ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (7): 365 - 374

doi: 10.30466/vrf.2024.2035817.4371

Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Establishment and application of dual isothermal amplification of *Pasteurella multocida* and *Streptococcus suis* in pigs

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Article Info

Article history:

Received: 17 July 2024
Accepted: 06 November 2024
Available online: 15 July 2025

Keywords:

Lateral flow dipstick
Partonella multocida
Recombinase polymerase amplification
Streptococcus suis

Abstract

Porcine respiratory disease complex is a clinically lethal condition and is the leading cause of mortality in weaned piglets as well as growing and fattening pigs. *Pasteurella multocida* (Pm) and *Streptococcus suis* (SS) are common respiratory pathogens in porcine respiratory disease complex. This study combined the recombinase polymerase amplification (RPA) technique with the lateral flow dipstick (LFD) technique. The dual Basic-RPA detection method for Pm and SS and the dual RPA-LFD rapid visualization detection method for Pm and SS were constructed, respectively. The detection limit of RPA-LFD was 10^{-6} ng µL^{-1}, which was higher than the detection limit of RPA at 10^{-5} ng µL^{-1} and much higher than the detection limit of polymerase chain reaction at 10^{-4} ng µL^{-1}. There was no cross-reactivity with other pathogens which indicated that the method had good specificity and high sensitivity. The detection rate of RPA-LFD was much higher than that of conventional Polymerase chain reaction in 60 clinical samples collected in 2023 with suspected Pm and SS. This method could avoid the complicated temperature cycling instruments and does not require professional laboratory skills, which makes it suitable for on-site detection.

Introduction

Porcine respiratory disease complex (PRDC), a disease caused by a combination of infections with multiple pathogens or non-infectious factors, is one of the most serious health challenges in modern swine production.[1] The general clinical signs of PRDC are reduced feed intake, fever, coughing and respiratory distress, which cause a decrease in feed conversion efficiency and growth rate.[2] *Pasteurella multocida* (Pm) and *Streptococcus suis* (SS) are important pathogens of PRDC. When pigs are exposed to two or more respiratory pathogens simultaneously, their interactions at the cellular and molecular level become complex. Infection of these pathogens with each other is far more harmful than infection alone. Given the high complexity of mixed infections, it is undoubtedly a challenging task to analyze in depth the interaction mechanisms between these pathogens.[2] Therefore, developing a sensitive, rapid and specific diagnostic tool is crucial for controlling these diseases.[3]

Pasteurella multocida is a zoonotic bacterium, a gram-negative, non-motile parthenogenetic globular or short rod-shaped bacterium of the family Pasteurellaceae.[4] These bacteria are often capable of causing disease when there is a change in the host immunocompetence or when the bacteria invade the interior of tissues using injuries caused by scratches and bites. Pigs are one of the most susceptible animals to Pm, which mainly causes lung disease in pigs.[5] *Pasteurella multocida* strains are categorized into five podoconiotic serogroups (A, B, D, E, and F) of which serotypes A, D, and B are frequently reported to cause disease in pigs.[6,7] In addition, it has been shown that Pm type A can be a major pathogen in swine pneumonia and septicemia, whereas, serotype B usually

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causes septicemic pasteurellosis.[8] Toxic capsular D serotypes and less common A strains can cause progressive atrophic rhinitis.[9] In addition, swine pneumonia is not only highly contagious and prone to spread rapidly in swine herds but also may cause a variety of other infectious diseases which can bring great economic losses to the farming industry and seriously affecting economic efficiency.[5] *Pasteurella multocida* is an essential cause of progressive atrophic rhinitis and pneumonic bartonellosis, an economically important disease that frequently leads to reduced feed utilization and stunted growth. Similarly, pneumonic bartonellosis has been reported to significantly reduce the growth rate of swine.[10]

Streptococcus suis is an important zoonotic pathogen that is prevalent worldwide and is considered one of the most important bacterial pathogens responsible for significant economic losses occurring in the pig industry.[11] *Streptococcus suis* infections are most commonly found in piglets and mainly lead to the death of weaned piglets between 5 and 10 weeks of age.[12,13] *Streptococcus suis* mainly colonizes the respiratory and digestive tracts of pigs.[14] Twenty-nine serotypes of SS strains have been identified and defined based on differences in the antigenicity of SS pod polysaccharide . Among them, SS type 2 is considered to be the most virulent and the serotype with the highest isolation rate, and serotype 2 strains pose a great threat to public health as zoonotic pathogens.[15-17] The most common clinical feature of SS infection is severe bacteremia with the presence of live bacteria in the blood of patients.[18]

