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

Kafkas Univ Vet Fak Derg
28 (5): 601-611, 2022
DOI: 10.9775/kvfd.2022.27691

RESEARCH ARTICLE

Rapid Visual Detection of *Streptococcus suis* and *Actinobacillus pleuropneumoniae* Through Duplex Recombinase Polymerase Amplification Combined with Lateral Flow Dipsticks

Shanshan ZHANG [1,a,†] Hongbing XIE [1,b,†] Mingcheng LIU [1,c] Airong ZHENG [2,d] Haixing YAN [1,e] Mingyuan DUAN [1,f] Xiaobing WEI [1,g] Zhanwei TENG [1,h] Jianhe HU [1,i] Huihui ZHANG [1,j (*)] Xiaojing XIA [1,k (*)]

[†] *These authors contribute equally to this work*

[1] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, CHINA

[2] Forage and Feed Station of Henan Province, Zhengzhou 450008, CHINA

ORCIDs: [a] 0000-0003-1288-0829; [b] 0000-0001-8178-9928; [c] 0000-0003-4923-1427; [d] 0000-0003-2544-3329; [e] 0000-0003-1199-201X; [f] 0000-0002-8163-777X; [g] 0000-0001-6114-2473; [h] 0000-0003-1160-2237; [i] 0000-0002-6768-0851; [j] 0000-0002-7396-2249; [k] 0000-0001-7717-8411

Article ID: KVFD-2022-27691 Received: 21.04.2022 Accepted: 04.09.2022 Published Online: 10.09.2022

Abstract: Primers and corresponding probes were designed for the glutamate dehydrogenase *(gdh)* gene of *Streptococcus suis* and the *Apx*IV gene of *Actinobacillus pleuropneumoniae* to establish a dual recombinant enzyme polymerase amplification (RPA)-lateral flow dipstick (LFD) detection method for the simultaneous rapid identification of *S. suis* and *A. pleuropneumoniae.* The specificity test showed that the amplification results for other pathogens were all negative, indicating that the method exhibited good specificity. The sensitivity test showed that the lowest nucleic acid concentration detectable with this method was 10^{-5} ng/μL, which was significantly higher than that observed with PCR and basic RPA. The results showed that this method detected all reference strains and clinical isolates, which was consistent with the PCR detection results. Among the 45 clinical samples, 19 cases of *S. suis,* 1 case of *A. pleuropneumoniae* and no mixed infections were detected. The detection rate was higher than that observed with bacterial isolation and the conventional PCR method, which indicated that this method is very practical and suitable for the rapid clinical detection of *S. suis* and *A. pleuropneumoniae.* Compared with the traditional method, the dual RPA-LFD method has several advantages, including high specificity, high sensitivity, fast speed and minimal requirement of instruments and equipment. In addition, the method can achieve the synchronous and rapid detection of *S. suis* and *A. pleuropneumoniae* and is helpful for the preliminary screening of clinical diseases.

Keywords: *Streptococcus suis, Actinobacillus pleuropneumoniae, Recombinase polymerase amplification, Lateral flow dipstick, Rapid detection*

Streptococcus suis ve *Actinobacillus pleuropneumoniae*'nin Lateral Flow Dipstick İle Kombine Edilmiş Dubleks Rekombinaz Polimeraz Amplifikasyonu Yoluyla Hızlı Görsel Tespiti

Öz: Primerler ve karşılık gelen problar, *Streptococcus suis* ve *Actinobacillus pleuropneumoniae*'nin eş zamanlı hızlı identifikasyonu sağlayan bir dual rekombinant enzim polimeraz amplifikasyon (RPA)-lateral flow dipstick (LFD) teşhis metodunun geliştirilmesi için ilgili bakterilerin sırasıyla glutamat dehidrojenaz *(gdh)* geni ve ApxIV geni için tasarlanmıştır. Yapılan özgüllük testinde, diğer patojenler için amplifikasyon sonuçlarının hepsinin negatif saptanması bu yöntemin iyi bir özgüllük sergilediğini gösterdi. Duyarlılık testi, bu yöntemle saptanabilen en düşük nükleik asit konsantrasyonunun10-5 ng/μL olduğunu gösterdi ve bu değer, PCR ve temel RPA ile saptananlardan önemli ölçüde daha yüksekti. Sonuçlar, bu yöntemin, PCR ile saptananlarla tutarlı olarak tüm referans suşları ve klinik izolatları belirlediğini gösterdi. 45 klinik örnek arasından 19'u *S. suis* ve 1'i *A. pleuropneumoniae* olarak saptandı ve miks enfeksiyon tespit edilmedi. Tespit oranı, bakteri izolasyonu ve geleneksel PCR yöntemine göre daha yüksekti, bu da bu yöntemin oldukça pratik olduğunu ve *S. suis* ve *A. pleuropneumoniae*'nin hızlı klinik tespiti için uygun olduğunu gösterdi. Geleneksel yöntemle karşılaştırıldığında, dual RPA-LFD yönteminin yüksek özgüllük, yüksek hassasiyet, hız ve minimum alet ve ekipman gereksinimi gibi birçok avantajı vardır. Ayrıca bu yöntem, *S. suis* ve *A. pleuropneumoniae*'nin eşzamanlı ve hızlı tanısını sağlayabilecek ve klinik enfeksiyonların ön taramasına yardımcı olabilecek niteliktedir.

Anahtar sözcükler: *Streptococcus suis, Actinobacillus pleuropneumoniae, Recombinase polymerase amplification, Lateral flow dipstick, Rapid detection*

How to cite this article?

Zhang S, Xie H, Liu M, Zheng A, Yan H, Duan M, Wei X, Teng Z, Hu J, Zhang H, Xia X: Rapid visual detection of *Streptococcus suis* and *Actinobacillus pleuropneumoniae* through duplex recombinase polymerase amplification combined with lateral flow dipsticks. *Kafkas Univ Vet Fak Derg,* 28 (5): 601-611, 2022. DOI: 10.9775/kvfd.2022.27691

[(*)] Corresponding Author

Tel: +86-17698229153 (H. Zhang), +86-18838765510 (X. Xia)
E-mail: chery2017@126.com (H. Zhang), quik500@163.com (X. Xia), xjxia@hist.edu.cn (X. Xia)

Introduction

Streptococcus suis is one of the main pathogens that causes significant economic losses in the swine industry and is present in almost 100% of large-scale pig farms worldwide [1]. The main route of infection in pigs is considered the respiratory tract, and the resulting infection mainly causes sepsis, meningitis, arthritis, pneumonia, endocarditis, and even sudden death in severe cases [1-3]. SS serotype 2 (SS2), the most virulent serotype, is the most commonly isolated in human infection cases (74.7% of cases) [1,4]. Furthermore, *S. suis* is a zoonotic pathogen that can infect humans through contact with carrier pigs or pork products and thereby causes septicemia, meningitis, shock-like syndrome, or even death [1]. Over the past 50 years, most *S. suis* infections in humans have involved sporadic cases of occupational contact, such as with farmers, veterinarians, butchers, food processors and other occupational groups [5]. However, the number of cases involving *S. suis* infection in humans has increased in recent years, exhibiting a trend toward the general population. Two large-scale outbreaks of *S. suis* infection, which included a total of 229 infections and 52 deaths, have occurred in Jiangsu Province and Sichuan Province, China; these outbreaks have attracted great attention in the field of public health and scientific research and have seriously threatened public health and safety [5-7].

