A rapid novel visualized loop-mediated isothermal amplification method for *Salmonella* detection targeting at fimW gene

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ABSTRACT *Salmonella* infection causes huge losses in the poultry industry worldwide. With the aim to prevent infectious diseases caused by *Salmonella* and to achieve rapid visualized *Salmonella* detection in poultry production, we used cresol red as an indicator to develop a novel visualized loop-mediated isothermal amplification method that targets the *Salmonella* fimW gene firstly in related field. The detection limit was 7.3×10^1 CFU/mL, and the method was highly specific and showed a high clinical detection rate. The entire reaction can be completed in about 40 min and only requires a water bath at 62°C, which makes the method extremely suitable for application to poultry production.

Key words: *Salmonella,* fimW gene, LAMP, novel visualized detection

INTRODUCTION

Salmonella is a zoonotic pathogen that has profound impacts globally. More than 2,600 identified serotypes display different pathogenicities and an extensive host range (Ma et al., 2018). In particular, poultry and poultry products have been considered main sources of *Salmonella* (Youn et al., 2017), which can cause many poultry diseases, such as pullorum disease and fowl typhoid, and has caused huge losses in the poultry industry worldwide (Dan et al., 2018). Therefore, the early prevention and control of *Salmonella* infection has received increasing attention and being an urgent need that exists to develop a rapid detection method to control the spread of *Salmonella* at an early stage (Draz and Lu, 2016).

Currently, the conventional gold standard culture methods for *Salmonella* require multiple subculture steps, followed by biochemical and serologic confirmation, which are labor intensive and time consuming

(Xuefei et al., 2009). Molecular methods available for the detection of *Salmonella*, such as PCR, real-time PCR, and ELISA, require expensive laboratory instruments and specialized expertise. Furthermore, these methods are too complicated to meet the needs, which renders them unable to meet the needs of the poultry industry for rapid on-site detection (Chayapa et al., 2010; Srisawat and Panbangred, 2015; Zhuang et al., 2015).

As a novel detection method, loop-mediated isothermal amplification (**LAMP**) provides excellent detection performance without the requirement for advanced instruments and professional expertise (Youn et al., 2017). It involves many different monitoring methods, such as gel electrophoresis, turbidity measurements, and the use of fluorescent dyes or colorimetry. These methods either require opening tubes that can easily lead to aerosol pollution or rely on inapparent turbidity and color change may cause false-negative results (de Avelar et al., 2019), hence not suitable for on-site use. Therefore, establishing a practical novel visualized *Salmonella* rapid detection method is significant.

Salmonella fimW gene is an ideal specific molecular detection target with a unique sequence, which is not found in other pathogens (Zhang et al., 2014). In this study, we targeted the *Salmonella* fimW gene, and according to the pH change resulting from the DNA amplification reaction, cresol red is selected as a suitable

indicator (red in alkaline; yellow-white in acidic conditions) to establish a novel visualized LAMP method. We evaluated the sensitivity and specificity of this method and performed a clinical test. The results showed that this method is a reliable and effective diagnostic tool that is extremely suitable for application to poultry farming and for on-site use.

MATERIALS AND METHODS

Bacterial Strains and Culture Preparation

A total of 54 bacterial strains comprising 40 different *Salmonella* serovars and 14 non-*Salmonella* species (Table 1) were used in this study. Clinical isolates were identified by and stored at the laboratory of the Veterinary College, South China Agricultural University, Guangzhou, China. The main reagents used for bacterial culture were as follows: Luria-Bertani broth, Luria-Bertani agar, Xylose Lysine Tergitol-4 Agar, buffered peptone water (**BPW**), and tetrathionate broth base (Huankai Microbiology Technology Corporation, Guangdong, China); PBS (Dingguo Changsheng Biotechnology Corporation, Beijing, China). Each strain was streaked on nutrient agar or blood agar plates, cultured in an incubator for a certain period of time, and the selected colony was inoculated into the broth and then shaken and cultured using a shaker to complete the enrichment.