Recombinase polymerase amplification (RPA) is a novel isothermal amplification technique for nucleic acids, that has the advantage of high sensitivity and specificity.[3,19] The RPA technique utilizes phage recombinase to form a complex with oligonucleotide primers, and this complex is highly efficient in facilitating the binding of the oligonucleotide primers to the homologous sequences of double-stranded DNA molecules. By binding tightly to single-stranded DNA binding protein and strand displacement polymerase, RPA can complete efficient DNA amplification in only 30 min at a constant temperature of about 37.00 ˚C, demonstrating its rapid and stable amplification ability.[20] Combining Basic-RPA with nucleic acid gel electrophoresis enables the analysis of the assay results. The detection of Basic-RPA products is mainly performed by the Lateral Flow Dipstick (LFD) for the interpretation of the results. The LFD assay requires a special nfo probe with fluorescein-amide (FAM) antigenic labeling at the 5' end and middle tetrahydrofuran (THF), and the 3' end has a blocker, typically a C3spacer.[21] In the RPA reaction, specific target nucleic acid sequences are efficiently amplified by nfo nuclease and the amplification products are labeled with fluorescence and biotin for subsequent detection. The RPA-LFD method is simple and portable, and it also has an efficient and accurate amplification performance, requiring only simple sample preparation, and the results can be observed directly by the naked eye without relying on complex or expensive equipment.[22] The RPA-LFD method has also been studied more in the detection of pathogens in experimental animals. Liu *et al.* designed a pair of primers based on the *invA* gene of Salmonella, and established an RPA-LFD technique for visual and rapid detection of Salmonella, which can achieve a constant temperature within 20 min, and the method can achieve a high sensitivity in the detection of Salmonella in a short time.[23] The method can achieve amplification in a constant temperature metal bath within 20 min with a sensitivity of 10 copies per µL and no cross-reactivity with *Staphylococcus aureus*, *Escherichia coli, Listeria monocytogenes,* and *Shigella*.[24] In addition, researchers have studied the RPA-LFD method for *Streptococcus agalactiae*,[25] *Fusarium oxysporum*,[26] *Porphyromonas gingivalis*,[27] Bovine Viral Diarrhea Virus and Bovine Herpesvirus 1,[28] Herpesvirus Carp 3,[29] Canine Distemper Virus,[30] and Porcine Sapropelus Virus.[31]

Materials and Methods

Strains and reagents. *Streptococcus suis* serotype 2 strain, *P*Pm, *Actinobacillus pleuropneumoniae* (APP), Enteropathogenic *E.coli*, *Glaesserella parasuis* (GPS), *L.monocytogenes*, *Salmonella*, and *Aeromonas hydrophila* were kept in our laboratory. The Bacterial genomic DNA rapid extraction kit was purchased from Ebixon Co. Ltd. (Shanghai, China) Biotechnology, the TwistAmp™ Basic kit was purchased from TwistDx (Maidenhead, UK), the polymerase chain reaction (PCR) Master Mix kit was purchased from Sangong Bioengineering Co. Ltd. (Shanghai, China), the LFD test strips were purchased from Hangzhou Yosida Biotechnology Co. Ltd. (Kyoto, Japan). Brain Heart Infusion, Todd Hewitt Broth, Luria-Bertani, Tryptic Soy Agar and Tryptic Soy Broth culture media were purchased from Solebao Technology Co. Ltd. (Beijing, China).

Genomic DNA extraction. *Pasteurella multocida*, SS, APP, enteropathogenic *E. coli*, *Salmonella*, *L. monocytogenes*, GPS, and *A. hydrophila* were placed in solid medium, incubated at 37.00 ˚C for 8 - 16 hr, and then single colonies were picked in liquid medium and incubated with shaking at 37.00 ˚C for 8 - 16 hr. The genomic DNA of Pm, SS, APP, Enteropathogenic *E.coli*, *Salmonella*, GPS, and *A. hydrophila* were extracted with the use of the Ezup Column Bacterial Genome DNA Rapid Extraction Kit (Absin Biotechnology Co. Ltd., Shanghai, China), and the concentration of DNA was measured by Nanodrop 2,000 (Thermo Scientific, Wilmington, USA) and stored at – 80.00 ˚C. Genomic DNA was extracted from 1.00 mL of fresh bacterial liquid using the Ezup column-type rapid genomic DNA extraction kit, and the concentration of DNA was determined using Nanodrop 2,000 and stored at – 80.00 ˚C. After the extraction, Pm and SS genomic DNA were diluted to 10.00 ng µL^{-1}, Pm, and SS were taken and mixed in the same volume, and then gradient dilution was carried out using the ten-fold multiplicative dilution method. The DNA concentrations were sequentially diluted to 10^{-1} ng µL^{-1}~10^{-8} ng µL^{-1}, set aside at – 20.00 ˚C.

Primer design and screening. Porcine Pm *kmt1* gene sequences and SS *gdh* genes were downloaded from GenBank® and analyzed by multiple comparisons on DNASTAR software (version 7.1; DNASTAR, Madison, USA) to look for specific fragments in the sequences. Three pairs of Pm primers and eight pairs of SS primers were designed using the Primer 5.0 Design Primers Software (Qingke, Beijing, China) according to the TwistAmp™ (TwistDx) DNA Amplification Kit Analysis Design Manual. The mixed genome of Pm and SS was used as the DNA template for RPA amplification, and the reaction products of RPA were subjected to agarose gel electrophoresis, the results were evaluated for specificity, and the optimal primers were selected (Table 1). To visualize the reaction results on flow measurement chromatography test strips, the primers were labeled with biotin at the 5' end. Two TwistAmp™ nfo probes were designed according to the instructions of the TwistAmp™ Basic kit (TwistDX). The 5' end of the probe was labeled with fluorescein-amide, and the 3' end was modified with C3-Spacer. Then, the THF site was placed in the middle position from the 5' end of the probe with one nucleotide substitution. At least 15 nucleotides were added at the 3' end after the THF residue. The primers and probes were synthesized by Sangon Biological Engineering Co., Ltd. According to the recommended reaction system and operation of the RPA-nfo kit manual, the reaction temperature and time after RPA optimization were used as the reaction conditions for the screening of probes (Table 1). The screening of unspecific primers by the RPA method is shown in Table 2.

