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Establishment of RPA-Cas12a-Based Fluorescence Assay for Rapid Detection of Feline Parvovirus

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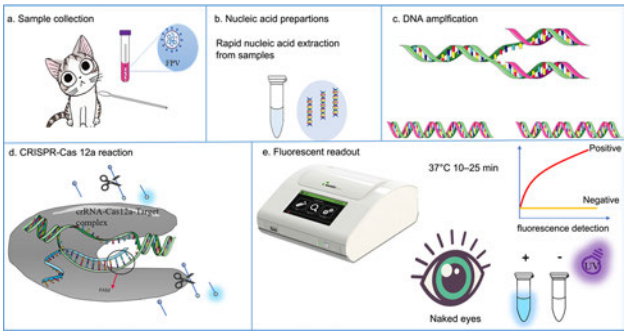
Abstract

Feline parvovirus (FPV) is highly infectious for cats and other Felidae and often causes severe damage to young kittens. In this study, we incorporated recombinase polymerase amplification (RPA) and Cas12a-mediated detection and developed an RPA-Cas12a-based real-time or end-point fluorescence detection method to identify the NS1 gene of FPV. The total time of RPA-Cas12a-based fluorescence assay is approximately 25 min. The assay presented a limit of detection (LOD) of 1 copies/μl (25 copies/per reaction), with no cross-reactivity with several feline pathogens. The clinical performance of the assay was examined using total genomic DNA purified from 60 clinical specimens and then compared to results obtained with qPCR detection of FPV with 93.3% positive predictive agreement and 100% negative predictive agreement. Together, the rapid reaction, cost-effectiveness, and high sensitivity make the RPA-Cas12a-based fluorescence assay a fascinating diagnostic tool that will help minimize infection spread through instant detection of FPV.



K e y w o r d s: CRISPR-Cas12a, detection, feline parvovirus, RPA, real-time fluorescence

Introduction

Feline parvovirus virus (FPV) is rampant around the world and is a non-enveloped virus containing a single-stranded DNA genome, which is approximately 5.1 kb in length (Stuetzer and Hartmann 2014; Liu et al. 2023). The genome of FPV includes two open reading frames (ORFs), which encode nonstructural protein 1 (NS1) and nonstructural protein 2 (NS2), respectively (Wang et al. 2017; Van Brussel et al. 2019). FPV is a potentially fatal pathogen that infects various species, including domestic cats, raccoons, and minks (Stuetzer and Hartmann 2014). FPV infection was typically characterized by vomiting, fever, leucopenia, and diarrhea (Pfankuche et al. 2018). Significantly, FPV poses a severe threat to young animals up to 6 months and is associated with a high mortality and morbidity rate in this population (Zhang et al. 2019). Moreover, FPV could persist in many cats as a subclinical infection, and cats infected with FPV can shed the virus for at least six weeks following infection (Stuetzer and Hartmann 2014). This highly contagious virus can typically spread through contact with infected secretions in the environment (Stuetzer and Hartmann 2014). As a result, a fast, affordable, and practical tool for the detection of FPV infection is urgently required to facilitate early intervention, treatment, and infection prevention.

Various methods for the detection of FPV infection have been exploited, including virus isolation, latex agglutination, immunochromatographic tests, electron

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microscopy, ELISA, and molecular testing (Veijalainen et al. 1986; Ikeda et al. 1998; Esfandiari and Klingeborn 2000; Decaro et al. 2008; DiGangi et al. 2011; Lane et al. 2016; Wang et al. 2019; Zhang et al. 2019). In recent years, though several detection methods have been developed commercially to detect FPV, nucleic acid testing is still the gold standard for FPV detection. Nucleic acid-based diagnostics relying on PCR have become one of the most widely used techniques in clinical molecular diagnostics. However, its application to pathogen detection necessitates specialized equipment and professional staff. Recently, various new nucleic acid detection methods, including isothermal amplification such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), and clustered regularly interspaced short palindromic repeat (CRISPR), have been developed (Oliveira et al. 2021). Among these methods, RPA/RAA is carried out at a relatively low and constant temperature between 37°C and 42°C without a bulky instrument and enables rapid amplification of nucleic acids within 20–30 min without the lengthy process of heating-denaturation, cooling-annealing, and heat-preservation-extension (Lobato and O'Sullivan 2018). A previous study that combined the RPA with lateral flow dipstick (LFD) showed promise as a faster testing alternative for detecting FPV (Wang et al. 2019). However, the RPA, along with the LFD method, requires the tube to be opened for amplification-product transfer at the end of the reaction, and the tube must be opened again for a strip test. An accumulation of amplification products in the laboratory environment may result in contamination or false-positive results. In addition, the use of strips results in an increase in costs.

In the study, the RPA-Cas12a-based fluorescence assay for the detection of FPV nucleic acid by targeting the conserved segment of the nonstructural protein 1 (NS1) gene was developed (Fig. 1A). As a rapid, ultrasensitive, specific, and inexpensive tool, it could be exploited to conduct the veterinary diagnostic testing for use in veterinary hospitals without sending animal samples out to a laboratory usually entails.

Experimental

Materials and Methods

Viruses, cells, and clinical samples. The feline parvovirus (FPV) used in this study was isolated from the fecal specimen from a domestic cat with clinical signs of suspected parvovirus infection in 2021 in Shanghai and validated by the Shanghai Animal Disease Control Center (SHADCC) according to a previous study (Bergmann et al. 2021). The FPV was seeded with F81 cells, cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco™, Thermo Fisher Scientific, Inc., USA) supplemented with 10% fetal bovine serum (FBS) with incubation at 37°C in 5% CO_2, and then stored in SHADCC. In contrast, other viruses were purchased from the American Type Culture Collection (ATCC®, USA). Sixty anal swabs samples were collected from felines with clinical signs suspicious of feline panleukopenia and treated at the animal hospital or healthy felines in Shanghai and kept at –80°C.

