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One-pot platform for rapid detecting virus utilizing recombinase polymerase amplification and CRISPR/Cas12a

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Abstract

The livestock industry has been deeply affected by African swine fever virus (ASFV) and *Capripoxvirus* (CaPV), which caused an enormous economic damage. It is emergent to develop a reliable detection method. Here, we developed a rapid, ultra-sensitive, and one-pot DNA detection method combining recombinase polymerase amplification (RPA) and CRISPR/Cas12a for ASFV and CaPV, named one-pot-RPA-Cas12a (OpRCas) platform. It had the virtue of both RPA and CRISPR/Cas12a, such as high amplification efficiency, constant temperature reaction, and strict target selectivity, which made diagnosis simplified, accurate and easy to be operated without expensive equipment. Meanwhile, the reagents of RPA and CRISPR/Cas12a were added to the lid and bottom of tube in one go, which overcame the incompatibility of two reactions and aerosol contamination. To save cost, we only need a quarter of the amount of regular RPA per reaction which is enough to achieve clinical diagnosis. The OpRCas platform was 10 to 100 times more sensitive than qPCR; the limit of detection (LOD) was as low as 1.2×10^{-6} ng/μL (3.07 copies/μL by ddPCR) of ASFV and 7.7×10^{-5} ng/μL (1.02 copies/μL by ddPCR) of CaPV with the portable fluorometer in 40 min. In addition, the OpRCas platform combined with the lateral flow assay (LFA) strip to suit for point-of-care (POC) testing. It showed 93.3% consistency with qPCR for clinical sample analysis. Results prove that OpRCas platform is an easy-handling, ultra-sensitive, and rapid to achieve ASFV and CaPV POC testing.

Key points

- *The platform realizes one-pot reaction of RPA and Cas12a.*
- *Sensitivity is 100 times more than qPCR.*
- *Three output modes are suitable to be used to quantitative test or POC testing.*

Keywords African swine fever virus (ASFV) · *Capripoxvirus* (CaPV) · Recombinase polymerase amplification (RPA) · CRISPR/Cas12a · Lateral flow assay (LFA)

Introduction

The plenty of viruses cause severe disease to livestock and pose a great threat to health and the economy, such as African swine fever virus (ASFV) and *Capripoxvirus* (CaPV) (Tuppurainen et al. 2017; Zhou et al. 2018). The spread of the ASFV and CaPV causes billions of livestock slaughtered for their high infectivity. It is crucial for customs to control the spread of diseases that developing a rapid, easy-handling diagnosis method. The classical methods for virus detection are divided into three categories, virological, immunological, and

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molecular methods (Oura et al. 2013). Firstly, the viral culture method needs too much time for isolating and authenticating. Moreover, it requires a large and well-established database to support the results, which have limited development in the field of rapid detection of viruses (Pelletier et al. 2011). In addition, enzyme-linked immunosorbent assay (ELISA) is the most typical immunological method, which has been approved for a lot of diseases detection by FDA and OIE (Kazakova et al. 2017; Qiu et al. 2021; Zhang et al. 2020). But many researchers had found that ELISA showed low sensitivity of 77.2% in the commercial kits, poor thermal stability, poor repeatability, and false positive due to the interference of autoantibodies (Gallardo et al. 2015). Research showed that it took 14 days for animals to develop antibodies from its primary infection (Armstrong et al. 2005). Thereby, the immunological method is hysteretic for virus detection, resulting in the infective range being enlarged. In contrast, the nucleic acid test is a timely and accurate method since nucleic acid can be detected in the blood within 24 h of infection (Ning et al. 2021). Currently, the nucleic acid test is flourishing in the diagnosis field, including variable temperature amplification and isothermal amplification methods (Cai et al. 2021; Choi et al. 2021b; Hussein et al. 2021; Watanabe et al. 2014; Zheng et al. 2019). The representative variable amplification methods, polymerase chain reaction (PCR), droplet digital PCR (ddPCR), and real-time quantitative PCR (qPCR), utilize the renaturation dynamics to control the DNA replication events, which shows brilliant detection ability within 35 cycles, 3 copies per reaction in theory (Aguero et al. 2003; Jia et al. 2020; Wang et al. 2020b). Even qPCR is defined as the golden standard method for nucleic acid detection. However, the requirement of expensive apparatuses and professional staff leads to the fact that they are restricted to the laboratory and not conducive to POC testing. For POC testing, the isothermal amplification is favored because it gets rid of equipment for temperature cycling control (Niemz et al. 2011). Among them, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are developing rapidly in the diagnosis field, owing to their significant specificity and efficiency. For instance, Tomotada (Iwamoto et al. 2003) successfully detected three *Mycobacterium* by LAMP, and Olaf (Piepenburg et al. 2006) applied RPA with a tetrahydrofuran probe to detect *Staphylococcus* aureus. However, the complex primer design limits the application of LAMP (Notomi et al. 2015). The multienzyme system guarantees the sensitivity of simple-design RPA at 37 °C for 20 min. Hence, the RPA is more convenient to apply in POC testing. Specifically, RPA utilized multiple enzymes to simulate PCR at a constant temperature, primers targeted dsDNA by recombinase rather than denaturation and annealing eliminating variable temperature, the single-stranded DNA-binding protein (SSB) stabilized the ssDNA availing next amplification event, and the DNA polymerase without 5′ to 3′ and 3′ to 5′ exonuclease activity replaced parent DNA. It was worth noting that several DNA polymerases could amplify one parent DNA at the same time rather than temperature cycle for one replication.

