Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Journal of Virological Methods 271 (2019) 113679

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of a recombinase polymerase amplification assay with lateral flow dipstick for rapid detection of feline parvovirus

Zhao-Hua Wang[a], Xiao-Jia Wang[a,*], Shao-Hua Hou[b]

[a] Key Laboratory of Animal Epidemiology of the Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing, 100193, China
[b] Beijing Veterinary Research Institute of Chinese Academy of Agricultural Sciences, Beijing, 100193, China

ARTICLE INFO

Keywords:
RPA
LFD
FPV
Detection

ABSTRACT

Feline panleukopenia caused by feline parvovirus (FPV), a single-stranded DNA virus, is typically highly contagious and often presents with lethal syndrome. The broad spectrum of possible hosts suggests its potential for transmission from animal to person through close contact with pets. FPV thus serves as an example of the importance of new rapid point-of-care field diagnostic tools for the control and prevention of transmission, especially among rare wild animals and pet cats. Recombinase polymerase amplification (RPA), as a real-time and isothermal method, could be a more affordable alternative to PCR when combined with a lateral flow dipstick (LFD) indicator. In this study, we report a novel FPV lateral flow dipstick RPA (LFD-RPA) instant detection method capable of detecting a range of different FPV strains. The LFD-RPA assay consists of specific primers, probe, and nucleic acid strip. It is capable of detecting 10^2 copies of target nucleic acid per reaction, which is one order of magnitude higher than the sensitivity of traditional PCR. The most suitable reaction conditions for this assay are at 38 °C for 15 min. This paper develops an efficient visual detection system that can eliminate the need for professional staff and expensive and sophisticated equipment for field detection.

1. Introduction

Feline parvovirus (FPV) was first isolated in 1961 by Bolion and subsequently identified as belonging to the *Parvoviridae* family (Cohen and Yohn, 1961; Siegl and Bates, 1985). The viral particles, with a genome of linear single-stranded nucleic acids of approximately 5000 bases, are spherical without envelope (Agbandje and McKenna, 1993). FPV has a wide range of hosts, including domestic cats and many different wild and captive carnivores, such as the families of *mustelidae*, *procyonidae*, and *viverridae* (Demeter and Gal, 2009; Allison and Harbison, 2012; Fei-Fei and Yong-Feng, 2017). Cats infected with FPV commonly display anorexia, vomiting, diarrhea, neutropenia, and lymphopenia, with a high fatality rate (Truyen and Parrish, 2013; Stuetzer and Hartmann, 2014). There has been significant evolution of the virus over the last 50 years, especially in the widely distributed pathogen canine parvovirus (CPV). CPV, which was first detected in 1978, evolved from FPV with mutated genes and has global distribution (Parrish, 1999). Cats once infected with FPV can carry the virus long term, which increase the possibility of virus transmission between infectious and healthy animals. Many cats undergo a subclinical infection without any clinical signs (Truyen and Parrish, 1992). In addition to the fecal-oral transmission route, this highly contagious virus can infect other cats by hiding in the environment (Steinel and Parrish, 2001). FPV has been the subject of several decades of research, but is still rampant around the world.

A number of different diagnostic methods for FPV infection have been described, including virus isolation, latex agglutination, immunochromatographic tests, electron microscopy, ELISA, and PCR (Veijalainen and Neuvonen, 1986; Schunck and Kraft, 1995a, 1995b; Ikeda and Miyazawa, 1998; Esfandiari and Klingeborn, 2000; Decaro and Desario, 2008; Digangi and Gray, 2011; Lane and Brettschneider, 2016). These methods are generally available for most viruses. With the development of new detection materials and methods, a series of improvements on the basic PCR method were reported. The programmed step electric field strength (PSEFS) method is more than 100 times faster than conventional slab gel electrophoresis, and on-line capillary electrophoresis method can enhance detection sensitivity (Shin and Lee, 2012; Nan and Yoo, 2013). Though all kinds of detection methods have been produced commercially, the nucleic acid testing of samples to confirm FPV is still the gold standard. Antigen and antibody detection do not show higher sensitivity and specificity than molecular biological detection. The virus isolation process is, however, complex, and electron microscopy is expensive and requires tedious operator manipulation. A convenient and efficient method of detecting FPV is urgently

* Corresponding author.
E-mail addresses: wangxj@cau.edu.cn (X.-J. Wang), houshaohua@126.com (S.-H. Hou).

https://doi.org/10.1016/j.jviromet.2019.113679
Received 7 March 2019; Received in revised form 18 May 2019; Accepted 10 June 2019
Available online 16 June 2019
0166-0934/ © 2019 Elsevier B.V. All rights reserved.