DNA extraction. According to the manufacturer's recommendations, total DNA was extracted from FPV-infected F81 cells using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen®, Corning, USA). In contrast, to prepare the total viral DNA of clinical samples, 500 µl of sterile phosphate-buffered saline (PBS) was added to each tube with the anal swabs sample, vortexed for 5 min, and then centrifuged at 12,000 rpm for 15 min at 4°C. Subsequently, the supernatants were transferred to the microcentrifuge tube and utilized for viral DNA extraction using the kit described above. All extracted DNA samples were suspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and stored at –80°C until further use.

Template design and construction of recombinant plasmid. To obtain the FPV-specific conserved sequences, genome sequences of fifteen strains of FPV (GenBank numbers: MW926316.1, MW926315.1, MW926314.1, MT614366.1, MT614366.1, MZ362883.1, MZ913313.1, MZ913314.1, MZ913315.1, MZ913316.1, MZ913317.1, MZ913318.1, MZ913319.1, OK128324.1, OK128325.1) were downloaded and aligned using ClustalW. After multiple alignments, the 404 bp fragment of the nonstructural gene NS1 was identified as the target template for molecular detection and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China) and then cloned into pBluscript II SK (+) vector (Fig. 1B) to construct the recombinant plasmid pBluscript-NS1. Subsequently, the resulting plasmid was transformed into *Escherichia coli* TOP10 cells, allowing us to prepare the NS1 detection template for FPV conveniently. The number of DNA copies was determined using the following equation:

$$DNA\ copy\ number = \frac{[M\ (ng/\mu l) \times 6.02 \times 10^{23}]}{(N \times 10^{9} \times 660)}$$

where M indicates the concentration of DNA in nanograms measured using B-500 Biophotometer (Metash Instruments, China), N represents the length of the DNA in base, and the average molecular weight of a base is defined as 660 Da.

Primer for RPA design and selection. Based on the 404 bp fragment of the NS1 gene identified as the target fragment for FPV detection according to the results described above, four pairs of primers and different

