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Rapid and accurate detection of African swine fever virus by DNA endonuclease-targeted CRISPR trans reporter assay

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Abstract

The first case of African swine fever (ASF) outbreak in China was reported in a suburban pig farm in Shenyang in 2018. Since then, the rapid spread and extension of ASF has become the most serious threat for the swine industry. Therefore, rapid and accurate detection of African swine fever virus (ASFV) is essential to provide effective strategies to control the disease. In this study, we developed a rapid and accurate ASFV-detection method based on the DNA endonuclease-targeted CRISPR trans reporter (DETECTR) assay. By combining recombinase polymerase amplification with CRISPR-Cas12a proteins, the DETECTR assay demonstrated a minimum detection limit of eight copies with no cross reactivity with other swine viruses. Clinical blood samples were detected by DETECTR assay and showed 100% (30/30) agreement with real-time polymerase chain reaction assay. The rapid and accurate detection of ASFV may facilitate timely eradication measures and strict sanitary procedures to control and prevent the spread of ASF.

Key words: African swine fever, African swine fever virus, recombinase polymerase amplification, CRISPR-Cas12a, DNA endonuclease-targeted CRISPR trans reporter assay

Introduction

The emergence and spread of African swine fever (ASF) in China since 2018 had caused devastating consequences for the pig industry. As of August 1, 2019, almost all provinces of China have officially reported outbreak of ASF cases and more than 1,000,000 pigs have been slaughtered [1]. Over 50% of the global pig population is in China; therefore, pig production and pork consumption are critical for the economics and agricultural development in China and around the world. Comprehensive and integrated strategies are urgently required to prevent and control the spread of ASF [2,3]. Under these circumstances, rapid and accurate detection of African swine fever virus (ASFV) is very important for the timely establishment of preventive measures and controlling policies [4]. Because there is no commercial vaccine or effective drug to treat ASF at present, the primary eradication strategies are accurate slaughter of infected pigs and strict implementation of sanitary procedures.

ASFV is a large, enveloped, and double-stranded DNA (dsDNA) virus that can cause acute hemorrhagic fever in domestic pigs and wild boars, while the native hosts of ASFV also include soft ticks. Domestic pigs infected with ASFV have a mortality rate of 100%; therefore, the World Organization for Animal Health (OIE) listed

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ASF as a notifiable contagious animal disease, and it is listed as predominantly infectious animal disease in China [4]. The genome of ASFV is large and complex with the DNA size ranging from 170 to 194 kb, which encodes more than 150 open reading frames (ORFs) of ~50 structural proteins and 100 host-induced immunoregulatory proteins [5,6]. Most structural proteins, such as pp220, pp62, p72, p54, p30, and CD2v, are major components of virus particles and are important in the process of virus attachment, entry, and replication [7,8]. The gene regions of p72, p54, p30, B602L, and 9GL have been used for genetic homogeneity analysis of different ASFV strains. Among them, the *p72* gene contains the most conserved regions, and more than 22 genotypes of ASFV strains have been identified by targeting the *p72* gene [9,10].

Timely and accurate detection of ASFV emergence is important for establishing an early surveillance system [11]. Two kinds of OIE-recommended polymerase chain reaction (PCR) assays for ASFV detection are conventional PCR and real-time PCR, and these detection methods are widely used in clinical tests [12]. However, both these assays are inconvenient for on-site diagnosis because they require complex instruments and expert operating techniques. Isothermal amplification technologies, such as recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and cross-priming amplification (CPA) have been developed for field detection of ASFV [13–15]. Although the detecting sensitivity of LAMP was much higher than that of CPA, further improvements in sensitivity are still required for ASFV detection [15]. Therefore, convenient and accurate detection methods are urgently needed for the control of ASF.

