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Rapid detection of porcine encephalomyocarditis virus (EMCV) by isothermal reverse transcription recombinase polymerase amplification assays

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ABSTRACT

In this study, we combined reverse transcription recombinase polymerase amplification assay with the fluorescence detection platform (qRT-RPA) and lateral flow biosensor (LFB RT-RPA) to allow for rapid detection of porcine encephalomyocarditis virus (EMCV). Primers and probes were designed to target the highly conserved region of 3D gene of porcine EMCV. The optimal reaction condition of qRT-RPA and LFB RT-RPA was set as 42 °C for 20 min. The assays were highly specific to EMCV and no cross-reactions were observed with seven other porcine viruses. With a 10-fold serially diluted EMCV genomic RNA as template, the limit of detection was 1.0×10^2 and 1.0×10^1 copies for qRT-RPA assay and LFB RT-RPA assay, respectively. A total of 92 samples from different sources were examined using qRT-RPA, LFB RT-RPA and qRT-PCR. We found 100% diagnostic agreement between qRT-RPA (23/92) and qRT-PCR (23/92), and 97.83% diagnostic agreement between LFB RT-RPA (25/92) and qRT-PCR (23/92). There was no significant difference in performance between the RT-RPA assays developed in this study and a previously described qRT-PCR. However, RT-RPA assays were rapid and easy to perform while LFB RT-RPA exhibited higher sensitivity for EMCV than qRT-PCR. Therefore, the developed EMCV RT-RPA assays provide an attractive and promising tool for effective detection of EMCV in low-resource settings.

1. Introduction

The encephalomyocarditis virus (EMCV) belongs to the genus *Cardiovirus* within the family *Picornaviridae*, and it is a non-enveloped virus with a positive-sense single-stranded RNA approximately 7.8 kb in size (Palmenberg et al., 1984). EMCV was first isolated from a gibbon in 1945 in Florida (Helwig and Schmidt, 1945). Since then, the virus has been detected in different domestic and wild animals globally, including pigs, cattle, wild boar, dog, rodents, raccoons, elephants, marsupials, alpacas, llama, tigers, lions and non-human primates, and even humans (Canelli et al., 2010; Carocci and Bakkali-Kassimi, 2012; Diallo et al., 2013; Liu et al., 2016, 2013; Luo et al., 2017; Murnane et al., 1960; O'Connor et al., 2020; Oberste et al., 2009; Philipps et al., 2012; Reddacliff et al., 1997; Romey et al., 2021; Yeo et al., 2013). Natural reservoirs (rodents) with EMCV infection typically present without clinical signs whereas most other animal species with EMCV develop myocarditis and encephalitis that may cause sudden death (Liu et al., 2016, 2013; O'Connor et al., 2020; Philipps et al., 2012; Romey et al., 2021). Rodents are considered natural hosts of EMCV, while pigs are considered the most susceptible domestic animal (Koenen, 2006). The first swine EMCV infection was detected in Panama in 1958 (Murnane et al., 1960). Since then, more EMCV infections have been reported in pig farms in

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Europe (Maurice et al., 2005), North America (Czechowicz et al., 2011; Dea et al., 1991; Feng et al., 2015a; Murnane et al., 1960), Australia (Acland and Littlejohns, 1975), Korea (An et al., 2009) and China (Feng et al., 2015a). EMCV causes myocarditis and encephalitis with sudden death in piglets and reproductive disorders in sows (Koenen, 2006), causing severe economic losses in the swine industry worldwide. Swine EMCV was first characterized in China in 2005, and as of 2016, at least 11 strains of porcine EMCV had been reported in China (Liu et al., 2016; Zhang et al., 2007).

