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Sensitive and rapid visual detection of *Salmonella* Typhimurium in milk based on recombinase polymerase amplification with lateral flow dipsticks

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ABSTRACT

Salmonella Typhimurium (*S.* Typhimurium) can cause serious foodborne diseases. In this study, an assay combining recombinase polymerase amplification (RPA) with lateral flow dipsticks (LFD) was developed to detect *S.* Typhimurium in milk. The RPA forward primers STF1, STF2, STF3, the reverse primer STR labeled with digoxin, and the probe STProb labeled with FAM were designed and screened to produce RPA products for LFD detection. The RPA reaction volume, temperature, and time were then optimized, and the sensitivity and specificity of the developed method were analyzed. Finally, the RPA–LFD method was evaluated using milk artificially contaminated with *S.* Typhimurium. Results indicated that the primer pair STF1/STR is the optimal combination for detecting the bacterium. The minimum volume, shortest time, and optimal temperature of the RPA reaction were 10 μL, 10 min, and 40–42 °C, respectively. The limit of detection of RPA-LFD for detecting the genomic DNA of *S.* Typhimurium was 1 fg, which is 5 and 10 times lower than the corresponding limits of RPA–agarose gel electrophoresis (AGE) and PCR–AGE, respectively. Testing with 29 other foodborne bacteria as controls revealed that RPA–LFD was highly specific for *S.* Typhimurium. RPA–LFD can detect *S.* Typhimurium at concentrations as low as 1.95 CFU/mL in artificially inoculated milk samples and is thus 10 times more sensitive than PCR. Hence, the RPA–LFD assay established in this study could be a potential point-of-care/need test for *S.* Typhimurium, especially in areas with limited resources.

1. Introduction

Salmonella Typhimurium (*S.* Typhimurium) is a zoonotic pathogen that can cause disease by people eating this pathogen contaminated foodstuffs. So contamination with *S.* Typhimurium can cause great economic losses to the food industry worldwide and result in drastic consequences to human health (Saikia et al., 2015). In 2014, salmonellosis led to 88,715 hospitalizations in the European Union (EFSA, 2015). In Australia, *S.* Typhimurium infection accounted for 34% of all salmonellosis-related hospitalizations (OzFoodNet, 2012). In the USA, the Centers for Disease Control and Prevention (CDC) reported around 1.2 million cases of salmonellosis involving 23,000 hospitalizations and 450 deaths (CDC, 2018). Approximately 75% of the 30 million cases of foodborne diseases in China are attributed to *Salmonella* infections (Wu et al., 2013). Clearly, *Salmonella*, especially the *S.* Typhimurium serotype, is an important risk factor and threat to food safety (Chandra et al., 2013). Therefore, developing rapid and reliable techniques for detecting *S.* Typhimurium in contaminated food is imperative.

Foodborne bacterial identification has mainly relied on the combination of conventional bacterial cultivation with biochemical methods (Stears et al., 2003). Unfortunately, these approaches not only require several steps, including enrichment, pure culture, and biochemical identification of bacteria, but are also costly and time-consuming, as some methods may take 5–7 days to complete (Ko and Grant, 2003). Immunological assays are relatively sensitive (Magliulo et al., 2007; Ohk and Bhunia, 2013; Shelby et al., 2017) in detecting foodborne bacteria. However, they also present many drawbacks, including preparation of specific antibodies, high rate of false positives, time- and

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labor-intensiveness, high cost, and requirement of well-trained operators. Therefore, currently available methods cannot rapidly detect foodborne bacteria.

Various PCR-based technologies to detect *Salmonella* have recently been developed, and these methods are generally more rapid, accurate, specific, sensitive, and stable than the traditional methods described above (Babu et al., 2013; Li et al., 2013; Wang et al., 2014). However, PCR-based detection technologies cannot conveniently and rapidly identify foodborne targets because they involve bulky instruments, time-consuming thermal cycling steps and their operation requires skilled technicians; thus, their application to complex food matrices and/or in resource-poor settings is limited (Asiello and Baeumner, 2011). Isothermal nucleic acid amplification technologies, including nucleic acid sequence-based amplification (Compton, 1991; Ma et al., 2018), strand displacement amplification (Lee et al., 2018; Walker et al., 1992), loop-mediated amplification (Notomi et al., 2000), rolling circle amplification (Le and Seo, 2018; Liu et al., 1996), helicase-dependent amplification (Vincent et al., 2004), and recombinase polymerase amplification (RPA) (Piepenburg et al., 2006), have been explored to avoid the thermal cycling steps required during gene amplification. RPA is a novel, highly rapid, and sensitive method for amplifying target DNA/RNA sequences at a relatively low temperature range (37–42 °C) (Chen et al., 2018; Gootenberg et al., 2018).

Frequently used endpoint assays directed against RPA products mainly consist of agarose gel electrophoresis (AGE) (Wang et al., 2016), chemiluminescence (Kunze et al., 2016), electrochemistry (Del Río et al., 2016), colorimetric readout (Koo et al., 2016), enzyme-linked immunosorbent assay (ELISA) (Santiago-Felipe et al., 2014), real-time fluorescence (Babu et al., 2017), and lateral flow dipsticks (LFD) (Sun et al., 2016). Of these, the first two methods are time consuming and labor intensive, which means their use is limited to a great extent in resource-limited environments. By contrast, LFDs are a simple tool used for endpoint tests; the sticks feature an antibody labeled with gold nanoparticles specific to some antigen to detect RPA products, thereby eliminating the need for a nucleotide cleanup step. LFD is read visually and can intuitively display results within 5–10 min (Wu et al., 2017).

