

Rapid on-site detection of *Salmonella pullorum* based on lateral flow nucleic acid assay combined with recombinase polymerase amplification reaction

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ABSTRACT Caused by *Salmonella pullorum*, pullorosis is a bacterial disease threatening the poultry industry and has been listed as the bacterial disease to be eliminated by the government. However, antibiotic treatment of pullorosis has become increasingly difficult, resulting in severe influences on the sustainable development of poultry. Abuse of antibiotics may cause global drug-resistant problems. Hence, early diagnosis of young chickens and accurate treatment of sick chickens are urgently needed. Traditional serotyping for *Salmonella* detection is costly and labor-intensive, whereas other commonly used plate agglutination test methods often cause physical injury of chickens. Therefore, a rapid and nondamaging detection method is of great significance for early diagnosis, which is the key step in accurate medication and elimination of pullorosis. In this study,

we propose a novel lateral flow nucleic acid assay (LFNAA) system combining recombinase polymerase amplification (RPA) for the detection of *S. pullorum*. In this method, the DNA of *S. pullorum* strains was quickly amplified by RPA under 37°C, and then, the RPA products were added onto the LFNAA sample pad until the final results could be observed by naked eyes within 3 min. The proposed assay is fast and delivers visible results to naked eyes in field test. The limit of detection for genomic DNA was 5×10^{-3} ng/ μ L, indicating high sensitivity. In addition, the proposed LFNAA system is cost-effective, efficient, and nondamaging to chicks in the field test. This system provides technical support for early diagnosis of *S. pullorum* in the poultry and paves a way for future precision medicine to avoid the global drug-resistance issues.

Key words: recombinase polymerase amplification (RPA), lateral flow nucleic acid assay (LFNAA), *Salmonella pullorum* (*S. pullorum*), early diagnosis

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INTRODUCTION

Salmonella pullorum can infect 10-day-old chickens, resulting in high mortality with severe systemic lesion (Barrow and Neto, 2011). Besides young chickens, the infected adult birds also exhibit symptoms of dysentery, weight loss, reduced egg laying, and deformity of the reproductive tract (Shivaprasad 2000). However, it is barely possible to eliminate *S. pullorum* infection as it can spread vertically and horizontally. The outbreak of *S. pullorum* can cause severe economic losses, and it has been listed as a bacterial disease to be eliminated

by the Chinese government (Berchieri et al., 2001). In various developing countries, antibiotic treatment, which seriously affects the sustainable development of poultry breeding and may cause transmission of highly resistant strains via food chain and environment, still serves as the main method for pullorosis treatment (Gong et al., 2013). Indeed, severe bacterial drug resistance, which is exacerbating owing to repeated utilization of antibiotics and irrational medication, is a severe threat nowadays. Therefore, a viable early diagnosis method for precision medicine is urgently needed.

Conventional detection of *S. pullorum* relies on serotyping, which is based on the White–Kaufmann–Le Minor scheme (Majchrzak et al., 2014). However, serotyping is a time-consuming and labor-intensive process. Typically, the entire experiment requires 5 or 6 d and a series of typing antisera (Bell et al., 2016). Another diagnostic method commonly used in chicken farms is plate agglutination test with diagnostic antigen. Plate

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agglutination test requires collection of blood samples, which causes physical injury (Ranieri et al., 2013). Recently, PCR has been widely used as a tool for bacterial detection, in virtue of its high throughput and excellent sensitivity and specificity (Karns et al., 2015). Encouragingly, recombinase polymerase amplification (RPA), which is a promising isothermal methodology, is as specific as PCR amplification (Lobato and O'Sullivan, 2018), yet much faster. Indeed, RPA results are typically obtained within 5–10 min. Unlike PCR, RPA requires no heating equipment or reagents as it functions well at 37–42°C (Stringer et al., 2018). Furthermore, a growing number of studies demonstrated excellent effectiveness of RPA in testing of many pathogens, including species of *Salmonella*, *Listeria*, *Vibrio*, and some strains of *Escherichia coli* (Gao et al., 2017).

