Agr. Nat. Resour. 55 (2021) 945–957

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Development of loop-mediated isothermal amplification (LAMP) SYBR Green I assay as screening test for detection of 4 strains of *Salmonella* spp. in feed and feed ingredients

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Article Info

Article history:
Received 12 August 2021
Revised 19 October 2021
Accepted 4 November 2021
Available online 20 December 2021

Keywords:
Feed ingredients,
*Inv*A gene,
LAMP,
LAMP-SYBR green I,
Salmonella spp.

Abstract

Salmonella spp. are a group of gram negative and pathogenic bacteria that cause food poisoning in humans and animals. Traditionally, the *Salmonella* causative source has been attributed to animal products, feed and feed ingredients. Conventional detection methods of *Salmonella* spp. are time-consuming since they take 5 d. Herein, a LAMP-SYBR green I test integrated with LAMP (loop-mediated isothermal amplification) was developed to increase the yield of the *Salmonella* invasion gene (*Inv*A) with the *Salinv*A01 primer set and identification of *Salmonella* spp. by pre-enrichment for 4 hr and a reaction time within 60 min at an optimized temperature of 62°C. Subject to these optimized conditions, the detection limits of the designed LAMP for *Salmonella* spp. pure culture were 1.33×10^3 colony forming units (cfu)/mL or 26.6 cfu/tube. LAMP-SYBR green I and LAMP-AGE showed no cross-reactivity with 15 isolates of non-*Salmonella* spp. including *Escherichi coli*, *Listeria monocytogenes*, *Staphylococcus aureus, Bacillus cereus*, *Micrococcus luteus, Microbacterium spp.*, *Corynybacter glutamicum*, *Pichia membranaefaciens*, *Rhodotorula mucilaginosa*, *Serratia marcescens* and *Proteus mirabilis*. Among the 24 samples of feed and feed ingredients that were used to evaluate artificial contamination and enrichment of *Salmonella* spp., LAMP-SYBR green I and LAMP-AGE demonstrated 100% accuracy compared to PCR and conventional methods. In conclusion, the LAMP-SYBR green I assay was confirmed to be highly specific, accurate, fast and convenient, and to have low detection limits. The results can be provided in 1 hr using isothermal conditions, so there is no need to use a thermocycler. Hence, the developed assay can be utilized for screening *Salmonella* spp. contamination in feed and feed ingredients.

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https://doi.org/10.34044/j.anres.2021.55.6.05

Introduction

Salmonella is considered the most common pathogens involved in foodborne diseases in humans and animals, causing economic losses through mortality, reduced production; consequently, contamination by *Salmonella* is a major concern globally, including in Europe (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021), the United States and Japan (Chen et al., 2017) and Thailand (Pelyuntha et al., 2021). Traditionally, the causative source for *Salmonella* has been attributed to animal products (Tadesse and Gebremedhin, 2015), including milk and chicken residues (Lamas et al., 2018), eggs, duck meat, chicken meat and other meat products (Siala et al., 2017). In recent years, outbreaks have been increasing of foodborne illness due to unconventional sources of *Salmonella*, involving vegetables, fruits and feed ingredients. The presence of *Salmonella* has been documented in more ingredient types such as grains, oilseed meals and fish meal (Brandl et al., 2013). *Salmonella* contamination in feed and feed ingredients can be spread and circulated to animal and food products from animals and throughout the food chain, resulting in major economic losses and importantly being a serious health hazard to humans and animals (Yang et al., 2017; Djeffal et al., 2018).

The conventional method of detecting *Salmonella* is a microbiological procedure which takes 5 to 7 d to identify contamination. This method is based on pre-enrichment and enrichment in selective media and confirmation using biochemical and serotyping methods. This approach is highly labor intensive but it has high accuracy for detecting *Salmonella* living cells (Food and Drug Administration, 2013; Andrews et al., 2018). Molecular methods such as polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) and loop-mediated isothermal amplification (LAMP) have been manipulated in *Salmonella* detection to achieve sensitivity, specificity and a short detection time (Yang et al., 2018; Samar et al., 2019). PCR, multiplex PCR and RT-PCR are considered the most suitable methods developed for the detection of pathogenic bacteria, with *Salmonella* being successfully studied during past decades because it involves a fast, simple, low sensitivity and high specificity assay. However, the amplification method based on PCR requires complex and specific instruments (Abubakar et al., 2007). For PCR application to feed ingredients, one problem is that the feed ingredients have a chemical composition that can interfere with the activity of DNA polymerase for PCR amplification (Pinto et al., 2017).

LAMP is a DNA amplification technique performed using the *Bst* DNA polymerase to increase the yield of a specific fragment of DNA and sophisticated instruments are not required, so it is more practical for microorganism detection (Notomi et al., 2000; Nagamine et al., 2001). The products of LAMP can be detected based on visible assessment by dropping a fluorescent DNA dye, such as SYBR green I, to reduce the detection time for assessment of the results. A positive result initiates a color reaction to fluorescent green, while a negative result leaves the dye unchanged from its original orange color (Dixit et al., 2018). Microorganism studies have been reported using LAMP applications for the detection of *Salmonella* in food and feed (Yang et al., 2018). The *hila, fimA, stn* and *inv*A genes are most regularly used for *Salmonella* detection involved with virulence (Reda et al., 2019; Wen et al., 2020). The *Inv*A gene of *Salmonella* has broad specificity for *Salmonella* and is similar to other *Salmonella* serovars (Hara-Kudo et al., 2005; Pal et al., 2017). The objective of the current research was to develop a LAMP-AGE and LAMP-SYBR green I technique to increase the yield of the *Salmonella* invasion gene (*inv*A) with greater specificity and a low detection limit based on rapid and simple detection of *Salmonella* in feed and feed ingredients.

Materials and Methods

Strains of bacterial for validation studies

All 19 bacterial strains (4 *Salmonella* serotypes and 15 non-*Salmonella* strains) were acquired from the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Sean campus, Thailand (VPHVETKU) and the Microbiology Department, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Thailand (MICROFLASKU), as shown in Table 1. A pure isolate of *Salmonella* Typhimurium was used as a pure culture source to optimize the LAMP assays, PCR and sensitivity tests. All strains were transferred from stock into 10 mL of TSB (Soybean-casein digest medium, Difco; USA) and incubated at 37°C for 24 hr.

