Development of an isothermal amplification-based assay for the rapid visual detection of *Salmonella* bacteria

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ABSTRACT

The efficient and timely detection of pathogens is a major concern worldwide. The aim of this study was to establish a rapid detection method for *Salmonella* bacteria in food samples to facilitate timely treatment. Widely used detection methods currently include culture-based methods and PCR-based methods. The former are time consuming, requiring 2 to 3 d, whereas the latter have higher accuracy but are typically complicated, requiring expertise and expensive instruments. In this study, a sensitive and rapid approach for the visual and point-of-use detection of *Salmonella* bacteria based on recombinase polymerase amplification (RPA) and a lateral-flow (LF) nucleic acid strip was established. We designed a pair of primers according to the *invA* gene of *Salmonella* bacteria: one was modified with digoxin, and the other was modified with biotin. In the presence of the biotin- and digoxin-modified primers and target DNA, the RPA produced a substantial amount of duplex DNA attached to biotin and digoxin. The products were detected using LF strips through immunoreaction: anti-digoxin antibodies on the gold nanoparticles, digoxin on the duplex, streptavidin on the LF test line, and biotin on the duplex. The developed RPA-LF assay allowed detection of *Salmonella* genomic DNA in less than 20 min with simple water bath equipment or portable thermal equipment. In addition, the RPA-LF assay was highly sensitive, with a detection limit as low as 20 fg of target DNA or 1.05×10^1 cfu of bacteria in pure culture, and highly specific, exhibiting no cross-reaction with *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Shigella*, *Enterobacter aerogenes*, or *Campylobacter jejuni*. Importantly, *Salmonella* could be detected in milk and chicken breast at concentrations as low as 1.05×10^0 cfu/mL or $1.05 \times$

10^0 cfu/g after enrichment for 2 h and in eggs at 1.05×10^0 cfu/g after enrichment for 4 h. Furthermore, RPA was more sensitive than PCR, which requires a thermal cycling device. In summary, this study describes a sensitive, simple, and point-of-use detection method for *Salmonella* bacteria.

Key words: *Salmonella*, recombinase polymerase amplification, lateral-flow strip, nucleic acid test

INTRODUCTION

Organizations and consumers are paying increasing attention to food safety due to the frequent food safety incidents occurring in many countries. The Centers for Disease Control and Prevention (2014) estimates that disease caused by contamination with foodborne pathogens affects 48 million people and results in 128,000 hospitalizations and 3,000 deaths every year in the United States. *Salmonella* is considered an important risk to poultry and consumer health worldwide (Kutay et al., 2016) and is responsible for numerous foodborne illnesses around the world (Millan-Sango et al., 2016).

The standard culture-based methods for *Salmonella* detection are time consuming and labor intensive, requiring 3 d to obtain a presumptive positive or negative result and another 2 to 4 d to identify a positive result (Khueankhanchaoen et al., 2016). Consequently, for food samples, the applications of conventional methods are limited. Polymerase chain reaction has been widely employed for the rapid detection of *Salmonella*. This technique requires expensive thermal cycling instruments and professional operators, which limit its application in point-of-use detection and resource-limited settings.

To enable point-of-use detection, new isothermal amplification technologies are being developed that are suitable for use in settings where infrastructure, expensive instruments, and technical expertise are not available (Gill and Ghaemi, 2008; Kim and Easley, 2011). Isothermal amplification involves the amplification of nucleic acids at a constant temperature. A variety of mechanisms have been developed, such as nucleic acid

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Table 1. Primers of basic recombinase polymerase amplification (RPA) and lateral-flow RPA assay used in this study

Assay	Primers ¹
Basic RPA reaction	F: 5'-CTACAAGCATGAAATGGCAGAACAGCGTCG-3' R: 5'-CAACCAGATAGGTAGGTAATGGAATGACGA-3'
Lateral flow RPA reaction	F: 5'-digoxinCTACAAGCATGAAATGGCAGAACAGCGTCG-3' R: 5'-biotin-CAACCAGATAGGTAGGTAATGGAATGACGA-3'

¹F = forward; R = reverse.