Few studies have reported the optimization of RPA primer combinations and reaction conditions, including time, temperature, and volume. In fact, the 50 μL RPA reaction volume reported in the majority of the papers available is not economical. Thus, the present study aims to develop an ultra-sensitive, specific, visual, and convenient RPA-LFD assay based on optimization of RPA primer combinations and reaction conditions for rapid point-of-care (POC) and point-of-need (PON) detection of *S.* Typhimurium in food samples.

2. Materials and methods

2.1. Experimental design

In this study, the experimental procedure was designed as shown in Fig. 1. Firstly, genomic DNA was extracted from the cultured bacteria and used as the template of RPA reaction; Secondly, the primer and probe were designed and labeled before the RPA products were detected by LFD; Thirdly, the primer screening was done prior to optimization of RPA reaction condition; Fourthly, the specificity and sensitivity of RPA-LFD were analyzed and lastly the RPA-LFD developed was evaluated in milk sample.

2.2. Bacterial strains and DNA extraction

Thirty-four bacterial strains were obtained and stored at −80 °C before use. Among these strains, five isolated strains within *S.* Typhimurium serotypes were used as the target bacteria, and the rest were used as experimental controls. Genomic DNA was extracted according to the instructions in the Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). The recombinant plasmid rpUCm-invA was constructed in our laboratory and extracted following the instructions of the SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China). The obtained DNA was subsequently quantified on a Nanodrop 2000 (ThermoFisher Scientific, Waltham, USA) and stored at −20 °C before use.

2.3. Detection principle of RPA–LFD

The basic RPA reaction includes five steps: (1) the recombinase/oligonucleotide primer complexes form and target homologous DNA; (2) strand exchange forms a D-loop structure; (3) the polymerase initiates synthesis; (4) parental strands separate and synthesis continues; and (5) two duplexes form. The TwistAmp™ nfo Kit (TwistDx, Cambridge, UK) was used to produce the labeled RPA products for LFD detection. The TwistAmp™ amplification reaction promoted by two oligonucleotides can generate targets for annealing of the TwistAmp™ LF probe. The nfo enzyme known as endonuclease IV can cleave the probe at the tetrahydrofuran residue (THF) position and generate a new 3′ hydroxyl group (effectively deblocking the probe); this procedure can be considered a priming action for polymerase extension, thus transforming the probe into a primer. The amplicon produced by the processed probe and the 5′ labeled amplification primer effectively co-joins the two antigenic residues in one DNA molecule, which can be detected by LFD (Milenia HybriDetect 2 T) (Milenia Biotec, Gießen, Germany).

2.4. Design of primers and probes

The primers and probes for LFD detection were designed based on the following principles recommended by the TwistAmp™ nfo Kit: the length range of primers was typically 30–35 bp and the RPA products flanked by the forward and reverse primers were between 100 and 500 bp. For the primer sequence, long “tracks” of guanines at the 5′ end (first 3–5 bp) were avoided, while cytosines (perhaps, pyrimidines in general) were preferred, possibly because the latter encourages the formation of recombinase filaments. Guanines and cytosines at the 3′ end of the primer (last 3 bp) tend to provide a stable “clamped” target for the polymerase. The RPA primer design should also conform to the basic principles of PCR primer design. The length of the lateral flow (LF) probes was ideally 46–52 bp, at least 30 bp of which is placed 5′ to the THF site, and at least 15 bp of which is located 3′ to it. Care must be taken to avoid the occurrence of primer–probe dimers due to overlap of the probes and the primers opposite the direction of the probes. Designing a probe located within the smallest candidate amplicon defined by the innermost primers in the forward and reverse groups of candidate primers is a good strategy. In this way, the probe could be used to test the performance of all “surrounding” primers.

2.5. Labeling of primers and probes

The conserved region of the *invA* gene of *S.* Typhimurium was analyzed. Briefly, the *invA* gene (Genbank No. M90846.1) was aligned with those of other isolated strains within the *S.* Typhimurium serotypes using the online BLASTn tool from the National Center for Biotechnology Information website. According to the design rules of the RPA primer and probe described earlier, the forward primers STF1, STF2, and STF3, the reverse primer STR, and the probe STProb were synthesized and/or labeled by Sangon Biotech (Shanghai, China) (Table 1). STR was labeled at the 5′ end with digoxin, while STProb was incorporated with three labels as follows: a 5′-carboxyfluorescein group, fluorescein amidite (FAM), an internal abasic nucleotide analogue (THF) that replaces a base (G) that would be present in the target sequence, and a polymerase extension blocking group (C3-Spacer). Here, the potential combinations of STF1, STF2, and STF3 with STR can produce the predicted RPA products of 273, 188, and 118 bp, respectively. STProb was dexterously designed within the smallest amplicon flanked by STF3 and STR and is therefore also located within the larger

