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Yersinia pestis detection by loop-mediated isothermal amplification combined with magnetic bead capture of DNA



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ABSTRACT

We developed a loop-mediated isothermal amplification (LAMP) assay for the detection of *Y. pestis* by targeting the 3a sequence on chromosome. All 11 species of the genus *Yersinia* were used to evaluate the specificity of LAMP and PCR, demonstrating that the primers had a high level of specificity. The sensitivity of LAMP or PCR was 2.3 or 23 CFU for pure culture, whereas 2.3×10^4 or 2.3×10^6 CFU for simulated spleen and lung samples. For simulated liver samples, the sensitivity of LAMP was 2.3×10^6 CFU, but PCR was negative at the level of 2.3×10^7 CFU. After simulated spleen and lung samples were treated with magnetic beads, the sensitivity of LAMP or PCR was 2.3×10^3 or 2.3×10^6 CFU, whereas 2.3×10^5 or 2.3×10^7 CFU for magnetic bead-treated liver samples. These results indicated that some components in the tissues could inhibit LAMP and PCR, and liver tissue samples had a stronger inhibition to LAMP and PCR than spleen and lung tissue samples. LAMP has a higher sensitivity than PCR, and magnetic bead capture of DNAs could remarkably increase the sensitivity of LAMP. LAMP is a simple, rapid and sensitive assay suitable for application in the field or poverty areas.

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Introduction

Plague is a zoonotic disease caused by Gram-negative bacterium *Yersinia pestis*, which is occasionally transmitted to humans from *Y. pestis*-infected rodents via the bites of infected fleas.¹ *Y. pestis* is thought to have evolved from a serotype O: 1b strain of *Y. pseudotuberculosis* about 6000–10,000 years

ago although these two species cause remarkably different diseases.^{2–3} *Y. pestis* is a highly virulent and infectious pathogen, and was classified as a Category A pathogen by the U. S. Centers for Disease Control and Prevention.⁴ Historically, *Y. pestis* has given rise to three major plague pandemics, leading to millions of human deaths. Recently, the increased outbreak of plague around the world is reported annually to

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Table 1 – Sequence of the primers for LAMP and PCR reactions.

Primer names	Type	Sequence (5'–3')	Primer length (bp)
3a-F3	Forward outer primer	ACTACCATCCCCTCAAGGT	20
3a-B3	Backward outer primer	GAGGGCGTTTGGTAGAGAA	20
3a-FIP	Forward inner primer	CACCGCGTTATCTCATCCCG-TTTTCGAGTAGGGTTAGGTGGGC	44
3a-BIP	Backward inner primer	CATGGACGTATGGCGGGTCA-TTTGTGATGCCGTCCAATGCA	42
3a-LF	Forward loop primer	ACCGCCATGAAATGGACAATG	21

the World Health Organization (WHO), and plague was classified as a re-emerging infectious disease by the WHO.^{5–6} In addition, plague has been attracting a considerable attention because its causative agent has always been recognized as one of the classical biological warfare or bioterrorism agents.^{7–8} To minimize these threats, development of a rapid method for the detection of *Y. pestis* is essential.

Y. pestis was often detected by bacterial isolation and microscopy observation,⁹ phage lysis assay,^{10–11} ELISA assays based on the detection of F1 antigen and antibodies against *Y. pestis*,^{12–15} conventional PCR assays,^{16–18} real-time quantitative PCR assays,^{19–28} biosensors based on fiber-optic or upconverting phosphor technology,^{29–31} solid-phase radioimmunoassay based on radiolabeled monoclonal antibody for the detection of plague antigen.³² All these methods are playing an important role in the diagnosis of plague, but these methods either are time-consuming and laborious, or require expensive equipment and personnel with a high level of technical expertise. However, permanent surveillance of the foci of plague located in the poor regions to predict future epizootics in rodents and exposure risk for humans or investigation of samples suspected of bioterrorism on site requires a simple, rapid and efficient diagnostic method. A colloidal gold particles-based lateral-flow (LF) strip detection method for *Y. pestis* has been developed based on antibodies to F1 and LcrV proteins.^{33–34} This dipstick test is a low-cost, easy-to-use and rapid screening method in the surveillance of plague or investigation of samples suspected of bioterrorism on site, but nucleic acid-based rapid detection technology could be a more powerful alternative for detecting *Y. pestis*.

Loop-mediated isothermal amplification (LAMP) technology has received considerable attention because it allows efficiently amplification of DNA with high specificity and sensitivity under isothermal conditions of 60–65 °C. The LAMP reaction can be accomplished within less than 1 h based on a set of four to six primers and the Bst DNA polymerase with strand displacement activity.³⁵ The method is more suitable for field applications, especially in poverty areas, because it does not require specialized or expensive equipment, and only a simple and inexpensive water bath or heating block can satisfy LAMP assay.^{36–37} In addition, LAMP results can be read by the naked eye, or the lateral flow dipstick (LFD) under natural light, which makes the detection results easy to be judged in the field. Currently, the LAMP technique has been widely used in the diagnosis of infectious diseases,³⁸ and it is more sensitive in detecting bacteria compared to the conventional polymerase chain reaction (PCR) method.^{39–40} In this study, we construct a simple and rapid LAMP method for detection of *Y. pestis* based on the specific sequence 3a (GenBank accession no. AF350075) that is a specific fragment located on *Y. pestis* chromosome found by using a comparative genomic

method.⁴¹ The specificity of the method was evaluated by using all 11 species of the genus *Yersinia*. The sensitivity was evaluated by using *Y. pestis* pure culture, and simulated tissue samples, and magnetic bead-treated simulated tissue samples.