Actinobacillus pleuropneumoniae infection can cause severe irreversible damage to the lungs [8]. In acute disease outbreaks, morbidity can range from 10% to 100%, and mortality of 1%-10%. The peracute form is characterized by a high mortality rate and sudden death. It can also show subacute and chronic course. *A. pleuropneumoniae* can infect pigs of all ages, and its clinical manifestations are characterized by fibrinous hemorrhagic and necrotizing pleuropneumonia [8]. The bacteria have many serotypes, and there is no cross-protection between serotypes during immunization with inactivated vaccines, which poses a great challenge in the prevention and control of the disease [9]. Currently, the disease is distributed worldwide [8]. *A. pleuropneumoniae* can be detected in all major swine-producing countries, but the virulence of local strains may vary [8].

Respiratory disease is the most important health problem that affects the swine industry worldwide and is commonly known as porcine respiratory disease syndrome (PRDC) [10]. Clinically, *A. pleuropneumoniae* and *S. suis* are common and important pathogens of the respiratory tract in pigs, and their mixed infection triggers PRDC that leads to lung lesions, which in turn results in large economic losses and impaired animal welfare [10,11]. In recent years, the number of cases of mixed infections with *S. suis* and *A. pleuropneumoniae* has gradually increased [10]. These infections are challenging to detect through traditional bacteriological diagnosis; thus, the establishment of a detection method for the rapid diagnosis and identification of these two bacteria is necessary. Such a method is very important for preventing and controlling the spread of the disease in a timely manner. At present, the combination of bacterial isolation and culture and serological typing remains the gold standard for detecting these two pathogens, but this method is time-consuming, demonstrates low sensitivity and has high operational requirements; thus, this method is unfavorable for the promotion of epidemic monitoring [12,13]. In the past two decades, polymerase chain reaction (PCR)-based molecular detection technology has been widely used in the molecular diagnosis of *A. pleuropneumoniae* and *S. suis*. Conventional PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-sequence characterized amplified region (PCR-SCAR), double PCR, real-time fluorescence quantitative PCR and other technologies are used for the amplification of either conserved genes or the capsular polysaccharide genes specific to different serotypes to achieve the rapid detection and identification of these two pathogens [13-15]. PCR-based molecular technology is an established and reliable method for the isolation and identification of *A. pleuropneumoniae* and *S. suis* but also has various characteristics, including extensive requirements for instruments and equipment, demanding experimental conditions and skilled laboratory personnel.

In recent years, isothermal amplification technology has received increasing attention for the detection of pathogens, and among the related techniques, loop-mediated isothermal amplification (LAMP) is a commonly used method [16]. However, LAMP technology has many problems, including the need for complex primer designs along with multiple pairs of primers; the occurrence of nonspecific amplification, which is due to the ease of interaction between products; and the inability to verify the accuracy of the amplification results by sequencing [17,18]. Therefore, there remains a clinical need for a simple, rapid, and sensitive method that can be used for the early diagnosis of *S. suis* infection. As an isothermal DNA amplification technology, recombinase polymerase amplification (RPA) has undergone rapid development in the field of molecular diagnostics [19]. Due to its simplified instrument requirements and shorter reaction time compared with those of other methods, RPA is considered the most applicable method for on-site diagnosis [19]. A lateral flow dipstick (LFD) is a simple detection device that can be used for the qualitative or semiquantitative detection of target nucleotides and can be used in the field or small regional laboratories without the help of instruments [20]. By labeling with fluorophores, the RPA amplification products can be combined with a LFD (i.e., RPA-LFD) to visually detect amplification products. RPA-

LFD technology has the following characteristics: simple operation, high sensitivity, strong specificity, and suitability for rapid on-site diagnosis [19]. Thus, this technology is suitable for clinical and field detection.

Glutamate dehydrogenase (Gdh), which is a bridge that connects carbon and nitrogen metabolism, plays an important role in the process of bacterial energy metabolism and thereby directly affects the pathogenicity of bacteria [21,22]. The Gdh protein of *S. suis* is expressed at the surface of bacterial cells and is an antigenic, extremely evolutionarily conserved and antigenic component; therefore, Gdh can be used as a diagnostic antigen for *S. suis* to establish a universal detection method for all *S. suis* serovars [22]. *Apx*IV is a species-specific toxin of *A. pleuropneumoniae*, and the *apx*IV sequence is highly conserved among all serotypes and is absent in other species of *Actinobacillus*, *Haemophilus*, and *Pasteurella multocida*; therefore, *apx*IV is often used as a specific clinical diagnostic gene for *A. pleuropneumoniae* [13]. In this study, we designed specific RPA primers targeting the *gdh* gene of *S. suis* and the *apx*IV gene of the *A. pleuropneumoniae* toxin to establish a dual RPA-LFD detection method for the simultaneous detection of *S. suis* and *A. pleuropneumoniae*.

Material and Methods

Strains

Streptococcus. suis serotype 1 (SS1) strain JZLQ036, SS2 strains CVCC606, CVCC1941, JZLQ022, ZY05719, 05ZYH33, and JZLQ019, SS7 strain JZLQ034, SS9 strain JZLQ035, *A. pleuropneumoniae* serotype 1 4074 strain (APP1, CVCC259) and serotype 5b L20 strain (APP5b, CVCC263) were kindly provided by Professor Shen Zhiqiang and Professor Lei Liancheng. Enteropathogenic *Escherichia coli*, *Glaesserella parasuis*, gen. nov., comb. nov., *Pasteurella*, *Salmonella* (ATCC 25922), *Staphylococcus aureus* (ATCC 49525), and *Aeromonas hydrophila* AH-1) were sourced from our Institute. The pMD-18T-gdh plasmid was preserved by our laboratory [23].

Primer Design and Synthesis

The *S. suis gdh* gene sequence (GenBank accession number: AF229683) and *A. pleuropneumoniae Apx*IV gene sequence (GenBank accession number: HM021153) were downloaded from GenBank and compared and analyzed using DNAStar software. Five pairs of *S. suis*-specific RPA primers and five pairs of *A. pleuropneumoniae*-specific RPA primers were designed, and these primers were then synthesized by Sangon Biological Engineering Co., Ltd.

Bacterial Genomic DNA Extraction

The genomic DNA templates of all strains used in the experiment were extracted from overnight cultures of bacteria using a bacterial DNA extraction kit. The concentration of the extracted DNA templates was uniformly diluted to 10 ng/μL for use.

To obtain bacterial DNA from tissues, 1 mL of PBS was added to 2 g of infected pig lung tissue, and a suspension produced by grinding. The suspension was centrifuged at 2000 g/min for 5 min, and a 500-μL aliquot of supernatant was collected and centrifuged at 12000 g/min for 5 min. The supernatant was discarded, and 100 μL of bacterial lysis buffer was added for resuspension. The suspension was maintained at 57°C for 1 h and then at 100°C for 10 min. The suspension was then centrifuged (12000 g/min, 5 min), and the supernatant was stored at -80°C until use for the RPA reaction.