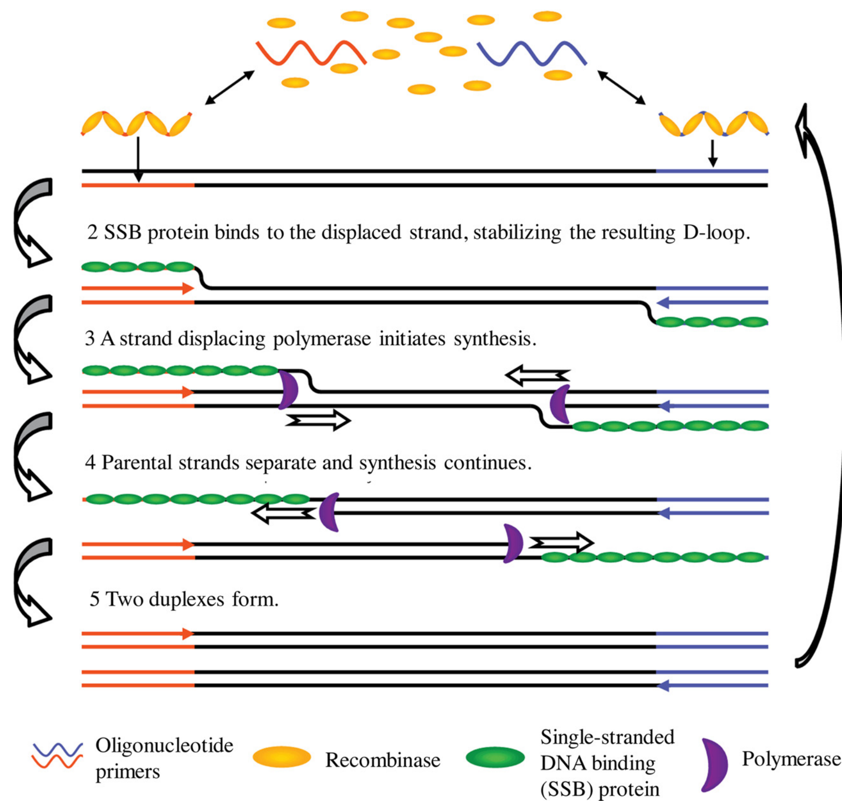

Fig. 1. Basic principles of RPA (TwistDxTM Limited, Cambridge, UK).

required.

The global spread of infectious diseases makes the development of rapid point-of-care diagnostic tools a major focus in addressing global health concerns. Although advances in PCR have streamlined this method significantly, sample preparation and detection of PCR requires a specialized work environment and professional staff. To address these issues, a number of new diagnostic methods for the detection of nucleic acid have been developed and are currently being refined. One of the more common technologies is recombinase polymerase amplification (RPA) assay.

RPA assay requires a constant temperature rather than thermo cycling (Figs. 1, 2). It relies on a synthetically engineered adaptation of a natural cellular process called homologous recombination, comprising three key proteins (recombinase, recombinase loading factor and single-stranded binding protein). The enzymatic activity catalyzes the reaction at a single optimum temperature between 37 ℃ and 42 ℃ (Daher and Stewart, 2016; Li and Macdonald et al., 2019). RPA can routinely generate results within 20 min or less. Here we describe the development and validation of an RPA assay for the detection of FPV nucleic acid. As a diagnostic tool, it could be applied to an outbreak in limited resource settings without complex advice and achieve rapid point-of-care detection.

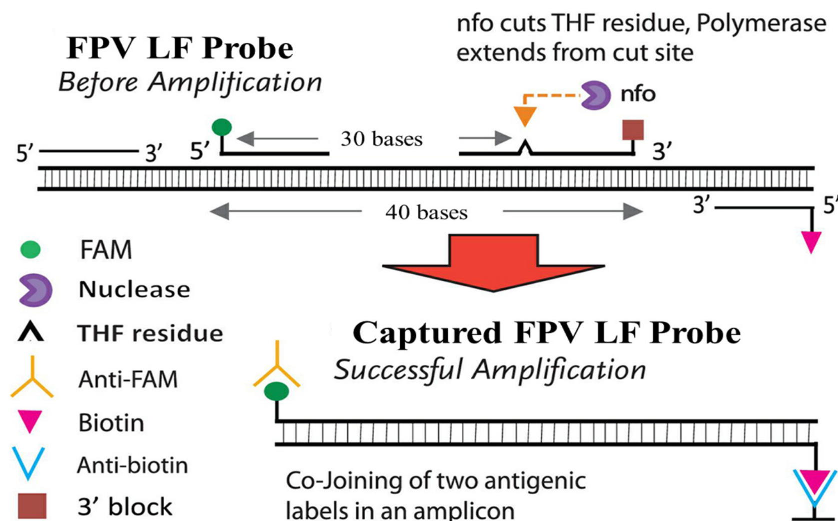

Fig. 2. Principles of LFD- RPA using TwistDx's proprietary probe systems.

2. Materials and methods

2.1. Virus, cells, and clinical samples

The feline parvovirus used in this study was isolated in our laboratory, and the identity was validated by aligning the VP2 gene sequence with reference sequences (GenBank: KC473946.1, KM624023.1, and KJ813895.1). The FPV was cultured by F81 cells maintained in our laboratory, and other viruses were bought from ATCC. F81 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), then incubated in 37℃ humidified incubator with 5% CO_2. Thirty anal swabs (n = 30) were collected from various breeds and ages of felines treated at the animal hospital of China Agricultural University. All the felines were suspected of being FPV infected. The anal swabs samples were stored at -80 ℃.

