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Original research article

Development of a recombinase polymerase amplification fluorescence assay to detect feline coronavirus

Xiaoliang Hu^{a,1}, Li Xiao^{b,1}, Xiao Cong^{c,1}, Yujun Zhu^b, Bihong Huang^{b,**}, Feng Cong^{b,*}

^a School of Life Sciences and Food Engineering, Yibin University, Yibin Key Laboratory of Zoological Diversity and Ecological Conservation, Yibin, 644000, China

^b Guangdong Laboratory Animals Monitoring Institute and Guangdong Provincial Key Laboratory of Laboratory Animals, Guangzhou, 510633, China

^c Anhui Science and Technology University, Chuzhou, 233100, China

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ABSTRACT

Feline coronavirus (FCoV) is classified into two pathotypes: the avirulent feline enteric coronavirus (FECV), and the virulent feline infectious peritonitis virus (FIPV). Rapid pathogen detection, which is efficient and convenient, is the best approach for early confirmatory diagnosis. In this study, we first developed and evaluated a rapid recombinase polymerase amplification (RPA) detection method for FCoV that can detect FCoV within 15 min at 39 °C. The detection limit of that assay was 233 copies/μL DNA molecules per reaction. The specificity was high: it did not cross-react with canine distemper virus (CDV), canine coronavirus (CCoV), canine adenovirus (CAV), feline calicivirus (FCV), feline herpesvirus (FHV), or feline parvovirus (FPV). This assay was evaluated using 42 clinical samples (30 diarrhea samples and 12 ascites samples). The coincidence rate between FCoV-RPA and RT-qPCR for detection in clinical samples was 95.2%. In summary, FCoV-RPA analysis provides an efficient, rapid, and sensitive detection method for FCoV.

1. Introduction

Feline coronavirus (FCoV) is an important pathogen of cats and wild felids [1,2]. It is classified into two pathotypes: the avirulent feline enteric coronavirus (FECV), and the virulent feline infectious peritonitis virus (FIPV). FCoV is a member of the virus family *Coronaviridae*, order *Nidovirales*, and possesses a single-stranded positive-sense RNA genome that is nearly 29 kb in length [3]. The FECV is highly prevalent in the cat family, and causes mild and transient diarrhea [4,5]. FIPV is a mutant of the avirulent FECV; the characteristic clinical manifestations are a fibrinous, granulomatous serositis and neurological signs [6]. Therefore, an efficient diagnostic method plays a key role in rapidly and correctly identifying the virus pathotype.

Polymerase chain reaction (PCR) [7,8], reverse transcription quantitative PCR (RT-qPCR) [9,10] and loop-mediated isothermal amplification (LAMP) [11,12] are all highly sensitive and specific at targeting and amplifying RNA from field and clinical samples. All these methods are widely used for the detection of microorganisms. However, they require complex instrumentation and well-trained staff, and are

time-consuming.

The recombinase polymerase amplification (RPA) assay has been considered as the second general isothermal method for efficient amplification of DNA under constant temperature conditions (between 37 and 42 °C) [13]. It depends on three enzymes, a recombinase, DNA polymerase, and DNA-binding proteins, for isothermal amplification [14]. Detection assays for RNA viruses have been rapidly developed in which RPA is coupled with reverse transcription [15,16]. In this report, we describe the initial development of a rapid and sensitive RPA assay for detecting FCoVs.

2. Materials and methods

2.1. Ethics statement

No animals were sacrificed specifically for this study. Samples of feces and ascites fluid were collected by the veterinary hospital. The owner of each cat took the initiative to send the sick animal to the veterinary hospital for treatment. When the veterinary hospital was

* Corresponding author.

** Corresponding author.

E-mail addresses: liang679@163.com (X. Hu), 997859951@qq.com (L. Xiao), 1049777843@qq.com (X. Cong), 837895642@qq.com (Y. Zhu), 269823434@qq.com (B. Huang), congcong521@126.com (F. Cong).

¹ Xiaoliang Hu, Li Xiao and Xiao Cong contributed equally as first authors.

unable to determine the nature of the pathogen, and with the client's consent for further diagnosis and experiments, samples were sent to Guangdong Laboratory Animal Monitoring Institute for confirmation. We reported the results to the veterinary hospital according to the diagnostic findings, and we were not involved in the collection of samples.