RPA-Basic

For screening of the primers, the RPA reaction was performed using 2 μL of the template and the conditions provided in the instructions for the TwistAmp® Basic kit (TwistDX, UK). The screening ensured that the primer reaction conditions for each *S. suis* or *A. pleuropneumoniae* pair were the same, and templates for positive and negative controls were set for each pair of primers.

After selection of the optimal primer pairs, the RPA reaction was performed using a 50-μL preliminary reaction system according to the instructions for the TwistAmp® Basic kit (TwistDX, UK). The single-plex reaction fraction consisted of 29.5 μL of reaction buffer, 2.4 μL of each of the *S. suis* and *A. pleuropneumoniae* forward and reverse primers, 2 μL of the template (DNA) and 6.4 μL of nuclease-free water. The above mixture was gently mixed, added to the lyophilized RPA reaction tube, shaken and mixed well to rehydrate the solid reactant in the lyophilized state. Afterward, 2.5 μL of 280 mM MgAc was added and incubated at 39°C for 20 min using a thermocycler, and the amplified products were detected by 2% agarose gel electrophoresis. To determine the optimal reaction conditions of dual RPA for RPA-Basic reactions, the RPA primer concentration ratio, temperature and time in the reaction system were optimized. First, the reaction system was used for the screening of different primer volume ratios, and the determined primer ratios were then used for the stepwise screening of different temperatures (30°C, 35°C, 37°C, 39°C, and 45°C) and durations (5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, and 40 min).

The dual RPA-Basic assay was evaluated using genomic DNA of six nontarget bacteria, namely, enteropathogenic *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*, to assess the specificity of the RPA-LFD. The template was subjected to 10-fold sequential dilutions to obtain 9 different concentrations (10^{0}-10^{-8} ng/μL). The sensitivity experiment

was performed using the established dual RPA-Basic assay to evaluate the lowest limit of detection of the method.

RPA-LFD

The RPA-nfo amplification reaction was performed according to the conditions provided with the DNA constant-temperature rapid amplification kit (TwistAmp RPA nfo kit; TwistDX), and after the reaction was completed, the results were interpreted using the Milenia GenLine HybriDetect kit. Specifically, 5 μL was added to a centrifuge tube containing 195 μL of HybriDetect Assay Buffer, and after even mixing, the sample end of the colloidal gold test strip was inserted into the centrifuge tube and allowed to reach equilibrium; the result was interpreted within 10 min. The same controlled reaction conditions were used for each pair of *S. suis* or *A. pleuropneumoniae* RPA-nfo reactions, and positive and negative controls were established for each pair of primers.

To improve the sensitivity of RPA-LFD, different primer and probe concentration ratios were evaluated. The reaction components included buffer A, template, primers and probes (the *S. suis* and *A. pleuropneumoniae* forward and reverse primers and probes were changed to obtain a ratio of 1:1:0.3, and a total of 7 groups of experimental groups and 1 group of negative controls were established). The above composition premix was added to a 0.2-mL RPA-nfo reaction tube containing lyophilized enzyme powder. Finally, 2.5 μL of buffer B was added to the cap of the reaction tube and fully mixed. After mixing well, the reaction solution was shaken to the bottom of the tube, and the reaction tube was then immediately incubated at 39°C for 20 min. At the end of the reaction, 5 μL of product was added to a centrifuge tube containing 195 μL of MGCBB and mixed well. The sample end of the colloidal gold strip was then inserted into the centrifuge tube and allowed to reach equilibrium. The results were interpreted by observing the control line and the test line within 10 min.

The specificity of this dual RPA-LFD method was assessed through experiments using the optimized dual RPA-LFD system with genomic DNA from a mixture of *S. suis* and *A. pleuropneumoniae*, *A. pleuropneumoniae*, *S. suis*, *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*. The template was diluted to 9 concentrations ranging from 10^{0} ng/μL to 10^{-8} ng/μL. A negative control that lacked any added DNA template was used. Sensitivity experiments were performed using the established dual RPA-LFD system to evaluate the minimum limit of detection of the method.

Sample Collection and Bacterial Isolation

A 40-day-old nursery piglet group in a large-scale pig farm in Henan was suspected of having an infectious disease. The body temperature of the sick pigs rose above 40°C, accompanied by obvious respiratory symptoms. The lung samples of 45 affected piglets were collected aseptically and inoculated with THB agar plates containing 5% (v/v) sheep blood and/or TSA agar plates containing 10% (v/v) newborn calf serum and 0.01% (v/v) NAD by streaking with an inoculation ring. After culturing for 24 h at 37°C in a CO_2 incubator, suspected colonies were picked for Gram stain microscopy.

Detection by Conventional PCR

A pair of primers was designed to specifically amplify a 650-bp fragment of the *Apx*IV gene of *A. pleuropneumoniae* [24]. A multiplex PCR was developed to detect all serotypes of *S. suis* [25].

Clinical Application Test

To further confirm the effect of using the dual RPA-LFD system in clinical application, 45 clinical lung tissues of pigs suspected of being infected with *S. suis* and/or *A. pleuropneumoniae* from Shandong and Henan provinces were collected in this study, and RPA-LFD was performed under the optimal reaction conditions using pMD-18T-gdh recombinant plasmid as a positive control, ddH_2O as a blank control, and total DNA from healthy pig lung tissues as a negative control. Additionally, the abovementioned lung tissue samples were subjected to traditional bacterial isolation and culture and conventional PCR detection [24,25], and the test results were compared and analyzed.

Results

Screening of the RPA-Basic Primers

The screening revealed that the *A. pleuropneumoniae* ApxIV2698F/ApxIV3020R primer pair and *S. suis gdh*1166F/GDH1346R primer pair yielded a clear and single band (at the 323-bp and 180-bp positions, respectively) after the RPA amplification reaction, and the controls yielded no bands *(Table 1, Fig. 1)*. Therefore, the primer pairs ApxIV2698F/ApxIV3020R and GDH1166F/GDH1346R were used for the subsequent experiments.

Optimization of the Dual RPA-Basic Amplification System

The optimization work showed that the two specific bands could be detected with volume ratio of *S. suis* and *A. pleuropneumoniae* primers equal to 2.4 μL:0.6 μL, 2.4 μL:1.2 μL, 2.4 μL:1.8 μL, 2.4 μL:2.4 μL, 1.8 μL:2.4 μL, and 1.2 μL:2.4 μL *(Fig. 2-A)*, and the volume ratio of the *S. suis* and *A. pleuropneumoniae* primers of 2.4 μL:1.2 μL was the preferred ratio for the subsequent assays. The optimal primer ratio was then used to screen the reaction time. The results showed that electrophoresis bands could be detected with RPA reaction durations of 15-35 min *(Fig. 2-B)*, but

Table 1. Primer pairs and probes for RPA-Basic

Primer/Probe Name	Primer sequence (5'→3')	Target Fragment Length (bp)
ApxIV2698F	AGCAGTGCTTCTGTCGTTAGAGTCACGCCTTC	323 bp
ApxIV3020R	CGAGAATAATCGGCTACCCAT TTCCCTTCG	
ApxIV323Pn	FAM-CAATTAAGTAGTATACGCAATGTAAAGCAT[THF]ATCCTACCGTTATGC-C3spacer	
gdh1166F	TTCGCTTGTCATGGACTCGTGAAGAAGTAG	180 bp
gdh1346R	TATACCAAACCTTGGGCAATCATGCTATCC	
gdh1225Pn	FAM-AAATACGACCTTGGTACAGACTACCTTGCAGG[THF]GCTAACATCGCAGCCT-C3spacer	

