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Rapid visual detection of *Pasteurella multocida* through recombinase polymerase amplification combined with lateral flow dipsticks*

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Summary

***Pasteurella multocida* (Pm) is a zoonotic pathogen that can cause severe diseases in humans. It is easily confused with swine fever and swine erysipelas in clinical diagnosis because of mixed infections. The aim was to develop a highly sensitive and specific clinical rapid method for the detection of Pm in swine. A specific recombinant enzyme polymerase amplification (RPA) primer was designed according to the *kmt1* gene sequence of Pm. By optimizing the reaction temperature and time of RPA, a Pm detection method based on RPA-LFD was developed. The sensitivity, specificity, and clinical application of the method were evaluated. The results showed that the rapid RPA-LFD for the detection of Pm in swine could be completed within 40 min at 39°C, with the lowest detection limit of 1×10^{-6} copies · μL^{-1} and no cross-reactivity with enteropathogenic *Escherichia coli*, *Staphylococcus aureus*, *Glaesserella parvoviridis*, *Aeromonas hydrophilus*, *Listeria monocytogenes*, *Actinobacillus pleuopneumoniae*, or *Streptocuccus suis*. The detection rate of the RPA-LFD method for 50 clinical samples was higher than that of the conventional PCR method and bacterial isolation. The RPA-LFD assay for Pm in swine developed in this study has the advantages of high specificity, high sensitivity, rapid detection, and ease of operation, which provides a new technical means for the field detection of Pm.**

Keywords: *Pasteurella multocida*, recombinase polymerase amplification, lateral flow test strips, rapid detection

Pasteurella multocida (Pm) is a Gram-negative, immobile, non-spore-forming coccobacillus that can cause hemorrhagic septicemia or contagious pneumonia in various livestock and poultry (14, 16). It is, moreover, a pathogen of zoonotic infectious diseases that can cause human bacteremia and seriously threaten human health (22). Pm can be classified into five serotypes, A, B, D, E, and F, according to specific capsular antigens, and 12 serotypes according to somatic antigens. Different serotypes have different host characteristics and pathogenic features (3). The serotypes prevalent in swine are mainly type A and D, which can cause progressive atrophic rhinitis, hemorrhagic septicemia, and porcine pneumonia (1, 15). Pm is widely prevalent in most countries and regions with developed pig industry. Pm infection can cause respiratory disorders in pigs, reduce feed reward, affect production performance, and even lead to deaths, causing enormous economic losses to pig industry (7). Rapid and accurate detection is important for the prevention and control of various diseases caused by Pm.

The traditional method for identifying Pm are still biochemical tests, but they are time-consuming, laborious, and have a low detection rate. With the development of molecular biology, more methods have been used to detect Pm. PCR and qPCR can be used

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to target specific genes of Pm, such as *kmt1*, *tox*A, *psl*, and *Fur* (4, 9, 17, 19, 24). Also, generalized 16SrRNA sequences can be used to identify Pm. These methods are effective in detecting pathogens, but they all require complex thermal cycling devices, and the reaction time is too long to meet the needs of field detection. In order to rapidly detect Pm in the field, loop-mediated isothermal amplification (LAMP) has been developed. LAMP can rapidly amplify DNA under isothermal conditions and is commonly used to detect animal pathogens. However, false positive results caused by contamination, primer sequence similarity, and primer dimerization in the amplification process limit its application (5).