In this study, we propose a novel lateral flow nucleic acid assay (LFNAA) system combining RPA for detection of *S. pullorum*. The proposed LFNAA system exhibits advantages such as good portability, easy operation, and visible results to naked eyes (Quesada-González and Merkoçi, 2015). Hence, lateral flow strips may be the ideal carrier for RPA in field tests. However, lateral flow immunoassay are limited by high cost of antibody modification and interferences caused by antibodies (Jauset-Rubio et al., 2016). To tackle these problems, we used primers modified by spacer C3-ssDNA tail. During the detection, tail nucleotide sequences can combine with gold-labeled probes, which could be captured by complementary sequences at the control line for chromogenic reaction. Compared with antibody-based lateral flow immunoassay, the nucleic acid-based LFNAA shows reduced cost and time of DNA amplification. This RPA combined with the LFNAA system is fast, highly sensitive, and delivers visible results to naked eyes in field tests.

Much importantly, the LFNAA system can realize nondestructive testing, compared with traditional plate agglutination test. In LFNAA, anal swab samples were collected instead of blood samples to greatly reduce damages to chickens and avoid unnecessary economic loss. The LFNAA system provides a feasible approach for early diagnosis of *S. pullorum* in chicken farms and paves a way for future precision medicine to avoid global drug-resistance issue.

MATERIALS AND METHODS

Bacterial Strains

Strains of *S. pullorum* (ATCC 19945) and non-*Salmonella* organisms, including *Salmonella* Enteritidis (*S. Enteritidis*) (BNCC103134), *Salmonella* Typhimurium (*S. Typhimurium*) (BNCC 103281), *Listeria monocytogenes* (BNCC 185988), *E. coli* (BNCC 336435), and *Staphylococcus aureus* (BNCC 186335) were purchased from commercial providers. All bacterial strains were bought from BNCC (BN-Bio Inc., Beijing).

The frozen isolate stocks were recovered in Luria-Bertani nutrient agar at 37°C for 12 h. The colonies were transferred to Luria-Bertani broth and

cultured for 12 h at 37°C with constant shaking (320 rpm). Genomic DNA were extracted from all strains using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit accordingly and then stored at –20°C.

Materials

A TwistAmp Basic Kit was purchased from TwistDx Ltd. (Cambridge, UK). All LFNAA materials, including backing cards, glass fiber sample pads, conjugation pads, nitrocellulose membranes, and absorbent pads, were purchased from JN-Bio Co., Ltd. (Shanghai, China). Gold, trisodium citrate, NaH₂PO₄, Na₂HPO₄, and NaCl were offered by Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). The TE buffer was from Tiangen (Beijing, China). ddH₂O, TAE, and PBS were provided by Solarbio (Beijing, China). Agarose and Cycle Pure Kit were bought from OMEGA (Omega Bio-Tek). The MiniBEST Bacteria Genomic DNA Extraction Kit was bought from TaKaRa (TaKaRa Bio-Inc., Japan).

DNA Extraction

We used the MiniBEST Bacteria Genomic DNA Extraction Kit to extract DNA from bacterial strains. NanoDrop 2000 (Thermo, China) was used to check the quality, purity, and concentration of the DNA template. As per the spectrophotometric evaluation results, the DNA template concentration was 10 ng/μL, which was suitable for high-purity PCR amplification with the A260/A280 ratio of 1.72–1.96 and 260/230 ratio ranging from 1.8 to 2.0.

Primer Design and Modification

The RPA primers were designed as per the nucleotide sequence of the *ipaJ* gene harbored in pSPI12 (Accession Number: GU949535) from *S. pullorum* using software Premier 5.0, as per the principles of RPA primer design. The specificity of primers was evaluated using the basic local alignment search tool. As shown in Figure 1, reverse primers (SEEP-R) in the LFNAA system were modified by spacer C3 at the 5'-end as stopper and connected to a segment of tail nucleotide sequence as a ssDNA tail. Meanwhile, forward primers (SEEP-F) were labeled with biotin, which can bind to streptavidin fixed on the test line. The primers and gold nanoparticle (AuNP) probes were synthesized in the Sangon Biotechnology laboratory (Shanghai, China), as listed in Table 1.

