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Rapid and sensitive detection of *Mycoplasma hyopneumoniae* by recombinase polymerase amplification assay

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ABSTRACT

Mycoplasma hyopneumoniae is the etiological agent of swine enzootic pneumonia, which is associated with high economic losses in swine production worldwide. In this study, recombinase polymerase amplification assays using real-time fluorescence detection (real-time RPA) and lateral flow strip detection (LFS RPA) were developed to detect *M. hyopneumoniae* based on the conserved region of the mhp165 gene. Real-time RPA was performed in Genie III at 39 °C for 20 min, while the LFS RPA was performed in an incubator block at 39 °C for 15 min, and the products were visible on the LFS inspected by the naked eyes within 2 min. Both assays were specific for *M. hyopneumoniae*, as there were no cross-reactions with other pathogens tested. The limit of detection of both RPA assay was 5.0×10^2 fg of *M. hyopneumoniae* DNA, which was the same as that of a real-time PCR assay. Of the 146 clinical samples, *M. hyopneumoniae* DNA was identified in 41, 42, and 47 samples by the real-time RPA, LFS RPA and real-time PCR, respectively. Compared to real-time PCR, the real-time RPA and LFS RPA assays showed diagnostic specificity of 100%, a diagnostic sensitivity of 87.23% and 89.36%, and a kappa value of 0.903 and 0.909, respectively. These results have demonstrated that the developed RPA assays are suitable for rapid and reliable detection of *M. hyopneumoniae* in diagnostic laboratory and at point-of-need facility.

1. Introduction

Mycoplasma hyopneumoniae is the primary etiological agent of enzootic pneumonia (EP) in pigs, and is also one of the primary agents involved in the porcine respiratory disease complex (PRDC) (Thacker and Minion, 2012). EP is characterized by sporadic, dry and non-productive cough, mild fever, weight gain loss, high morbidity, and low mortality (Sibila et al., 2009). Presently, EP is one of the most worldwide common respiratory diseases and responsible for considerable economic loss in swine production (Thacker and Minion, 2012).

Early diagnosis of infection with *M. hyopneumoniae* is extremely important for timely intervention to limit the effects of EP, and is a key to monitor herd-negative status. Bacteriological culture of *M. hyopneumoniae* is the gold standard diagnostic method (Thacker and Minion, 2012), however, culture is too laborious, time-consuming and difficult (Okada et al., 2005; Sibila et al., 2009; Strait et al., 2008), which making it impractical for use and generally not attempted. Serologic testing *via* ELISA is the most common, economical, and generally convenient method for *M. hyopneumoniae* herd surveillance. However, seroconversion to *M. hyopneumoniae* is often delayed after natural infection, which makes the serology less effective in detecting early-stage infection (Kurth et al., 2002; Sorensen et al., 1993) Current serological methods are further hampered by cross-reactions which have been reported between *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (Armstrong et al., 1987; Freeman et al., 1984). A series of nucleic acid amplification-based assays have been developed and reported to be sensitive and specific for *M. hyopneumoniae*, such as polymerase chain reaction (PCR), nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP), which are widely used for detection of *M. hyopneumoniae* in pig(Cai et al., 2007; Calsamiglia et al., 1999; Dubosson et al., 2004; Li et al., 2013; Liu et al., 2015; Strait et al., 2008). PCR is considered as the gold standard in molecular detection filed, however, implementation of the PCR assays is limited in under-equipped laboratories and at the point-of-need (PON) diagnosis due to

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the requirements of expensive thermocycler, a centralized laboratory facility and experienced technicians. Compared to the PCR assays, the isothermal amplification methods show the advantages in respects to convenience, minimal equipment requirement, and rapid "sample to answer". Although the developed LAMP assays did not require specialized equipment, they are difficult to design as at least 4 primers were required, and the reaction time was 30 or 45 min (Li et al., 2013; Liu et al., 2015). An ideal diagnostic method would combine the sensitivity, specificity and flexibility with the speed and ease of use. A simpler and more convenient method is still needed for rapid and reliable detection of *M. hyopneumoniae* at PON diagnosis and in laboratories without access to real-time PCR instrumentation.