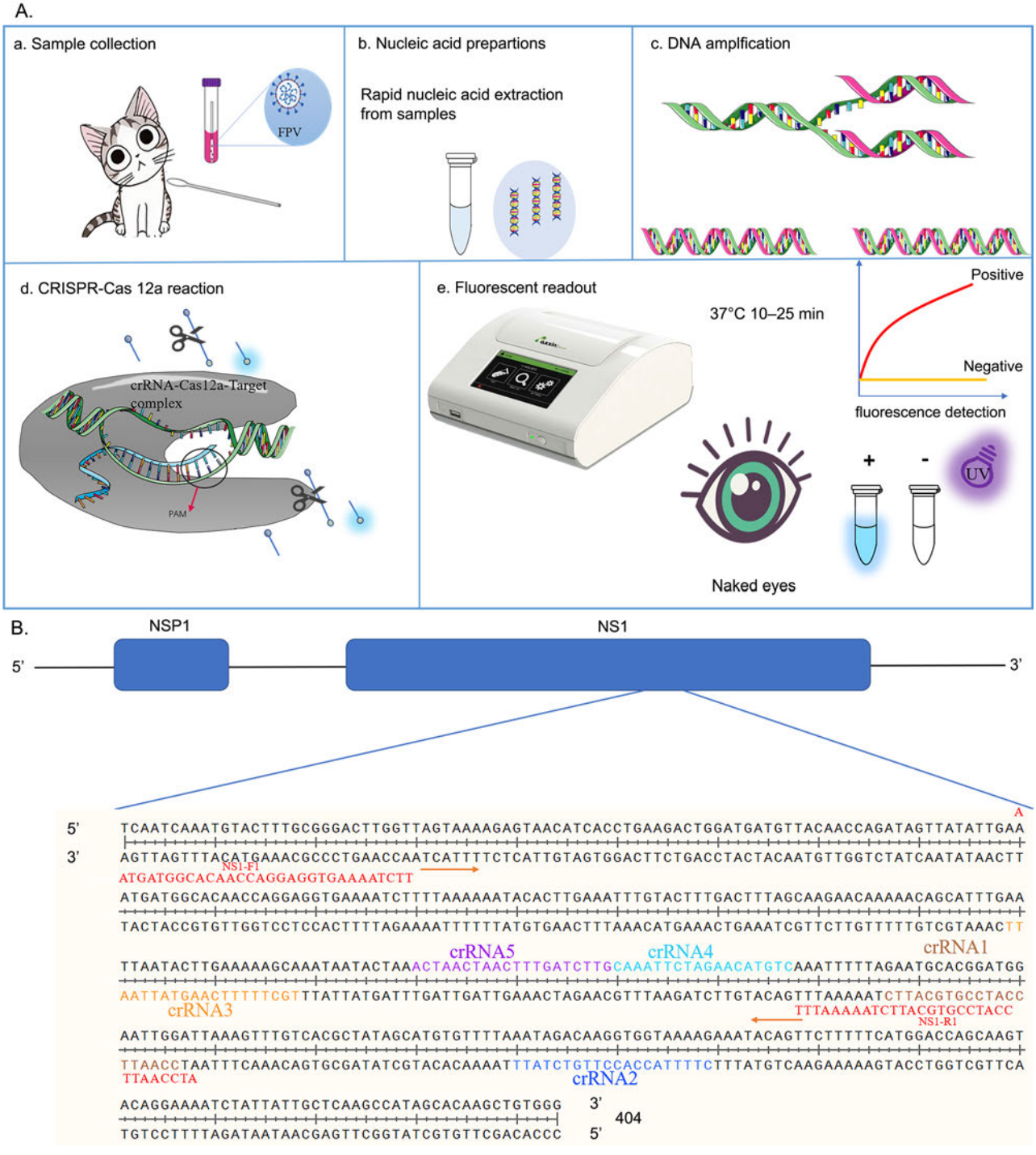

Fig. 1. The schematic flow of RPA-Cas12a-based fluorescence assay for the detection of feline parvovirus virus (FPV) by combining recombinase polymerase amplification (RPA) and CRISPR/Cas12a (A). The genomic DNA of FPV was extracted from clinical samples and amplified using RPA to prepare double-stranded DNA (dsDNA) at 37°C. Then, CRISPR/Cas12a was activated by the complex of amplified products of the target gene, Cas12a, and crRNA, and the single-stranded DNA molecules (ssDNA reporter) were cut to produce fluorescence signals. Finally, the readout of the RPA-Cas12a-fluorescence assay was obtained using real-time or end-point fluorescence and naked eyes using the corresponding simple device. Visualization of primers for RPA and crRNA spacer sites to detect Cas12a within the target nonstructural protein 1 (NS1) gene of FPV genome (B). Primers and crRNA are indicated by red and yellow colored text, respectively.

primer pairs (NS1-F1/NS1-R1, NS1-F2/NS1-R2, NS1-F3/NS1-R3, NS1-F4/NS1-R4, NS1-F3/ NS1-R1, NS1-F2/ NS1-R4, NS1-F1/ NS1-R3) for RPA amplification were designed according to the principles of RPA primer design, and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China). Meanwhile, the specificity of RPA primers was assessed using Nucleotide BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Then, different

Table I
The oligonucleotide sequences for primers and crRNA for the RPA-Cas12a-based fluorescence assay of FPV.

Oligonucleotide name	Sequence (5'–3')
NS1-F1	AATGATGGCACAACCAGGAGGTGAAAATCTT
NS1-R1	ATCCAATTCCATCCGTGCATTCTAAAAATTT
NS1-F2	TTAGAATGCACGGATGGAATTGGATTAAAG
NS1-R2	CCACAGCTTGTGCTATGGCTTGAGCAATAA
NS1-F3	TGATGGCACAACCAGGAGGTGAAAATCTTT
NS1-R3	AATCCAATTCCATCCGTGCATTCTAAAAAT
NS1-F4	TTTAGAATGCACGGATGGAATTGGATTAAAG
NS1-R4	CACAGCTTGTGCTATGGCTTGAGCAATAAT
ssDNA reporter	FAM-TTATTATT-BHQ
crRNA1-F	GAAATTAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATGAATGCACGGATGGAATTGG
crRNA1-R	CCAATTCCATCCGTGCATTCATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTAATTTC
crRNA2-F	GAAATTAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATAATAGACAAGGTGGTAAAAG
crRNA2-R	CTTTTACCACCTTGTCTATTATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTAATTTC
crRNA3-F	GAAATTAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATAATTAATACTTGAAAAAGCA
crRNA3-R	TGCTTTTTCAAGTATTAATTATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTAATTTC
crRNA4-F	GAAATTAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATGACATGTTCTAGAATTTGCA
crRNA4-R	TGCAAATTCTAGAACATGTCATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTAATTTC
crRNA5-F	GAAATTAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATCAAGATCAAAGTTAGTTAGT
crRNA5-R	ACTAACTAACTTTGATCTTGATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTAATTTC

primer pairs were examined using the kit (LeSun Bio, China) to screen for the optimal primer pair. The optimal primer pair was applied in subsequent experiments. Briefly, RPA reaction mixtures were composed of 25 µl of 2 × reaction buffer, 2 µl of each primer (10 µM), 5 µl of DNA template (1×10^4 copies/µl), 13 µl of nuclease-free water and 3 µl of magnesium acetate (280 mM). Then RPA reaction was conducted at 37°C for 25 min in an Axxin T8-isothermal instrument (T8-ISO; Axxin Pty., Ltd., Australia). The amplified products were resolved on a 1% (w/v) agarose gel and visualized under UV light.

crRNA design and selection. To ensure the high efficiency of RPA-Cas12a-fluorescence assay for FPV, five crRNA sequences were designed based on the 404 bp fragment of the NS1 gene, and their corresponding primer pair for crRNA in vitro synthesis was synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China). For crRNA preparation, their corresponding primer pairs for crRNA preparation *in vitro* were synthesized by GENEWIZ Germany GmbH (Germany) and annealed to produce the double-stranded DNA (dsDNA) using an annealing buffer. The generated dsDNA was extracted using the TIANgel Midi Purification Kit (TIANGEN Biotech (Beijing) Co., Ltd., China) and used as the in vitro transcription reaction template (IVT). IVT was carried out using the IVT T7 Kit (Takara Biomedical Technology (Beijing) Co., Ltd., China) at 37°C. The IVT reaction mixture consisted of 5 µl of 10 × transcription buffer, 5 µl of each NTP solution (10 µM), 1 µl of RNase inhibitor (1 µM), 5 µl of T7 RNA polymerase (1 µM), 6.5 µl of RNase-free water and 6.25 µl of each single-stranded primer (10 µM). After 2 hours of incubation, a final concentration of 10 units of DNase I was added to the reaction mixture and further incubated at 37°C for 30 minutes to digest the template dsDNA fully. Subsequently, the crRNA was collected by centrifugation at 15,000 rpm for 20 min at 4°C, and the crRNA concentration was measured using B-500 Biophotometer. To screen for the optimal crRNA, crRNA target sites in the 404 bp fragment of NS1 gene were presented in Fig. 1A. Details of primer sets and ssDNA reporter are shown in Table I.