DNA Amplification by RPA Reaction

To verify the applicability of primers, we optimized the RPA system. RPA reaction was conducted using TwistAmp DNA amplification kits in an isothermal PCR PTC-100 instrument or a thermostat water bath. For each reaction, a 50-μL reaction mixture consisting of 1.2 μL forward primer (10 μM), 1.2 μL reverse primer

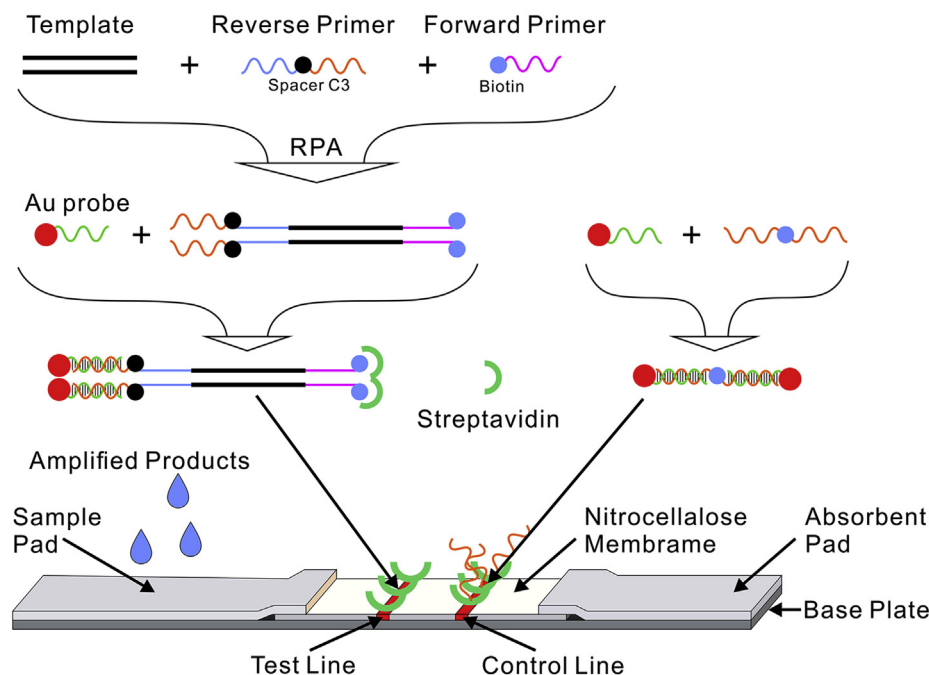


Figure 1. Schematic of the LFNA (lateral flow nucleic acid assay). Abbreviation: RPA, recombinase polymerase amplification.

(10 μ M), 2 μ L DNA template, 41.5 μ L buffer A and 1.6 μ L ddH₂O was involved and 2.5 μ L buffer B was added into the reaction mixture upon dissolution of powders in the solution. Then, tubes were kept at 40°C for 20 min. The amplified products were investigated using 2% agarose gel electrophoresis (AGE) or the LFNA system.

Construction of the LFNA System

The lateral flow nucleic acid strip consisted of a base plate, glass fiber sample pads, nitrocellulose membranes, and sample absorbent pads. The nitrocellulose membrane was the reaction zone sprayed on 1 test line and 1 control line. Streptavidin modified by a complementary ssDNA sequence was sprayed using a colloidal gold point system (Biodot XYZ3060) to obtain a streptavidin-biotinylated control line. The 1 mg/mL streptavidin was allowed to react with 100 μ M ssDNA (control probe) of the same volume for 2 h at room temperature (Xu et al., 2018). Similarly, 5 mg/mL streptavidin was sprayed directly on the test line. Subsequently, the complete lateral flow assay was dried at 37°C for 2 h, cut into 4 mm strips, and then stored at 4°C.

Preparation of Gold-Labeled Probes

The 25 nm AuNP were synthesized using the method proposed by Liu et al., with minor modification (Liu et al., 2010). All glassware were soaked in aqua regia and rinsed with deionized water before utilization. Then, 1 mL of 1 mmol HAuCl₄ was added into 99 mL DI water, and 2.0 mL of 194 mmol aqueous solution of sodium citrate was rapidly added into the boiling HAuCl₄ solution under intense agitation. The solution color changed from purple to red, indicating that AuNP were obtained. The solution was kept boiling for another 5 min under vigorous stirring. Eventually, the solution was naturally cooled to room temperature and then stored at 4°C.