The CRISPR-Cas system has been applied for genome editing to create targeted, dsDNA breaks. In contrast to Cas9, Cas12a (Cpf1) recognizes a T-rich protospacer adjacent motif (PAM) and catalyzes guide RNA (crRNA) maturation to generate dsDNA breaks [16]. However, activated Cas12a enzymes also exhibit indiscriminate single-stranded DNA (ssDNA) cleavage activity that can degrade ssDNA molecules. By combining the target-activated and nonspecific ssDNA cleavage property of Cas12a with a fluorophore quencher (FQ)-labeled reporter, the rapid and specific molecular detection platform DNA endonuclease-targeted CRISPR trans reporter (DETECTR) assay was developed [17]. Generally, LbCas12a (*Lachnospiraceae bacterium* Cas12a) and AsCas12a (*Acidaminococcus* sp. Cas12a) can only recognize the 5′-TTTN-3′ PAM; therefore, the recognizing sequences of these two types of Cas12a are significantly limited for precision site-specific genome editing application. Currently, all developed detection methods of ASFV based on CRISPR/Cas12a technology only use the LbCas12a [18–21]. However, the FnCas12a (*Francisella novicida* Cas12a) can recognize a more suitable 5′-TTN-3′ PAM without detectable levels of nuclease-induced indels at certain locus [16,22]. Moreover, FnCas12a possesses stronger DNA cleavage activity and has much more widespread application [23]. Therefore, in the current study, we chose FnCas12a as the genome editing tool in ASFV detection for the first time and developed a rapid diagnostic method targeting the conserved region of ASFV *p72* gene by combining RPA with CRISPR-Cas12a enzymes. This rapid, specific, and convenient detection method might facilitate timely eradication of ASFV.

Materials and Methods

Virus samples

ASFV-positive samples were collected by the National Research Center for Exotic Animal Diseases, China Animal Health and Epidemiology Center (Qingdao, China). ASFV-positive samples were inactivated and transferred to a BSL-2 lab for genomic DNA extraction and detection. For specificity assays, porcine viruses used were as follows: classical swine fever virus (CSFV) Chinese vaccine strain (AY382481), porcine circovirus type 2 (PCV2) JXL strain SH (AY686763), pseudorabies virus (PRV) strain Bartha (EU719641), porcine parvovirus (PPV) strain nanjin-1 (AY739664), Japanese encephalitis virus (JEV) strain SD12 (MH753127), and porcine reproductive and respiratory syndrome virus (PRRSV) strain HUN4 (EF635006).

Preparation of positive DNA standards

Highly conserved regions of the *p72* gene from ASFV SY18 strain (GenBank: MH766894) were chosen for detecting targets with the forward primer, p72-SY18-F (5′-TTAGGTACTGTAACGCAGCA-3′), and the reverse primer, p72-SY18-R (5′-ATGGCATCAG GAGGAGC-3′). PCRs of 20 μl contained 1× Ex Taq PCR Mix (TaKaRa, Tokyo, Japan), 0.2 μM primers, and 2 μl genomic DNA. Amplification products were cloned into pMD-18T vectors (TaKaRa), which were used as DNA standards for assays.

Primers and probes design for RPA

Combinations of primers and probes for RPA reactions were designed according to the highly conserved regions of ASFV SY18 strain *p72* gene (**Table 1**). All primers and probes were synthesized by HuaGene Biotech. RPA reactions were performed mainly according to manufacturer's guidelines for TwistAmp Exo kits (TwistDx, Cambridge, UK). Briefly, 50 μl reactions contained 0.24 μM forward and 0.24 μM reverse primers, 0.12 μM probe, 1× rehydration buffer, 13.2 μl sample and water, and 14 mM magnesium acetate added to RPA mix. RPA reactions were carried out in a fluorescence plate reader (BioTek Synergy 2; BioTek, Winooski, USA) at 37°C for