Preparation of bacterial culture and DNA extraction

Nutrient agar (Himedia; India) was used to culture all bacterial strains, which were incubated at 37°C overnight. The boiling DNA extraction method was used to extract DNA from all microorganisms (Ahmed and Dablool, 2017). A single

bacterial colony from nutrient agar was picked and inoculated in TSB and incubated at 37°C overnight. Then, 1 mL of cultured cells in TSB was centrifuged at 10,000 revolutions per minute (rpm) for 1 min, supernatants were discarded and 100 µL of sterile dH_2O was added to the pellets and mixed well. The mixed pellet in a microcentrifuge tube was boiled in 100°C water for 10 min prior to placing on ice for 1 min. The microcentrifuge tube was centrifuged at 10,000 rpm for 1 min and the supernatant was subsequently transferred to a new sterile microcentrifuge tube for use as the DNA template for all reactions.

Table 1 *Salmonella* spp. and non-*Salmonella* spp. used in this study

Microbial strain	Source
Salmonella spp. (*n* = 4)	
1. *Salmonella* Typhimurium	VPHVETKU
2. *Salmonella* Enteritidis	VPHVETKU
3. *Salmonella* Choleraesuis	VPHVETKU
4. *Salmonella* Typhi *1417*	VPHVETKU
Non-*Salmonella* spp. (*n* =15)	
5. *Escherichia coli*	VPHVETKU
6. *Escherichia coli* 527	VPHVETKU
7. *Bacillus cereus*	MICROFLASKU
8. *Bacillus cereus* lab KPS	MICROFLASKU
9. *Bacillus cereus* 2372	MICROFLASKU
10. *Listeria monocytogenes*	VPHVETKU
11. *Staphylococcus aureus*	MICROFLASKU
12. *Staphylococcus aureus* 2329	MICROFLASKU
13. *Micrococcus luteus*	MICROFLASKU
14. *Microbacterium* 1413	MICROFLASKU
15. *Corynybacter glutamicum* 461	MICROFLASKU
16. *Pichia membranaefaciens* 5108	MICROFLASKU
17. *RhodotorµLa mucilaginosa* 5861	MICROFLASKU
18. *Serratia marcescens*	MICROFLASKU
19. *Proteus mirabilis*	MICROFLASKU

Design of loop-mediated isothermal amplification primers

The PrimerExplorer V5 software (http://https://primerexplorer.jp/lampv5) was used to generate all LAMP primer sets in this work, which were based on the *Inv*A gene (Genbank: DQ644633.1) with a length of 1,918 bp from *Salmonella bongori*. The CLUSTALW multiple alignment program (https://www.genome.jp/tools-bin/clustalw) was used to align the sequences of *Inv*A genes. Each LAMP primer set contained a backward outer primer (B3), forward outer primer (F3), backward inner primer (BIP) and forward inner primer (FIP). All *Salmonella* LAMP primers in this work were protected by petty patent submission number 2003002289. The LAMP and PCR products sizes are presented in Table 2.

Optimization of loop-mediated isothermal amplification reaction

All LAMP primer sets were synthesized by Ward Medic IDT, Thailand. First, each LAMP reaction of the *inv*A gene was performed in a total volume of 25 µL containing 0.8 µM each of the LAMP inner primers (BIP and FIP), 0.4 µM each of LAMP outer primers (B3 and F3), 1.2 mM dNTPs (Thermo Fisher Scientific Inc.; Lithuania), 0.8 M betaine, 4 mM $MgSO_4$, 1X of *Bst* polymerase buffer (Biolab Inc.; UK), 8 U of *Bst* DNA polymerase, 2 µL of DNA template and made up with sterile deionized water to 25 µL. The optimum temperature was determined using the LAMP conditions by testing at 65°C, 64°C, 63°C, 62°C, 61°C, 60°C, 59°C, 58°C, 57°C, 56°C and 55°C for 60 min. Under the identified optimal temperature, the optimization time was evaluated at 10 min, 20 min, 30 min, 40 min, 50 min and 60 min. Each LAMP product was assessed based on 2% agarose gel electrophoresis (AGE) visible to the naked eye by adding 2µL of SYBR green I into the LAMP product. Two microliters of sterile H_2O were used as the negative control (NC).

Table 2 Loop-mediated isothermal amplification (LAMP) product sizes of 5 primer sets with and without primer and size of polymerase chain reaction (PCR) product

Primer set	Start 3'–5'	LAMP product size with primer (bp)	LAMP product size without primer (bp)	PCR product (bp)
*Salinv*A01	448–551	102	181	199
*Salinv*A02	554–695	142	225	227
*Salinv*A03	681–785	105	187	187
*Salinv*A04	439–551	113	196	199
*Salinv*A05	461–551	91	172	180

Specificity tests of loop-mediated isothermal amplification assays

The specificity of the LAMP assays was determined under the optimized conditions based on the *Inv*A gene and testing using the 19 bacterial isolates (Table 1) including *Salmonella* spp. and non-*Salmonella* spp. Each microbial DNA template was amplified using LAMP with *Salinv*A primers and the products from amplification were analyzed based on 2% AGE and SYBR green I.

Method detection limits of loop-mediated isothermal amplification and polymerase chain reaction assays

The lowest concentrations of analyte (method detection limits, MDLs) were measured by dilutions of *S*. Typhimurium cells cultured using ten-fold serial dilutions and the standard guidelines (Ben-David and Davidson, 2014). The starting concentration of the *S*. Typhimurium cultures containing 1.33×10^8 colony forming units (cfu/mL were diluted to 1×10^7 cfu/mL, 1×10^6 cfu/mL, 1×10^5 cfu/mL, 1×10^4 cfu/mL, 1×10^3 cfu/mL, 1×10^2 cfu/mL and 1×10^1 cfu/mL) before boiling the DNA extraction as previously mentioned in the Materials and Methods section. The LAMP products were assessed using 2% AGE and SYBR green I.