Establishment of RPA. According to the instruction manual of the TwistAmp™ Basic kit (TwistDx), 50.00 µL Basic-RPA reaction system was established. The RPA reaction system was configured as follows: 2.40 µL of forward and reverse primers, 29.50 µL of reaction buffer, 2.20 µL of template (DNA), the remaining was made up with ddH_2O water, the solid reactant was mixed with the solution thoroughly and then finally 2.50 µL of magnesium acetate was added to the lid of the reaction tube and placed in a metal bath at 39.00 ˚C for 20 min. 2.50 µL was added to the cap of the reaction tube and placed in a 39.00 ˚C metal bath reaction for 20 min while setting ddH_2O as a negative control. At the end of the reaction, an equal volume of phenol-chloroform solution was added and the

Table 1. Primers and probes for Basic-RPA and RPA-LFD amplification.

Primers	Sequence（5→3'）	Fragment size (bp)
KMT1F	GGCTCGTTGTGAGTGGGCTTGTCGGTAGTCT	129
KMT1Rn	Biotin-GTCCAATCAGTTGCGCCGTTGTCAAGGAAG	
KMT1Pn	FAM-TGGCTTGTGGCAAAGAAAAGCACAGTTTTG [THF]TGGGCGGAGTTTGG3spacer	
GDH1166F	TTCGCTTGTCATGGACTCGTGAAGAAGTAG	147
GDH1340Rn	Biotin-AAACCTTGGGCAATCATGCTATCCGACATT	
GDH1225Pn	FAM-AAATACGACCTTGGTACAGACTACCTTGCAGG [THF]TGGGCGGAGTTTGG3spacer	

Table 2. Pm and SS primer screening.

Name	Sequence（5→3'）	Fragment size (bp)
PLPEF	ATGGCAGTTATGGACAACCTTCATCAGA	169
PLPER	CCAACTCAGTTTACATCACTTAATACGG	
OMPHF	TGGTTTCACATTTGGTGGTGCGTATGTCTT	184
OMPHR	GTGCTGCTGGCGGATTCTGTTCAACTTCTT	
GDH162F	TATCGTTGAGCCTGAGCGTAT	78
GDH240R	GCCACGGTTGACTTGAACAT	
GDH158F	CTCGTATCGTTGAGCCTGAG	99
GDH257R	TTGAACTGAACACGGTAGCC	
GDH874F	GACAGAATACGCTGCAGA AAA ATC	128
GDH1002R	GGCAGCAGCTTGTTTGCCGTTGATCTCATT	
GDH556F	CGCCTCCGCCAGTTTGATGCAGGTGTCTTG	150
GDH706R	AGTTTGGTCTTTGAAGGATTTACCGTTTGC	
GDH1166F	TTCGCTTGTCATGGACTCGTGAAGAAGTAG	180
GDH1346R	TATACCAAACCTTGGGCAATCATGCTATCC	
GDH1110	AGCTGCCAACGCTGGTGGTGTAGCTGTATC	188
GDH1298R	CCTGCAAGGTAGTCTGTACCAAGGTCGTATT	
GDH812	ACGAAACTGGTATCGACTTCGACCTCTTGG	190
GDH1002R	GGCAGCAGCTTGTTTGCCGTTGATCTCATT	

RPA amplification products were purified by centrifugation at 12,000 rpm for 5 min. After centrifugation, the supernatant was analyzed by 2.00% agarose gel electrophoresis.

Optimization of RPA reaction conditions. To improve the amplification efficiency, primer ratio, temperature and time were optimized for RPA. *Pasteurella multocida* and SS volume ratios were set at (µL): (0.00 / 2.40), (0.40 /2.40), (0.90 / 2.40), (1.30 / 2.40), (1.90 / 2.40), (2.40 / 2.40), (2.40 / 1.90), (2.40 / 1.40), (2.40 / 0.90) and the optimal priming ratio was selected. Six reaction temperature gradients were set at 25.00, 30.00, 35.00, 37.00, 39.00, and 45.00 ˚C, respectively, and the optimal temperature was determined by incubation for 20 min, different reaction times were set at 10, 15, 20, 25, 30, 35, 40, and 45 min, respectively and the results were observed to select the shortest and optimal RPA reaction. The results were observed to select the shortest and the best time for the RPA reaction.

