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Original Article

Development of a rapid, one-step-visual method to detect *Salmonella* based on IC-LAMP method

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Abstract

Background: *Salmonella* can cause serious human gastroenteritis and is frequently isolated from various food samples. The cell culturing, immunoassay, and polymerase chain reactions (PCR) are the current methods to detect such pathogenic agents. However, these methods are time-consuming and labor-intensive, and thus unavailable for rapid-monitoring of *Salmonella*. **Aims:** This study aimed to develop an immunocapture-loop-mediated isothermal amplification (IC-LAMP) for rapid and sensitive detection of *Salmonella*. **Methods:** *Salmonella* was used as antigen to produce monoclonal antibody (mAb) and mAbs were prepared via subcloning three times. The mAb 1B12 with high affinity was coated on the surface of the immuno-magnetic beads (IMBs) to capture *Salmonella*. The enriched products (IMBs-*Salmonella*) were used for LAMP using the special primers targeted the conserved *invA* gene of *Salmonella*. **Results:** The IC-LAMP was developed based on mAb 1B12 and LAMP. Targeting the conserved *invA* gene of *Salmonella*, the detection time was shortened to 50 min from three days. If the reaction contains *Salmonella*, the green fluorescence and the trapezoidal strip can be clearly observed. Importantly, the method combines the specificity of antibody and LAMP with a detection limit of 5 CFU/ml in artificially contaminated water and milk. The specificity of this method was demonstrated by testing other similar bacteria. The results indicate that the IC-LAMP reacts only with *Salmonella* and does not cross-react with other similar bacteria. **Conclusion:** The IC-LAMP assay developed here is a rapid, sensitive, one-step-visual method to screen for the presence of *Salmonella* in food samples. This method is faster than traditional PCR, LAMP, and other methods, and can be used as a primary screening method for the detection.

Key words: Detection, LAMP, Magnetic Immunocapture, Monoclonal antibody, *Salmonella*

Introduction

Salmonella is an important bacterial pathogen worldwide, infecting humans and a variety of animals. Infection can spread through habitats, feeding activities, or reservoirs (Zhang *et al*., 2003; Alves *et al*., 2015). *Salmonella* may cause several diseases including typhoid fever and salmonellosis, when it is ingested (Royer and Pare, 2003; Salzman *et al*., 2003). *Salmonella* infects approximately 94 million people globally each year, including 80.3 million food-borne cases and 155,000 deaths (Majowicz *et al*., 2010). In 2013, the Economic Research Service (ERS) estimated that the monetary costs of countering salmonellosis in the United States were over $3,666 million. Improved rapid and accurate detection methods are strongly needed. Culture-based technology was long considered the gold standard for detecting *Salmonella* in all kinds of samples. However, this process requires multiple subculture steps, and biochemical and serological confirmation is also needed. Together, these steps require about seven days, and is a time-consuming and labor-intensive process (Zhao *et al*., 2017). Loop-mediated isothermal amplification (LAMP) was described by Notomi *et al*. (2000). Loop-mediated isothermal amplification is a novel nucleic acid amplification method, that amplifies DNA with high specificity, sensitivity, rapidity and efficiency, and has been applied for the detection of various pathogens, including fungi, parasites, viruses, and bacteria (Mori and Notomi, 2009; Law *et al*., 2015; Song *et al*., 2016; Khan *et al*., 2018; Liu *et al*., 2018). A positive reaction generates white magnesium pyrophosphate precipitate, which can be easily observed by the naked eye, without the requirement of electrophoresis (Mori *et al*., 2001). This advantage allows the method to be performed as an on-site test using simple reaction equipment (Kanitkar *et al*., 2017). However, this method still has some limits, as it cannot enrich cells and it is typically necessary to enrich *Salmonella* before detection. Thus, it is difficult to apply this technology to various important samples, such as milk, seafood, and faeces.

In this study, LAMP was combined with magnetic

immuno-capture technology in a process that showed a higher detection limit than that of polymerase chain reactions (PCR) and was more specific than the culture-based method. Use of the immunocapture-loop-mediated isothermal amplification (IC-LAMP) method for the detection of *Salmonella* was efficient, rapid, and showed high specificity.

