Rapid and Sensitive Detection of *Salmonella* in Chickens Using Loop-Mediated Isothermal Amplification Combined with a Lateral Flow Dipstick^S

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Received: December 6, 2017

Revised: March 16, 2018

Accepted: March 21, 2018

First published online
April 6, 2018

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Supplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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Salmonellosis is a highly contagious bacterial disease that threatens both human and poultry health. Tests that can detect *Salmonella* in the field are urgently required to facilitate disease control and for epidemiological investigations. Here, we combined loop-mediated isothermal amplification (LAMP) with a chromatographic lateral flow dipstick (LFD) to rapidly and accurately detect *Salmonella*. LAMP primers were designed to target the *Salmonella invA* gene. LAMP conditions were optimized by adjusting the ratio of inner to outer primers, MgSO₄ concentration, dNTP mix concentration, amplification temperature, and amplification time. We evaluated the specificity of our novel LAMP-LFD method using six *Salmonella* species and six related non-*Salmonella* strains. All six of the *Salmonella* strains, but none of the non-*Salmonella* strains, were amplified. LAMP-LFD was sensitive enough to detect concentrations of *Salmonella enterica* subsp. *enterica* serovar Pullorum genomic DNA as low as 89 fg/μl, which is 1,000 times more sensitive than conventional PCR. When artificially contaminated feed samples were analyzed, LAMP-LFD was also more sensitive than PCR. Finally, LAMP-LFD gave no false positives across 350 chicken anal swabs. Therefore, our novel LAMP-LFD assay was highly sensitive, specific, convenient, and fast, making it a valuable tool for the early diagnosis and monitoring of *Salmonella* infection in chickens.

Keywords: *Salmonella*, *invA* gene, loop-mediated isothermal amplification, lateral flow dipstick, detection

Introduction

Salmonellosis in chickens is caused by *Salmonella*, a common zoonotic pathogen. This pathogen poses a serious threat to China's socio-economic and public health [1]. The number of *Salmonella*-related deaths and infections increases every year; therefore, salmonellosis is an important public health concern [2]. Indeed, even low doses of *Salmonella* can cause a wide range of human and animal diseases [3]. The

worldwide annual cost of medical treatment for *Salmonella* infections is about \$11.4 billion [4]. Consumption of the *Salmonella* pathogen via contaminated food or water causes most human infections [5]; it is estimated that 95% of *Salmonella* infections are due to the consumption of contaminated foodstuffs [6]. *Salmonella* is most common in meat, processed meats, processed foods containing meat, eggs, and foods contaminated during processing [7]. *Salmonella* outbreaks following the consumption of chicken

eggs contaminated with *Salmonella enterica* are relatively common [8, 9]. Therefore, the rapid and accurate detection of *Salmonella* infections in chickens is of critical importance to animal husbandry, food safety, and human health.

The rapid detection method, which combines a polymerase chain reaction (PCR), a quantitative real-time PCR (qPCR), and an enzyme-linked immunosorbent assay (ELISA), is gradually becoming more widely used for the differential diagnosis of *Salmonella* and other foodborne pathogens [10, 11]. However, this method also has some disadvantages. For example, PCR and qPCR analyses will only detect pathogens above a certain concentration; these methods also cannot detect whether or not the bacteria was alive at the time of the analysis [11]. ELISAs require expensive instruments, and ELISA results are both unstable and highly influenced by the technique of the experimenter, therefore limiting the wider application of this method [12].

Several additional detection methods have been used for the detection of *Salmonella*, including gene chips, PCR-pyrosequencing, pulsed-field gel electrophoresis (PFGE), ribotyping, intergenic sequence (IGS) typing, matrix-associated laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and chemiluminescence [13, 14]. Unfortunately, these techniques require access to expensive laboratory instruments and specialized personnel; most also require large sample sizes. These methods are often time-consuming, labor-intensive, and complicated [15], making such tests difficult to popularize and promote in the field. Therefore, the development of new, user-friendly tests for *Salmonella* and other bacterial food pathogens is of great practical importance to public health and veterinary medicine.

In 2000, Notomi *et al.* [16] developed the easy and LAMP uses 4–6 specially designed primers and a strand-displacing Bst DNA polymerase to quickly perform isothermal amplification of target DNA [17] within one hour. The advantages of LAMP include high specificity, high sensitivity, and simple equipment requirements. The products of LAMP amplification are usually detected with gel electrophoresis, followed by either staining with ethidium bromide (EB) or the addition of calcein; contamination of the amplification product could produce false positives [18]. Unfortunately, these shortcomings reduce the practicality of the LAMP technique in the field.