AuNP modified by probes were prepared using a modified method on the basis of a previous one (Liu et al., 2013). Specifically, 20 μ L of 100 μ M thiol-modified AuNP probe was gently added to 500 μ L of prepared AuNP. After exposure to darkness for 16 h, 56 μ L of 10 mmol PB buffer (NaH₂PO₄/Na₂HPO₄, pH = 7.4) was added and 2 mol NaCl solution was changed to 0.3 mol by dropwise addition. After 8 h, the mixture was centrifuged for 30 min (4°C, 16,100 rpm). The supernatant was removed, and the precipitate was rinsed with 0.3 mol NaCl (pH 7.0) and 10 mmol PB solution. After rinsing twice, the colloids were resuspended in 0.01%

Table 1. The sequences of the primers and AuNP probes.

Primer	Sequence (5' to 3')	5'-end	3'-end
Control probe	TTGGTCGTGGTGGTGGTTT	5'Biotin	
SEEP-F	CGTACAATAAGGGATTATGTAAACCACG	5'Biotin	
SEEP-R	TTGGTCGTGGTGGTGGTTT/iSpC3/ TGTTAATTCAGAGTAAAGACCAGTTAACAC		
AuNP probe	AAACCACCACCACGACCAATTTTTTTTTTTTTTTT		3'HS-SH C6