Recombinase polymerase amplification (RPA) is a simple, rapid technology that can be used to accurately detect pathogens in samples on site (2). This technique uses a special DNA polymerase that can perform nucleic acid amplification at room temperature, eliminate the multiple reaction steps of traditional PCR, and has the characteristics of good specificity and high sensitivity. Thus, the reaction speed and efficiency are improved. The RPA reaction system consists of three enzymes: recombinant enzyme, DNA polymerase, and single-chain binding protein (SSB). The recombinant enzyme forms a nucleoprotein microfilament complex with the primer and probe. After the primer acts on the homologous site, the complex searches for the target gene fragment and causes the primer to undergo a chain exchange reaction with the template. The target gene fragment is then amplified by DNA polymerase, while SSBs bind to the replaced single-stranded DNA, preventing it from reverting back to the double-stranded structure again, and the process is repeated until the target gene is amplified exponentially (8, 13). RPA can amplify the target DNA within 40 minutes at 37~42°C (11). The combination of RPA and LFD yields an RPA-LFD detection method whose results can be seen by the naked eye without the need for any equipment and is suitable for field inspection. RPA-LFD has been successfully tested for a variety of pathogens, including *Brucella* and *Staphylococcus aureus* (6).

In this study, RPA was combined with LFD, and the species-specific *kmt1* gene of Pm was used as the target to develop a rapid detection method for Pm characterized by high sensitivity and specificity as well as simplicity of operation, which would provide new technological support for clinical diagnosis and molecular epidemiological investigation of Pm.

Material and methods

Strains and reagents. All types of *Streptococcus suis* (SS, isolated strain), *Pasteurella multocida* (C44-1), *Actinobacillus pleuopneumoniae* (APP, CVCC259), enteropathogenic *Escherichia coli* (*E. coli*, isolated strain), *Glaesserella parvovirus* (GPS, isolated strain), *Listeria monocytogenes* (*L. monocytogenes*, isolated strain), *Staphylococcus aureus* (*S. aureus*, ATCC49525), and *Aeromonas hydrophila* (*A. hydrophila*, AH-1) are preserved at the Laboratory of Preventive Veterinary Medicine of the Henan Institute of Science and Technology. The bacterial genome DNA extraction kit was purchased from Ebixon (Shanghai) Biotechnology Co., Ltd.; the TwistAmp™ Basic and nfo kit was purchased from TwistDx (UK) Co.; the PCR Master Mix kit was purchased from Sangong Bioengineering (Shanghai) Co., Ltd.; Flow Chromatography Strips were purchased from Hangzhou Yousida Biotechnology Co.; THB and TSB culture media were purchased from Beijing Solebaum Technology Co.

Bacterial culture and genomic DNA extraction. The strains were streaked and cultured according to their respective solid growth media, and single colonies were selected in liquid medium and cultured overnight in a shaking bed at 37°C. The genomic DNA of SS, APP, GPS, Pm, *E. coli*, *L. monocytogenes*, *S. aureus*, and *A. hydrophila* was extracted from the logarithmic growth period using the Ezup Column Bacterial Genomic DNA Rapid Extraction Kit and preserved at –80°C.

Design and synthesis of primers. The Pm *kmt1* gene sequence was downloaded from GenBank and analyzed by multiplex comparison with the DNAStar software (DNASTAR, Madison, Wisconsin, USA) to search for specific fragments in the sequence. Primers were designed for synthesis using the Primer 5.0 software according to the TwistAmp™ DNA Amplification Kit Analysis Design Manual (Tab. 1). Based on the 3 pairs of primers designed, RPA amplification was performed using the Pm genome as a DNA template, and the reaction products of RPA were subjected to agarose gel electrophoresis to select the optimal primers. In order to visualize the reaction results on lateral flow chromatography test strips, the 5' ends of the primers were labeled with biotin. Two TwistAmp™ nfo probes were designed according to the instructions of the TwistAmp™ Basic kit (TwistDX, UK). The 5' end of the probe was labeled with carboxylin (FAM) and the 3' end was modified with C3-Spacer. Then, the THF (tetrahydrofuran) site was placed 30 bp from the 5' end of the probe and 1 nucleotide was replaced. At least 15 nucleotides were added to the 3' end of the THF residue (Tab. 1).