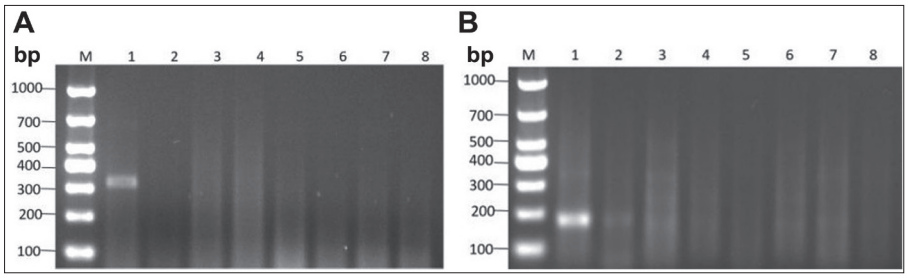
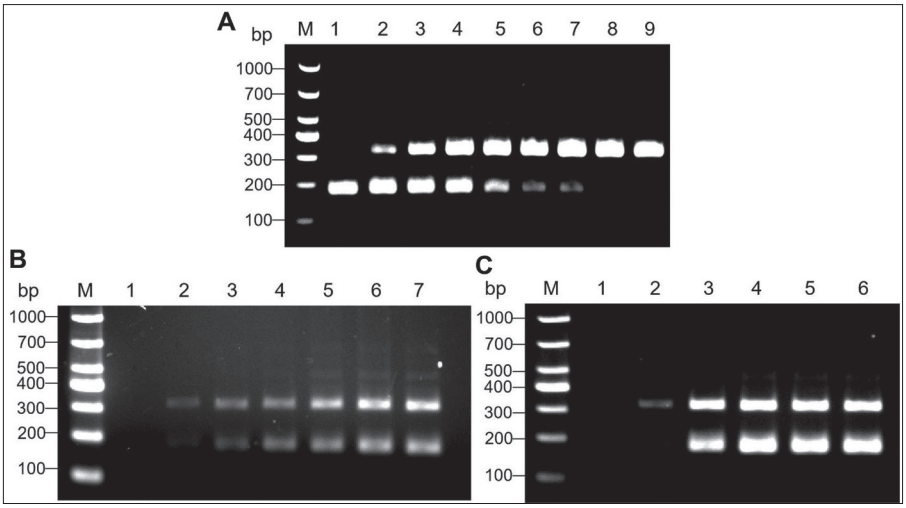


Fig 1. Results of the different primers detected by RPA-Basic. **A.** The results of APP primers detected by RPA-Basic. M. 1000 DNA Ladder, lanes 1-8 are: APP, negative control, enteropathogenic *E. coli*, *Salmonella*, *H. parasuis*, *S. aureus*, *Pasteurella*, *A. hydrophila*. **B.** The results of SS primers detected by RPA-Basic. M. 1000 DNA Ladder; lanes 1-8 are: SS, negative control, enteropathogenic *E. coli*, *Salmonella*, *H. parasuis*, *S. aureus*, *Pasteurella*, *A. hydrophila*

Fig 2. Results from the optimization of the dual RPA-Basic amplification system. **A.** Optimization of the RPA-Basic SS-APP primer volume ratio (μL). M, 1000 DNA Ladder; lanes 1-9, 2.4:0, 2.4:0.6, 2.4:1.2, 2.4:1.8, 2.4:2.4, 1.8:2.4, 1.2:2.4, 0.6:2.4, and 0:2.4, respectively; **B.** Optimization of the RPA-Basic reaction time. M,1000 DNA Ladder; lanes 1-7, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, and 35 min, respectively; **C.** Optimization of the RPA-Basic reaction temperature. M, 1000 DNA Ladder; lanes 1-6, 25°C, 30°C, 35°C, 37°C, 39°C, and 45°C, respectively



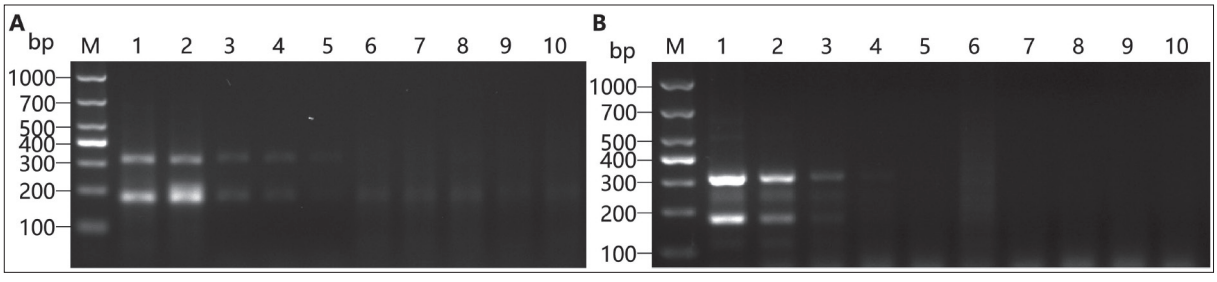


Fig 3. Comparison of the specificity and sensitivity of the dual RPA-Basic system and PCR. **A.** Results from the analysis of the sensitivity of Basic-RPA. M, 1000 DNA Ladder; lanes 1-10, nucleic acid concentrations of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng/μL, and negative control, respectively; **B.** Results from the analysis of the sensitivity of PCR. M, 1000 DNA Ladder; lanes 1-10, nucleic acid concentrations of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng/μL, and negative control, respectively

no significant difference in the brightness of the test line was observed after 20 min. Therefore, 20 min was selected as the optimal reaction time for the subsequent tests. The reaction temperature was then optimized by determining the optimal primer concentration and reaction time. The results showed that when the temperature was between 30°C and 45°C, the agarose gel could specifically detect RPA amplification products *(Fig. 2-C)*. Considering the detection application, 37°C was selected as the reaction temperature for the subsequent experiments.

Specificity and Sensitivity of the Dual RPA-Basic System

The optimized dual RPA-Basic reaction system yielded amplification products only with the *S. suis* and/or *A. pleuropneumoniae* groups, and no amplification products were observed with the other 6 groups or the negative control group *(Fig. 3),* confirming the specificity of the dual RPA-Basic detection method. To determine the sensitivity of the dual RPA-Basic system, the template was sequentially diluted 10-fold to obtain 10 concentrations ranging from 10^{0} to 10^{-9} ng/μL. The results showed that the nucleic acid detection limit of RPA-Basic was 10^{-3} ng/μL, which was higher than that of PCR (10^{-2} ng/μL) *(Fig. 3).*

Screening of Probes for the Dual RPA-LFD System

The selected reverse primers of *S. suis* and *A. pleuropneumoniae* were labeled with biotin, and the designed probes were labeled with 6-Carboxyfluorescein under these conditions. This labeling was only applied to detect a single colloidal gold test strip under this condition. To screen the optimal probe for the dual RPA-LFD system, the same template and reaction conditions were used for each *S. suis* and *A. pleuropneumoniae* RPA-nfo reaction; additionally, each pair of primers was tested using a positive control, negative control, and enteropathogenic *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*. The final screening combination is shown in *Table 1,* and the respective reaction results are shown in *Fig. 4.* The test and control lines of the *S. suis* and *A. pleuropneumoniae* RPA-LFD detection groups both appeared, whereas the other groups yielded only a control line, indicating that this primer-probe combination could be used for the subsequent experiments.