As an isothermal DNA amplification technique, recombinase polymerase amplification (RPA) is rapid, reliable and easy to perform. RPA assays have been developed and used widely for the detection of different pathogens (Daher et al., 2016; Piepenburg et al., 2006; Wang et al., 2017, 2018), and RPA is considered to be the most applicable approach for PON diagnosis and field applications (Amer et al., 2013). RPA reaction uses enzymes called recombinases that form complexes with oligonucleotide primers and pair the primers with homologous sequences in DNA. A single-stranded DNA-binding protein binds to the displaced DNA strand and stabilizes the resulting D-loop. The primer then initiates DNA amplification by a strand-displacing DNA polymerase (Piepenburg et al., 2006). RPA offers several amplification product detection strategies either in real-time with fluorescent probes or post amplification by agarose gel electrophoresis or by direct visual detection. Real-time detection of RPA products could be performed through additional exonuclease III (exo) and exo probes in the reaction system. During the RPA reaction, the fluorescent signal is measured real-time using fluorescence detection equipment. As an alternative to real-time detection, direct visual detection of the RPA products depends on endonuclease IV (nfo), the LF probe and the opposing amplification primer labeled at the 5′ end with biotin, and the amplicons are then detected by the naked eyes in a 'sandwich' assay format, such as a lateral flow strip (LFS), which uses anti-FAM gold conjugates and biotin-ligand molecules.

In this study, the real-time RPA and LFS RPA assays were developed for rapid, specific and sensitive detection of *M. hyopneumoniae*, and the performance of the assays were evaluated by detecting the clinical samples.

2. Materials and methods

2.1. Virus, bacteria strains and clinical samples

Mycoplasma hyopneumoniae and a panel of other pathogens considered dangerous to pigs were used in the study. *Mycoplasma hyopneumoniae* (strain 168) was obtained from the commercial attenuated live vaccine. Porcine circovirus 2 (PCV2, strain HB-MC1), pseudorabies virus (PRV, strain SH151218), *M. hyorhinis* (strain HB-BD1), *M. flocculare* (strain HB-XS3), *Haemophilus parasuis* (strain F187G3), *Actinobacillus pleuropneumoniae* (strain CVCC266), *Pasteurella multocida* (strain F91G3) and *Klebsiella pneumoniae* (strain F21W3) were isolated and maintained in our laboratory.

A total of 146 clinical samples (60 nasal swabs and 86 fresh lungs) were collected in Hebei Province from November 2017 to August 2018. The nasal swabs were collected from the pigs with respiratory symptom of 2 different pig farms, and the fresh lungs with or without lesions of enzootic pneumonia were obtained from the markets for agricultural products in Hebei. The swabs were inoculated and vortexed in 1 mL sterile phosphate-buffered saline (PBS, pH 7.4), and centrifuged at 10,000*g* for 10 min at 4 °C. The lung samples were homogenized with phosphate-buffered saline (PBS, pH 7.4) as a 10% (w/v) suspension and centrifuged for 10 min at 10,000*g* at 4 °C. The precipitate was collected for DNA extraction.

2.2. DNA extraction

All the mycoplasma and bacteria genomic DNA were extracted using the TIANamp Bacteria DNA kit (Tiangen, Beijing, China), and viral DNA were extracted using the TIANamp Virus DNA kit (Tiangen, Beijing, China), which were all performed according to the manufacturer's instructions, respectively. Total DNA extracted from clinical samples was finally eluted in 50 μL of nuclease-free water. All DNA were quantified using a ND-2000c spectrophotometer (NanoDrop, Wilmington, USA) and stored at −80 °C until use.