Recent studies have shown that LAMP-LFD (lateral flow dipstick) is based on LAMP amplification principles, but it uses a test strip to display the results [19]. That is, LAMP-LFD uses a biotin-labeled LAMP product hybridized with a digoxin-labeled DNA probe and complexed with a gold-

labeled anti-digoxin antibody [20]. This hybridization product is trapped by a biotin ligand and bound to a lateral flow test strip, which forms an immune complex. Non-hybridized digoxin-labeled probes pass through the test line and bind to the goat anti-mouse immunoglobulin G (IgG) antibody control line [21]. The use of digoxin-labeled probes increases the detection specificity and sensitivity, reduces the number of false positives caused by non-specific amplification, and ensures the accuracy of the results [22]. LAMP-LFD takes 10–15 min compared to 35–50 min required for gel electrophoresis [23]. This method also reduces the dependence on equipment and avoids the use of the carcinogen EB. Although LAMP-LFD detection methods have been successfully developed for a variety of pathogenic microorganisms [23–26], no LAMP-LFD method is yet available for the detection of *Salmonella*.

Here, we developed a novel LAMP-LFD method to rapidly detect *Salmonella* by targeting the *invA* gene. We evaluated the sensitivity, specificity, and reproducibility of our LAMP-LFD assay using pure *Salmonella* cultures, artificially contaminated feed, and clinical samples. Our LAMP-LFD assay is a reliable and effective diagnostic tool for the detection of avian salmonellosis in farm-based laboratories.

Materials and Methods

Reagents and Materials

We purchased a bacterial genome DNA extraction Kit, 10 × PCR buffer, and dNTP mix from Sangon Biotech Co., Ltd. (China). We bought Bst 2.0 DNA polymerase large fragment (M0538) from New England Biolabs, Ltd. (China). We obtained buffered peptone water (BPW) and Rappaport Vassiliadis *Salmonella* Enrichment Broth (RV) from Hopebio-Technology Co. (Qingdao, China). The LFD was provided by the Nucleic Acids Labeling and Detecting Lab, Key Laboratory of Ministry of Education with Provincial Co-Construction of Local and Ethnic High Incidence in Xinjiang (Shihezi, China). We purchased calcein, betaine, and MgSO₄ from Sigma (USA).

Bacterial Strains and Samples

All strains of *Salmonella* and non-*Salmonella* bacteria used in this study were from stocks maintained at the Laboratory of Animal Zoonosis at Shihezi University (China; Table 1). We used *S. enterica* subsp. *enterica* serovar Pullorum (hereafter referred to as *S. Pullorum*) to test the specificity and sensitivity of our LAMP-LFD assay, but all *Salmonella* species were used for the feed-contamination experiments. We bought feed and chicken eggshells from a farm in Shihezi, Xinjiang, China. We confirmed that the feed and eggshells were not contaminated with *Salmonella* using the culture method. We collected 350 anal swabs from

Table 1. Serovars of *Salmonella enterica* subsp. *enterica* and other pathogenic bacterial species used in this study.

Classification	Bacteria	Strains	Source	Location of separation
Target bacterial species	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovars			
	Pullorum	CVCC ^a 530	CVCC ^a	USA
	Pullorum	CVCC ^a 1791	CVCC ^a	Jiangsu, China
	Typhimurium	CVCC ^a 542	CVCC ^a	Zhejiang, China
	Enteritidis	CVCC ^a 3378	CVCC ^a	Beijing, China
	Gallinarum	CICC ^b 21510	CICC ^b	Czech Republic
	Pullorum	CVCC ^a 1802	CVCC ^a	Jiangsu, China
Negative control bacterial species	<i>Pasteurella multocida</i>	Serotype 5	Isolated from raw chicken	
	<i>Escherichia coli</i>	EC-xj 01	Isolated from raw chicken	Xinjiang, China
	<i>Staphylococcus aureus</i>	CVCC ^a 1885	CVCC ^a	Beijing, China
	<i>Avian aspergillus</i>	CVCC ^a 3596	CVCC ^a	
	<i>Pseudomonas aeruginosa</i>		Isolated from raw chicken	
	<i>Haemophilus paragallinarum</i>	Type A	Isolated from raw chicken	

^aCVCC, China Veterinary Culture Collection Center.

^bCICC, China Center of Industrial Culture Collection.

chickens on several large chicken farms around Urumqi, Xinjiang, China. All of the strains and specimens were stored at -80°C .

