SIMPLE, SPECIFIC, SENSITIVE AND RAPID LOOP-MEDIATED METHOD FOR DETECTING *YERSINIA ENTEROCOLITICA*

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Abstract. *Yersinia enterocolitica* (YE) is a main pathogenic bacterium causing diarrhea and yersiniosis occurs in both developed and developing countries with high incidence. YE in contaminated food is able to survive for a long duration even under cold storage, thereby enhancing the risk of food infection. In this study, a new loop-mediated isothermal amplification (LAMP) method showing the characteristics of simplicity, rapidity, high specificity and sensitivity was established by targeting *outL* of pathogenic YE. Two inner-primers and outer-primers were designed and LAMP reaction was optimized for Mg^{2+}, betaine, dNTPs and inner primers concentrations, reaction temperature and time. Sensitivity and specificity of the LAMP assay was evaluated using YE genomic DNA and those of 44 different bacteria strains, respectively. Validation of LAMP detection method was by employing meat samples spiked with varying CFU of YE. The optimized LAMP assay was specific, capable of detecting 97 fg of genomic DNA (equivalent to 37 genome copies) of YE (100-fold more sensitive than PCR) and 80 CFU/ml of YE-spiked meat samples based on ethidium bromide stained amplicon bands on agarose gel-electrophoresis and on GelRed fluorescence of the LAMP reaction solution, respectively. This rapid, sensitive and specific LAMP technique should enable application in field inspection of *Y. enterocolitica* in food.

Keywords: *Yersinia enterocolitica,* detection, loop-mediated isothermal amplification, optimization

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INTRODUCTION

Yersinia enterocolitica (YE) belonging to gram-negative bacteria is the third most common food-borne bacterium in Europe after *Salmonella enterica* and *Campylobacter jejuni* (Hanifian and Khani, 2012). Most yersiniosis is caused by YE and can lead to a variety of symptoms, such as fever, abdominal pain and diarrhea (Aziz and

Aziz, 2011; Kumar and Virdi, 2012). YE is a frequent cause of diarrhea in both developed and developing countries, and contributes to a considerable economic loss.

Yersiniosis is regarded as a specific zoonotic disease according to the European Council Directive 92/117/EEC (The Council of the European Communities, 1993). The epidemiology of yersiniosis is not yet fully understood, and YE infection is thought to be caused through the consumption of undercooked animal products or YE-contaminated water (Thoerner *et al*, 2003; Trcek *et al*, 2010; Ong *et al*, 2012). As a result, detection of YE is necessary for supervising food safety.

Many methods have been developed for detecting pathogenic YE strains. Immunology techniques and traditional culture have been employed (Riber and Jungersen, 2007; Balakrishna *et al*, 2010; Laukkanen *et al*, 2010; Savin *et al*, 2012). Furthermore, methods utilizing a combination of traditional bacterial culture, immunological and PCR techniques in a one-time detection of YE in food have been reported (Estrada *et al*, 2012). However, PCR and quantitative (q)PCR are considered as being the most efficient, sensitive and specific tools for identifying strains of YE (Lambertz *et al*, 2008; Gómez-Duarte *et al*, 2009).

Loop-mediated isothermal amplification (LAMP) is an isothermal (60º-65ºC) technique of DNA amplification, making use of *Bst* DNA polymerase and 4 primers designed from 6 regions of target DNA (Notomi *et al*, 2000). High efficiency, specificity, rapidity, sensitivity and simplicity are the characteristics of LAMP (Notomi *et al*, 2000; Dai *et al*, 2012).

In this study, the LAMP method for detecting YE was established by targeting the general secretion pathway protein L gene (*outL*), which is also named *Yst2L* and encodes the enterotoxin Yst (Yersinia stable toxin) that is one of the important virulence markers (Bancerz-Kisiel *et al*, 2012). Cultured YE and other bacterial strains were used to evaluate sensitivity and specificity of the LAMP method, and the performance of the LAMP method was determined by comparing with conventional PCR method. For testing the ability of LAMP method to detect YE in food, meat samples spiked with varying YE amounts were employed.