Optimization of the Dual RPA-LFD Detection Reaction System

The RPA-LFD primer and probe concentration ratio in the reaction system was optimized using the selected primers and probes listed in *Table 1.* The selected *S. suis* reverse primer was labeled with biotin, the *A. pleuropneumoniae* reverse primer was labeled with digoxigenin digoxin, and both probes were labeled with FAM fluorescein, corresponding to the double colloidal gold detection test strip. The results showed that the colors of the *A. pleuropneumoniae* test line and *S. suis* test line were most similar when the *A. pleuropneumoniae* and *S. suis* upstream and downstream primers and probes were added to the systems containing *A. pleuropneumoniae* volumes of 2, 2, or 0.6 μL and *S. suis* volumes of 0.5, 0.5, or 0.15 μL in the seven experimental groups *(Fig. 5);* notably, the negative control only yielded the control line; thus, the reaction systems consisting of 2, 2, or 0.6 μL of *A. pleuropneumoniae* and 0.5, 0.5, or 0.15 μL of *S. suis* were selected for the subsequent experiments.

Specificity and Sensitivity of Dual RPA-LFD Detection

The optimized dual RPA-LFD system was used for specificity experiments using genomic DNA from a mixture of *S. suis* and *A. pleuropneumoniae*, *A. pleuropneumoniae*, *S. suis*, the negative control, *E. coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *S. aureus*, *Pasteurella*, and *A. hydrophila*. As shown in *Fig. 6-A*, the results showed that the double-positive and single-positive results for *A. pleuropneumoniae* and/or *S. suis* all displayed both the appropriate test line(s) and the control line. For the controls and the nontarget bacteria, only the control line was displayed; thus, the specificity of the method was demonstrated. To determine the sensitivity of the dual RPA-LFD system, the template was sequentially diluted 10-fold to obtain 10 concentrations (10^{0}-10^{-9} ng/μL). The results showed that the colors of the test lines of *A. pleuropneumoniae* and *S. suis* gradually faded with decrease in the concentrations from 10^{0} to 10^{-5} ng/μL, and the test line completely disappeared with lower concentrations (10^{-6} ng/μL and less) *(Fig. 6-B).* In addition, only the control line was observed with the negative control. Therefore, the limit of detection of the dual RPA-LFD established in this study was 10^{-5} ng/μL, which is 100 times that of the constructed dual RPA-Basic system and 1.000 times that of PCR.

Isolation and Identification of *S. suis* and *A. pleuropneumoniae*

A total of 10 strains of *S. suis* were isolated from different pig tissue samples at different time points in the affected

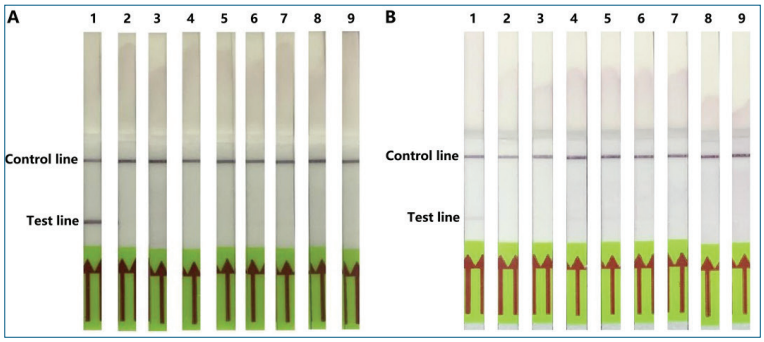


Fig 4. Screening of detection probes for the dual RPA-LFD system. **A.** Screening of probe for SS. Lanes 1-9, *Streptococcus suis*, negative control, enteropathogenic *Escherichia coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *Staphylococcus aureus*, *Pasteurella*, *Bacillus*, *Aeromonas hydrophila*, and *Actinobacillus pleurisy*, respectively; **B.** Screening of probe for APP. Lanes 1-9, *Actinobacillus pleurisy*, negative control, enteropathogenic *Escherichia coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *Staphylococcus aureus*, *Pasteurella*, *Aeromonas hydrophila*, and *Streptococcus suis*, respectively

pig farm, and the bacteria were small round, gray-white and translucent colonies on the THB agar plates. The isolated bacterial smear was Gram-stained and observed under an oil microscope with a light microscope, and it was a blue-purple brevis. *A. pleuropneumoniae* was not isolated from any lung tissue samples *(Table 2)*.

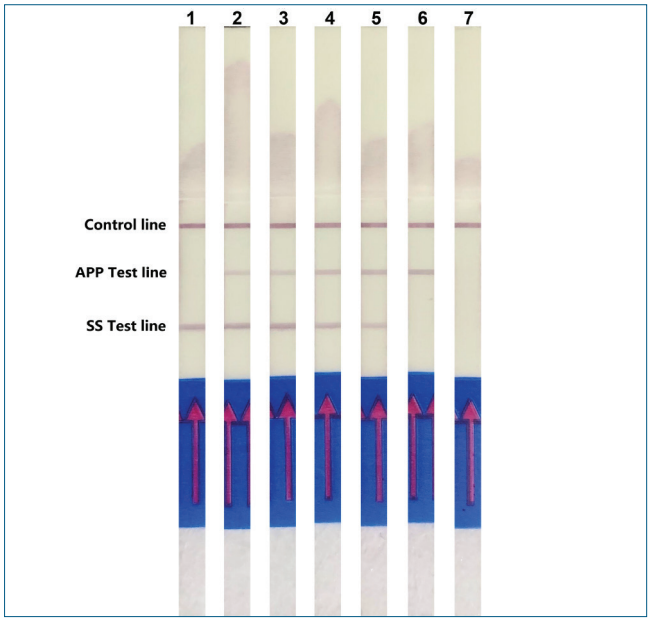


Fig 5. Optimization results of the dual LFD-RPA reaction system. 1. APP (0, 0, 0), SS (2, 2, 0.6). 2. APP (2, 2, 0.6), SS (2, 2, 0.6). 3. APP (2, 2, 0.6), SS (1.5, 1.5, 0.45). 4. APP (2, 2, 0.6), SS (1, 1, 0.3). 5. APP (2, 2, 0.6), SS (0.5, 0.5, 0.15). 6. APP (2, 2, 0.6), SS (0, 0, 0). unit (μL). 7. negative control

Conventional PCR Detection

Among 45 clinical samples suspected of *S. suis* or *A. pleuropneumoniae* infection, a total of 18 *S. suis*-positive samples were detected by conventional PCR detection, including 7 strains of serotype 2, 3 strains of serotype 7 and 2 strains of serotype 9, accounting for 15.2%, 6.7% and 4.4% of the total samples, respectively *(Table 2)*. One strain of *A. pleuropneumoniae* was detected.