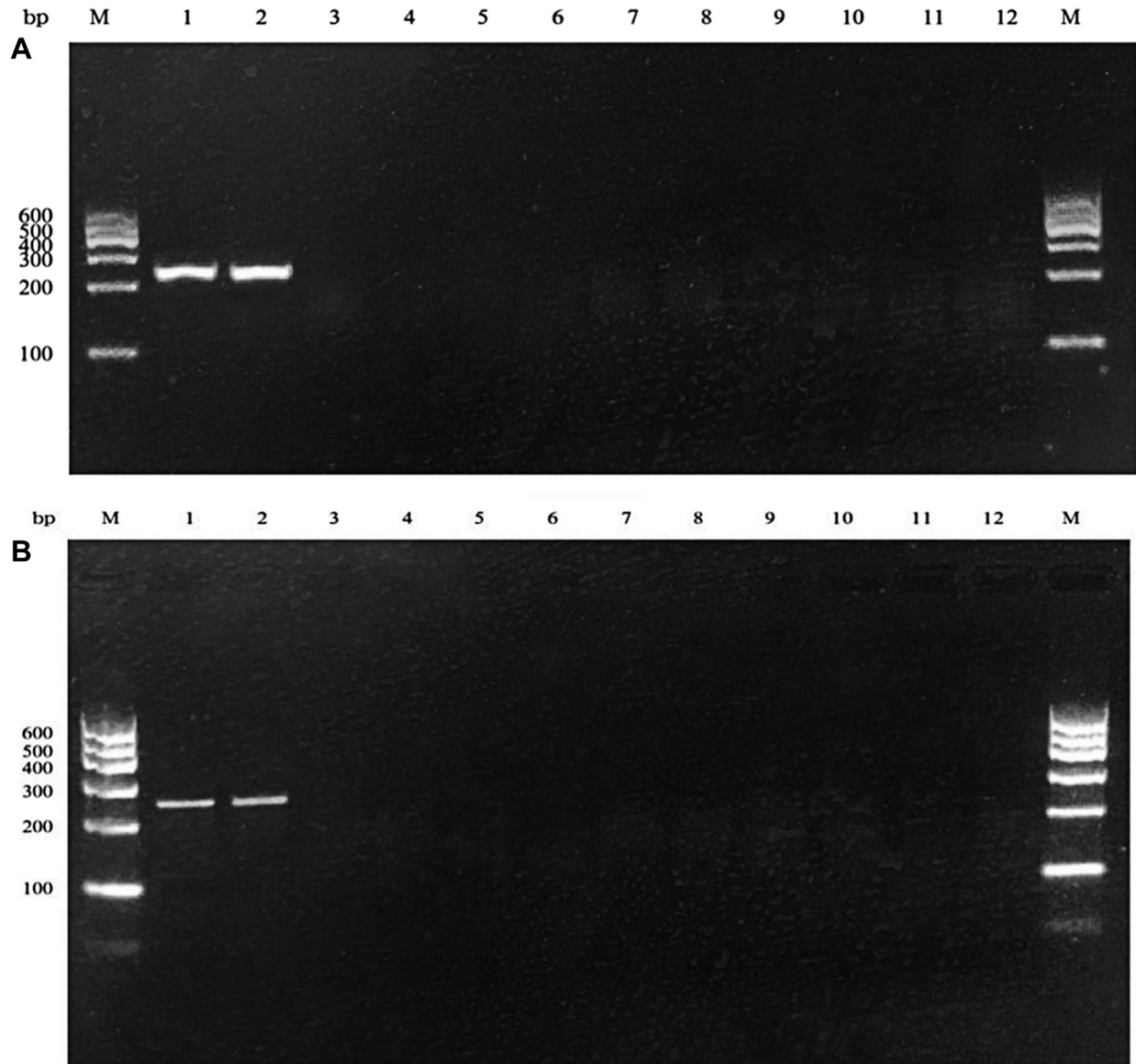


Figure 2. (A) The specificity of the RPA primers. The template species from left to right are *Salmonella pullorum*, *Salmonella Enteritidis*, *Salmonella Typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*, with 2 parallel lines for each species. (B) The specificity of the modified RPA primers. The template species from left to right are *S. pullorum*, *S. Enteritidis*, *S. Typhimurium*, *L. monocytogenes*, *E. coli*, and *S. aureus*, with 2 parallel lines for each species. Abbreviation: RPA, recombinase polymerase amplification.

azide, 10 mmol PB solution with 0.3 mol NaCl and then stored at 4°C.

Specificity of the LFNA System

To determine the specificity of the LFNA system, DNA samples of strains of *S. pullorum*, *S. Enteritidis*, *S. Typhimurium*, *L. monocytogenes*, *E. coli*, and *S. aureus* were evaluated by RPA reactions with AGE and the LFNA system, respectively.

Sensitivity of the LFNA System

A variety of dilutions (10, 1, 10^{-1} , 5×10^{-2} , 10^{-2} , 5×10^{-3} , 10^{-3} , 5×10^{-4} ng/ μ L) of the extracted genomic DNA were prepared and detected by RPA reactions with AGE and LFNA system, respectively. In

this way, the limit of detection (LOD) of the LFNA system was verified.

Clinical Sample Analysis

The LFNA system was assessed using genomic DNA from *Salmonella* isolates (10 *Salmonella* isolates) collected from the chicken farm. The results were then compared with those delivered by the traditional serotyping and PCR results with AGE.

RESULTS AND DISCUSSION

Design of the LFNA System

In this study, we propose a LFNA system combined with RPA amplification, including a control line based