sequence-based amplification (Liu et al., 2016), loop-mediated isothermal amplification (Chen et al., 2016), helicase-dependent amplification (Chen et al., 2015), rolling circle amplification (Li et al., 2016), recombinase polymerase amplification (RPA; Chao et al., 2015), and strand displacement amplification (Hellyer and Nadeau, 2004). Compared with these technologies, RPA has several advantages: RPA is faster (<30 min) and operates at a lower temperature (37–42°C). In the RPA method, a recombinase complex is used to introduce primers to specific DNA sites. These primers are elongated by strand-displacing DNA polymerase, and the intermediates are stabilized by single-stranded DNA-binding proteins (Krölov et al., 2014). For the visual detection of RPA production, compact instruments that are small and lightweight can be used. For point-of-use detection in a resource-limited setting, RPA in combination with a lateral-flow (LF) strip is a desirable option. The LF strips are simple devices that can be used to detect the target analyte in samples. The advantages of LF over other detection methods include its rapidity, low cost, ease of use, and long-term stability (Dzantiev et al., 2014). Colloidal gold is a commonly used label in LF detection. The mobile phase is driven by capillary action, which rehydrates the antibody-conjugated colloidal gold. When it is passed through the test zone, the target antigen is captured by the antibody-conjugated nanoparticles and accumulates, thereby allowing visual detection by the unaided eye (Hwang et al., 2016). This LF-based detection method does not require an electronic device to generate visual inspection results.

In the present study, we used the RPA reaction to amplify the *Salmonella invA* gene followed by LF to detect the result. The accumulation of gold nanoparticles (AuNP) produced a characteristic band on the test line, enabling the visual detection of *Salmonella* bacteria. After determining of the sensitivity and specificity of the assay, it was applied to test selected food samples. We compared the sensitivity of RPA-LF with that of PCR-LF. Based on our results, we conclude that RPA-LF is rapid, simple, and suitable for point-of-use detection.

MATERIALS AND METHODS

Reagents and Instrumentation

The RPA basic amplification kits were obtained from TwistDx Ltd. (Babraham, UK). All primers (Table 1) were obtained from Genewiz (Suzhou, China) and were suspended in water at a concentration of 10 µM. The DNA extraction kit was purchased from Tiangen (Beijing, China). Mouse anti-digoxin monoclonal antibody was purchased from Abcam (Cambridge, UK). Gold chloride trihydrate, trisodium citrate dihydrate, streptavidin, PBS (0.01 M), Tween 20, polyethylene glycol 20,000, BSA, and agarose were obtained from Sigma-Aldrich (St. Louis, MO). The plastic backing, sample pad, absorption pad, and nitrocellulose (NC) membrane were acquired from Millipore (Billerica, MA). Water was purified with a Milli-Q system (Millipore, Madrid, Spain). All solvents and other chemicals were analytical reagent grade.

Bacterial Strains

A total of 5 *Salmonella* strains and 6 other bacterial strains were used to determine the specificity of RPA (Table 2). These strains were stored in the Key Laboratory of Food Nutrition and Safety of the Ministry of Education, Tianjin University of Science and Technology (Tianjin, China). Stock cultures were stored at –80°C in 0.8 mL of Luria-Bertani broth (Beijing Land Bridge Technology Co., Ltd., Beijing, China) and 0.2 mL of 80% glycerol. The DNA templates were extracted with the TIANamp Bacteria DNA kit (Tiangen).

Primer Design and Screening

Primers specific to the *Salmonella invA* gene (Abouzeed et al., 2000) were designed based on guidelines provided by TwistDx (Babraham, UK). A variety of forward and reverse primer candidates were chosen, and the amplification effects were observed. The set of primers that produced the brightest bands were modified with end-tags to make them compatible with LF detection. The primers are summarized in Table 1.

Table 2. Bacterial strains used in our study

Species	Source
<i>Salmonella cholerae</i>	CICC21494
<i>Salmonella enteritidis</i>	CICC10867
<i>Salmonella paratyphi</i>	CICC21501
<i>Salmonella enterica</i> ssp. <i>enterica</i>	ATCC13076
<i>Salmonella typhi</i>	CICC10982
<i>Escherichia coli</i>	CICC10305
<i>Listeria monocytogenes</i>	CICC21633
<i>Shigella</i>	ATCC21535
<i>Enterobacter aerogenes</i>	CICC10293
<i>Staphylococcus aureus</i>	ATCC 25923
<i>Campylobacter jejuni</i>	ATCC33560