Clinical Application of Dual RPA-LFD Detection

Using the established dual LFD-RPA method to detect each serotype strain of *S. suis* and *A. pleuropneumoniae*, the test lines could be clearly observed *(Fig. 7)*. Among 45 clinical samples suspected to involve *S. suis* or *A. pleuropneumoniae* infection, a total of 19 *S. suis*-positive samples and 1 *A. pleuropneumoniae*-positive sample were detected by LFD-RPA (without mixed infection). The dual RPA-LFD method resulted in a higher detection rate of *S. suis* compared with the conventional PCR method *(Table 2)*.

Discussion

A. pleuropneumoniae and *S. suis* are important pathogenic bacteria that endanger the pig industry [9]. Both bacteria

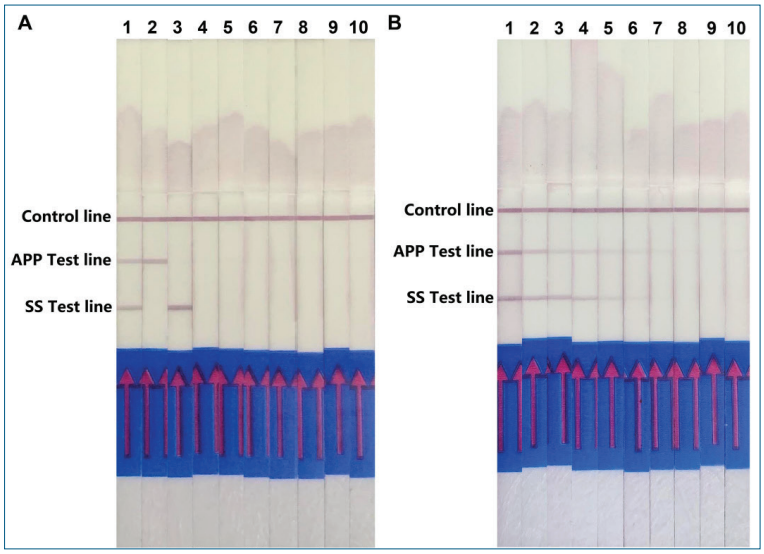


Fig 6. Specificity and sensitivity of the dual RPA-LFD detection method. **A.** Specificity of RPA-LFD. Lanes 1-10, SS and APP positive control, APP positive control, SS positive control, negative control, *Escherichia coli*, *Salmonella*, *Glaesserella parasuis*, gen. nov., comb. nov., *Staphylococcus aureus*, *Pasteurella*, and *Aeromonas hydrophila*, respectively. **B.** Sensitivity of RPA-LFD. Lanes 1-9, nucleic acid concentrations of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} ng μL, respectively; lane 10, negative control

Fig 7. Clinical sample results with the dual PRA-LFD system. Lanes 1-4, SS and APP positive control, SS positive control, APP positive control, and negative control, respectively; Lanes 5-13, clinical test samples

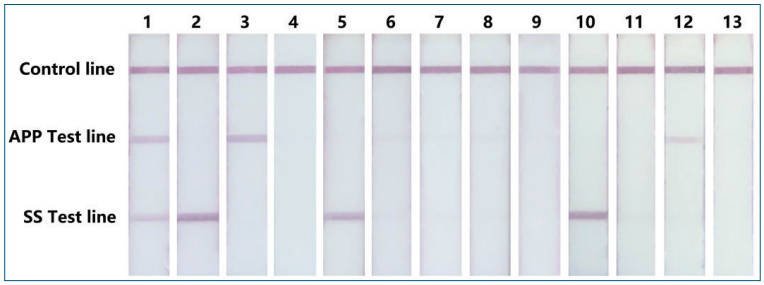


Table 2. *Application of different methods for the detection of actual samples*

Isolates	*S. suis*						*A. pleuropneumoniae*		
	Bacterial Isolates	Serotype-specific PCR				Dual RPA-LFD	Bacterial Isolates	PCR	Dual RPA-LFD
		2	7	9	Other Serotype				
XXHX001									
XXHX002									
XXHX003		+				+			
XXHX004					+	+			
XXHX005	+	+				+			
XXHX006									
XXHX007				+		+			
XXHX008									
XXHX009	+	+				+			
XXHX010					+	+			
XXHX011	+		+			+			
XXHX012	+	+				+			
XXHX013									
XXHX014	+	+				+			
XXHX015									
XXHX016									
XXHX017									
XXHX018					+	+			
XXHX019									
XXHX020									
XXHX021	+	+				+			
XXHX022									
XXHX023									
XXHX024	+				+	+			
XXHX025									
XXHX026									
XXHX027									
XXHX028	+	+				+		+	+
XXHX029									
XXHX030			+			+			
XXHX031									
XXHX032									
XXHX033	+			+		+			
XXHX034									
XXHX035					+	+			
XXHX036									
XXHX037						+			
XXHX038									
XXHX039									
XXHX040	+		+			+			
XXHX041									
XXHX042									
XXHX043					+	+			
XXHX044									
XXHX045									

often concurrently infect pig herds and can cause respiratory symptoms in pigs, which makes the condition complex and results in great difficulty in obtaining clinical differential diagnoses [9]. Therefore, to improve the efficiency and accuracy of detection, this study selected the highly conserved *S. suis gdh* gene and *A. pleuropneumoniae Apx*IV gene as diagnostic antigens and developed a dual RPA-LFD visual rapid detection method that resulted in the highly sensitive, highly specific and simultaneous detection of these two bacteria. Therefore, this method can be conveniently applied in clinical laboratories and/or used for field diagnosis.

In molecular biology detection methods, the accurate selection of target genes is crucial and can directly affect the reliability of the reaction results. *S. suis* strains exhibit extensive genetic heterogeneity within and among serotypes, which increases the difficulty of establishing genetic diagnostic methods [21]. The *gdh* gene of *S. suis* is highly conserved in all capsular serotypes and can be used as a diagnostic antigen [21]. In our previous study, Gdh was selected as a diagnostic antigen for developing PPA-ELISA and dot-PPA-ELISA diagnostic methods, which have been demonstrated to be suitable for the large-scale and on-site/field diagnosis of *S. suis* infections [2,26]. *Apx*IV is one of the virulence factors of *A. pleuropneumoniae* and belongs to the RTX toxin family. *Apx*IV is present in all serotype strains of *A. pleuropneumoniae* but not in other closely related species of bacteria and is a good target gene for the detection of *A. pleuropneumoniae* species [27,28]. Based on these observations, in this study, we selected the *gdh* and *Apx*IV genes as targets for the establishment of a dual RPA-LFD visualization rapid detection method.