Materials and Methods

Strains used in this study

All strains used in this study are shown in Supplementary Table (ST1). In addition, *Shigella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus* were used for specificity testing.

Production of polyclonal antibody (pAb) and monoclonal antibody (mAb) against *Salmonella*

The production of pAb and mAb and against *Salmonella* was performed as was previously described (Zhang *et al*., 2019; Zhang *et al*., 2019). We use 10^7 CFU of *Salmonella* that was dissolved in phosphate buffer saline (PBS) and inactive for 1 h at 80°C as antigen to immunized mice (Zhang *et al*., 2018). The first immunization was performed using 10^7 CFU of *Salmonella* mixed with Freund's complete adjuvant. The second and third immunizations were performed using 10^7 CFU of *Salmonella* mixed with Freund's incomplete adjuvant. The booster immunization was performed using 10^7 CFU of *Salmonella* dissolved in PBS, after three days of the third immunization. Through three times normal immunizations and a booster immunization, the pAb were prepared. Then, the spleen cells of the mice were collected and fused with mouse myeloma cells (SP2/0) (Sun *et al*., 2015). The indirect enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of *Salmonella*-specific antibodies in the hybridoma supernatants (Encheng *et al*., 2013; Du *et al*., 2016; Datta *et al*., 2018). The positive hybridomas will also be screened, after subcloning several times.

Purification and titration of Ascitic fluid

The female BALB/c mice (7-week-old) were used to produce ascites against *Salmonella*. Briefly, the mice were injected intraperitoneally with 0.5 ml of sterile paraffin oil. Seven days later, 10^6 cells of each hybridoma suspended in RPMI 1640 medium were used to inject each mouse. Ascitic fluid of mice was collected and purified using protein A affinity chromatography. The purified ascites were confirmed by Western blot and ELISA (Xia *et al*., 2016).

Western blot analysis

Western blot was used to identify the reactivity and specificity of ascites (Hemmatzadeh and Kazemimanesh, 2017). Briefly, the *Salmonella* were harvested and suspended in water and then inactivated. The resulting cellular lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the proteins were transferred from the gel to a nitrocellulose (NC) membrane, and blocked with blocking buffer for 2 h at 37°C. The Ascitic fluid against *Salmonella* (1:2,000) were added and incubated for 2 h at 37°C. After incubation, the goat anti-mouse immunoglobulin G (IgG) heavy chain and light chain (H+L) (1:4,000; GenScript, USA) was used as the second antibody and was incubated for 1 h at 37°C. Finally, the NC membrane was scanned and analyzed with a chemiluminescence imaging system (BIOTOP Biosciences).

Genomic DNA extraction

All strains were grown on Luria-Bertani (LB) solid plates (Solarbio, China) first and then cultivated in LB liquid medium. The genomic DNA was extracted using a Bacteria Genomic DNA kit (Zomanbio, China), according to the manufacturer's instructions.

The LAMP assay

The LAMP reaction was performed in 25 µL, containing a set of four primers (F3: 5´-CGC GGC CCG ATT TTC TCT-3´, B3: 5´-GGC AAT AGC GTC ACC TTT G-3´, FIP: 5´-GCG CGG CAT CCG CAT CAA TAT TTT GGT ATG CCC GGT AAA CAG AT-3´, and BIP: 5´- GCG AAC GGC GAA GCG TAC TGT TTT CAT CGC ACC GTC AAA GGA A-3´), 1 µL of *Bacillus stearothermophilus* (Bst) DNA polymerase (Eiken Chemical Co., Ltd., Tokyo, Japan), 2 µL of DNA template, 12.5 µL of 10 × LAMP buffer (Eiken Chemical Co., Ltd., Tokyo, Japan), 4 µL of dNTP mixture, and water. The mixture was maintained at 63°C for 40 min and terminated at 85°C for 5 min (Deb *et al*., 2017). The LAMP products were analyzed by separation on 3% agarose gel.

