Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
31 (1): 81-89, 2025
DOI: 10.9775/kvfd.2024.32899

Research Article

Establishment and Application of Dual RPA-Basic and RPA-LFD Detection Method for *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*

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How to cite this article?

Li J, Wei X, Li S, Liu M, Zhu Q, Wang K, Wang L, Cao Y, Chang M, Zhu C, Teng Z, Liu X, Zhang H, Xia X, Ding K: Establishment and application of dual RPA-basic and RPA-LFD detection method for *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. *Kafkas Univ Vet Fak Derg*, 31 (1): 81-89, 2025.
DOI: 10.9775/kvfd.2024.32899

Article ID: KVFD-2024-32899
Received: 28.08.2024
Accepted: 21.12.2024
Published Online: 24.12.2024

Abstract

Based on recombinase polymerase amplification (RPA) detection technology (combined with (lateral flow dipstick, LFD)), it is aimed to establish a dual recombinase polymerase amplification method for the rapid identification of *Pasteurella multocida* (Pm) and *Actinobacillus pleuropneumoniae* (APP). The conserved fragments of Pm *kmt1* gene and APP *ApxIV* gene were selected for the amplification of target fragments. Eight pairs of primers for Pm and APP, and one probe for KMT1Pn and APP323Pn were designed. A single RPA-Basic primer screening test was performed. The reaction time and temperature of double RPA were optimized. The optimal primers and probe matching systems of dual RPA-LFD were explored. Dual RPA sensitivity and specificity tests were performed. The method was used to detect 60 clinical samples. The results of the primer screening test showed that the primers had the strongest specificity and the highest amplification efficiency for ApxIV2698F/ApxIV3020R and KMT1F/KMT1R. The method had the best amplification efficiency at a reaction temperature of 37ºC and a reaction time of 35 min. The optimal primer ratio of KMT1F/KMT1R and ApxIV2698F/ApxIV3020R was 2 µL : 1.5 µL, and the optimal probe ratio of KMT1Pn and APP323Pn was 0.6 µL : 0.4 µL. The minimum detection limit of dual RPA-Basic and RPA-LFD sensitivity test was 10^{-6} ng/µL. The specific test results showed no cross-reaction with enteropathogenic *Escherichia coli, Salmonella, Glaesserella parasuis, Staphylococcus aureus, Streptococcus suis, Aeromonas hydrophila*. Using 60 clinical samples of suspected Pm and/or APP infection to evaluate the detection system, the detection rate of dual RPA-Basic and RPA-LFD is higher than that of PCR, indicating that they have strong practicability. This study successfully established a dual RPA-Basic and RPA-LFD detection method for Pm and APP, which can be used for the rapid differential diagnosis of Pm and APP mixed infection in clinical.

Keywords: *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, Recombinase polymerase amplification, Lateral flow dipstick, Rapid detection

Introduction

Actinobacillus pleuopneumoniae (APP) belongs to the family *Bartonellaceae*, the genus *Actinobacillus*, has pods and is mostly hemolytic. There are many serotypes of APP, and the serotypes correlate with different courses of disease [1]. Based on differences in capsular antigens, APP can be classified into serotypes 1-15. The researchers found serotypes 16-19 in strains that could not be typed [2-4]. All serotypes of APP can cause disease, but the strength of pathogenicity varies. The main clinical features of APP infection in pigs are hemorrhage, necrosis and fibrinous exudation in lungs [5]. The acute form has a high morbidity and mortality rate, which can be up to 80%~100% [6]. Some sick pigs are often accompanied by chronic pneumonia after recovery, resulting in growth stagnation and long-term bacterial and becoming a source of infection for other pigs, causing large economic losses to the pig industry [7].

Pasteurella multocida (Pm) is a Gram-negative zoonotic

pathogen that can cause disease in pigs, cattle, sheep, chickens, ducks, rabbits and other hosts, causing a variety of animal and human infections [8]. Pm usually exists in the nasal cavity, tonsils, lungs and other parts of pigs, and can cause diseases such as swine plague and infectious atrophic rhinitis of pigs [9]. Swine plague is an acute septicemic infection usually characterized by sepsis and hemorrhagic inflammation of tissues and organs [10]. Pm can cause atrophic rhinitis in pigs with clinical signs of sneezing, nosebleeds, and growth retardation [11]. The serious harm of Pm is also manifested in that pigs are susceptible to secondary infection when the body's defense ability is weakened after infection with other disease, and its incidence is generally not affected by seasonal interference, causing significant economic losses to the pig industry [12].