Table 1. Primers and probes for RPA reactions

Primer	Name	Sequence (5′→3′)
Sets 1	Primer-1-F	GGTATGTAAGAGCTGCAGAACT
	Primer-1-R	TAAGCTTTCAGGATAGAGATACAGC
	Probe-1	AGAGCTGCAGAACTTTGATGGAAATTTA[FAM-dT]C[THF]A[BHQ-dT]AAGATTGATACCA[3′-block]
Sets 2	Primer-2-F	GTTCGCTGCGTATCATTTTCA
	Primer-2-R	TACCGAAACTTGGTTTACTACTGC
	Probe-2	GGAGAGGGCCACTGGTTCCCTCCACCGA[FAM-dT]A[THF]C[BHQ-dT]CCTGGCCAACCAA[3′-block]
Sets 3	Primer-3-F	GTTCGCTGCGTATCATTTTCATCGG
	Primer-3-R	TACCGAAACTTGGTTTACTACTGCGAATAC
	Probe-3	TTGCGCACAAGCGTTGTGACATCCGAAC[FAM-dT]A[THF]A[BHQ-dT]TCGTCTAGGGAAT[3′-block]

30 min, and the relative fluorescence intensity was measured every 5 min at λ_{ex} of 485 nm and λ_{em} of 525 nm.

Protein expression and purification

A plasmid with the *Cas12a* gene was obtained from Addgene (Addgene plasmids #69976; Addgene, Watertown, USA), and *Escherichia coli* BL21 (DE3) containing Cas12a-expressing plasmids were grown in Luria-Bertani medium at 16°C for 14 h. For Cas12a protein purification, cell pellets were harvested by centrifugation at 8000 *g* for 10 min at 4°C and resuspended in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole, 0.5 mM PMSF, and 0.25 mg/ml lysozyme). After 30 min of sonication on ice, lysates were centrifuged at 12,000 *g* for 20 min at 4°C, and the protein was purified using Ni-NTA resin according to the manufacturer's instructions.

Detection of ASFV by FQ-labeled reporter assays

Detecting ASFV by an FQ-labeled reporter assays was performed as previously described [17] with slight modifications. Three guide RNAs targeting ASFV template (**Table 2**) were designed and synthesized by HuaGene Biotech. FnCas12a-crRNA complexes (200 nM FnCas12a:250 nM crRNA:4 nM activator) were pre-assembled at 37°C for 30 min, and reactions were started by diluting to a final concentration of 50 nM FnCas12a:62.5 nM crRNA:1 nM activator in 1× NEB#3 Buffer and ultrapure water to a total volume of 20 µl with 50 nM custom ssDNA FQ reporter (5′-FAM/TTATT/3′-TAMRA) substrates. The FQ cleavage reaction mixture was incubated for ∼60 min in a fluorescence plate reader (BioTek Synergy 2) at 37°C with relative fluorescence intensity measured every 5 min at λ_{ex} of 485 nm and λ_{em} of 525 nm. For sensitivity assays, standard pMD18-p72 recombinant plasmids diluted from 8×10^5 to 8×10^0 copies were evaluated. To evaluate specificity assays, ASFV-positive samples and other virus samples (CSFV, PCV2, PRV, PPV, and JEV) were also tested simultaneously.

Detection of clinical samples by DETECTR assays

Detection of ASFV by DETECTR assays was performed using RPA reactions combined with CRISPR-Cas12a enzymes (**Fig. 1**).

Table 2. Guide RNAs for fluorophore quencher-labeled reporter assays

Name	Sequence
crRNA1	AAUUUCUACUGUUGUAGAU **UCGAUAAGAUUGAUACCAUGA**
crRNA2	AAUUUCUACUGUUGUAGAU **CCUCCACCGAUACCUCCUGGC**
crRNA3	AAUUUCUACUGUUGUAGAU **CGCACAAGCGUUGUGACAUCC**

TwistAmp Basic kits were used for the RPA reactions. Briefly, 0.48 µM forward and reverse primer, 1× rehydration buffer, 2 µl sample, and 11.2 µl ultrapure water were added to 47.5 µl, and RPA reactions were initiated with addition of 14 mM magnesium acetate with incubation at 37°C for 20 min. Subsequently, 18 µl of RPA reaction product was added to 20 µl FQ-labeled reporter assay reactions (50 nM FnCas12a:62.5 nM crRNA:50 nM custom ssDNA-FQ reporter), and the reaction mixtures were incubated in a fluorescence plate reader (BioTek Synergy 2) at 37°C for 30 min with fluorescence measured every 5 min at λ_{ex} of 485 nm and λ_{em} of 535 nm [17].