Sensitivity and specificity analysis. The specificity and sensitivity examination of the RPA-Cas12a-fluorescence assay for FPV was conducted under the optimized reaction conditions described above. RPA-amplified products were briefly added to the CRISPR/Cas12a reaction mixture. Then 5 µl of RPA products were added to 20 µl of the CRISPR/Cas12a reaction mixture, which consisted 2 µl of crRNA (80 nM), 0.5 µl of Cas12a (20 nM; New England Biolabs, UK), 4 µl of ssDNA reporter (160 nM), 2.5 µl of 10 × NEB 2.1 buffer (New England Biolabs, UK) and 11 µl of RNase-free water. Finally, the reaction mixtures were incubated in the Axxin T8-isothermal instrument for 20 min at 37°C to evaluate fluorescent signals.

For the sensitivity assessment, the detection limit was evaluated using the 10-fold dilutions of the recombinant plasmid pBluscript-NS1 ranging from 100 to 0.1 copies/μl as the reaction template for the RPA-Cas12a-fluorescence assay. The limit of detection was determined according to the reaction consisting of the minimum amount of DNA template that produced a fluorescent signal. For the specificity analysis, each DNA or cDNA sample of 1.0×10^4 copies/μl from feline calicivirus/Cat/Shanghai/01/2021 (FCV), feline herpesvirus-1/Cat/Shanghai/01/2014 (FHV-1), feline infectious peritonitis virus ATCC® VR-990™ (FIPV), feline *Mycoplasma felis* ATCC® 23391™ (MYC), and *Chlamydia psittaci* ATCC® VR-120™ (CP) were applied as the templates for the RPA-Cas12a-fluorescence assay. FCV and FHV-1 strains were previously isolated from nasal swabs of feline in the animal relief center in Shanghai and stored by SHADCC. FIPV, MYC, and CP were bought from the American Type Culture Collection (ATCC®, USA).

Examination of clinical samples. The viral DNA was extracted from clinical samples and performed using RPA-Cas12a-fluorescence assay. The results were compared with those obtained by qPCR as previously described (Van Brussel et al. 2019). The assay was carried out using the ABI 7500 instrument (Applied Biosystems).

Statistical analysis. The level of agreement between the two methods was determined using Cohen's "kappa" (κ) analysis via the DiagnosticTest using openepi software (Dean et al. 2013), showcasing a robust agreement with a κ-value of ≥ 0.750, coupled with a highly significant p-value of < 0.0005.

Results

Schematic Diagram of FPV Detection Based on CRISPR/Cas12a. The principles of the RPA-Cas12a-based FPV detection assay are displayed in Fig. 1A. First, for nucleic acid amplification, the amplification reactions of the target gene fragment of FPV were conducted using an RPA kit at a constant temperature of 37°C. RPA consists of DNA recombinase and single-strand DNA binding protein to substitute for the usual heat denaturation step in conventional PCR, and the amplification reaction was carried out by the DNA recombinase with strand-displacement activity necessary to extend the primer to form the protein-DNA complex. The complex can retrieve homologous sequences in the target genome in the reaction mixture and then exponentially amplify the specific region of DNA within 20 min. The reaction was conducted using a portable fluorescence constant-temperature amplification instrument (Axxin T8-isothermal instrument), and the amplified products were detected by agarose electrophoresis. Secondly, the amplified products were added into the CRISPR-Cas12a/crRNA complex for nucleic acid detection to generate a Cas12a-crRNA-target triplex. Following the triplex formation, the Cas12a endonuclease was activated, performing the promiscuous cleavage activity of collateral ssDNAs trans-cleavage. Then, the trans-cutted non-specific ssDNA reporter emitted a strong fluorescence signal. Moreover, the results can be simply observed with the naked eye under a UV light.

Screening of RPA primers for FPV detection. In order to obtain the optimal primer pairs for RPA-Cas12a-fluorescence assay, five primer pairs for targeting the NS1 gene were designed and analyzed using an RPA kit at 37°C for 25 min. The generated products of RPA were checked using agarose gel electrophoresis. As shown in Fig. S1, RPA-amplified products with the expected size were manufactured using seven pairs of primers, respectively. In contrast, the primer pair FPV-F1/PV-R1 yielded the bright and clear band on the gel without tailing and non-specific bands, while others were less efficiently amplified by comparison. This indicates that the primer pair NS1-F1/NS1-R1 is the appropriate selection for RPA amplification as the optimal primer pair. Consequently, the primer pair NS1-F1/NS1-R1 was selected for the following experiment.