2.3. RPA primers and probes

Nucleotide sequence data for different *M. hyopneumoniae* strains available in GenBank were aligned to identify the conserved regions in the mhp165 gene, which was determined as the molecular target for RPA. According to the reference sequences of *M. hyopneumoniae* (Acession number: AE017332, AE017243, AE017244, CP002274, EU658730, EU658731, EU658732), the primers, exo probe and LF probe were designed following RPA manufacturer guidelines (TwistDx. Cambridge, UK). Primers and probes are listed in Table 1 and were synthesized by Sangon Biotech Co., Shanghai, China.

2.4. Real-time RPA assay

Real-time RPA reactions were performed in 0.2 mL reaction tube containing a freeze-dried enzyme pellet, and the total reaction volume was 50 μL containing 29.5 μL of rehydration buffer and 2.5 μL of magnesium acetate (280 mM) from the ZC BioScience™ exo kit (ZC BioScience, Hangzhou, China). Other components included 420 nM each RPA primers (mhp165-*exo*-F and mhp165-exo-R), 120 nM exo probe (mhp165-exo-P), and 1 μL of genomic DNA or 5 μL of sample DNA. The real-time RPA reactions were performed at 39 °C for 20 min in the Genie III scanner device (OptiGene Limited, West Sussex, UK). Samples produced an exponential amplification curve above the threshold of the negative control were considered positive.

Table 1
Sequences of the primers and probes for *M. hyopneumoniae* real-time PCR and RPA assays.

Assay	Primers and probes	Sequence 5′-3′	Amplicon size (bp)	References
Real-time PCR	mhp165-F	TGCCCAGGATATTTCCGATCCAGA	132	Strait et al. (2008)
	mhp165-R	AGACCTGAAGAACGTGCATGGAGA		
	mhp165-P	FAM-GCGATCTCAACAAATACCGGGA TTGGT-BHQ1		
Real-time RPA	mhp165-*exo*-F	TCAGAAGTTGATCCAAAAACAGGACTAAAA	134	This study
	mhp165-exo-R	CCCGTAGTTTTAAAAAATGGCGAGGAGGCT		
	mhp165-exo-P	CTACCAAACAAAACAAGGTAGTTTTATTGGA (FAM-dT)(THF)(BHQ1-dT)TCCCAATCATCCCGA -C3-spacer		
LFS RPA	mhp165-LF-F	TCAGAAGTTGATCCAAAAACAGGACTAAAA	134	This study
	mhp165-LF-R	Biotin-CCCGTAGTTTTAAAAAATGGCGAGGA GGCT		
	mhp165-LF-P	FAM-CTACCAAACAAAACAAGGTAGTTTTAT TGGAT(THF) TTCCCAATCATCCCGA-C3-spacer		

2.5. LFS RPA assay

LFS RPA reactions were performed in a 50 μL volume containing 29.5 μL of rehydration buffer and 2.5 μL of magnesium acetate (280 mM) from the TwistAmp™ nfo kit (TwistDX, Cambridge, UK). Other components included 420 nM each RPA primers (mhp165-LF-F and mhp165-LF-R), 120 nM LF probe (mhp165-LF-P), and 1 μL of genomic DNA or 5 μL of sample DNA. The tubes were vortexed briefly, spun down once again and immediately incubated in an incubator block at 39 °C for 5, 10, 15 and 20 min. LFS (USTAR, Hangzhou, China) was used to detect the amplicons that were dual-labeled with FAM and biotin. The RPA products, which were dual labeled with FAM and biotin, were detected using LFS as described previously (28, 29). A testing sample was considered positive when both the test line and the control line were visible, negative when only the control line was visible, and invalid when the control line was invisible.

2.6. Analytical specificity and sensitivity analysis

The real-time and LFS RPA assays were carried out to amplify the nucleic acids of a panel of pathogens including *M. hyopneumoniae, M. hyorhinis*, *M. flocculare*, *H. parasuis*, *A. pleuropneumoniae, P. multocida*, *K. pneumoniae*, PCV2, and PRV, which are considered to be dangerous to the swine respiratory system. Five independent reactions were performed.