APP and Pm are both common and important pathogens of porcine respiratory disease syndromes and are often mixed infections, which makes actual production prevention and control face a great challenge [13]. At the same time, the use of overdose or non-sensitive antibiotics has a negative impact on the control of animal diseases. The misuse or abuse of antibiotics can lead to the development of bacterial resistance and increased antibiotic residues in pigs and their products [14]. The establishment of rapid detection methods for App and Pm is the key to the successful control of these diseases. The commonly used bacterial detection methods include bacterial isolation, indirect ELISA, indirect hemagglutination, PCR and immunofluorescence, etc. [15,16]. Bacterial isolation and PCR methods are most commonly used. Bacterial isolation takes at least 2~3 d to produce identification results, which is time-consuming and laborious, not conducive to taking measures for timely treatment, and the sensitivity is not high and the results are inaccurate [17]. Although PCR based detection methods can quickly detect pathogens, they require professional technicians and expensive instruments, and is difficult to realize point-of-care testing (POCT). Serologic and immunologic methods are prone to false negative results [15,16]. Recombinase polymerase amplification (RPA) is a nucleic acid isothermal amplification technology developed by the British company TwistDx Inc. in 2006, which has received widespread attention in recent years [18]. This technology eliminates the limitations of conventional PCR requiring precise thermal cycling. RPA utilizes certain specific proteins and enzymes to achieve a target-specific amplification process for the purpose of detecting specific pathogens [19-21]. Due to these characteristics, RPA does not require higher temperature denaturation, annealing and extension steps in the amplification reaction, thus making the nucleic acid amplification more convenient and faster [22]. Compared with other conventional PCR technology, it can be carried out in the temperature range of 20~45ºC, and the reaction speed is faster. Usually, the detection process only takes 15~25 min [18,23,24]. RPA amplification can be combined with agarose gel electrophoresis, lateral flow chromatography (LFD) and fluorescence signal analysis to reflect the amplification results. Among them, RPA electrophoresis (RPA-Basic) has the advantage of low cost, while RPA-LFD has the advantage of visible results to the naked eye and is one of the more common RPA amplification binding methods [25]. The current study has not established a dual RPA detection method with APP and Pm as the research objects. So this study designed primers and probes for APP and Pm based on genes *ApxIV* and *kmt1*, respectively, and established a dual RPA detection method to realize fast APP and Pm detection, and made a preliminary application to provide a new means for veterinary clinical diagnosis and epidemiological investigations.

Material and Methods

Ethical Statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the Henan Institute of Science and Technology with approval code number: 202009.

Experimental Strains

Pasteurella multocida (Pm, C44-1), *Actinobacillus pleuopneumoniae* (APP, CVCC259), *Streptococcus suis* (CVCC606), Enteropathogenic *Escherichia coli (E. coli*, isolated strain), *Glaesserella parasuis* (GPS, isolated strain), *Listeria monocytogenes (L. monocytogenes*, isolated strain), *Salmonella* (CVCC541), *Staphylococcus aureus (S. aureus*, ATCC49525), *Aeromonas hydrophila (A. hydrophila*, AH-1) are all preserved in our laboratory [26].

Primer Design and Synthesis

The gene sequences of APP *ApxIV* (GenBank accession number: HM021153), *16S rRNA* gene (GenBank accession number: D30030.1), disulfide bound formation protein E *(dsbE)* gene (GenBank accession number: AF458420.1), Pm outer membrane protein *(ompH)* gene (GenBank accession number: U50907.1), lipoprotein E *(plpE)* gene (GenBank accession number: GU108958.1) and hydrolase family protein *(kmt1)* gene (GenBank accession number: MN518176.1) published in GenBank were analyzed and compared, and primers and nfo probes were designed based on the principle of designing RPA primers and nfo probes [16]. The 5' end of the probe was labeled with carboxyl (FAM) and the 3' end was modified with C3-Spacer, and the THF site was placed in the middle of the 5' end of the probe. At least 15 nucleotides were added to the 3' end of the probe after the THF residue. The primers

Table 1. *Probe and primers used for RPA-LFD detection*

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	
APP2698F	AGCAGTGCTTCTGTCGTTAGAGTCACGCCTTC	323
APP3020R	Dig-CGAGAATAATCGGCTACCCATTTCCCTTCG	
APP323Pn	FAM-CAATTAAGTAGTATACGCAATGTAAAGCAT [THF]ATCCTACCGTTATGC-C3spacer	
APP3F	ATGGCATTATTTGGCACTGACGGTGATGAT	453
APP3R	GGCCATCGACTCAACCATCTTCTCCACCTG	
ApxIV680F	CTGAACATGAGGATTTGTTTCTCGGTGGTG	680
ApxIV680R	CCCATTATTTCCGTCCGGTTTATTCAGGTC	
16S444F	AAGTTCTTTCGGTAGCGAGGAAGGTATCAA	444
16S444R	GATTTACTACGTTAGCTTCGGGCACCAGAC	
dsbE18F	GCTTCCATACTTGCCTTATTCGGTTATCGT	399
dsbE399R	CGCATCCTTCGGTTGATCGGAATAGGTAAG	

and probes were synthesized by Sango Biotech (Shanghai) Co., Ltd *(Table 1)*.

