China, and the method of ISO 6579:2002 was used to determine that the samples were not con-

taminated with *Salmonella*. After that 1 mL aliquots of the serial 10-fold dilutions of the target bacterial *Salmonella* Typhimurium ATCC 14028 was mixed with 8 mL of buffer peptone water (BPW), and then 1-g aliquots of PIF were added into the mixture to form the serial concentrations ranging from 2.2×10^8 to 2.2×10^{-2} cfu/g. Then, the template DNA of 1 mL of the different concentrations of reconstituted PIF were extracted via the method previously described. Aliquots of the 2- μ L template DNA were used to detect the sensitivity of the LAMP-LFD and PCR methods in PIF without enrichment.

PMA Treatment for Carryover Contamination of LAMP

The stock solution of PMA (20 mM in nuclease-free water; Biotium Inc., Hayward, CA) was diluted in nuclease-free water to achieve different concentrations. The principle of PMA to eliminate the carryover contamination of LAMP is shown in Figure 3. Various concentrations of PMA (18 μ L) were mixed with 2 μ L of different concentrations of LAMP products (1,000 ng/ μ L, 100 ng/ μ L, 10 ng/ μ L, 10 pg/ μ L, 10 fg/ μ L, and 10 ag/ μ L), respectively. In other words, the mass of

Figure 2. The schematic illustration of the lateral flow dipstick. FITC = fluorescein isothiocyanate; AuNP = gold nanoparticles; FIP and BIP = 2 inner primers. Color version available online.

Figure 3. The principle of propidium monoazide (PMA) to eliminate the carryover contamination of loop-mediated isothermal amplification (LAMP). The treatment process includes 2 stages. In stage 1, PMA touches the LAMP amplicons in a contaminated environment at ambient temperature and then is exposed to halogen light of 1×10^5 lx to activate PMA. The photoreactive azide group in PMA is converted to an active nitrene group. In stage 2, the LAMP amplicons in the contaminated environment are covalently linked with the activated PMA permanently and will not be amplified, whereas the residual active PMA will become the hydroxylamine by reacting with water, with no capability to covalently bind to LAMP amplicons. Color version available online.

2 μ L of different concentrations of LAMP products is equivalent to the mass ranging from 2,000 ng (a level 10^{11} times higher than the carryover contamination of aerosol droplets) to 20 ag (attograms; the level of the carryover contamination of aerosol droplets). The mixtures (the various concentrations of PMA mixed with the different concentrations of LAMP products) were mixed in a 0.2-mL transparent microcentrifuge tube, and the tubes were placed horizontally at ambient temperature and exposed to a 650-W halogen light source at a distance of 20 cm (illuminance = 111,500 lx) for 2 min. The illuminance was measured by an LX-101 Digital Light Lux Meter (ESUN, Kaohsiung City, Taiwan). To evaluate the results, 2- μ L aliquots of the treated mixtures were subjected to LAMP amplification. The results were analyzed by electrophoresis on a 2% agarose gel.

Photolysis of PMA

It is crucial to test that excessive levels of PMA can undergo complete photolysis under the light exposure conditions used here, and such inactivation of PMA does not affect amplification reactions. The LAMP amplicons were added to 20 μ L of the different con-

centrations (450, 126, 90, 63, and 45 μ M) of PMA solutions to reach the final concentrations of 10 ag/ μ L, followed by exposing the different concentrations of PMA to a illuminance of 111,500 lx at 10 min, at intervals to 120 min. Mixtures were treated as mentioned above on ice to induce PMA crosslinking with the LAMP amplicons. Then aliquots of the 2 μ L mixtures were used as templates for LAMP reactions and the results were observed by electrophoresis on a 2% agarose gel.

PMA Applied in LAMP-LFD Detection

We used 18 μ L of nuclease-free water and 140 μ M PMA mixed with 2 μ L of LAMP products (at 5 ag/ μ L, the minimum mass of products can occur the LAMP amplification) and then exposed to light (111,500 lx) for 100 min, to demonstrate that the LAMP carryover contaminants treated by PMA can be reasonably applied in a practical test and the high concentration of PMA will not cause interference to the practical test results. Afterward, each of the 2- μ L mixtures was used as a template for re-amplification. Subsequently, a PMA-treated (18 μ L of 140 μ M PMA mixed with 2 μ L of 5 ag/ μ L of LAMP products exposed to 111,500 lx

for 100 min) mixture mixed with 1 ng/ μ L of the target DNA was used as a template for re-amplification. The results were observed by the detection methods of LAMP-AGE and LAMP-LFD.