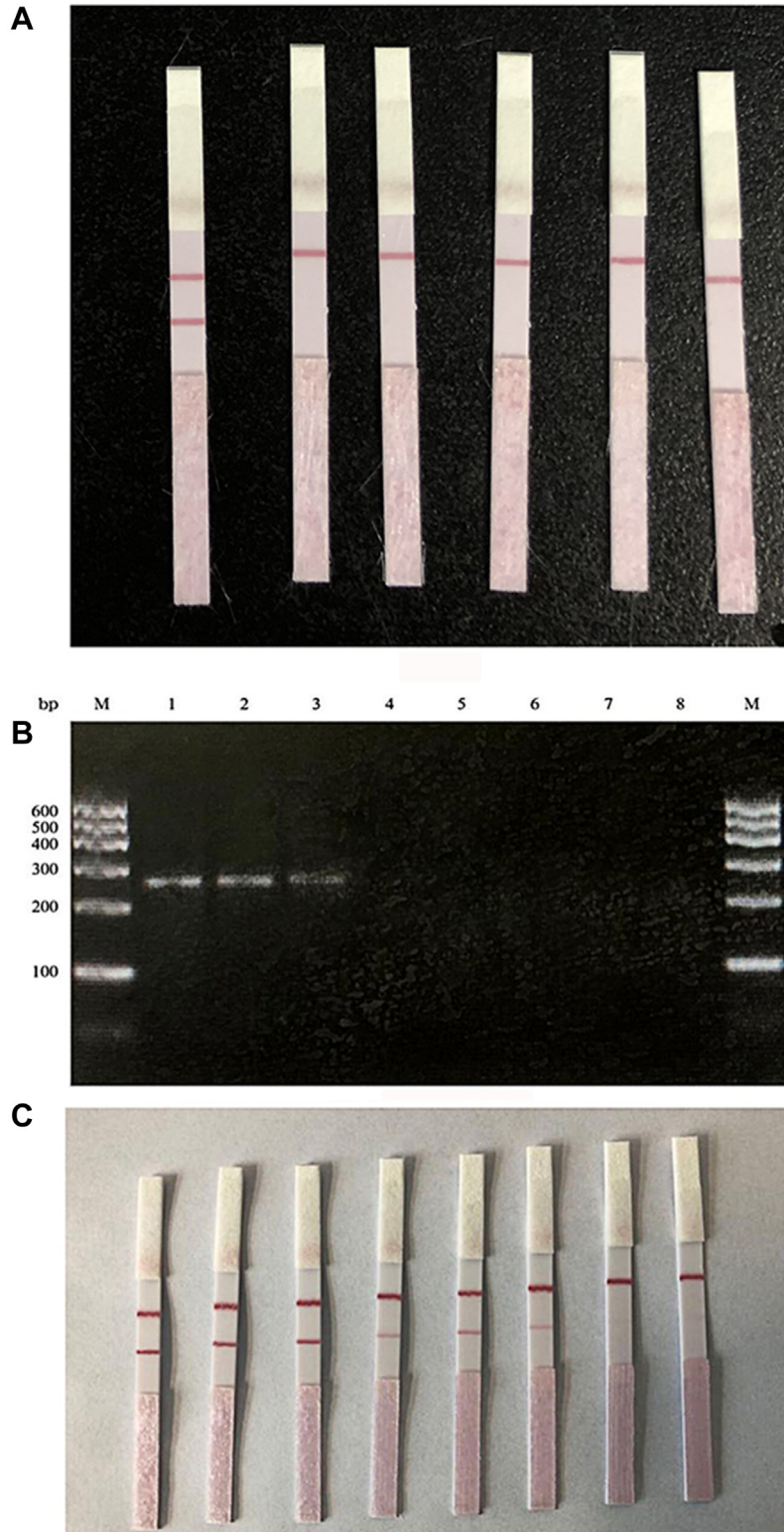


Figure 3. (A) The specificity of the RPA primer to different species, analyzed with the LFNAA system. The template species from left to right are *Salmonella pullorum*, *Salmonella Enteritidis*, *Salmonella Typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*. (B) Detection of a series of concentrations (10, 1, 10^{-1} , 5×10^{-2} , 10^{-2} , 5×10^{-3} , 10^{-3} , 5×10^{-4} ng/ μ L) by RPA primers with agarose gel electrophoresis. (C) Detection of a series of concentrations (10, 1, 10^{-1} , 5×10^{-2} , 10^{-2} , 5×10^{-3} , 10^{-3} , 5×10^{-4} ng/ μ L) by RPA primers with the LFNAA system. Abbreviations: LFNAA, lateral flow nucleic acid assay; RPA, recombinase polymerase amplification.

