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A pH sensitive, loop-mediated isothermal amplification assay for detection of *Salmonella* in food

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ABSTRACT

Salmonella is a common foodborne pathogen. This study aimed to develop a rapid detection method for *Salmonella* spp. in food by using a pH sensitive, loop-mediated isothermal amplification (LAMP) assay. Firstly, a suitable pH indicator was selected to follow the change in pH of LAMP reactions. Then, the best primer set was selected from among three previously approved primer sets specific to the genes *invA*, *invE*, and *phoP*. The amplification-positive reactions were easily detectable with neutral red at a concentration of 15 mM (color change from faint orange to red). The best primers were those specific to *phoP* when incubated at 63.5°C for 1.5 h. The developed assay was validated for detection of *Salmonella* Typhimurium in spiked minced chicken meat and liquid egg with a sensitivity of 2 CFU/g or 2 CFU/mL. With this novel, pH-sensitive LAMP assay, *Salmonella* can be detected in food in 6 h.

Keywords: LAMP; rapid detection; *Salmonella*

1. INTRODUCTION

Salmonella is a facultative anaerobic, non-sporulating, gram-negative bacillus in the family *Enterobacteriaceae*. This bacterium is a common foodborne pathogen which can be transmitted by contaminated poultry, egg products, and vegetables. Various serovars of *Salmonella* have resulted in the greatest burden of foodborne disease worldwide. Together, they accounted for an estimated 6.43 million disability-adjusted life years lost due to illness caused by contaminated food (Kirk et al., 2015). The infectious dose was $\geq 10^5$ cells in healthy adults, but this could be as low as 15-20 cells in susceptible persons (Kothary and Babu, 2001). Although *Salmonella* is classified into at least 2,500 serovars (Popoff et al., 2000), only a limited number of serovars can cause human infection. *S.* Enteritidis is the predominant serovar causing *Salmonella* outbreaks related to the consumption of poultry and their products (Techathuvanan and D'Souza, 2012). *S.* Typhimurium is mostly found in pork meat (Techathuvanan et al., 2010). An increase in consumer health awareness and economic impacts of *Salmonella* infection has led to the development of cost-efficient, sensitive, and rapid detection methods.

The "gold standard" for detection of *Salmonella* in food is a time-consuming and labor-intensive method that can take up to 5 days (FDA, 2017). The standard method includes pre-enrichment, selective enrichment (depending on the background microorganism load of the sample), and spreading on differential media prior to biochemical confirmation tests. In the food industry, sensitive, rapid, and reliable methods to detect microbial

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contamination in finished products are necessary. Many studies have focused on the development and validation of rapid methods for screening and detection of *Salmonella*.

Enzyme-linked immunosorbent assay (ELISA) can be applied with some limitations due to their low specificity and sensitivity (Nowak et al., 2007; Eriksson and Aspan, 2007). Other methods based on conventional polymerase chain reaction (PCR), real-time PCR, and DNA microarrays have also been studied with respect to their sensitivity and specificity for detecting *Salmonella* in food matrices (Almeida et al., 2013; Wang et al., 2014; Kim and Lee, 2016).

Loop-mediated isothermal amplification (LAMP) is a technique by which DNA can be amplified at a constant temperature. It was developed in 2008 by Tomita and coworkers. The technique is isothermal due to the use of *Bst* DNA polymerase, an enzyme originally isolated from *Bacillus stearothermophilus* that has a strand displacement activity, eliminating the need for a thermocycler. Moreover, the stem-loop structure allows a self-sustained sequence replication, resulting in a rapid and efficient LAMP reaction (Tomita et al., 2008). Recently, there have been an increased number of studies on the application of LAMP for identifying and detecting pathogens in foods. LAMP detection methods developed for *Salmonella* have targeted several species-specific genes such as invasion genes (*invA* and *invE*), flagellin-associated gene (*fliC*), and a transcriptional regulator gene (*phoP*) (Li et al., 2009; Ye et al., 2011; Kumar et al., 2014; Chen et al., 2015). The methods showed high specificity with the least detection limit of 2 CFU/25 g of food sample (Zhang et al., 2011).