RPA Reaction

The RPA reaction was performed according to the manufacturer's recommended protocols (Cordray and Richards-Kortum, 2015). A variety of reaction temperatures and incubation times were tested to determine the optimal reaction conditions. For each reaction, 29.5 μ L of rehydration buffer, 10.7 μ L of sterile water, 2.4 μ L of forward and reverse primers (10 μ M), and 2.5 μ L of template were mixed. One enzyme pellet from the kit was added to each reaction; then, 2.5 μ L of magnesium acetate solution was added to the tube to start the reaction. The tubes were then briefly centrifuged and vortexed to thoroughly mix the samples. Subsequently, the tubes were incubated in a water bath at 36, 37, 38, 39, 40, or 41°C (Lutz et al., 2010) for 2.5, 5, 10, 15, 20, or 25 min. After the RPA reaction, the products were heated at 82°C [slightly below the product melting temperature (T_m)] for 5 min to denature the primer dimer. The products were analyzed by both gel electrophoresis and LF strip. For LF strip detection, 90 μ L of PBS was mixed with 10 μ L of RPA reaction product. Then, the mixture was transferred to the test strip, and the band was visualized within 5 min. For agarose gel electrophoresis detection, 5 μ L of RPA reaction product was transferred to 2% agarose gel and visualized by staining with ethidium bromide.

Synthesis of Antibody-Conjugated AuNP

Gold nanoparticles (nanospheres) with diameters of 15 ± 3 nm were prepared according to previously reported methods (Hwang et al., 2016) and characterized by field emission electron microscopy (JEM-2010 FEF, JEOL, Tokyo, Japan) and UV/visible absorption spectroscopy (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-12566>). The anti-digoxin antibody was conjugated to functionalized AuNP according to a previously described method (Zhao et al., 2004). Under gentle stirring, 12 μ L of 1-mg/mL mouse anti-digoxin monoclonal antibody was added to 1 mL of

colloidal gold solution (pH 8.5). After incubation for 1 h at 4°C, 20 μ L of 20% (wt/vol) BSA and 10 μ L of 20% polyethylene glycol 20,000 were added to the solution, which was then incubated for another 15 min at 4°C. Subsequently, the mixture was centrifuged at 300 g/min for 15 min at 4°C, and the supernatant was transferred to a new microcentrifuge tube. Next, the new mixture was centrifuged again at 8,600 g/min for 30 min. The pellet was resuspended in 250 μ L of buffer (5% BSA, 0.025% Tween 20, and 1% sodium azide).

Assembly of the Paper-Based LF

The principle of the LF strip is illustrated in Figure 1. The LF strips consisted of plastic backing, an NC membrane, a conjugation pad, a sample pad, and an absorption pad. The conjugation and sample pads were both made from glass fibers. The conjugate pad was saturated with antibody-conjugated AuNP and dried at room temperature. Prior to assembly, a goat anti-mouse antibody (1 ng/cm) and streptavidin (0.7 ng/cm) were sprayed onto the NC membrane to produce the control and test lines, respectively. Then, the NC membrane was dried overnight in an oven at 37°C. To assemble the test strip, the NC membrane was placed in the center of the plastic backing and overlapped with the absorption pad and sample pad on each side. The conjugate pad containing anti-digoxin antibody that was labeled with AuNP was placed between the sample pad and the NC membrane. The assembled card was cut into 3.7-mm-wide strips using a programmable cutter (Zeta Corporation, Gunpo, South Korea) and stored in desiccator at room temperature.

Sensitivity and Specificity of the RPA-LF Assay

The sensitivity and specificity of the RPA-LF assay were determined at the optimal reaction time and temperature. The DNA from an overnight culture of *Salmonella* was extracted using the TIANamp Bacteria DNA kit (Tiangen). The concentration of total isolated DNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RPA assay was performed in duplicate using 20 ng to 20 fg of DNA as the template. The sensitivity of the RPA assay was determined using 10-fold serial dilutions of the *Salmonella* culture. The DNA templates were prepared from the different concentrations of *Salmonella* cultures by heating the bacteria at 95°C for 10 min (Yang et al., 2016). The specificity of the RPA method was evaluated by cross-reactivity analysis using 5 *Salmonella* and 6 non-*Salmonella* species, which are listed in Table 2. Each assay was repeated 3 times. The

detection limit was defined as the lowest concentration to test positive in all 3 repeat assays.