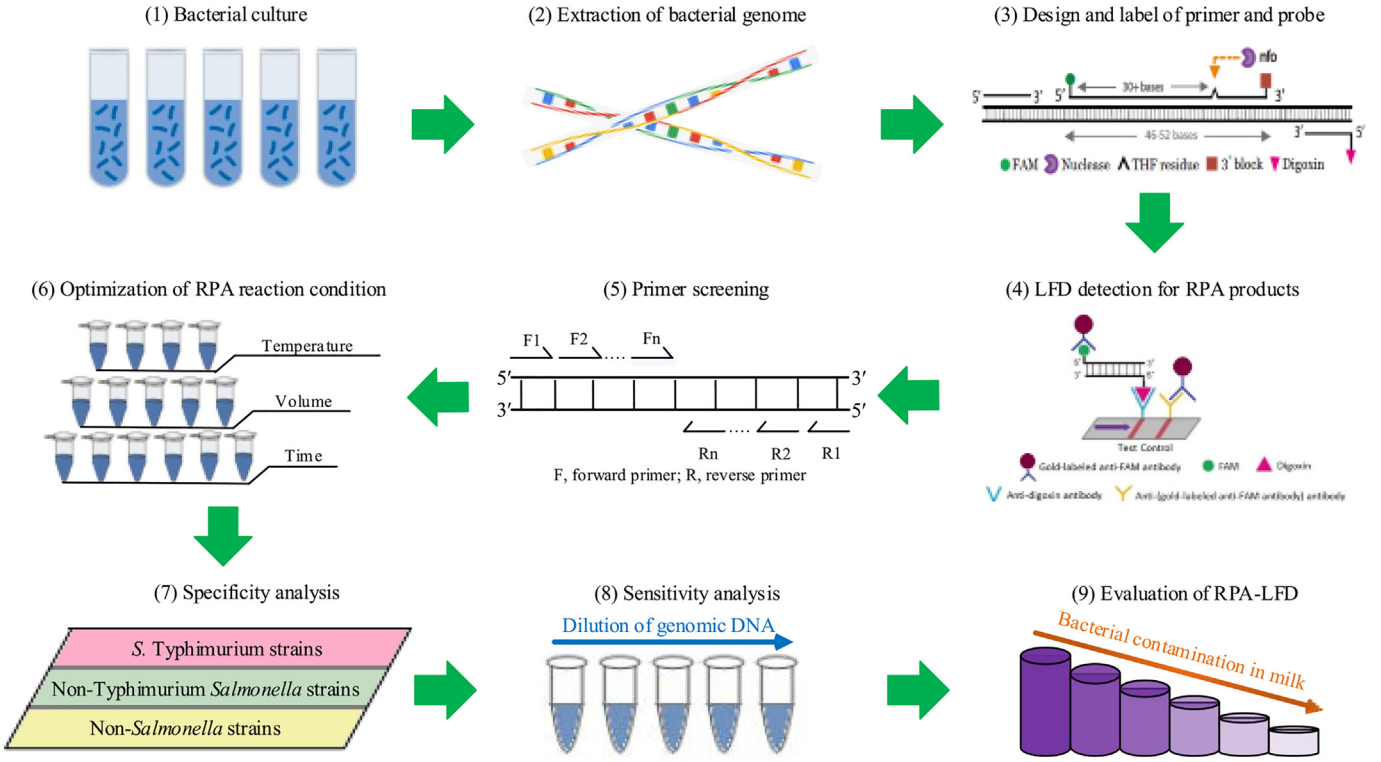

Fig. 1. Schematic diagram of experimental procedure.

candidate amplicon resulting from either STF1/STR or STF2/STR primer combination.

2.6. Screening of primers

PCR and RPA were used to screen the optimal combination of primers. PCR amplification was carried out using 1 μL of 20 pg genomic DNA of *S.* Typhimurium as a template in a total volume of 25 μL containing 12.5 μL of Taq PCR Master Mix (Sangon Biotech, Shanghai, China), 1 μL of 10 pM forward/reverse primer, and 9.5 μL of ddH_2O. The PCR reaction was performed in a gradient thermocycler (PTC-200, BioRad, CA, USA) with the following conditions: predenaturation at 95 °C for 5 min and 95 °C for 25 s, 60 °C for 30 s, and 72 °C for 45 s for 30 cycles, with a final elongation step at 72 °C for 10 min. RPA amplification was conducted in accordance with the specifications of the TwistAmp™ nfo Kit with a total volume of 10 μL. In brief, 29.5 μL of rehydration buffer was mixed with 12.2 μL of ddH_2O and used to resuspend the lyophilized enzyme pellet in a tube. The mixture was divided into five aliquots (8.34 μL each) in 0.2 mL tubes following gentle vortex mixing. Subsequently, a premixture containing 0.42 μL of 10 μM forward/reverse primer and 0.12 μL of 10 μM STProb was added to each aliquot followed by 0.2 μL of 20 pg genomic DNA of *S.* Typhimurium as a template. Finally, 0.5 μL of 280 mM magnesium acetate solution as an initiator was pipetted into the lid of the reaction tubes before transient centrifugation to induce all of the reactions to start at the same time. Reactions were performed at 40 °C for 20 min in a thermostatic water bath (HH-6, Jieruier Electric Appliance, Jintan, China). Subsequently, the PRA products were cleaned up using SanPrep Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China). Thereafter, the PCR and purified RPA products were electrophoresed on 2% AGE after 4S Green Plus (Sangon Biotech, Shanghai, China) staining and then detected under ultraviolet light. The unpurified RPA product was directly detected by LFD (Milenia HybriDetect 2 T). In brief, 2 μL of the RPA product was transferred to a well of a microtiter plate containing 100 μL of assay solution (0.1 M Tris-Cl, pH 8.0) and mixed using a micropipette. Afterward, dipsticks were placed into the solution in an upright position and incubated for about 5 min. This experiment was done in duplicate. As described in the instructions of Milenia HybriDetect 2 T, the RPA reaction was considered positive when the Control (C) line and the Test (T) line were simultaneously visible on the LFD, negative when only the C line was visible on the LFD, and invalid if no band was visible on the LFD. Each experiment was done in duplicate. The primer combination that amplified the RPA products the most after evaluation by AGE and LFD was considered the optimal combination for RPA reaction, meanwhile, the primer combination that amplified the PCR products the most by AGE was considered the optimal combination for PCR

Table 1
Primers and probe used in this study.