Genomic DNA Extraction

According to the growth characteristics of Pm, APP, SS, GPS, Enteropathogenic *E. coli*, *L. monocytogenes*, *S. aureus*, *A. hydrophila*, *Salmonella*, the bacteria were cultured to the logarithmic stage and genomic DNA was extracted using the Ezup Column Bacterial Genomic DNA Extraction Kit (Absin Shanghai Biotechnology Co., Ltd.). The concentration of DNA was determined using Nanodrop 2000 and DNA was stored at -80ºC for use.

RPA-Basic Reaction

Single RPA-Basic Primer Screening: The mixture of Pm and APP genomic DNA prepared as above was used as the template for RPA amplification, while ddH_2O was set as the negative control. According to the instruction manual of TwistAmpTM Basic kit (TwistDx (UK) Company), a 50 µL reaction system was set up: 2.4 µL each of forward and reverse primers, 2.2 µL of template (DNA), 29.5 µL of reaction buffer, 2.5 µL of magnesium acetate solution, and ionized water were added to 50 µL, and the reaction was carried out in a metal bath at 39ºC for 20 min. The amplification products were appropriately diluted and detected by electrophoresis on 2 % agarose gel to screen the best primer.

Establishment of Dual RPA-Basic Reaction System and Optimization of Reaction Conditions: The above 50 µL reaction system was slightly modified by adding a pair of primer of 4.8 µL, and deionized water was reduced accordingly. Six reaction temperature gradients of 25°C, 30°C, 35°C, 37°C, 39°C and 45°C were set to determine the optimal reaction temperature. The RPA reaction was carried out according to 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min and 45 min, respectively, to optimize the optimal reaction time. The primer volume ratios were set as (µL): Pm (0, 0) and APP (2.4, 2.4), Pm (0.4, 0.4) and APP (2.4, 2.4), Pm (0.9, 0.9) and APP (2.4, 2.4), Pm (1.3, 1.3) and APP (2.4, 2.4), Pm (1.9, 1.9) and APP (2.4, 2.4), Pm (2.4, 2.4) and APP (2.4, 2.4), Pm (2.4, 2.4) and APP (1.9, 1.9), Pm (2.4, 2.4) and APP (1.4, 1.4), Pm (2.4, 2.4) and APP (0.9, 0.9), and the primer ratios were screened for use in subsequent studies.

Dual RPA-Basic Specificity and Sensitivity Detection: The constructed reaction system was used to detect GPS, Enteropathogenic *E. coli*, *L. monocytogenes, S. aureus, A. hydrophila* and *Salmonella* genomic DNA. Sterile deionized water was used as a negative control to evaluate the specificity of this reaction. The template was successively diluted from 10^{-1} ng/µL to 10^{-8} ng/µL in a 10-fold ratio. The sensitivity of the proposed dual RPA-Basic system was tested and the minimum detection limit of the method was evaluated.

RPA-LFD Reaction

Screening of Dual RPA-LFD Probes: The RPA-nfo amplification reaction was performed according to the recommended reaction conditions for the TwistAmpTM nfo kit (50 µL system) : 29.5 µL reaction buffer, 2.1 µL forward and reverse primers, 0.6 µL probe (10 µM), 2.2 µL template (DNA), and 11 µL nuclease-free water. After the reaction, the results were interpreted using flowmeter chromatographic strips (Ustar Biotechnology (Hangzhou) Co., Ltd.). Specifically, 5 µL was added into a centrifuge tube containing 195µL MGCBB, and after mixing evenly, the sample end of the colloidal gold test strip was inserted into the centrifuge tube for balance, and the results were interpreted within ten minutes. The same reaction conditions were controlled for each pair of Pm or APP RPA-nfo reaction, and positive control and negative control were set for each pair of primers.

Optimization of Dual RPA-LFD Reaction System: In order to improve the sensitivity of RPA-LFD reaction, different primer and probe concentration ratios were set to optimize the RPA-LFD reaction. The reaction components included 40.9 µL A buffer and 2 µL template. The forward and reverse primers and probes of Pm and APP varied according to the ratio gradient of 1 : 1 : 0.3, and a total of 7 experimental groups and negative control group were set up. The above consisted of pre-mixing solution, which was added to 0.2 mL RPA-nfo reaction tube containing lyophilized enzyme powder. Finally, 2.5 µL B buffer was added to the lid of the reaction tube and thoroughly mixed. After mixing, the reaction liquid was thrown (or rapidly centrifuged) to the bottom of the tube, and then the reaction tube was immediately incubated at 39ºC in a constant temperature device for 20 min. After the reaction was over, 5 µL was taken and added into a centrifuge tube containing 195 µL MGCBB, and after mixing evenly, the sample end of the colloidal gold test strip was inserted into the centrifuge tube for balance, and the quality control line and detection line were observed. The results were interpreted within 10 minutes.