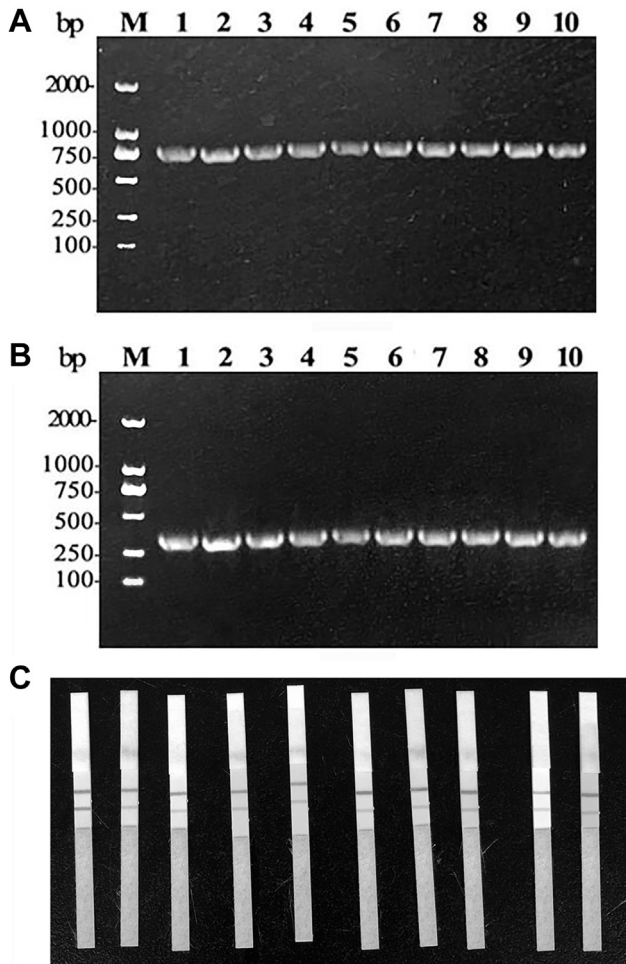


Figure 4. (A) Detection of 10 samples of *Salmonella pullorum* strains by PCR primers with agarose gel electrophoresis. (B) Detection of 10 samples of *S. pullorum* strains by RPA primers with agarose gel electrophoresis. (C) Detection of RPA primer detecting 10 samples of *S. pullorum* strains by RPA primers with the LFNA system. Abbreviations: LFNA, lateral flow nucleic acid assay; RPA, recombinase polymerase amplification.

on complementary nucleic acids and a test line in accordance with a biotin–streptavidin double sandwich assay. The LFNA system is illustrated in Figure 1. Reverse primers were modified with spacers C3 at the 5′-end as stopper and connected to a segment of a tail nucleotide sequence as ssDNA tail. After RPA amplification, the ssDNA tail was combined with a gold-labeled probe,

which contained a complementary nucleic acid sequence. In presence of the target DNA, biotin at one end of the RPA amplification product binds to streptavidin immobilized on the test line and the tail at the other end, which is bound to the gold-labeled detection probe, delivers chromogenic reaction on the test line. In absence of the target DNA, RPA amplification is not initiated, and gold-labeled detection probes are not captured by streptavidin on the test line. Hence, no chromogenic reaction occurs on the test line, and the reagent will flow directly to the control line. In both cases, the gold-labeled detection probes bind to the complementary ssDNA sequences immobilized on the control line of the strip.

Initial Design and the Specificity of RPA Primers

The design of RPA primers have specific requirements: a pair of primers should be composed of 30 bp to 34 bp, and primer sequences should be strictly complementary to the amplification fragments. The 3–5 nucleotides at the 5′-end are preferably cytosine to facilitate recombination, and the nucleotides at the 3′-end are usually G or C to maintain the stability of the polymerase and avoid dimmer hairpin structures. In this way, an ideal amplification band can be obtained. As per these principles, primers are designed and examined by the National Center for Biotechnology Information.

To determine the specificity of the RPA assay, *S. pullorum* strains and other strains were examined (Figure 2A). No cross-reactivity was observed with the genomic DNA from other strains detected by AGE. In addition, a positive result was obtained only with the DNA of *S. pullorum*, whereas negative results were observed from other strains by AGE.

The determined optimized primer pair was modified as per previous instructions. Spacer C3 and tail sequences were introduced to reverse primers, and forward primers were modified with biotin. Afterward, positive amplification of RPA reaction was performed on both unmodified and modified primers, and RPA reactions with modified primers were analyzed by AGE. As shown in Figure 2B, amplification products of modified primers were slightly longer than their unmodified peers owing to

Table 2. *Salmonella pullorum* strains isolated from chicken farms to evaluate the clinical applicability of the LFNA system.