Establishment and optimization of RPA-LFD. The RPA-nfo amplification reaction was screened for primer and probe combinations according to the conditions recommended by the DNA Thermostatic Rapid Amplification Kit (TwistAmp™ nfo kit; TwistDX). The RPA-LFD system is shown in Table 3. After completion of the reaction, 5.00 µL was added to a closed centrifuge tube containing 200 µL of ddH_2O and the results were interpreted using the Milenia GenLine HybriDetect kit (Milenia, Gießen, Germany). After mixing, the sample end of the colloidal gold test strip was inserted into a closed centrifuge tube and the results were read within 5 min. The primer ratios were optimized for RPA-LFD following the conditions provided in the DNA Thermostatic Rapid Amplification Kit (TwistAmp™ nfo kit; TwistDX), with the Pm and SS probe additions (µL) set to (0.00, 0.00, 0.00 : 2.00, 2.00, 0.60), (1.00, 1.00, 0.60 : 2.00, 2.00, 0.60), (2.00, 2.00, 0.60 : 2.00, 2.00, 0.60), (2.00, 2.00, 0.60 : 1.00, 1.00, 0.60), (2.00, 2.00, 0.60 : 1.50, 1.50, 0.60), (2.00, 2.00, 0.60 : 0.50, 0.50, 0.60). At the end of the reaction, GenLine HybriDetect kit (Milenia) was used to interpret the results and select the optimal primer volume ratio.

Analysis of RPA and RPA-LFD specificity. The prepared Pm and SS genomic DNAs were utilized as positive controls for RPA and RPA-LFD and the extracted genomic DNAs of SS, Pm, APP, enteropathogenic *E. coli*, GPS, *L. monocytogenes*, *Salmonella*, and *A. hydrophila* were used as negative controls. Additionally, ddH_2O was set as a negative control. The RPA and RPA-LFD reactions were performed under optimal conditions using the screened optimal primers and probes to evaluate the pathogen specificity of the RPA and RPA-LFD assays for the target Pm *kmt1* gene.

Table 3. Recombinase polymerase amplification-lateral flow dipstick detection method system

Components	Volume (µL)
A buffer	40.90
Upstream primer	2.00
Downstream primer	2.00
Probe	0.60
Template	2.00
B buffer	2.50
Total volume	50.00

Recombinase polymerase amplification and RPA-LFD sensitivity analyses. Mixed Pm and SS DNA templates at a concentration of 10.00 ng μL^{-1} were diluted in a 10-fold gradient dilution to 10^{-1}~10^{-7} ng μL^{-1} and negative control was set up to perform a double RPA, RPA-LFD and PCR sensitivity assay under the optimal conditions with RPA-LFD detected using a sidestream laminar test strip and RPA and PCR detected by agarose gel electrophoresis and comparing the sensitivity differences between RPA, RPA-LFD, and PCR.

Clinical sample testing. A total number of 60 clinical samples of pharyngeal swabs suspected to be infected with Pm and/or SS from an animal hospital were collected in this study and bacterial culture was taken to extract genomic DNA as a template for the RPA-LFD assay. The extracted DNA was used as a positive control for Pm/SS, and ddH_2O was used as a negative control for this study. A double RPA-LFD assay was carried out under the optimal reaction conditions obtained from the study. The results of the assay were compared and analyzed with those of the conventional PCR assay.

Results

Design and screening results of RPA primers. The specific primers designed with *kmt* and *gdh* genes as targets were used for Basic-RPA and RPA-LFD amplification of Pm/SS genomic DNA. As shown in Figure 1. Only *kmt1* showed clean, bright and single bands after the Basic-RPA reaction of the three pairs of Pm primers designed,[32] with no non-specific amplification and a relatively weak primer dimer which had a better amplification effect and the other two pairs of primers did not amplify any bands, therefore, *kmt1* was selected as the best primer for RPA and used in the subsequent experiments. Four sets of SS primers (GDH1166F/ GDH1346R, GDH874F/GDH812, GDH556F/GDH706R, and GDH162F/GDH240R) were initially selected based on the RCR reaction and then further screened using the Basic-RPA reaction. Among them, primers GDH1166F/ GDH1346R and GDH874F/GDH812R had clear electrophoretic bands and their negative controls had no bands while the positive amplification of the other primers showed stray bands, no bands or dragging bands, therefore, primers GDH1166F/GDH1346R were used in the SS Basic-RPA assay portion of this study.[33]

Optimization results of RPA reaction conditions. As shown in Figure 2, the optimized results of the Pm and SS primer volume ratio indicated that the electrophoretic band brightness of Pm and SS was more consistent when the primer volume ratio of Pm and SS was 2.40 / 2.40 µL. After repeating the experiments, the primer volume ratio of 2.40 / 2.40 µL for Pm and SS was finally selected as the optimal volume ratio in this study. As shown in Figure 3A, the RPA amplification products could be specifically detected at 35.00 ~ 49.00, and 37.00 ˚C was selected as the optimal reaction temperature in this study in consideration of the later application of clinical testing. As shown in Figure 3B, the electrophoresis results showed that under the uniform reaction temperature of 37.00 ˚C, the RPA method could detect the target product in the range of 10 ~ 35 min, however, there was no significant difference in the brightness of electrophoresis bands after 35 min. Therefore, to reduce the time consumed for the detection, this study chose 35 min as the optimal reaction time.