Nucleic acid isothermal amplification technology provides simplified nucleic acid artificial replication conditions that require only a constant temperature without thermal cycling. Nucleic acid isothermal amplification technology not only reduces the time of amplification by eliminating repeated heating and cooling steps but also can achieve multiple molecular reactions that proceed asynchronously, which greatly improves the efficiency of nucleic acid amplification [19]. At present, the widely used nucleic acid isothermal amplification methods include nucleic acid sequence-based amplification (NASBA), LAMP, strand displacement amplification (SDA) and RPA [29-32]. Compared with traditional PCR detection methods and other DNA isothermal amplification technologies, RPA can amplify target DNA to detectable levels without special equipment in a shorter time and at lower temperatures [33]. In addition, it has been shown that RPA has a sensitivity similar to that of PCR detection, and the sensitivity of some RPA systems is even 10 or 1.000 times greater than that of PCR detection [34,35]. Detection methods such as real-time fluorescence quantitative RPA, RPA-LFD, and RPA-ELISA that were developed in combination with this technology have been widely used in diagnosis, medical treatment, agriculture, food safety and other fields [7,36,37]. Among these methods, LFD technology is a visual detection tool that can be performed without expensive and complex instruments and trained personnel, and the results can be observed within 5-10 min. This study combined RPA and LFD technology to establish a method for the simultaneous detection of *A. pleuropneumoniae* and *S. suis*. This rapid RPA-LFD visual detection method can be used in resource-constrained laboratories or for on-site/field diagnosis and can be considered a convenient method for the detection of distant, less portable samples. In addition, the limit of detection of the RPA-LFD detection method established in this study was as low as 10^{-5} ng/μL, which is 100 times that of the constructed dual RPA-Basic detection method and 1.000 times that of conventional PCR detection technology.

The practicability of RPA-LFD detection technology is an important indicator of its application and promotion [38]. On the one hand, in this study, RPA-LFD was applied for the analysis of collected disease materials, and the detection rate was higher than that of conventional PCR. On the other hand, experimental screening revealed that the expected RPA amplification bands could appear at the optimal reaction temperature of RPA (between 30°C and 45°C), which indicated that the reaction conditions needed for RPA are relatively flexible and that the reaction can be completed by heating in a water bath. These results indicate that RPA-LFD technology is very practical for the rapid dual detection of *S. suis* and *A. pleuropneumoniae*, which is conducive to the achievement of rapid on-site detection or field detection. In terms of detection efficiency, RPA-LFD detection can be completed within 30 min, whereas conventional PCR and LAMP technology usually take 2.5 h and 1 h, respectively. Therefore, the detection efficiency of RPA-LFD technology is significantly higher than that of PCR or LAMP technology [36]. In addition, RPA-LFD amplification requires only two primers, which greatly reduces the difficulty and complexity of primer design compared with that needed for LAMP technology, which requires six primers [33]. The reliability of the detection technology depends on its specificity, and the analysis of the specificity of the dual RPA-LFD visualization rapid detection system established in this study showed that the detection method did not cross-react with six other common pathogenic microorganisms, which demonstrated that the method exhibits good specificity.

In summary, the dual RPA-LFD detection method for *A. pleuropneumoniae* and *S. suis* established in this study has several advantages, including high sensitivity, high specificity and fast speed. Additionally, using this method, the results can be directly observed by the naked eye,

and large-scale precision instruments are not necessary. Therefore, this method is very suitable for on-site or field detection and has broad application potential.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding authors (X. Xia and H. Zhang).

Acknowledgements

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

Financial Support

This research work was funded by the National Natural Science Foundation of China (Grant Nos. 32172876 and 31972715), the Program for Innovative Talents (in Science and Technology) in University of Henan Province (23HASTIT046), the Young Talent Lifting Project in Henan Province (Grant No. 2021HYTP038), the Youth Backbone Teacher Project of Colleges and Universities of Henan Province (Grant No. 2020GGJS163) and the Key Specialized Research and Development Breakthrough Program in Henan Province (Grant No. 202102110101).

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Authors' Contributions

XX and HZ designed the research and project outline. SZ, HX, ML and AZ carried out the experiments and analysis the data. HY, MD, XW, ZT, JH, SZ and HX drafted the manuscript. XX, HZ and JH revised the manuscript. All authors have read and approved the final manuscript.

References

1. Goyette-Desjardins G, Auger J, Xu J, Segura M, Gottschalk M: *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. *Emerg Microbes Infect*, 3, 1-20, 2014. DOI: 10.1038/emi.2014.45

2. Xia X, LI S, Wang J, Chen J, Xie J, Jiang S, Shen Z: Development of PPA-ELISA for the detection of antibodies against *Streptococcus suis* using recombinant GDH protection as antigen. *China Vet Sci*, 42, 932-937, 2012. DOI: 10.16656/j.issn.1673-4696.2012.09.013

3. Xia X, Wang X, Wei X, Jiang J, Hu J: Methods for the detection and characterization of *Streptococcus suis*: From conventional bacterial culture methods to immunosensors. *Anton Leeuw Int J G*, 111, 2233-2247, 2018. DOI: 10.1007/s10482-018-1116-7

4. Haas B, Grenier D: Understanding the virulence of *Streptococcus suis*: A veterinary, medical, and economic challenge. *Med Mal Infect*, 48 (3): 159-166, 2018. DOI: 10.1016/j.medmal.2017.10.001

5. Gottschalk M, Xu J, Lecours MP, Grenier D, Fittipaldi N, Segura M: *Streptococcus suis* infections in humans: What is the prognosis for Western countries? (Part II). *Clin Microbiol Newsl,* 32, 97-102, 2010. DOI: 10.1016/j.clinmicnews.2010.06.001

6. Gottschalk M, Segura M, Xu J: *Streptococcus suis* infections in humans: The Chinese experience and the situation in North America. *Anim Health Res Rev,* 8, 29-45, 2007. DOI: 10.1017/S1466252307001247

7. Wertheim HFL, Nghia HDT, Taylor W, Schultsz C: *Streptococcus suis*: An emerging human pathogen. *Clin Infect Dis*, 48, 617-625, 2009. DOI: 10.1086/596763

8. Gottschalk M, Broes A: Actinobacillosis. **In,** Diseases of Swine. 11th ed., 749-766, Hoboken NJ: John Wiley & Sons, Inc., 2019. DOI: 10.1002/9781119350927.ch48

9. Nahar N, Turni C, Tram G, Blackall PJ, Atack JM: *Actinobacillus pleuropneumoniae*: The molecular determinants of virulence and pathogenesis. *Adv Microb Physiol*, 78, 179-216, 2021. DOI: 10.1016/bs.ampbs.2020.12.001

10. Opriessnig T, Gimenez-Lirola LG, Halbur PG: Polymicrobial respiratory disease in pigs. *Anim Health Res Rev*, 12, 133-148, 2011. DOI: 10.1017/S1466252311000120

11. Fablet C, Marois-Crehan C, Simon G, Grasland B, Jestin A, Kobisch M, Madec F, Rose N: Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: A cross-sectional study. *Vet Microbiol*, 157, 152-163, 2012. DOI: 10.1016/j.vetmic.2011.12.015

12. Nakayama T, Zhao J, Takeuchi D, Kerdsin A, Chiranairadul P, Areeratana P, Loetthong P, Pienpringam A, Akeda Y, Oishi K: Colloidal gold-based immunochromatographic strip test compromising optimised combinations of anti-*S. suis* capsular polysaccharide polyclonal antibodies for detection of *Streptococcus suis*. *Biosens Bioelectron*, 60, 175-179, 2014. DOI: 10.1016/j.bios.2014.03.074