Traditionally, fluorescent dyes such as SYBR Green I and gel electrophoresis are employed for detecting amplicons in laboratories. The former shows a high non-specificity and the latter requires pre-treatment with protease digestion in the RPA experiment. Therefore, it is vital to introduce a sensitive and specific end-point signal output method. As an emerging technology, clustered regularly interspaced short palindromic repeats/CRISPR-associated 12a system (CRISPR/Cas12a) has been widely used in gene editing and biosensors. Specifically, Cas12a and CRISPR RNA (crRNA) form ribonucleoprotein (RNP), which recognizes protospacer adjacent motif (PAM) site with "TTTN" motif and binds double-stranded DNA (dsDNA) complemented to crRNA to form a ternary complex. The same as Cas9, target dsDNA cleavage events happen but only for non-complemental strand. After target dsDNA cut, indiscriminate collateral cleavage activity is activated for a long time that Cas12a non-specifically shears single-stranded DNA (ssDNA) reporter labeled fluorophore and quencher (Chen et al. 2018). This lays the foundation for the signal construction of the sensor. James (Broughton et al. 2020) set up DETECTR platform which combined RT-LAMP and CRISPR/Cas12a for SARS-CoV-2 diagnosis. It is powerful for Cas12a to be applied to virus detection. Choi et al. utilized Cas12a to cut up the ssDNA bridge, which linked two AuNPs, to achieve Cell-free DNA (cfDNA) colorimetric detection (Choi et al. 2021a). Zhang lab provided step-by-step instructions for RNA or DNA detected by RT-RPA and T7 transcription (Kellner et al. 2019).However, the method combined RPA with CRISPR/Cas12a has great challenges in practical application. Firstly, there was interference between RPA and CRISPR/Cas12a reagents (Guanghui et al. 2020). Secondly, high amplification efficiency of RPA would cause aerosol pollution for step by step methods.

To solve the above problems, we constructed a one-pot detection platform based on RPA and CRISPR/Cas12a, named One-pot-RPA-Cas12a (OpRCas) platform for rapid and accurate detection of ASFV and CaPV. In this platform, RPA replicated target exponentially, the Cas12a solved the RPA non-specific amplification with a selective crRNA. The RPA reagent and the CRISPR/Cas12a system were assembled on the bottom and lid of the tube, respectively. A simple spinning after RPA, the Cas12a reagent was mixed to amplicons without opening lid. This way avoided aerosol pollution. In addition, it was built on the LFA strip to achieve portable and visual detection. Taking full use of the efficient RPA and trans-cleavage activity of Cas12a, the LOD of ASFV and CaPV were as low as 1.2×10^{-5} ng/μL (3.07 copies/μL by ddPCR) and 7.7×10^{-5} ng/μL (1.02 copies/μL by ddPCR). In the actual sample tests, the OpRCas platform

had high sensitivity and accuracy with 93.3% (28/30) consistency with qPCR. Therefore, the OpRCas platform is a potential diagnostic tool for ASFV and CaPV.

Materials and methods

Reagents and instrumentations

All primers and the crRNA used in this study were offered by Sangon Biotech (Shanghai, China) and were shown in Table S1. The basic CEA kit was purchased from GenDx Biotech Co. for RPA reaction, Ltd. The 2×premix Ex Taq (Probe qPCR) with UNG was purchased from TaKaRa. The LbCas12a and 10×NEBuffer 2.1 were offered by New England Biotechnology Co., Ltd. The nucleic acid of the lumpy skin disease virus (LSDV) wild strain (NI 2490) was donated by Dr. Emmanuel Albina, France; LSDV vaccine strain (LW 1959) was purchased from South Africa (Bio Onderstepoort); goat pox virus (GTPV) vaccine strain (CVCC AV41) and sheep pox virus (SPPV) vaccine strain (CVCC AV42) were purchased from the China Veterinary Drug Administration; genomic DNAs of vaccinia virus (VV) and *Suipoxvirus* (SPV) were purchased from the ATCC; the nucleic acids of bluetongue virus (BTV), and peste des petits ruminants virus (PPRV) was gifted by Dr. Ai from the Kunming Customs Technology Center. The negative pork, African swine fever virus (ASFV), and classical swine fever virus (CSFV) were provided by Chongqing Customs Technology Center. Fluorescence thermostatic amplifier GS8 was purchased from GenDx Co., Ltd, China. CRISPR-based lateral flow test strips were offered by Warbio Biological Co., Ltd, China. ABI Pro Flex™ PCR Amplifier was purchased from life technology™, USA.

Designing specific primers and crRNA

The primers and the crRNA were designed for the conserved genes *B646L* in ASFV and *LSD74* in CaPV. Notably, primers of CaPV can simultaneously amplify LSDV, GTPV, and SPPV since they shared 97% nucleotide identity (Gupta et al. 2020). The qPCR primers were obtained by SN/T 1559–2010 and SN/T 5197–2019 to verify ASFV and CaPV.