Materials and methods

Bacterial strains, reagents, instruments and animals

Y. pestis EV vaccine strain, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. ruckeri*, and *Y. aldovae* were used to evaluate the specificity of LAMP and PCR; 10× thermopol buffer, MgSO₄ (100 mM), and Bst DNA Polymerase, Large Fragment (8000 U/ml) were purchased from NEB (Beijing, China); Calcein and real-time turbidity meter LA-320c were purchased from Eiken China CO., LTD.; Taq DNA polymerase (5 U) and dNTPs (2.5 mM) were purchased from TaKaRa (Dalian, China); SM3-P100, amino-modified silica-coated magnetic beads, was purchased from Shanghai Allrun Nano Science & Technology CO., LTD. BALB/c were obtained from Laboratory Animal Research Center, Academy of Military Medical Science, China (licensed from the Ministry of Health in General Logistics Department of Chinese People's Liberation Army, Permit No. SCXK-2007-004). The protocols were approved by Committee of the Welfare and Ethics of Laboratory Animals, Beijing Institute of Microbiology and Epidemiology. The experiments were conducted strictly in compliance with the Regulations of Good Laboratory Practice for nonclinical laboratory studies of drug issued by the National Scientific and Technologic Committee of People's Republic of China.

LAMP and PCR assays

For LAMP reaction, a set of five primers was designed according to the published sequence of 3a in *Y. pestis* KIM D46 (GenBank accession no.: AF350075) using Primer Explorer version 4. A forward inner primer (FIP), a backward inner primer (BIP), two outer primers (F3 and B3), and a loop forward primer (LF) were used for LAMP amplification. The sequences of the primers are shown in Table 1. LAMP reaction was performed in a total volume of 25 µL mixture containing 10× Thermopol buffer (2.5 µL), 100 mM MgSO₄ (1.5 µL), 2.5 mM dNTP Mix (14 µL), 100 mM 3a-FIP (0.4 µL), 100 mM 3a-BIP (0.4 µL), 10 mM 3a-F3 (0.5 µL), 10 mM 3a-B3 (0.5 µL), 10 mM 3a-LF (0.2 µL), 8000 U/mL of Bst DNA Polymerase, Large Fragment (1 µL), water (3 µL), template DNA (1 µL). The LAMP reaction was carried out at 65 °C for 60 min and inactivated at 80 °C for 5 min in water bath. For direct visual detection of DNA