The standard IC-LAMP assay

The immuno-magnetic beads (IMBs) were prepared as described in our previous study (Zhang *et al*., 2018). After preparation of IMBs, they were used to capture *Salmonella* in different samples at 37°C for 0.5 h. Next, the remaining samples were removed and the IMBs were washed three times with phosphate buffer saline containing 0.05% Tween-20 (PBS-T). The enriched products (IMBs-*Salmonella*) were then lysed with 10 µL of solution A (125 mM NaOH, 1 mM EDTA, 0.1% Tween-20) and incubated for 10 min at 65°C. Finally, the reaction was terminated by addition of 10 µL of solution B (125 mM HCl, 10 mM Tris) (Zhang *et al*., 2019). Then, the LAMP reaction was performed as described above.

Specificity and sensitivity of LAMP, and IC-LAMP

Common foodborne pathogenic bacteria (Supplementary Table (ST1)) were cultured and used for the extraction of genomic DNA. Loop-mediated isothermal amplification and IC-LAMP assays were conducted to identify the specificity of the designed

primers. Additionally, the sensitivity of the LAMP and IC-LAMP assays were verified using the different concentrations of *Salmonella* and plasmids.

Practical application of IC-LAMP to *Salmonella* detection in milk and water

To further verify the applicability of IC-LAMP technology, *Salmonella* was mixed with pasteurized milk samples and water. The *Salmonella* strains were then diluted into the milk and water samples for final concentrations of 5×10^7 to 5×10^0 CFU/ml. The milk and water samples were mixed with the IMBs in a tube and then incubated for 30 min at 37°C. The specificity of the IC-LAMP was determined in both processed milk and sterile water. To do this, bacterial mixtures were prepared with *Salmonella* (a mixture of *Salmonella*, *E. coli*, *S. aureus*, *Shigella*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*) or the bacterial mixtures without *Salmonella*. All strains were mixed together and diluted into skim milk or sterile water. The bacterial mixtures were captured with IMBs. The compounds of bread-cell were washed thoroughly and lysed as described above. Next, 2 µL of the bacteria lysate were used for IC-LAMP. Non-contaminated milk and water samples were used as negative controls.

Results

Characterization of *Salmonella*-specific mAbs

After the booster immunization, the sera of mice were collected and the titer of the pAb was tested by ELISA (Fig. 1A). Through three rounds of subcloning, two stable positive hybridomas were prepared and named as 1B12 and 2F1. Then, the hybridomas of 1B12 and 2F1 were used to produce ascites. The immunoglobulin isotypes of mAb 2F1 and 1B12 were determined by mouse mAb isotyping kit. The results indicated that mAb 1B12 and 2F1 were isotypes IgG3, and the light chains of the two mAbs belonged to the kappa chain family (Table 1). The titers of two mAbs were detected by ELISA (Fig. 1B). The concentrations of the two mAbs are shown in Table 1. ELISA analysis demonstrated that mAb 1B12 bound *Salmonella* specifically with high affinity (K_d = 1.126 ± 0.15 nM) (Fig. 1CA). The purity of the purified mAbs were confirmed by SDS-PAGE (Fig. 1D). The results of Western blot showed that mAb 1B12 only recognized *Salmonella* (Fig. 1E, Lanes 1 and 2) and showed no reactivity with the other similar bacteria.

Table 1: Characterization of the mAbs against *Salmonella*

mAbs	Isotype	mAb titer	mAb concentration (mg/ml)
1B12	IgG3, κ chain	10^6	2.5
2F1	IgG3, κ chain	10^5	1.8

Specificity and sensitivity of LAMP

To measure the specificity, we tested the ability of the LAMP assay to generate signals from other bacterial samples. Based on the *invA*, two pairs of primers were designed successfully and could specifically amplify *Salmonella*, but no fragments were amplified from the other bacterial strains (Fig. 2A). To evaluate the sensitivity of LAMP, the plasmids were serially diluted from 10^9 to 10^0 copies/µL. The results of the LAMP showed that the trapezoidal strip was clearly observed even at a concentration of 10^0 copies/µL (Fig. 2B).