The DNA product yielded from LAMP is very high (up to 10^9 copies within an hour), resulting in a rapid release of the pyrophosphate ion by-product. Initially, positive LAMP reactions were determined by the formation and accumulation of insoluble magnesium pyrophosphate (Tomita et al., 2008). Alternative visualization methods for LAMP used the color shift of a metal ion-sensitive indicator, e.g., calcein (orange to green), hydroxynaphthol blue or HNB (violet to sky blue), and malachite green (dark blue to light blue) (Tomita et al., 2008; Goto et al., 2009; Jothikumar et al., 2014). Color changes using calcein and HNB are difficult to distinguish by eye. Intercalating nucleic acid dyes, e.g., SYBR green, can be used for real-time visualization but require a UV illuminator (Barbau-Piednoir et al., 2013). Besides pyrophosphate, hydrogen ion is also released as a by-product during DNA polymerization. In a recent study, pH-sensitive dyes were used to detect LAMP reactions (Tanner et al., 2015). However, this method has not yet been validated in food samples.

This study aimed to optimize and validate an in-house pH-sensitive LAMP assay for detecting *Salmonella* in food samples. The LAMP assay combined with enrichment and DNA extraction was accurate, sensitive, and inexpensive. This novel pH-sensitive LAMP assay can potentially be used as a rapid method for primary screening for *Salmonella* in the food industry.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

S. Typhimurium TISTR 292 and *Escherichia coli* TISTR 780 were obtained from Thailand Institute of Scientific and Technological Research, Thailand. Bacterial strains were cultivated in nutrient broth or on nutrient agar (HIMEDIA, India) at 37°C.

2.2 Preparation of DNA

The DNA template was prepared by heat treatment. Briefly, 1 mL of overnight culture was centrifuged at 10,000 rpm for 5 min. After the supernatant was discarded, the pellet was resuspended in 1 mL of nuclease-free water. The resulting cell suspension was heated at 95°C for 5 min and recentrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a fresh tube and used as the DNA template for LAMP.

2.3 Selection of pH-sensitive dye

Bromothymol blue (BTB), neutral red (NR), and phenolphthalein (PT) were tested for colorimetric visualization of LAMP. All indicator dyes were obtained from Amresco (USA) and dissolved in ethanol to obtain 100-mM stock and then diluted to the desired concentrations in water. One microliter of dye was added to 25 μL of reaction mixture. The LAMP reactions were visually inspected, and images were recorded using a smartphone.

2.4 LAMP reaction and condition optimization

Three sets of previously approved primers specific to the genes *invA*, *invE*, and *phoP* were compared. All primers are listed in Table 1. The standard LAMP reaction was performed in a 25-μL volume using 8 U *Bst* DNA polymerase, large fragment M0275 (New England Biolabs, USA), and the reaction ingredients were added as suggested by the company (FIP and BIP, 1.6 μM each; F3 and B3, 0.2 μM each; LF and LB, 0.4 μM each; dNTPs, 1.4 mM each; 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 8 mM $MgSO_4$, 0.1% Triton® X-100, and 2 μL of DNA template). The reaction mixture was mixed thoroughly, centrifuged, and then incubated at 65°C for 60 min in a water bath. To optimize the assay, the following parameters were varied: ratio of inner: outer primer from 1:1 to 8:1, incubation temperature from 60 to 65°C, and incubation time from 45 to 90 min.

Table 1 Primers used in this study and their references

Primer	Sequence (5′ → 3′)	Reference
invE FIP	AGGATTCGTCTCCAGGGGCGCCGCTGACATTTCGTCCG	Chen et al., 2015
invE BIP	TGCGGCCTGTTGTATTTCCGCTTGTCCCGGCAGACATCT	
invE F3	GTTACGAAATTGCGCCAGC	
invE B3	TGGCTCAACCTCCGGTAT	
invA FIP	CCCAGATCCCCGCATTGTTGATTTTTCCGCCCCATATTATCGCTAT	Ye et al., 2011
invA BIP	GACCATCACCAATGGTCAGCATTTTATTGGCGGTATTTCGGTGGG	
invA F3	GTTCAACAGCTGCGTCATGA	
invA B3	CGCTATTGCCGGCATCATTA	
phoP FIP	GGCGTGAGAGATCCACCTGGAATGCGCCGTAATAGCGGTC	Li et al., 2009
phoP BIP	CACCATTATGGAAACGCTTATCCGCCGGATACAGCTGAAGCATC	
phoP F3	GCCATTCCACATCGAAGAGGT	
phoP B3	ATGAGAACATCAATGGTATGGC	
phoP LF	CAGGTGATCAACATCCCGCC	
phoP LB	CGGTAAAGTGGTCAGCAAAGAT	