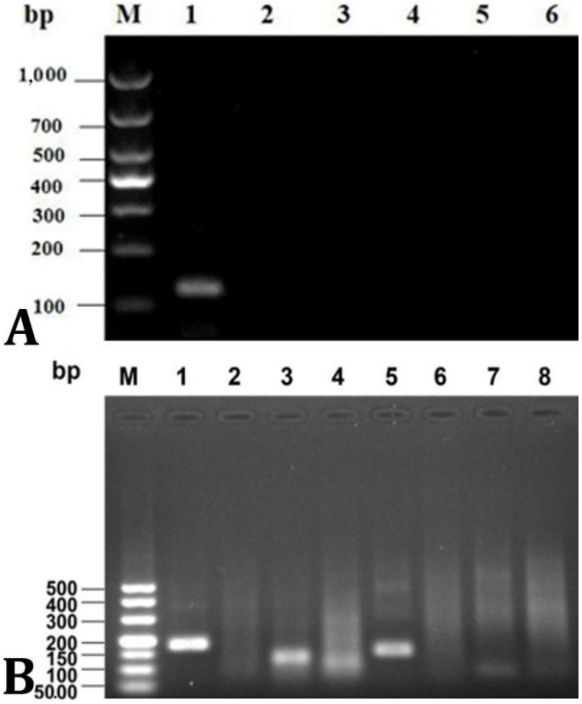

Fig. 1. The results of different primers detected by recombinase polymerase amplification. **A)** *Pasteurella multocida* primer screening results; Lane M: DL1000 DNA Marker; Lanes 1 - 6: primer KMT1F/KMT1R, negative control, primer ompHF/ompHR, negative control, primer plpEF/plpER, negative control, respectively. **B)** *Streptococcus suis* screening results; Lane M: DL500 DNA Marker; Lanes 1 - 8 are: primer GDH1166F/GDH1346R, negative control, primer GDH874F/GDH1002R, negative control, primer GDH556F/GDH706R, negative control, primer GDH162F/GDH240R, negative control, respectively.

Optimization results of the RPA-LFD system. The optimization of the dual RPA-LFD reaction system was mainly focused on the primer-probe volume ratio, and the optimization results are shown in Figures 4, with obvious dual detection lines in experimental groups 2, 3, 4, and 5. To maintain the same color depth of SS and Pm detection lines and to facilitate the subsequent detection, the combination of (2.00, 2.00, 0.60) and (1.00, 1.00, 0.60) was selected for the subsequent experiments.

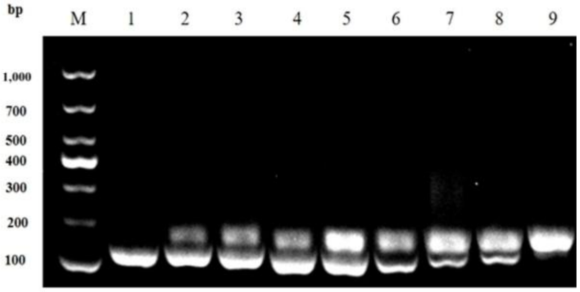

Fig. 2. Optimization of dual recombinase polymerase amplification primer volume ratios. Lane M: DL1000 DNA Marker; Lanes 1 - 9 Primer volume ratios are (µL): (0.00 / 2.40), (0.40 / 2.40), (0.90 / 2.40), (1.30 / 2.40), (1.90 / 2.40), (2.40 / 2.40), (2.40 / 1.90), (2.40 / 1.40), (2.40 / 0.90), respectively.

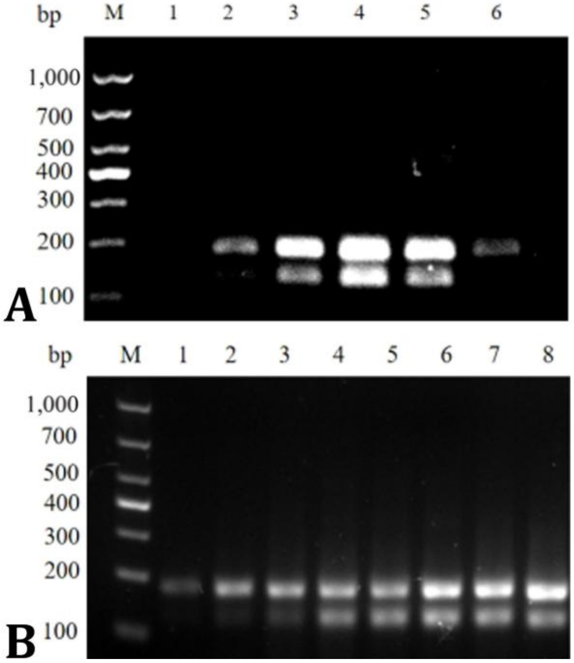

Fig. 3. Optimization of dual recombinase polymerase amplification (RPA) reaction conditions. **A)** Optimization of RPA reaction temperature. Lane M: DL1000 DNA Marker; Lanes 1 - 6: 25.00, 30.00, 35.00, 37.00, 39.00 and 45.00 ˚C. **B)** Optimization of RPA reaction time. Lane M: DL1000 DNA Marker; Lanes 1 - 8: 10, 15, 20, 25, 30, 35, 40 and 45 min, respectively.