Preparation of *Salmonella*-Spiked Samples and Simulated Sample Detection

Salmonella was cultured in 9 mL of Luria-Bertani broth at 37°C for 24 h, harvested by centrifugation at $4,000 \times g$ for 20 min at 4°C, and washed 3 times with PBS (0.01 M phosphate, 0.137 M NaCl, pH 7.3). The final pellet was then added to milk, chicken breast, or egg using 10-fold serial dilutions to achieve different concentrations (cfu/g or cfu/mL): 1.05×10^0 , 1.05×10^1 , and 1.05×10^2 . The milk, chicken breast, and egg, which were verified to be free of *Salmonella* according to the National Standard GB/47894-2010 (People's Republic of China, 2010), were purchased from a local supermarket. Inoculated samples (25 g or 25 mL) were added to the Luria Bertani enrichment broth (Beijing Land Bridge Technology Co., Ltd., Beijing, China) in a 1:10 ratio to obtain the first dilution. To assess the detection limits at different enrichment times, which

were aimed at increasing the bacterial concentrations to detectable levels, the samples were incubated at 37°C for 0, 1, 2, 3, or 4 h. Then, 1-mL aliquots were collected at each time point and boiled for 10 min to release the DNA. The obtained liquid was used as the template for the subsequent RPA assay. Each food sample was tested twice, and each assay was repeated 3 times.

RESULTS

Operating Principle of the RPA-LF

The operating principle of the RPA-LF assay is based on sandwich-type reactions (Figure 1). The RPA was used to generate numerous biotin- and digoxin-labeled duplex DNA. The product solution was diluted 10 times with PBS (pH 8.0) and pipetted onto the sample pad. This solution, driven by capillary action, migrated through the conjugation pad and rehydrated the anti-digoxin antibody-conjugated AuNP. The digoxin on the duplex DNA reacted with the anti-digoxin on the surface of the AuNP to form a biotin-duplex DNA-di-

Figure 1. Schematic diagram of the recombinase polymerase amplification (RPA) lateral-flow (LF) nucleic acid biosensor for the detection of double-labeled (digoxin/biotin) *invA* gene. NC = nitrocellulose; AuNP = gold nanoparticles. Color version available online.

goxin-anti-digoxin-AuNP complex, which continued to migrate along the strip. The complex was captured by streptavidin immobilized on the test line, which led to an increase in the number of AuNP complexes and the development of a visual band on the test line. Excess antibody-conjugated AuNP continued to migrate and were captured by the secondary antibody immobilized on the control line. Finally, in the presence of the target, 2 visual bands appeared (positive), whereas for negative samples, only one visual band was observed on the control line (negative). The visual band on the control line indicates that the LF strip functioned properly. To measure the sensitivity of the *Salmonella* RPA-LF assay, the detection limit was defined as the minimum target concentration that produced a visual band at the test line that was significantly different from the test line on the negative control strip. The LF strips were stable for at least 6 mo at room temperature. As shown in Supplemental Figure S2 (<https://doi.org/10.3168/jds.2017-12566>), the brightness of the bands did not significantly differ between newly (LF1) and previously (LF2) prepared LF strips. To the best of our knowledge, this is the first RPA-LF assay developed to detect *Salmonella* bacteria.

Performance of RPA

The RPA reaction was performed according to the Quick Guide provided by the manufacturer. All reactions produced a visual band at 188 bp after gel electrophoresis. The brightness of the band was directly proportional to the amount of template added to the RPA reaction (Figure 2B, 2D).

Optimization of the RPA-LF Conditions

To determine the optimal temperature for the RPA reaction, the RPA assay was tested at temperatures ranging from 36 to 41°C. First, we assessed this range of temperatures using an incubation time of 10 min and analyzed the amplification products using LF strips. As shown in Figure 3A, weak test lines were obtained at 36, 37, and 38°C, and no differences in amplification were detected among the temperatures of 39, 40, and 41°C. Consequently, 39°C was selected arbitrarily as the reaction temperature for subsequent experiments. Next, we tested the effect of incubation time on amplification efficiency. We performed the RPA assay at 39°C and incubated for 2.5, 5, 10, 15, 20, or 25 min. As shown in Figure 3B, as the amplification time increased from 2.5 to 10 min, the brightness of the strip increased. No differences in amplification were observed between the amplification times of 10 and 15 min. When the amplification time was 20 or 25 min, the band was brighter, but the reaction time was longer, and false-positive results were more likely to occur due to unexpected amplification or primer-dependent artifacts (Piepenburg et al., 2006). Thus, amplification times of 5 and 10 min were selected for the *Salmonella* RPA-LF assay in this study.