The OpRCas platform for detecting ASFV and CaPV

Three premixes were required in this OpRCas platform, RPA mix, Cas12a mix and sample mix. One RPA mix was consisted of 20 μL ddH_2O, 20 μL solvent, 2.5 μL each of 10 μM forward and reverse primer, and a tube-lyophilized CEA reagent. To save cost, each RPA mix was divided into four reactions of 10 μL. Cas12a mix was prepared by mixing 2 μL of 1 μM LbCas12a, 2 μL of 1 μM crRNA, 1×NEB buffer 2.1, and 1 μL 10 μM of ssDNA reporters for each reaction. Before reaction, sample mix was premixed by 2 μL sample and 1 μL activator. A 10 μL/reaction RPA mix and 7 μL/reaction Cas12a mix were respectively loaded on the bottom of the tube and on the lid without opening lid in process of detection. Then, 3 μL/reaction sample mix was added into RPA mix and closed the lid quickly. The tube was incubated at 37 °C for 20 min for amplification. Next, Cas12a mix fell into the bottom by simply spinning. Finally, it was incubated in a portable fluorescence thermostatic amplifier GS8 at 37 °C for 30 min. The products of RPA should be digested by proteinase K at 65 °C for 5 min if gel electrophoresis was needed.

The LFA strip combined with the OpRCas platform

The RPA amplification and detection of CRISPR/Cas12a were the same as described above. It was worth noting that the ssDNA reporter was replaced with the reporter modified with FAM and biotin. After the reaction, 80 μL ddH_2O was added. The strip was inserted into the tube waiting for result.

The qPCR for ASFV and CaPV detection

Each group of samples were tested by qPCR for comparing. The qPCR was carried out according to SN/T 5197–2019 (LSDV) and SN/T 1559–2010 (ASFV). In short, the qPCR reaction system was mixed by 12.5 μL Premix Ex Taq (Probe qPCR) with UNG, 1 μL of 10 μM forward primer-SN/T, 1 μL of 10 μM reverse primer-SN/T, 0.5 μL of 10 μM probe, 5 μL ddH_2O, and 5 μL sample. And it was performed in condition as followed: 25 °C for 10 min, 95 °C for 30 s and 40 cycles for amplification. For CaPV, the cycle was set as 95 °C for 10 s and 60 °C for 34 s. For ASFV, the cycle was set as 95 °C for 10 s and 58 °C for 1 min.

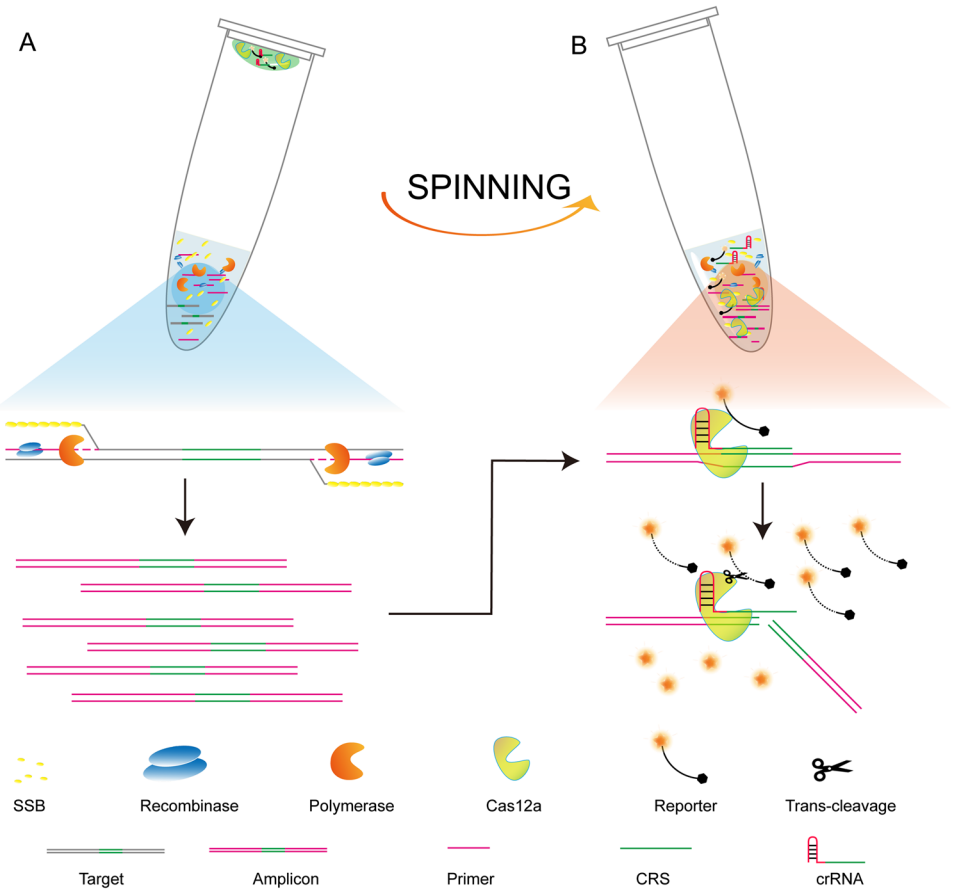
Clinical sample detection

The nucleic acid of fourteen samples of ASFV and sixteen samples of CaPV were tested by OpRCas platform. Meanwhile, qPCR as standard method was employed for analyzing the results.