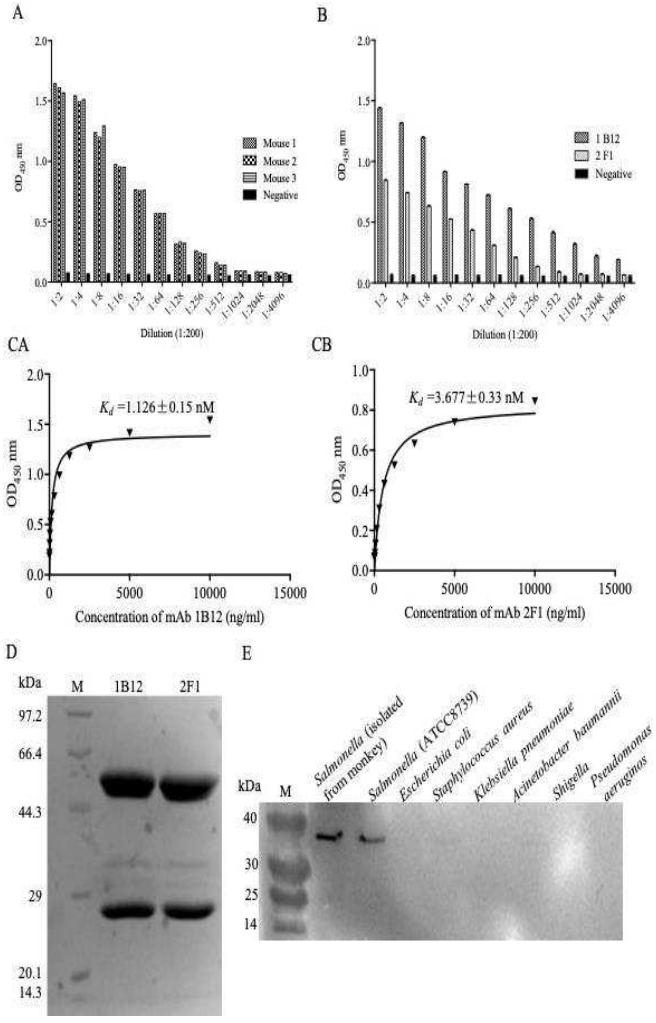

Fig. 1: Characterization of anti-*Salmonella* pAb and mAb. (**A**) The titer of pAb, (**B**) The titer of mAb 1B12 and 2F1, (**C**) The saturation curve to determine the dissociation constants for mAb 1B12 (CA) and 2F1 (CB), (**D**) SDS-PAGE analysis of purified mAb 1B12 and 2F1. M: Protein marker, and (**E**) Western blot analysis of mAb 1B12. M: Protein marker. OD: Optical density

Specificity and sensitivity of IC-LAMP

Staphylococcus aureus, *E. coli*, *Shigella*, *P. aeruginosa*, *Salmonella*, *A. baumannii*, and *K. pneumoniae* were captured by IMBs. The sample of mixed bacteria lacking *Salmonella* was also captured by IMBs and the enriched products were used as a template to determine the specificity of the assay. The results showed that the trapezoidal strips clearly appeared when the mixed bacteria contained *Salmonella* (Fig. 3A). Next, the sensitivity of the IC-LAMP assay was tested with *Salmonella* serially diluted into sterile water (from 5 ×

10^6 to 5×10^0 CFU/ml). The results showed that the trapezoidal strip was clearly observed even at a concentration of 5×10^0 CFU/ml (Fig. 3B).

Detection of *Salmonella* contamination of milk using the IC-LAMP assay

The practical application of IC-LAMP was confirmed using artificial contamination of milk. The green fluorescence and trapezoidal bands were obviously observed when the milk samples contained mixed bacteria including *Salmonella* (Figs. 4A, C). Next, *Salmonella* were serially diluted into skim milk (from 5×10^5 to 5×10^0 CFU/ml) to assess the sensitivity of the IC-LAMP assay. Results showed that the amplified fragments and the green fluorescence were clearly observed even at a concentration of 5×10^0 CFU/ml (Figs. 4B, D).