2.5 Sensitivity evaluation

For the sensitivity test, minced chicken meat and liquid egg white were used as the food matrices. Prior to spiking with the serially diluted *S.* Typhimurium, 25 g of minced chicken meat or 25 mL of liquid egg white was mixed with 225 mL of buffered peptone water (BPW) (HIMEDIA, India), homogenized, and autoclaved. Then, 1 mL of the serially diluted *S.* Typhimurium was added to obtain 2, 2×10^2, 2×10^4, 2×10^6, and 2×10^8 CFU/g or CFU/mL of food. The spiked samples were incubated for 12 h at 37°C. Every 3 h, 1 mL of the enriched sample was removed for DNA preparation.

2.6 Assay validation

To validate the assay, 40 samples of chicken parts, including minced meat, fillet, gizzard, liver, and

heart (8 samples each), were randomized from fresh markets in Nakhon Pathom province, Thailand. Chicken parts were kept on ice and transported to our laboratory within 1 hour. All samples were processed immediately after arrival. Briefly, each sample was aseptically divided into two 25-g portion. The first portion was added to 225 mL of BPW, homogenized, and incubated at 37°C for 3 h. Then, 1 mL of sample was removed for DNA preparation. The resulting supernatant was used as the DNA template in the optimized LAMP assay. The second portion was used for detection with the 3M *Salmonella* express (SALX) systems (3M, USA). The 3M SALX system includes primary and secondary enrichment, plating on Petrifilm SALX, and biochemical confirmation, which took about 3 days.

3. RESULTS AND DISCUSSION

3.1 pH-sensitive dye suitable for visualization

To select pH-sensitive dyes, a standard LAMP reaction was applied, and *invE* primer sets were used. Firstly, the pH of the LAMP reaction was checked using pH indicator strips. The initial pH of the reaction mixture was close to 9, and the final pH was shifted to approximately 6-7 in the positive control using *S.* Typhimurium DNA as the template and remained at approximately 8-9 in the negative control using *E. coli* DNA as the template. The pH values of LAMP reactions were consistent with those in a previous study, in which a decrease in pH was observed, from the initial pH of 8.8 to a final pH of 6.0-6.5 (Tanner et al., 2015). One microliter of each dye (10, 15, and 20 mM) was added to the reaction mixture before or after incubation, referred to as one-pot or endpoint visualization, respectively. Addition of PT to the reaction mixture produced a pink color for a few seconds, which gave way to turbidity immediately after mixing, indicating the insolubility of PT under the test conditions. Thus, PT was not used in further experiments. The color transition of BTB from blue-green to light green was observed at all concentrations. However, the difference between blue-green and light green was difficult to discern by eye. NR shifted from faint orange to red at all concentrations, but 15 mM showed the greatest contrast (Figure 1). No difference was observed between one-pot and endpoint visualization. Since re-opening the reaction tube to add pH indicator after incubation may enhance the risk of cross-contamination, one-pot visualization is preferable. Therefore, NR at a concentration of 15 mM was selected for one-pot visualization of LAMP.