Tab. 1. Primer and probe sequences used in the experiments

Primer/probe	Sequence (5'→3')	Fragment size/bp
KMT1F	GGCTCGTTGTGAGTGGGCTTGTCGGTAGTCT	129
KMT1Rn	Biotin-GTCCAATCAGTTGCGCCGTTGTCAAGGAAG	
KMT1Pn	FAM-TGGCTTGTGGCAAAGAAAAGCACAGTTTTG[THF]TGGGCGGAGTTTGG3spacer	
PLPEF	ATGGCAGTTATGGACAACCTTCATCAGA	169
PLPER	CCAACTCAGTTTACATCACTTAATACGG	
OMPHF	TGGTTTCACATTTGGTGGTGCGTATGTCTT	184
OMPHR	GTGCTGCTGGCGGATTCTGTTCAACTTCTT	

The primers and probes were both synthesized by Shanghai Sangong Bioengineering Co.

Construction and optimization of RPA-Basic reaction system. According to the instruction manual of the TwistAmp™ Basic kit, a 50 µL RPA reaction system was developed for amplification. The RPA reaction system consisted of 2.4 µL each of forward and reverse primers, 29.5 µL of reaction buffer, 2.2 µL of template (DNA), and 11 µL of deionized water. After mixing the solid reactants with the solution thoroughly, 2.5 µL of magnesium acetate solution was taken and added to the inner side of the cap of the reaction tube, and the tube was turned up and down 8-10 times for mixing, and placed in a metal bath for reaction at 37°C for 20 min. The amplification products were detected by electrophoresis on a 2% agarose gel. In order to improve the amplification efficiency, the reaction temperature (25, 30, 35, 37, 39, and 45°C) and time (10, 20, 25, 30, 35, 40, and 45 min) of RPA-Basic were optimized. The optimized reaction temperatures and times were used for subsequent RPA-nfo experiments.

Construction of RPA-LFD detection system. On the basis of RPA-Basic, a rapid nucleic acid detection method for Pm RPA-LFD was developed. According to the instructions for the DNA constant temperature rapid amplification kit, a 50 µL reaction system was constructed: 40.9 µL A buffer, 2 µL each of forward and reverse primers, 0.6 µL probe (10 µmol · L^{-1}), and 2 µL template (DNA). After sufficient mixing, 2.5 µL of buffer B was added to the inner side of the lid of the reaction tube. The reaction tube was turned up and down 8-10 times for mixing, and the reaction was carried out in a metal bath at 39°C for 20 min. After the reaction was completed, the 10 µL amplification product was poured into a closed tube containing 600 µL of dd H_2O, and after sufficient mixing, the sample end of a colloidal gold test strip was inserted into the tube, and the results of the detection line and quality control line were observed within 5 min.

Specificity test. Under optimal reaction conditions, the genomic DNA of eight strains of SS, APP, GPS, Pm, *E. coli*, *L. monocytogenes*, *S. aureus*, and *A. hydrophila* was used as RPA template, and distilled water was used as the negative control to amplify the genomic DNA of the bacteria. The specificity of the RPA-LFD method was evaluated.

Sensitivity test. The extracted DNA was diluted into 8 concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} copies · μL^{-1} by 10-fold ploidy dilution, and the concentrations were detected with the optimal primer probe and reaction conditions in ascending order by RPA-Basic and RPA-LFD methods to determine the minimum detection limit. The PCR assay was maintained with the amount of template added for RPA-LFD, and finally the detection limit obtained for RPA-LFD was compared with that for the PCR assay.

Clinical application. Fifty nasopharyngeal swabs from suspected Pm infections were collected from different pig farms in Henan Province for clinical detection and analysis with RPA-LFD. Positive control, negative control, and blank control were Pm DNA, total DNA of healthy animal tissue, and dd H_2O, respectively, which were detected by the RPA-LFD method developed in this study. At the same time, the above samples were also detected by traditional bacterial isolation and conventional PCR. Finally, the experimental results obtained by various methods were compared and analyzed to verify the actual clinical detection effectiveness of the RPA-LFD method.