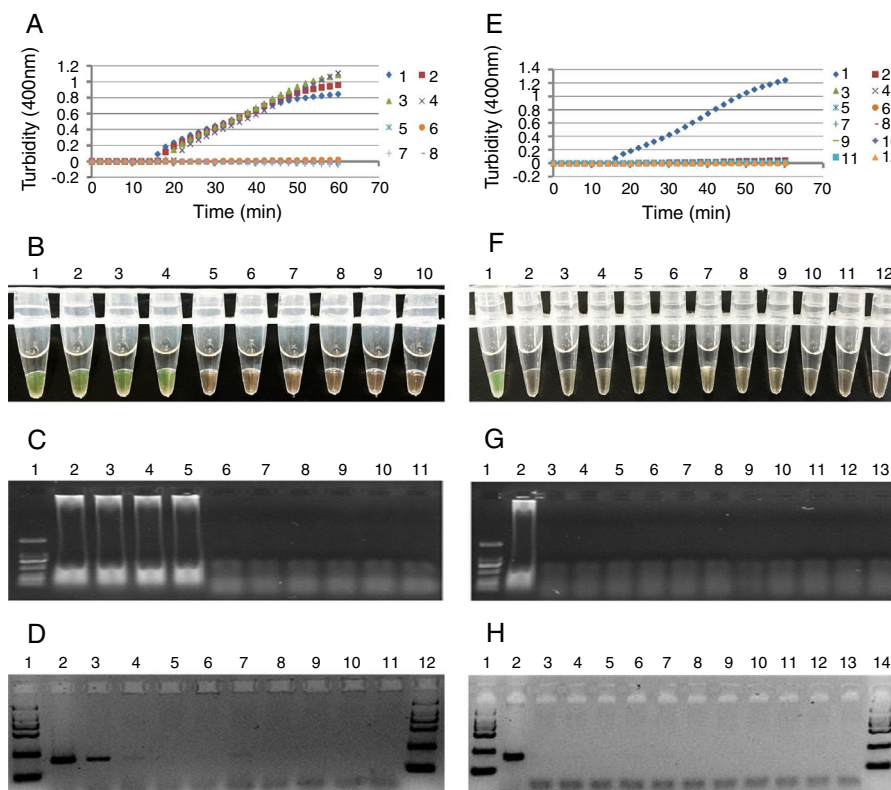


Fig. 1 – The sensitivity (A–D) of the LAMP or PCR for the detection of *Y. pestis* using serial dilutions of extracted DNA, and the specificity (E–H) of the LAMP or PCR for the detection of *Y. pestis* using 20 ng DNA from each bacterium of the genus *Yersinia*. (A) Detection of LAMP products with a real-time turbidity meter. 1–8: 20 ng–0.002 pg; 10: negative control. (B) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–9: 20 ng–0.0002 pg; 10: negative control. (C) Detection of LAMP products with agarose gel electrophoresis. Lane 1: marker; 2–10: 20 ng–0.0002 pg; 11: negative control. (D) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; 2–10: 20 ng–0.0002 pg; 11: negative control; 12: marker. (E) Detection of the LAMP products with a real-time turbidity meter. 1: *Y. pestis*; 2: *Y. pseudotuberculosis*; 3: *Y. enterocolitica*; 4: *Y. frederiksenii*; 5: *Y. intermedia*; 6: *Y. kristensenii*; 7: *Y. bercovieri*; 8: *Y. mollaretii*; 9: *Y. rohdei*; 10: *Y. ruckeri*; 11: *Y. aldovae*; 12: negative control. (F) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1: *Y. pestis*; 2: *Y. pseudotuberculosis*; 3: *Y. enterocolitica*; 4: *Y. frederiksenii*; 5: *Y. intermedia*; 6: *Y. kristensenii*; 7: *Y. bercovieri*; 8: *Y. mollaretii*; 9: *Y. rohdei*; 10: *Y. ruckeri*; 11: *Y. aldovae*; 12: negative control. (G) Detection of LAMP products with agarose gel electrophoresis. Lane 1: marker; 2: *Y. pestis*; 3: *Y. pseudotuberculosis*; 4: *Y. enterocolitica*; 5: *Y. frederiksenii*; 6: *Y. intermedia*; 7: *Y. kristensenii*; 8: *Y. bercovieri*; 9: *Y. mollaretii*; 10: *Y. rohdei*; 11: *Y. ruckeri*; 12: *Y. aldovae*; 13: negative control. (H) Electrophoretic analysis of PCR products with 1.5% agarose gel. Lane 1: marker; 2: *Y. pestis*; 3: *Y. pseudotuberculosis*; 4: *Y. enterocolitica*; 5: *Y. frederiksenii*; 6: *Y. intermedia*; 7: *Y. kristensenii*; 8: *Y. bercovieri*; 9: *Y. mollaretii*; 10: *Y. rohdei*; 11: *Y. ruckeri*; 12: *Y. aldovae*; 13: negative control; 14: marker.

amplification, 1 μ L of Fluorescent Detection Reagent containing calcein (EIKEN CHINA CO., LTD.) was added to the reaction tube before the LAMP reaction. For a positive reaction, a color change from orange to green was observed through the naked eye under natural light, whereas a negative reaction mixture remained orange. Alternatively, specific DNA amplification was monitored spectrophotometrically by a real-time turbidity meter. To confirm that color change in reaction tubes is a result of DNA amplification, LAMP product was also detected by 0.8% agarose gel electrophoresis and ethidium bromide staining.

Conventional PCR reaction was performed in a total volume of 25 μ L mixture containing 10 $\times$ buffer (3 μ L), 2.5 mM dNTPs (0.6 μ L), 10 mM 3a-F3 (0.5 μ L), 10 mM 3a-B3 (0.5 μ L), 5 U

of Taq DNA polymerase (0.1 μ L), deionized water (15.3 μ L) and template DNA (5 μ L). F3 and B3 primers were used for the conventional PCR amplification (Table 1). The reaction was subjected to an initial denaturation at 95 $^{\circ}$ C for 5 min, followed by 35 cycles of denaturation at 95 $^{\circ}$ C for 30 s, annealing at 56 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 1 min and a final extension at 72 $^{\circ}$ C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Extraction of bacterial genomic DNA

For preparation of high-quality genomic DNA, *Y. pestis* EV vaccine strain and other bacteria were cultured in Luria broth