Results

Principle of the OpRCas platform

The OpRCas platform was based on RPA and CRISPR/Cas12a, as shown in Scheme 1. Targets were amplified billions of times by RPA at the bottom of the tube (Scheme 1A), and Cas12a recognizes and bound the amplicon by pairing



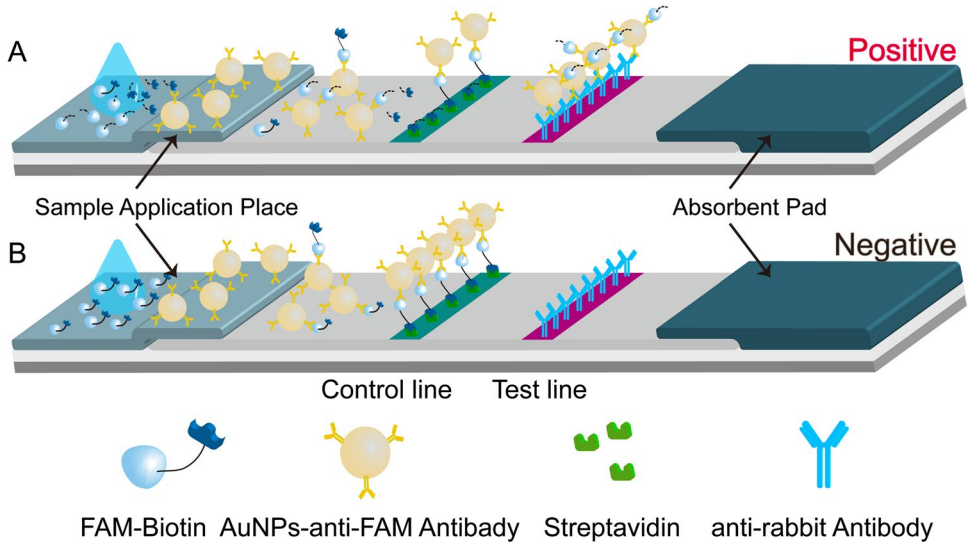
Scheme 1 The principle of the OpRCas platform, **A** RPA reaction before mixing; **B** Cas12a reaction after mixing

with the crRNA, activating its cis-cleavage and trans-cleavage activities (Scheme 1B). Specifically, a fluorescence signal increased because Cas12a trans-cleavage was activated by target dsDNA to non-specifically cut ssDNA reporters whose ends were modified fluorophore and quencher. Cas12a not only achieved specific detection by the crRNA, which solved the false positives caused by non-specific RPA amplification, but also realized signal amplification and output. Noteworthily, 7 μL of CRISPR/Cas12a system on the lid of tube was small enough to be held by surface tension avoiding aerosol pollution (Scheme 1A). After the RPA reaction, CRISPR/Cas12a system was mixed with the RPA system by centrifugation for detection. Therefore, the platform is a simple and sensitive tool in the detection of nucleic acids.

In addition, we designed the LFA strip (Scheme 2) to eliminate the use of a fluorimeter by modifying FAM and biotin on the reporters rather than fluorophore and quencher. The intact reporters bound with gold nanoparticles on the conjugate pad via the affinity between the FAM and anti-FAM antibody, and the intact reporters-AuNPs were captured by streptavidin on the control line (C-line) (Scheme 2A and B). The severed reporters could pass the C-line without biotin, so that AuNPs were caught by the anti-rabbit antibody (second antibody) labeled on test line (T-line) (Scheme 2A). In a word, the presence of T-line means positive, and vice versa. and C-line indicates that the strip is in normal use. This is a portable tool for the POC testing in areas where large instruments are constraints.

Specificity of the OpRCas platform for ASFV and CaPV detection

Specificity was the primary feature of the detection platform. In Fig. 1, the nucleic acids of a variety of viruses were used to verify the specificity of the the OpRCas platform, which were ASFV, CaPV, SPPV, GTPV, LSDV (vaccine strain, v), LSDV (wild strain, w), CSFV, VV, SPV, BTV, and PPRV. For ASFV detection, Fig. 1A and C shows that only ASFV had obvious fluorescence, while other viruses had no fluorescence. For CaPV assay, SPPV, GTPV, LSDV(v), and LSDV(w) had distinct fluorescence in Fig. 1B and D. It was worth noting that the CaPV genus contained SPPV, GTPV, and LSDV. And the primers were designed to target their common conserved areas, so three viruses could be detected. While other kinds of viruses had no fluoresce. It indicated that the OpRCas platform had good specificity for ASFV and CaPV. In addition, the LFA strip was used to detect ASFV



Scheme 2 The principle of the LFA strip combined with the OpRCas platform, **A** positive; **B** negative