Results and discussion

Screening of primer probes. In the screening stage, Pm DNA was used as the positive control template for each primer, and sterilized deionized water was used as the negative control. Three sets of primers (KMT1F/KMT1R, OMPHF/OMPHR, PLPEF/PLPER) were designed according to the RPA design principle, amplified using the RPA-Basic reaction, and then evaluated by 2% agarose gel electrophoresis. One of the primers, KMT1F/KMT1R, showed a clear target band at 129 bp. Its negative control had no band, and the target band was not amplified by other primers. Therefore, primer KMT1F/KMT1R was selected for further study (Fig. 1). Subsequent studies confirmed that KMT1F/KMT1R and its probe had good specificity in RPA-LFD detection. Therefore, primers KMT1F/KMT1R were used in the RPA-Basic detection of Pm in this study.

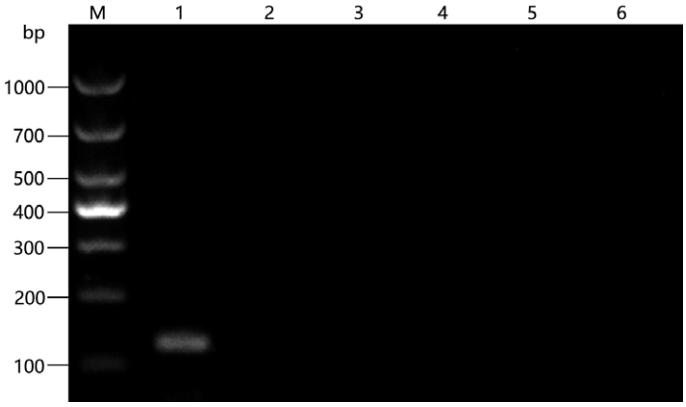

Fig. 1. Results of different primers detected by RPA-Basic
Explanations: M. 1000 bp DNA Ladder; 1. Primer KMT1F/KMT1R; 2. Negative control; 3. Primer OMPHF/OMPHR; 4. Negative control; 5. Primer PLPEF/PLPER; 6. Negative control.

Construction and optimization of RPA detection system. Once the optimal primers were determined, the reaction temperature and time were further optimized. In this study, mainly the two aspects of temperature and time were optimized. The results showed that electrophoretic bands were brightest when the reaction temperature was 39°C. When the reaction temperature continued to rise to 45°C, the bands either did not change significantly or their brightness would actually weaken, so 39°C was selected as the optimum reaction temperature (Fig. 2A). At the optimum temperature, with the extension of reaction time, the brightness of electrophoretic bands gradually increased, and basically reached the same level after 40 min. Considering the timeliness of the reaction system, the reaction time was chosen to be 40 min (Fig. 2B). In summary, the