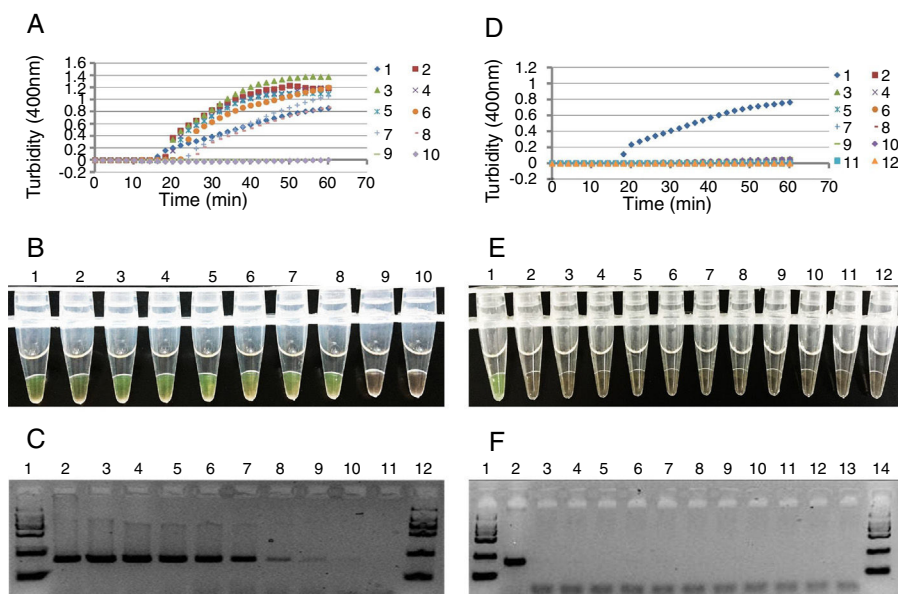


Fig. 2 – The sensitivity (A–C) of the LAMP and PCR for the detection of *Y. pestis* by using the DNA extracted crudely from serial dilutions of bacterial solutions, and the specificity (D–F) of the LAMP and PCR for the detection of *Y. pestis* using crudely extracted DNA from each bacterium (2.3×10^7 CFU) of the genus *Yersinia*. (A) Detection of the LAMP products with a real-time turbidity meter. 1–9: 2.3×10^7 – 2.3×10^{-1} CFU of *Y. pestis*; 10: negative control. (B) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–9: 2.3×10^7 – 2.3×10^{-1} CFU of *Y. pestis*; 10: negative control. (C) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–10: 2.3×10^7 – 2.3×10^{-1} CFU of *Y. pestis*; 11: negative control; 12: marker. (D) Detection of LAMP products with a real-time turbidity meter. 1: *Y. pestis*; 2: *Y. pseudotuberculosis*; 3: *Y. enterocolitica*; 4: *Y. frederiksenii*; 5: *Y. intermedia*; 6: *Y. kristensenii*; 7: *Y. bercovieri*; 8: *Y. mollaretii*; 9: *Y. rohdei*; 10: *Y. ruckeri*; 11: *Y. aldovae*; 12: negative control. (E) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1: *Y. pestis*; 2: *Y. pseudotuberculosis*; 3: *Y. enterocolitica*; 4: *Y. frederiksenii*; 5: *Y. intermedia*; 6: *Y. kristensenii*; 7: *Y. bercovieri*; 8: *Y. mollaretii*; 9: *Y. rohdei*; 10: *Y. ruckeri*; 11: *Y. aldovae*; 12: negative control. (F) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; 2: *Y. pestis*; 3: *Y. pseudotuberculosis*; 4: *Y. enterocolitica*; 5: *Y. frederiksenii*; 6: *Y. intermedia*; 7: *Y. kristensenii*; 8: *Y. bercovieri*; 9: *Y. mollaretii*; 10: *Y. rohdei*; 11: *Y. ruckeri*; 12: *Y. aldovae*; 13: negative control; 14: marker.

(LB) at 26 °C and 37 °C, respectively, and then collected by centrifugation. The collected bacterial pellet was resuspended in SE buffer (0.15 M NaCl, 0.1 M EDTA-2Na-2H₂O, adjusted to pH 8.0 by 10 M HCl), added to a final concentration of 2% SDS, and then placed in a water bath at 60 °C for 10 min. Then, an equal volume of phenol, chloroform and isoamyl alcohol mixture (25:4:1) was added and the solution was mixed by slowly inverting or rotating to denature proteins, then to remove proteins. The aqueous phase was added a final concentration of 50–100 µg/ml of RNase to degrade RNAs at 37 °C for 30–60 min. Then, an equal volume of chloroform was added, shaking vigorously. The aqueous phase was precipitated with two times volume of ethanol, and then the DNA was spooled out with a glass rod, rinsed with 70% ethanol, and dried in air. Finally, the DNA was dissolved into TE buffer (10 mM Tris-HCl, 1 mM EDTA-2Na-2H₂O, adjusted to pH 8.0 by 10 M HCl) for use.

For simple and rapid isolation of bacterial DNA, the bacterial cells were resuspended in deionized water, and then genomic DNA was released by boiling the bacterial suspension at 100 °C for 10 min. The supernatant was collected and used as the DNA template in both LAMP and conventional PCR reactions.

Capture of bacterial genomic DNA by magnetic beads

Bacterial genomic DNA was released by boiling bacterial suspension at 100 °C for 10 min, 300 µL of the supernatant was mixed with the same volume of binding buffer [4 M NaCl and 20% polyethylene glycol (PEG) 8000], followed by addition of 0.2 mg magnetic nanoparticles SM3-P100. The suspension was vibrated vigorously for 15 s, followed by standing at room temperature for 5 min. The magnetic beads were collected by using an external magnet, and the supernatant was discarded. The magnetic pellet was washed two times with 200 µL of 70% ethanol, and then dried completely at room temperature. Finally, the magnetic pellet was resuspended in 100 µL of deionized water to elute bound DNA at room temperature for 5 min with continuous agitation. The supernatant containing the genomic DNA was used as the DNA template in both LAMP and conventional PCR assays.