The two outer primers (F3 and B3) of each LAMP primer sets were used to amplify the *inv*A genes based on PCR. The PCR reaction consisted of 2.5 µM of each outer primer, 0.15 mM dNTPs, 1.25 mM $MgCl_2$, 1.5 Unit *Taq* polymerase, 2 µL DNA templates and adding dH_2O to make up to 20 µL. The five steps of the PCR cycling commenced with 7 min for the initial denaturation step at 95°C, followed by 40 cycles of the amplification step, including denaturation at 95°C for 30 sec. After the denaturation step, the temperature was reduced to 62°C for 1 min (annealing). The last step (extension) used the optimal temperature at 72°C for 1 min and then 5 min at 72°C for the end of the reaction. Three microliters of each PCR product were examined based on 2% AGE and 2 µL of sterile dH_2O was included as the negative control (NC).

Samples of feed and feed ingredients

Twenty-four samples of feed and feed ingredients (7 feed samples and 17 feed ingredients) were randomly collected from the Feed Analysis-2 Laboratory, Department of Animal Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus in Nakhon Pathom, Thailand from June to July 2019. All samples were processed for bacterial identification according to standard protocols as outlined by Bacteriological Analytical Manual in Chapter 5 *Salmonella* Section (Food and Drug Administration, 2017).

Duration of pre-enrichment for loop-mediated isothermal amplification assay

Before the LAMP analysis, the durations were determined of the pre-enrichment step of *Salmonella*-contaminated feed and feedstuffs. Samples (25 g each) of feed and feedstuff were mixed with 1×10^8 cfu of *Salmonella* Typhimurium. Then, 25 g of mixed samples were add to 225 mL of water peptone buffer (WPB) and incubated at 37°C with agitation at 200 rpm. Samples (1 mL each) of pre-enrichment were collected in sterile microtubes at 2 hr, 4 hr, 6 hr, 10 hr, 14 hr, 18 hr and 24 hr. Samples collected at each time interval were extracted for DNA using the boiling method. Then, the LAMP products were analyzed using 2% agarose gel electrophoresis.

Artificial contamination and enrichment of Salmonella spp. in feed and feed ingredients

A sample (1 mL) of *S*. Typhimurium was injected into 25 g samples of each of the 24 feed and feed ingredient samples from pure cultures of *S*. Typhimurium containing 1×10^8 cfu/mL. The artificially-contaminated samples were prepared before enrichment, with 25 g of contaminated artificial sample mixed with 225 mL of buffer peptone water before being incubated at 37°C for 24 hr. Then, 1 mL of supernatant samples was collected before centrifugation and genomic DNA extraction. PCR and LAMP were used to analyze the DNA extracted from samples. For traditional culture detection, the enrichment solution was used in accordance with the ISO 6579:2002 Horizontal Method (ISO, 2002). Following pre-enrichment, selective enrichment was undertaken using a pipette to transfer 1 mL of supernatant of the samples to 9 mL Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn Himedia; India) broths and 0.1mL to 10 mL Rappaport Vassiliadis Soy Broth (RVS Himedia; India) using a pipette and incubating at 37°C (MKTTn) and 42°C (RVS) for 24 hr. After that, 10 µL of RVS and MKTTn were spread on xylose lysine deoxycholate agar (Himedia; India) and brilliant green agar (Himedia; India), followed by 24 hr of incubation at 37°C. Presumptive *Salmonella* colonies were isolated, chosen, purified and tested biochemically using triple sugar iron, urea broth and lysine iron assays.

Results

Optimization of loop-mediated isothermal amplification assay

Five LAMP primer sets for detection of the *Salmonella inv*A gene fragment were evaluated. The LAMP optimum temperature was assessed for amplification of the *Salinv*A01, *Salinv*A02, *Salinv*A03, *Salinv*A04 and *Salinv*A05 primer sets. Fig. 1 presents the results when equal concentrations of the LAMP reaction master mix were used and assessed based on 2% AGE and SYBR green I. For the *Salinv*A01 primer set, the optimum temperature was 61°C (Fig. 1A), whereas for the *Salinv*A02, *Salinv*A03, *Salinv*A04 and *Salinv*A05 primer sets, the optimum temperatures were similar at 61°C (Figs. 1B, 1C, 1D and 1E, respectively). No amplification occurred in the negative controls (NC). The optimum reaction time for LAMP amplification of the *Salinv*A01–*Salinv*A05 primer sets was 60 min. Fig. 2 shows the results when equal LAMP reactions at 61°C were used and assessed based on 2% agarose gel electrophoresis and SYBR green. For all the primer sets, the optimum time was similar at 60 min (Fig. 2A), whereas for the *Salinv*A02, *Salinv*A03, *Salinv*A04, and *Salinv*A05 primer sets, the optimum temperatures were similar at 61°C (Figs. 2A, 2B, 2C, 2D and 2E, respectively). NC (negative controls) had no amplification. Therefore, these results presented that the LAMP assay using the *Salinv*A01 primer set for detection of *Salmonella* beneficially amplified the *inv*A genes, and the LAMP products had lower optimized temperatures than the other LAMP primer sets products (61°C and 62°C) within 60 min.

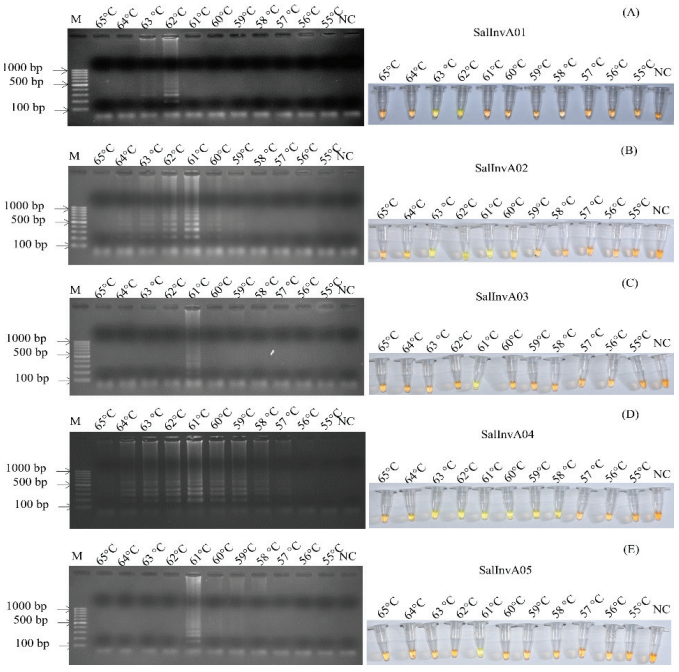

Fig. 1 Optimization of loop-mediated isothermal amplification (LAMP) reaction temperature, for *SalinvA*01–05 LAMP assays optimized for temperature and assessed based on 2% agarose gel electrophoresis (left) and SYBR green (right): (A) *Salinv*A01; (B) *Salinv*A02; (C) *Salinv*A03; (D) *Salinv*A04; (E) *Salinv*A05, where M = 100 bp DNA ladder marker and NC = negative control (without DNA template)