RESULTS

Optimization of the LAMP Assay

To optimize the LAMP assay, we performed for the concentration of each reagents, reaction temperature, and reaction time. The optimal LAMP reaction conditions were detected by electrophoresis on 2% agarose gel and evaluated by the clearest characteristic ladder-like pattern. The results showed that the optimal ratio of outer primer to inner primer was 1:8 and the optimal conditions are 1.8 mM deoxynucleoside triphosphate, 4 mM MgSO_4 , and 0.9 μ L of *Bst* 2.0 WarmStart DNA polymerase (data not shown). The reaction was incubated at various temperature from 60 to 65°C for 1 h, respectively. When the reaction was incubated at 65°C, the LAMP products appeared to have the strongest signals (data not shown). Thus, the 65°C was selected as the optimal incubation temperature. Then the reaction was incubated for 15, 30, 45, 60, and 75 min, respectively. When the reaction was incubated for 45 min, the LAMP amplicons showed the clearest characteristic ladder-like pattern. Thus, we selected 45 min as the optimal reaction time (data not shown).

Optimization of the LFD Methods

To improve the sensitivity of the LAMP-LFD, we systematically optimized the LFD components by varying each parameter individually. In this study, we did a sensitivity test for each factor, and each parameter with the lowest sensitivity was chosen as the final LFD detection setting. The different concentrations of Tris (50, 100, 150, and 200 mM) were used to prepare the running buffer to determine the detection limits of LAMP-LFD, respectively. It was found that the Tris concentration of 100 mM obtained the lowest detection limits; thus, 100 mM Tris is the optimal concentration in running buffer (data not shown). Then the pH value of Tris was prepared ranging from 6.5 to 9.0 in running buffer to determine the detection limits, respectively. The result was shown that the pH 8.0 of Tris is the optimal condition for the running buffer. Following optimization, another factors of the running buffer are the 0.2% BSA (wt/vol), 0.05% casein (wt/vol), 4% trehalose (wt/vol), 0.5% Tween 20, and 0.05% sodium azide (wt/vol), which provided a stable environment for LFD (Figure 4).

Specificity and Sensitivity of LAMP-LFD for the Pure Culture

In all analysis replicates, the results of *Salmonella* strains appeared as the typical ladder-like structure using 2% AGE, and the LFD revealed all expected positive results (2 red lines in the detection region). At the same time, all the results of non-*Salmonella* strains did not elicit amplification, and the LFD detection region only displayed the single control line (Tables 1 and 2).

The LAMP-LFD and LAMP-AGE detection limit is the same, being 3.7×10^0 cfu/mL for the pure culture, whereas the detection limit of conventional PCR is 3.7×10^2 cfu/mL (Figure 5).

Figure 4. The methods of loop-mediated isothermal amplification-lateral flow dipstick (A) and loop-mediated isothermal amplification-agarose gel electrophoresis (B) for detection of *Salmonella* Typhimurium ATCC 14028. M: 2,000-bp DNA ladder; lane 1: double-distilled H_2O ; lane 2: genomic DNA of *Salmonella* Typhimurium ATCC 14028. Color version available online.

Figure 5. The sensitivity of different detection methods for the pure culture. (A) The detection method of loop-mediated isothermal amplification-agarose gel electrophoresis (LAMP-AGE). (B) The detection method of loop-mediated isothermal amplification-lateral flow dipstick (LAMP-LFD). (C) The detection method of conventional PCR. M: marker. Lanes 1 to 11: the genomic DNA of the concentrations of *Salmonella* Typhimurium ATCC 14028 ranging from 3.7×10^8 to 3.7×10^{-2} cfu/mL, respectively; lane 12: negative control (double-distilled H_2O).

Sensitivity and Applicability of the LAMP-LFD in Artificially Contaminated PIF

Three different methods have been reported to extract the template DNA in PIF: the boiling method (Li et al., 2016), the commercial Gen Elute Bacterial Genomic DNA extraction kit, and use of the chelating agent EDTA (Kumar and Mondal, 2015). We chose the best method to extract the template DNA in PIF by comparing the quality of the extraction DNA. The results verified that the commercial DNA extraction kit demonstrated the best extraction efficiency from PIF for detection of *Salmonella* (data not shown).