Serovar	Isolate number	ipaJ-positive or negative	Dulcitol fermentation	Ornithine decarboxylase
<i>S. pullorum</i>	1	Positive	-	+
<i>S. pullorum</i>	2	Positive	-	+
<i>S. pullorum</i>	3	Positive	-	+
<i>S. pullorum</i>	4	Positive	-	+
<i>S. pullorum</i>	5	Positive	-	+
<i>S. pullorum</i>	6	Positive	-	+
<i>S. pullorum</i>	7	Positive	-	+
<i>S. pullorum</i>	8	Positive	-	+
<i>S. pullorum</i>	9	Positive	-	+
<i>S. pullorum</i>	10	Positive	-	+

Abbreviation: LFNA, lateral flow nucleic acid assay.

the presence of tail sequences. In addition, the results demonstrated that the tail sequences would not affect product amplification.

Specificity of the LFNA System

The specificity of the RPA primers used in the LFNA system was evaluated by 6 RPA reactions, including DNA from *S. pullorum*, *S. Enteritidis*, *S. Typhimurium*, *L. monocytogenes*, *E. coli*, and *S. aureus*. After purification, the amplified products were investigated by the LFNA system. As shown in Figure 3A, the primers amplified the *S. pullorum* DNA and showed negative results to other common strains.

Sensitivity of the LFNA System

A series of concentrations (10 , 1 , 10^{-1} , 5×10^{-2} , 10^{-2} , 5×10^{-3} , 10^{-3} , 5×10^{-4} ng/ μ L) were involved to assess the LOD of the LFNA system. The RPA amplification products were investigated by AGE and the LFNA system. As shown in Figures 3B and 3C, AGE could detect the DNA template of 10^{-1} ng/ μ L, whereas the LFNA could generate a weak chromogenic reaction even if the concentration of the template was 5×10^{-3} ng/ μ L, indicating that the LOD of LFNA was 5×10^{-3} ng/ μ L.

Application of the LFNA System

To evaluate the diagnostic possibilities of the established LFNA system, 10 samples of *S. pullorum* were isolated from naturally contaminated samples from a chicken farm. The PCR results showed that all the 10 samples contained the specific 741-bp target band of *ipaJ* (Figure 4A) (Xu et al., 2018), suggesting that these strains are *S. pullorum*. Meanwhile, the PCR results were consistent with those delivered by the traditional biochemical reactions and serotyping (Table 2).

The isolated *S. pullorum* strains were examined by the AGE method (Figure 4B) and the proposed LFNA system (Figure 4C). The AGE results showed that 10 samples contained *S. pullorum* strains. Similarly, clear chromogenic reaction was observed on the test line of the strip in the LFNA. The LFNA system was validated by screening for *S. pullorum* in samples isolated.

Discussion

A novel LFNA system combined with RPA amplification is established for detection of *S. pullorum*. The proposed LFNA consists of a control line based on the principle of complementary nucleic acids and a test line based on an antigen-antibody double sandwich reaction. The proposed LFNA was used to detect nucleic acid extracts of *S. pullorum*, and the LOD was 5×10^{-3} ng/ μ L. Compared with other colloidal gold test strips, the LFNA proposed assay not only reduced the cost but also mitigated the influence of disturbance factors because after RPA amplification, the ssDNA tail was

combined with the gold-labeled capture probe, and the forward primer labeled with biotin was bound to streptavidin on the test line as expected, which formed a stable combination of nucleic acid and AuNP. For early detection of *S. pullorum* in chicken, a rapid screen using the proposed LFNA system can greatly reduce the need for antisera, and it facilitates future noninvasive detections. In addition, the proposed LFNA can be readily extended to detection of other DNA sequences as it is facile, rapid, sensitive, cost-effective, and portable. The whole process consisting of RPA amplification and product detection could be finished within 40 min, with no need for expensive equipment or trained personnel.

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DISCLOSURES

The authors declare no conflict of interest. The authors confirm that all data underlying the findings are fully available without restriction and declare that they have no competing interests.

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