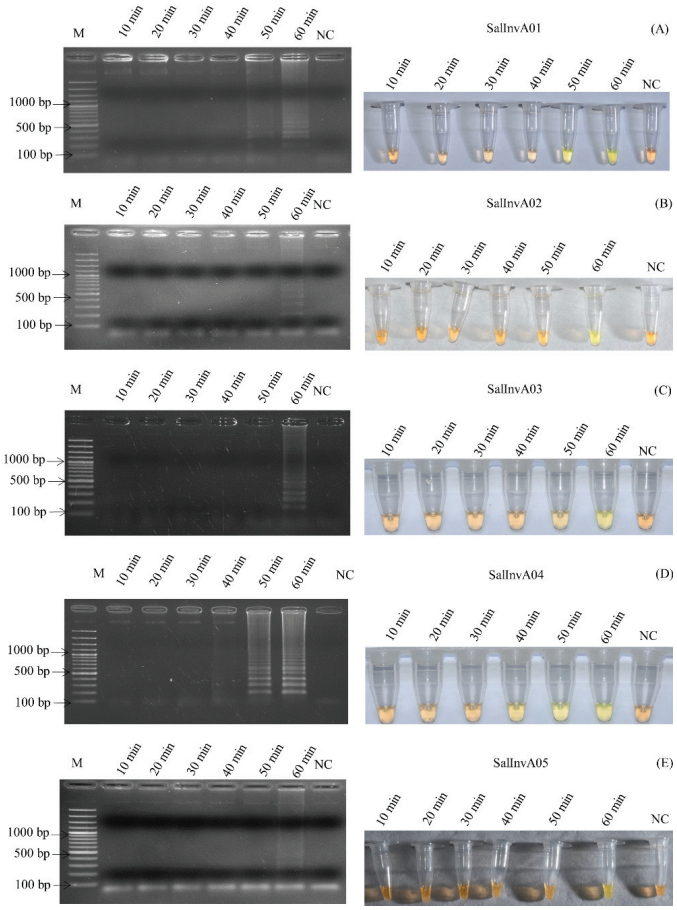

Fig. 2 Optimization of loop-mediated isothermal amplification (LAMP) reaction time for *Salinv*A01–05 LAMP assays optimized for temperature and assessed based on 2% agarose gel electrophoresis (left) and SYBR green (right): (A) *Salinv*A01; (B) *Salinv*A02; (C) *Salinv*A03; (D) *Salinv*A04; (E) *Salinv*A05, where M = 100 bp DNA ladder marker and NC = negative control (without DNA template)

Specificity of t loop-mediated isothermal amplification method

The analytical specificity tests of the LAMP-AGE and LAMP-SYBR green I (Fig. 4) assays for the detection of *Salmonella* species using all primers sets are shown in Fig. 3 and Fig. 4, respectively targeting the *inv*A genes. There was no cross amplification of the target genes in the non-*Salmonella* strains. These results indicated that the LAMP-SYBR green I and LAMP-AGE assays based on all primer sets of *inv*A genes were very efficient and highly specific for the detection of *Salmonella* spp.

Method detection limits

The detection limits of the LAMP-AGE, LAMP-SYBR green I and PCR reactions using each primer sets for amplification of the *inv*A gene (*Salmonella*) are presented in Fig. 5 (*SalinvA01* primer set). The specific products like-ladders were amplified from all primer sets *SalinvA01–SalinvA05* (*Salmonella* spp.) in the LAMP-AGE assay (Fig. 5A) and assessed based on SYBR green I (LAMP-SYBR green I assay) as shown in Fig. 5B. The PCR products of the *Salinv*A01–*Salinv*A05 primer sets were 199 bp (Fig. 5C), 227 bp, 187 bp, 199 bp and 180 bp in size. This study considered using ten-fold serial dilutions to estimate the MDLs for the standard guidelines (Ben-David and Davidson, 2014). The initial concentration of cultures of

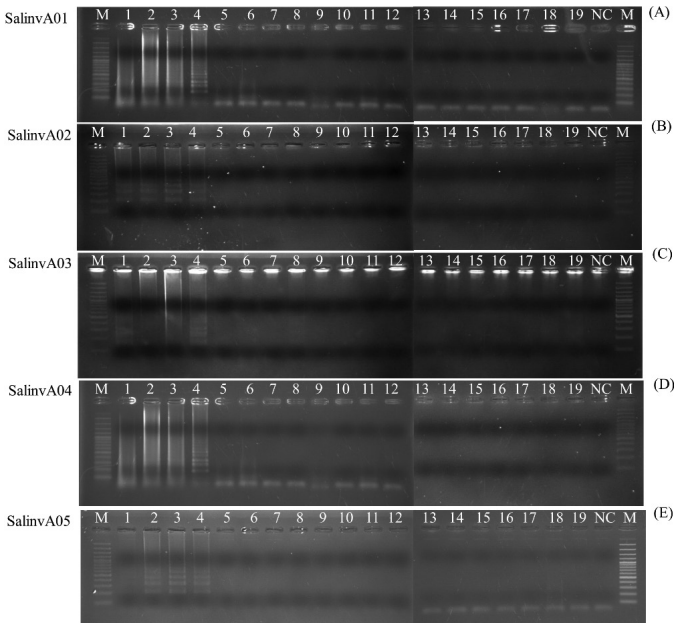

Fig. 3 Specificity tests for detection of *Salmonella* spp. and non-*Salmonella* based on all primer sets *inv*A genes using LAMP-AGE: (A) *Salinv*A01, (B) *Salinv*A02, (C) *Salinv*A03, (D) *Salinv*A04 and (E) *Salinv*A05, where lanes 1–4 represent *Salmonella* spp., *S.* Typhimurium, *S.* Enteritidis, *S.* Choleraesuis and *S.* Typhi 1417, lanes 5–19 represent *Salmonella* spp., *E. coli*, *E. coli* 527, *B. cereus*, *B. cereus* lab KPS, *B. cereus* 2372, *L. monocytogenes*, *S. aureus*, *S. aureus* 2329, *Micrococcus luteus*, *Microbacterium* 1413, *Corynybacter glutamicum* 461, *Pichia membranaefaciens* 5108, *Rhodotorula mucilaginosa* 5861, *Serratia marcescens* and *Proteus mirabilis* and NC = negative control (without DNA template)