We selected PIF samples that were not already contaminated with *Salmonella* to artificially contaminated with varying densities of *Salmonella* Typhimurium ATCC 14028 without enrichment to determine the sensitivity of each detection method. The results showed that the detection methods of LAMP-AGE and LAMP-LFD have the same detection limit as low as 2.2×10^0 cfu/g and the PCR detection limit is 2.2×10^2 cfu/g (Figure 6).

To further determine the applicability of the LAMP-LFD method, we detected 60 positive PIF samples artificially contaminated with *Salmonella* Typhimurium ATCC 14028 and 20 negative PIF samples that were not contaminated with *Salmonella*, respectively. From the results, we concluded that all 60 positive PIF samples could be detected and all 20 negative PIF samples could not be detected. In other words, the method of LAMP-LFD had a high specificity (100%) for detection of *Salmonella* in PIF.

PMA Treatment and Carryover Contamination

We defined the lowest concentration without LAMP amplification bands as PMA concentration to eliminate the carryover contamination of LAMP. The final concentration (450, 126, 126, 90, 63, and 45 μM) of PMA can completely inhibit the re-amplifications of previous LAMP products (100 ng/ μL , 10 ng/ μL , 1 ng/ μL , 1 pg/ μL , 1 fg/ μL , and 1 ag/ μL), respectively (Figure 7). In other words, 18 μL of different concentrations (500, 140, 100, 70, and 50 μM) of PMA solutions can completely inhibit the re-amplifications of 2 μL of previous LAMP products (1,000 ng/ μL , 100 ng/ μL , 10 ng/ μL , 10 pg/ μL , 10 fg/ μL , and 10 ag/ μL), respectively.

PMA Photolysis

We used the LAMP amplicons (2- μL concentrations of 10 ag/ μL) as the template for re-amplification and defined PMA to be completely inactivated when the brightness of amplification bands was measured as be-

ing no lower than the positive control. In this study, we selected the shortest time for this as the photolysis time of PMA. Results showed that the concentration of 450 μM PMA does not completely undergo photolysis in less than 120 min; the other concentrations (126, 90, 63, and 45 μM) of PMA required 100, 70, 50, and 40 min to finish complete photolysis, respectively (Figure 8).

PMA Applied in LAMP-LFD Detection

The LAMP products (2 μL of 5 ag/ μL) treated by PMA did not result in re-amplification, but when treated by nuclease-free water, re-amplification did occur. The target DNA was successfully detected while the contaminants were removed simultaneously (Figure 9). These results verified that PMA can be effectively applied in eliminating LAMP carryover contaminants without affecting the LFD color observation. Meanwhile, the inactivated PMA has no detectable influence on the LAMP reaction efficiency.

DISCUSSION

Previous studies have reported that many LAMP-oriented methods can detect *Salmonella*, but with low specificity and not combined with the LFD method (Ziemer and Steadham, 2003; Soli et al., 2013). In this study, we used the very specific and conserved *Salmonella* target gene *siiA* to establish the LAMP-LFD detection method to detect *Salmonella* in PIF.

In previous studies, using real-time PCR (Hyeon et al., 2010) and LAMP-AGE (Zhang et al., 2012a) to detect *Salmonella* required about 2 h and 1 h 45 min, respectively. Meanwhile, the LAMP monitoring methods of SYBR Green I (Zhang et al., 2012a), calcein (Li et al., 2016), and real-time turbidity (Zhuang et al., 2014) achieve the results within 1 h, but require better detection conditions or expensive instruments. In our study, the detection time of the LAMP-LFD method to detect *Salmonella* is less than 1 h, and the condition requirements for monitoring the results are more general. Meanwhile, the sensitivity of LAMP-LFD for the pure cultures was 3.7 cfu/mL, which is lower than previously reported such as the detection limit of 8×10^2 cfu/mL (Zhang et al., 2012a), 6.3×10^3 cfu/mL (Zhang et al., 2012b), 5 cfu/mL (Zhuang et al., 2014), and 4.1 cfu/mL (Li et al., 2016). At the same time, we used the Bst 2.0 WarmStart DNA polymerase to improve the stability of the LAMP experiment and reduce the nonspecific amplification (Tanner et al., 2012). So the LAMP-LFD method we established is more rapid, convenient, and sensitive. To our best knowledge, no previous studies have used the LAMP method for detection of *Salmo-*

Figure 6. The sensitivity of different detection methods for artificial contamination of powdered infant formula (PIF). (A) The detection method of loop-mediated isothermal amplification-agarose gel electrophoresis (LAMP-AGE). (B) The detection method of loop-mediated isothermal amplification-lateral flow dipstick (LAMP-LFD). (C) The detection method of conventional PCR. M: marker. Lanes 1 to 11: the PIF samples were artificially contaminated with concentrations of the *Salmonella* Typhimurium ATCC 14028 ranging from 2.2×10^8 to 2.2×10^{-2} cfu/g, respectively; lane 12: negative control (double-distilled H₂O). Color version available online.