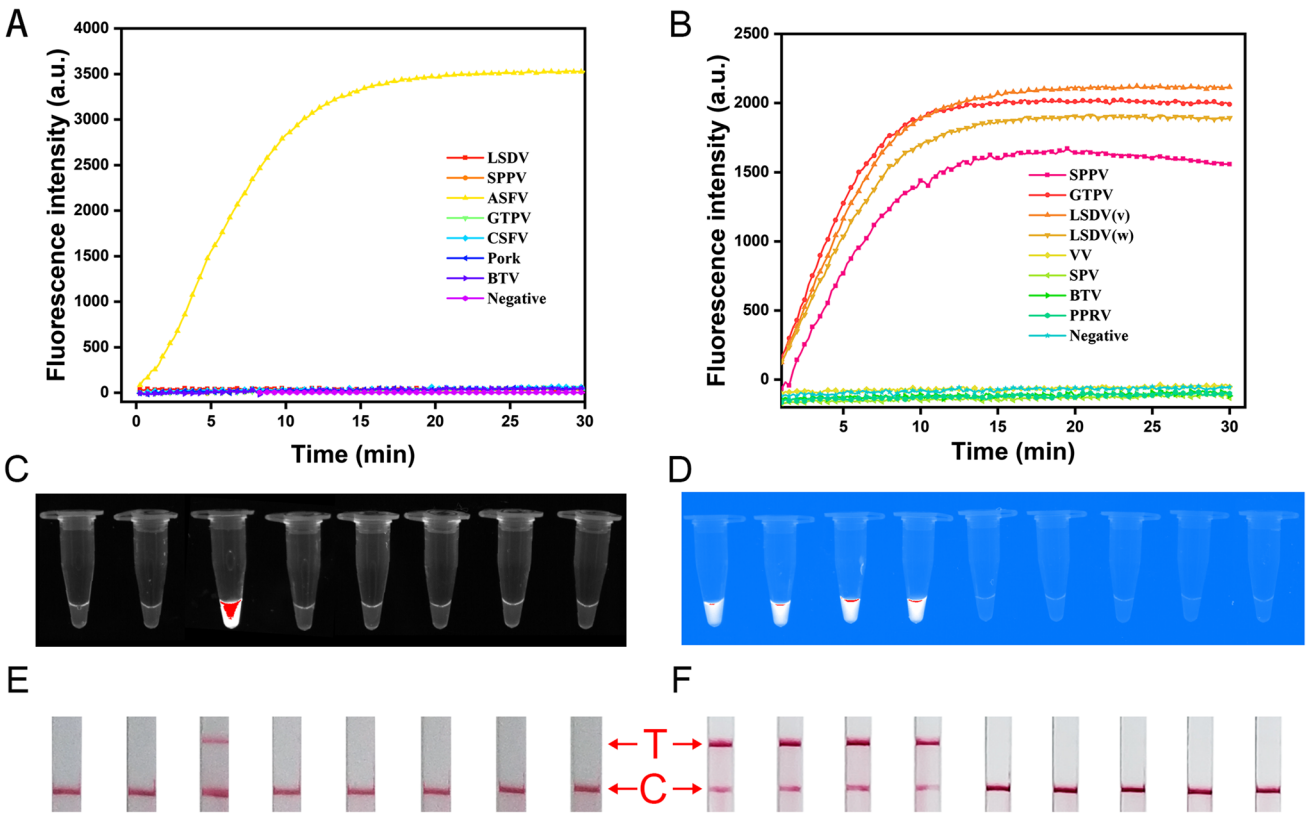


Fig. 1 The specificity of the OpRCas platform detecting ASFV (**A**, **C**, **E**) and CaPV (**B**, **D**, **F**). Samples were LSDV, SPPV, ASFV, GTPV, CSFV, pork, BTV, and negative control group from left to right in ASFV test, and SPPV, GTPV, LSDV(v), LSDV(w), VV, SPV, BTV, PPRV, and negative control group from left to right in CaPV test, respectively. **A**, **B** The real-time fluorescence intensity; **C**, **D** the corresponding fluorescent image; **E**, **F** the corresponding LFA strip

and CaPV, and the results were the same as the fluorescence results in Fig. 1E and F. Only the ASFV and CaPV (SPPV, GTPV, LSDV(v), and LSDV(w)) showed obvious bands on the T-line, while other viruses only emerged C-line. For comparing OpRCas platform with RPA alone, amplicons of RPA were detected by 2% gel electrophoresis for verifying the specificity of RPA reaction (Fig. S1A and B). Furthermore, the qPCR was implemented according to SN/T

1559–2010 (ASFV) and SN/T 5197–2019 (CaPV) to verify their specificity of ASFV and CaPV, respectively (Fig. S2A and B). The above results proved the great specificity of the OpRCas platform in ASFV and CaPV detection.

Sensitivity of the OpRCas platform

To verify sensitivity of the OpRCas platform, different concentrations of nucleic acids were detected (Fig. 2). As