The genomic DNA of *M. hyopneumoniae*, ranging from 1.0×10^7 to 1.0×10^0 fg/μL, was prepared in nuclease-free water and used for the RPA analytical sensitivity analysis. One microliter of each dilution was amplified by RPA to determine the limit of detection (LOD) of the assay, and five independent reactions were performed. LFS RPA assay was performed independently by five different technicians.

2.7. Real-time PCR

A real-time PCR assay was performed on a ABI 7500 instrument (Applied Biosystems, Foster City, California) described previously (Strait et al., 2008). Sequences for the primers and probe are provided in Table 1. The Premix Ex Taq (Takara, Dalian, China) was applied in real-time PCR assay and the reaction was performed as follows: 95 °C for 10 min; then 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.8. Validation with clinical samples

The real-time and LFS RPA assays were assessed on 60 swine nasal swabs and 86 swine fresh lungs, and all samples tested by RPA assays were also tested by a real-time RT-PCR(Strait et al., 2008), which was run in parallel.

3. Results

3.1. Analytical specificity and sensitivity of the real-time RPA assay

Specific amplification was observed with *M. hyopneumoniae*, and there was no cross-detections of other pathogens tested (Fig. 1A). Five independent reactions were repeated and similar results were observed, demonstrating the good repeatability of the assay. The RPA assay was performed eight times on the molecular standard, in which 1.0×10^7–1.0×10^3 fg DNA molecules were detected in 8/8 runs, 1.0×10^2–1.0×10^0, 0/8 (Fig. 1B). Two-fold serial dilutions of the genomic DNA were made from 1.0×10^3 to 2.5×10^2 fg/μL. The above two-fold dilutions of the genomic DNA were further used in the RPA, and the LOD of the assay was 5.0×10^2 fg per reaction.

3.2. Performance the LFS RPA assay

The results from performing the LFS RT-RPA test with different reaction times are shown in Fig. 2A. No amplified products were observed in the reactions incubated for 5 min, and weakly products were observed after 10 min incubation. No clear differences between the products were observed after 15 and 20 min incubations. Similar results were observed in three independent reactions. Therefore, the optimal incubation time for LFS RPA assay was set at 15 min.

For the analytical specificity analysis, only *M. hyopneumoniae* was detected by the LFS RPA and the other pathogens were not detected (Fig. 2B). As shown in Fig. 2C, the LOD of the LFS RPA was 5.0×10^2 fg per reaction, which was the same as the real-time PCR and real-time RPA. The LFS RPA assay was performed three times by three different technicians, and results similar to those described above were obtained, demonstrating the good specificity and acceptable sensitivity.

3.3. Validation of RPA assays on clinical samples

Of the 110 clinical samples, 41 (28.08%), 42 (28.77%), and 47 (32.19%) samples were positive for *M. hyopneumoniae* by the real-time RPA, LFS RPA and real-time PCR, respectively (Table 2). Furthermore, the diagnostic performance of the real-time RPA and LFS RPA was compared to the real-time PCR by their respective diagnostic sensitivity (DSe), diagnostic specificity (DSp), positive predictive value (PPV), negative predictive value (NPV) and kappa coefficient. Compared to real-time PCR, the real-time RPA and LFS RPA assays showed DSp of 100%, DSe of 87.23% and 89.36%, PPV of 100%, NPV of 96.15% and 97.40%, and kappa value of 0.903 and 0.909, respectively (Table 3). It took less than 20 min in the RPA assays to obtain the positive results, while it took 30–50 min in the real-time RT-PCR with the Ct values ranging from 21.05 to 33.21. These results indicated that the performance of the RPA assays was comparable to real-time PCR, but the RPA assays are faster.