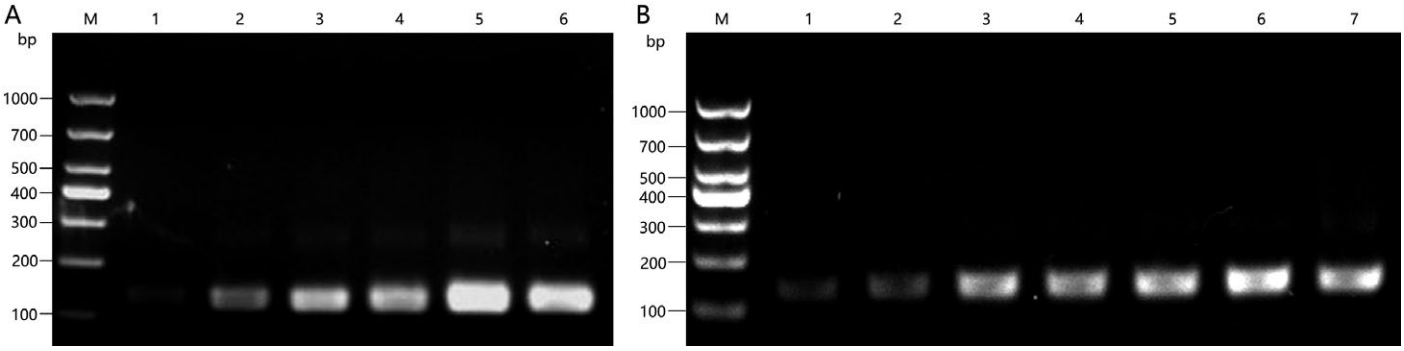

Fig. 2. RPA-Basic reaction temperature and time optimization
Explanations: A. Reaction temperature optimization; M. 1000 bp DNA Ladder; 1-6. Reaction temperatures are 25°C, 30°C, 35°C, 37°C, 39°C, 45°C; B. Reaction time optimization; M. 1000 DNA Ladder; 7-13. Reaction times are 10 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min.

optimal reaction temperature was 39°C, and the optimal reaction time was 40 min.

Specificity of the RPA-LFD detection method. The DNA of eight common porcine respiratory pathogens including SS, APP, GPS, Pm, *E. coli*, *L. monocytogenes*, *S. aureus*, and *A. hydrophila* were detected as described in the Methods section. Dd H_2O was used as the negative control. RPA-Basic and RPA-LFD methods were used to determine the specificity of screening primers KMT1F/KMT1R. The results showed that, except for a single and bright band in the positive control, no bands appeared in all other lanes, demonstrating that the specificity of the primer and its detection method were good (Fig. 3).

Sensitivity of the RPA-LFD detection method. The results of PCR and the RPA-LFD method for Pm are shown in Figure 4. With the PCR method, the bands could be seen at a DNA concentration of

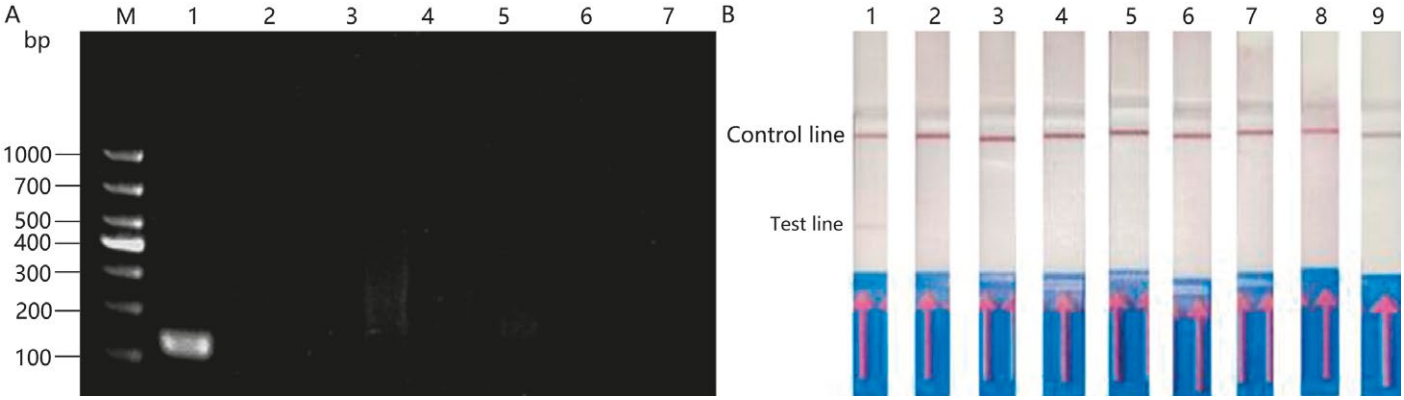

Fig. 3. Specificity evaluation of the RPA-LFD detection method for *Pasteurella multocida*
Explanations: A. RPA-Basic; B. RPA-LFD; M. 1000 bp DNA Ladder; 1. Positive control; 2-8. SS, APP, GPS, *Pasteurella*, *E. coli*, *L. monocytogenes*, *S. aureus*, and *A. hydrophila*; 9. Negative control.