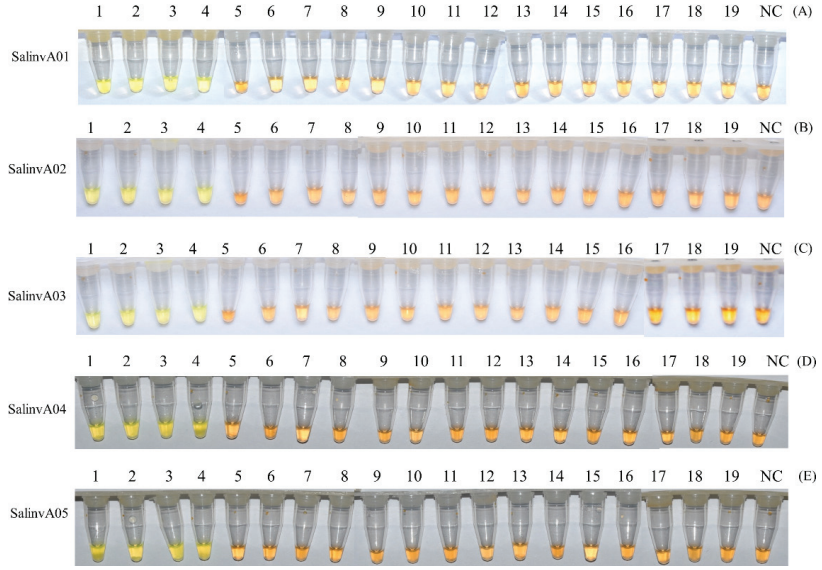

Fig. 4 Specificity tests for detection of *Salmonella* spp. and non-*Salmonella* based on primer sets *inv*A genes using LAMP-SYBR green I: (A) *Salinv*A01; (B) *Salinv*A02; (C) *Salinv*A03; (D) *Salinv*A04 and (E) *Salinv*A05; where tubes 1–4 represent *Salmonella* spp., *S.* Typhimurium, *S.* Enteritidis, *S.* Choleraesuis and *S.* Typhi 1417, tubes 5–19 represent *Salmonella* spp., *E. coli*, *E. coli* 527, *B. cereus*, *B. cereus* lab KPS, *B. cereus* 2372, *L. monocytogenes*, *S. aureus*, *S. aureus* 2329, *Micrococcus luteus*, *Microbacterium* 1413, *Corynybacter glutamicum* 461, *Pichia membranaefaciens* 5108, *RhodotorμLa mucilaginosa* 5861, *Serratia marcescens* and *Proteus mirabilis* and NC = negative control (without DNA template)

Salmonella spp. containing 1.33×10^8 cfu/mL were diluted to 1×10^7 cfu/mL, 1×10^6 cfu/mL, 1×10^5 cfu/mL, 1×10^4 cfu/mL, 1×10^3 cfu/mL, 1×10^2 cfu/mL and 1×10^1 cfu/mL before DNA extraction and amplification. The sensitivities of LAMP and PCR were the same using the *Salinv*A01 primers. The MDLs of the LAMP-AGE, LAMP-SYBR green I assays and PCR using pure culture based on the *Salinv*A01 primer sets of *inv*A gene were 1.33×10^3 cfu/ml corresponding to 26.6 cfu/tube.

Duration of pre-enrichment for loop-mediated isothermal amplification assay

The duration of the pre-enrichment step of *Salmonella*-contaminated feed and feed ingredients were determined based on the LAMP method. Samples were used of 25 g of cassava pulp, poultry meal and swine feed 333 spiked with 1×10^8 cfu of *Salmonella* Typhimurium pre-enrichment processing in WPB. The 1 mL samples of pre-enrichment were collected in microtubes for LAMP assay after incubation for 2 hr, 4 hr, 6 hr, 10 hr, 14 hr, 18 hr and 24 hr. The data revealed that LAMP could detect *Salmonella* in cassara pulp at 4–24 hr (Fig. 6A), poultry meal at 2–24 hr (Fig. 6B) and swine feed #333 at 2–24 hr (Fig. 6C). The positive control was DNA of *Salmonella* Typhimurium. Hence, the recommended pre-enrichment time interval before LAMP assay was 4 hr.

Evaluation of artificial contamination of Salmonella spp. in feed and feed ingredients

Using culture-based examination, the 24 randomly collected feed and feed ingredient samples were contaminated with *Salmonella* spp. Evaluation of the 24 feed and feed ingredients samples showed that all 24 samples were positive based on LAMP-AGE and LAMP-SYBR green I amplification (Figs. 7B and 7C, respectively), with 20 samples having specifically positive bands based on PCR (Fig. 7A) and 24 samples having colonies based on conventional methods, as shown in Table 3. LAMP-AGE and LAMP-SYBR green I were successfully detected with the *Salinv*A01 primer set that was specific to the amplified *inv*A gene of *Salmonella* spp.