Specificity and Sensitivity of Dual RPA-LFD: In order to explore the specificity of RPA, Pm, APP, SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella* genomic DNA were extracted as detection objects, respectively. The specificity of the method was evaluated by RPA-LFD detection under the optimized double RPA-LFD optimal conditions. The template was diluted at a multiplicity of 10^{-1} ng/µL ~10^{-7} ng/µL. Sensitivity experiments were performed using the established dual RPA-LFD system to evaluate the lowest detection limit of the method.

PCR Detection

The PCR assay (Sango Biotech (Shanghai) Co., Ltd.) was performed with a 25 µL system, and the amplification program was 94°C pre-denaturation for 5 min, denaturation at 94°C for 40 s, annealing at 63°C for 45 s, and extension at 72°C for 90 s, with a total of 35 cycles. The amplified PCR products were verified on a 2% agarose gel.

Clinical Sample Test

To further confirm the effect of using the dual RPA-Basic and RPA-LFD in clinical application, 60 clinical lung tissues of pigs suspected of being infected with Pm and/or APP from Henan and Shandong provinces were collected, and dual RPA methods were performed under the optimal reaction conditions using pMD-18T-kmt1 and pMD-18T-ApxIV recombinant plasmid as a positive control, ddH_2O as a blank control, and total DNA from healthy pig lung tissues as a negative control. The results were compared to those obtained from a conventional PCR assay conducted on DNA extracted from the respective clinical samples.

Statistical Analysis

SPSS 24.0 statistical software was used for statistical analysis, with the operating procedures for testing Actinobacillus pleuropneumoniae in pigs (local standard in Anhui Province) and the diagnostic techniques for porcine brucellosis (agricultural industry standard in the People's Republic of China) as the gold standards. The detection efficiency of RPA-LFD and PCR were calculated separately, and Kappa test was used to analyze the consistency of the two results. Kappa<0.4 indicates low consistency; 0.75≥Kappa≥0.4 indicates moderate consistency; When 1.0 ≥Kappa>0.75, it is considered highly consistent.

Results

Screening of Primers

The study designed a total of 8 primer pairs for Pm *kmt1*, *OmpH*, and *PlpE* genes along with APP *ApxIV*, 16S rRNA, and *dsbE* genes *(Table 1)*. The results showed that APP ApxIV2698F/ApxIV3020R primer pairs and Pm KMT1F/KMT1R primer pairs could obtain clear and single bands at positions 323 bp and 129 bp on electropherograms after RPA amplification reactions, while the other primer pairs were amplified without bands, dragging, or non-specific bands *(Fig. 1-A,B)*. Genomic DNA of SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella* were used as positive controls (ddH_2O was used as a negative control) for the Pm/APP, Pm, and APP Basic-RPA assay. The results are shown in *Fig. 1-C*, and no bands appeared in all lanes except for the positive control, which showed bright bands. It indicated that the specificity of the APP ApxIV2698F/ApxIV3020R primer pair and the Pm KMT1F/KMT1R primer pair was

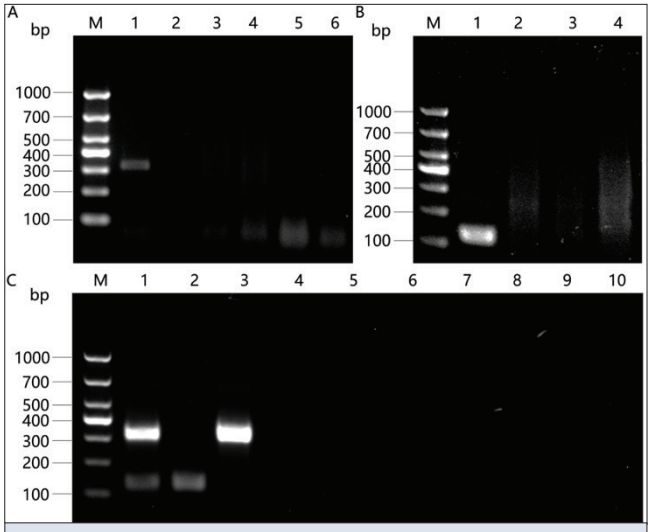

Fig 1. RPA-Basic primer screening results. A. Screening results of APP primer RPA-Basic. M: DL1000 DNA Marker; 1~6 are: primer KMT1F/KMT1R, negative control, primer dsbE18F/dsbE399R, primer ApxIV680F/ApxIV680R, primer 16S444F/16S444R, primer APP3F/APP3R; B. Screening results of Pm primer RPA-Basic. M: DL1000 DNA Marker; 1~4 are: primer KMT1F/KMT1R, primer OmpHF/OmpHR, negative control, primer PlpEF/PlpER; C. Specific results of primer selected by Pm and APP. M: DL1000 DNA Marker; 1~10 are: Pm/APP, Pm, APP, SS, GPS, Enteropathogenic *E. coli*, *L. monocytogenes*, *S. aureus*, *A. hydrophila*, *Salmonella*, negative control