MATERIALS AND METHODS

Bacteria, culture and genomic DNA preparation

Forty-four bacterial strains, including 2 YE strains (CMCC52225 and CMCC52208), used in the study are listed in legend to Fig 2. Bacterial strains were streaked on nutrient agar (OXOID, Hamshire, UK) and a single clony was selected and cultured in LB broth at 37ºC for 16-18 hours, except for *Vibrio parahaemolyticus*, which was cultured in LB broth containing 3% sodium chloride at 30ºC overnight and *Brucella suis* S2 strain in Tryptone Soy Broth (TSB, OXOID, Hamshire, UK) at 37ºC for 48 hours. Genomic DNA (gDNA) was extracted using AxyPrepTM Bacterial Genetic DNA Miniprep kit (Axygen, Union City, CA) according to the manufacturer's instructions.

Design of LAMP primers and optimization of LAMP reaction

YE *outL* (GenBank accession no. AM286415) was chosen as the target DNA. The 4 YE-specific primers, designed using PrimerExplorer V4 software program (http://primerexplorer.jp/elamp4.0.0/index.html), and synthesized by Sangon Biotech (Shanghai, China) are listed in Table 1.

LAMP reaction parameters required for optimization included concentrations of Mg^{2+}, betaine, dNTPs, and inner primers (FIP and BIP), and the reaction temperature and time. LAMP reaction in a 25 µl volume, in addition to the above optimized reagents contained 0.5 µl of 10 µM outer-primers (each), 1 µl (8 U) of *Bst* DNA polymerase (New England Biolabs, Beverly, MA), 2.5 µl of 10X thermpol reaction buffer (New England Biolabs, Beverly, MA) and 2 µl (97 ng) of template gDNA (water was used in negative control). Reactions were terminated by heating at 80ºC for 4 minutes and 5 µl aliquots were analyzed by 2% agarose gel-electrophoresis and staining with ethidium bromide, after the reaction solutions were centrifuged briefly**.**

Specificity of LAMP assay

Extracted gDNA (97 ng per tube) of 2 YE strains and those of 42 non-YE bacterial strains (97 ng per tube) were used as templates in the optimized LAMP assay and amplicons analyzed by gel-electrophoresis as described above.

Sensitivity of LAMP compared with conventional PCR assay

One ml aliquot of YE cultured in 5 ml of LB broth at 37ºC overnight was centrifuged at 12,000*g* for 1 minute and the bacterial pellet was used for gDNA extraction. Concentration of YE gDNA was measured using Take 3 procedure (Biotech Epoch, Winooski, VT) and 10-fold serially diluted in distilled water, which were then amplified under the optimized LAMP reaction condition and by conventional PCR (amplifying 351 bp of YE *Ail*). The latter reaction was carried out in 25 µl volume containing 12.5 µl of Premix Ex Taq Version 2.0 (TaKaRa), 0.5 µM each primer (sense primer: TAATGTGTACGCTGCGAG and antisense primer: GACGTCTTACTTGCACTG) and 2 µl of the same gDNA used in LAMP assay. Thermocycling (EastWin, EDC-810, Beijing, China) conditions were as follows: 95ºC for 10 minutes; 25 cycles of 95ºC for 15 seconds, 57ºC for 30 seconds, and 72ºC for 30 seconds; then a final step at 72ºC for 10 minutes (Thoerner *et al*, 2003). Experiments were carried out in triplicate. The amounts of LAMP amplicons were observed in terms of turbidity by eye, and then were stained with 1% (2 µl) red nucleic acid fluorescent dye (GelRed, Biotium, Hayward, CA) and observed under ultraviolet light. Both LAMP and PCR amplicons (5 µl) were analyzed also by agarose gel-electrophoresis as described above. The lowest DNA concentration with a positive reaction was considered as the limit of detection.