4. Discussion

Rapid and convenient diagnostic tools are critical for veterinarians to take appropriate measures to prevent the outbreak of diseases or control it as early as possible. The developed real-time RPA and LFS RPA assays for detection of *M. hyopneumoniae* demonstrated to be rapid, specific, sensitive, and easy to perform. Furthermore, the RPA reaction reagents are lyophilized for cold-chain independence and long-term storage, and the user-friendly PON detection platform for real-time RPA and the independence of sophisticated incubation instrument for LFS RPA make the assays readily suitable for field applications.

Several researchers had demonstrated the efficacy of the PCR and LAMP to detect 16S rRNA and other conserved regions of genomic DNA of *M. hyopneumoniae* in different clinical specimens (Calsamiglia et al., 1999; Dubosson et al., 2004; Kurth et al., 2002; Li et al., 2013; Liu et al., 2015; Stark et al., 1998; Strait et al., 2008; Verdin et al., 2000). Genetic diversity among strains of *M. hyopneumoniae* was also demonstrated, which could affect the detection results of the molecular diagnosis methods (de Castro et al., 2006; Madsen et al., 2007; Mayor et al., 2007; Stakenborg et al., 2006). A previous study had demonstrated that some *M. hyopneumoniae* PCR assays did not detect all isolates, while the real-time PCR assay based on the mhp165 gene was highly specific and capable of detecting all isolates of *M. hyopneumoniae* (Strait et al., 2008). The developed LAMP assay based on the mhp165 gene also demonstrated the good performance (Li et al., 2013). In this study, the RPA primers and probe were also designed based on the mhp65 gene. Through *in silico* analysis, there was no mismatch in the forward primer