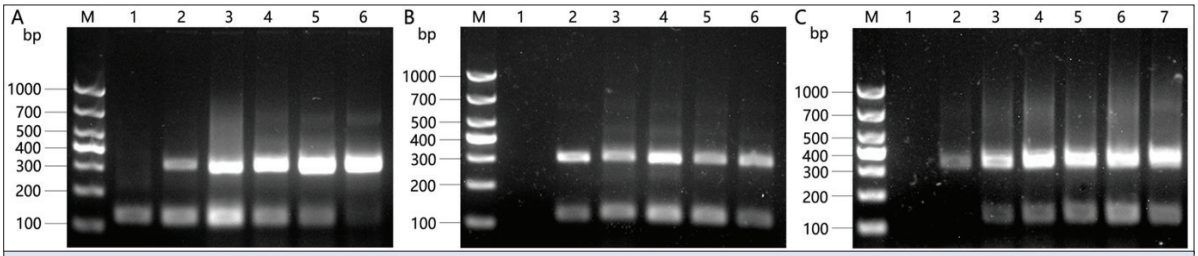

Fig 2. Optimization of dual RPA-Basic amplification conditions. A. RPA-Basic primer ratio screening results. M: DL1000 DNA Marker; The volume ratio of primer 1-6 (μL): Pm (0.2, 0.2) and APP (2.4, 2.4), Pm (0.4, 0.4) and APP (2.4, 2.4), Pm (0.7, 0.7) and APP (2.4, 2.4), Pm (1.2, 1.2) and APP (2.4, 2.4), PM (2.4, 2.4) and APP (2.4, 2.4), Pm (1.7, 1.7) and APP (2.4, 2.4); B. Reaction temperature optimization results. M: DL1000 DNA Marker; The reaction temperatures from 1 to 6 are: 25ºC, 30ºC, 35ºC, 37ºC, 39ºC, 45ºC; C. Reaction time optimization results. M: DL1000 DNA Marker; The reaction time from 1 to 7 are 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min

good, so ApxIV2698F/ApxIV3020R and KMT1F/KMT1R primer pairs were identified to be used in the subsequent experiments.

Optimization of Dual RPA-Basic Reaction Conditions

The results of RPA-Basic reaction showed that the brightness of electrophoretic bands was relatively consistent when the primer volume ratio was 2.4 μL upstream and downstream of Pm and 0.7 μL upstream and downstream of APP *(Fig. 2-A)*. After repeating the experiments, the optimal volume ratio of Pm and APP primer was finally selected as 2.4 μL: 0.7 μL. The optimal primer volume ratio was used for the screening of RPA-Basic reaction time and temperature. As shown in *Fig. 2-B*, the RPA-Basic amplification products could be specifically detected when the temperature was between 30 and 45°C. Considering the later application of clinical detection, 37°C was chosen as the optimal reaction temperature in this study. As shown in *Fig. 3-C*, the electrophoresis results showed that the RPA-Basic method could detect target product in the range of 15~40 min at the reaction temperature of 37°C, but there was no significant difference in the brightness of electrophoretic bands after 35 min. In order to ensure the timeliness of the detection, 35 min was chosen as the optimal reaction time in this study.

Specificity and Sensitivity of the Dual RPA-Basic Assay

The specificity of the dual RPA-Basic assay was performed using optimized reaction conditions. The results are shown in *Fig. 1-C*, where no bands appeared in all lanes except for the positive control, which appeared as bright bands, indicating that the specificity of this RPA-Basic assay was good. After determining the specificity, the sensitivity of the dual RPA-Basic detection system was studied. The