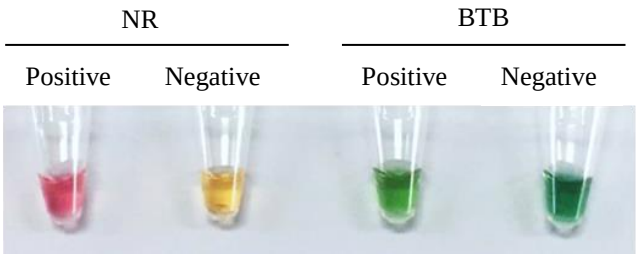

Figure 1 Color transition of LAMP reaction when 15 mM of neutral red (NR) or bromothymol blue (BTB) was added to reaction mixture before incubation (one-pot visualization)

3.2 Optimized conditions of the LAMP assay

The optimal concentration of inner:outer primer was 8:1. Incubation at 62.5°C for up to 90 min was not sufficient to generate a clear positive result (data not shown). The optimal incubation conditions were 63.5°C for 90 min. LAMP reactions containing *invA* and *phoP* primers showed a clear color transition of NR, while those containing *invE* primers were barely distinguishable (data not shown). Thus, *invA* and *phoP* primers were selected for evaluation of assay sensitivity under the optimal conditions. The *invA* gene has been studied extensively as the target gene for detecting *Salmonella* by both LAMP and PCR-based techniques (Chen et al., 1997; Isogai et al., 2005; Ohtsuka et al, 2005; Nucera et al., 2006, Jacobsen and Holben, 2007; Malorny et al., 2007, Ye et al., 2011).

3.3 Sensitivity of the LAMP assay for detection of *Salmonella* in food

S. Typhimurium was diluted in BPW and inoculated into 25 g (or mL) of minced chicken meat and liquid egg white to obtain 2, 2×10^2, 2×10^4, 2×10^6, and 2×10^8 CFU/g or CFU/mL of food. The spiked foods were mixed with 225 mL of BPW and homogenized. The mixtures were incubated for 12 h. During the incubation, 1-mL sample was removed from each mixture every 3 h for DNA extraction. The DNA extracts were subjected to the optimized LAMP assay with *invA* or *phoP* primers. The results from minced chicken meat samples showed that the detection limit was 2 CFU/g with 12 h of enrichment incubation when *invA* primers were used (Figure 2a). In contrast, *phoP* primers required only 3 h of enrichment incubation to detect 2 CFU/g *S.* Typhimurium (Figure 2b), indicating a much higher efficiency of *phoP* primers under our conditions. The results for sensitivity in liquid egg white were similar (data not shown). Evidently, the enrichment step is necessary to assure high sensitivity. Enrichment for 24 h increased the sensitivity from 10^4-10^6 CFU/25 g to 1-3 CFU/25 g of spiked meat (Yang et al., 2016). Previous studies demonstrated that the detection limit of *phoP* primers was 35 CFU/25 mL of food samples (Li et al., 2009), while *invA* primers have a detection limit of 1 CFU/cm^2 on eggshells (Ye et al., 2011). The disagreement of our results with previous studies was probably due to differences in the LAMP conditions and food matrices used. The additional loop primers in the *phoP* primer set might have enhanced the overall performance of the reaction. In this study, the sensitivity was tested in food matrices. The assay sensitivity might differ if a pure culture or purified DNA was used.

It is notable that the color of the pH indicator changed slightly even in the negative control containing *E. coli* DNA (Figure 2c). This effect was also reported by Tanner and coworkers (2015). In that study, they attributed this intermediate color change to non-template amplification. Nevertheless, the difference between the red color of the positive reactions and the slight orange color of the negative reactions was sufficient to allow discrimination.

3.4 Validation of the LAMP assay

Forty samples of naturally contaminated foods were examined with the in-house optimized pH-sensitive LAMP assay in parallel with the 3M SALX system. The LAMP assay using *phoP* primers and NR in combination with enrichment in BPW for 3 h could detect *Salmonella* in 33 out of 40 samples (Table 2). The results from our in-house LAMP assay were consistent with those of the 3M SALX system after the confirmation step. A total of 7 samples (sample codes 21, 23, 25, 27, 28, 33, and 34) tested negative by both methods. The high accuracy of the LAMP assay could be attributed, at least in part, to the previously reported high specificity of the *phoP* primers for *Salmonella*. Li and coworkers (2009) demonstrated that *phoP* primers correctly identified 66 strains of *Salmonella*, including 31 type strains and 32 isolates from all 6 subspecies of *S. enterica* and also 3 isolates of *S. bongori*, while 73 non-*Salmonella* strains tested negative. Visualization of the LAMP products by agarose gel electrophoresis could confirm the applicability of the in-house pH-sensitive LAMP assay. The 3M SALX system has already proven comparable to FDA/BAM methods (Bird et al., 2014). When 3M SALX was considered as the reference method, neither false-positive nor false-negative results were detected by the pH-sensitive LAMP assay developed in this study. Future studies should include a conventional culture-based method in parallel with the 3M SALX system to calculate positive and negative predictive value.