2.2. DNA extraction

Total DNA extraction from F81 cells growth medium containing feline parvovirus was extracted using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, United States) according to the manufacturer's instructions. For viral DNA extraction from the anal swabs, the swabs were inoculated in 500 μl sterile phosphate-buffered saline and centrifuged at 12,000 rpm for 8 min at 4 ℃. The supernatant was collected and used for viral DNA extraction using the kit described above. All DNA samples were stored at -20 ℃ until used.

2.3. Primer, probe, template design and preparation

The VP2 gene was selected as the target gene. Polymerase chain reaction (PCR) primers of VP2 gene were designed using the Primer Premier 5.0 software package. Four pairs of primers (Vp2-F1/ Vp2-R1, Vp2-F2/ Vp2-R2, Vp2-F2/ Vp2-R3, Vp2-F1/ Vp2-R4) were designed to ensure high efficiency of obtaining the complete VP2 gene. The sequences of VP2 gene from nine publicly available FPV GenBank numbers (KC473946.1, KM624023.1, KJ813895.1, KJ813894.1, KJ813893.1, KX434462.1, KX434461.1, JX048608.1, JN867596.1) were analyzed using DNAMAN version 9 software and supplemented by Nucleotide BLAST online (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The area of greatest sequence conservation was selected and used to design primers and probes for RPA assay. A series of gradient forward and reverse primers and probes were designed using the Primer Premier 5.0 software package according to the principles of RPA primer design, and the specificity was evaluated by Nucleotide BLAST online. The PCR primer pair used for comparing the sensitivity between the RPA and PCR method was designed according the published reference (Schunck and Kraft, 1995a, 1995b). All of the above primers and probes were synthesized by Sangon Biotech (Shanghai, China); the sequences are shown in Table 1. DNA probes for LFD assays carry a FAM antigenic marker at the 5′ end, the 3′ end was blocked with a C3-spacer (polymerase extension blocking group), and a base analog tetrahydrofuran (THF) was inserted between the 30th and 31st base.

Table 2
Clinical performance of the FPV-RPA-LFD assay in comparison with traditional PCR.

	PCR		RPA-LFD	
	Positive	Negative	Positive	Negative
suspected infectious cases (n = 30)	7	23	8	22
suspected healthy cases (n = 13)	0	13	0	13

2.4. Construction of recombinant plasmid

Feline parvovirus DNA was amplified with primers Vp2-F1/Vp2-R1, Vp2-F2/Vp2-R2, Vp2-F2/Vp2-R3, Vp2-F1/Vp2-R4 respectively in a 25 μl volume comprising 12.5 μl of 2× Taq PCR Mix (Mei5, China), 1.0 μl of forward and reverse primers (10 μM, total of 2.0 μl of primers), 1 μl of template DNA, and 4.5 μl of double-distilled H_2O. PCR amplification of the VP2 gene was performed under the following conditions: initial denaturation at 95 ℃ for 5 min, denaturation at 95 ℃ for 45 s, annealing at 57, 52, 55, 56 ℃ respectively for 45 s, extension at 72 ℃ for 140 s, and a final elongation step at 72 ℃ for 10 min. 30 cycles were set to amplify the VP2 gene. The generated fragments were resolved on a 1% (W/V) agarose gel.

We choose the primers amplified accurate fragment amplified by primers to generate the VP2 gene using 2xPfu MasterMix (CWBIO, China). The PCR reaction was conducted as specified in the manufacturer's instructions. Then, the amplified fragment was purified using a Gel Extraction Kit (Omega, United states) and linked to a pEASYBlunt zero Cloning Kit (Transgen biotech, China). Trans1-T1 phage resistant chemically competent cells (Transgen biotech, China) were transformed with ligation product and incubated on the LB agar medium with ampicillin by agar streak culture. The next preparation of recombinant plasmid was manufactured following a standard progress of picking up the monoclonal colony, sequencing the gene, and extracting the recombinant plasmid pEB-VP2 from the bacterial suspension containing accurate gene sequence. The concentration of the recombinant plasmid pEB-VP2 was measured and then the copies were calculated.

2.5. Establishment and optimization of lateral flow strip RPA assay

The TwistAmp RPA nfo kit (TwistDx™ Limited, Cambridge, UK) was

Table 1
Details regarding the primers and probes for PCR and RPA.

Usages	Primers or probes	Direction	Nucleotide sequences (5'-3')
PCR	Vp2-F1	Forward	CCAATGAGTGATGGAGCAGTTCAACCAGAC
	Vp2-F2	Forward	CACCAATGAGTGATGGAGCAGTTC
	Vp2-R1	Reverse	CTTAACATATTCTAAGGGCAAACC
	Vp2-R2	Reverse	ACCTTATAGACAGTATACGAGACC
	Vp2-R3	Reverse	TAGGTGCTAGTTGATATGTAATAAACA
	Vp2-R4	Reverse	ATTCTAAGGGCAAACCAACC
RPA	RPA-F1	Forward	CACTTACTAAGAACAGGTGATGAATTTGCTACAG
	RPA-F2	Forward	GTGCCAGTACACTTACTAAGAACAGGTGATG
	RPA-F3	Forward	'TGTCAGAAATGAAAGAGCTACAGGATCTGGGAACG
	RPA-R1	Reverse	CCAAGCATTTGCATCAACCAATGACCAAGGTGTTA
	RPA-R2	Reverse	CAGATTGAGGCAAAGAATTTAGAAATGGTGGT
	RPA-R3	Reverse	AGTCTGTATTTCCCATTTGAG TTACACCACGTCT
	RPA-Pe-1	Forward	(FAM)GATTGTAAACCATGTAGACTAACACATACA (THF) GGCAAACAAA TAGAG (C3 Spacer)
PCR	Primer M1	Forward	GAAAACGGATGGGTGGAAAT
	Primer M2	Reverse	AGTTGCCAATCTCCTGGATT