Comparison of LAMP and PCR methods in the detection of YE-spiked samples

After culturing in 5 ml of LB broth for 16-18 hours at 37ºC, YE was collected as described above and re-suspended in sterile phosphate-buffered saline (PBS) at a concentration of $8.0x10^7$ CFU. This stock bacterial suspension was 10-fold serially diluted in sterile PBS (from 10^{-1} to 10^{-7}), then plated on 3 LB agar plates and incubated at 37ºC for 24 hours for CFU counting. An aliquot of 100 µl each diluted bacterial solution was added to 900 µl of beef homogenate, prepared by homogenizing 100 mg of beef (obtained from a local supermarket in Changchun, China) with LB (2,000 rpm for 10 minutes under aseptic condition in Ultra-Turrax disperser T10, IKA). Then 1 ml aliquot of each YE-spiked meat sample was centrifuged at 2500*g* for 10 minutes and the pellet was re-suspended in 2.25 ml of digestion buffer (10 mM Tris pH 8.0, 10 mM EDTA and 0.05% SDS). Then the

samples were incubated with 0.8 mg/ml pronase (Boehringer Mannheim, Germany) at 40ºC for 3 hours and centrifuged at 2500*g* for 15 minutes at 4ºC (Allmann *et al*, 1995). The pellets were used for gDNA extraction and subjected to the optimized LAMP method in comparison with PCR as described above. There were a total of 28 YE-spiked beef samples as assay of each spiked sample was conducted in triplicate.

RESULTS

LAMP primers and reaction optimization

DNA fragment of YE *outL* was amplified with expected molecular size of 240 bp using outer primers (B3 and F3) and the PCR amplicon obtained was verified by cloning and sequencing (results not shown). Sequences of LAMP primers and that of *outL* fragment were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/) in order to assess the efficiency of the designed LAMP primers. The identities of LAMP primers showed up to 100% identity with YE *outL* fragment, and the DNA homologies of the amplification products were analyzed by means of Clustalx1.83 software showing maximum identity of 98% (results not shown).

In order to establish the LAMP method, optimal reaction conditions were determined for such parameters as [Mg^{2+}] (1-6 mM), [dNTPs] (0.8-2.6 mM), [betaine] (0-1.6 M), [FIP and BIP] (0.2-1.2 µM), time (45-75 minutes) and temperature (55-65ºC). Based on the intensity and clarity of the ethidium bromide-stained amplicon bands separated by agarose-gel electrophoresis, the optimal LAMP conditions for detecting YE gDNA were 5 mM Mg^{2+}(Fig 1A), 1.8 mM dNTPs (Fig 1B), 1 M betaine (Fig 1C), 0.8 mM each FIP and BIP (1:4 ratio of outer to inner primers) (Fig 1D, 60 minutes, for economy of time (Fig 1E) and 63ºC (Fig 1F).

Specificity and sensitivity of LAMP assay

Using the optimized LAMP assay conditions, 97 ng of gDNA of the 2 YE strains yielded positive results, and LAMP assay did not amplify equivalent amounts of gDNA of the 42 unrelated bacteria (Fig 2). The 2 YE strains were also detected by conventional PCR method (data not shown).

Amplicons of LAMP assay could be readily observed as a turbid solution with 97 fg gDNA of YE in positive tube (Fig 3A). When analyzed by agarose gel-electrophoresis, the limit of detection by LAMP was 97 fg/tube (equivalent to 37 copy/tube) of YE gDNA (Fig 3B), 100-fold more sensitive than that by conventional PCR (Fig 3C). The quantity of one gene copy is equivalent to 2.63 fg (Lambertz *et al*, 2008). The limit of detection by the turbidity or fluorescence was the same as that by agarose gel-electrophoresis.

Assessment of the LAMP method of YE-spiked meat samples

In order to assess the sensitivity of the developed LAMP method to detect YE in beef samples, these samples were artificially contaminated with various amounts of YE. The detection limit of the LAMP method was 80 CFU/ml of YE in YE-spiked beef samples using both agarose gel-electrophoresis and GelRed fluorescence analyses (Fig 4). Each experiment was repeated at least 3 times. The presence of YE in the spiked beef samples were also confirmed by PCR, which could detect only 32% of the samples (data not shown).