Name	Sequence (5′ → 3′)	Sense	Length (bp)	Application
STF1	TTCCTGATTTACTTAAAGAAGTGCTCAGACATGCC	+	35	PCR/RPA
STF2	TTCCGTGCGTAATATGAAGTTAATTATGGAAGCGC	+	35	PCR/RPA
STF3	AACCTTGTGGAGCATATTCGTGGAGCAATGGCGCG	+	35	PCR/RPA
STR	DIG-CTTTGCGAATAACATCCTCAACTTCAGCAGATACC	−	35	PCR/RPA
STProb	FAM-TTATATTTGTCATAAATTCGCCAATGGCGGC-THF-AATTACGAGCAGTAAT-C3-Spacer	+	47	RPA

DIG: Digoxin; FAM: Carboxyfluorescein group; THF: Tetrahydrofuran, internal a basic nucleotide analogue, replacing a base (G) that would be present in the target sequence of RPA product; C3-Spacer: polymerase extension blocking group; All primers were used in both PCR and RPA reactions and while LF probe STProb was only used in RPA reaction; "+": Forward direction; "−": Reverse direction.

reaction used as method control in the follow up experiment.

2.7. Optimization of RPA reaction

Ten volume gradients of 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μL; six temperature gradients of 37, 38, 39, 40, 41, and 42 °C; and six time gradients of 5, 10, 15, 20, 25, and 30 min were successively conducted to determine the optimal RPA reaction condition. When one parameter was optimized, the two other optimal parameters were held constant. The RPA products originating from parameter optimization were evaluated by AGE and/or LFD as described previously. This experiment was conducted in duplicate.

2.8. Specificity analysis of the RPA reaction

The genomic DNA of 34 bacterial strains and the recombinant plasmid rpUCm-invA were used as templates of the RPA reaction to verify the cross-reactivity of the optimal primer combination of *S.* Typhimurium in the RPA reaction with non-target bacterial strains. The resulting RPA products were analyzed by LFD directly and by AGE post-purification following the procedures described above. This experiment was conducted in duplicate.

2.9. Sensitivity analysis of RPA-LFD for genomic DNA of S. Typhimurium

Genomic DNA was diluted to 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 5 fg, or 1 fg as the template of the RPA and PCR reactions to obtain the limit of detection (LOD) of RPA-LFD for the genomic DNA of *S.* Typhimurium resulting from pure bacterial culture. PCR and purified RPA products were analyzed by 2% AGE, and RPA products were directly analyzed by LFD. This experiment was carried out in duplicate. Traditional PCR assay was employed in this study as the control for sensitivity comparison between RPA–LFD and PCR.

2.10. Evaluation of RPA-LFD in artificially contaminated milk

Raw milk purchased from a local small dairy farm (Henan Huahua Cattle Husbandry Technology Co, Ltd., Xinxiang, Henan Province, China) and that had been proven to be negative for *S.* Typhimurium by a biochemical identification method based on the instructions of the national standard (GB4789.4-2016) in China was used as a inoculated food sample to evaluate the influence of a liquid milk matrix on the RPA-LFD assay. In brief, 25 mL of raw milk was poured into an aseptic homogeneous cup with 225 mL of buffered peptone water (BPW) (10 g/L peptone, 5 g/L sodium chloride, 9 g/L disodium hydrogen phosphate dodecahydrate, 1.5 g/L potassium dihydrogen phosphate, pH 7.2) and thoroughly mixed by shocking before inoculating with 1 mL of 10-fold serial dilutions (10^0–10^7) of bacterial culture or 1 mL BPW medium as the negative control. After mixing, the milk sample corresponding to each dilution was immediately spread onto a bismuth sulfite agar (10.0 g/L peptone, 5.0 g/L beef paste, 5.0 g/L glucose, 0.3 g/L ferrous sulfate, 4.0 g/L disodium hydrogen phosphate, 0.025 g/L brilliant green, 2.0 g/L ammonium bismuth citrate, 6.0 g/L sodium sulfite, 20.0 g/L agar, pH 7.5) plate for *S.* Typhimurium colony counting. The genomic DNA from each dilution was also immediately extracted as described above and used as the template of RPA and PCR reactions. The resultant PCR and purified RPA products were analyzed with 2% AGE, and unpurified RPA products were analyzed by LFD as described previously. This experiment was done in duplicate.

3. Results

3.1. Determination of the optimal primer combination

As shown in Fig. 2, specific fragments of 273, 188, and 118 bp were obtained on AGE from the STF1/STR, STF2/STR, and STF3/STR primer combinations, respectively. The PCR–AGE result (the most brilliant band of 188 bp) was not consistent with those of the RPA–AGE (the most brilliant band of 273 bp) and RPA–LFD methods. However, the results of the latter two methods were consistent, which means the primers appropriate for the PCR reaction were not necessarily suitable for the RPA reaction. Thus, using PCR instead of RPA to screen RPA primers is not feasible, whereas using the RPA–AGE method to screen the RPA primers with or without RPA–LFD is feasible. The primer combination STF1/STR and STF2/STR were considered the optimal candidate combinations of RPA and PCR reactions, respectively.

3.2. Optimal conditions of the RPA reaction

Several critical variables, including reaction volume, temperature, and time, were optimized for the RPA reaction. The RPA band utilizing a reaction volume of 5 μL was distinctly dimmer than those using all other volumes with nearly the same luminance of RPA product regardless of the application of RPA–AGE (Fig. 3A) or RPA–LFD (Fig. 3B). This finding indicates that 10 μL is the minimum reaction volume and is corroborated by the limited results published in the literatures. AGE (Fig. 4A) and LFD (Fig. 4B) results confirmed that the RPA products obtained were not obviously different between 40 and 42 °C, which means 40–42 °C can be considered the optimal reaction temperature range. The luminance of the RPA product analyzed by AGE (Fig. 5A) and LFD (Fig. 5B) remained relatively unchanged with time from 10 min to 30 min; this result indicates that 10 min is the shortest possible reaction time. Thus, the optimal RPA reaction parameters of 10 μL, 40 °C (41 and 42 °C were also chosen), and 10 min were applied throughout the rest of the study.