Preparation of simulated samples and magnetic bead capture of DNA

The spleen, liver and lungs were collected aseptically from pathogen-free BALB/c mice, and then blended with the

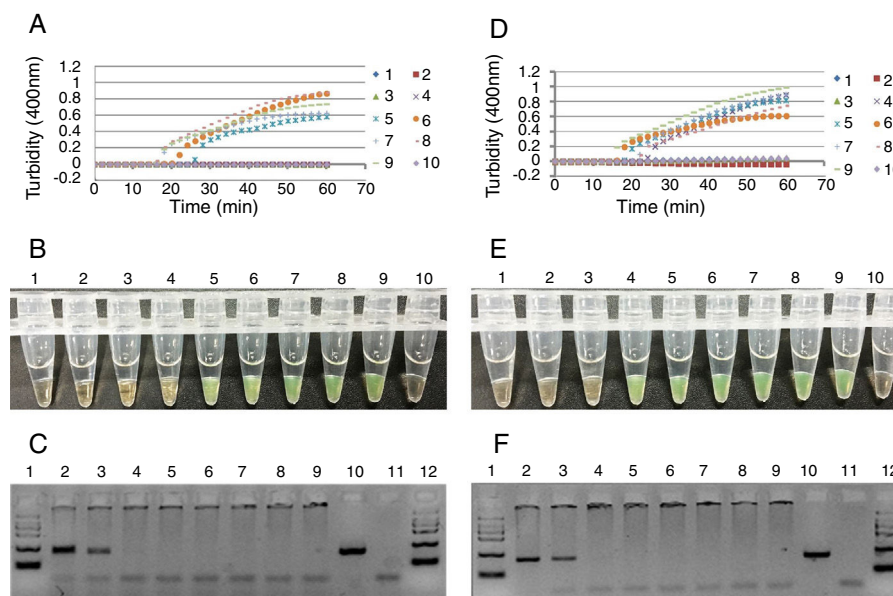


Fig. 3 – Detection of *Y. pestis* in simulated spleen samples by LAMP and PCR. The sensitivity of LAMP or PCR was determined by using the DNA extracted by boiling simulated spleen sample for 10 min (A–C) or by using the DNA captured by magnetic beads (D–F). (A) Detection of LAMP products with a real-time turbidity meter. 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (B) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (C) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–9: 2.3×10^7 – 2.3×10^0 CFU of *Y. pestis*; 10: positive control; 11: negative control; 12: marker. (D) Detection of LAMP products with a real-time turbidity meter. 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (E) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (F) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–9: 2.3×10^7 – 2.3×10^0 CFU of *Y. pestis*; 10: positive control (20 ng DNA); 11: negative control; 12: marker.

appropriate volume of PBS buffer to prepare tissue homogenates. *Y. pestis* was cultured in LB, collected by centrifugation, and then resuspended in deionized water to prepare serial 10-fold dilutions of bacterial suspension. The spleen, liver and lung tissue homogenates were respectively inoculated with the same volume of serial 10-fold dilutions of bacterial suspension to obtain different concentrations of artificially contaminated samples (2.3×10^7 – 2.3×10^{-1} CFU). Noninoculated tissue homogenates were mixed with the same volume of deionized water to be used as a negative control. Artificially contaminated samples for each tissue were boiled at 100 °C for 10 min, and then centrifugated at $10,000 \times g$ for 2 min. The supernatants can be directly used as the DNA template in both LAMP and conventional PCR assays. The supernatants can be further used to isolate bacterial DNA by magnetic beads according to the protocol described above, and then used in both LAMP and conventional PCR assays.

Determination of LAMP or PCR sensitivity

The sensitivity of LAMP or PCR for the detection of *Y. pestis* was evaluated with the high-quality genomic DNA. Serial 10-fold dilutions of DNA were made in sterile deionized water, and used as the DNA templates to perform LAMP and PCR assays.

The sensitivity of LAMP or PCR was evaluated with *Y. pestis* pure culture. Serial 10-fold dilutions of *Y. pestis* cells were made in sterile deionized water, boiled at 100 °C for 10 min, and the supernatants were used for LAMP and conventional PCR reactions. The sensitivity of LAMP or PCR was evaluated with the simulated samples prepared by using mouse spleen, liver and lungs. Serial 10-fold dilutions of *Y. pestis* cells were respectively spiked into the same volume of tissue homogenates, and boiled at 100 °C for 10 min. The supernatants were directly used for LAMP and PCR assays. In addition, the bacterial DNA was captured from the supernatants by magnetic beads, and then used to determine the sensitivity of LAMP and PCR assays.

Evaluation of specificity of LAMP or PCR

The specificity of LAMP or PCR for the detection of *Y. pestis* was evaluated by using the high-quality bacterial DNAs (20 ng) from 11 species of bacteria in the genus *Yersinia*. The specificity of the LAMP or PCR was also evaluated with pure cultures from 11 species of bacteria in the genus *Yersinia*. Each bacterium was diluted to 10^7 CFU in sterile deionized water, boiled at 100 °C for 10 min, and the supernatants were used for LAMP and PCR reactions.