DISCUSSION

YE can survive and proliferate at temperatures as low as 0ºC (Smith, 1971;

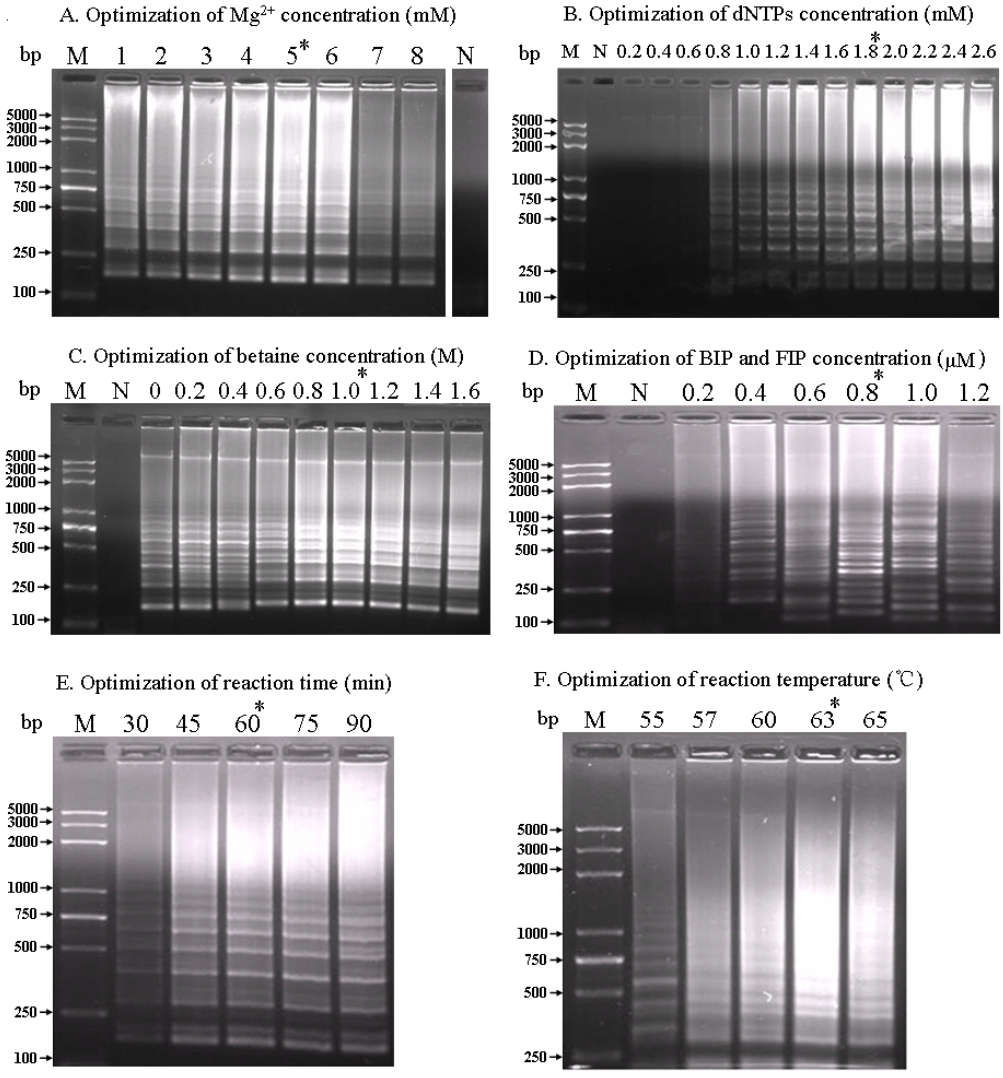

Fig 1–Optimization of LAMP reaction parameters. All optimized parameters of components were concentrations in 25 µl of the reaction system. The parameters optimized were: (A) 5 M [$MgSO_4$] in the presence of 1.8 mM dNTPs (2.5 mM each), 0.8 M betaine, 0.8 µM BIP and FIP, at 65°C for 60 minutes; (B) [dNTPs] with optimal [$MgSO_4$] and the other conditions described for (A); (C) [betaine] with optimal [$MgSO_4$] and [dNTPs] and the other conditions described for (A); (D) [BIP and FIP] with optimal [$MgSO_4$], [dNTPs] and [betaine] and the other conditions described for (A); (E) time, with optimal [$MgSO_4$], [dNTPs], [betaine], and [BIP and FIP] at 65°C; and (F) temperature with optimal [$MgSO_4$], [dNTPs], [betaine], and [BIP and FIP] for 60 minutes. LAMP reaction was conducted in 25 µl containing (in addition to the above reagents) (0.2 µM) outer-primers, 1 µl (8 U) of *Bst* DNA polymerase, 2.5 µl of 10X thermpol reaction buffer and 2 µl (97 ng) of template gDNA. Lane M: DL 5000 DNA marker; lane N: negative control. *optimal parameter.

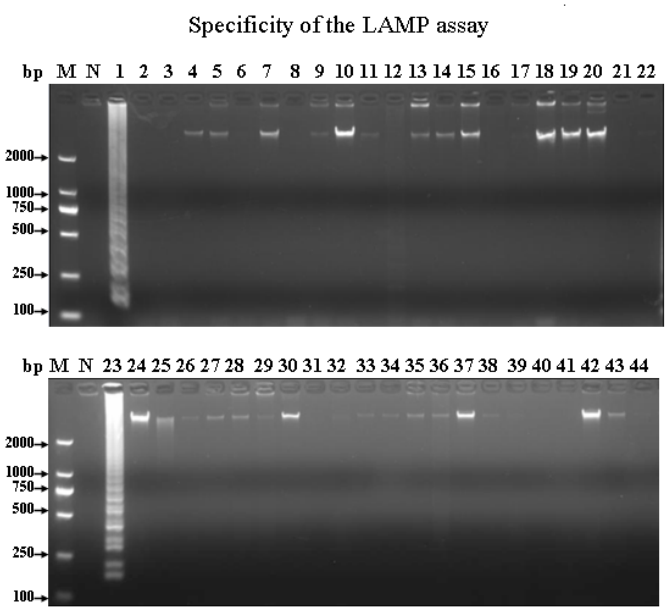

Fig 2–Specificity of LAMP assay. LAMP assays were conducted under optimized conditions using 97 ng of bacterial gDNA and the amplifications were analyzed by agarose gel-electrophoresis as described in Materials and Methods. Lane M: DL2000 DNA marker; lane N: negative control; lane 1: *Yersinia enterocolitica* (CMCC 52225); lane 2: enterotoxigenic *Staphylococcus* (CGMCC1.2465); lane 3: *Luteus micrococcus* (CGMCC 1.193); lane 4: *Brucella suis* 2 (CVCC 22); lane 5: *Staphylococcus aureus* (CGMCC 1.128); lane 6: *S. typhimurium* (CGMCC 1.1194); lane 7: *Pseudomonas aeruginosa* (CICC 21636); lane 8: *Bacterium paratyphosum* C (CMCC50118); lane 9: *Salmonella paratyphosa* (CMCC 50093); lane 10: *Bacillus cereus* (CGMCC 1.195); lane 