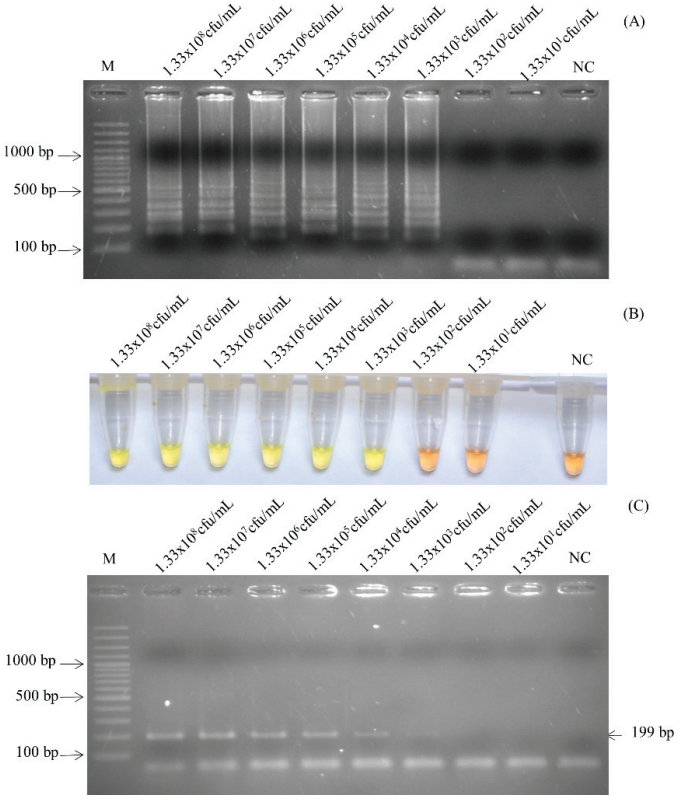

Fig. 5 Method detection limits of primer set *Salinv*A01 for detection of *Salmonella* spp. pure culture using: (A) LAMP-AGE; (B) LAMP-SYBR Green; (C) polymerase chain reaction assays, where M = 100bp DNA ladder marker and NC = negative control (without DNA template)

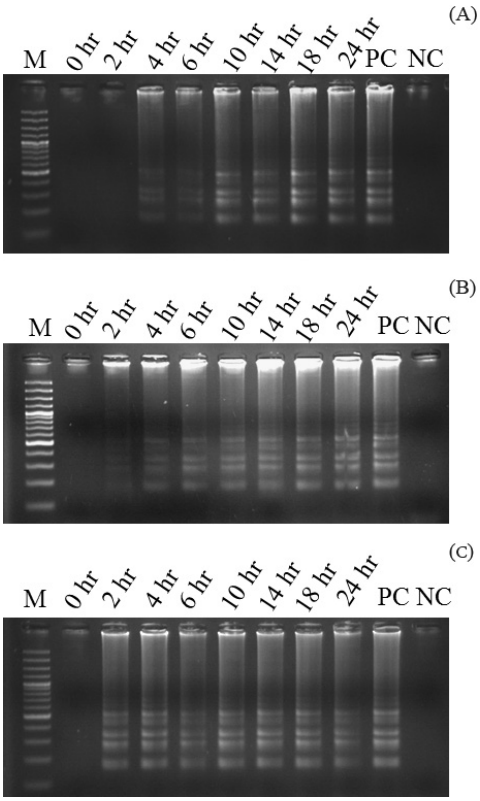

Fig. 6 Length of pre-enrichment time for loop-mediated isothermal amplification assay based on inoculation *S.* Typhimurium in: (A) cassava pulp; (B) poultry meal; (C) swine feed into BWP analyzed using the *SalInv*A01 primer and demonstrated on 2% AGE, where M = 100 bp DNA ladder plus marker, PC = positive control (genomic DNA of *S.* Typhimurium) and NC = negative control (without DNA template)

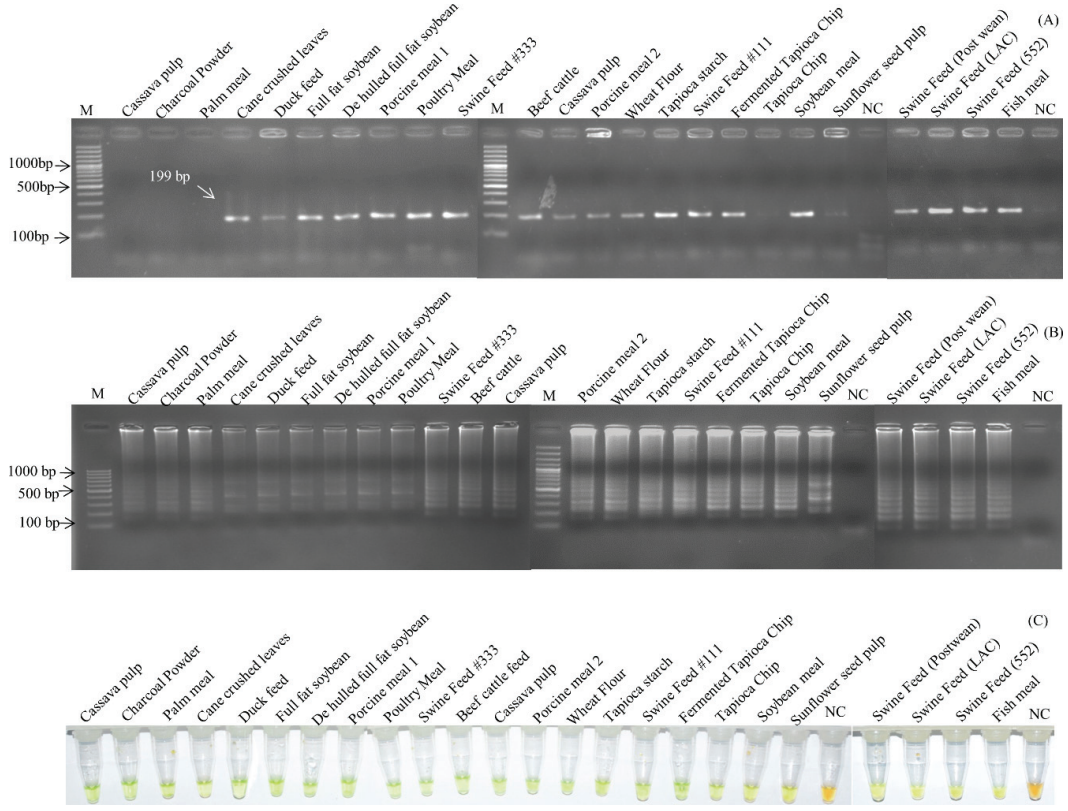

Fig. 7 Detection of artificial contamination of *Salmonella* spp. in 24 feed and feed ingredients based on *Salinv*A01 primer set using: (A) polymerase chain reaction; (B) LAMP-AGE; (C) LAMP-SYBR green I assays, where NC = negative control (without DNA template)