Detection Limit of the RPA-LF Assay

The *Salmonella* RPA assay was performed at 39°C with incubation times of 5 and 10 min. Ten-fold serial dilutions of template DNA were prepared, corresponding to 20 ng to 20 fg of DNA. The products were ana-

Figure 2. (A) Lateral-flow (LF) strips showing the detection limit of recombinase polymerase amplification (RPA) with an RPA incubation time of 5 min. (B) Agarose gel image of RPA amplicons with an RPA incubation time of 5 min. (C) Lateral flow strips showing the detection limit of RPA with an RPA incubation time of 10 min. (D) Agarose gel image of RPA amplicons with an RPA incubation time of 10 min. NC = negative control; M = D2000 DNA ladder used as size marker.

Figure 3. Optimization of the recombinase polymerase amplification (RPA) reaction temperature (A) and incubation time (B) using target DNA.

lyzed using LF strips and agarose gel electrophoresis. The 5-min RPA incubation had detection limits of 20 and 200 pg by LF and agarose gel electrophoresis, respectively (Figure 2A, 2B). In contrast, the 10-min RPA incubation resulted in detection limits as low as 20 fg and 2 pg by LF and agarose gel electrophoresis, respectively (Figure 2C, 2D). Furthermore, the color of the visual band on the test line observed using 20 fg of target DNA of *Salmonella* differed significantly from that observed for the negative control without *Salmonella*. Therefore, 20 fg of target DNA was considered the visual detection limit of the RPA-LF assay.

The RPA-LF assay and PCR-LF assay were applied to pure cultures with different concentrations of *Salmonella* ranging from 1.05×10^0 to 1.05×10^8 cfu/mL. As shown in Figure 4, 1.05×10^1 cfu/mL of *Salmonella* could be detected using the RPA-LF assay, whereas 1.05×10^5 cfu/mL of *Salmonella* was required for detection using the PCR-LF assay. Therefore, the RPA-LF assay had a lower detection limit than did the PCR-LF assay.

Specificity of the Protocol

To assess the specificity of the RPA-LF assay under the optimal conditions determined above, 5 *Salmonella* and 6 non-*Salmonella* strains that frequently contami-

nate food were amplified by RPA and observed using LF strips. As shown in Figure 5, the 5 *Salmonella* strains produced a visual band at the test line, whereas the 6 non-*Salmonella* strains only generated a visual band at the control line. Therefore, no cross-reactions were observed in the *Salmonella* strains examined, and the RPA-LF assay was specific for the detection of *Salmonella* bacteria.

Application to Food Samples

The developed RPA-LF assay was evaluated with milk, chicken breast, and egg samples spiked with *Salmonella* at concentrations ranging from 1.05×10^0 to 1.05×10^2 cfu/mL or cfu/g. The spiked samples were subsequently incubated for 0 to 4 h to determine the optimal enrichment time for different concentrations of *Salmonella*. In milk and chicken breast, positive results were obtained for the samples containing 1.05×10^2 cfu/mL or 1.05×10^2 cfu/g of *Salmonella* immediately after the sample was added to the enrichment culture (Figure 6). The RPA-LF assay could detect 1.05×10^1 cfu/mL, 1.05×10^1 cfu/g, and 1.05×10^2 cfu/g in milk, chicken breast, and egg, respectively, after at least 1 h of enrichment at 37°C. The RPA-LF assay detected 1.05×10^0 cfu/mL, 1.05×10^0 cfu/g, and 1.05×10^1 cfu/g of *Salmonella* after 2 h of enrichment in milk,

Figure 4. The sensitivity of PCR lateral flow (LF; A) and recombinase polymerase amplification (RPA)-LF (B) assays using DNA extracted from 1.05×10^1 to 1.05×10^8 cfu/mL in pure culture by boiling in sterile water. N represents negative control.