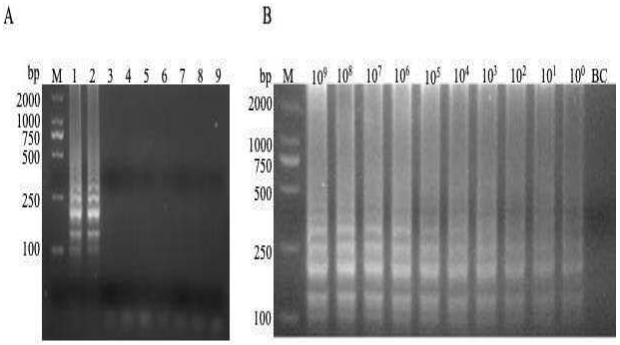

Fig. 2: Specificity and sensitivity of the LAMP assay against *Salmonella* or the plasmid containing the *Salmonella invA* gene. (**A**) Specificity of the *invA*-LAMP assay. M: 2000 bp DNA marker; 1: *Salmonella* (isolated from monkey); 2: *Salmonella* (ATCC8739); 3: *Escherichia coli*; 4: *Staphylococcus aureus*; 5: *Klebsiella pneumoniae*; 6: *Acinetobacter baumannii*; 7: *Pseudomonas aeruginos*; 8: *Shigella*; 9: Blank control, and (**B**) Sensitivity of the *invA*-LAMP assay (8×10^9 to 8×10^0 copies). M: 2000 bp DNA marker, and BC: Blank control

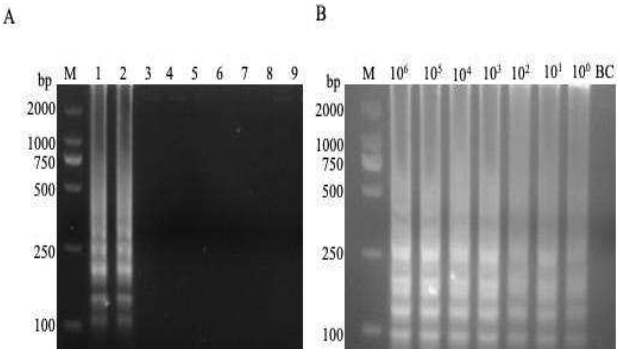

Fig. 3: Specificity and sensitivity of the IC-LAMP assay tested for artificially contaminated water captured by IMBs. (**A**) Specificity of the IC-LAMP assay. M: 2000 bp DNA marker; 1: *Salmonella* (isolated from monkey); 2: *Salmonella* (ATCC8739); 3: *Escherichia coli*; 4: *Staphylococcus aureus*; 5: *Klebsiella pneumoniae*; 6: *Acinetobacter baumannii*; 7: *Pseudomonas aeruginos*; 8: *Shigella*; 9: Blank control, and (**B**) Sensitivity of the IC-LAMP assay (5×10^6 to 5×10^0 CFU/ml). M: 2000 bp DNA marker, and BC: Blank control