Table 3 Detection of artificial contamination of *Salmonella* spp. in 24 feed and feed ingredients based on primer set *Salinv*A01 using polymerase chain reaction (PCR), LAMP-AGE and LAMP-SYBR Green I assays against standard culture

Feed and feed ingredients	Culture-based	PCR	LAMP-AGE	LAMP-SYBR Green I
Feed				
1. Duck feed	+	+	+	+
2. Swine feed #333	+	+	+	+
3. Beef cattle feed	+	+	+	+
4. Swine feed #111	+	+	+	+
5. Swine feed post wean	+	+	+	+
6. Swine feed 550 LAC	+	+	+	+
7. Swine feed 550 KPF	+	+	+	+
Feed ingredients				
8. Cassava pulp 1	+	-	+	+
9. Charcoal powder	+	-	+	+
10. Palm meal	+	-	+	+
11. Cane crushed leaves	+	+	+	+
12. Full fat soybean	+	+	+	+
13. DE hulled full fat soybean	+	+	+	+
14. Poultry meal	+	+	+	+
15. Porcine meal 1	+	+	+	+

Table 3 Continued

Feed and feed ingredients	Culture-based	PCR	LAMP-AGE	LAMP-SYBR Green I
16. Cassava pulp 2	+	+	+	+
17. Porcine meal 2	+	+	+	+
18. Wheat flour	+	+	+	+
19. Tapioca starch	+	+	+	+
20. Tapioca chip	+	-	+	+
21. Fermented tapioca chip	+	+	+	+
22. Soybean meal	+	+	+	+
23. Sunflower seed pulp	+	+	+	+
24. Fish meal	+	+	+	+
Total positive samples	24	20	24	24

LAMP = loop-mediated isothermal amplification

The specificity, detection limit and accuracy values of the LAMP-SYBR green I and LAMP-AGE assay were 100% compared to PCR and the culture methods (Table 4). These data suggested that the LAMP-SYBR green I assay was an efficient tool for the detection of *Salmonella* spp.

Discussion

Previously, species-specific genes have been identified, such as the *inv*A gene for *S.* Typhimurium, the *bcfD* gene for *S.* Newport, the *phoP* gene for *S.* Paratyphi A, the *siiA* gene for *S.* Typhimurium, the *g62* gene for *S.* Choleraesuis and the *ttr* locus for *S*. Typhimurium (Kreitlow et al., 2020). The specificity of the LAMP assay was confirmed using 46 strains of *Salmonella* and 32 strains of non-*Salmonella* species. However, the primers designed using *inv*A, *siiA*, *bcfD*, *phoP*, and g62 failed to detect *S. enterica* subsp IV, while the *ttr* primer detected all *Salmonella* (Kreitlow et al., 2020). Many PCR-based *Salmonella* monitoring systems use the *inv*A gene as a popular target molecule (Barletta et al., 2013; Li et al., 2014). The *inv*A gene is found on the chromosome and codes for a bacterial inner membrane protein that is required for epithelial cell invasion; it is highly conserved in two *Salmonella* species (Chaudhary et al., 2015). The *inv*A gene has broad specificity for *Salmonella* and is similar to other *Salmonella* serovars (Pal et al., 2017). Of the 2,058 total nucleotide sites in the *inv*A gene, 308 nucleotide sites (15.0%) were reported to be mutated (Buehler et al., 2019).

Bai et al. (2018) used Primer3 to design a PCR primer for the detection of *Salmonella* from *Salmonella enterica* based on the *inv*A gene, while Liu et al. (2019) used PrimerExplorer v3 to design a LAMP primer for the detection of *Salmonella enterica* serovar Typhi based on the conserved *inv*A location in *Salmonella enterica* and used the Allele ID software to generate internal, external, and loop primers (Fatemeh et al., 2020). Herein, five primer sets (*Salinv*A01–*Salinv*A05) were designed from the *inv*A gene on the nucleotide sequence of *Salmonella bongori* strain CNM-256 (GenBank Accession number: DQ644633.1), having identities greater than 95% among other *Salmonella* serovars. The current work showed

Table 4 Comparison of specificity, sensitivity, accuracy and process time of LAMP-SYBR green I and LAMP-AGE for detection of *Salmonella* spp. in feed and feed ingredients against standard culture and polymerase chain reaction (PCR) assays

Diagnosis methods	Positive results of *Salmonella* spp.	Sensitivity (%)	Specificity (%)	Accuracy (%)	Process time	Unit cost of one sample test (USD)
Culture-based	24	100	100	100	4 days	10.71
PCR-based	24	100	100	83.33	2.30 hr	2.75
LAMP-AGE	24	100	100	100	1.30 hr	2.14
LAMP-SYBR green I	24	100	100	100	1.00 hr	1.5350

LAMP = loop-mediated isothermal amplification;
USD 1 = THB 32.67 in November, 2021

that the LAMP reaction to amplify the *inv*A gene of *Salmonella* spp. was at an optimized temperature of 62°C for 60 min, which was a longer optimizing time than in other studies. For example, Vichaibun and Kanchanaphum (2020) designed inner and outer primers of LAMP and recognized the *inv*A gene from *Salmonella* Typhimurium reactions cultured at 65°C for 45 minutes. Notably, the 60 min optimized time in the current study was similar to the times reported in Zhuang et al. (2014), Mei et al. (2019) and Kreitlow et al. (2020). One possible explanation could be that the concentration of loop primers and *Bst* DNA polymerase was less than previously reported. However, it was proposed that loop primers and *Bst* DNA polymerase be added to the process to enhance amplification (Nagamine et al., 2002). The MDLs of the developed LAMP for *Salmonella* pure culture were 1.33×10^3 cfu/mL or 26.6 cfu/tube in this investigation, which were higher than in past studies, while the MDLs of the LAMP assay using genomic DNA of *S*. Enteritidis were 4 cfu/reaction (Gong et al., 2016), 2.2–6.3 cfu/reaction (Hara-Kudo et al., 2005) and 12 cfu/reaction (Hu et al., 2018). One possible explanation for the MLD values recorded in the current study being higher than in previous studies could be the DNA extraction boiling method and centrifuge step that were used in the current study. DNA extraction is a key step in increasing the detection limit, purity, yield, level of contamination and scalability. Therefore, the DNA extraction boiling method may be a popular option because it is easy, the most cost effective and requires the least time. However, this method had a lower detection limit and less purity, yield, contamination, and scalability than extraction using commercial kits (Yamagishi et al., 2016). The current study used 10,000 rpm for 1 min in the centrifuge step. The detection limit and yield of DNA could be increased by adjusting the speed in the centrifuge step to 13,000 rpm for 1 min.