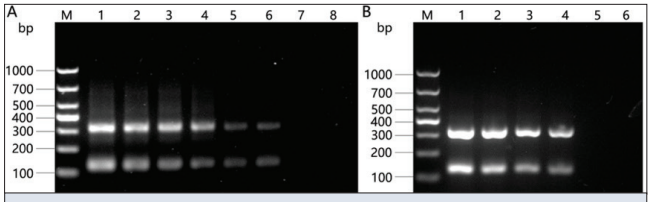

Fig 3. Comparison of the specificity and sensitivity of the dual RPA-Basic assay with the PCR assay. A. Basic-RPA sensitivity test results. M.1000 DNA Ladder; Nucleic acid concentrations in lanes 1-8 are (ng/μL): 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, negative control; B. PCR sensitivity test results. M.1000 DNA Ladder; Nucleic acid concentrations in lanes 1-6 were in the order of (ng/μL): 10-1, 10-2, 10-3, 10-4, 10-5, negative control

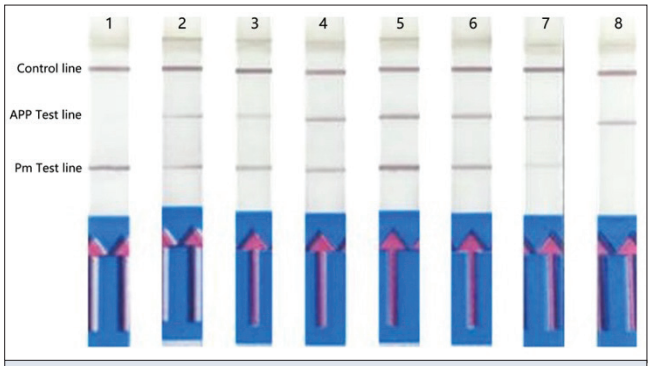

Fig 4. Screening results of dual RPA-LFD detection probes. Lanes 1-9 are: M: DL1000 DNA Marker; primer volume ratios from 1 to 8 are (μL): APP (0, 0, 0) and Pm (2, 2, 0.6), APP (0.7, 0.7, 0.4) and Pm (2, 2, 0.6), APP (1.5, 1.5, 0.4) and Pm (2, 2, 0.6) , APP (2, 2, 0.6) and Pm (1, 1, 0.6), APP (2, 2, 0.6) and Pm (1.5, 1.5, 0.4), APP (2, 2, 0.6) and Pm (1, 1, 0.4), APP (2, 2, 0.6) and Pm (0.5, 0.5, 0.4), APP (2, 2, 0.6) and Pm (0, 0, 0.4)

results are shown in *Fig. 3*, which shows that as the mixed template concentration gradually decreased from 10^{-1} ng/μL, the color of the detection line of APP and Pm also gradually became lighter until it disappeared at 10^{-7} ng/μL template concentration. Tt can be concluded that the RPA-Basic reaction system can detect as low as 10^{-6} ng/μL, which is higher than the lowest detection concentration of PCR (10^{-4} ng/μL).

Screening of Dual RPA-LFD Detection Probes

The corresponding probes were designed according to the primers obtained from RPA-Basic screening. The selected reverse primers for Pm and APP were labeled with biotin and digoxin, respectively, and the designed probes were labeled with FAM fluorescein. The results are shown in *Fig. 4*, and obvious double detection lines can be seen in experimental groups 2, 3, 4, 5, 6 and 7. In order to keep the color depth of APP and Pm detection lines consistent and facilitate the subsequent detection, the combinations of Pm upper and lower primer probe volumes of 2 μL, 2 μL, and 0.6 μL and APP upper and lower primer probe volumes of 1.5 μL, 1.5 μL, and 0.4 μL were comparatively selected for the subsequent experiments.

Specificity and Sensitivity of the Dual RPA-LFD Assay

Using the optimized dual RPA-LFD system with Pm and APP, Pm, APP as positive control, ddH_2O as negative

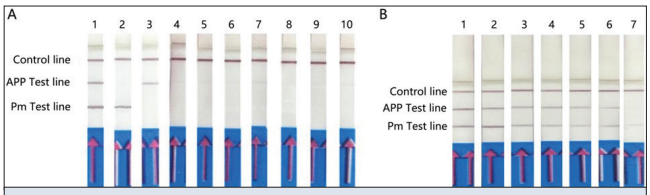

Fig 5. Specificity and sensitivity of RPA-LFD. A. Specificity and sensitivity of RPA-LFD. 1-10 are: Pm/APP positive control, Pm positive control, APP positive control, negative control, SS, GPS, Enteropathogenic *E. coli*, *L. monocytogenes, S. aureus, A. hydrophila, Salmonella*. B. RPA-LFD sensitivity. 1-7 Nucleic acid concentrations are (ng/μL): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}