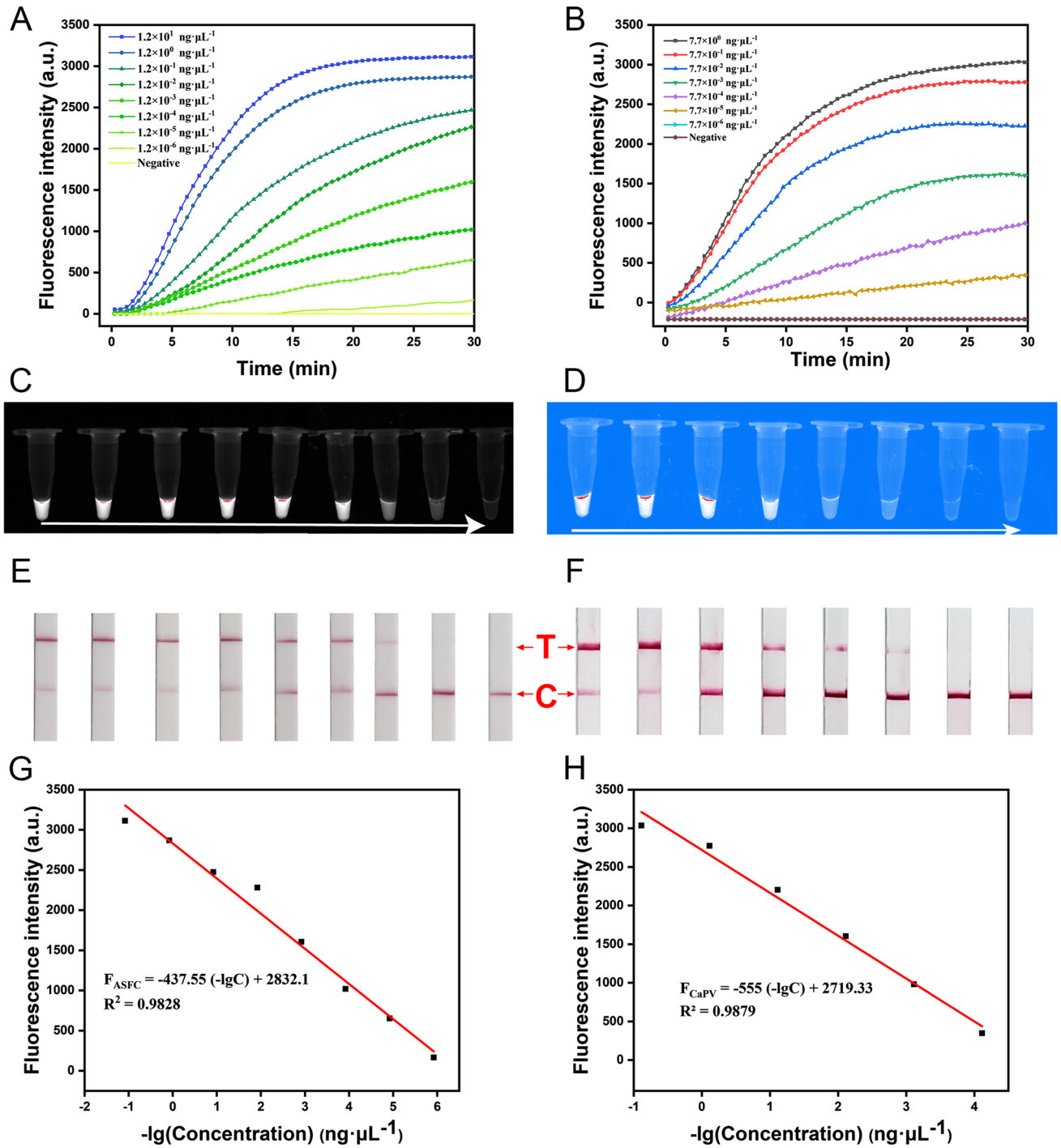


Fig. 2 The sensitivity of the OpRCas platform for detecting ASFV (**A**, **C**, **E**, **G**) and CaPV (**B**, **D**, **F**, **H**). The concentrations were from 12 ng/μL to 1.2×10^{-6} ng/μL of ASFV, and from 7.7 ng/μL to 7.7×10^{-6} ng/μL of CaPV. A negative control was set at the end of each group of tests. **A**, **B** The fluorescence intensity; **C**, **D** the corresponding fluorescent image; **E**, **F** the corresponding LFA strip. **G**, **H** The liner relationship between fluorescence intensity and concentration, $F_{ASFV} = -437.55\times(\text{-lgC}) + 2832.1$ ($R^2 = 0.9828$) and $F_{CaPV} = -555\times(\text{-lgC}) + 2719.33$ ($R^2 = 0.9879$), respectively

the concentration of ASFV increased from 1.2×10^{-6} to 1.2×10^{1} ng/μL (Fig. 2A and C), the fluorescence intensity gradually increased. Linear fitting was performed on the fluorescence intensity at 30 min and the logarithm of the concentration, obtaining a good linear relationship in Fig. 2G, $F_{ASFV} = -437.55$ (-LgC) + 2832.1, $R^2 = 0.9828$. Its actual detection limit was as low as 1.2×10^{-6} ng/μL (3.07 copies/μL by ddPCR). Actually, there was significant difference within 20 min after the Cas12a reaction was activated. This indicated that the OpRCas platform had good sensitivity in detecting ASFV. In addition, LFA strips detected different concentrations of ASFV in Fig. 2E. Obviously, LFA couldn't reveal the T-line when the concentration was lower than 1.2×10^{-5} ng/μL. The real detection limit of LFA was as low as 1.2×10^{-5} ng/μL, while Fig. 3A revealed the qPCR could only detect ASFV samples at concentrations greater than 1.2×10^{-4} ng/μL. The OpRCas platform was 100 times more sensitive than the standard method qPCR in ASFV detection.

Similarly, as the concentration of CaPV increased from 7.7×10^{-6} to 7.7 ng/μL, the fluorescence intensity gradually increases in Fig. 2B and D. The LOD was as low as 7.7×10^{-5} ng/μL (1.02 copies/μL by ddPCR) through the fluorescence signal, $F_{CaPV} = -555\times$ (-lg C) + 2719.33, $R^2 = 0.9879$ (Fig. 2H). The real detection limit of LFA was as low as 7.7×10^{-5} ng/μL in Fig. 2F. It was the same result that the sensitivity of the OpRCas platform was higher than that of qPCR with the LOD of 7.7×10^{-3} ng/μL for CaPV detection in Fig. 3B. Furthermore, our method showed many advantages over other methods, more sensitive, rapid, or one-step (Table S2). Therefore, the combination of CRISPR/Cas12a endowed the OpRCas platform with a simpler and more sensitive signal output.