The DNA extraction process in the current study depended on centrifugation to precipitate cells and sludge from feed ingredients, which may have introduced added complication. Methods for obtaining DNA without centrifugation may also be possible, such as using immunomagnetic separation of *Salmonella* from the sample sediment, where the magnetic beads have anti-*Salmonella* attached on the surface to capture *Salmonella* contaminated in samples before DNA extraction without centrifugation. Nevertheless, the optimal solution temperature and process time would still be required for the capture of *Salmonella* using magnetic beads (Zheng et al., 2014). However, the centrifugation step in the current study removed large amounts of sludge that may have interfered with the LAMP assay. Therefore, a portable centrifuge could be used in this step.

For the detection of *Salmonella* contaminated in samples by conventional methods, the pre-enrichment procedure of at least 24 hr was required to allow for sufficient organisms (Andrews et al., 2018). Additionally, investing less time in the process of pre-enrichment reduces the time for screening the *Salmonella* using the LAMP method. Srisawat and Panbangred (2015) inoculated *Salmonella* in food samples mixed with 225 mL of lactose broth with 5 hr for pre-enrichment before detection using the LAMP method. In the current study, LAMP method was able to detect *Salmonella* contaminated in a plant-based feed ingredient (cassava pulp) after 4 hr of pre-enrichment and in an animal-based feed ingredient (poultry meal) after 2 hr of pre-enrichment. Hence, the pre-enrichment step was beneficial for detecting *Salmonella* in feed and feed ingredients because it multiplied viable cells, reduced interference from powdered substances through dilution and eliminated the issue of false-positive results (Wang et al., 2012).

The specificity results using LAMP-SYBR green I and LAMP-AGE in the current study showed high specificity by non-amplified product with 15 other strains, including *E. coli*, *B. cereus*, *L. monocytogenes*, *S. aureus, M. luteus, Microbacterium spp.*, *Corynybacter glutamicum*, *Pichia membranaefaciens*, *Rhodotorula mucilaginosa*, *Serratia marcescens* and *Proteus mirabilis*. Zhuang et al. (2014) reported the results of the LAMP primer specific for *bcfD* sequences was effective for the amplification of 44 strains of *Salmonella*, but not for non-*Salmonella* strains. Hu et al. (2018) reported that specific results positive for all *Salmonella* except *Prot6E* and only specific on *Salmonella* Enteritidis. Conversely, genes such as *fimA, stn, pipA* and *iroB* may not be detected in all *Salmonella* strains (Wang et al., 2013). These studies indicated that the conserving region of the *Salmonella inv*A gene was successful in the detection of each of the *Salmonella* strains but not for any of the other non-*Salmonella* strains such as LAMP primers (Zhao et al., 2010; Kreitlow et al., 2020), PCR primers (Barletta et al., 2013; Li et al., 2014) and DNA sequencing (Chaudhary et al., 2015; Pal et al., 2017; Buehler et al., 2019).

The results of evaluating the artificial contamination and enrichment of *Salmonella* spp. in feed and feed ingredients indicated that among the 24 samples of feed and feed ingredients, LAMP-SYBR green and LAMP-AGE demonstrated 100% accuracy compared to conventional methods and PCR. The LAMP primer *Salinv*A*01* detected *Salmonella* in 24 feed and feed ingredients and showed positive results in all 24 samples and therefore had the same detection rate as

the conventional method. PCR detected *Salmonella* contamination in 20 of the 24 samples, with negative results in cassava pulp, charcoal powder, palm meal, and tapioca chip. Feed and feed ingredients have a chemical composition of dry matter (carbohydrates, proteins, lipids, vitamins) and inorganic matter (Moran, 2005) and the chemical compounds in the feed ingredients were not entirely removed during the DNA extraction using the boiling extraction method and this may have caused problems in the subsequent PCR analysis or affected the integrity of the DNA (Piskata et al., 2017). Hence, inhibitor substances can obstruct PCR by reducing or totally inhibiting DNA polymerase activity (Pinto et al., 2017). Kaneko et al. (2007) found that LAMP was extremely resistant to biological contaminants and could amplify the target DNA from food and animal feed (Yang et al., 2014; Yang et al., 2016; Ge et al., 2019). Thus, the enrichment step is very helpful for detecting *Salmonella* in feed and feed ingredients because it multiplies viable cells, reduces interference from powdered substances through dilution and eliminates the issue of false-positive results. The dead cells can cause false-positive results which become negligible after enrichment (Wang et al., 2012).

LAMP-SYBR green I and LAMP-AGE were highly accurate compared to conventional methods and PCR. LAMP-SYBR green I was considerably specific, sensitive, convenient, fast and accurate. The conventional method for detecting *Salmonella* is a labor-intensive procedure that takes 5–7 d and requires numerous subculture phases, complex biochemical and serological tests and is time-consuming; however, it has excellent accuracy for *Salmonella* live cells (Hernandez et al., 2005; Food and Drug Administration, 2017). In most cases, PCR takes 1–2 hr and requires the use of a thermocycler to generate target DNA and about 20 times less DNA is produced (Mashooq et al., 2016). The conventional method for detection and identification of *Salmonella* is a time-consuming microbiological procedure (5–7 d), involving many subculture steps and complicated biochemical and serological testing as well as being very labor intensive; however, it has high accuracy for *Salmonella* living cells (Hernandez et al., 2005; Food and Drug Administration, 2017).

In conclusion, the authors successfully developed a LAMP-SYBR green I assay to detect *Salmonella* spp. The developed assay was highly specific, sensitive, accurate, fast and convenient to use and the results could be assessed in 1 hr without requiring any complex instruments under isothermal conditions. Hence, the LAMP-SYBR green I assay could be used for screening of *Salmonella* spp. contamination in feed and feed ingredients

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This research was supported by the Center for Agricultural Biotechnology (CAB) and the Center of Excellence on Agricultural Biotechnology (AG-BIO/MHESI).

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