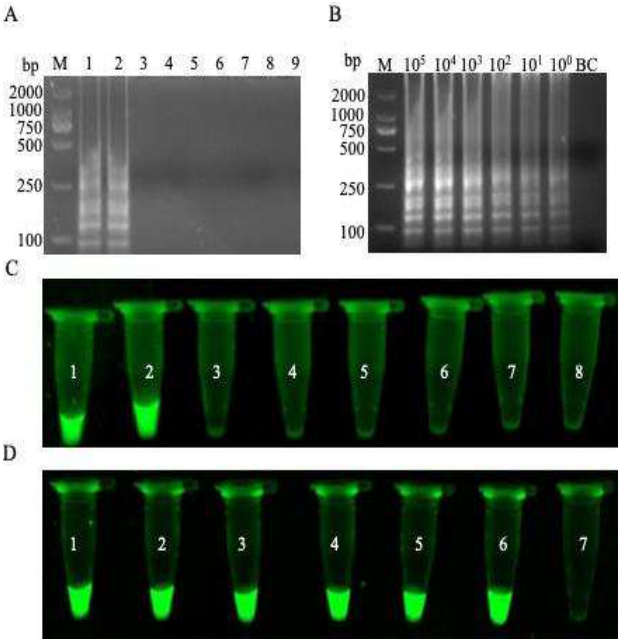

Fig. 4: Specificity and sensitivity of the IC-LAMP assay tested by artificial contamination of milk captured by IMBs. (**A**) Specificity of the IC-LAMP assay. M: 2000 bp DNA marker; 1: *Salmonella* (isolated from monkey); 2: *Salmonella* (ATCC8739); 3: *Escherichia coli*; 4: *Staphylococcus aureus*; 5: *Klebsiella pneumoniae*; 6: *Acinetobacter baumannii*; 7: *Pseudomonas aeruginos*; 8: *Shigella*; 9: Blank control, (**B**) Sensitivity of the IC-LAMP assay (5×10^5 to 5×10^0 CFU/ml). M: 2000 bp DNA marker, and BC: Blank control, (**C**) Specificity of IC-LAMP assay based on SYBR Green I detection. 1: *Salmonella* (isolated from monkey); 2: *Salmonella* (ATCC8739); 3: *Escherichia coli*; 4: *Staphylococcus aureus*; 5: *Klebsiella pneumoniae*; 6: *Acinetobacter baumannii*; 7: *Pseudomonas aeruginos*; 8: *Shigella*, and (**D**) Sensitivity of the IC-LAMP assay based on SYBR Green I detection. 1-6: 5×10^5 to 5×10^0 CFU/ml, and 7: Blank control

Discussion

Presenting a significant food safety risk, *Salmonella* is a common cause of food poisoning worldwide (Handley *et al*., 2015; Karp *et al*., 2018). Culture-based techniques and biochemical assays are the most commonly used detection assays, but these methods require a long enrichment time and subsequent identification, and so cannot be used for rapid detection (Elzein *et al*., 2013; Soria *et al*., 2013). Polymerase chain reactions technologies can be used to assess whether various food products and environment samples contain *Salmonella* (Law *et al*., 2014; Singh *et al*., 2017). However, complex instruments are required to denature the template DNA and to detect the PCR products, greatly limiting the wide application of these technologies in under-resourced settings and in field laboratories (Hapuarachchi *et al*., 2019). Recently, the use of LAMP was reported as a tool to detect *Salmonella* (Shehata and Lu, 2016). Although these methods can detect *Salmonella*, there are still many shortcomings for this approach, because these methods cannot enrich cells,

especially for some specialty samples.

In this study, we used *Salmonella* as the antigen to produce mAbs. Two mAbs were screened through three cycles of subcloning. The specificity of mAb 1B12 was evaluated by Western blot. The results of Western blot showed that mAb 1B12 bound *Salmonella* specifically and the other similar microbial strains including *Shigella*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, and *S. aureus* were not recognized. We used mAb 1B12 with high affinity to develop and validate an IC-LAMP assay for the detection of *Salmonella*. This approach was rapid, sensitive, visual, and specific for the detection of *Salmonella* in contaminated milk and water. The overall detection requires about 50 min, including the enrichment of *Salmonella*. Moreover, the specificity and sensitivity of IC-LAMP reactions were successfully demonstrated. The IC-LAMP assay combined the specificity of antibody and LAMP exhibits a detection limit of 5×10^0 CFU/ml for processed milk and water samples and 10^0 copies/μL for plasmid, which is higher than direct PCR (10^3 CFU/ml) (Chin *et al*., 2017), real-time PCR coupled with immunomagnetic separation or centrifugation (10^5 CFU/ml for 30 min) (Zheng *et al*., 2016), and LAMP (5 CFU of *Salmonella* pure culture or 200 CFU of artificially spiked faeces) (Zhuang *et al*., 2014). Besides, compared with culture-based techniques and biochemical assays, PCR, real-time PCR, and normal LAMP, the IC-LAMP assay developed here only needed a portable heating apparatus without the processes of the enrichment of cells followed by extraction of the genome or plasmid, any special equipment, or professional skills.

In conclusion, this work demonstrates a reliable method to detect *Salmonella* based on LAMP combined with magnetic immunocapture assay IC-LAMP. The procedure is simple, rapid, and can be used for on-site detection of *Salmonella*.

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Online Material

Refer to web version on PubMed Central® (PMC) for Supplementary Material.