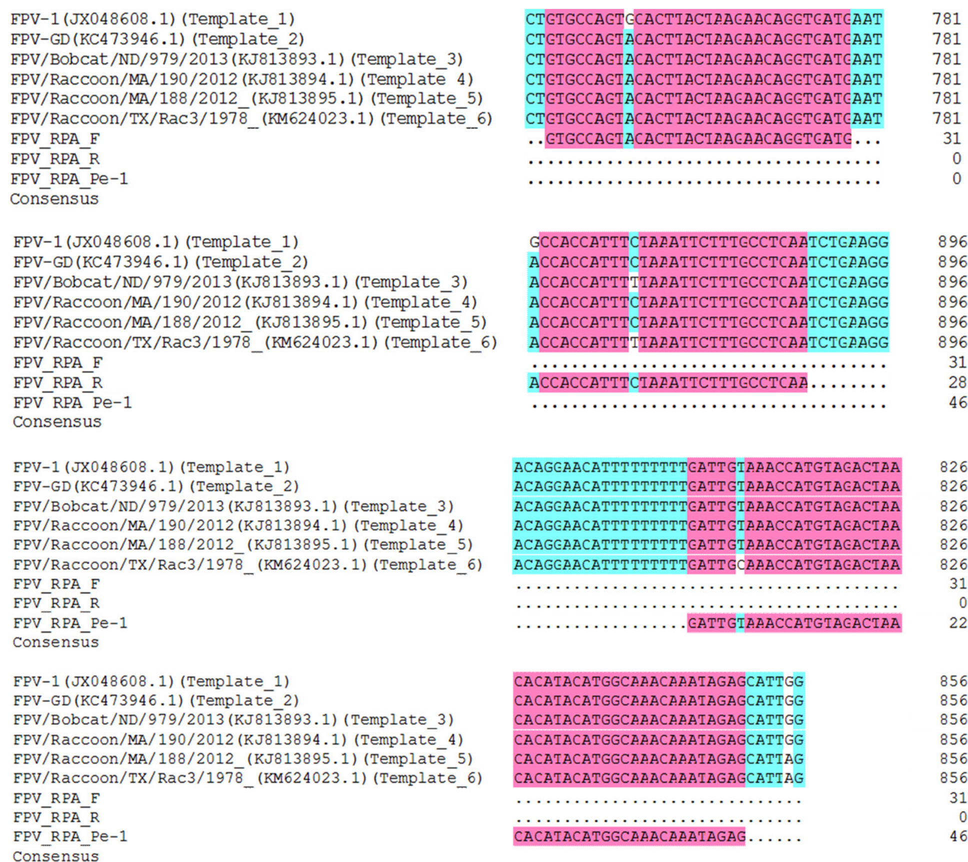

Fig. 3. Alignment of FPV from GenBank with FPV RPA-LFD primers and probe indicating the RPA target region. Lines 1 to 6 represent FPV VP2 genes from six different isolation strains. Lines 7, 8, and 9 represent, respectively, the forward primer, reverse primer and probe sequence position.

used to screen for the optimal primer pair. The primer groups were combined as follows: RPA-F1/ RPA-R2, RPA-F2/ RPA-R2, RPA- F1/ RPA-R3, RPA-F3/ RPA-R1, RPA-F2/ RPA-R3. Briefly, the RPA reaction composition contained 29.5 μl of rehydration buffer, 2.1 μl of 10 μM RPA forward or reverse primers, 1 μl of pEB-VP2, 0.6 μl of 10 μM probe, and 12.2 μl of ddH_2O. The above components were mixed together and added to PCR micro-tubes with a dried enzyme pellet, 2.5 μl 280 mM MgAc was added to PCR Tube caps, then vortex and spun simultaneously to ensure the reaction was initiated at the same time. The reaction was performed at 38 °C for various times (5, 10, 15, and 20 min), and then purified using the Gel Extraction Kit according to the manufacturer's instructions. The purified products were electrophoresed in a 1.0% (W/V) agarose gel and visualized under UV light.

For analysis of the amplified product, the lateral flow dipstick (USTR, China) was used. We mixed 1 μl of amplified product to buffer solution, then diluted at a ratio of 1 to 1000 in the 1.5 ml centrifuge tube. The strips were immersed in the dilute amplified product in the corresponding well. After incubating 2 to 5 min at room temperature, we detected the result by naked eye from the test line (T line) and the control line (C line) on the strips. The red line displayed at the T line indicates a positive result. The C line is always red; if only the T line was developed, the strip malfunctioned. To optimize the RPA-LF reaction conditions, the test was performed at a series of temperatures (25, 30, 32, 34, 36, 38, 40 °C), times (5, 10, 15, 20, 30 min) and plasmid concentrations (1×10^6 copies μl^{-1}, 1×10^5 copies μl^{-1}, 1×10^4 copies μl^{-1}, 1×10^3 copies μl^{-1}, 1×10^2 copies μl^{-1}, 1×10^1 copies μl^{-1}, 1×10^0 copies μl^{-1}). 1 μl RPA reaction product was visualized by lateral flow strip, and the surplus was purified by Gel Extraction Kit and detected using 1.0% (W/V) agarose gel electrophoresis.