11: *Micrococcus lysodeikticus* (CGMCC 1.634); lane 12: *Bacterium paratyphosum* B (CMCC 50094); lane 13: *Salmonella choleraesuis* (CGMCC 1.1859); lane 14: *Eberthella typhosa* (CMCC 50071); lane 15: *Salmonella anatis* (CMCC 50774); lane 16: *Shigella flexneri* (CGMCC 1.1868); lane 17: *Bacillus subtilis* (CGMCC1.1630); lane 18: *Vibrio parahaemolyticus* (CGMCC 1.1616); lane 19: *Escherichia coli* (CMCC 44108); lane 20: *E. coil* O157:H7 (CICC 21530); lane 21: *Vibrio fluvialis* (CGMCC 1.1611); lane 22: mode strain of *Escherichia coli* (CMCC 44817); lane 23:*Yersinia enterocolitica* (CMCC 52208); lane 24: epidermal *Staphylococcus* (ATCC 12228); lane 25: *Edwardsiella tarda* (ATCC 15947); lane 26: *Pseudomonas fluorescens* (CGMCC 1.867); lane 27: Hofmann's bacillus (CMCC 38203); lane 28: invasive *E. coli* (CMCC 44102); lane 29: *Pseudomonas stutzeri* (CGMCC 1.202); lane 30: *Serratia marcescens (CGMCC 1.589);* lane 31: *Aeromonas hydrophila* (CGMCC 1.1816); lane 32: *Klebsiella pneumoniae* (CGMCC 1.1736); lane 33: hive Hough Caledonia bacteria (CGMCC 1.2712); lane 34: *Corynebacterium glutamicum* (CGMCC 1.1736); lane 35: *Vibrio alginolyticus* (CICC 21611); lane 36: *Streptococcus agalactiae* (CGMCC 1.1481); lane 37: *Aeromonas sobria* (CMCC 10502); lane 38: *Acinetobacter baumanii* (ATCC 19606); lane 39: *Listeria monocytogenes* (CMCC 54002); lane 40: *Listeria monocytogenes* (CVCC 1599); lane 41: *Listeria monocytogenes* (CVCC 1598); lane 42: *Vibrio harveyi* (CGMCC 1.1593); lane 43: *Vibrio alginolyticus*; lane 44: *E. coli* DH5α (CGMCC 1.1369). ATCC, American Type Culture Collection 10801 University Boulevard Manassas (VA), USA; CGMCC, China General Microbiological Culture Collection Center, Beijing, China; CICC, China Center of Industrial Culture Collection, Beijing, China; CMCC, China Center of Medical Microbiology Culture Collective, Beijing, China; CVCC, China Veterinary Culture Collection Center, Beijing, China.