3.3. Specificity analysis of RPA-LFD

The specificity of the primer combination STF1/STR was examined by RPA–AGE and RPA–LFD assays. As shown in Table 2, both AGE and LFD assays were positive when the genomic DNA derived from five isolates of *S.* Typhimurium serotype and the positive control rpUCm-invA were used as templates of the RPA reaction. Regardless of the application of AGE or LFD, no RPA product was detected for the other 29 control bacteria, including three non-Typhimurium serotypes of *Salmonella* species designated as *S. enteritidis, S. infantis*, and *S. paratyphi*. This result reveals that RPA–LFD based on the STF1/STR primer combination is highly specific for *S.* Typhimurium.

3.4. Sensitivity analysis of RPA-LFD for genomic DNA of S. Typhimurium

A total of 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 5 fg, 1 fg, or 100 ag of *S.* Typhimurium genomic DNA was used as the templates for the PCR and RPA reactions for the sensitivity test. The LODs of PCR–AGE (Fig. 6A), RPA–AGE (Fig. 6B), and RPA–LFD (Fig. 6C) were 10, 5, and 1 fg, respectively. The sensitivity of RPA–LFD was 5 and 10 times higher than those of RPA–AGE and traditional PCR–AGE, indicating that RPA–LFD is an ultra-sensitive method for detecting the genomic DNA of *S.* Typhimurium.

3.5. Application of RPA-LFD in artificially contaminated milk samples

Genomic DNA of bacteria at contamination levels of 1.95×10^7, 1.95×10^6, 1.95×10^5, 1.95×10^4, 1.95×10^3, 1.95×10^2, 1.95×10^1, and 1.95×10^0 CFU/mL determined via plate counting was extracted and detected by PCR–AGE, RPA–AGE, and RPA–LFD. While the LOD of PCR–AGE was 1.95×10^1 CFU/mL (Fig. 7A), those of RPA–AGE and RPA–LFD were identical at 1.95 CFU/mL (Fig. 7B and C). Thus, the LOD of the developed RPA–LFD method is one order of magnitude (10 times) lower than that of the traditional PCR–AGE method.

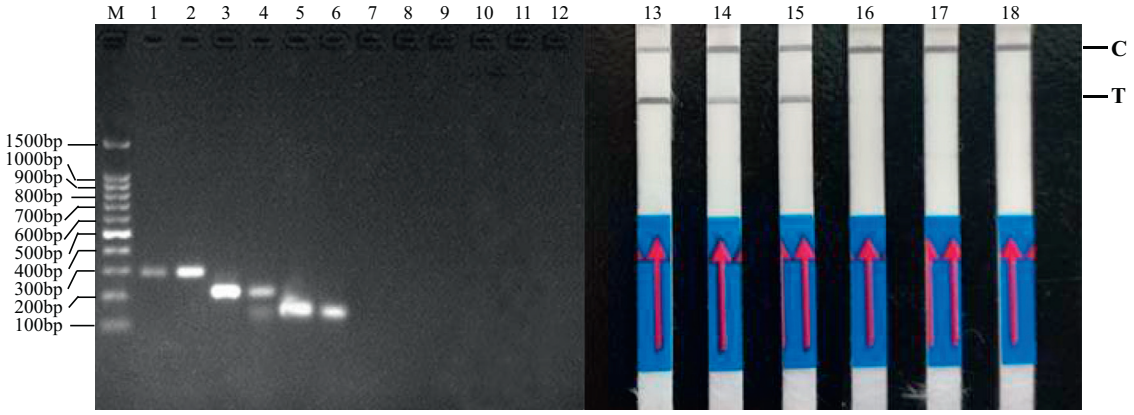

Fig. 2. Screening of different RPA primer combinations by using PCR and RPA. PCR–AGE screening of lanes 1, 3, 5, and 7–9; RPA–AGE screening of lanes 2, 4, 6, and 10–12; RPA–LFD screening of lanes 13–18. Lanes 7, 8, and 9 are the negative controls of 1, 3, and 5, respectively; lanes 10, 11, and 12 are the negative controls of 2, 4, and 6, respectively; and lanes 16–18 are the negative controls of 13–15, respectively. Genomic DNA of *S.* Typhimurium and the three primer pairs STF1/STR, STF2/STR, and STF3/STR were used as the template and primer combinations for RPA amplification, respectively. M: DNA marker; 1, 2, 7, 10: STF1/STR (273 bp); 3, 4, 8, 11: STF2/STR (188 bp); 5, 6, 9, 12: STF3/STR (118 bp); C: Control line; T: Test line.