DNA Extraction

Genomic DNA was extracted using the TIANamp Bacteria DNA Kit (TIANGEN Biochemical Technology Corporation, Beijing, China) or via the boiling method (Youn et al., 2017), and the DNA samples were stored at -20°C until further analysis.

Primer Design

The LAMP primers were designed in accordance with the *Salmonella* fimW gene sequence (GenBank: AP019375.1) using software PrimerExplorer V5 (Table 2). All primers were synthesized by Sangon Biotech (Shanghai) Co. Ltd. and were used at a final concentration of 10 μM and stored at -20°C until use.

Establishment and Optimization the LAMP Reaction System

To optimize the reaction system, the following steps were performed: The substances used in the reaction were filtered, and the optimum primer ratio of the external primer, inner primer, and loop primer was established; the concentration and dosage of the reagent was optimized; the optimum reaction temperature (58°C–65°C in one-degree increments) and the reaction time (15 min–75 min) were screened. All the reactions were monitored using a fluorescence curve. The LAMP reaction consisted of 12.5 μL LAMP Mix, including

Bst enzyme, MgSO$_4$ (New England Biolabs, Inc.), (NH$_4$)$_2$SO$_4$, KCl, Tween-20, cresol red, KOH, dNTP, betaine (Sigma-Aldrich), outer primer, inner primer, and loop primer in an optimum ratio of 1:6:2 and 2.5 μL genomic DNA in a total volume of 25 μL with ddH$_2$O.

Sensitivity Test

The typhimurium standard strain ATCC 14028 was used for testing via the plate count method. The concentration of the bacterial culture ranged from 7.3×10^0 CFU/mL to 7.3×10^7 CFU/mL. Extracted genomic DNA was visualized via the LAMP method. Fluorescence curves were generated to monitor the progress of the reaction and were compared with those generated using the Chinese national standard PCR method (No. GB/T 28642-2012).

Specificity Test

Genomic DNA was extracted from 54 bacterial strains and was tested using the optimized visualized LAMP reaction system. The results of the reaction were observed with the naked eye and were compared with the results from the national standard PCR method.

Clinical Test

A total of 61 clinical samples were collected (the sources included chickens and ducks) and were tested using the visualized LAMP method as described in this study. Samples were also assayed by gold standard culture method (No. GB 4789.4-2016) and the results of both methods were compared with those from the Chinese national standard PCR method. The bacterial solution used for national standard PCR and visualized LAMP detection were taken from samples after preenriched by BPW.

RESULTS

Establishment of the Visualized LAMP Reaction System

The visualized LAMP reaction system was established as shown (Figure 1): yellow represents a positive reaction and red, a negative one. The optimum reaction temperature and time was 62°C and 40 min, respectively (Figure 2).

Sensitivity Test Results

The detection limit of the visualized LAMP method was 7.3×10^1 CFU/mL and was verified by a fluorescence curve. Compared with the national standard PCR method, the sensitivity of the PCR method using agarose gels was 1,000 times lower than that of the LAMP method (Figure 3).

Table 1. Bacterial strains used in this study and specificity test results for the national standard PCR method and the visualized LAMP method.