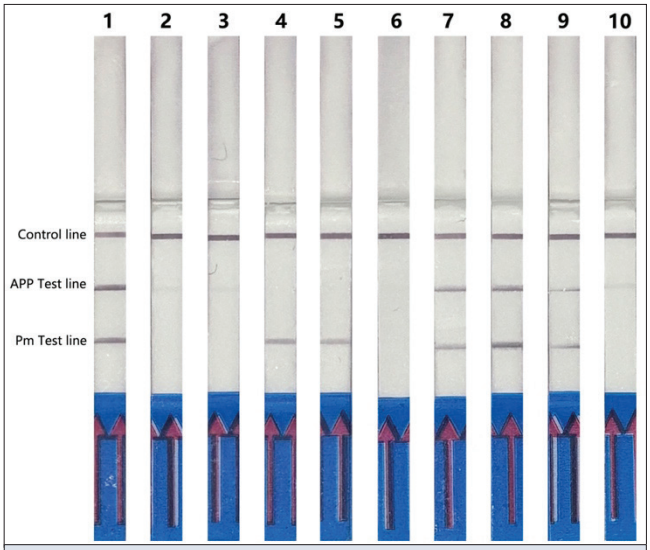

Fig 6. Clinical sample results with the dual PRA-LFD system. Lane 1, Pm and APP positive control, Lane 2, negative control, Lanes 3-10, clinical test samples

control, SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella* as detection objects for RPA-LFD assay. As shown in *Fig. 5-A*, the double positive and single positive results of Pm and APP were established, and the other groups and negative controls showed only the quality control line, which determined that the specificity of the assay was established. To determine the sensitivity of the dual RPA-LFD, the template was sequentially diluted to seven concentrations ranging from 10^{-1} to 10^{-7} ng/μL in 10-fold ratios. The sensitivity experiments were carried out using the established optimal dual RPA-LFD system, and the results were shown in *Fig. 5-B*. The color of the detection lines of APP and Pm gradually faded from 10^{-1} to 10^{-5} ng/μL, and the detection lines completely disappeared after 10^{-6} ng/μL, and only the quality control line appeared in the negative control. Therefore, the detection limit of the dual RPA-LFD assay established in this study was 10^{-6} ng/μL, which is higher than the lowest detection concentration of PCR (10^{-4} ng/μL).

Clinical Sample Test Results

Sixty clinical samples were tested by PCR, double RPA-Basic and double RPA-LFD, and the results of the three

Table 2. Application of different methods for the detection of actual sample

Detection Methods	Number of Positive Cases			Sensitivity (%)			Specificity (%)			Positive Predictive (%)			Negative Predictive (%)			Kappa		
	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP
PCR	7 (11.7%)	8 (13.3%)	2 (3.3%)	76.9	68.2	66.7	100	100	100	100	100	100	94.3	86.5	94.9	0.795	0.631	0.592
RPA-Basic	9 (15.0%)	15 (25.0%)	4 (6.7%)	90.9	100	100	100	100	100	100	100	100	98.0	100	100	0.936	1.0	1.0
RPA-LFD	9 (15.0%)	15 (25.0%)	4 (6.7%)	90.9	100	100	100	100	100	100	100	100	98.0	100	100	0.936	1.0	1.0

methods were statistically analyzed. Using the established dual LFD-RPA method to detect each serotype strain of Pm and APP, the test lines could be clearly observed *(Fig. 6)*. As shown in *Table 2*, a total of 7 Pm-positive samples, 8 APP-positive samples and 1 Pm/APP double-positive sample were detected by conventional PCR. Dual RPA-LFD detected a total of 9 Pm-positive samples, 15 APP-positive samples and 4 Pm/APP-positive samples. In summary, the dual RPA-LFD method has a higher detection rate than PCR. From the point of the difficulty and time spent on the operation of the three methods, RPA-LFD takes the shortest time, and the operation process is relatively simple. There is no need to learn the operation skills of large-scale instrumentation, and only need to master the basic experimental techniques to complete the whole experiment.

Discussion

In recent years, with the rapid development of pig farming and the increasing degree of intensification of farming, the impact of bacterial diseases on pig farming has been highlighted. The intensive environment of pig farming provides very favorable conditions for pathogens to multiply and infect pig herds, which brings great difficulties in disease prevention and control. APP is a typical bacterium that infects and attacks the lungs through the respiratory tract, and its infection leads to severe irreversible damage to the lungs, resulting in acute death. Respiratory infectious diseases caused by APP have always been one of the most important bacterial infectious diseases plaguing the pig farming industry [27]. Pm is the main representative bacterium of the genus Pasteurella, which can cause a variety of diseases such as atrophic rhinitis and hemorrhagic septicemia in pigs. At present, a variety of diseases caused by Pm have different degrees of occurrence and prevalence in many areas, and the harm to the pig industry is becoming more and more serious. This study selected the highly conserved the conserved fragments of Pm *kmt1* gene and APP *ApxIV* gene as the diagnostic target and developed RPA-Bacic and RPA-LFD detection method with high sensitivity, strong specificity, and wide detection range, which can be conveniently and quickly applied in laboratories, especially in POCT.