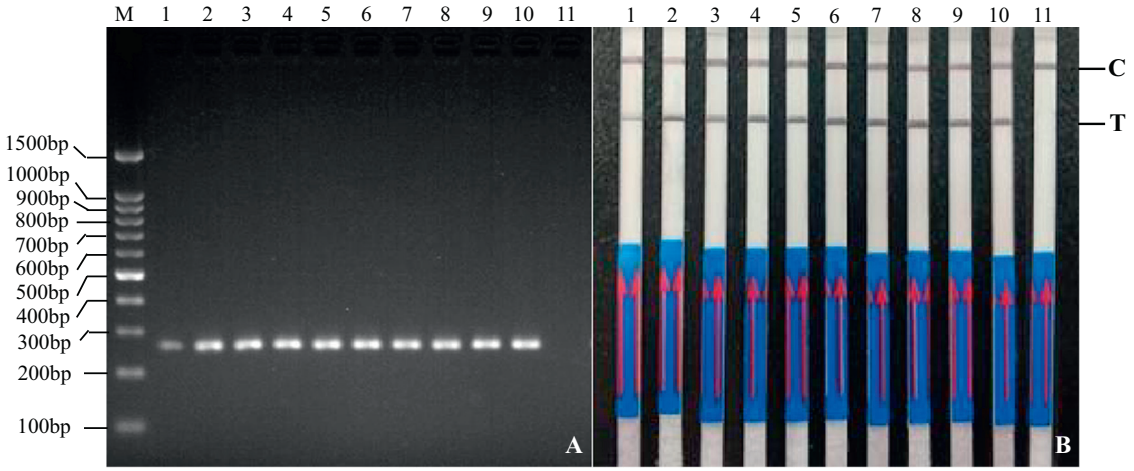

Fig. 3. Optimization of RPA reaction volume. (A) AGE and (B) LFD detection of RPA products. M: DNA marker, 1: 5 μL, 2: 10 μL, 3: 15 μL, 4: 20 μL, 5: 25 μL, 6: 30 μL, 7: 35 μL, 8: 40 μL, 9: 45 μL, 10: 50 μL, 11: Negative control, C: Control line, T: Test line.

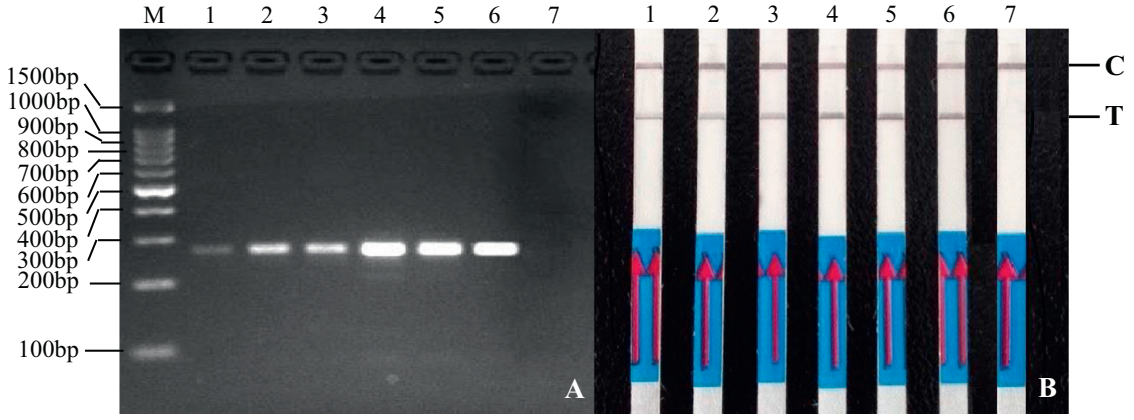

Fig. 4. Optimization of RPA reaction temperature. (A) AGE and (B) LFD detection of RPA products. M: DNA marker, 1: 37 °C, 2: 38 °C, 3: 39 °C, 4: 40 °C, 5: 41 °C, 6: 42 °C, 7: Negative control, C: Control line, T: Test line.

4. Discussion

In general, inappropriate RPA primer/probe combinations may lead to false positives or low sensitivity. To solve this problem, the design rules of the RPA primer/probe, including the nucleotide composition (especially at the 5′-end), sequence length, and interplay between forward primer/probe and the reverse primer, were carefully considered. Overlapping of the FAM-labeled STProb with the digoxin-labeled reverse forward STR may cause the formation of dimers and eventually lead to a false positive result. Different primer combinations designated

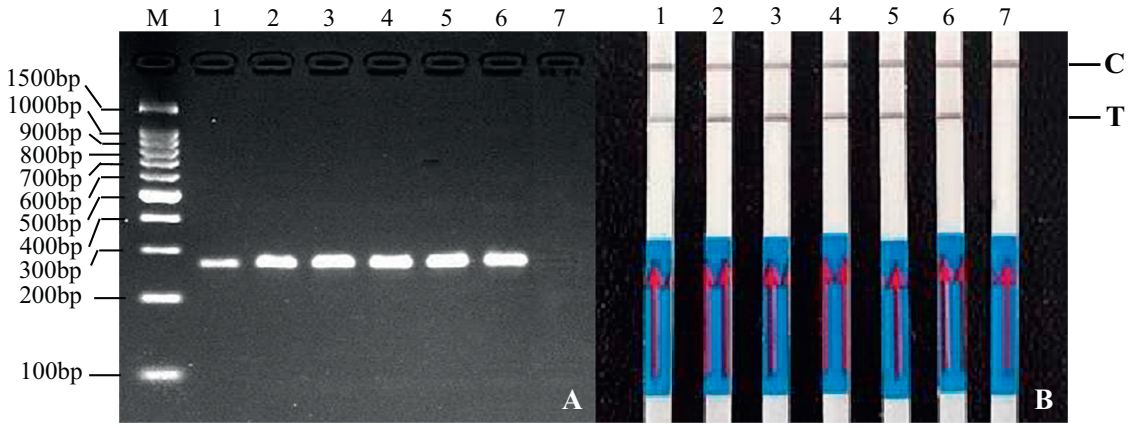

Fig. 5. Optimization of RPA reaction time. (A) AGE and (B) LFD detection of RPA products. M: DNA marker, 1: 5 min, 2: 10 min, 3: 15 min, 4: 20 min, 5: 25 min, 6: 30 min, 7: Negative control, C: Control line, T: Test line.