The application of OpRCas platform to clinical diagnosis

The application ability in actual samples is the main feature of the detection platform. Fourteen ASFV samples (A1 to A14) and sixteen CaPV samples (C1 to C16) were analyzed by the OpRCas platform (Table S3). As shown in Fig. 4A and E, 7 samples were positive, A7, A8, A9, A10, A11, A12, and A13; the others were negative. In addition, Fig. 4C revealed the results of the LFA strip. Clearly, the samples from A7 to A13 showed obvious bands on the T-line, while the other samples only had bands on the C-line. These results were the same as the fluorescence signals. To explore the accuracy of the test, ASFV samples were tested by the standard method qPCR and found that the results of qPCR were consistent with the fluorescence signal in Fig. 4G. The above data indicated that the OpRCas platform had an accuracy rate of 100% in detecting ASFV (Table S3). The CaPV samples were tested using the platform and found that C1, C2, C3, C7, C8, C9, C10, C11, C13, and C15 samples had strong fluorescence; C4 showed weak positive since it had a slightly weaker fluorescence intensity, and others were negative in Fig. 4B and F. In the LFA strip testing, most of the results were the same as the fluorescence signal that positive samples had obvious bands on the T-line and negative samples had only band on the C-line in Fig. 4D. However, the C4 sample showed a different result that it came back a negative result. That was because its concentration was lower than the limit of detection of the strip. In the validation process of qPCR, both C4 and C10 showed negative results, indicating that the sensitivity of qPCR may be not enough to accurately detect them. Overall, the OpRCas platform had good detecting capability and better sensitivity than qPCR

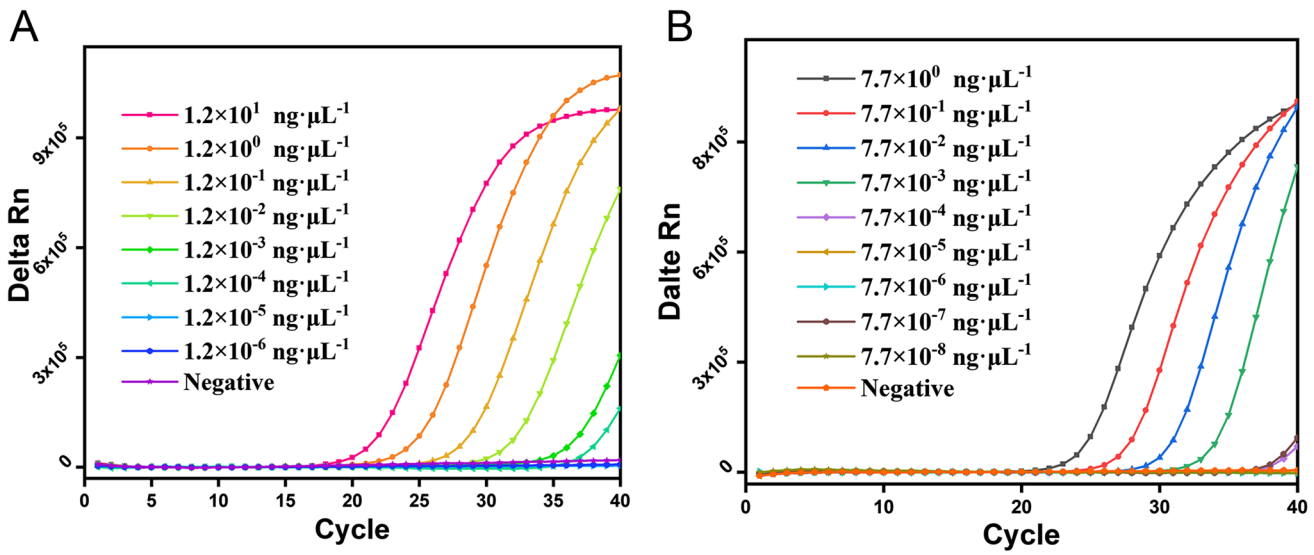


Fig. 3 the results of ASFV (**A**) and CaPV (**B**) at different concentrations detected by qPCR

in detecting CaPV. To sum up, the OpRCas is a clinically robust technique for DNA virus detection at a constant temperature, which is more convenient and sensitive than qPCR in clinical application.

Discussions

ASFV and CaPV are worldwide threats to husbandry, but they have not received as much attention as other diseases.