Considering the public health significance and the considerable economic loss of EMCV, a rapid, reliable and easy method for diagnosis is required for implementing the effective prevention and control measures to prevent its epidemics. Various laboratory assays have been developed for the EMCV infection, including the virus isolation(Yuan et al., 2014a) and a different series of nucleic acid testing assays (NAT) (Vanderhallen and Koenen, 1997; Wang et al., 2012; Yuan et al., 2014b, 2014c). All these methods are crucial to the diagnosis of EMCV infection. EMCV isolation assay is relatively easy to perform and rapid, providing a definitive result for most isolates in 24–48 h. However, the requirement for cell culture facilities in the laboratory restricts its application as a routine method. At present, the routine laboratory detection of EMCV involves NAT assays, including conventional reverse-transcription PCR (RT-PCR), real-time RT-PCR (RT-qPCR) based on SYBR Green or Taq-Man probe and reverse-transcription LAMP (RT-LAMP) (Vanderhallen and Koenen, 1997; Wang et al., 2012; Yuan et al., 2014b, 2014c).

Recombinase polymerase amplification (RPA) is an isothermal nucleic acid testing technology first described in 2006. RPA is now applied widely in nucleic acid testing, including in pathogen detection (Li et al., 2018; Piepenburg et al., 2006). To our knowledge, no RPA assay has been developed for the detection of EMCV to date. In this study, we developed reverse transcription RPA assays combining with fluorescence signal analysis (qRT-RPA) and lateral flow biosensor (LFB RT-RPA) that target the highly conserved region of 3D gene for rapid and accurate detection of porcine EMCV. In addition, RPA assays were evaluated using organ samples from artificially challenged mice and clinically diseased pigs.

2. Materials and methods

2.1. Virus strains and samples

EMCV and a panel of other viruses of great significance in pig industry were used in this study. EMCV (strain BD2) was isolated from newborn piglets that with acute myocarditis in Hebei province and kept in our laboratory (Yuan et al., 2014a). Porcine circovirus-2 (PCV-2, strain HB-MC1) and respiratory and reproductive syndrome virus (PRRSV, strain HB-Xl) were also isolated and kept in our laboratory. Porcine epidemic diarrhea virus (PEDV, strain CV777), classical swine fever virus (CSFV, strain AV1412), pseudorabies virus (PRV, strain Bartha-K61), and porcine parvovirus (PPV, strain BJ-2) were obtained from the commercial attenuated live vaccines. Denatured cell-free extracts of foot-and-mouth disease virus (FMDV) type O were obtained from the commercial Liquid-phase Blocking ELISA Kit (Lot 101/23, Lanzhou Veterinary Research Institute, Lanzhou, China).

Thirty-two mice organs, including heart, brain, spleen and liver, were used in this study. Of these, 8 were collected from 2 eight-week-old BALB/c mice inoculated with 0.1 mL Dulbecco's modified Eagle medium and 24 were collected from 6 eight-week-old BALB/c mice inoculated with 0.1 mL EMCV BD2 suspension and died after three days. Details of EMCV inoculation experiment with BALB/c mice had been described previously (Yuan et al., 2015). Samples of lung, spleen, liver, tonsil and lymph node were collected from 60 pigs with reproductive disorders in the Bio-Safety Disposal Centers for Dead Livestock and Poultry in Hebei Province in December 2021. Twenty mg of each organ from each pig were separately collected and pooled together to form a single sample (pooled swine organs, 100 mg) before nucleic acid extraction.

2.2. DNA/RNA extraction

Genomic RNA of EMCV, PRRSV, CSFV, PEDV and FMDV, and DNA of PRV, PCV2 and PPV were extracted using the TIANamp Virus DNA/RNA kit (Tiangen, Beijing, China) following the manufacturer's instructions. The RNA or DNA extracted was eluted in 50 μL nuclease-free water and quantified using a ND-2000c spectrophotometer (NanoDrop, Wilmington, USA). One hundred mg of individual mice organ samples and 100 mg of pooled swine organ samples were all homogenized with 1 mL phosphate-buffered saline (PBS, pH 7.4) and a 10% (w/v) suspension was prepared. After centrifugation at 8000 g for 5 min at 4 °C, 200 μL of the supernatant was collected and the RNA were extracted using the Magnetic Virus DNA/RNA Extraction kit (Tianlong, Xian, China) on automated nucleic acid extraction platform (NP968, Tianlong, Xian, China). The RNA/DNA extracted from each clinical sample was finally eluted in 100 μL of nuclease-free water. All DNA/RNA templates were stored at − 80 °C until use.