2.6. Sensitivity and specificity analysis

The specificity and sensitivity analysis of FPV RPA-LFD was performed at the optimized reaction conditions described above. For the sensitivity analysis, we diluted the recombinant plasmid pEB-VP2 to obtain a series of DNA concentrations, ranging from 10^5 copies μl^{-1} to 10^0 copies μl^{-1}. 1 μl standard DNA of every gradient dilution was added to the amplification system, and reacted at 38 °C for 15 min. The amplified products were tested by lateral flow strip as described above. For the specificity analysis, DNA or cDNA of feline herpes virus (FHV), feline enteric coronavirus (FECV), feline syncytium forming virus (FeSFV), and feline immunodeficiency virus (FIV) were used as the templates for the RPA-LFD assay.

2.7. Detection of feline samples

We extracted viral DNA from clinical samples and detected by RPA-LFD. The results were compared with those obtained by PCR as previously described. The statistics are displayed at the Table 2.

3. Results

3.1. Screen of FPV RPA primers

In order to search for the most efficient primer pairs for RPA-LFD assay, we first designed five primer pairs to amplify a high level of sequence conservation at the FPV-VP2 standard plasmid by sequence alignment of strains on the GenBank. VP2 genes from the strains of FPV-1 (NCBI accession number JX048608.1), FPV-GD(12/09/YGP) (NCBI accession number KC473946.1), FPV/Bobcat/ND/979/2013 (NCBI accession number KJ813893.1), FPV/Raccoon/MA/190/2012 (NCBI

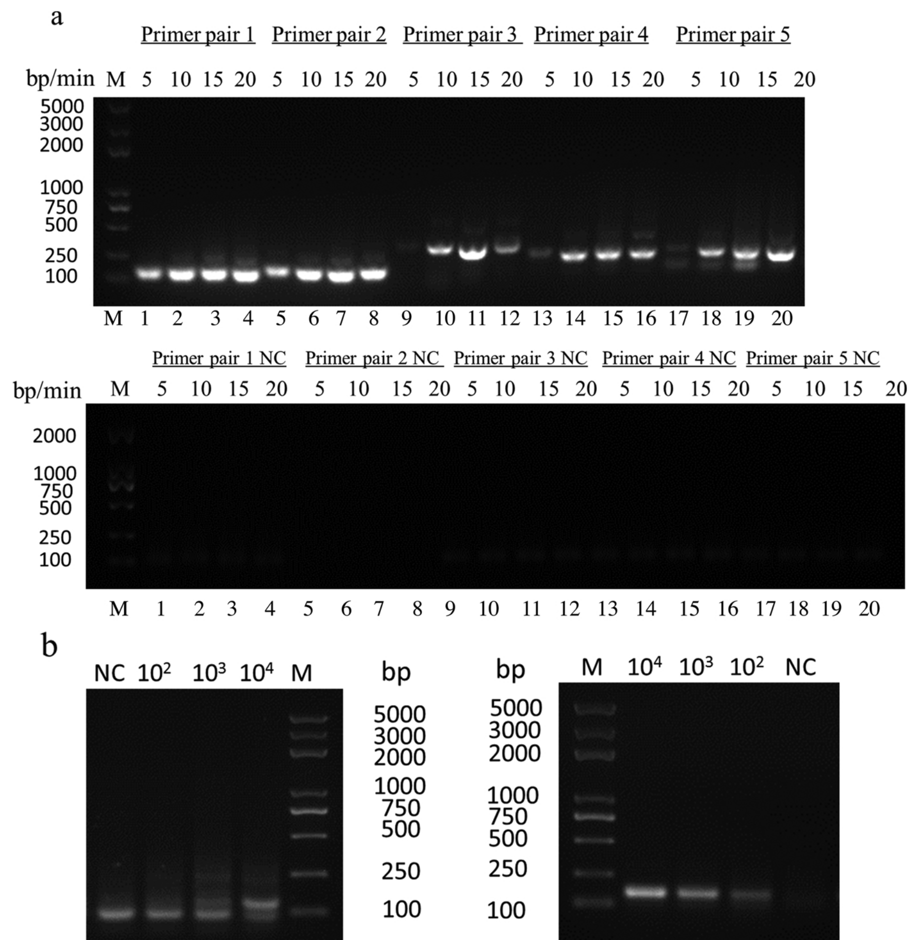

Fig. 4. Screening of FPV RPA primer pairs and probes. (a) primer pairs (numbers 1–5 corresponding to RPA- F1/ RPA- R2, RPA- F2/ RPA- R2, RPA- F3/ RPA- R1, RPA- F1/ RPA- R3, RPA- F2/ RPA- R3) were selected to amplify the pEB-VP2 standard plasmid, at concentration of 1×10^5 copies μl^{-1}. The assays were performed at 38 °C for 5, 10, 15, and 20 min in turn. The reaction products were detected by 1% (W/V) agarose gel electrophoresis (AGE). (b) Two probes designed for amplification assay. Left - assay used by Probe 1. Right - assay used by Probe 2. The assay was performed at 38 °C for 20 min with primer pair 2.

accession number KJ813894.1), FPV/Raccoon/MA/188/2012 (NCBI accession number KJ813895.1), and FPV/Raccoon/TX/Rac3/1978 (NCBI accession number KM624023.1) share a high level of sequence identity with the FPV RPA target region (Fig. 3).