Table 2
Specificity analysis of RPA-LFD for *S.* Typhimurium.

Strain name	Strain code	Applied assays	
		RPA-AGE	RPA-LFD
Bacillus cereus	CMCC 63303	−	−
Campylobacter jejuni	CICC 22936	−	−
Citrobacter koseri	ATCC 27156	−	−
Clostridium perfringens	ATCC 13124	−	−
Cronobacter sakazakii	ATCC 29544	−	−
Enterobacter aerogenes	ATCC 13048	−	−
Enterobacter cloacae	ATCC 13047	−	−
Enterococcus faecium	ATCC 27270	−	−
Escherichia coli	CICC 10032	−	−
Escherichia coli (OneShot Top10)[a]	−	+	+
Escherichia coli O157:H7	ATCC 43895	−	−
Klebsiella pneumoniae	ATCC 27336	−	−
Listera monocytogenes	ATCC 19115	−	−
Proteusbacillus vulgaris	CMCC 49027	−	−
Pseudomonas aeruginosa	ATCC 27853	−	−
Pseudomonas fluorescens	ATCC 13525	−	−
Salmonella enteritidis	CMCC 50041	−	−
Salmonella infantis	ATCC 51741	−	−
Salmonella paratyphi	ATCC 9150	−	−
Salmonella Typhimurium	ATCC 13311	+	+
	ATCC 14028	+	+
	ATCC 50115	+	+
	ATCC 700720	+	+
	BNCC 108207	+	+
Serratia odorifera	ATCC 33077	−	−
Serratia marcescens	ATCC 14040	−	−
Shigella boydii	ATCC12028	−	−
Shigella dysenteriae	CMCC 51592	−	−
Shigella flexneri	CMCC 51572	−	−
Shigella sonnei	CMCC 51105	−	−
Staphylococcus aureus	BNCC 337755	−	−
Streptococcus hemolytic-β	CICC 10373	−	−
Streptococcus hermophilus	CICC 6063	−	−
Vibrio parahaemolytieus	ATCC 17802	−	−
Yersinia enterocolitica	CMCC 52204	−	−

CMCC: China Center for Medical Culture Collection; CICC: China Center of Industrial Culture Collection; ATCC: American Type Culture Collection; BNCC: BeNa Culture Collection; *Escherichia coli* (OneShot Top10)[a]: Gene engineering Oneshot Top10 strain of *Escherichia coli* harbouring the recombinant plasmid rpUCm-invA; "+": Positive; "−": Negative.

as STF1/STR, STF2/STR, and STF3/STR were examined by PCR–AGE, RPA–AGE, and RPA–LFD to evaluate RPA productivity. The combination of RPA primers yielding the maximum amount of RPA product using both AGE and LFD was considered the potential optimal combination and used for subsequent RPA–LFD test. Only one reverse primer STR was used in this work because of the difficulty of designing other reverse primers based on the design principles of RPA primers and sequence characteristics of the *invA* gene of *S.* Typhimurium. However, the reverse primer STR can combine with all three forward primers, which means the STProb probe can easily be integrated into the minimum amplicon. The FAM antigen was used to label the forward primer STF1 instead of the STProb probe. Unfortunately, a false positive result was observed without addition of bacterial genomic DNA to the RPA reaction system (data not shown). This finding confirms that introduction of the LF probe labeled with the FAM antigen is necessary to produce double antigenic RPA products for LFD detection (Yan et al., 2014).

Thus far, few investigations have been conducted to understand the effect of RPA reaction volume on the RPA products, and the RPA reaction volume recommended in the majority of papers reporting on this topic and the TwistAmp™ Kit is 50 μL (Gao et al., 2017; Sun et al., 2016; Wu et al., 2017). In the current study, the results of RPA–AGE and RPA–LFD confirmed that 10 μL is the optimal volume of the RPA reaction. Such a small reaction volume can greatly reduce the cost of the RPA application and may promote the wider application of RPA technology in food safety detection. The RPA reaction time was only 10 min, which is rarely reported in the literature (Jaroenram and Owens, 2014a) but meets the demands of rapid POC detection. As described in the instructions of the TwistAmp™ Kit, the RPA reaction could amplify detectable RPA products at temperatures between 37 °C and 42 °C (Zhao et al., 2015), as confirmed. The finding also showed that the same amount of RPA products were obtained at temperatures varying from 40 °C to 42 °C and the optimal temperature for RPA reaction was considered as 40–42 °C.

The LOD of RPA–LFD was 1 fg for genomic DNA and 1.95 CFU/mL for *S.* Typhimurium in artificially inoculated food, both of which are lower than those reported in the literatures (Kersting et al., 2014; Sun et al., 2016). To the best of our knowledge, this difference in method sensitivity may be due to dimer formation of the primer/primer (probe) and the characteristics of the nucleotides used for the RPA reaction templates. Thirty-four bacterial strains representative of different species causing foodborne illness were used for specificity analysis to guarantee the accuracy of the RPA-LFD assay. A recombinant plasmid rpUCm-invA was utilized as the positive control to ensure that the RPA reaction and/or LFD detection worked well in the specificity experiments.