2.3. RPA primers and probes

The 3D gene is highly conserved in the EMCV genome (Vanderhallen and Koenen, 1997; Wang et al., 2012; Yuan et al., 2014b, 2014c). In this study, EMCV-specific RPA primers, exo probe and nfo probe were designed following RPA guidelines from TwistDx (Cambridge, United Kingdom) and synthesized by Generay Biotechnology (Shanghai, China) to amplify a highly conserved sequence within the 3D gene of EMCV. 3D gene sequences from 13 different swine EMCV strains (Accession numbers: KF709977, DQ464062, DQ464063, FJ897755, KF771002, KJ524643, MH191297, KF836390, EU780148, EU780149, LC508268, AF356822, X74312) were aligned and compared using the CLUSTALW within DNASTAR 6.0 program. Both real-time RPA and LFB RPA primers amplified a 189 bp fragment from a highly conserved region of the 3D gene of EMCV. The specificity of RPA primers and probes was also tested in silico with nucleotide sequences from EMCV isolates from wild boar (KF836387), south china tiger (KF293299), dog (KU664327), elephant (MT250508), mouse (KF836388, DQ288856, JX257003, X87335), and non-human primates (M81861, L22089, KC310738, KC310737). Details of RPA primers, exo probe and nfo probe are shown in Table 1.

2.4. EMCV-specific qRT-RPA assay

EMCV specific qRT-RPA reactions were performed in 0.2 mL reaction tube containing freeze-dried enzyme pellet from the ZC BioScience™ RT-exo kit (ZC BioScience, Hangzhou, China). The total reaction volume was 50 μL, containing 25 μL of rehydration buffer, 2.5 μL of magnesium acetate (280 mM), 2.0 μL of EMCV-exo-F (10 μmol/L), 2.0 μL of EMCV-exo-R (10 μmol/L), 0.6 μL of EMCV-exo-P (10 μmol/L), 1 μL of standard viral RNA or 5 μL of sample viral RNA, 16.9 μL or 12.9 μL of ddH2O. qRT-RPA reactions were performed at 42 °C for 20 min in portable and battery-chargeable Genie III scanner device (OptiGene Limited, West Sussex, UK). Samples producing an exponential amplification curve above the threshold of the negative control were considered positive.

2.5. EMCV-specific LFB RT-RPA assay

EMCV specific LFB RT-RPA reactions were performed in 0.2 mL reaction tube containing freeze-dried enzyme pellet from the GenDx™ RT-LFB kit (GenDx, Suzhou, China). The total reaction volume was 50 μL, containing 20 μL of rehydration buffer, 2.0 μL of magnesium acetate (280 mM), 2.1 μL of EMCV-exo-F (10 μmol/L), 2.1 μL of EMCV-exo-R (10 μmol/L), 0.6 μL of EMCV-exo-P (10 μmol/L), 1 μL of standard viral RNA or 5 μL of sample viral RNA, 22.2 μL or 18.2 μL of ddH2O. RPA reactions were optimized in a portable metal bath incubator at 39–45 °C for 20 min to determine the optimal reaction temperature. Then, RPA was

Table 1
Sequences of the primers and probes for EMCV qRT-RPA, LFB RT-RPA and qRT-PCR assays.

Assay	Primers and probes	Sequence 5′–3′	Amplicon size (bp)	Reference
qRT-RPA	EMCV-exo-F	GACTGCCTTCGGTGTCGCCATGCAAGGTTTT	189	This study
	EMCV-exo-R	TCAAACGCATGGGTTGAAATGGCTAATGAC		
	EMCV-exo-P	AGCGTGTCTACGATGTGGACTACTCCAAC (FAM-dT)(THF)(BHQ1-dT)GATTCGACCCATTCG-C3-spacer		
LFB RT-RPA	EMCV-nfo-F	GACTGCCTTCGGTGTCGCCATGCAAGGTTTT	189	This study
	EMCV-nfo-R	Biotin-TCAAACGCATGGGTTGAAATGGCTAATGAC		
	EMCV-nfo-P	FAM-AGCGTGTCTACGATGTGGACTACTCCAACT (THF)TGATTCGACCCATTCG -C3-spacer		
qRT-PCR	EMCV-F	TCATTAGCCATTTCAACCCA	135	Yuan et al. (2014c)
	EMCV-R	GAGATACAAACCCGCCCTAA		
	EMCV-P	FAM–TCCATCATGAAGTGCGACGTCG-TAMRA		