2.2. Viruses and sample collection

Canine parvovirus (CPV), canine distemper virus (CDV), canine coronavirus (CCoV), canine adenovirus (CAV), feline coronavirus (FCoV), feline calicivirus (FCV), feline herpesvirus (FHV), and feline parvovirus (FPV) were stored in the Guangdong Laboratory Animal Monitoring Institute. Forty-two samples of feline diarrhea and ascites fluid were received by the institute.

Viral RNA was extracted using TRIzol reagent (TaKaRa Biotechnology, Dalian, China) and viral DNA was extracted using the DNA Mini Kit (50) (Omega Bio-tek, Norcross, GA, USA), following the manufacturers' protocols. Viral genomic DNA and/or RNA were stored at -80°C . Construction of FCoV positive standard plasmids was performed using a Plasmid Mini Kit I (200) (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's protocol.

2.3. Primer design and PCR analysis

The FCoV sequences based on the membrane gene were aligned using Lasergene software. Eight sets of primers and probes based on the membrane gene of FCoV (KY566209.1) were designed according to the recommendations from TwistDx Co., Ltd, following the manufacturer's protocol (TwistDx Assay Design Manual). All primer/probe sequences used in this study are listed in Table 1, and were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.4. Cloning of standard recombinant plasmids

DNA fragments of the membrane gene (m-F, m-R, Table 1), were amplified from FCoV. The PCRs were conducted in a total of volume of 20 μL containing 10 μL of $2 \times$ Premix rTaq, 7 μL of ddH₂O, 1 μL of forward primer ($c = 10 \text{ nM}$), 1 μL reverse primer ($c = 10 \text{ nM}$), and 1 μL (10 ng) of DNA. The PCR products were purified using the Axygen Gel Extraction Kit (Axygen, USA) and then cloned into the pMD18-T vector (TaKaRa, Tokyo, Japan). The plasmids were sequenced using an ABI 3730XL Sanger-based Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). The positive plasmids were extracted, concentrated, and

Table 1

Source of FCoV primers and probes used in the experiment.

Names	Sequences(5'-3')	Size
F27	GTTACTACTCAGTCATCTGCTTAATCTTAC	153bp
R27	AGTAATCTAGTTTCTGCTAAAACCTTGTGT	
F28	GAAATCTATATGCTGAAGGTTTCAAATGG	132bp
R28	TAGTTGCTTTAATTGTTTCCCAACTAATG	
F29	AAACCTGCTTTGAACGTGGTGATCTTATT	140bp
R29	CATTTAATGCCATAAACGAGCCAGCTAAA	
F30	AGTGCGTTAATCACACAGATTCAAGTTGTC	180bp
R30	ACATGATCAGCATTTAATGCCATAAACGA	
F31	TCAGCTGGTCTGTAATATTGATTGTTTTA	179bp
R31	CGAACATTACATATCTGGAACCTTGATACT	
F32	GAGATTTTGTCCCAAAGGAATAGGTAATA	172bp
R32	ATCAATCTGTCTTTAAATTTAGCATCAGC	
F33	CTATTGTTCTAGCGCTTACGATTTTAAATG	139bp
R33	AGCTGAATAGATCTCACAAAATACATCATC	
F34	GAACCTCAGATATACCATGACAGAGATTTTC	143bp
R34	GTTAAAAACTTTTAGCTTCTCACTAAAGCA	
M-F	ATGAAGTACATTTGTTAACACTCG	789bp
M-R	CATGAGAAAATTTATFACATATGGTGTA	
Probes	TCTTACCATGCGACATTTACCTAAATATG(FAM-dT)(THF)A (BHQ-dT)GATTGCTACACCT(C3spacer)	

stored at -20°C .

2.5. Development of RPA assays

The RPA reactions were performed using RPA basic kits (ZC Bioscience, Hangzhou, China) according to the manufacturer's instructions. Briefly, a total of volume of 50 μL containing 2.5 μL of Mg^{2+} buffer ($c = 280 \text{ mM}$), 300 nM of each primer, 120 nM of probe, 1 μL (10 ng) of DNA template and sterile water were added and mixed with one tube of the basic reaction unit. The reaction procedure of RPA was performed at 39°C in a Deaou-308C Tubes Canner (DEAOU Biotechnology, China).