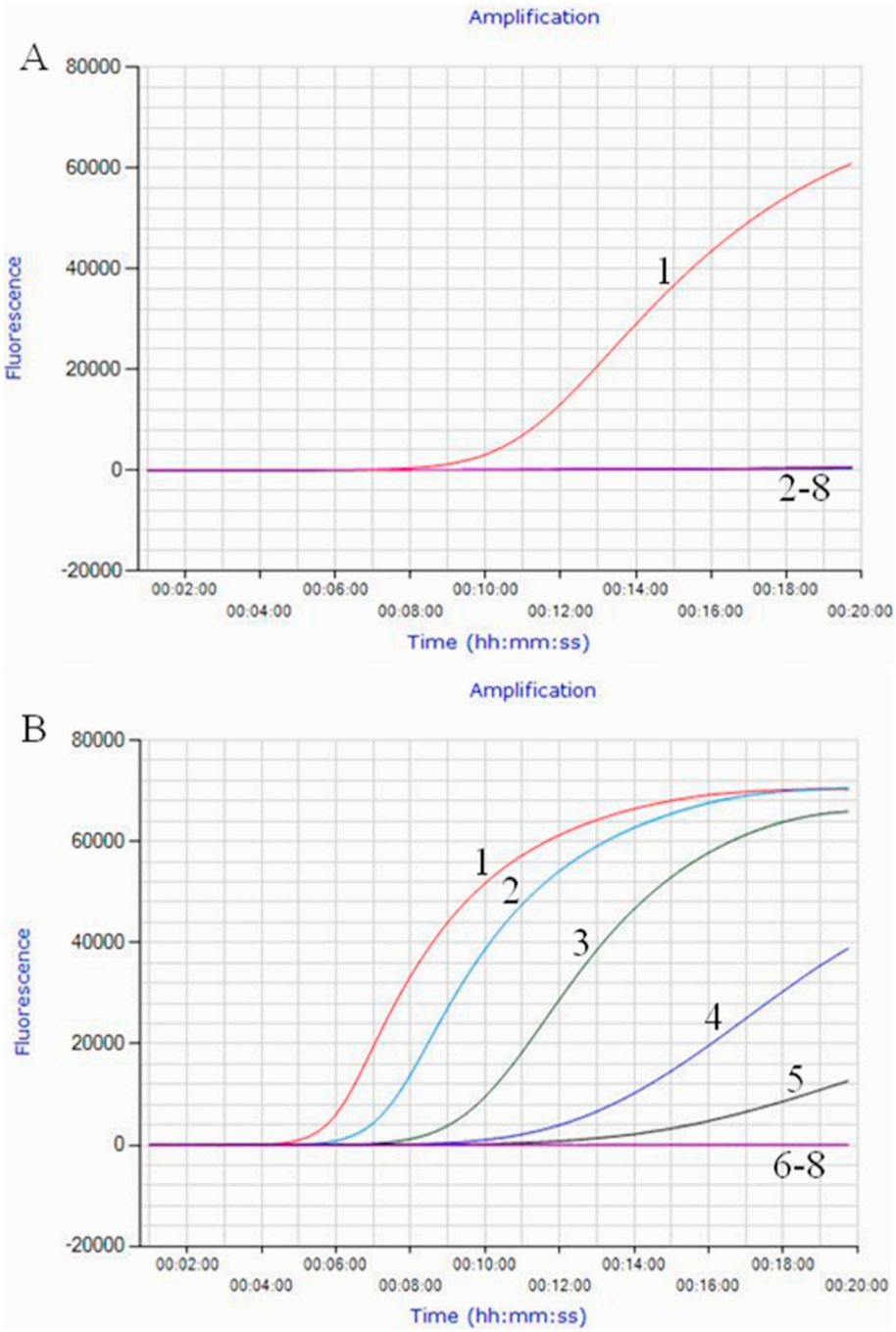

Fig. 1. Performance of *M. hyopneumoniae* real-time RPA assay. (A) Analytical specificity of the real-time RPA assay. Only the *M. hyopneumoniae* was amplified, but not other pathogens tested (n = 5). lane 1, *M. hyopneumoniae*; lane 2, *M. hyorhinis*; lane 3, *M. flocculare*; lane 4, *H. parasuis*; lane 5, *A. pleuropneumoniae*; lane 6, *P. multocida*; lane 7, PCV2; lane 8, PRV. (B) Fluorescence development over time using a dilution range of 1.0×10^7–1.0×10^0 fg of *M. hyopneumoniae* genomic DNA. lane 1, 1.0×10^7 fg; lane 2, 1.0×10^6 fg; lane 3, 1.0×10^5 fg; lane 4, 1.0×10^4 fg; lane 5, 1.0×10^3 fg; lane 6, 1.0×10^2 fg; lane 7, 1.0×10^1 fg; lane 8, 1.0×10^0 fg.

in the circulating strains, only one mismatch in the reverse primer in the strains 06MP2501 (EU658730), 95MP1509 (EU658731), and 96MP0001 (EU658732), and there were 2 mismatches in the probe in the strains 06MP2501 (EU658730), 95MP1509 (EU658731), 96MP0001 (EU658732) and 168 (CP002274). The RPA could tolerate less than 9 mismatches in primer and probe showing no influence on the performance of the assay (Abd El Wahed et al., 2013; Boyle et al., 2013), and mismatches toward the 5′ end appear to be more easily tolerated by RPA (Daher et al., 2015). Based on the above facts, the developed RPA assays would perform well despite the presence of the mismatches in the reverse primer and probe. It is assumed the assay would detect all the circulating *M. hyopneumoniae* based on the above facts, but this should be further confirmed by testing more *M. hyopneumoniae* DNA extracts or clinical samples from various regions worldwide.