Note: W: A or T , S: G or C

performed at optimal reaction temperature for 5, 10, 15 and 20 min to determine optimal reaction time. RPA products dual labeled with FAM and Biotin were detected using the lateral flow biosensor (GenDx, Suzhou, China) following the manufacturer's instructions. A testing sample was considered positive when red bands were visible on both the test line and control line, negative when red band was visible only on the control line and invalid when red band was invisible on the control line.

2.6. Analytical specificity analysis

The analytical specificity of developed EMCV-specific qRT-RPA and LFB RT-RPA assays was determined using 1.0×10^4 copies of EMCV RNA or 6.08×10^7-5.15×10^9 copies of PRRSV, CSFV, PEDV, FMDV, PRV, PPV and PCV2 RNA or DNA, which are the important swine viruses. The specificity analysis was performed five times independently with these templates.

2.7. Analytical sensitivity analysis

The analytical sensitivity or limit of detection (LOD) of the developed EMCV-specific qRT-RPA and LFB RT-RPA assays was determined using 10-fold serial dilutions of EMCV RNA as the template, with a concentration ranging between 1.0×10^6 and 1.0×10^0 copies/μL. The LOD of RPA assays was determined as the highest dilution of the virus detectable using the assays. A previously described qRT-PCR assay was run concurrently with RPA assays for each dilution to compare the analytical sensitivity of the tests (Yuan et al., 2014c). The sensitivity analysis of qRT-RPA and LFB RT-RPA assays were repeated five times independently.

2.8. Validation with the clinical samples

To confirm the potential applicability of the EMCV-specific qRT-RPA and LFB RT-RPA assays in clinical diagnostics, the developed RPA assays were directly applied to total RNA extracted from 32 individual mice organ samples and 60 pooled swine organ samples. The qRT-PCR assay was run concurrently with RT-RPA for these samples (Yuan et al., 2014c).

3. Results

3.1. Analytical specificity and sensitivity of EMCV-specific qRT-RPA assay

In the analytical specificity analysis of qRT-RPA assay, only the EMCV RNA was amplified as indicated by a typical fluorescent curve, and no other viruses tested were amplified (Fig. 1 A). LOD of qRT-RPA assay was determined by using a 10-fold serial dilutions of the EMCV RNA in the range of 1.0×10^5 to 1.0×10^0 copies/μL. The real-time RPA assay was repeated five times, in which 1.0×10^5-1.0×10^2 copies of