According to the manufacturer, a sample was confirmed positive if the amplification curve was above three and a half standard deviations (3.5 SD) from the background in the course of a valid time range (i.e. after 17–18 min of amplification). A threshold time range of 0–4 min and 30 s was used.

2.6. Repeatability and precision variations of the RPA assay

The standard plasmid was diluted to 10^6 copies/ μL , and was tested by three independent RPA reactions in one day or in two days. The coefficient of variation was analyzed using Graph prism 5.0.

2.7. Analysis of sensitivity and specificity

To evaluate the sensitivity of FCoV RPA, a positive plasmid was serially diluted 10-fold (from 10^6 copies/ μL to 10^1 copies/ μL), and tested in three replicates each time to assess day-to-day variation. A probit regression was performed to determine the limit of detection (LOD) at 95% probability using SPSS Statistics software (IBM Corporation, New York, USA).

The specificity of the FCoV RPA assay was evaluated by testing a panel of viruses with a fixed amount of nucleic acid (10 ng): canine distemper virus (CDV), canine coronavirus (CCoV), canine adenovirus (CAV), feline calicivirus (FCV), feline herpesvirus (FHV), and feline parvovirus (FPV). Genome extraction and RPA were performed as described above. Nuclease-free water served as the negative control.

2.8. Testing of field samples

The 42 field samples, including 30 feline diarrhea samples and 12 ascites samples, were tested by the FCoV RPA assay and the results compared with RT-qPCR to check any nonspecific amplification. The nucleic acid of FCoV was used as the positive control.

3. Results

3.1. Screening of primer sets

The relative performance of eight candidate primer sets was evaluated and compared, and the products were evaluated on 2.0% agarose gels. Of the primer pairs, the pair F28 and R28 was chosen because of the high yield of amplicon and the lowest formation of primer dimers. In addition, the other primer sets were not chosen according to findings regarding the specificity and background of the assay (Fig. 1A).

3.2. Sensitivity and specificity of RPA

To evaluate the performance of the RPA, the sensitivity was determined using serial dilutions of a standard plasmid, which was 10-fold serially diluted, ranging from 10^6 to 10^1 copies/ μL . As shown in Fig. 1B, the detection limit of the FCoV-RPA was 10^2 copies/ μL of target DNA, which is consistent with RT-qPCR. Amplification curves of 10^6 – 10^1 copies/ μL were successfully detected within 6.0–16.0 min (Fig. 1B). The limit of detection was further analyzed using six independent RPA tests, and the sensitivity was 233 copies per reaction at