Bacterial strain	Source	No.	PCR results	LAMP results
Salmonella typhimurium	ATCC 14028,	2	+	+
	Clinical isolate		+	+
Salmonella Enteritidis	ATCC 9120,	2	+	+
	ATCC10398		+	+
Salmonella Gallinarum	CICC21510,	2	+	+
	Clinical isolate		+	+
Salmonella Pullorum	Clinical isolate	2	+	+
Salmonella Choleraesuis	Clinical isolate	2	+	+
Salmonella Corvallis	Clinical isolate	2	+	+
Salmonella Kentucky	Clinical isolate	2	+	+
Salmonella Derby	Clinical isolate	2	+	+
Salmonella Rissen	Clinical isolate	2	+	+
Salmonella Indiana	Clinical isolate	2	+	+
Salmonella Albany	Clinical isolate	2	+	+
Salmonella Infantis	Clinical isolate	2	+	+
Salmonella London	Clinical isolate	2	+	+
Salmonella Weltevreden	Clinical isolate	2	+	+
Salmonella Agona	Clinical isolate	2	+	+
Salmonella Saintpaul	Clinical isolate	2	+	+
Salmonella Panama	Clinical isolate	2	+	+
Salmonella Havana	Clinical isolate	1	+	+
Salmonella Cerro	Clinical isolate	1	+	+
Salmonella Mbandaka	Clinical isolate	1	+	+
Salmonella Goldcoast	Clinical isolate	2	+	+
Salmonella Meleagridis	Clinical isolate	1	+	+
Escherichia coli	ATCC25922,	2	−	−
	C600		−	−
Pseudomonas aeruginosa	ATCC27853,	2	−	−
	Clinical isolate		−	−
Staphylococcus aureus	CMCC26003	1	−	−
Campylobacter jejuni	NCTC 11168,	2	−	−
	Clinical isolate		−	−
Campylobacter coli	Clinical isolate	2	−	−
Riemerella anatipestifer	Clinical isolate	2	−	−
Listeria monocytogenes	Clinical isolate	1	−	−
Listeria iuanuii	Clinical isolate	1	−	−
Shigella	Clinical isolate	1	−	−

Abbreviation: LAMP, loop-mediated isothermal amplification.

Specificity Test Results

Different serotypes of *Salmonella* strains were successfully positively amplified, whereas non-*Salmonella* strains, such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Campylobacter jejuni, Campylobacter coli, Riemerella anatipestifer, Listeria monocytogenes, Listeria iuanuii,* and *Shigella* were not amplified (Table 1). This result confirmed the specificity of the visualized LAMP assay, which was consistent with that of the standard PCR method.

Clinical Test Results

Among the 61 clinical samples, the positive detection rates for *Salmonella* were as follows: 42 of 61 by using gold standard culture methods; 39 of 61 (3 missed

inspections) by using the visualized LAMP method, and only 24 of 61 by using the national standard PCR method (18 missed detections). The detailed detection results for the clinical samples showed that the efficacy of the visualized LAMP test was very similar to that of the gold standard culture methods (Figure 4).

DISCUSSION

Many LAMP methods have been published, which have targeted different *Salmonella* genes, including inva, fimY, phoP, stn, and Sdf. However, some target genes were not specific enough, or the observation methods were not sufficiently practical (Xuefei et al., 2009; Yang et al., 2010; Shao et al., 2011; Zhang et al., 2012; Srisawat and Panbangred, 2015). The selection

Table 2. LAMP primers used in this study.

| Primer name	Sequence (5′–3′)	Length (bp)
F3	CTGGATGATGATTGGTTCAG	20
B3	GAAGGGACGCTATGTCGA	18
FIP	ACATGAGCTTTTCTTTATCGCATTTTATCAGATACCTATGCATACCCA	48
BIP	CAGACCATGTCTGTATATGCTGCCTGTAAGATCAATATCATTTTCCGG	48
LF	TACAAATAATCGCCCGTAGCTGAT	24