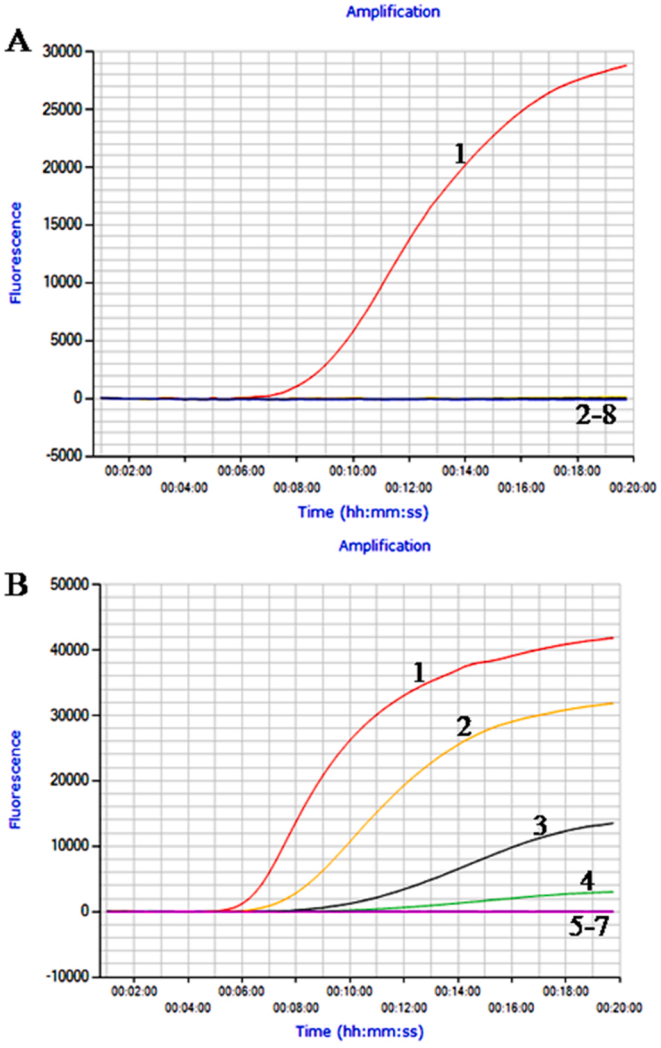

Fig. 1. Performance of EMCV-specific qRT-RPA assay. Only the EMCV RNA was amplified, and the limit of detection of the assay was 1.0×10^2 copies. A. Analytical specificity of the qRT-RPA assay evaluating on the viruses important in pig. Line 1, EMCV; line 2, PRRSV; line 3, CSFV; line 4, PEDV; line 5, FMDV; line 6, PRV; line 7, PCV2; line 8, PPV; B. Analytical sensitivity of the qRT-RPA assay using a dilution range of 1.0×10^5-1.0×10^0 copies of EMCV RNA. Line 1, 1.0×10^5 copies; line 2, 1.0×10^4 copies; line 3, 1.0×10^3 copies; line 4, 1.0×10^2 copies; line 5, 1.0×10^1 copies; line 6, 1.0×10^0 copies; line 7, ddH2O.

EMCV RNA were detected in 8/8 runs, 1.0×10^1 and 1.0×10^0, 0/8 (Fig. 1B). The LOD of qRT-RPA was 1.0×10^2 copies, which was consistent with LOD of the described qRT-PCR. Similar results were obtained in five independent repeats, demonstrating the good reliability and consistency of EMCV-specific qRT-RPA assay.

3.2. Optimization of the reaction condition of EMCV-specific LFB RT-RPA assay

Using 1.0×10^5 copies of EMCV genomic RNA as the template, optimal reaction conditions for EMCV-specific LFB RT-RPA was estimated. The optimal reaction temperature was estimated at 39 °C – 45 °C, as shown in Fig. 2 A. The red bands (test lines) were clearer when the reaction temperature exceeded 40 °C. However, no significant differences in the visibility of red bands were observed among 41 °C, 42 °C and 43 °C. The optimal reaction temperature set was 42 °C. The estimated optimal reaction time for 5, 10, 15 and 20 min at 42 °C were shown in Fig. 2B. The 3D gene of EMCV was amplified successfully at 42 °C for more than 15 min. The red bands (test lines) were clearer after incubation for 20 min and 30 min, with no significant difference between thema. The optimal reaction time was set as 20 min

Similar results were obtained in five independent repeats. The optimal reaction condition set for EMCV-specific LFB RT-RPA assay was set as 42 °C for 20 min