Analysis of dual RPA and RPA-LFD specificity. As shown in Figure 5A, no bands appeared in all lanes except for the positive control group which showed bright bands. It indicated that the specificity of the RPA assay was good. To ensure the specificity of the probe, seven other common pathogenic bacteria strains were used as controls for RPA-LFD specificity analysis. The specificity results of KMT1F/KMT1R, GDH1166F/GDH1340R primers and probes KMT1Pn and GDH1225Pn obtained from the screening are shown in Figure 5B. Except for the positive control test strips which showed bands on the detection line, no cross-reactivity was appeared on the detection lines of the other controls or the negative control group. In summary, it could be concluded that the RPA-LFD assay had strong specificity.

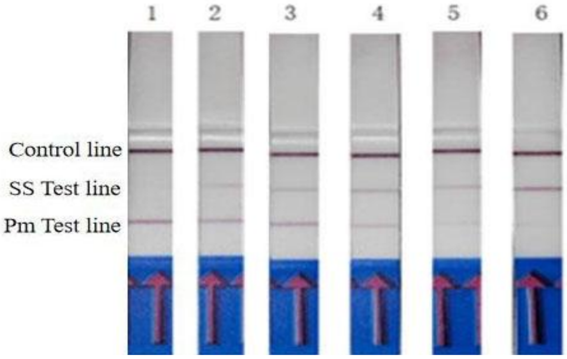

Fig. 4. Optimization of dual recombinase polymerase amplification-lateral flow dipstick *Pasteurella multocida* (Pm) - *Streptococcus suis* (SS) primer volume ratio. Lanes 1 – 6: Primer volume ratios are (µL): (0.00, 0.00, 0.00 / 2.00, 2.00, 0.60), (1.00, 1.00, 0.60 / 2.00, 2.00, 0.60), (2.00, 2.00, 0.60 / 2.00, 2.00, 0.60), (2.00, 2.00, 0.60 / 1.00, 1.00, 0.60), (2.00, 2.00, 0.60 / 1.50, 1.50, 0.60), (2.00, 2.00, 0.60 / 0.50, 0.50, 0.60), respectively.

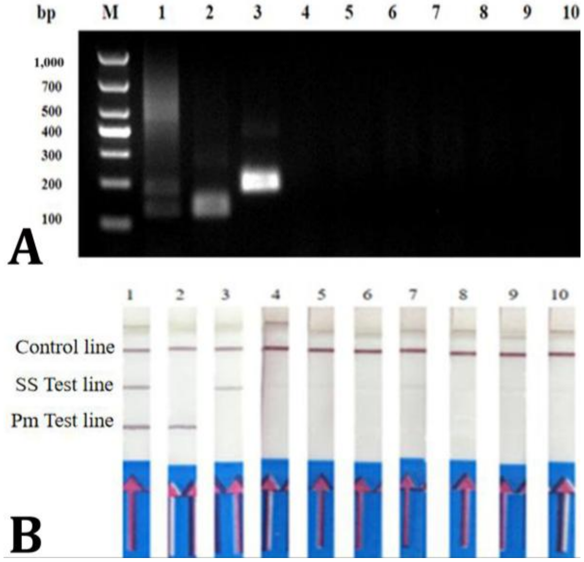

Fig. 5. Dual recombinase polymerase amplification (RPA) and RPA- lateral flow dipstick (LFD) method-specific assay results. **A)** Results of *Pasteurella multocida* (Pm) and *Streptococcus suis* (SS) specificity detection by RPA method. Lane M: DL1000 DNA Marker; Lanes 1 - 10: Pm/SS, Pm, SS, Actinobacillus pleuropneumoniae, enteropathogenic Escherichia coli, Salmonella, Listeria monocytogenes, Glaesserella parasuis, Aeromonas hydrophila, respectively. **B)** Results of Pm and SS specificity detection by RPA- LFD method. Lanes 1 - 10: Pm/SS, Pm, SS, *Actinobacillus pleuropneumoniae*, enteropathogenic *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Glaesserella parasuis*, *Aeromonas hydrophila*, respectively.

Analysis of dual RPA and RPA-LFD sensitivity. The PCR sensitivity test showed the lowest detection concentration is only 10^{-4} ng µL^{-1} (Fig. 6A), the lowest detection limit of RPA-LFD was 10^{-6} ng µL^{-1} (Fig. 6B), and the lowest detection limit of RPA was 10^{-5} ng µL^{-1} (Fig. 7), which indicated that the sensitivity of RPA-LFD was higher than that of RPA by 10 times and much higher than that of PCR by 100 times.