Bacterial Culture and DNA Extraction

All of the bacterial strains were cultured on common ordinary agar (*Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*), potato dextrose agar (avian *Aspergillus*), or blood agar (*Pasteurella multocida* and *Haemophilus paragallinarum*) at 37°C for 20–48 h. *Haemophilus influenzae* were grown under facultative anaerobic conditions (10% CO_2). We used the plate-

colony counting method to determine the concentration of each culture. We used 10-fold serial dilutions with phosphate buffered solution (PBS) to dilute all of the bacterial cultures to approximately 10^1 – 10^8 CFU/ml. DNA was extracted from 1 ml of diluted bacterial culture using the Bacterial Genome DNA Extraction Kit following the manufacturer's instructions. DNA concentration and quality were verified using UV spectrophotometric analysis at $A_{260\text{ nm}}$ and $A_{280\text{ nm}}$. *Salmonella* DNA was used in the LAMP reactions directly.

Table 2. Specific primers and probes designed for *Salmonella invA* detection.

Primer name	Type	Sequences (5' → 3')	Length (bases)
PCR			
invAF	Forward primer	AAAAGAAGGGTCGTCGTTAGG	21
invAR	Backward primer	CCACTCGCATCAAATCAAAA	20
LAMP			
invAF3	Forward-outer primer	AGCTGTTGAACAACCCATTG	20
invAB3	Backward-outer primer	TCGGCACAAGTAATATCAAC	19
invAFIP ^a	Forward-inner primer	CTCAATACTGAGCGGCTGCTC	45
(F1c+tttt+F2)		ttttGTTGTTACGGCTATTTTGA	
invABIP	Backward-inner primer	GGTGGTTTTAAGCGTACT	43
(B1c+tttt+B2)		ttttGTCTCTGTAGAGACTTTAT	
invALP	Loop-backward primer	TCCGTGAAGCAAAACGTAGCGCCG	24
Probe for LAMP-LFD			
invA-HP ^b	Hybridization probe	CGGTGGTTTTAAGCGTACTC	20

^a5'-Labeled with biotin when used in the LAMP-LFD assay.

^b5'-Labeled with digoxin when used in the LAMP-LFD assay.

LAMP Primers and Probe

To ensure assay specificity, five primers and a probe were designed to identify eight regions of the target gene (*invA*). All of the *invA* sequences were aligned with DNAMAN 4.0 [22], and the conserved regions were used for analysis across the *Salmonella* species. We designed LAMP primers using PrimerExplorer v3 (<http://primerexplorer.jp>; [27]), based on the *invA* genes in the previously published *S. enterica* genome (GenBank Accession No: NC003197.1). We designed two outer primers (F3 and B3), two inner primers (FIP and BIP), a loop primer (LP), and a probe (HP). FIP was labeled with biotin at the 5' end, and HP was labeled with digoxin at the 5' end (Table 2). All of the primers and the probe were synthesized and labeled by Sangon Biotech (China).

PCR Assay

We made up specific PCR mixtures (50 μ l) for each of the *Salmonella* strains. PCR mixtures contained 20 μ M invAF, 20 μ M invAR, 5 U ExTaq DNA polymerase, 5 μ l 10 $\times$ PCR buffer, 2.5 mM dNTPs, and 1 μ l DNA from the given strain. All of the reaction cycles were 95°C for 5 min; 30 cycles of 95°C for 40 sec, 59°C for 30 sec, and 72°C for 20 sec; and a final extension of 72°C for 10 min. After PCR amplification, we analyzed 5 μ l of the PCR product with 2.5% agarose gel electrophoresis (AGE) using 0.6 μ g/ml EB. The target amplicon was a 278-bp fragment of the *Salmonella invA* gene. All of the experiments were repeated at least three times.

LAMP Assay

Following a previous study [25], LAMP was performed in a 25- μ l reaction mixture containing 1 μ l of the inner primers (20 mol/l), 1 μ l of the outer primers (10 mol/l), 1 μ l of the LP primer (15 mol/l), 2.5 μ l 10 $\times$ ThermoPol buffer, 3.5 μ l dNTPs, 2.5 μ l betaine (10 mol/l), 1 μ l Bst 2.0 DNA polymerase, 1 μ l calcein, 7.5 μ l ddH₂O, and 1 μ l DNA template. We used the genome of *S. Pullorum* as a positive control (8.9 ng/ μ l), and the non-*Salmonella* genome as the negative control. Reaction mixtures were briefly centrifuged, then incubated in a 60°C water bath for 50 min. We analyzed 5 μ l of the amplified product with 3% AGE and the fluorescent dye method.