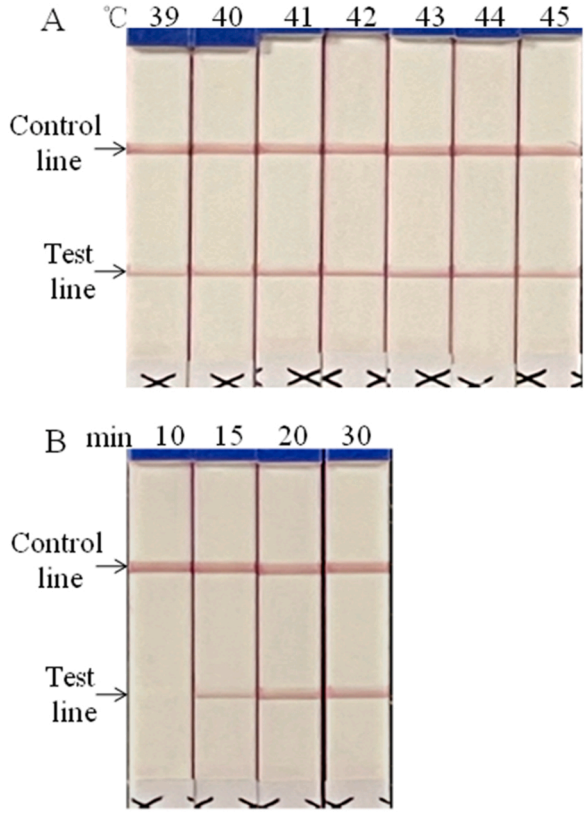

Fig. 2. Optimization of the reaction temperature and time of EMCV-specific LFB RT-RPA assay. The optimal reaction condition was set to be performed at 42 °C for 20 min. A. Optimization of the reaction temperature. The target gene of EMCV was amplified successfully at 39 °C – 45 °C. The red bands (test lines) were clearer when the temperature was higher than 40 °C, and there were no clear differences between 41 °C, 42 °C and 43 °C. The optimal reaction temperature was set as 42 °C. B. Optimization of the reaction time. The target gene of EMCV was amplified successfully at 42 °C for more than 15 min. The red bands (test lines) were clearer after incubation for 20 min and 30 min, and there were no clear differences for them. The optimal reaction time was set as 20 min.

3.3. Analytical specificity and sensitivity of EMCV-specific LFB RT-RPA assay

In the LFB RT-RPA, the red band on the test line was only observed when EMCV RNA was used as the template (Fig. 3 A). In the analytical sensitivity analysis, the red band on the test line was observed when 1.0×10^6-1.0×10^1 copies of EMCV RNA were used as the template in the LFB RT-RPA assay (Fig. 3B). The LOD of LFB RT-RPA was 1.0×10^1 copies, which was 10 times lower than LOD of the described qRT-PCR. Similar results were observed in five independent repeats, demonstrating the good reliability and consistency of the EMCV-specific LFB RT-RPA assay.

3.4. Performance of EMCV-specific RT-RPA assays on clinical samples

For the 26 brain, heart, spleen and liver samples collected from EMCV-inoculated mice, 21 samples were positive for EMCV using qRT-RPA assay (threshold time ranging from 3.57 to 15.30 min) and qRT-PCR assay (Ct value ranging from 15.00 to 34.56, with the EMCV RNA copy number between 3.2×10^4 and 9.3×10^9 copies/100 mg). In contrast, positive EMCV results were obtained in 23 samples using LFB RT-RPA assay (Table 2). All eight samples collected from control mice were EMCV RNA negative in all tests (Table 2). For the 60 mixed swine organs, three tests gave similar results, in which two samples were EMCV RNA positive (Table 2). The detection results for qRT-RPA, LFB RT-RPA and qRT-PCR were also similar across all samples except for two liver samples from EMCV-inoculated mice. Both liver samples were positive for EMCV RNA using the developed LFB RT-RPA assay but negative using the other two assays. Compared with qRT-PCR, the developed EMCV-specific qRT-RPA and LFB RT-RPA assays showed a