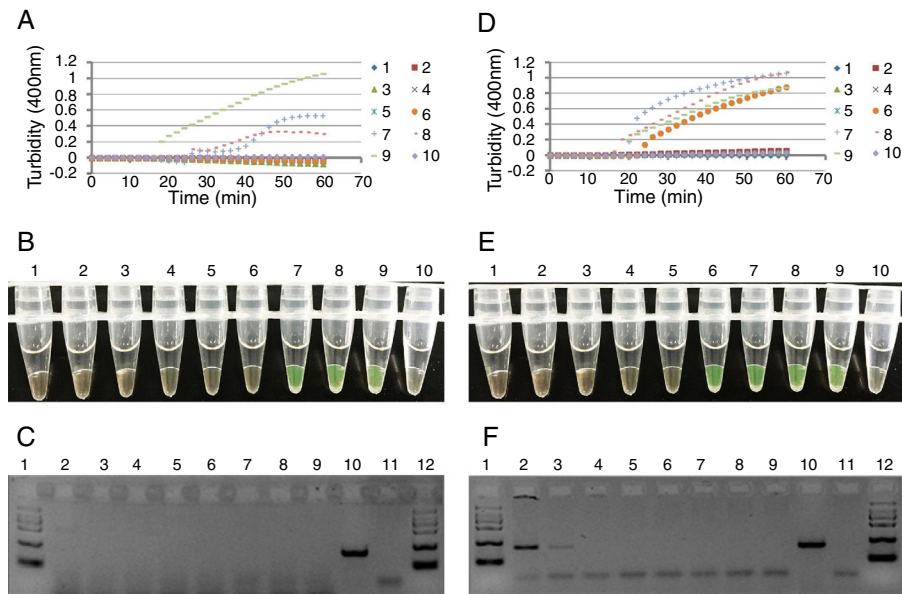


Fig. 4 – Detection of *Y. pestis* in simulated liver samples by LAMP and PCR. The sensitivity of LAMP or PCR was determined by using the DNA extracted by boiling simulated liver sample for 10 min (A–C) or by using the DNA captured by magnetic beads (D–F). (A) Detection of LAMP products with a real-time turbidity meter. 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (B) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (C) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–9: 2.3×10^7 – 2.3×10^0 CFU of *Y. pestis*; 10: positive control (20 ng DNA); 11: negative control; 12: marker. (D) Detection of LAMP products with a real-time turbidity meter. 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (E) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (F) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–9: 2.3×10^7 – 2.3×10^0 CFU of *Y. pestis*; 10: positive control (20 ng DNA); 11: negative control; 12: marker.

Results

The sensitivity of detection of *Y. pestis* by LAMP and PCR assays

To determine the sensitivity of LAMP or PCR assay, serial 10-fold dilutions of high-purity *Y. pestis* DNAs were tested. The results showed that the lowest detection limit for LAMP was 0.02 ng of *Y. pestis* DNA when the product was detected by the naked eye, a real-time turbidity meter or agarose gel electrophoresis and ethidium bromide staining, whereas the sensitivity for PCR was 0.2 ng of *Y. pestis* DNA (Fig. 1A–D). These results indicated that LAMP had a higher sensitivity than PCR for the detection of *Y. pestis* DNA, and two LAMP detection methods had the same sensitivity. When *Y. pestis* was determined by using serial 10-fold dilutions of bacterial solution, the sensitivity for LAMP was 2.3 CFU of *Y. pestis* when the product was detected by a real-time turbidity meter, the naked eye under natural light or agarose gel electrophoresis, whereas the sensitivity for PCR was 23 CFU of *Y. pestis* (Fig. 2A–D). Three LAMP detection methods also had the same sensitivity, and had a higher sensitivity than PCR.

The specificity of detection of *Y. pestis* by LAMP and PCR assays

To determine the specificity for the detection of *Y. pestis* by LAMP or PCR assay, 11 species of the genus *Yersinia* were tested.

When the high-purity DNAs extracted from 11 species of the genus *Yersinia* were used for the detection of *Y. pestis*, respectively, the LAMP product was only detected in the reaction tube of *Y. pestis*, and no LAMP amplicons appeared in the reaction tubes of other *Yersinia* strains. Similarly, when the high-purity DNAs extracted from 11 species of the genus *Yersinia* were respectively used to detect *Y. pestis* by PCR, specific band was only observed in *Y. pestis* EV vaccine strain (Fig. 1D–F). When *Y. pestis* was determined by using 10^7 CFU of pure culture from 11 species of the genus *Yersinia*, LAMP product was only detected in the reaction tube of *Y. pestis*, and no LAMP amplicons appeared in the reaction tubes of other *Yersinia* strains. Similarly, when 11 species of the genus *Yersinia* were used to detect *Y. pestis* by PCR, specific band was only observed in *Y. pestis* EV vaccine strain (Fig. 2D–F).

Detection of *Y. pestis* in simulated samples by LAMP and PCR assays

To determine the sensitivity for the detection of *Y. pestis* in simulated spleen, liver and lung samples by LAMP or PCR. Artificially contaminated spleen, liver and lung samples containing different concentrations of *Y. pestis* were prepared by mixing spleen, liver and lung tissue homogenates with the same volume of serial 10-fold dilutions of *Y. pestis* suspension. Artificially contaminated spleen, liver and lung samples were boiled for 10 min, and then the supernatants were directly