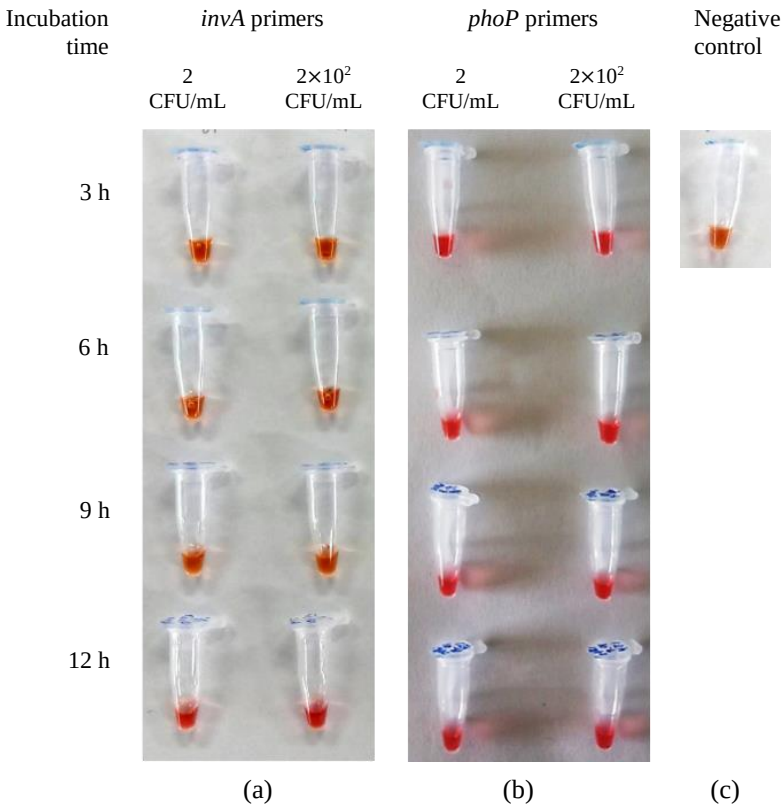

Figure 2 Sensitivity of the pH-sensitive LAMP assay for detection of *S.* Typhimurium in minced chicken meat

Table 2 Comparative analysis of *Salmonella* contamination in 40 samples of chicken parts using the in-house optimized pH-sensitive LAMP assay and 3M SALX system

Sample code	LAMP	3M SALX	Sample code	LAMP	3M SALX
01	+	+	21	−	−
02	+	+	22	+	+
03	+	+	23	−	−
04	+	+	24	+	+
05	+	+	25	−	−
06	+	+	26	+	+
07	+	+	27	−	−
08	+	+	28	−	−
09	+	+	29	+	+
10	+	+	30	+	+
11	+	+	31	+	+
12	+	+	32	+	+
13	+	+	33	−	−
14	+	+	34	−	−
15	+	+	35	+	+
16	+	+	36	+	+
17	+	+	37	+	+
18	+	+	38	+	+
19	+	+	39	+	+
20	+	+	40	+	+

4. CONCLUSION

In this work, we have developed a pH-sensitive, one-pot LAMP assay for detecting *Salmonella* in food. Our LAMP assay, in combination with a short enrichment and an easy DNA extraction step, demonstrated high sensitivity at 2 CFU/g or CFU/mL of food. Under optimal conditions, the detection of *Salmonella* in both spiked and naturally contaminated food products was successful. Moreover, this LAMP assay showed 100% diagnostic accuracy when compared with the widely accepted culture-based 3M SALX system. With further validation and approval by authorized organizations, this LAMP assay can potentially be used in diagnostic food laboratories.

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