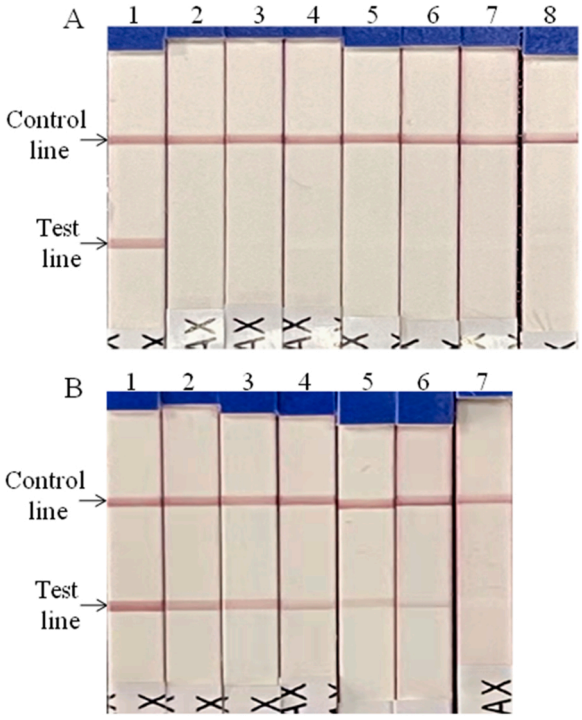

Fig. 3. Performance of EMCV-specific LFB RT-RPA assay. Only the EMCV RNA was amplified, and the limit of detection of the assay was 1.0×10^1 copies. A. Analytical specificity of the LFB RT-RPA assay evaluating on the viruses important in pig. Lane 1, EMCV; lane 2, PRRSV; lane 3, CSFV; lane 4, PEDV; lane 5, FMDV; lane 6, PRV; lane 7, PCV2; lane 8, PPV; B. Analytical sensitivity of the LFB RT-RPA assay using a dilution range of 1.0×10^6-1.0×10^0 copies of EMCV RNA. Lane 1, 1.0×10^6 copies; lane 2, 1.0×10^5 copies; lane 3, 1.0×10^4 copies; lane 4, 1.0×10^3 copies; lane 5, 1.0×10^2 copies; lane 6, 1.0×10^1 copies; lane 7, 1.0×10^0 copies.

Table 2
Comparison of qRT-RPA, LFB RT-RPA and qRT-PCR assays for detection of EMCV RNA in different clinical samples.

Samples	Number	qRT-RPA		LFB RT-RPA		qRT-PCR	
		P	N	P	N	P	N
Organs from inoculated mice	24	21	3	23	1	21	3
Organs from uninfected control mice	8	0	8	0	8	0	8
Organs from diseased pigs	60	2	58	2	58	2	58
T	92	23	69	25	67	23	69

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

high diagnostic specificity (DSp) of 100% and 92.0%, respectively, diagnostic sensitivity (DSe) of 100%, positive predictive value (PPV) of 100%, negative predictive value (NPV) of 100% and 97.1%, respectively, and kappa coefficient value of 1.0 and 0.944, respectively (Table 3).

4. Discussion

The RPA technology was first reported in 2006. Since 2014, it has been widely adopted for clinical diagnosis due to its isothermal nucleic amplification, simplified equipment requirements and fast reaction times (Li et al., 2018). In this study, EMCV-specific RPA assays that amplified 3D gene were developed to rapidly and accurately detect EMCV in clinical samples within 20 min. Both qRT-RPA assay using exo probe and LFB RT-RPA assay using nfo probe combined with lateral flow biosensor demonstrated high specificity and sensitivity to EMCV and comparable performance in detecting clinical samples. The RPA reagents are provided as lyophilized powder, which preserves full activity at 25 °C for up to 12 weeks (Lillis et al., 2016). Furthermore, the portable detection platforms and minimization of sophisticated instruments made the developed EMCV-specific RT-RPA assays a promising and efficient method for point-of-need diagnosis of EMCV infection. To our knowledge, this is the first study to develop RPA assay for rapid detection of EMCV.

The performance of the RPA largely depends on the characteristics of the primers and probe, which determine the specificity and sensitivity of the assay. In this study, primers and probes were designed to amplify the 3D gene of EMCV, which is used for the diagnosis of EMCV infection by RT-PCR, RT-qPCR and LAMP (Vanderhallen and Koenen, 1997; Wang et al., 2012; Yuan et al., 2014b, 2014c). Sequence homology of the primers and probes was analyzed by nucleotide–nucleotide search using the basic local alignment search tool (BLAST) of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The designed primers and probes were highly homologous to all EMCV isolates from China, Korea, Japan, Belgium (AF356822), and a few strains isolated from Germany (X74312) and USA (M81861, DQ288856). No mismatch was detected between primers and probes for all these EMCV strains, which clustered under lineage 1 based on whole genome or 3D gene phylogenetic analysis. In theory, the RT-RPA assay developed in this study could detect all the lineage 1 EMCV isolates. In addition, the developed qRT-RPA and LFB RT-RPA performed well with BD2 genomic RNA, and no cross-reactivity was observed with the PRRSV, CSFV, PEDV, FMDV, PRV and PCV2, demonstrating the high specificity of these assays. The topology of the neighbor-joining (NJ) trees revealed that all EMCV isolates clustered into five lineages (lineages 1, 2, 3, 4 and 5) (Feng et al., 2015b; Liu et al., 2016). Unfortunately, 3–10 nucleotide mismatches were detected in the EMCV isolates belonging to lineages 2, 3, 4 and 5, comprising most strains isolated from non-human primates and some strains isolated from pigs in Italy (M37588) and USA (AY296731, M22457, M22458). Although previous studies demonstrated that RPA could tolerate 5–9 mismatches without affecting the performance of the assay (Abd El Wahed et al., 2013; Daher et al., 2016), it was difficult to successfully detect the lineages 2, 3, 4 and 5 EMCV using homology analysis and the research experience in our laboratory. Our results indicated that the developed qRT-RPA and LFB RT-RPA assays were specific to lineage 1 EMCV and exhibited no cross-reactions with other significant swine pathogens we tested.