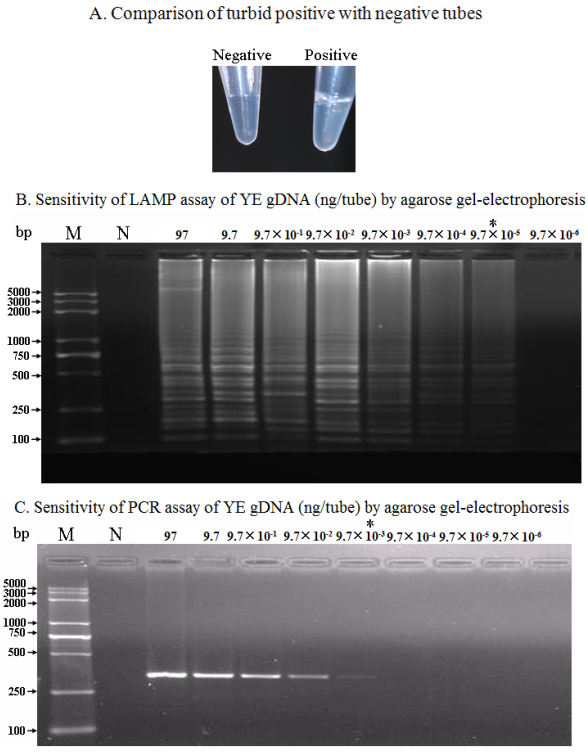

Fig 3–Sensitivity of LAMP assay. Varying amounts of YE gDNA were subjected to the optimized LAMP and PCR assays as described in Materials and Methods. LAMP results were evaluated by eye (A) showing a typical positive turbidity result using 97 fg of YE gDNA. Amplifications from both the LAMP (B) and PCR (C) assays were analyzed by agarose gel-electrophoresis. Lane M: DL5000 DNA marker; lane N: negative control. *Limit of detection.