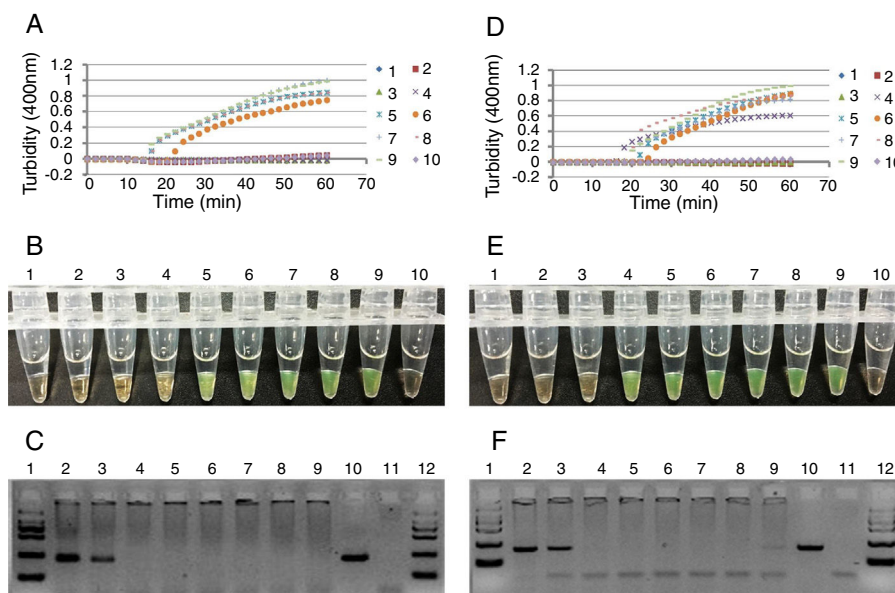


Fig. 5 – Detection of *Y. pestis* in simulated lung samples by LAMP and PCR. The sensitivity of LAMP or PCR was determined by using the DNA extracted by boiling simulated lung samples for 10 min (A–C) or by using the DNA captured by magnetic beads (D–F). (A) Detection of LAMP products with a real-time turbidity meter. 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (B) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (C) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–9: 2.3×10^7 – 2.3×10^0 CFU of *Y. pestis*; 10: positive control (20 ng DNA); 11: negative control; 12: marker. (D) Detection of LAMP products with a real-time turbidity meter. 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (E) Visualization of LAMP products stained with calcein and inspected under natural light. Tube 1–8: 2.3×10^0 – 2.3×10^7 CFU of *Y. pestis*; 9: positive control; 10: negative control. (F) Electrophoretic analysis of PCR products with agarose gel. Lane 1: marker; Lane 2–9: 2.3×10^7 – 2.3×10^0 CFU of *Y. pestis*; 10: positive control (20 ng DNA); 11: negative control; 12: marker.

determined by LAMP and PCR assays, respectively. The sensitivity for the artificially contaminated spleen by LAMP or PCR assay was 2.3×10^4 or 2.3×10^6 CFU of *Y. pestis*, respectively. The LAMP assay was 100-fold more sensitive than PCR assay (Fig. 3A–C). The lowest detection limit for the artificially contaminated liver samples by LAMP was 2.3×10^6 CFU of *Y. pestis*, but PCR assay didn't detect 2.3×10^7 CFU of *Y. pestis* in the simulated liver samples (Fig. 4A–C). The lowest detection limit for the artificially contaminated lung samples by LAMP and PCR assay was 2.3×10^4 and 2.3×10^6 CFU of *Y. pestis*, respectively. The LAMP assay was 100-fold more sensitive than PCR assay (Fig. 5A–C). To increase the sensitivity for the detection of *Y. pestis* in the simulated samples, the supernatants obtained by boiling the artificially contaminated spleen, liver and lung samples were respectively mixed with the magnetic beads to capture the DNAs for LAMP and PCR assays. After the DNAs in the simulated spleen samples were captured by magnetic beads, the sensitivity for LAMP or PCR assay was 10^3 or 10^6 CFU of *Y. pestis*, respectively. LAMP assay was 1000-fold more sensitive than PCR assay (Fig. 3E and F). The lowest detection limit for the artificially contaminated liver by LAMP or PCR was 2.3×10^5 or 2.3×10^7 CFU of *Y. pestis*. LAMP assay was 100-fold more sensitive than PCR assay (Fig. 4E and F). The sensitivity for the artificially contaminated lungs by LAMP or PCR assay was 10^3 or 10^6 CFU of *Y. pestis*. LAMP assay was 1000-fold more sensitive than PCR assay (Fig. 5E and F).

Discussion

Y. pestis strains usually contain three virulence plasmids pCD1, pMT1 and pPCP1, of which pCD1 was inherited from its ancestor *Y. pseudotuberculosis*. The plasmid pCD1 mainly encodes type three secretion system (T3SS), which plays an essential role in the pathogenesis of *Y. pestis*.^{42–43} The newly acquired pPCP1 encodes a surface proteinase, plasminogen activator (Pla), that is responsible for the increase of the pathogen's invasive ability in bubonic plague and the replication of *Y. pestis* rapidly in the lungs.^{44–45} Another plasmid pMT1 encodes F1 antigen, one of the major protective antigens in *Y. pestis*, and the murine toxin (Ymt) required for survival of *Y. pestis* in the midgut of flea.⁴⁶ Immunological diagnosis of *Y. pestis* was mainly based on F1 antigen or its antibodies, but this method could not detect the virulent *Y. pestis* strains lacking F1 antigen.⁴⁷ In addition, our recent studies found some natural isolates of *Y. pestis* with one or two plasmids. By contrast, the target genes on chromosome might be more stable. In this study, we constructed a LAMP method to detect *Y. pestis* based on the 3a sequence on chromosome, which might recognize a greater amount of samples than previous studies. Currently, there have been two reports that described the detection of *Y. pestis* by LAMP.^{48–49} Compared with these two reports, our method had a similar sensitivity with Nune's method for the