Figure 5. Molecular specificity of the recombinase polymerase amplification (RPA) lateral-flow (LF) assay using 6 non-*Salmonella* bacteria (A) and 5 *Salmonella* bacteria (B). *E. coli* = *Escherichia coli*; *S. aureus* = *Staphylococcus aureus*; *C. jejuni* = *Campylobacter jejuni*; *E. aerogenes* = *Enterobacter aerogenes*; *L. monocytogenes* = *Listeria monocytogenes*.

chicken breast, and egg, respectively. In addition, egg containing 1.05×10^0 cfu/g *Salmonella* produced a positive result after enrichment for 4 h.

DISCUSSION

Currently, the high prevalence of *Salmonella* poses a great risk to public health (Abirami et al., 2016). Rapid detection methods that can shorten the detection time for *Salmonella* and limit the dissemination of *Salmonella* along the food chain are needed (Abirami et al., 2016). However, current detection methods require

expensive instruments or inconvenient, time-consuming procedures that severely limit their suitability for point-of-use detection (Rohde et al., 2016). Recombinase polymerase amplification is a recently described isothermal amplification technique that can amplify target DNA to detectable levels in less time and at a lower constant temperature than other isothermal amplification procedures, such as helicase-dependent amplification (Ramalingam et al., 2009), loop-mediated isothermal amplification (Cornelissen et al., 2016), and rolling circle amplification (Zhu et al., 2014). In addition, RPA is tolerant to impure samples (Krölov et

Figure 6. Typical photographs of the analytical performance of recombinase polymerase amplification (RPA) lateral flow (LF) in milk (A, D, G), chicken breast (B, E, H), and egg (C, F, I) after inoculation with 10^0 , 10^1 , or 10^2 cfu/mL or cfu/g *Salmonella* after different enrichment times. (A), (B), and (C): RPA-LF result of incubation at 37°C for 0 h after inoculation; (D), (E), (F): RPA-LF result of incubation at 37°C for 1 h after inoculation; (G) and (H): RPA-LF result of incubation at 37°C for 2 h after inoculation; and (I): RPA-LF result obtained from egg after incubation for 4 h. N represents the negative control.

al., 2014). Thus, DNA purification from samples is not required, which eliminates the cost and time required for DNA extraction. These advantages make RPA particularly suitable for use in remote regions (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12566>). Currently, the cost of the RPA-LF assay is higher than that of PCR-LF; however, this cost is expected to decrease with increased development and usage (Silva et al., 2015). The LF strips are promising tools that translate isothermal amplification into a visual test and eliminate the need for trained personnel, expensive materials, and other external apparatus. An assay combining RPA and LF strips would have many advantages, such as simple operation, fast detection, rapid reaction, and low energy consumption (Kolm et al., 2015; Rivas et al., 2015). The LF strips were stable for at least 6 mo at room temperature. As shown in Supplemental Figure S2 (<https://doi.org/10.3168/jds.2017-12566>), the brightness of the bands did not significantly differ between newly (LF1) and previously (LF2) prepared LF strips. Thus, the RPA-LF assay is suitable for the point-of-use diagnosis of *Salmonella* infection.

The RPA-LF assay is based on the sandwich-type hybridization reaction, which relies on the antigen-antibody reaction. Under suitable conditions, RPA produces amplification products concurrently labeled with digoxin and biotin. The streptavidin on the test line captures biotin, attached on one end of the RPA products, and digoxin, attached on the other end, is captured by anti-digoxin antibody coated on AuNP. The accumulation of AuNP on the test line produces a visual band. The assay was capable of detecting as few as 1.05×10^1 cfu and had a detection limit of 20 fg of DNA per test. This detection limit is in the same range as those of other nucleic acid amplification-based techniques (Chen et al., 2015, 2016; Cornelissen et al., 2016). Thus, the RPA-LF assay is much more sensitive than culture-based methods, and the PCR-LF assay has a detection limit 4 orders of magnitude higher than that of the RPA-LF assay. The culture enrichment of samples before target detection is necessary because the contamination levels are often below the detection limit. Food samples were artificially inoculated with *Salmonella* (spiked level: 1.05×10^0 to 1.05×10^2 cfu/g or 1.05×10^0 to 1.05×10^2 cfu/mL), and the RPA-LF assay produced a positive result at 0 h for milk and chicken breast containing an initial bacteria concentration of 1.05×10^2 cfu/mL or 1.05×10^2 cfu/g. Additionally, following a 1-h incubation, the RPA-LF assay allowed detection of an initial bacteria concentration of 1.05×10^2 cfu/g in egg. The chicken breast and milk matrices did not affect the analytical sensitivity of RPA, and the limit of detection was in the same range as that for pure bacterial culture. However, our study