The sensitivity of DETECTR assay was evaluated using standard pMD18-p72 recombinant plasmids diluted from 8×10^5 to 8×10^0 copies. To evaluate the specificity of DETECTR assays, ASFV-positive samples and other virus samples (CSFV, PCV2, PRV, PPV, and JEV) were tested simultaneously.

Comparison of DETECTR assays with real-time PCR assays

To compare the performances of different methods, 30 clinical blood samples (20 positive and 10 negative) were detected using DETECTR assays and OIE-recommended real-time PCR assays. Real-time PCR was performed as previously described [24,25]. Standard pMD18-p72 recombinant plasmids were used in the following PCR conditions: 95°C for 1 min, and 40 cycles of 95°C for 20 s, and 60°C for 55 s. The threshold cycle value (Ct) was measured with the StepOne Plus real-time PCR system (ABI7500; Applied Biosystems, Foster City, USA).

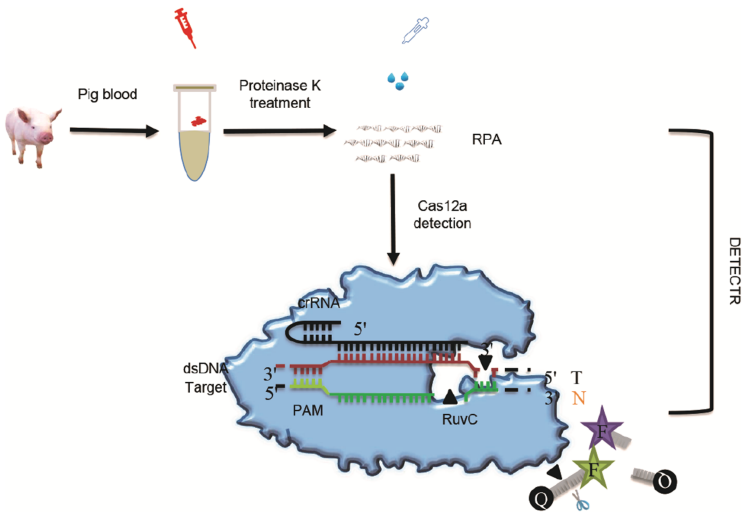


Figure 1. Schematic diagram of the DETECTR assay The system contains three steps: sample preparation, RPA, and Cas12a cleavage reaction.

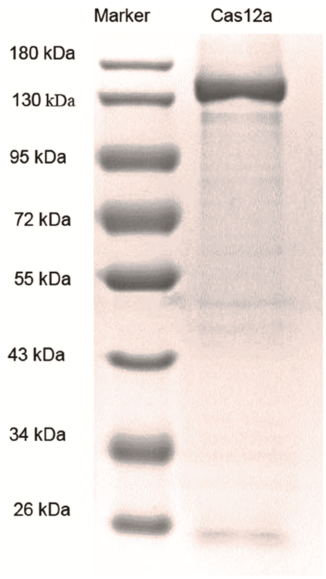


Figure 2. Isolation and purification of Cas12a protein The molecular mass of eluted protein was 139.9 kDa and the purity of predominant single band was 90.5%. Eluted protein peak was collected for further use.

Results

Expression and purification of Cas12a protein

To obtain Cas12a protein for DETECTR assays, Cas12a protein tagged with His was expressed in *E. coli* and purified using Ni-NTA resin. Eluted protein-rich peaks were analyzed by SDS-PAGE. Eluted protein was 139.9 kDa, corresponding to the molecular mass of the Cas12a protein with a predominant single band with a purity of 90.5%, measured by Image Lab software (**Fig. 2**). The N-terminal sequence of purified Cas12a was confirmed by automated Edman degradation (data not shown).