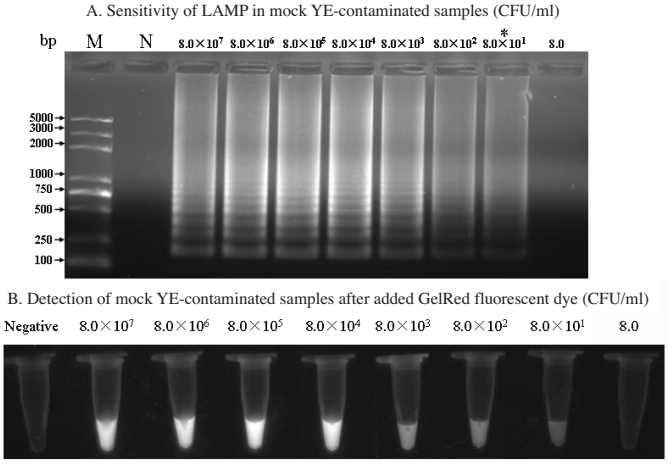

Fig 4–Evaluation of LAMP assay on YE-spiked meat samples. Meat samples were spiked with YE of varying CFUs as described in Materials and Methods and subsequently extracted YE gDNA were detected using the LAMP method. Amplicons were determined by agarose gel-electrophoresis (A) and by direct visualization under ultraviolet light after adding GelRed fluorescent dye (B). Lane M: DL5000 DNA marker; lane N: negative control. *Limit of detection.

Table 1
Primers used in LAMP-mediated amplification of *Y. enterocolitica outL*.

Primer name	Primer sequence
Backward outer primer (B3)	5′-CAACTCTAATCTTTTGTCCTGAA-3′
Forward outer primer (F3)	5′-AGGAATTCAAACACATCACG-3′
Backward inner primer (BIP)	5′-ATTTGCCATAATGCGTAATACATCGttt CGTGTTTATAACCAAACTTCGG-3′
Forward inner primer (FIP)	5′-AGGTCGATATTATCGCCATAGGAAAttt AGTAAACAGACCTAAAAAAAGAAGC-3′

Trcek *et al*, 2010). Asian children aged below 5 years old have the highest incidence of yersiniosis because meat (pork) food is often prepared at home and such children have much more chance of being exposed to YE-contaminated meat (pork) products (Ong *et al*, 2012). Thus, an efficient method for detecting YE in food is urgently needed for the benefit of protecting consumer health.

There are 4 isolation and culture methods to identify pathogenic YE, but they require at least 5 days to obtain the result (Laukkanen *et al*, 2010). Although immunological assays have been used to distinguish pathogenic YE from other microorganisms (Jungersen *et al*, 2006), false-positive results from cross-reaction with brucellosis infection is an important disadvantage, especially in detecting serotype O:9 YE, the most common strain causing yersiniosis, as this YE strain contains O-antigen lipopolysaccharide similar to that of *Brucella abortus* (Riber and Jungersen, 2007). PCR and qPCR methods do not require time-consuming culturing, are more sensitive (1.0 pg of YE gDNA /μl of PCR) and more specific than immunology assays (Lambertz *et al*, 2008; Gómez-Duarte *et al*, 2009). However, these methods require relatively expensive instruments and complicated procedures, making them unsuitable for on-site use.

On the other hand, the LAMP method for amplifying DNA is cost-saving and convenient, requiring only a simple heating block or water bath to maintain isothermal condition, and LAMP results as turbid solutions are readily observable by the naked eye or by adding a fluorescent dye into the reaction system (following sedimentation) and viewing under ultraviolet light (Wang *et al*, 2009; Huy *et al*, 2012). LAMP method which is very sensitive (Lau *et al*, 2011; Suwanampai *et al*, 2011; Sowmya *et al*, 2012; Wang *et al*, 2012) allowing detection of at least 6 copies of the haploid soybean genomic DNA within an hour (Guan *et al*, 2010). As a result, the LAMP assay is easier to operate and portable, which is desirable for on-site studies.

In this study, the optimized LAMP assay developed for specific detection of *Yersinia enterocolitica* had a sensitivity of 97 fg of YE gDNA (equivalent to 37 genome copies) and 80 CFU/ml of YE-spiked meat sample based on ethidium bromide stained amplicon bands on agarose gel-electrophoresis and on GelRed fluorescence of the LAMP reaction solution, respectively. Thus this rapid, sensitive and specific LAMP technique should be

further standardized to enable its application in field inspection of *Y. enterocolitica* -contaminated food.

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