Figure 7. Concentration of propidium monoazide (PMA) eliminates carryover contamination of loop-mediated isothermal amplification (LAMP). Panels A to F show the different concentrations of LAMP products (1 ag/ μL , 1 fg/ μL , 1 pg/ μL , 1 ng/ μL , 10 ng/ μL , and 100 ng/ μL , respectively) eliminated by different concentrations of PMA. M: marker. Panel A: lanes 1 to 7, the final concentration of PMA is 27, 36, 45, 54, 63, 72, and 81 μM , respectively; lane 8, negative control (NC). Panel B: lanes 1 to 9, the final concentration of PMA is 27, 36, 45, 54, 63, 81, 90, 99, and 108 μM , respectively; lane 10, NC. Panel C: lanes 1 to 9, the final concentration of PMA is 45, 54, 63, 72, 81, 90, 99, 108, and 117 μM , respectively; lane 10, NC. Panel D: lanes 1 to 9, the final concentration of PMA is 63, 72, 81, 90, 99, 108, 117, 126, and 135 μM , respectively; lane 10, NC. Panel E: lanes 1 to 7, the final concentration of PMA is 99, 108, 117, 126, 135, 144, and 153 μM , respectively; lane 8, NC. Panel F: lanes 1 to 9, the final concentration of PMA is 45, 99, 180, 270, 360, 450, 540, 630, and 720 μM , respectively; lane 10, NC. Panel G: the varied final concentration (450, 126, 126, 90, 63, and 45 μM) of PMA can completely inhibit subsequent re-amplifications of LAMP products (100 ng/ μL , 10 ng/ μL , 1 ng/ μL , 1 pg/ μL , 1 fg/ μL , and 1 ag/ μL , respectively). All of the experiments were repeated in triplicate.

Figure 8. Time needed for complete photolysis of propidium monoazide (PMA). Panels A to D: the different final concentration of PMA (45, 63, 90 and 126 μM) can be completely photolysis. M: marker. Lane 1: positive control; lanes 2 to 14: exposing the different concentration of PMA to a illuminance of 111,500 lx in 10-min intervals ranging from 0 to 120 min; lane 15: negative control. Panel E: the different concentrations (45, 63, 90, and 126 μM) of PMA required 40, 50, 70, and 100 min to finish photolysis, respectively. All of the experiments were repeated in triplicate.

Figure 9. Propidium monoazide (PMA) can completely eliminate the carryover contamination of loop-mediated isothermal amplification (LAMP) without inhibiting LAMP amplification. M = marker. Lanes 1 and 2, the mixtures (nuclease-free water and PMA were respectively mixed with LAMP products and then exposed to light) were used as a template for re-amplification; lane 3, PMA-treated mixture mixed with the target DNA was used as a template for re-amplification. Lane 4, negative control. Panels A and B used lateral flow dipstick and 2% agarose gel electrophoresis to observe the results, respectively. All of the experiments were repeated in triplicate. Color version available online.

nella in PIF. Only a single known study has established the method of real-time PCR to detect *Salmonella* in artificially contaminated PIF, with a detection limit of 10^3 cfu/mL without enrichment (Hyeon et al., 2010). In our study, the method of LAMP-LFD detection limit of artificially contaminated PIF without enrichment was 2.2 cfu/g, which is 1,000 times lower than the previous study and 100 times lower than the conventional method of PCR (Figure 5). In summary, the detection system we have established for detection of *Salmonella* is more rapid, accurate, sensitive, and suitable for on-site inspection of the PIF or dairy products than the previous studies.

It has been previously reported that the *siiA* gene is specific to *Salmonella* (Hassena et al., 2015). Because the *siiA* gene plays an extremely important role in the field of *Salmonella* pathogenesis, we believe it is more suitable for detecting *Salmonella*. In our current study, we used 21 *Salmonella* strains and 31 non-*Salmonella* strains to determine the specificity of the detection method, which we established is 100%. At the same time, aiming to further determine the applicability of the LAMP-LFD method, we detected 60 positive PIF samples that were artificially contaminated with *Salmonella* and 20 negative PIF samples, respectively. The results verified that the LAMP-LFD method of our development have a high diagnostic specificity (100%) for detecting *Salmonella* in PIF.