crRNA optimization of RPA-Cas12a-fluorescence assay. Previous studies have exhibited that the optimal crRNA can improve the efficiency and fidelity of the Cas12 system. Nevertheless, previous studies have come to inconsistent conclusions about crRNA engineering, indicating that the crRNA engineering method is not universal and needs to be experimentally validated for efficacy after designing by the software. To obtain the optimal crRNA sequence, a more flexible approach could be used to design multiple crRNA specific for the DNA region of interest, enabling us to experimentally validate the efficacy of crRNA after designing by the software. Here, five crRNA sequences were designed and evaluated in the RPA-Cas12a-fluorescence assay. As shown in Fig. 2A, the apparent fluorescence curve was observed in the crRNA5, the mixture with equimolar of crRNA3 and crRNA5 (crRNA3&crRNA5) or the mixture with equimolar of crRNA1 and crRNA5 (crRNA1&crRNA5) treated group. Meanwhile, the fluorescence curve could reach the plateau in 20 min when crRNA5, crRNA3&crRNA5 or crRNA1&crRNA5 was used for detecting the target gene. In addition, the fluorescence value mediated by Cas12a-crRNA-target triplex in the crRNA5, the mixture with equimolar of crRNA1 and crRNA5 (crRNA1&crRNA5), as well as the mixture with equimolar of crRNA3 and crRNA5 (crRNA3&crRNA5) treated group exhibited a ignificant statistical difference in fluorescence intensities compared

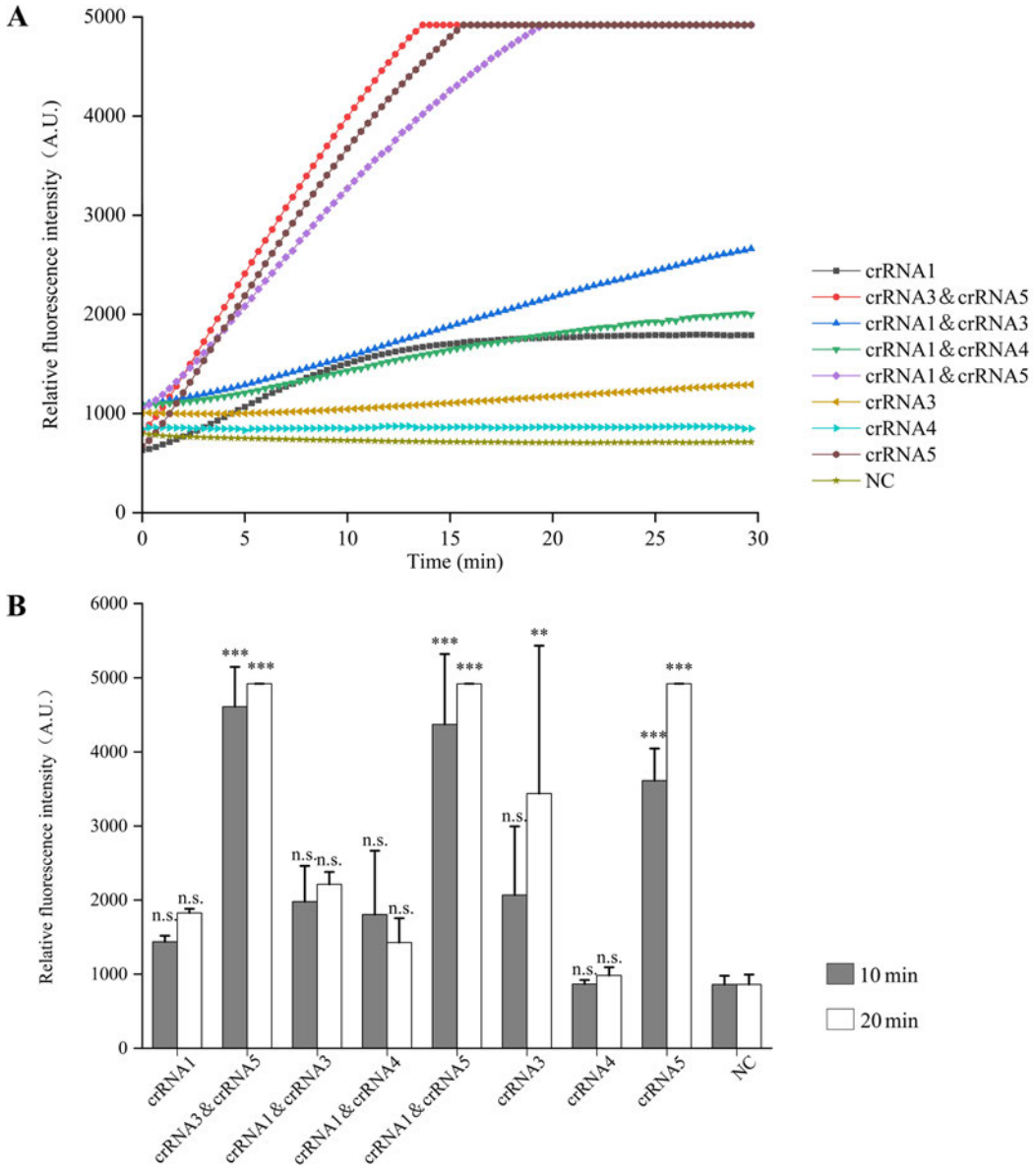

Fig. 2. Examination of optimal crRNA for RPA-Cas12a-based fluorescence assay for the detection of FPV. The fluorescence readouts of RPA-Cas12a-fluorescence real-time (A) and end-point (B) assay were shown using different crRNA or a combination of crRNA for 30 min at 37°C, respectively. Error bars represent the standard deviation of the data (n = 3). Statistical analysis was applied to evaluate the statistical significance between treated groups and the negative control (NC). $^{**}p < 0.05$, $^{***}p < 0.001$, and n.s. – no significant differences

with that in the no-crRNA group for more than 10 min (Fig. 2B). Interestingly, the crRNA3&crRNA5-mediated group achieve the desired high-efficiency cutting reaction compared with other crRNA-mediated groups, and the fluorescence signal saturated at approximately 12 min (Fig. 2B). Therefore, crRNA3&crRNA5 was used in the subsequent RPA-Cas12a-mediated experiments.