detection of *Y. pestis* DNA, but showed a higher sensitivity than Dong's method for the detection of *Y. pestis* pure culture. All these three methods had a higher specificity. However, the two previous reports are based on the target of F1 antigen gene encoded on the pMT1 for the detection of *Y. pestis* pure culture and high-quality genomic DNA. Evidently, these two methods cannot detect *Y. pestis* strains lacking F1 antigen on the plasmid pMT1, and a LAMP method is also needed to detect *Y. pestis* in actual samples. Based on the LAMP method we constructed in this study, we determined simulated spleen, liver and lung tissue samples combined with magnetic bead capture of *Y. pestis* DNA.

The genus *Yersinia* contains 11 species, of which *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Y. pestis* are pathogenic for humans and animals, whereas other eight species, formerly called *Y. enterocolitica*-like isolates, *Yersinia frederiksenii*, *Yersinia intermedia*, *Yersinia kristensenii*, *Yersinia bercovieri*, *Yersinia mollaretii*, *Yersinia rohdei*, *Yersinia ruckeri*, and *Yersinia aldovae* are opportunistic pathogens mostly found in the environment.¹ To evaluate the specificity of the primers based on the 3a sequence for the detection of *Y. pestis*, all 11 species of the genus *Yersinia*, closely related organisms, were tested in the experiments. The results showed that the primers designed in this study had a high level of specificity for *Y. pestis* by LAMP or PCR. However, in our recent study, 3a-negative natural isolates of *Y. pestis* were found in Dingbian County, Shaanxi Province, China,⁵⁰ indicating that a 3a-based LAMP or PCR method is also not reliable for the detection of *Y. pestis*. Therefore, a multi-target LAMP or PCR detection method should be developed for the reliable identification of *Y. pestis* in the future.

To determine the sensitivity for the detection of *Y. pestis*, we first use serial 10-fold dilutions of high-quality DNA to perform LAMP or PCR assay. The results showed that the lowest detection limit of LAMP or PCR assay was 0.02 or 0.2 ng of *Y. pestis* DNA, indicating that LAMP assay was 10-fold more sensitive than conventional PCR assay. Then, serial 10-fold dilutions of *Y. pestis* pure culture were used to crudely extract DNA by boiling, and then detected by LAMP and PCR. The results showed that the sensitivity of LAMP or PCR assay was 2.3 or 23 CFU of *Y. pestis*, indicating that LAMP assay was 10-fold more sensitive than PCR assay. In addition, the results also showed that the detection of LAMP products by the naked eye was as sensitive as that by a real-time turbidity meter. LAMP amplification can be accomplished within 60 min at an isothermal temperature of 65 °C. Therefore, LAMP assay is a simple, rapid and sensitive method suitable for application in the field, especially in poverty areas.

To evaluate the reliability for the determination of *Y. pestis* by LAMP and PCR assays in actual samples, we determined *Y. pestis* in the simulated spleen, liver and lung samples. The results showed that the sensitivity of three simulated samples were much lower than that of *Y. pestis* pure culture and DNA, indicating that some components in the spleen, liver and lung tissues might inhibit LAMP and PCR reactions. This result was similar to the previous studies in which clinical samples, such as serum, plasma and urine, had an inhibition to both LAMP and PCR assay.^{51–52} In addition, our results also showed that the simulated liver sample had a lower sensitivity than the simulated spleen and lung samples for the detection of *Y.*

pestis by LAMP and PCR. This result indicated that there might be more bioactive components in liver tissue that could inhibit LAMP and PCR reactions. Compared with PCR for the detection of three simulated samples, LAMP assay was 100-fold more sensitive than PCR assay, indicating that LAMP was less affected by inhibitory substances present in mouse spleen, liver and lung tissue samples than PCR. LAMP is not only more simple, rapid and sensitive than PCR, but also more suitable to detect spleen, liver and lung tissue samples than PCR.

To further enhance the sensitivity for the detection of actual samples, magnetic beads were used to capture *Y. pestis* DNAs from three simulated samples. After the DNAs were captured from the simulated spleen and lung samples with magnetic beads, the sensitivity of these two samples were increased for the detection of *Y. pestis* by LAMP, but the sensitivity of these two samples were unchanged for the detection of *Y. pestis* by PCR, and LAMP was 1000-fold more sensitive than PCR. After the DNAs were captured from simulated liver samples with magnetic beads, the sensitivity of LAMP and PCR were equally increased, and the sensitivity of LAMP was 100-fold more sensitive than that of PCR. These results indicated that magnetic bead capture of DNAs could remarkably increase the sensitivity of LAMP for the detection of *Y. pestis* in spleen, liver and lung tissue samples.

Conflicts of interest

The authors declare that they have no competing interests.

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