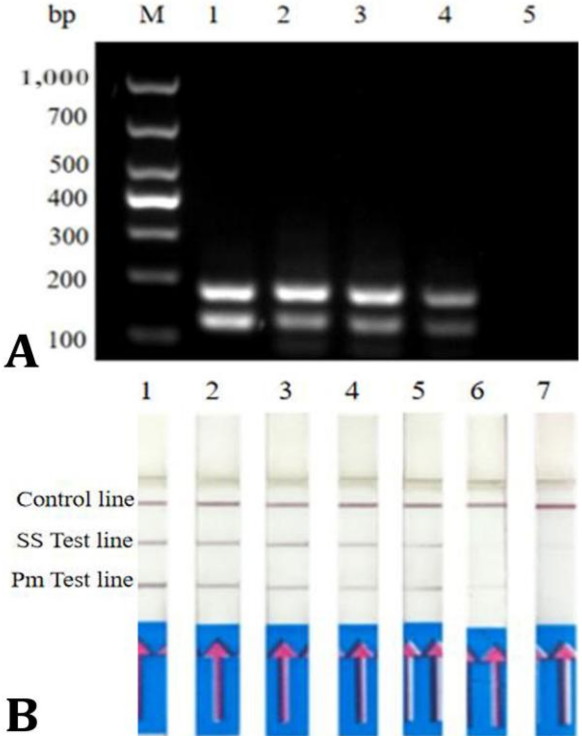

Fig. 6. Dual polymerase chain reaction (PCR) and recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) method sensitivity assay results. **A)** Sensitivity results of dual detection of *Pasteurella multocida* (Pm) and *Streptococcus suis* (SS) by PCR method; Lane M: DL1000 DNA Marker; Lanes 1 - 5 nucleic acid concentration (ng µL^{-1}): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively. **B)** Sensitivity results of dual detection of Pm and SS by RPA-LFD method; Lanes 1 - 7 Nucleic acid concentration (ng µL^{-1}): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}, respectively.

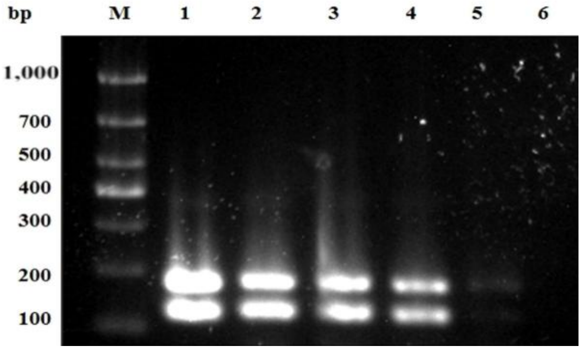

Fig. 7. Dual recombinase polymerase amplification method sensitivity results. Lane M: DL1000 DNA Marker; Lanes 1 - 6 nucleic acid concentrations (ng µL^{-1}): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, respectively.

Results of clinical sample detection. When the established dual RPA-LFD method was used to detect Pm and SS strains, the strains to be detected could be precisely identified (Fig. 8). Sixty samples collected from the clinic were tested by PCR and RPA-LFD methods, and the results of the two methods were statistically analyzed, as shown in Table 4. A total number of two Pm and SS positive samples were detected by conventional PCR, and a total number of four Pm and SS positive samples were detected by RPA-LFD. In summary, the RPA-LFD method had a higher detection rate, which was superior to the PCR method in terms of the ease of operation and the time consumed by the two methods. The RPA-LFD consumed the least amount of time and the operation process was relatively simple, therefore, there was no need to learn the techniques of operation of large-scale instruments, and only basic laboratory techniques need to be mastered. It is not necessary to learn how to operate a large-scale instrument, one only needs to master the basic experimental techniques to complete the whole experiment.

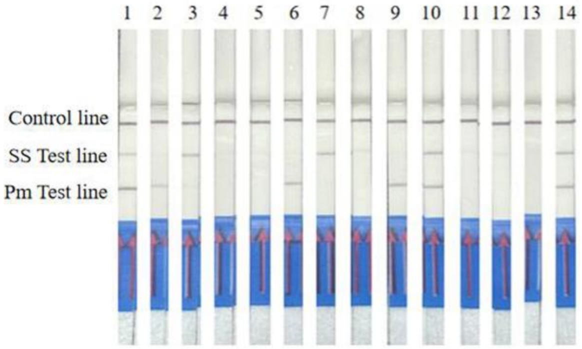

Fig. 8. Clinical sample recombinase polymerase amplification-lateral flow dipstick method detection results. Lanes 1 - 4, *Streptococcus suis* (SS) and *Pasteurella multocida* (Pm) positive control, SS positive control, Pm positive control and negative control, respectively. Lanes 5 - 14, clinical test samples.

Discussion

With the booming development of China social economy, people pursuit of quality of life is also increasing, especially with the growing demand for high-quality pork. However, porcine respiratory diseases have always been a difficult problem to ignore in pig farming, which seriously affects the health and growth of pigs, leading to more serious clinical outcomes and causing serious economic losses.[34] Two important respiratory pathogens in China swine farming industry, Pm and SS will invade the respiratory systems of pigs at the same time, triggering mixed infections.[1] Pm often leads to an acute bacterial disease characterized by bronchopneumonia, sepsis and sudden death. Infection with SS results in a high mortality rate characterized by meningitis, endocarditis, pneumonia, arthritis and sepsis. In addition to the health effects on

Table 4. Statistics of clinical test results.