To determine the optimal LAMP conditions, we altered the ratio of the inner primer to the outer primer (5:1–2:1), the concentration of dNTPs (0.1–1.4 mM), and the concentration of MgSO₄ (1–8 mM). We also varied the amplification temperature (54°C–64°C) and the amplification time (20–60 min).

LFD Assay

To detect the LAMP products with LFD, a gold-labeled anti-digoxin antibody and an anti-biotin antibody were embedded in an LFD. After the LAMP reaction was complete, 20 pmol of the digoxin-labeled *invA*-HP probe was added to the reaction solution. The mixture was held at 60°C for 5 min and then allowed to hybridize with a digoxin-labeled oligonucleotide probe at 80°C for 5 min. After hybridization, 5 μ l of the hybridized product was added to a clean tube containing 100 μ l LAMP reaction buffer. We

Fig. 1. Overview of the LAMP-LFD assay.

(A) The location of the primer pairs and probe, which were designed based on the conserved region of the *Salmonella invA* gene. (B) The triple-labeled product (biotinylated LAMP amplicon hybridized with the digoxin-labeled probe forming a complex with the gold-conjugated anti-digoxin antibody) is bound by the biotin ligand embedded on the test line and/or with anti-mouse antibody embedded on the control line. (C) Bands at both the control and test lines are required for a positive result. A single band at the control line is interpreted as a negative result.

then applied 80 μ l of the new mixture to the LFD strips with the sample pad. The hybridization results were visible to the naked eye within 5 min. When two bands appeared on the LFD (at the test line and at the control line), it indicated a positive result. A sample producing a single band at the control line indicated a negative result (Fig. 1). If no band appeared at the control line, the test strip was considered to be invalid.

Specificity, Repeatability, and Sensitivity of LAMP-LFD Assay

To determine the specificity of the LAMP-LFD assay under optimized conditions, we extracted genomic DNA from six *Salmonella* species and several negative control species (Table 1) that potentially exist in chicken [28]. All of the bacterial strains were tested at 10^4 CFU/ml. To determine the reproducibility of the LAMP-LFD assays, five parallel samples of *S. Pullorum* at 89 pg/ μ l and 8.9 pg/ μ l were analyzed with LAMP and LAMP-LFD. We added *E. coli* genomic DNA to the negative control instead of using a positive template. The LAMP reaction products were analyzed with 3% AGE and LFD. To determine the sensitivity of LAMP-LFD, we performed 10-fold serial dilutions of *Salmonella* DNA with PBS, diluting samples from 89 ng/ μ l to 8.9 fg/ μ l. These serial dilutions were used as the DNA template (1 μ l) for the LAMP assays and PCR. The LAMP products were analyzed with 3% AGE and LFD. The PCR products were analyzed with 2.5% AGE.

Detection of *Salmonella* in Feed Samples

We analyzed two types of chicken feed: eggshells and feed. We tested for *Salmonella* using the conventional culture method. Negative samples were used to test the sensitivity of the LAMP-LFD to artificial pollution. Test portions (25 g) were inoculated with 225 ml of the respective BPW. A similar sample, without added *Salmonella*, was used as the negative control. Samples were cultured at 37°C with 180 rpm/min shaking for 18–24 h. We then added 10 ml of the sample culture to 100 ml of RV medium. This new mixture was cultured at 37°C with 180 rpm/min shaking for 24 h. After culturing, the *Salmonella* concentration spiked, ranging from 10^8 to 10^4 CFU/25 g based on aerobic plate counts. We then centrifuged 1 ml of the final culture mix in sterile test tubes at 900 $\times g$ for 3 min to remove the large particles. We used the Bacterial Genome DNA Extraction kit to extract DNA for use as a detection template (following Wang [29]). We used 2 μ l of this template for LAMP-LFD, conventional LAMP, and PCR assays; these assays were repeated three times each.

Evaluation of the LAMP-LFD Assay with Clinical Samples

To evaluate the performance of the LAMP-LFD assay, we established a reference standard that combined the results of the GB/T4789.4-2008 method and PCR. A sample was considered to be positive when either the GB/T4789.4-2008 method or PCR showed positive results. Our LAMP-LFD assay was conducted on the chicken anal swabs that we collected. We compared our LAMP-LFD results with those of the conventional PCR assay. The

Fig. 2. Verification of the *Salmonella* LAMP-LFD assays.