Five primer pairs were used to amplify the VP2 gene at 38 °C for 5, 10, 15, and 20 min. The primer pair 1, 2 yielded clear product on the gel when amplified for only 5 min (Fig. 4a, Lanes 2–9), others were less efficiently amplified by comparison, and began to show evident DNA fragmentation at 10 min (Fig. 4a, Lanes 10–21). This result indicates that primer pair 1 and primer pair 2 are appropriate selections for amplification as DNA templates. We designed the probe for the amplified products of primer pairs 1 and 2 using the Primer Premier 5.0 software package. Considering that the probe design was no less than 45 bp and the probes for primer pair 1 revealed poor characteristics as analyzed by the Primer Premier 5.0 software, we chose primer pair 2 to optimize the probe results. Two probes were designed and added into the RPA amplification system. As shown in Fig. 4b, Probe 1 reduced the detection sensitivity to only 10^5 copies and showed more serious primer dimer compared with Probe 2. Thus we chose Probe 2 for the following study.

3.2. Establishment of RPA-LFD assay and optimization

To achieve rapid detection of products, we used a lateral flow dipstick (LFD) nucleic acid strip as the indicator. The forward primer of primer pair 2 was reconstituted carrying a biotin antigenic marker at the 5′ end, while the probe carried an FAM antigenic marker at the 5′ end. The amplification product can carry both biotin antigenic marker and FAM antigenic marker and is detectable by the LFD. We first explored the optimal amplification time of FPV RPA-LFD by controlling the temperature at 38 °C; the standard plasmid usage was 1×10^5 copies. As shown in Fig. 5, a faint band started to show at 10 min (Fig. 5a), which was later than agarose gel detection without probe addition Fig. 5b). We can ignore the influence of probe addition, considering that the LFD system greatly reduces detection time and process duration. The LFD indicator displayed a good visible result over a reaction time of 10–30 min (Fig. 5a).

3.3. Temperature optimization of the established RPA-LFD assay

The optimal reaction temperature was obtained by evaluating a temperature range from 25 °C to 45 °C for a 15 min reaction (Fig. 6. Both agarose gel (Fig. 6a) and LFD (Fig. 6b) began to show a clear faint band at 34 °C (lane 3). Adding probe to the RPA system produced no obvious change of amplification efficiency and even slightly reduced the sensitivity at temperatures up to 42 °C or higher (Fig. 6a, lanes 7, 8). The optimal temperature for amplification was found to be in the range of 36 °C to 40 °C (lanes 4–6).

3.4. FPV RPA-LFD assay detects 10^2 copies of target DNA

To investigate the sensitivity of the FPV RPA-LFD system, the pEB-VP2 was 10-fold serially diluted to achieve DNA concentrations ranging from 10^5 to 10^0 copies μl^{-1}. This detection method allowed for a more