Abbreviation: LAMP, loop-mediated isothermal amplification.
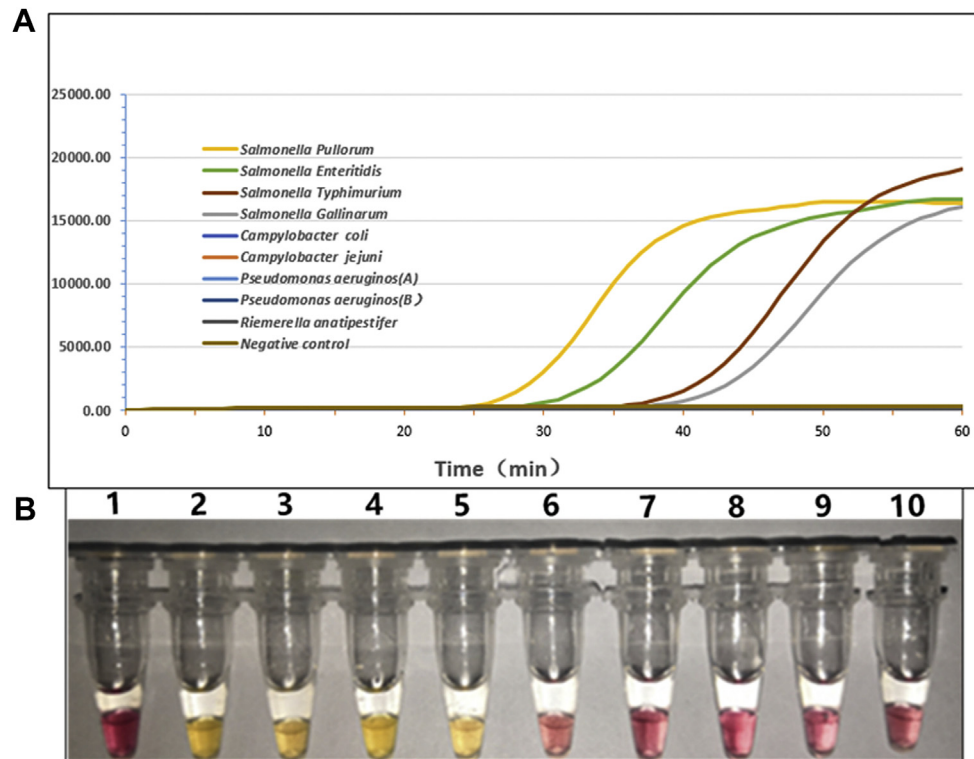
Figure 1. Establishment of the LAMP reaction system. (A) Fluorescence curve characterization of the visualized LAMP method. (B) Visualized LAMP reaction using different bacterial strains and species: (1) negative control; (2) *Salmonella* Pullorum; (3) *Salmonella* Enteritidis; (4) *Salmonella* Typhimurium; (5) *Salmonella* Gallinarum; (6) *Campylobacter coli*; (7) *Campylobacter jejuni*; (8) and (9) *Pseudomonas aeruginosa*; (10) *Riemerella anatipestifer*. Abbreviation: LAMP, loop-mediated isothermal amplification.