Some prior work has reported that the time of AuNP conjugated with antibody needed 2 h 30 min (Najian et al., 2015), 3 h 20 min (Chen et al., 2016), or 2 h (Peng et al., 2014). But in our study, the method we established to finish the conjugate process only required 45 min, which will greatly shorten the production time of LFD and the experimental cycle. Previous research has used LFD reader to determine the optimization LFD components, but the LFD reader cannot guarantee good parallelism because of the differences among batches of the test strip. Thus, we used the detection limits of LFD under different conditions to determine the optimization LFD components. Therefore, the methods we developed provide a new platform for rapid and accurate establishment of a multi-detection LAMP-LFD system for pathogen detection in the dairy industry.

After repeated experiments, we found that the method of LAMP-LFD was easily contaminated. In other words, false-positive results will appear in the LAMP-LFD detection method after several times of detection in the same place. Thus, it is of great significance to establish a method to eliminate contamination. Previous studies have reported that 1 ng/ μ L of the dead cell genomic DNA was completely inactivated by 5 μ M PMA, and 3 μ M PMA can be completely inactivated by light exposure for 2 min (Nocker et al., 2006;

Schnetzinger et al., 2013). In our current study, the results confirmed that the incubation process and steps involving ice can be omitted, which will not affect PMA when used to eliminate unwanted nucleic acid (data not shown). At the same time, we demonstrated that inactivated 1 ng/ μ L of LAMP products required 126 μ M PMA (25.2 times higher than PMA treated with genomic DNA), the appropriate final concentration of PMA was positively but not linearly correlated with the concentration of LAMP products, and the masses of the re-amplification products achieved by amplifying 2 μ L of each concentration of products are similar (data not shown). The reasons for this might lie in each stem-loop DNA in the cauliflower-like structure of LAMP products that can be used as the new templates for re-amplification. In this study, the results verified that when the PMA does not become inactivated, the corresponding band of the LAMP products is fainter (Figure 7). As a previous study has suggested, PMA can damage the DNA polymerase due to the organic azides of their energy-rich nature (Schnetzinger et al., 2013). Therefore, we speculated that the free PMA interfere with the LAMP amplification by damaging the *Bst* 2.0 WarmStart DNA polymerase. Thus, it is very necessary to ensure PMA are completely inactivated and determine the relationship between the concentration of PMA and the time required for complete photolysis. The result of our study verified a positive correlation between the concentration of PMA and photolysis time. In summary, the appropriate concentration of PMA diluted by water can be applied to the working environment of any easily contaminated area and adequate light exposure conditions can complete the decontamination process. After the decontamination stage, the rest of the PMA can be deliquidated by spraying enough water to the environment treated by PMA to reduce the concentration of the free PMA and then the photolysis time of PMA can be shortened. Previous studies only measured the distance and used a blue light-emitting diode or halogen light power to evaluate the treatment conditions. Thus, our current study is the first to employ illuminance instead of the previous conditions, which can be more flexible and accurate.

Although the results of real-time LAMP can be visualized, PMA will emit a strong fluorescence when bound to nucleic acids (Venkateswaran and Smiley, 2011), and a high concentration of PMA was required for eliminating the LAMP carryover contamination. Thus, it is easy to measure a stronger signal interference in real-time LAMP. Therefore, we selected the conventional AGE method to illustrate the results. The results verified that the approach we established to eliminate carryover contamination will not affect the visual detection

method of LAMP-LFD and is more effective (reducing the step of preparing the reaction solution and avoiding the inhibition of LAMP amplification) than other previous methods. Thus, the study will expand PMA application in the field of detection using LAMP and represents a powerful option for pathogen detection in dairy products.

CONCLUSIONS

In summary, we developed a visual LAMP-LFD method targeting the *Salmonella* *stiA* gene, which has a high specificity (100%) and effectively shortens the detection time in a model dairy food system. At the same time, we optimized the detection method by using PMA to eliminate carryover contamination. Therefore, the methods described herein provide a rapid, accurate, and convenient detection system for detecting *Salmonella* in PIF, and likely several other dairy products.

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