Performance of RPA reactions for ASFV amplification

Isothermal amplification reaction conditions were optimized using different sets of primers and probes, and those with the highest amplification efficiency were selected. An ASFV-positive clinical sample was used as a template to determine the optimum RPA reaction conditions. ASFV-positive signals were observed within 15 min using primers1 and probe1 (**Fig. 3**). The relative fluorescence intensity for primers2 and probe2 was increased slightly after 25 min, while relative fluorescence intensity of primers3 and probe3 did not change, even at 30 min. Therefore, the results showed that the primers1 and probe1 combination was the optimum condition for ASFV-positive reactions.

Development of FQ-labeled reporter assays for ASFV detection

To develop methods for ASFV detection using FQ-labeled reporter assays, different crRNAs were designed and optimized. Three crRNAs activated Cas12a enzyme activity with different patterns (**Fig. 4**). The cleavage activity of the Cas12a enzyme was activated by crRNA1 within 10 min but did not increase after that.

Compared with the negative control, the cleavage activity of the Cas12a enzyme was activated by crRNA1 within 10 min but did not increase after that. The cleavage activity of the Cas12a enzyme was not activated by crRNA2. The activity was increased when

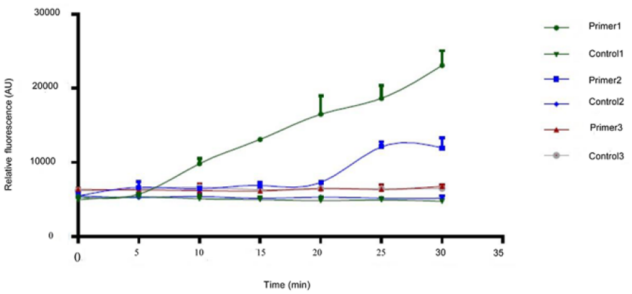


Figure 3. Performance of RPA reactions for ASFV Three sets of primers and probes were tested and the primer1 and probe1 combination was certified to be optimal.

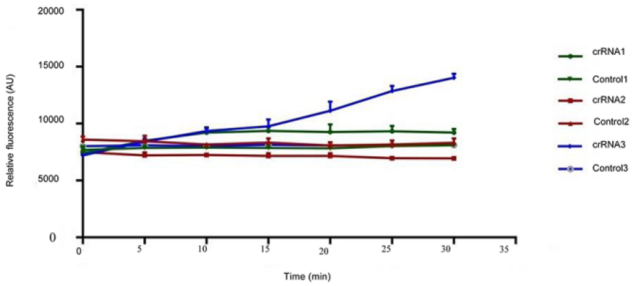


Figure 4. crRNAs used for fluorophore quencher-labeled reporter assays

activated by crRNA3, which provided relative fluorescence signals for the ASFV-positive sample; therefore, the crRNA3 was selected as the guide RNA for FQ-labeled reporter assays.

To evaluate the sensitivity of ASFV fluorophore quencher-labeled reporter assays, standard pMD18-p72 recombinant plasmids diluted from 8×10^5 to 8×10^0 copies were evaluated. Sensitivity was sufficient to detect 10^0 copies per reaction (**Fig. 5**).

Figure 6 showed that no cross reaction was observed in ASFV with other pig virus strains (CSFV, PCV2, PRV, PPV, and JEV), indicating excellent specificity of the ASFV FQ-labeled reporter assays.

DETECTR assays for detection of ASFV

To evaluate the sensitivity of DETECTR assays, RPA isothermal amplification combined with Cas12a ssDNase activation was performed. Standard pMD18-p72 recombinant plasmids diluted from 8×10^5 to 8×10^0 copies were evaluated. The detection limit of DETECTR assays was 8×10^0 copies per reaction (**Fig. 7**).

ASFV-SY18 strains tested positive with the DETECTR assays and non-ASFV viruses (CSFV, PCV2, PRV, PPV, and JEV) were negative (**Fig. 8**).