The sensitivity of RPA-Cas12a-fluorescence assay. To assess the sensitivity of the RPA-Cas12a-fluorescence assay, final concentrations of FPV DNA templates ranging from 100 to 1 copies/μl were investigated in the RPA-Cas12a-fluorescence assay. As presented in Fig. 3, the RPA-Cas12a-based fluorescence assay could detect as low as 1 copies/μl FPV DNA templates using real-time or endpoint visual detection at 37°C for 20 min. Specifically, when the template concentration was greater than or equal to 1 copies/μl, the fluorescence intensity was proportional to the template concentration. After 20 min of reaction, the endpoint fluorescence values exhibited a statistically significant difference between the experimental group with a final concentration greater than or equal to 1 copies/μl and the negative group (Fig. 3B). Significantly, in addition to a relatively low reaction temperature of 37°C, the positive result of clear green fluorescence signals were also clearly visualized via a portable UV light illuminator (Fig. 3C). In contrast, the negative group exhibited blue fluorescence signals. This indicates that the RPA-

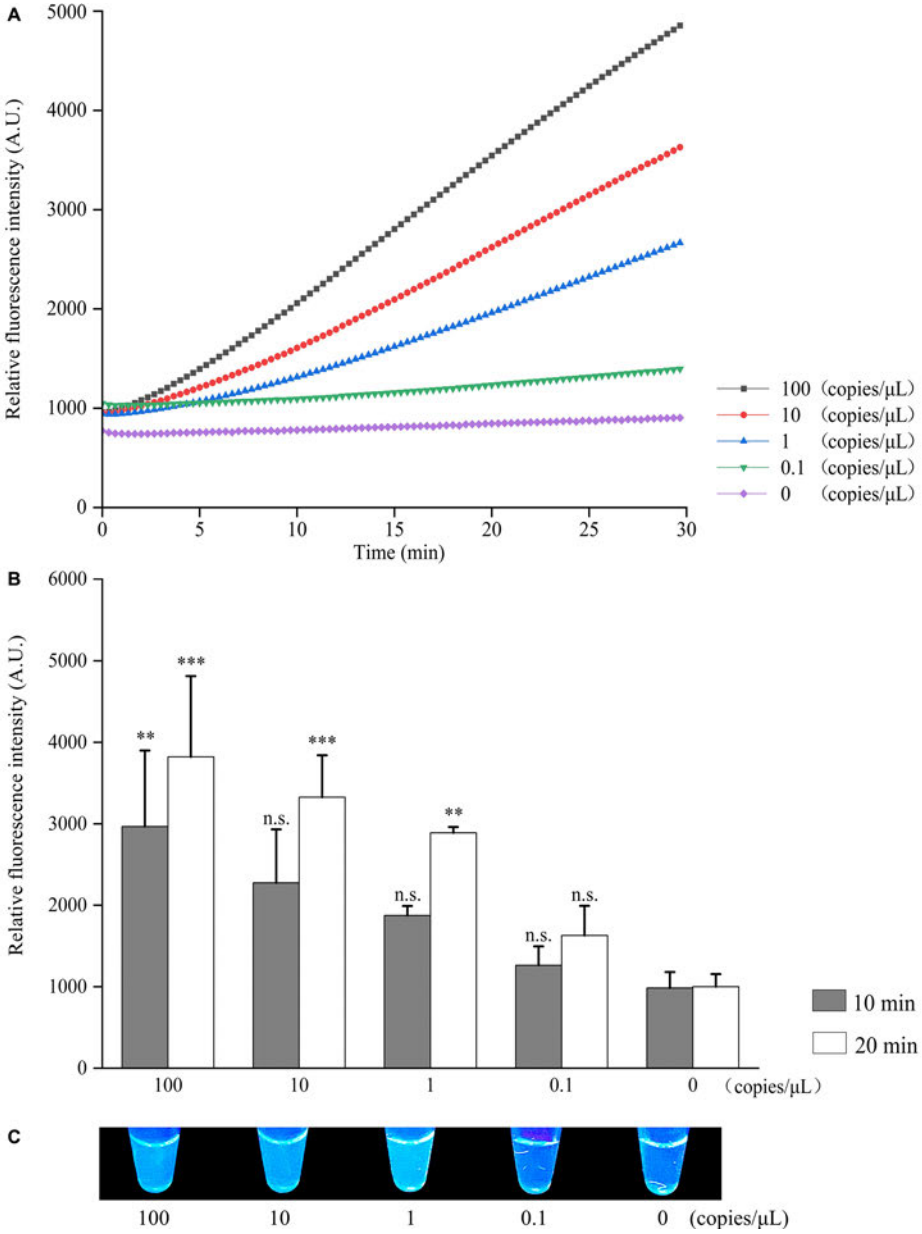

Fig. 3. Examination of sensitivity of RPA-Cas12a-based fluorescence assay. An increase in the fluorescence intensity with increasing the template concentration of the target gene from 0.1 to 100 copies/μl was observed according to real-time (A) and end-point (B) fluorescence readouts. In addition, the color shift between 1 or higher and 0.1 or lower copies/μl was visualized by the naked eye (C). Error bars represent the standard deviation of the data (n = 3). Statistical analysis was used to assess the difference between treated groups and the corresponding time point of negative control (NC).