(A) Agarose gel electrophoresis of LAMP products. Lane M: DNA markers; Lane 1; LAMP products; and Lane 2; negative control. (B) Visual inspection of LAMP products using calcein. (C) The LAMP-LFD assay.

PCR products were analyzed with 2.5% AGE, while the LAMP reaction products were analyzed with 3% AGE and LFD.

Results

Salmonella Detection with LAMP-LFD

To develop the LAMP-LFD method, we used the *S. Pullorum* genomic DNA as the target template. Our electrophoresis results indicated that templates positive for *Salmonella* had bands with a ladder-like pattern, while negative control templates did not have any bands (Fig. 2A). Our analysis of LAMP with calcein indicated that templates positive for *Salmonella* fluoresced green, while blank control templates fluoresced orange (Fig. 2B). Finally, our LAMP-LFD analysis indicated that templates positive for *Salmonella* had red bands in both the detection and the control areas; negative control templates had only a single red band in the control space (Fig. 2C).

Optimization of *Salmonella* LAMP

We measured the brightness and consistency of the ladder-like LAMP product bands obtained via electrophoresis, as well as the gray values, with Image LabTM 2.0 software (Bio-Rad Laboratories, USA), following the manufacturer's instructions. To determine the optimal amplification conditions after experimentation, we found that an inner primer to outer primer ratio of 3:1 resulted in maximum amplification of the LAMP product (Fig. S1A). The optimum concentrations of dNTPs and Mg²⁺ in the master mix were

0.5 mM (Fig. S1B) and 4 mM (Fig. S1C), respectively. Thus, our optimal *Salmonella* LAMP master mix contained 2.5 μ l 10 $\times$ ThermoPol buffer, 4 mM MgSO₄, 0.8 mM dNTPs, 1 μ l calcein, 1.5 mM betaine, 0.7 μ M each of outer primer (F3 and B3), 2.1 μ M each of inner primer (FIP and BIP), 0.8 μ M LP primer, and 1 μ l of target DNA.

To optimize the amplification temperature, we maintained our optimal LAMP reaction mixture with 1 μ l *S. Pullorum* DNA (58 ng/ μ l) template under isothermal conditions (54°C–64°C) for 50 min. After LAMP amplification at 60°C, four bright bands were visible; these bands were brighter than those observed after amplification at other temperatures (Fig. S1D). We thus considered 60°C the optimal temperature for the *Salmonella* LAMP assay.

Next, to determine the optimal reaction time, we held our optimal LAMP reaction mixture with 1 μ l *S. Pullorum* positive control template at 60°C for 20–60 min. After 20 min, LAMP amplification products were detectable by

electrophoresis. After 40 min, the amplification product was saturated (Fig. S1E). We thus considered 40 min the optimal duration for the *Salmonella* LAMP assay.

LAMP-LFD Specificity, Repeatability and Sensitivity

As for specificity, all of the six *Salmonella* species tested positive with a clear visible test line on the strip, while all non-*Salmonella* (negative control) species tested negative (Figs. S2A and S2B). Thus, the specificity of LAMP-LFD was sufficient. The repeatability of the LAMP-LFD was evaluated through comparison of the results obtained from five parallel samples. For 89 pg/ μ l and 8.9 pg/ μ l synthetic DNA, the five parallel samples obtained test results. For 8.9 pg/ μ l synthetic DNA, five out of five test samples had identical results, which indicated that the LAMP-LFD was reproducible. The sensitivities of the LAMP, LAMP-LFD, and PCR assays were also tested using a 10-fold dilution of *S. Pullorum* DNA. The LAMP plus gel electrophoresis

Fig. 3. Sensitivity of (a) LAMP, (b) LAMP-LFD, and (c) conventional PCR in the eggshells artificially contaminated with various serovars of *Salmonella enterica* subsp. *enterica*.