Table 3
Diagnostic sensitivity, diagnostic specificity, predictive value, and kappa value of EMCV qRT-RPA, LFB RT-RPA and qRT-PCR assays.

		qRT-PCR		
		P	N	T
qRT-RPA	P	23	0	23
	N	0	69	69
	T	23	69	92
		DSe:100%	DSp:100%	K:1.0
		PPV:100%	NPV:100%	
LFB RT-RPA	P	23	2	25
	N	0	67	67
	T	23	69	92
		DSe:92.0%	DSp:100%	K:0.944
		PPV:100%	NPV:97.1%	

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

The developed RT-PCR assays based on different target genes have contributed to the control of EMCV (Qin et al., 2018; Vanderhallen and Koenen, 1997; Wang et al., 2012; Yuan et al., 2014c). However, due to the requirement of a well-equipped laboratory for PCR assays, clinical samples must be transported to laboratories in suitable cold-chain conditions, delaying diagnosis and critical clinical decisions. In this study, we performed qRT-RPA assay on portable OptiGene Genie III and the LFB RT-RPA assay was on portable metal bath incubator. Both assays showed high EMCV detection performance comparable to the qRT-PCR for the clinical samples. Nevertheless, the developed RT-RPA assays for EMCV significantly reduced assay turnaround times, minimized the need for complicated instrumentation and experienced laboratory personnel, demonstrating great potential as a diagnostic tool in the field. Seemingly, RPA may not replace PCR for years to come, but it could be a versatile complement for PCR for the rapid detection of EMCV.

The field-amenable sample preparation methods, including concentration, extraction and purification, are needed to facilitate a complete RPA assay for on-site application. We still needed a high-speed centrifuge for viral RNA extraction in this study, which hampered actual on-site application in the real sense. Several studies demonstrated that RPA assays could work well with the nucleic acid extracted using different commercial kits (Faye et al., 2015; Inoshima et al., 2017). In a follow-up study, we will focus on optimizing nucleic acid extraction from clinical samples in the field.

In conclusion, the developed EMCV qRT-RPA and LFB RT-RPA assays targeting 3D gene are rapid, specific, sensitive and easy to perform for accurate detection of lineage 1 EMCV as an alternative to qRT-PCR. The assays demonstrate considerable potential as diagnostic tools in the field. However, further optimization and verification are warranted to evaluate the detection performance of these EMCV qRT-RPA and LFB RT-RPA assays.

Ethical approval

The mice samples were all obtained from a mice inoculation experiment, which was approved by the Institutional animal care and ethics committee of Hebei Agricultural University with certificate IACECHEBAU20110509. All the swine samples used were diagnostic samples submitted by clients for diagnostic services.

CRediT authorship contribution statement

Jinfeng Wang: Conceptualization, Methodology. **Sinan Chen**:

Methodology, Investigation. **Qingan Han**: Resources, Validation. **Kairui Wang**: Validation. **Libing Liu**: Validation. **Xiangdong Xu**: Data curation, Writing – original draft. **Wanzhe Yuan**: Funding acquisition, Resources. **Jianchang Wang**: Project administration, Writing – review & editing.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of interest statement

The authors declare that they have no competing interests.

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