13. Tobias TJ, Bouma A, Klinkenberg D, Daemen AJJM, Stegeman JA, Wagenaar JA, Duim B: Detection of *Actinobacillus pleuropneumoniae* in pigs by real-time quantitative PCR for the *apxIVA* gene. *Vet J*, 193, 557-560, 2012. DOI: 10.1016/j.tvjl.2012.02.004

14. Turni C, Singh R, Schembri MA, Blackall PJ: Evaluation of a multiplex PCR to identify and serotype *Actinobacillus pleuropneumoniae* serovars 1, 5, 7, 12 and 15. *Lett Appl Microbiol*, 59, 362-369, 2014. DOI: 10.1111/lam.12287

15. Watt AE, Browning GF, Markham PF, Marenda MS: Detection of naturally aerosolized *Actinobacillus pleuropneumoniae* on pig farms by cyclonic air sampling and qPCR. *Vet Microbiol,* 250:108856, 2020. DOI: 10.1016/j.vetmic.2020.108856

16. Wong YP, Othman S, Lau YL, Radu S, Chee HY: Loop-mediated isothermal amplification (LAMP): A versatile technique for detection of microorganisms. *J Appl Microbiol,* 124 (3): 626-643, 2018. DOI: 10.1111/jam.13647

17. Yang W, Chen P, Guo H, Chen Y, Liu H, He Q: Loop-mediated isothermal amplification targeting the *apx*IVA gene for detection of *Actinobacillus pleuropneumoniae*. *FEMS Microbiol Lett*, 300, 83-89, 2009. DOI: 10.1111/j.1574-6968.2009.01779.x

18. Zhang J, Zhu J, Ren H, Zhu S, Zhao P, Zhang F, Lv H, Hu D, Hao L, Geng M, Gong X, Pan X, Wang C, Qi Z: Rapid visual detection of highly pathogenic *Streptococcus suis* serotype 2 isolates by use of loop-mediated isothermal amplification. *J Clin Microbiol*, 51, 3250-3256, 2013. DOI: 10.1128/JCM.01183-13

19. Li J, Macdonald J, von Stetten F: A comprehensive summary of a decade development of the recombinase polymerase amplification. *Analyst*, 144 (1): 31-67, 2019. DOI: 10.1039/C8AN01621F

20. Wu W, Zhao S, Mao Y, Fang Z, Lu X, Zeng L: A sensitive lateral flow biosensor for *Escherichia coli* O157:H7 detection based on aptamer mediated strand displacement amplification. *Anal Chim Acta*, 861, 62-68, 2015. DOI: 10.1016/j.aca.2014.12.041

21. Okwumabua O, O'Connor M, Shull E: A polymerase chain reaction (PCR) assay specific for *Streptococcus suis* based on the gene encoding the glutamate dehydrogenase. *FEMS Microbiol Lett*, 218, 79-84, 2003. DOI: 10.1111/j.1574-6968.2003.tb11501.x

22. Okwumabua O, Persaud JS, Reddy PG: Cloning and characterization of the gene encoding the glutamate dehydrogenase of *Streptococcus suis* serotype 2. *J Allergy Clin Immunol*, 8 (2): 251-257, 2001. DOI: 10.1128/CDLI.8.2.251-257.2001

23. Xia X, Shen Z, Jiang S, Li S, Wu L Ma Z, Cheng L: Prokaryotic

expression of gene encoding glutamate dehydrogenase of *Streptococcus sui*s serotype 2 and preparation of polyclonal antibodies against its expressed products. *Anim Husbandry Feed Sci*, 3, 15-19, 2011.

24. Diao Y, Ding J, Jiang S, Cui Z, Zhuo B: Identification and detection of *Actinobacillus pleuropneumoniae* by PCR. *Chin J Vet Sci*, 26, 379-381, 2006. DOI: 10.3969/j.issn.1005-4545.2006.04.011

25. Kerdsin A, Akeda Y, Hatrongjit R, Detchawna U, Sekizaki T, Hamada S, Gottschalk M, Oishi K: *Streptococcus suis* serotyping by a new multiplex PCR. *J Med Microbiol*, 63, 824-830, 2014. DOI: 10.1099/jmm.0.069757-0

26. Xia X, Wang L, Shen Z, Qin W, Hu J, Jiang S, Li S: Development of an indirect Dot-PPA-ELISA using glutamate dehydrogenase as a diagnostic antigen for the rapid and specific detection of *Streptococcus suis* and its application to clinical specimens. *Anton Leeuw Int J G*, 110, 585-592, 2017. DOI: 10.1007/s10482-016-0825-z

27. Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, Gottschalk M, Nicolet J, Frey J: Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apx*IVA. *Vet Microbiol*, 79, 47-62, 2001. DOI: 10.1016/s0378-1135(00)00345-x

28. Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, Maclnnes JI, Segers RPAM, Frey J: Characterization of *apx*IVA a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology*, 145, 2105-2116, 1999. DOI: 10.1099/13500872-145-8-2105

29. Gill P, Ghaemi A: Nucleic acid isothermal amplification technologies-A review. *Nucleosides Nucleotides Nucleic Acids*, 27, 224-243, 2008. DOI: 10.1080/15257770701845204

30. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T: Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*, 28, e63-e63, 2000. DOI: 10.1093/nar/28.12.e63

31. Nycz CM, Dean CH, Haaland PD, Spargo CA, Walker GT: Quantitative reverse transcription strand displacement amplification: Quantitation of nucleic acids using an isothermal amplification technique. *Anal Biochem*, 259, 226-234, 1998. DOI: 10.1006/abio.1998.2641

32. Piepenburg O, Williams CH, Stemple DL, Armes NA: DNA detection using recombination proteins. *PLoS Biol*, 4:e204, 2006. DOI: 10.1371/journal.pbio.0040204

33. Daher RK, Stewart G, Boissinot M, Boudreaua DK, Bergeron MG: Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology. *Mol Cell Probes*, 29, 116-121, 2015. DOI: 10.1016/j.mcp.2014.11.005

34. Jiao Y, Xu C, Li J, Gu Y, Xia C, Xie Q, Xie Y, An M, Xia Z, Wu Y: Characterization and a RT-RPA assay for rapid detection of Chilli Veinal mottle virus (ChiVMV) in tobacco. *Virol J*, 17, 1-9, 2020. DOI: 10.1186/s12985-020-01299-w

35. Kapoor R, Srivastava N, Kumar R, Sharma SK, Rai R, Kumar S, Baranwal VK: Detection of episomal banana streak Mysore virus by reverse transcription-recombinase polymerase amplification assay. *J Plant Pathol*, 102, 499-503, 2020. DOI: 10.1007/s42161-019-00424-1

36. Reid MS, Le X, Zhang H: Exponential isothermal amplification of nucleic acids and assays for proteins, cells, small molecules, and enzyme activities: An EXPAR example. *Angew Chem Int Ed Engl*, 57, 11856-11866, 2018. DOI: 10.1002/ange.201712217

37. Santiago-Felipe S, Tortajada-Genaro LA, Puchades R, Maquieira A. Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis. *Anal Chim Acta*, 811, 81-87, 2014. DOI: 10.1016/j.aca.2013.12.017

38. Craw P, Balachandran W: Isothermal nucleic acid amplification technologies for point-of-care diagnostics: A critical review. *Lab Chip*, 12, 2469-2486, 2012. DOI: 10.1039/c2lc40100b