Assay evaluation in the eggshells artificially contaminated with (A) *S. enterica* subsp. *enterica* Pullorum, (B) *S. enterica* subsp. *enterica* Enteritidis, and (D) *S. enterica* subsp. *enterica* Typhimurium. Lane M: DNA markers; Lane 1: negative control (no template); Lane 2: 1.0×10^6 CFU/25 g; Lane 3: 9.0×10^5 CFU/25 g; Lane 4: 8.0×10^5 CFU/25 g; Lane 5: 7.0×10^5 CFU/25 g; Lane 6: 6.0×10^5 CFU/25 g; Lane 7: 9.0×10^3 CFU/25 g; Lane 8: 8.0×10^3 CFU/25 g; Lane 9: 7.0×10^3 CFU/25 g; Lane 10: 6.0×10^3 CFU/25 g; Lane 11: 5.0×10^3 CFU/25 g; and Lane 12: 4.0×10^3 CFU/25 g. Assay evaluation in the eggshells artificially contaminated with (C) *S. enterica* subsp. *enterica* Gallinarum. Lane M: DNA markers; Lane 1: negative control (no template); Lane 2: 1.0×10^7 CFU/25 g; Lane 3: 9.0×10^6 CFU/25 g; Lane 4: 8.0×10^6 CFU/25 g; Lane 5: 7.0×10^6 CFU/25 g; Lane 6: 7.0×10^5 CFU/25 g; Lane 7: 9.0×10^4 CFU/25 g; Lane 8: 8.0×10^4 CFU/25 g; Lane 9: 7.0×10^4 CFU/25 g; Lane 10: 6.0×10^4 CFU/25 g; Lane 11: 5.0×10^4 CFU/25 g; and Lane 12: 4.0×10^4 CFU/25 g.

method (Fig. S3A), the calcein visual LAMP method (Fig. S3B), and the LAMP-LFD method (Fig. S3C) had similar degrees of sensitivity, detecting concentrations of genomic DNA as low as 89 fg/ μ l. The detection limit of conventional PCR was 89 pg/ μ l of genomic DNA (Fig. S3D). Therefore, LAMP and LAMP-LFD detected concentrations 1,000 times lower than conventional PCR. Therefore, our *Salmonella* LAMP-LFD assay was suitable for *Salmonella* detection.

Detection of *Salmonella* in Spiked Feed Samples

In the artificially contaminated eggshells, the detection limit of LAMP-LFD and LAMP was approximately 8×10^3 CFU/25 g, with the exception of *S. enterica* subsp. *enterica* serovar Gallinarum (hereafter referred to as *S. Gallinarum*),

for which the detection limit was 10^5 CFU/25 g (Figs. 3A–3D). Across the three repeats, LAMP (once) and LAMP-LFD (twice) returned a positive band at a *Salmonella* concentration of 8×10^3 CFU/25 g. In contrast, PCR detected some species of *Salmonella* in some repeats at concentrations of 7.0×10^5 CFU/25 g (*S. Pullorum*, *S. enterica* subsp. *enterica* serovar Enteritidis, and *S. enterica* subsp. *enterica* serovar Typhimurium), but only detected *S. Gallinarum* at concentrations greater than 10^6 CFU/25 g. In the artificially contaminated chicken feed, LAMP-LFD and LAMP detected all of the species of *Salmonella* at concentrations of 8.0×10^4 CFU/25 g. PCR detected all of the species, except for *S. Pullorum*, at 8.0×10^4 CFU/25 g; *S. Pullorum* was detected by PCR at 8.0×10^6 CFU/25 g (Figs. 4A–4D).

Fig. 4. Sensitivity of (a) LAMP, (b) LAMP-LFD, and (c) conventional PCR in the chicken feed artificially contaminated with various serovars of *Salmonella enterica* subsp. *enterica*.

Assay evaluation in the chicken feed artificially contaminated with (A) *S. enterica* subsp. *enterica* Pullorum. Lane M: DNA markers; Lane 1: negative control (no template); Lane 2: 1.0×10^8 CFU/25g; Lane 3: 9.0×10^7 CFU/25g; Lane 4: 8.0×10^7 CFU/25g; Lane 5: 7.0×10^7 CFU/25g; Lane 6: 6.0×10^7 CFU/25g; Lane 7: 9.0×10^4 CFU/25g; Lane 8: 8.0×10^4 CFU/25g; Lane 9: 7.0×10^4 CFU/25g; Lane 10: 6.0×10^4 CFU/25g; Lane 11: 5.0×10^4 CFU/25g; Lane 12: 4.0×10^4 CFU/25g. Assay evaluation in the chicken feed artificially contaminated with (B) *S. enterica* subsp. *enterica* Enteritidis and (C) *S. enterica* subsp. *enterica* Gallinarum. Lane M: DNA markers; Lane 1: negative control (no template); Lane 2: 1.0×10^7 CFU/25g; Lane 3: 9.0×10^6 CFU/25g; Lane 4: 8.0×10^6 CFU/25g; Lane 5: 7.0×10^6 CFU/25g; Lane 6: 6.0×10^6 CFU/25g; Lane 7: 9.0×10^4 CFU/25g; Lane 8: 8.0×10^4 CFU/25g; Lane 9: 7.0×10^4 CFU/25g; Lane 10: 6.0×10^4 CFU/25g; Lane 11: 5.0×10^4 CFU/25g; and Lane 12: 4.0×10^4 CFU/25g. Assay evaluation in the chicken feed artificially contaminated with (D) *S. enterica* subsp. *enterica* Typhimurium. Lane M: DNA markers; Lane 1: negative control (no template); Lane 2: 1.0×10^7 CFU/25g; Lane 3: 9.0×10^6 CFU/25g; Lane 4: 8.0×10^6 CFU/25g; Lane 5: 7.0×10^6 CFU/25g; Lane 6: 6.0×10^6 CFU/25g; Lane 7: 5.0×10^6 CFU/25g; Lane 8: 8.0×10^5 CFU/25g; Lane 9: 7.0×10^5 CFU/25g; Lane 10: 6.0×10^5 CFU/25g; Lane 11: 5.0×10^5 CFU/25g; and Lane 12: 4.0×10^5 CFU/25g.