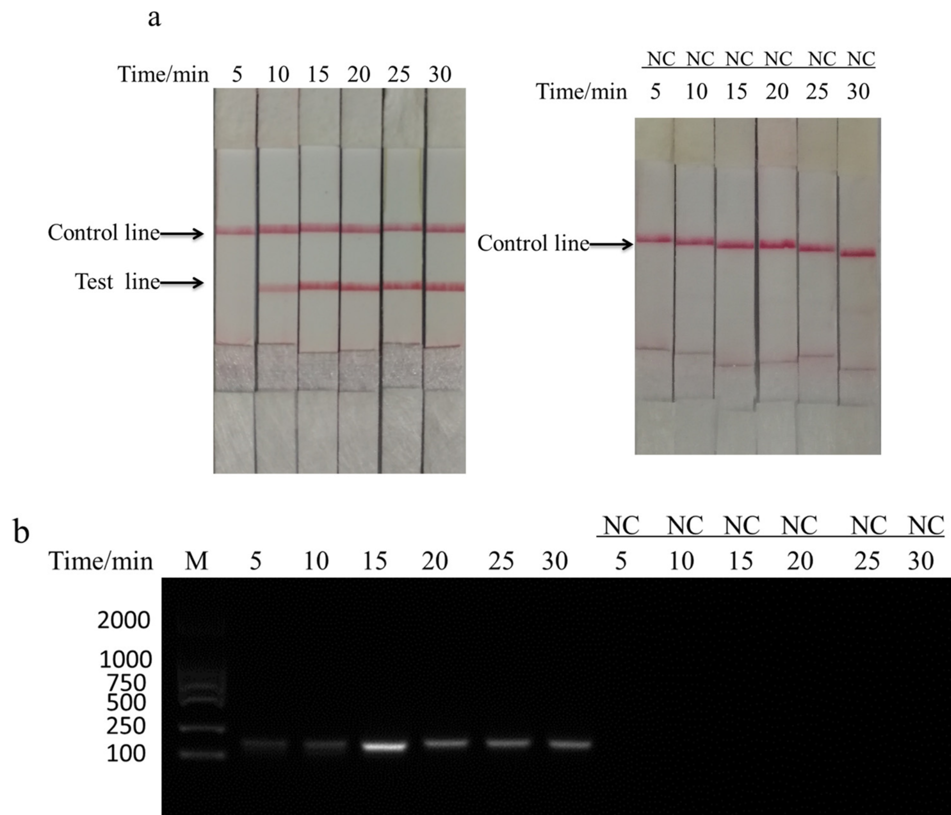

Fig. 5. Optimization of RPA-LFD reaction time. The RPA assay was performed at 38 ℃ for 5, 10, 15, 20, 25, and 30 min. The concentration of pEB-VP2 was 1×10^5 copies μl^{-1}. The reaction products were detected by (a) lateral flow dipstick and (b) 1% (W/V) agarose gel electrophoresis (AGE).

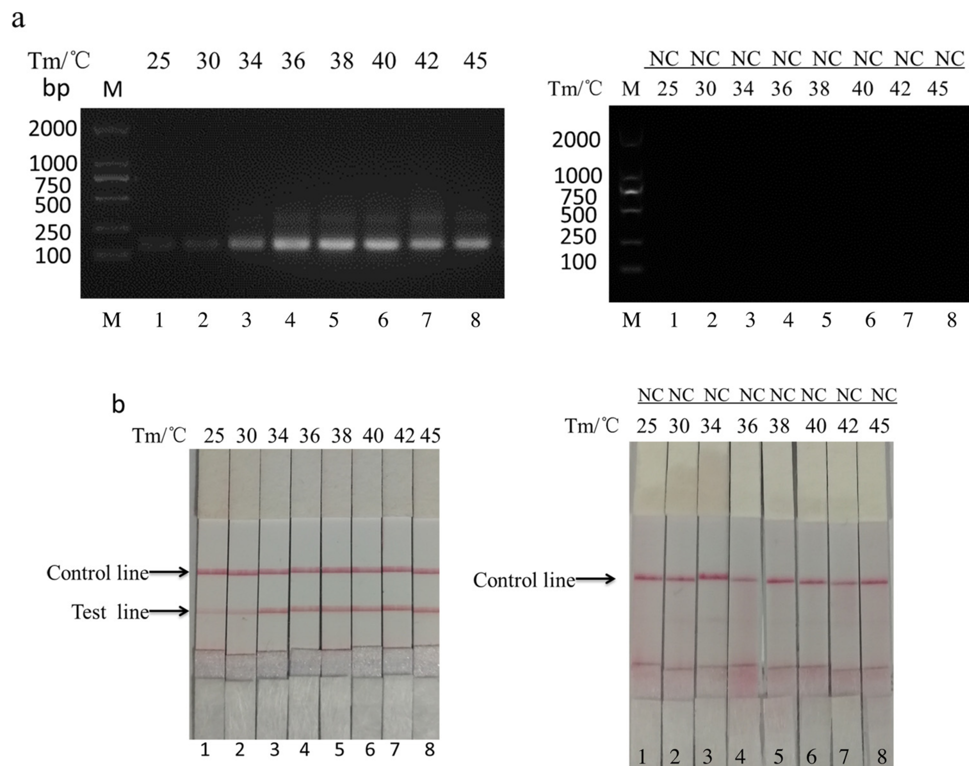

Fig. 6. Temperature optimization of established RPA-LFD method. Amplification performance of RPA-LFD assays conducted at 25, 30, 34, 36, 38, 40, 42, and 45 ℃ (lanes 1–8) for 15 min. The amplification products were detected by (a) 1% (W/V) AGE and (b) lateral flow strip.