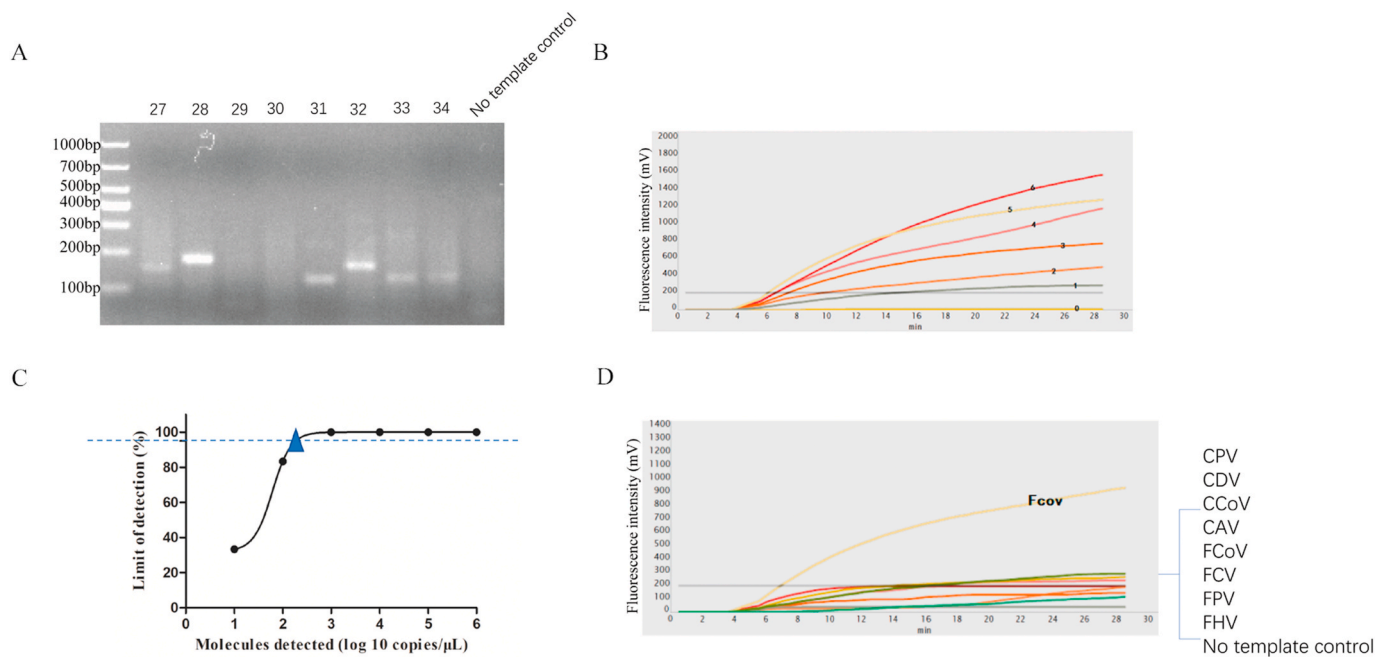


Fig. 1. FCoV-RPA assays. (A) The screening of the primer sets. (B) Sensitivity assessment of the FCoV-RPA assay was performed with 10^6 – 10^1 copies/ μ L DNA standard dilutions. 6: 10^6 copies/ μ L, 5: 10^5 copies/ μ L, 4: 10^4 copies/ μ L, 3: 10^3 copies/ μ L, 2: 10^2 copies/ μ L, 1: 10^1 copies/ μ L, 0: indicates negative control. (C) Analytical sensitivity of FCoV-RPA assay (triangle). (D) Specificity of the FCoV-RPA assay was observed. Nucleic acids of FCoV, CDV, CCoV, CAV, FHV, FPV, and FCV were tested to assess specificity.

95% probability (Fig. 1C).

To test the specificity of the FCoV-RPA assay, six other feline and canine pathogens were used as templates. As shown in Fig. 1D, positive products were amplified only from FCoV, and the curves for the CDV, CCoV, CAV, FCV, FHV, and FPV samples showed no amplification. The specificity testing of FCoV-RPA revealed that the assay showed no cross-reactivity with other canine and feline pathogens, indicating that the assay had high specificity.

3.3. Variation in repeatability and precision of the RPA assay

In order to verify the stability of the FCoV-RPA assay, standard plasmids were diluted to 10^6 copies/ μ L, and three independent RPA reactions in one day or in two days were carried out. The coefficient of variation was less than 5%, which showed that the FCoV-RPA assay was repeatable and precise (Table 2).

3.4. Testing of field samples

A total of 42 field samples, which were collected by the Guangdong Laboratory Animals Monitoring Institute, were tested for FCoV by this RPA assay and the results compared with RT-qPCR [10]. Fourteen of 30 diarrhea samples and 4 of 12 ascites samples were identified to be positive by FCoV-RPA; the samples negative by FCoV-RPA were also

Table 2

The day-to-day and within-day variations of the RPA assay. Data listed in this table were from three independent RPA reactions using 10^6 copies/ μ L of standard plasmid.

Within-day variation			Day-to-day variation		
TT	mean	CV%	TT	mean	CV%
6:00	6:06	2.5%	5:90	6:11	3.0%
6:23			6:18		
5:95			6:24		

CV: coefficient of variation; RPA: recombinase polymerase amplification; TT: threshold time.

negative by RT-qPCR. However, only 16 of the 18 FCoV-positive samples as determined by FCoV-RPA were also identified by RT-qPCR. The amplicons of the two discordant samples were purified and cloned into the pMD18-T vector for sequencing. The result demonstrated that the two samples were positive for FCoV. The coincidence rate between RT-qPCR and FCoV-RPA was 95.2% (Table 3). In order to determine the authenticity of the RPA results, the PCR products of the 18 positive samples were sequenced. The results showed that the sequences were 99% identical to that of the membrane gene of FCoV.