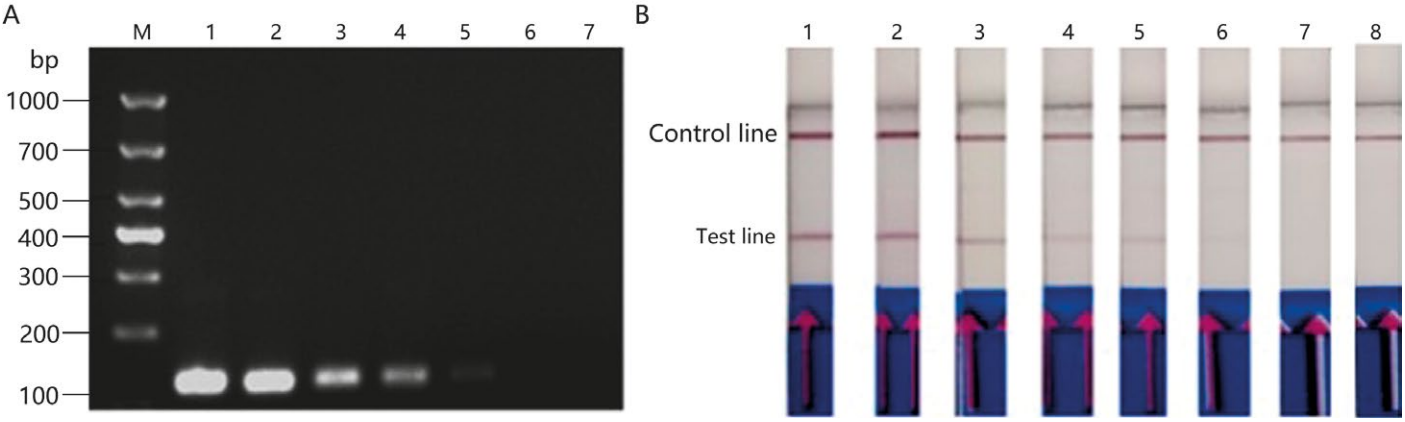

Fig. 4. Sensitivity evaluation of PCR and the RPA-LFD detection method
Explanations: A. PCR sensitivity; M. 1000 bp DNA Ladder; 1-6. Template DNA concentrations are 1×10^{-1} copies · μL^{-1}, 1×10^{-2} copies · μL^{-1}, 1×10^{-3} copies · μL^{-1}, 1×10^{-4} copies · μL^{-1}, 1×10^{-5} copies · μL^{-1}, 1×10^{-6} copies · μL^{-1}; 7 Negative control; B. The sensitivity of the RPA-LFD detection method; 1-7. Template DNA concentrations are 1×10^{-1} copies · μL^{-1}, 1×10^{-2} copies · μL^{-1}, 1×10^{-3} copies · μL^{-1}, 1×10^{-4} copies · μL^{-1}, 1×10^{-5} copies · μL^{-1}, 1×10^{-6} copies · μL^{-1}, 1×10^{-7} copies · μL^{-1}, 8; Negative control.

1×10^{-4} copies · μL^{-1}, but the brightness was very weak. With the continuous reduction of concentration, the color of the test strip detection line also faded. The RPA-LFD method still showed a clear detection line at a DNA concentration of 1×10^{-6} copies · μL^{-1}, but when the concentration was reduced to 1×10^{-7} copies · μL^{-1}, the detection line was almost invisible to the naked eye. The results show that the sensitivity of the RPA-LFD method was 100 times as high as that of PCR.

Test results for clinical samples. Test results for the 50 clinical samples collected are shown in Table 2. Among them, 19 samples were found positive for Pm by bacterial isolation, with a detection rate of 38%. A total of 20 positive samples were detected by ordinary PCR, with a detection rate of 40%. A total of 25 samples were detected as positive by RPA-LFD, with a detection rate of 50%. The clinical detection rate of RPA-LFD was higher than that of PCR and traditional bacterial isolation.