The *kmt1* gene is a species-specific gene of Pm and has been used as a target gene for PCR and LAMP methods to detect all subspecies of Pm [28,29]. The *plpE* gene is present in all serotypes of Pm and is a specific conserved gene of the bacterium. The sequence similarity of the *plpE* gene in different serotypes of Pm is more than 92%, which makes it suitable for the detection of Pm pathogen [30]. *OmpH* is a major protein presented on the outer membrane of Pm. Comparison of the *OmpH* sequences of 15 serotypes of Pm shows that this protein is highly conserved (72-100% homology) [31,32]. Both purified natural *OmpH* and whole bacteria can induce high levels of antibodies, and the induced protection rate is comparable [31,32]. In this study, three primers were designed for Pm-conserved *kmt1, plpE,* and *OmpH* genes, and the best primers with the highest amplification efficiency, namely KMTF1/KMTR1 (and corresponding nfo probes), were analyzed and selected by RPA method for subsequent RPA experiments. The *Apx IV* gene can be found in all serotypes of APP, so it can be used as a target gene for detecting APP species-specific [16]. Primers were also designed for APP 16S rRNA and *dsbE* genes in this study, but they were not effective. Finally, this study established dual RPA-Basic and RPA-LFD assays based on the conserved genes *Apx IV* of APP and *kmt1* of Pm.

It can be seen from this study that specific amplification bands can be shown after 15 min from the beginning of the RPA-Basic reaction, and the amplification effect is better at 25~45 min, and the best amplification effect is achieved at 35 min, which indicates the superiority of the RPA technology in terms of the detection time. The amplification bands of the RPA reaction have obvious bands in the range from 25 to 45°C, which shows that the amplification effect is better in this temperature range. When the temperature exceeded 37°C, the amplification bands showed a tendency to weaken, which may be caused

by the higher temperature reducing the enzyme activity in the reaction. In this experiment, the optimal reaction temperature was set at 37ºC and the optimal reaction time was set at 35 min. It was found that the lowest detection limit of Basic-RPA and RPA-LFD reached 10^{-6} ng/μL, which was higher than that of the conventional PCR assay and had good sensitivity. In addition, the primers/probes designed in this study were used to amplify RPA with SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella*, and the results showed that no specific bands were generated, which verified that the method had good specificity. The RPA assay is convenient, short and simple and can be applied to rapid detection in the front line of farm production. Finally, the dual RPA methods and conventional PCR methods were used to test the DNA extracted from clinical samples. The results show that using industry standards and local standards as the gold standard, dual RPA methods detection of Pm and APP has a specificity of 100% and a sensitivity of 90.9%. PCR technology showed 100% specificity and a sensitivity of 76.9%. The sensitivity of dual RPA methods is higher than that of PCR technology, which demonstrated the reliability and practicality of the dual RPA methods.

In conclusion, based on the exploration and optimization of RPA reaction conditions, the APP/Pm dual RPA-Basic and RPA-LFD rapid detection methods were successfully established. These two methods can complete the detection within 50 min, which is faster than the traditional PCR method. It does not require special instruments and experimental conditions, such as thermal cycler, which is suitable for basic units and on-site testing environments with a lack of instruments. The equipment is portable and simple to operate, without the need for professional operators, and has a high degree of specificity. In conclusion, this study is expected to provide a faster and more reliable detection technology for the implementation of APP and Pm detection and monitoring as well as prevention in farms, customs entry-exit animal disease quarantine laboratories and other institutions, as well as to provide a reference for the development of other animal disease detection technologies.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (X. X and K. D).

Acknowledgements: We thank all authors for their significant contributions to this study.

Financial Support: This research work was funded by the Central Plains Thousand Talents Program-Central Plains Science and Technology Innovation youth top talent, the Program for Innovative Talents (in Science and Technology) in University of Henan Province (23HASTIT046), the Excellent Youth Science Foundation of Henan Province (232300421031), the National Natural Science Foundation of China (Grant Nos. 32172876 and 32473070), the Key Specialized Research and Development Breakthrough Program in Henan Province (32102110123 and 232102110108), the College Students' Innovative Entrepreneurial Training Plan Program (202410467019) and the China Postdoctoral Science Foundation (2020 M672230).

Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): Not applicable.

Ethical Statement: All procedures performed in studies involving animals were in accordance with the ethical standards of the Henan Institute of Science and Technology with approval code number: 202009.

Author Contributions: XX and KD designed the research and project outline. JL, XW, SL and ML carried out the experiments and analysis the data. QZ, KW, LW, YC, MC, CZ, ZT, XL and HZ drafted the manuscript. HZ, XX and KD revised the manuscript. All authors have read and approved the final manuscript.

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