of an appropriate target gene for *Salmonella* detection is key to developing a great visualized LAMP method. *Salmonella* possesses many types of pili; type I pili are widely distributed among various serotypes, and the fimW gene is one of the major regulatory genes of type I pilus subunit proteins (Supreet et al., 2009; Zeiner et al., 2012). A search of sequences in GenBank using the fimW gene sequence as a query showed that fimW
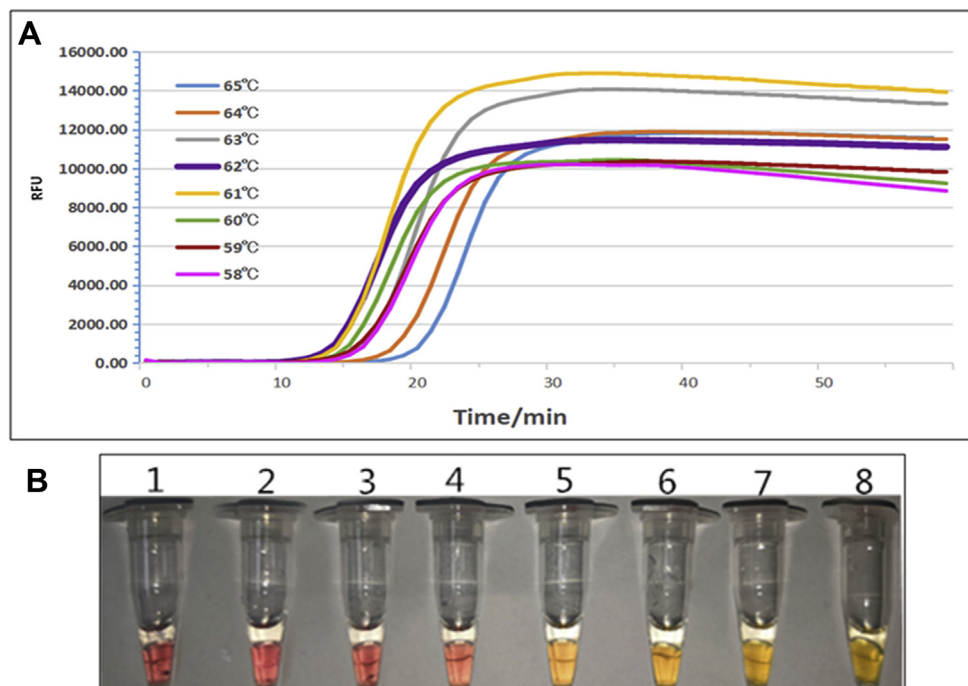
Figure 2. Optimization of the reaction temperature and time. (A) Screening for optimum reaction temperature, from 58°C–65°C. (B) Screening for the optimum reaction time: (1) 15 min; (2) 20 min; (3) 25 min; (4) 30 min; (5) 40 min; (6) 45 min; (7) 60 min; (8) 75 min.
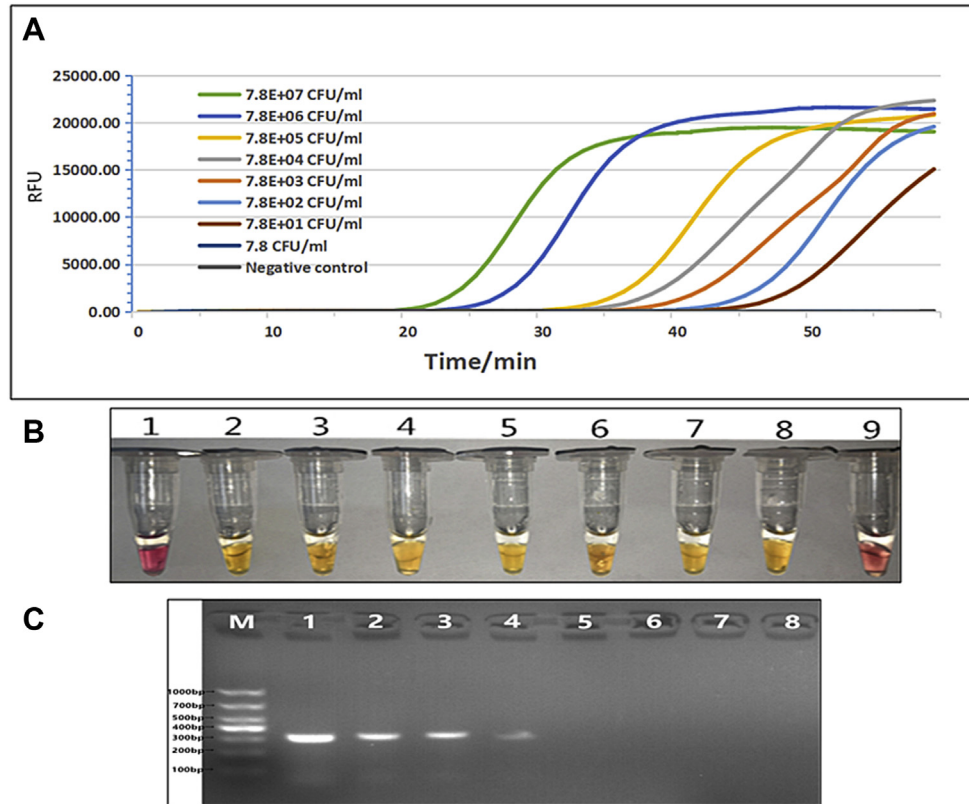
Figure 3. Comparison of the sensitivity of the visualized LAMP and national standard PCR methods. (A) Fluorescence curve characterization of the visualized LAMP method. (B) The novel visualized LAMP method. (1) Negative control; (2)–(9) a decreasing concentration gradient of the starting bacterial culture, 7.3×10^7 CFU/mL to 7.3×10^0 CFU/mL. (C) National standard PCR test. (M) DL1,000 DNA Marker; (1)–(8) 7.3×10^7 CFU/mL to 7.3×10^0 CFU/mL). Abbreviation: LAMP, loop-mediated isothermal amplification.

is a unique gene that is only present in different *Salmonella* serotypes, and no homologous sequences were found in other pathogens; therefore, we selected the fimW gene as an ideal target for the method.