Comparison of detection performance between DETECTR and real-time PCR assays

To compare the detection performance between DETECTR assays and TaqMan real-time PCR assays, 30 clinical blood samples (20 positive and 10 negative) were tested. The results of DETECTR assays were consistent with those of TaqMan real-time PCR assays (**Table 3**), indicating that these methods are comparable. However the DETECTR assays were completed in 50 min, while real-time PCR needed more than 2 h, indicating that the DETECTR assay was more efficient and rapid than the real-time PCR method.

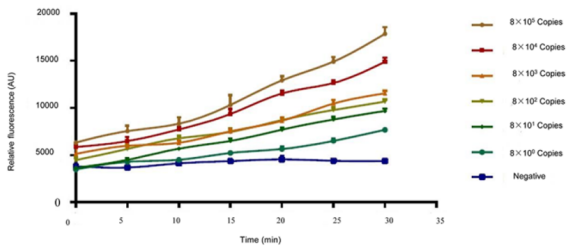


Figure 5. Sensitivity of ASFV detection by fluorophore quencher-labeled reporter assays Standard pMD18-p72 recombinant plasmids were diluted from 8×10^5 to 8×10^0 copies and evaluated.

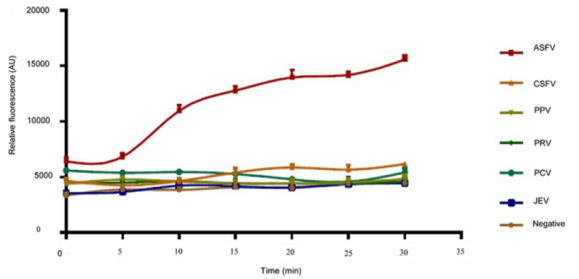


Figure 6. Specificity of ASFV detection by fluorophore quencher-labeled reporter assays Several pig viruses (CSFV, PCV2, PRV, PPV, and JEV) were examined and compared with ASFV, and no cross reaction was observed.

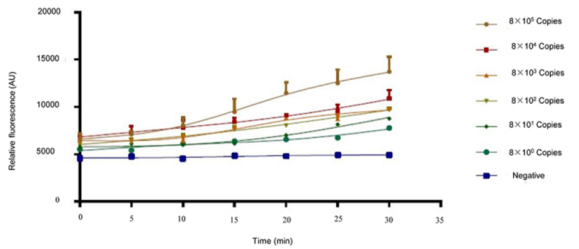


Figure 7. Sensitivity of ASFV detection evaluated by DETECTR assays Standard pMD18-p72 recombinant plasmids were diluted from 8×10^5 to 8×10^0 copies.

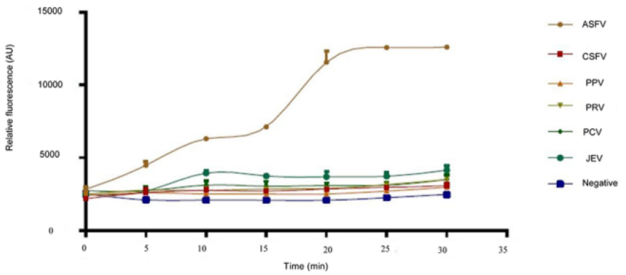


Figure 8. Specificity of ASFV detection by DETECTR assays No cross reactivity was observed with other viruses (CSFV, PCV2, PRV, PPV, and JEV).

Discussion

ASF-infected domestic pigs and wild boars are mainly characterized by high fever, skin cyanosis, and severe lymph nodes hemorrhages. Currently, the quick spread and extension of ASFV is a serious challenge for the swine industry in China [26]. ASFV has a remarkable capacity for transboundary and transcontinental spread with the rapid development of international trade and modern logistics [27,28]; hence, effective ASF risk management with comprehensive and integrated approaches is urgently needed [29–31]. Therefore, convenient, rapid, and accurate detection of ASFV is essential for the timely control strategies.