Diagnostic Application of LAMP-LFD

Of the 350 clinical samples (chicken anal swabs), our LAMP-LFD assay identified 45 as positive for *Salmonella*, with a positive rate of 12.86%; the parallel PCR identified 42 as positive, with a positive rate of 12.00%. LAMP-LFD was 100% consistent with the results of the Chinese National Standard method, while PCR was 93.31% consistent. This indicated that our LAMP-LFD assay would be appropriate for use in the field.

Discussion

Salmonella is a common pathogen that often causes bacterial food poisoning [30]. This bacteria therefore not only damages the livestock industry but also poses a serious threat to human health and safety. Although avian salmonellosis severely impacts the Chinese poultry industry, few effective vaccines and drugs are available to prevent and cure this disease. Methods for the early detection of *Salmonella* contamination are needed for the effective prevention and monitoring of this disease.

Sensitivity, convenience, practicality, efficiency and cost are the main factors that determine whether a pathogen detection method will be useful in the field [31]. Accurate diagnoses with the traditional microbial detection methods take at least one week, and typically require enrichment culture [32]. In contrast, we have shown that our LAMP-LFD assay is a fast, simple, effective, specific and sensitive technique for identifying *Salmonella* contamination. Although LAMP-LFD was based on the traditional LAMP method, LAMP-LFD has several advantages over LAMP. For example, the probe we used increased the detection specificity of LAMP-LFD, while the immunochromatographic strip was fast and convenient. The sensitivity of LAMP-LFD to *Salmonella* nucleic acids is consistent with that of LAMP, indicating that LAMP-LFD is suitable for use with clinical samples. LAMP-LFD returned LAMP amplification results with detection that was sensitive to 89 fg/ μ l of *Salmonella* gDNA within 5 min. Indeed, LAMP-LFD was 1,000 times more sensitive than conventional PCR, and the detection limit of LAMP-LFD was equivalent to that of LAMP-calcein gel electrophoresis. We validated the LAMP-LFD method using 350 clinical samples from poultry farms. LAMP-LFD identified 12.86% of the samples as positive for *Salmonella*, consistent with the results of the Chinese National Standard method. LAMP-LFD was slightly less sensitive to *Salmonella* DNA in artificially contaminated feed samples, suggesting that the type and origin of *Salmonella* contamination directly affects LAMP-

LFD sensitivity. LAMP-LFD assays accurately detected *Salmonella* contamination as low as 8×10^3 CFU/25 g across all feed types. This is consistent with the single previous study of LAMP sensitivity to *Salmonella* DNA in artificially contaminated feed samples [33]. It is worth noting that the sensitivity of LAMP-LFD varied both when different serotypes of *Salmonella* contaminated otherwise identical feed samples and when different types of feed were infected with identical *Salmonella* serotypes (Fig. 3). This was consistent with a previous study, which suggested that *invA* gene polymorphisms in different *Salmonella* serovars might cause the observed differences in the samples [32].

Temperature strongly affected the sensitivity of our LAMP assay. The amplification temperature of LAMP using Bst DNA polymerase is typically 60°C–65°C [16], but our LAMP assay was most sensitive at 60°C. When the amplification temperature was increased, the assay sensitivity decreased. The activity of Bst DNA polymerase depends on Mg^{2+} concentration [34]. In our LAMP reaction, the final Mg^{2+} concentration was 4 mM, and the Mg^{2+} concentration for PCR was between 0.5 mmol/l and 2.5 mmol/l [35], indicating that high concentrations of Mg^{2+} improved assay sensitivity. Similarly, the Bst DNA polymerase used in the LAMP reaction had a higher inhibitor tolerance than of the Taq MasterMix used for PCR [36]. In addition, dNTP concentration is closely related to LAMP amplification efficiency [34]; the final dNTP concentration for PCR is typically between 50 μ M and 200 mM [35]. Here, LAMP-LFD was most sensitive at 0.5 mM dNTP; when the dNTP concentration was increased above 0.5 mM, assay sensitivity decreased. Thus, LAMP reaction conditions directly affected detection specificity and sensitivity of the LAMP-LFD method. It is therefore critical to optimize the LAMP reaction system to improve the efficacy of LAMP-LFD.