Isolates	*Pasteurella multocida*		*Streptococcus suis*	
	PCR	RPA-LFD	PCR	RPA-LFD
Y1-4				
Y2-4				
Y3-4			+	+
Y4-4				
Y28-3			+	+
Y40-1				
Y40-2				
Y40-3	+	+		
6Y43-1				
Y43-2	+	+		
Y43-3	+	+		
Y45-1				
Y45-2			+	+
Y45-3				
2-4-1				
2-4-2				
2-4-3				
2-8-1				
2-8-2	+	+		+
2-8-3			+	+
2-8-4				
2-11-1	+	+	+	+
2-11-2		+	+	+
2-11-3				
2-11-4				
2-12-1			+	+
2-12-2				
2-12-3				
2-13-1				
2-13-2				
2-13-3				
2-14-1				
2-14-2				
2-14-3				
2-15-1				
2-15-2				
2-15-3				
2-20-1	+	+		
2-20-2				
2-20-3	+	+	+	+
2-30-1				
2-30-2				
2-30-3				
2-31-1				
2-32-1				
2-32-2				
2-32-3				
2-32-4			+	+
2-40-1				
2-40-2				
2-47-1				
2-47-2				
2-47-3				
2-48-1			+	+
2-48-2				
2-48-3				
1-2N				
1-3N				
1-4N				
N31-3			+	+

+ is a positive detection result.

infected animals, SS is an emerging zoonotic pathogen in humans.[35] Therefore, there is a need for further surveillance and early warning of Pm and SS pathogens and the establishment of a rapid visual portable diagnostic method that combines sensitivity, efficiency and specificity is essential for improving clinical outcomes and ensuring public health safety. Over the past decades, several diagnostic methods have been developed including reverse transcription (RT) PCR, multiplex RT-PCR or real-time RT-PCR-dependent DNA amplification, immuno-capture reverse transcription loop-mediated isothermal amplification, enzyme-linked immunosorbent assay, and small RNA sequencing. However, immune-related diagnostics and small RNA are too expensive for on-site testing. Traditional RT-PCR is limited in its ability to rapidly detect viruses due to its reliance on complex laboratory conditions and equipment. This efficiency has driven the rapid development and widespread use of RPA. In addition, RT-PCR-dependent DNA amplification and reverse transcription (RT) loop-mediated isothermal amplification (RT-LAMP) assays require high temperatures of 65.00 ˚C, which largely reduces their utility for on-site detection. In this study, a dual rapid detection method for Pm and SS was established by combining the characteristics of RPA and LFD. After combining RPA and LFD, the results showed that the detection was more sensitive and that the results could be realized in 40 min with the naked eye. The method can be completed within 40 min at 39.00 ˚C, is not interfered with by several other bacteria and has strong specificity. Compared with the common PCR assay, the detection limit of the RPA-LFD method was 10^{-6} ng µL^{-1}, which was about 100-fold higher than the lowest PCR detection limit of 10^{-4} ng µL^{-1}. The results of clinical samples showed that compared to other methods, RPA-LFD had the advantages of a higher detection rate, a greatly shortened reaction time, simple experimental operation, no need to use other precision instruments such as temperature cycling, etc., which is of great value in resource-limited areas as well as field testing in the field.

In this study, a dual RPA-LFD rapid detection method for Pm and SS was established after optimizing the RPA-LFD reaction conditions and system. After experimental validation, RPA had the highest reaction efficiency at a temperature of 37.00 ˚C. At the same time, the optimal reaction time was determined to be 35 min, which could ensure a sufficient reaction and avoid excessive waiting time. The optimal dosage of Pm primer was 2.40 / 2.40 µL, while the optimal dosage of SS primer was likewise 2.40 / 2.40 µL, and the optimization of the ratios could maximize the sensitivity and accuracy of the assay. In addition, we also optimized the probe ratios and determined the optimal ratios of 2.00, 2.00, and 0.60 µL for Pm primer probes and 1.00, 1.00 and 0.60 µL for SS primer probes, which not only improved the sensitivity of the assay but also ensured the stability of the results. In the specificity test, no cross-reactivity with the control bacteria was detected. In the RPA-LFD sensitivity test, the lowest detection limit was 10^{-6} ng µL^{-1}, which was 10 times higher than that of RPA and 100 times higher than that of the PCR method indicating that the detection results were more sensitive and specific after combining RPA with LFD. Finally, 60 clinical samples were tested and the results showed that the positive detection rate of the method was higher than that of the PCR method which further validated the accuracy and reliability of the method.

Acknowledgments

This work was funded by the Natural Science Foundation of Henan (232300421031), the National Natural Science Foundation of China (32172876 and 32302798), the Program for Innovative Talents (in Science and Technology) in University of Henan (23HASTIT046), Central Plains Thousand Talents Program-Central Plains Science and Technology Innovation youth top talent and the Key R & D and promotion projects of Henan (252102111001).

Conflict of interest

The authors declare no conflicts of interest. Shuang Li (First author) and Jingjing Li (Second author), contributed equally to this study.

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