To date, LFD is utilized for only a single or two targets at one time because of the limitation of the LFD detection principle (Jaroenram and Owens, 2014b). LFD cannot distinguish more than two nucleotide amplicons in one RPA reaction system, which may pose challenges for the high-throughput detection of foodborne pathogens. This problem may be resolved in future work by integrating a series of RPA reactions aimed at different foodborne targets into a microfluidic device to

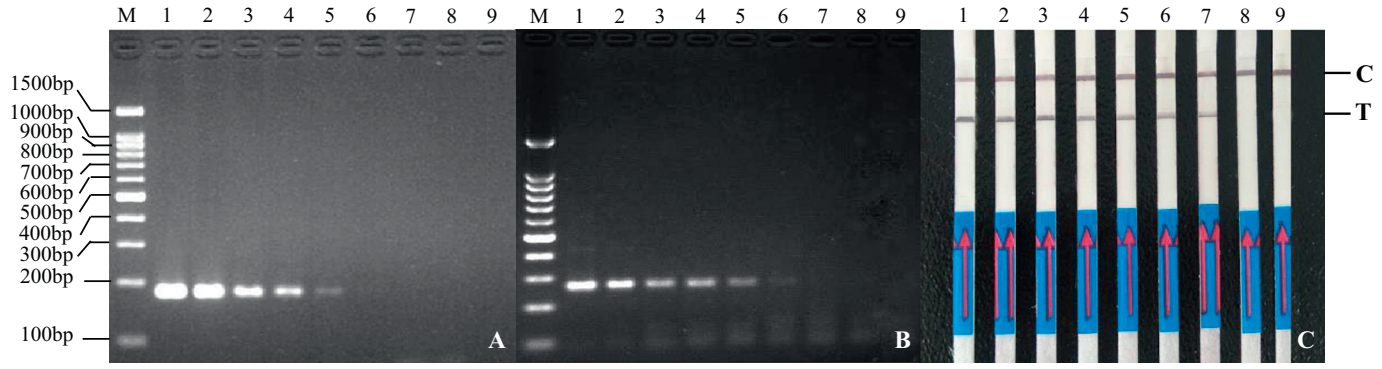

Fig. 6. Sensitivity analysis of RPA-LFD. (A) AGE detection of the PCR product. (B) AGE and (C) LFD detection of RPA products. Tenfold serially diluted genomic DNA of *S.* Typhimurium used as the template for RPA amplification. M: DNA marker, 1: 0.1 ng, 2: 10 pg, 3: 1 pg, 4: 100 fg, 5: 10 fg, 6: 5 fg, 7: 1 fg, 8: 100 ag, 9: Negative control, C: Control line, T: Test line.

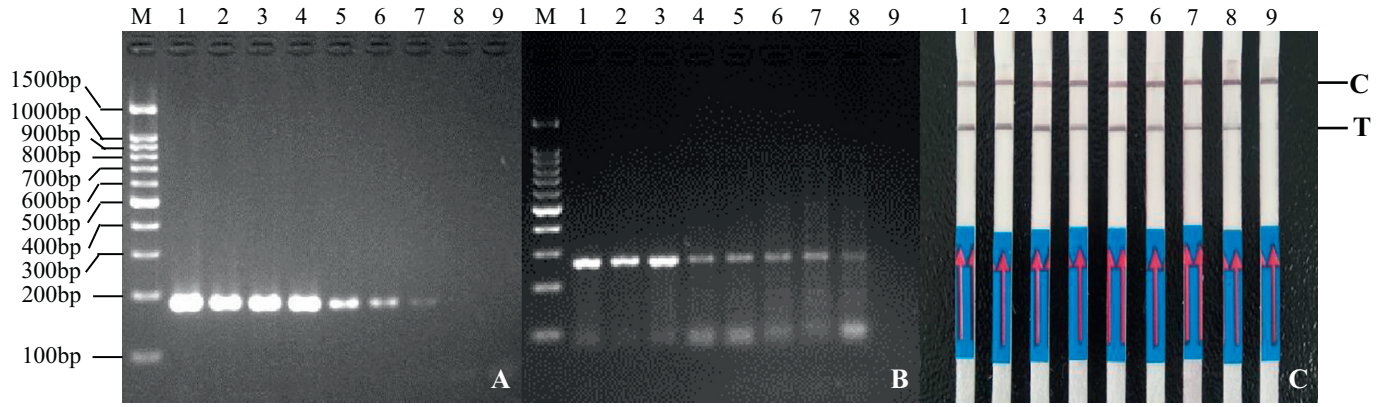

Fig. 7. Evaluation of RPA-LFD for detecting raw milk artificially contaminated with *S.* Typhimurium. (A) AGE detection of the PCR product. (B) AGE and (C) LFD detection of RPA products. Tenfold serially diluted genomic DNA extracted from *S.* Typhimurium-contaminated raw milk used as the template for RPA amplification. M: DNA marker, 1: 1.95×10^7 CFU/mL, 2: 1.95×10^6 CFU/mL, 3: 1.95×10^5 CFU/mL, 4: 1.95×10^4 CFU/mL, 5: 1.95×10^3 CFU/mL, 6: 1.95×10^2 CFU/mL, 7: 1.95×10^1 CFU/mL, 8: 1.95×10^0 CFU/mL, 9: Negative control, C: Control line, T: Test line.

realize the parallel detection of RPA products by using LFD (Renner et al., 2017).

5. Conclusions

In the current study, an RPA–LFD method for the rapid detection of *S.* Typhimurium was developed. The RPA products detected were obtained from only a 10 μL reaction volume and 10 min of reaction time at the temperature range 40–42 °C. Judgment via the naked eye could be accomplished via LFD endpoint detection. The RPA–LFD technique established in this study is an ultrasensitive assay with LODs of 1 fg and 1.95 CFU/mL for the genomic DNA of *S.* Typhimurium and *S.* Typhimurium in artificially contaminated raw milk, respectively. The developed method is a practical and effective tool to rapidly, specifically, sensitively, and conveniently detect *S.* Typhimurium in milk samples at POC and/or PON especially in resource-limited settings with low availability of laboratory equipment.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any study with human participants or animals performed by any of the authors.

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