As there is a correlation between *Salmonella* contamination and the most serious food safety hazards, we focused on the *S. enterica* subsp. *enterica* serovars Pullorum, Gallinarum, Typhimurium, and Enteritidis. LFDs performed after the LAMP amplification of the *invA* gene in *Salmonella*-contaminated samples were positive, while LFDs performed after LAMP amplification of non-*Salmonella* strains were negative. Therefore, LAMP-LFD was sufficiently specific. Although the specificity and sensitivity of LAMP-LFD were high, our samples were obtained from only a few poultry farms in one area of China, and might not have accurately represented the known diversity of *Salmonella* serovars (> 2,500; [37]). Therefore, the popularity and reliability of this detection method must be confirmed in

future studies, preferably encompassing samples from several areas. Sample sizes should also be increased.

LAMP analysis requires accurate primer design, a stable reaction system, and a sterile working area [38]. The selection of the target genes is the key to LAMP technology. To design our *Salmonella* LAMP assay, we first designed primers for the *Salmonella* *invA* gene based on a previously published *Salmonella* genome. The gene *invA* is highly conserved and found only in pathogenic *Salmonella* species. This gene is thus often used as a diagnostic marker to rapidly and accurately detect *Salmonella* contamination in food [39–41]. LAMP has been used worldwide to detect pathogenic microorganisms, clinically diagnose diseases, and determine embryo sex [42]. Thus, LAMP-LFD is potentially useful for the rapid, sensitive detection of *Salmonella*. Previous studies have shown that the sensitivity of PCR pathogen detection was affected by the presence of PCR inhibitors in the samples tested [43]. The development of PCR for rapid clinical diagnosis has also been hampered by a lack of adequate validation techniques [44]. LAMP results are easier to generate and interpret than those of qPCR or PCR [45]; LAMP is also approximately 2 times faster than qPCR [46]. More importantly, the LAMP-LFD procedure is simple: the entire reaction takes place at a constant temperature (60°C) in a reasonable amount of time, rendering a thermocycler or qPCR system unnecessary. Furthermore, the results can be interpreted directly with the naked eye. The addition of primers in the LAMP method is controversial because specific staircase bands might also be produced without the use of external primers [47]. The loop primers in the LAMP method shorten the reaction time and increase the efficiency of amplification, possibly because ring primers hybridized with the stem loop structure, starting strand displacement DNA synthesis. At present, the use of the LAMP assay is widespread and increasing, but several problems and defects of this assay remain to be clarified [48]. For example, LAMP amplification can also occur with invariant DNA templates, but the underlying mechanism is unclear. The explanation for this phenomenon might help to further simplify LAMP operation. In addition, the LAMP assay has a high rate of false positives, it has strict environmental requirements, and it may easily be contaminated. These factors have slowed the rate of adoption of the LAMP technique. However, as long as the standard LAMP procedure is performed according to the requirements, contamination can effectively be avoided.

In conclusion, the *Salmonella* LAMP-LFD detection method developed in this study had several advantages

over the traditional LAMP method. It was faster and more convenient than the traditional culture detection method. The use of LAMP-LFD might save time and manpower in health centers that test for *Salmonella* contamination. LAMP-LFD is a novel, rapid and efficient diagnostic tool for the primary identification of *Salmonella* contamination in the field.

Acknowledgments

This research was supported by grants from the Co-Innovation Center for the High Incidence of Zoonotic Disease Prevention and Control in Western China (No. 2013-179) and the National Natural Science Foundation of China (No. 31360226). The authors would like to express their gratitude to the Laboratory of Zoonosis at Shihezi University for their important contributions. The authors would like to thank Professor Xudong Cao for providing excellent technical support. We thank all of the participants who provided chicken anal swab samples for this study. We would also like to thank LetPub (www.LetPub.com) for providing linguistic assistance during the preparation of this manuscript.

Conflict of interest

The authors have no financial conflicts of interest to declare.

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