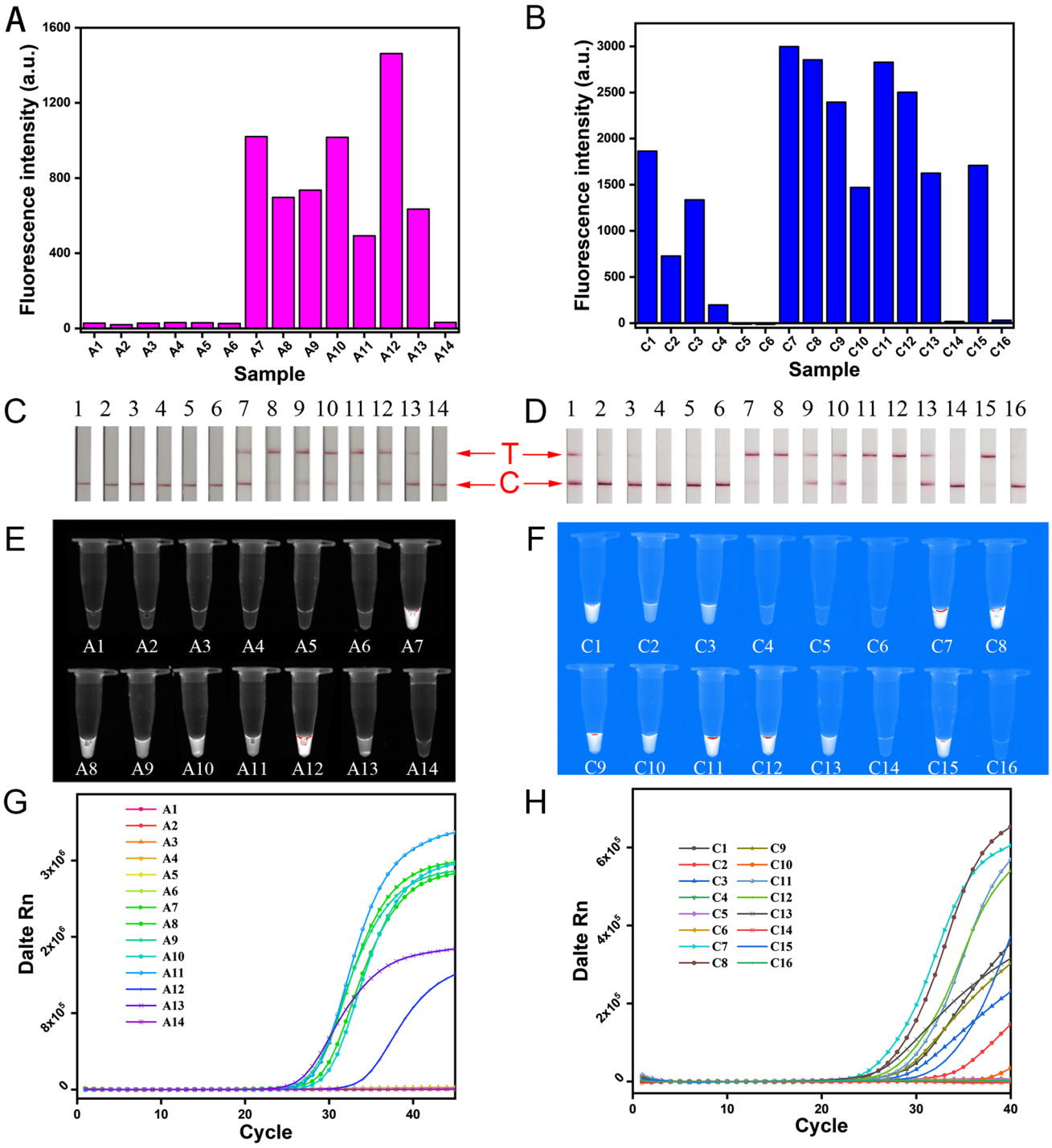


Fig. 4 Detection of clinical sample of ASFV (**A**, **C**, **E**, **G**) and CaPV (**B**, **D**, **F**, **H**) by the OpRCas platform and qPCR. **A**, **B** The fluorescence intensity; **C**, **D** the corresponding LFA strip; **E**, **F** the corresponding fluorescent image; **G**, **H** the results of ASFV and CaPV samples detected by qPCR

A time-consuming qPCR often reduces the efficiency of import and export trade. We have established a one-pot ultra-sensitive and specific platform successfully, which is based on RPA combining with CRISPR/Cas12a to rapidly detect ASFV and CaPV in one tube. By specific recognition ability of CRISPR/Cas12a, the OpRCas platform presents excellent specificity. Under the action of RPA with the high efficiency and CRISPR/Cas12a with the strong signal amplification capability, the OpRCas platform shows good sensitivity with the LOD as low as 1.2×10^{-6} ng/μL (3.07 copies/μL by ddPCR) of ASFV and 7.7×10^{-5} ng/μL (1.02 copies/μL by ddPCR) of CaPV in 40 min. This is even more sensitive than the standard method qPCR. Our one-pot reaction system shows great advantages, while there are lots of CRISPR/Cas12a detection developed (Lu et al. 2020; Wang et al. 2020a). For example, we have solved the problem of RPA in practical application, that is, the pollution caused by uncovering the lid. By adding samples at the bottom and the top of the tube respectively, the OpRCas platform achieves one tube test. Most importantly, we have introduced this rapid detection method to ASFV and CaPV, which are emergent to be diagnosed on field. In order to facilitate the detection of viruses in remote mountainous areas, the OpRCas platform combines the LFA strip for ASFV and CaPV detection, which is more convenient and gets rid of the limitations of large instruments. Moreover, in clinical sample testing, the OpRCas platform performs well.

The development of this OpRCas platform reveals a great significance in applied microbiology and biotechnology. First, this flexible platform has the versatility of nucleic acid detection with primers and crRNA substituted. Then, when CRISPR is mentioned, the first thing that comes to mind is gene editing, but it is verified again that the multi-function CRISPR/Cas system makes great achievement in improving detection methods. Meanwhile, the OpRCas have solved the incompatibility of two reactions and aerosol contamination, and saved cost by using a quarter of the volume of regular RPA per reaction. Last but not least, there were still many pathogens with poorly studied that their detections are too traditional and cumbersome to detect on field. Here, we provide a definite detection platform for CaPV and ASFV, which has practical application significance. Therefore, the ultra-sensitive and rapid detection platform is worth promoting.

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Author contribution YX and GC conceived, designed research, and analyzed data. XC, JY, and MS conducted experiments. YW and FN contributed new reagents or analytical tools. DH and CH wrote the manuscript. All authors read and approved the manuscript.

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Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

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

Conflict of interest The authors declare no competing interests.

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