In addition to the selection of suitable target genes, the visualized LAMP method described in this study is also practically applicable. Existing methods of LAMP observation mainly include turbidity analysis, gel electrophoresis, the addition of fluorescent dyes, and colorimetry. Furthermore, some methods also require special expensive monitoring equipment or the opening of tube lids after the reaction (Yang et al., 2018). This can easily cause aerosol contamination and is not suitable for clinical use. Research has shown that DNA amplification can release hydrogen ions as by-products, which cause a decrease in the pH (Tanner et al., 2015). Based on this principle, colorimetry is receiving increasing attention. Previous methods have used methyl green, malachite green, or hydroxynaphthol as pH indicators for the detection of products, but these reagents lead to color changes that are not unequivocal and are prone to missed inspection and are therefore not suitable for rapid detection and clinical application (Thapa et al., 2019).

Currently, only a PCR detection method that targets the *Salmonella* fimW gene has been published, a method that has a detection limit of 10^2 CFU (Zhang et al., 2014), and few other studies concerning this gene have
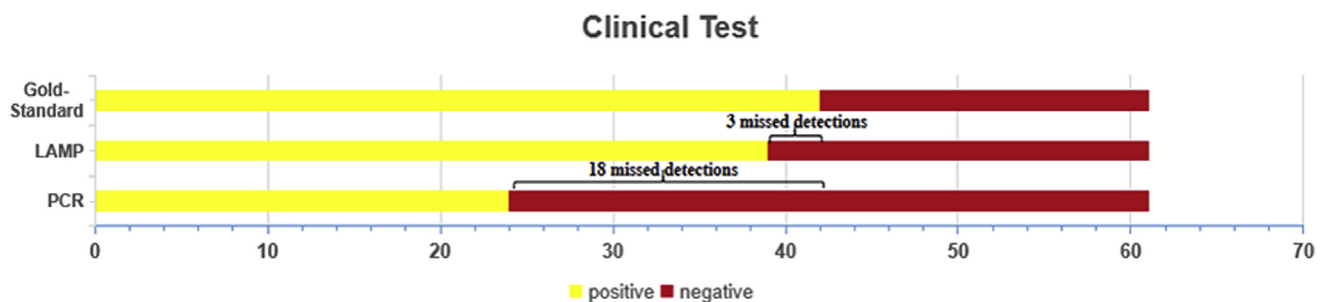
Clinical Test

Figure 4. Comparison of clinical test results. The number of positive and negative test results and number of false negatives (missed detections) for the gold standard culture method (Gold-Standard), the visualized LAMP method here (LAMP), and the Chinese national standard PCR method (PCR). Abbreviation: LAMP, loop-mediated isothermal amplification.

been performed. In this study, we target the *Salmonella* fimW gene to designed LAMP primers and used cresol red as an indicator included in the reaction mixture. This reduces the risk of aerosol contamination caused by opening the tube lids, realized true sense close-tube detection, and causes a color change from red to yellow, which is easy to discriminate. Results indicate that this method is as specific as standard PCR methods and is extremely sensitive. The detection limit is 7.3×10^1 CFU/mL, which is more sensitive than that in many previous reports (Yang et al., 2018) and is 1,000-fold greater than that in the national standard PCR method. The comparison between results of the clinical test used the visualized LAMP method, and those using the national standard PCR method showed that both methods caused missed detections. These could be resolved by increasing the incubation time because the bacterial cultures used for the test only contained a low concentration after simple incubation by BPW. Despite this, the visualized LAMP method is extremely practical and efficacious and has a detection compliance rate that is close to that of the gold standard.

In this study, we have developed a novel visualized LAMP method for the rapid and sensitive detection of *Salmonella*. Compared with gold standard culture methods, which are complex and require several days, the visualized LAMP method here can generate a result in only 40 min at 62°C, thus representing rapid detection. Moreover, its excellent detection performance and can observed directly without other operations are extremely suitable for extensive application in the poultry industry.

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