4. Discussion

Two serotypes of FCoV are recognized: type 1, which is found in naturally infected cats and causes mild clinical signs and transient diarrhea; and type 2, which arose following recombination events between type1 FCoV and CCoV and causes fatal disease [17]. Thus, rapid, convenient diagnosis of FCoV plays a key role in intervention and the implementation of preventive measures. In this report, we firstly describe a simple, rapid, sensitive, and specific diagnostic tool for FCoV detection using the RPA assay.

In general, the FCoV colloidal gold method is widely used in veterinary clinics and field testing. It is able to detect the presence of antigens in a sample within 5 min. Trace levels of antigen, however, are below its detection range, because its sensitivity is lower than that of the PCR or RT-qPCR methods.

Given that FCoV has significant virulence and poses a serious threat to cats and other animals, rapid and sensitive diagnosis is needed. Classical

Table 3

Coincidence rate of RPA and RT-qPCR.

		RT-qPCR		CR	
		Positive	Negative	Total	
RPA	Positive	16	2	18	95.2%
	Negative	0	24	24	
	Total	16	26	42	

CR: Coincidence rate. CR = (16 + 24)/42 \times 100%.

diagnostic methods, including indirect immunofluorescence antibody tests [18,19], virus neutralization (VN) assays [18,20], enzyme-linked immunosorbent assays [21], immunohistochemistry [22], and rapid immunochromatographic tests (RIM) [18,20], have been developed. Molecular diagnostic methods, for example PCR [7,8], quantitative PCR (qPCR) [9, 10] and loop-mediated isothermal amplification (LAMP) [11,12] have also been developed. However, these are time-consuming (taking at least 1 h) and require professional equipment.

In this study, a second type of isothermal nucleic acid amplification technology, the FCoV-RPA detection system, was developed, which demonstrates great advantages over LAMP and other existing detection methods. 1) The limit of detection was consistent with the RT-qPCR assay, as few as 233 copies/ μ L DNA molecules per reaction; 2) the detection time was <15 min, only one-quarter that of the RT-qPCR assay; 3) reaction conditions close to room temperature allow the FCoV-RPA assay to be performed using simpler devices. Moreover, a comprehensive assessment of sensitivity and specificity for 42 clinical samples (30 diarrhea samples and 12 ascites samples) showed that FCoV-RPA is more sensitive than RT-qPCR. This indicates that the FCoV-RPA assay is more suitable for rapid testing of clinical samples in veterinary hospitals.

As previously reported, the RPA assay can directly test original samples, such as blood, nasal swabs, pleural fluid or culture medium, without nucleic acid purification, and is conducive to spot detection [23–25]. However, a variety of PCR inhibitors, such as alkaline lysis and high genomic DNA concentrations may delay the generation of a fluorescence signal or inhibit the enzyme activity in the RPA reaction system [24,26]. In the present study, PBS buffer and nuclease-free water were used to dilute the fecal samples and ascites fluid. If a low level of fluorescence signal is observed (data not shown), it suggests that the samples contain a variety of components that inhibit the activity of recombinase proteins, and the RPA reaction will be affected. Further experimentation is needed to identify a suitable diluent for successful testing of crude DNA extracts from feces and ascites fluid.

To summarize, a real-time recombinase polymerase amplification (RPA) assay was developed to amplify the membrane gene of FCoV. It can detect FCoV within 15 min at 39 °C, with a detection limit of 233 copies/ μ L. Furthermore, the FCoV-RPA does not cross-react with CDV, CCV, CPV, CAV, FCV, FHV, and FPV. Compared with qRT-PCR, the RPA assay is more convenient and less time-consuming. It provides the possibility of rapid clinical detection of FCoV infection.

Author statement

Under supervision by Ph. D. Feng Cong and Li Xiao, Xiaoliang Hu and Bihong Huang performed sample preparation, data analysis and mechanics modelling and analysis. Xiaoliang Hu, Xiao Cong and Yujun Zhu performed calculations. All authors read and contributed to the manuscript.

Declaration of competing interest

The authors state that they have no competing interests.

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