$^{**}p < 0.05$, $^{***}p < 0.001$, and n.s. – no significant differences

-Cas12a-fluorescence assay explored here may be used for instant field detection without any other expensive or sophisticated equipment. All these results suggest that the RPA-Cas12a-fluorescence assay exhibited excellent sensitivity for FPV DNA detection.

The specificity of RPA-Cas12a-fluorescence assay. The nucleic acid samples extracted from pathogens causing the most common feline infectious diseases, including FIPV, FCV, FHV-1, MYC, and CP, were applied to examine the specificity of the RPA-Cas12a-fluorescence assay. The nucleic acid of RNA viruses was reversed transcribed to cDNA for RPA-Cas12a-fluorescence assay. As presented in Fig. 4A and 4B, in RPA-Cas12a-fluorescence assay with the optimal reaction condition described above, only the reaction composed of the target sequence of FPV, crRNA, Cas12a, and RPA products produced strong real-time and endpoint positive signals, and no cross-reactivity with other viruses was observed. Similarly, after incubation at 37°C for more than 10 min, only the reaction containing the target sequence of FPV yielded green fluorescence signals, which could be directly visible

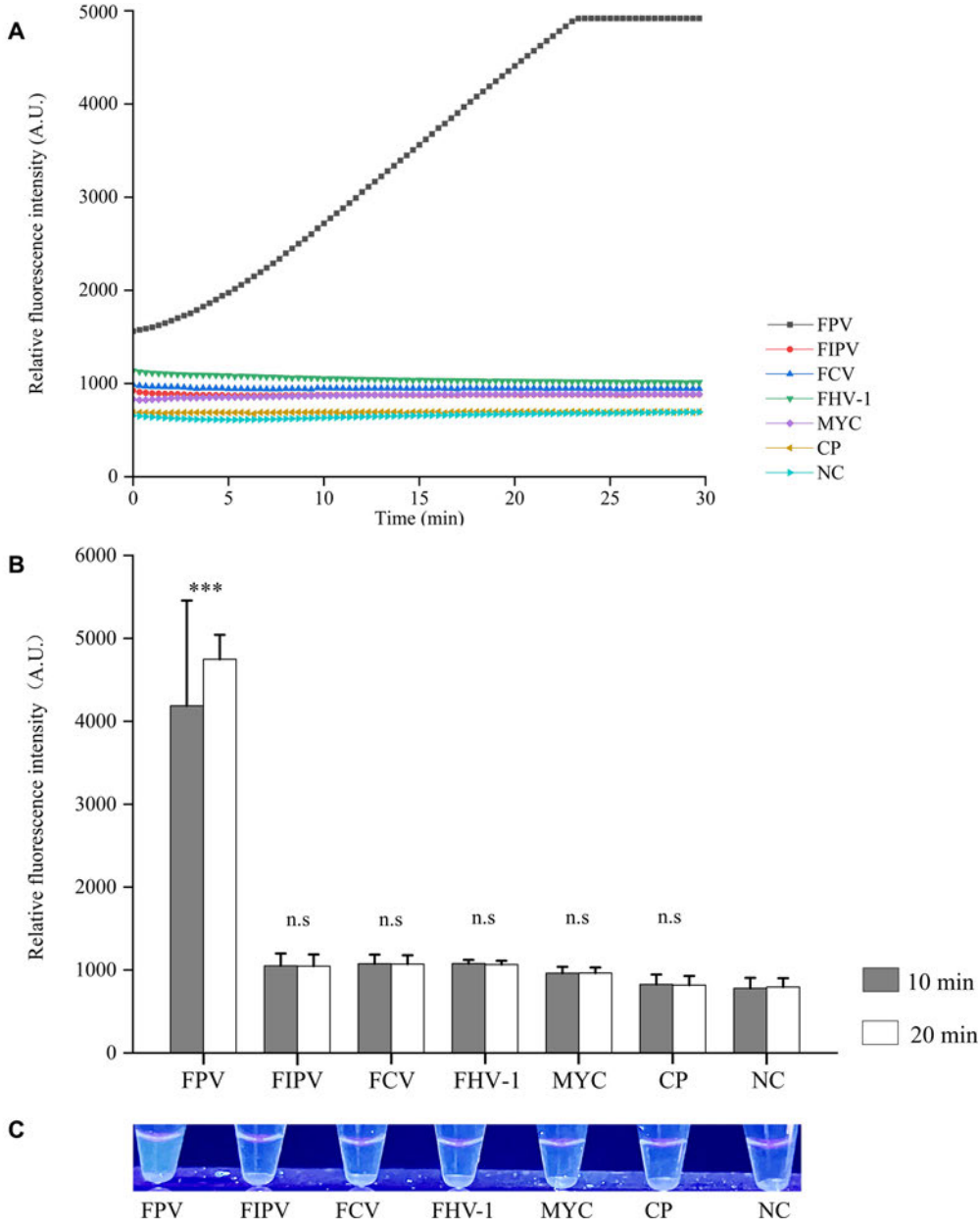

Fig. 4. Assessment of specificity of RPA-Cas12a-based fluorescence assay. The specificity of RPA-Cas12a-based fluorescence assay for FPV detection was investigated using genomic DNA extracted from FPV, feline calicivirus/Cat/Shanghai/01/2021 (FCV), feline herpesvirus-1/Cat/Shanghai/01/2014 (FHV-1), feline infectious peritonitis virus VR-990 (FIPV), feline *Mycoplasma felis* ATCC® 23391™ (MYC), and *Chlamydia psittaci* ATCC® VR-120™ (CP) as the template. Only FPV produced fluorescence signals according to real-time (A), end-point (B) fluorescence, and the naked eye (C) readouts. Error bars indicate the standard deviations of the data (n = 3). Statistical analysis was used to examine the difference between treated groups and negative control (NC). ***$p < 0.001$, and n.s. – no significant differences

by the naked eye when exposed to UV light (Fig. 4C). These results suggest that the RPA-Cas12a-fluorescence assay explored here can detect specifically FPV.