The developed PCR assays for *M. hyopneumoniae* required either the agarose gel electrophoresis or the expensive thermal cycler devices, which made their application in the field and the resource-limited settings difficult (Cai et al., 2007; Calsamiglia et al., 1999; Dubosson et al., 2004; Strait et al., 2008). In this study, the developed real-time RPA assay was performed on the portable tube scanner Genie III, which weighs only 1.75 kg and can be charged by battery for working a whole

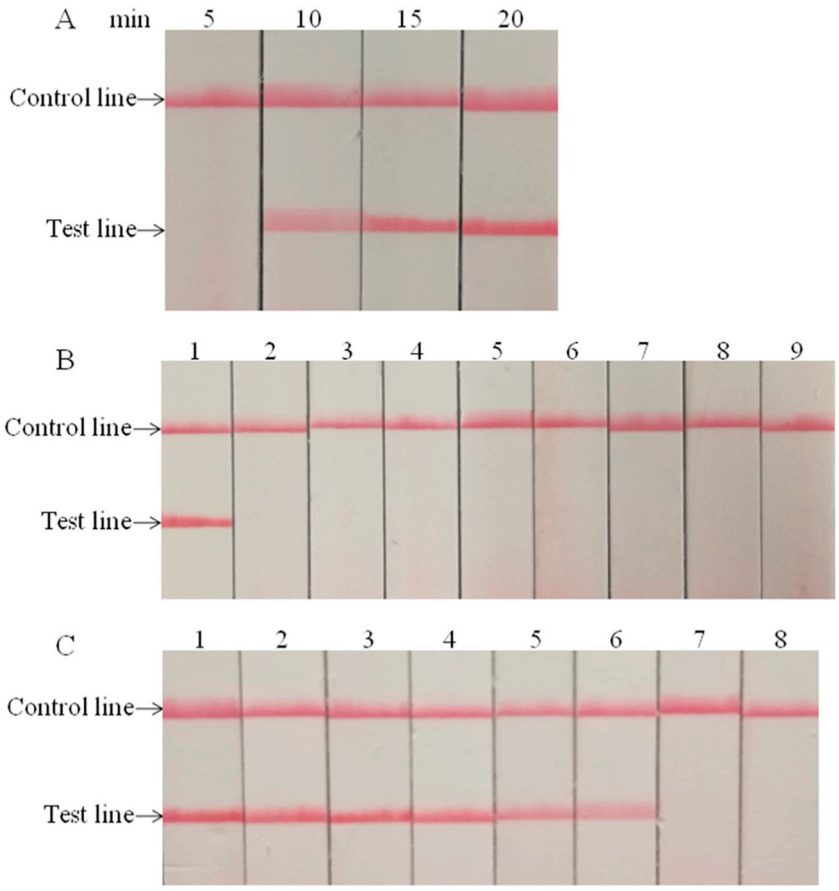

Fig. 2. Performance of *M. hyopneumoniae* LFS RPA assay. (A) Optimization of LFS RT-RPA reaction time. The test line was visible when the amplification time was longer than 10 min. (B) Analytical specificity of the real-time RPA assay. Only the *M. hyopneumoniae* was amplified, but not other pathogens tested (n = 5). lane 1, *M. hyopneumoniae*; lane 2, *M. hyorhinis*; lane 3, *M. flocculare*; lane 4, *H. parasuis*; lane 5, *A. pleuropneumoniae*; lane 6, *P. multocida*; lane 7, *K. pneumoniae*; lane 8, PCV2; lane 9, PRV. C. Analytical sensitivity of the LFS RPA assay. Lane 1, 1.0×10^7 fg; lane 2, 1.0×10^6 fg; lane 3, 1.0×10^5 fg; lane 4, 1.0×10^4 fg; lane 5, 1.0×10^3 fg; lane 6, 5.0×10^2 fg; lane 7, 2.5×10^2 fg; lane 8, 1.0×10^2 fg.

Table 2
Comparison of *M. hyopneumoniae* real-time RPA, LFS RPA and real-time PCR assays for detection of clinical samples.

Samples	Number of samples	Real-time RPA		LFS RPA		Real-time PCR	
		P	N	P	N	P	N
Nasal swabs	60	10	50	10	50	12	48
Fresh lungs	86	31	55	32	54	35	51
T	146	41	105	42	104	47	99

Note: P, positive; N, negative; T, total.