Table 3. Parallel testing results for DETECTR assays and TaqMan real-time PCR assays for clinical samples

	Positive	Negative
TaqMan real-time PCR assays	20	10
DETECTR assays	20	10

Advances in genome editing technology associated with CRISPR and CRISPR-associated (Cas) proteins bring novel insights to molecular diagnostics fields for infectious diseases [32]. By binding to a guide-complementary DNA, Cas12a-crRNA complexes have robust and nonspecific ssDNA *trans*-cleavage activity [17]. The molecular detection platform of DETECTR was developed by combining RPA reactions with CRISPR-Cas12a enzymes. In this study, optimum RPA reaction conditions were determined by comparing the performances of different sets of primers and probe targeting conserved regions of the *p72* gene. Primers and probes with the highest amplification efficiency were selected for RPA reactions (**Fig. 3**). To determine the ASFV dsDNA site-specificity for the cleavage activity of FnCas12a, crRNAs targeting the *p72* gene were designed and optimized for FQ-labeled reporter assays (**Fig. 4**). Although LbCas12a have been reported to have dsDNA breaking activity [18–21], the application of LbCas12a for ASFV detection may be dramatically limited because LbCas12a require a 5′-TTTN-3′ PAM sequence, which is usually hard to select in the potential targets. However, FnCas12a used in the current research may be better suited for accurate genome editing than LbCas12a. Because LbCas12a can recognize a more suitable 5′-TTN-3′ PAM sequence, it may have greater potential for the development of an ASFV detection platform [16,22,23]. Detection sensitivity of FQ-labeled reporter assays was evaluated using diluted concentrations of standard pMD18-p72 recombinant plasmids, and the detection limit was certified to be 8×10^0 copies (**Fig. 5**). For the specificity of ASFV detection by FQ-labeled reporter assays, no cross reactivity were observed with other main swine viruses, including CSFV, PCV2, PRV, PPV, and JEV (**Fig. 6**). By comparing the performance of DETECTR and real-time PCR assays, we found that the results of DETECTR assays were consistent with those of real-time PCR assays (**Table 3**). The detection sensitivity of DETECTR assays reached a limit of 8×10^0 copies (**Fig. 7**), which was much more sensitive than real-time PCR assays (800 copies; data not shown). Meanwhile, in the DETECTR assays, no cross reactions were found in ASFV with non-ASFV viruses (CSFV, PCV2, PRV, PPV, and JEV) (**Fig. 8**), indicating good specificity of the DETECTR assay.

Other formats for ASFV detection include ELISA, fluorescent antibody, PCR and isothermal assays [33–37]. The sensitivity of DETECTR assays was 8×10^0 copies per reaction, which was much more sensitive than real-time PCR, LAMP, or CPA. The gold nanoparticle-based lateral flow strip based on CRISPR/Cas12a technology is very convenient for field detection of ASFV. Generally, an FAM-Biotin ssDNA reporter specifically conjugated with gold nanoparticles can bind to streptavidin at the control line. When the FAM-Biotin ssDNA reporter is degraded by Cas12a, the complex could pass through the control line and can bind to the IgG antibody on the test line. However, the concentrations of FAM-Biotin ssDNA

reporters and gold nanoparticle used for lateral flow detection are very difficult to control, and the false-positive detection results are hard to avoid. OIE-recommended real-time PCR is the most widely used method for detecting ASFV; however, the specialized instructions and expensive equipment are not suitable for field detection. Moreover, DETECTR assays can detect samples within 1 h, while real-time PCR takes more than 2 h.

The outbreak of ASF causes great economic losses, and the demand for live pigs and pork products is still increasing. Although several research groups in China are making great efforts to generate effective vaccines against ASFV, early detection and eradication measures are still important for the control and prevention of the disease under the current circumstances [38]. In this study, we developed a rapid and accurate method for ASFV detection by combining RPA reactions with a CRISPR-Cas12a system, which may provide feasible eradication strategies and facilitate strict implementation of sanitary procedures.

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