Tab. 2. Results of tests on clinical samples for different detection methods

Detection methods	Number of positive cases	Positive detection rate (%)
Bacterial isolation	19	38
Ordinary PCR assay	20	40
RPA-LFD	25	50

Pm is the main pathogen that causes various respiratory diseases in pigs and can easily cause infections secondary to other diseases. It affects the growth and development of pigs and seriously hinders the development of the pig industry. Therefore, it is particularly important to develop a rapid, efficient, and convenient detection method. Detection methods for Pm are becoming more diversified, and PCR, qPCR, ELISA, and LAMP have been widely used (23). Zhao et al. (17) developed a PCR diagnostic method for Pm based on the *toxA* gene, which could detect Pm. Liu et al. (11) developed a dual real-time fluorescent quantitative PCR method for SS and Pm, which had a minimum detection concentration for the recombinant plasmid standard of 3.97×10^2 · copies µL. LAMP technology achieves efficient amplification of target genes at constant temperature, eliminates dependence on special equipment, such as PCR instruments, and has high specificity and sensitivity. Sun et al. (18) developed a LAMP detection method for Pm based on the *kmt1* gene, with a minimum detection limit of 10 cfu/mL, which was slightly less sensitive than the PCR method. Sun et al. (19) developed a LAMP method based on the *PlpB* gene of Pm, and the sensitivity of this method was 25 cfu/mL, with no cross-reaction with other pathogens.

Although PCR-based amplification has been regarded as an important standard for molecular diagnosis, it requires specialized and expensive equipment and well-trained professionals, which severely limits its use in the production line of pig farms. RPA is a novel nucleic acid isothermal amplification technique that can complete the isothermal amplification of the target fragment in 10 to 40 min at 37~42°C, with the advantages of a short detection time, high sensitivity, and simple operation. RPA has been widely used in the on-site detection of a variety of bacteria. Wang et al. (21) developed an RPA-LFD method for the detection of *Citrobacter freundii*, and the detection limits for pure cultures and genomic DNA of *Citrobacter freundii* were 1.5×10^2 cfu/mL and 200 fg/µL, respectively, which were 10 times as high as those of conventional PCR. Zhang et al. (25) developed an RPA-LFD method for detecting *Streptococcus suis*, with a minimum detection limit of 100 copies · μL^{-1}, which was higher than that of the conventional PCR method.

Reaction temperature and time determine the field applicability of the detection method. The RPA-LFD method developed in this study started to show target bands at 10 min, but the best results were obtained after 40 min. Compared with the other methods, this method greatly shortened the detection time, did not require other thermal cycling instruments, and the results could be read directly on the LFD after 40 min of amplification at 39°C in a metal bath, which is very suitable for field or on-site detection. Two other important technical parameters of detection methods are sensitivity and specificity. The sensitivity of the RPA-LFD detection method developed in this study was 1×10^{-6} copies · μL^{-1}, which is 100 times as high as that of conventional PCR, greatly improving the detection rate of the samples. In the detection of clinical samples, the detection rate of RPA-LFD was higher than that of conventional PCR. In terms of specificity, the method showed no cross-reaction with the other eight common respiratory pathogens, indicating that it is highly specific, accurate and reliable.

The design of primers is very crucial in developing an RPA-LFD method. Unlike conventional PCR primers, the primer length of RPA is generally 30~35 bp. Too short a length will reduce the binding rate with recombinant enzymes, and a too long one will make it easy to form dimers (26). The GC content is between 40% and 60%. On the other hand, due to the high sensitivity of the RPA-LFD method, aerosols are likely to be generated during operations such as sampling and loading, resulting in false positives, so the operation should be carried out in a well-ventilated and open environment (20).

In this study, a rapid RPA-LFD detection method for Pm was developed. It is characterized by high specificity, high sensitivity, and easy operation, and the results can be directly observed by the naked eye, making it especially suitable for field and on-site detection. This method provides a new technical means for the molecular epidemiological investigation of Pm and has broad application prospects.

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