Table 3
Diagnostic sensitivity, specificity, predictive value, and kappa value of real-time RPA, LFS RPA and real-time PCR assays for diagnosing *M. hyopneumoniae* infection.

		Real-time PCR		
		P	N	T
Real-time RPA	P	41	0	41
	N	6	99	105
	T	47	99	146
		DSe:87.23%	DSp:100%	K:0.903
		PPV:100%	NPV:94.29%	
LFS RPA	P	42	0	42
	N	5	99	104
	T	47	99	146
		DSe:89.36%	DSp:100%	K:0.919
		PPV:100%	NPV:95.19%	

Note: P, positive; N, negative; T, total; DSe, diagnostic sensitivity; DSp: diagnostic specificity; K: kappa value; PPV: positive predictive value; NPV: negative predictive value.

day. Furthermore, the common reaction tubes for real-time PCR could be used in Genie III for China market, and there was no need for the special tubes in this study. The developed LFS RPA assay combined RPA technology with lateral flow strip, which required only a simple incubator block, and the testing results were visible. Moreover, RPA reagents are cold chain independent and RPA is tolerant to common PCR inhibitors (Daher et al., 2016; Lillis et al., 2016; Moore and Jaykus, 2017). The above characteristics make the developed RPA assays ideal for PON detection of *M. hyopneumoniae*, which is especially important for pig farms located in rural areas.

The diagnostic performances of the developed RPA assays were compared to a real-time PCR assay, which is considered to be the gold standard of molecular detection methods. Based on the analysis data in this study, the performance of the real-time RPA and LFS RPA assays were comparable to real-time RT-PCR, while the RPA assays have the distinct advantages of rapidness and convenience. Although the above results are inspiring, the RPA assays need be further validated by testing of more *M. hyopneumoniae* DNA positive clinical samples. As in the real-time PCR, DNA extraction is still necessary in the RPA assays developed in this study. One of the main reasons for developing such assay is its potential use in the field or in the absence of a reliable power supply. Furthermore, a simple, rapid and effective genomic DNA extraction method is still needed to make the developed RPA assays applicable in the field. Presently, there are several kinds of commercial nucleic acid extraction kit being used in the field without the need of complex instruments, such as the SpeedXtract Nucleic Acid Kit (Qiagen, Hilden, Germany), innuPREP MP basic kit A (Jena Analytik, Jena, Germany), Punch-it™ NA-Sample Kit (NanoHelix, Daejeon, South Korea) and 1st tech DNA/RNA release kit (FIRSTECH, Beijing, China). A RT-RPA assay combined with the SpeedXtract Nucleic Acid Kit had been used in the point-of-care detection of Ebola virus in sera and swab samples in Guinea (Faye et al., 2015). Real-time RPA and LFS RPA assays

combined with innuPREP MP basic kit were also developed for the rapid detection of peste des petits ruminants virus, sheeppox virus and goatpox virus in the field (Yang et al., 2017a,b). Using the nucleic acid extracted by those simple methods as the template demonstrated the same performance when compared with the routine commercial nucleic acid extraction kits in the above RPA assays(Faye et al., 2015; Yang et al., 2017a; Yang et al., 2017b). In our laboratory, the nucleic acid extraction efficacy and the potentiality to being used in the field were also evaluated for the above four simple extraction kits to make the developed RPA assays for *M. hyopneumoniae* in the real sense of being applicable in the field.

In conclusion, the developed RPA assays with high specificity and sensitivity are simple, rapid and reliable for *M. hyopneumoniae* detection. The features of the developed RPA assays make them suitable to be potentially applied in the rapid detection of *M. hyopneumoniae* in underequipped diagnostic laboratory and the PON diagnosis at quarantine stations and farms, which are of great importance to control enzootic pneumonia in pig herds.

Conflicts of interest statement

The authors declare that they have no competing interests.

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