Clinical samples can be detected using the RPA-Cas12a-fluorescence assay. Sixty clinical samples, including 30 anal swabs from FPV-infected cats and 30 anal swabs from healthy cats, were applied to compare the performance of qPCR and the RPA-Cas12a--fluorescence method. As displayed in Table II, 30 samples from the anal swab of healthy cat were tested negative by both methods, with a 100% concordance rate between RPA-Cas12a-fluorescence assay and qPCR. Concerning 30 suspected positive samples, 28 samples tested positive and two samples negative using RPA-Cas12a-fluorescence assay, while 30 samples tested positive by qPCR. The consistency rate in the results between the RPA-Cas12a-fluorescence assay and qPCR for detecting FPV was 96.67% (58/60). Cohen's "kappa" (κ) analysis revealed a good agreement between the two methods, with a κ-value of 0.9333 and a *p*-value

Table II
Statistical analysis of FPV detection in clinical samples using the RPA-Cas12a-based fluorescence assay and qPCR. The difference was statistically significant, as determined via the DiagnosticTest using openepi software.

qPCR		Positive	Negative	Total	Consistency rate
RPA-Cas12a-based fluorescence	Positive	28	0	28	96.67%
	Negative	2	30	32	
	Total	30	30	60	

of 0.000. These results indicated that the RPA-Cas12a--fluorescence assay explored here is a useful tool for rapid, convenient, and reliable detection of FPV in a resource-limited environment.

Discussion

Nowadays, the population of pet cats has steadily risen globally (Kennedy et al. 2020). FPV is the most common viruses among felines, and its high infectivity and transmissibility have caused multiple epizootic outbreaks of feline panleukopenia in the unvaccinated cat population (Jenkins et al. 2020), especially in those aged under 1-year-olds (Battilani et al. 2011). In particular, FPV generally causes mild symptomatic or asymptomatic infection in most mature cats. However, FPV excretion in swabs from infected mature cats may cause a variety of potentially severe infections for newborn cats, even life-threatening (Isaya et al. 2021). Thus, a faster, simpler, and more cost-effective detection method for FPV has become increasingly significant to protect vulnerable and high-risk cats, especially in resource-limited settings. The last decade has witnessed rapid development in the combination of the nucleic acid isothermal amplification technique used to amplify nucleic acids at a constant temperature with the trans-cleavage activity of Cas12a to single-stranded DNA and its application (Gootenberg et al. 2017; Gootenberg et al. 2018; Qian et al. 2021).

Recently, a simple RPA-based RPA-LFD has been exploited for FPV detection with a limit of detection of 10^2 copies/per reaction (Wang et al. 2019). Nevertheless, such a test with LFD readout lacks high sensitivity and thus may not be capable of detecting FPV in infected cats with clinical symptoms. In contrast, asymptomatic carriers with low virus titers essentially go undetected using the RPA-LFD-mediated method. To improve the diagnosis of FPV, this study aimed to develop a more sensitive and specific RPA-Cas12a-based fluorescence detection test.

In this study, the RPA-Cas12a-based real-time or end-point fluorescence method for more sensitive detection of FPV was exploited by targeting the conserved fragment of the FPV NS1 gene. Primer design and selection is the key step in achieving efficient and robust DNA products using RPA in the RPA-Cas12a-based method. The optimal primer pair was screened to examine the effect of diverse primer sets on the RPA efficiency. Secondly, five crRNA for screening for the optimal crRNA were designed and examined to optimize Cas12a cutting efficiencies.

In this study, through the optimization of the primer for RPA amplification and Cas12 guide RNA (crRNA), the RPA-Cas12a-based real-time fluorescence assay can produce precise real-time fluorescence curves within 25 min, which avoids opening the reaction tube after amplification for LFD detection, thereby minimizing pollution exclusions into the environment risk. Furthermore, the RPA-Cas12a-based end-point fluorescence method developed here may be preferable for a simple read-out of the test results using a compact and handheld UV lght in limited-resource settings.

The standard plasmid of the conserved fragment of target FPV NS1 gene was diluted from 100 to 0.1 copies/μl, and in the RPA-Cas12a-based real-time or end-point fluorescence assay, the detection limit is 25 copies/per reaction, which is much more sensitive than the RPA-LFD method (100 copies/per reaction). In addition, in the RPA-Cas12a-based real-time or end-point fluorescence assay, there was no cross-reaction with the amplification products with the genome of other viruses, including FIPV, FCV, FHV-1, MYC, and CP.

Conclusions

In summary, the established novel RPA-Cas12a-based real-time or end-point fluorescence assay is more effective and sensitive for the detection of FPV. The assay can be completed within 25 min at 37°C. The detection limit of each reaction (25 copies/per reaction) is lower than that of existing diagnostic methods, such as the RPA-LFD assay (100 copies/per reaction), and the assay is highly specific to FPV. The RPA-Cas12a-based real-time or end-point fluorescence assay significantly reduced reliance on exceptional staff and advanced instruments. It would be beneficial in detecting FPV at early stages of asymptomatic or subclinical infection, especially in resource-poor settings.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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