revealed that the sensitivity of the RPA-LF assay was reduced in eggs relative to milk or chicken breast. According to our study, the selenite cystine broth (Beijing Land Bridge Technology Co., Ltd.), which was used to selectively enrich *Salmonella*, significantly affected the RPA, resulting in a large number of primer dimers and leading to false-positive results on the LF strips. Therefore, in the food sample detection, LB broth was used for enrichment culturing rather than selenite cystine broth.

Additional benefits of the RPA-LF assay are its short incubation time, avoidance of DNA purification steps, and low, single incubation temperature (35–45°C), which can be slightly higher than the surrounding temperature (Lillis et al., 2014). Thus, a water bath kettle is sufficient to achieve accurate detection (Crannell et al., 2014). This assay does not require the purification of DNA from samples. Therefore, heating the sample at 95°C for 10 min was sufficient to release the target DNA and certify the presence of the target pathogen. The omission of the DNA purification step before RPA amplification made the assay less time consuming and less expensive. The RPA-LF assay produces a visual band on the test line, which can be observed without magnification by untrained personnel in less than 30 min; this time includes time required for preparing the genomic DNA, and performing the RPA reaction and strip detection. Furthermore, the RPA reaction and LF test do not require any expensive equipment, which is important for pathogen detection in remote areas. The RPA reaction scheme, similar to the PCR reaction, uses only 2 primers, and it can be easily adapted to detect other pathogens. It also has the potential for use in multiplex reactions.

The RPA-LF assay is an attractive detection method that is suitable for point-of-use diagnosis and the mass monitoring of food. However, before the RPA assay can be widely applied, improvements are required. One drawback of the RPA-LF assay is that false-positive results can occur because of the primer dimers that simultaneously carry the biotin and digoxin. As in the PCR reaction, they are formed with similar efficiencies as the target sequence (Kolm et al., 2015). This problem can be solved by selecting the most suitable primers and heating slightly below the T_m of the RPA product. The T_m of the product is the temperature at which half of the complementary base pairs in the product become melted. Thus, heating below the T_m can dissolve the primer dimer without affecting the product. As shown in Supplemental Figure S3 (<https://doi.org/10.3168/jds.2017-12566>), false positives can be eliminated by optimizing the heating temperature and heating time. After heating at 82°C for 5 min, no primer dimers carrying both biotin and digoxin were detected;

under these conditions, the detection of *Salmonella* on the LF strip is not affected by primer dimers. Another drawback is that false-positive results can be caused by low levels of contamination with previously amplified nucleic acid fragments. The operator should ensure that the cap of the RPA amplification tube is not opened in the sample-handling area to prevent the formation of aerosol pollution. Therefore, the enrichment of bacteria in culture and the RPA reaction should be performed with care to avoid accidental laboratory contamination (Binet et al., 2014).

CONCLUSIONS

This study combined the RPA reaction with a nucleic acid LF immunostrip for the rapid detection of *Salmonella* bacteria. This immunostrip detection method, which uses inexpensive capillary action based on an overlapping paper membrane system, offers rapid visual detection results. The RPA-LF assay is highly specific and has a detection limit of 1.05×10^1 cfu in pure culture or 20 fg of DNA within 30 min using simple water bath equipment. Compared with conventional PCR and other isothermal amplification methods, the RPA reaction is much simpler, requires a shorter incubation time, and avoids the need for specialized personnel or expensive instruments. This sensor can be used to rapidly test food samples, such as samples of chicken breasts, eggs, and milk, without any sample pretreatment or technical expertise. Additionally, we were able to eliminate false positives caused by primer dimer generation during amplification by heating at 82°C for 5 min. The combination of RPA and LF is thus a promising on-site detection technology to monitor *Salmonella* infection.

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