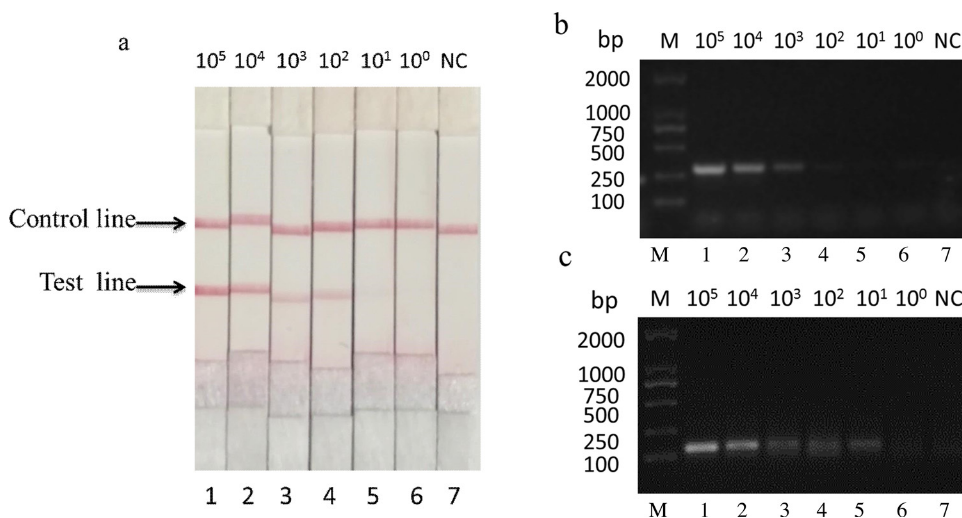

Fig. 7. Comparison of the sensitivity of RPA-LFD and PCR methods. (a) RPA assay, 1 μl RPA product was separated into buffer solution at 1:1000 to be detected by LFD. (b) PCR assay, the PCR reaction products were detected by 1% (W/V) AGE. (c) 40 μl RPA product was purified by gel kit and then 1 μl RPA product was detected by 1% (W/V) AGE. Samples show in Lanes 1–6 are with pEB-VP2 copies from 10^5 to 10^0 copies μl^{-1}. The Lane 7 showed negative control (NC).

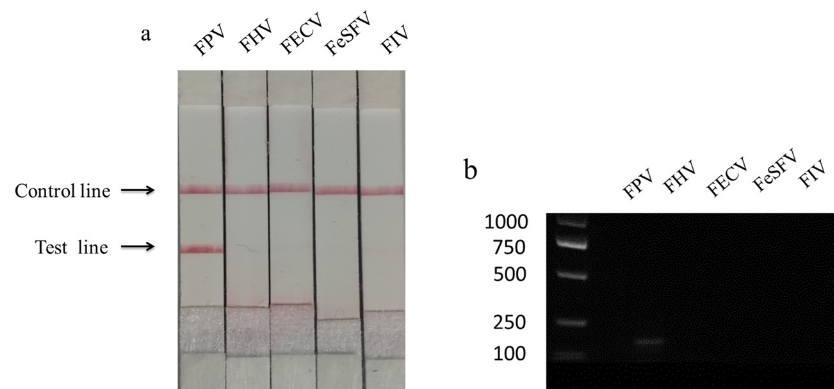

Fig. 8. Specificity of RPA assay. Product detection using lateral flow dipsticks and agarose gel electrophoresis. RPA assay was used to detect FPV, FHV, FECV, FeSFV, and FIV cDNA. The reaction products were detected by (a) lateral flow dipstick and (b) 1% (W/V) agarose gel electrophoresis (AGE).

accurate quantification of copy number, and greater precision in determining the limit of detection of the FPV RPA-LFD assay than what could have been obtained using extracted viral nucleic acid. The FPV RPA-LFD detected 10^2 copies of target DNA (Fig. 7a), and this is 10 times more sensitive than the traditional PCR, which was sensitive only to 10^3 copies μl^{-1} (Fig. 7b). The agarose gel electrophoresis can even detect 10^1 copies of target DNA (Fig. 7c). All of the above FPV RPA-LFD system tests were performed at 38 °C for 15 min, and this indicates that instant field detection may be achieved without any other expensive or sophisticated equipment.

3.5. FPV RPA-LFD assay specifically detects FPV

We extracted nucleic acid from viruses related to feline diseases including feline herpes virus (FHV), feline enteric coronavirus (FECV), feline syncytium forming virus (FeSFV), and feline immunodeficiency virus (FIV). The nucleic acid of RNA viruses was reversed transcribed to cDNA for RPA-LFD detection. None of the above viruses DNA were detected, and only FPV DNA yielded a positive signal at the test line (Fig. 8a, 8b). This FPV RPA-LFD system was specific to FPV and did not detect other pathogens that cause common infectious disease in cats.

3.6. Extracted nucleic acid from clinical samples can be detected by the FPV RPA-LFD assay

Anal swabs from 30 suspected infectious cases and 13 suspected healthy cases gathered from an animal hospital were tested by polymerase chain reaction (PCR) and the RPA-LFD method. The suspected healthy cat anal swab samples were all negative by both methods; for samples of suspected cases, PCR detected 7 positive samples and 23 negative samples. RPA-LFD measured 8 positive and 22 negative samples, where one positive sample detected by RPA-LFD was negative when measured by RCR (Table 2).

4. Discussion

FPV has been reported to infect various domestic pets and wild animals, but instant diagnosis is very difficult in underdeveloped regions. Protecting vulnerable and high-risk individuals requires the development of rapid pathogenic diagnosis. In addition, a wide range of field detection would enable infection control.

As next generation isothermal nucleic acid amplification technology, the RPA-LFD detection system demonstrates great advantages over PCR. The RPA requires the length of the forward and reverse primer to be no less than 30 bp. We prepared five primer pairs for screening for optimal primer. According to the gene sequence of amplified product with the optimal primer pair, we designed two probes for the next step. Primer pair 2 and Probe 2 displayed advantages and were the final choice (Fig. 4). The forward primer and the probe combined with Biotin and FAM respectively at the 5′ successfully combines the amplification product with the corresponding antibody placed at LFD. A faint band is observed if we detect a positive sample (Figs. 1,2). The addition of the probe not only facilitates the detection of products by LFD, but also enhances the specificity of FPV detection. RPA-LFD can detect 10^2 copies of the plasmid template (Fig. 7). A wide range of detection conditions, including reaction time from 15 to

30 min and temperature from 36 °C to 40 °C, produced no significant difference in amplification (Figs. 5,6). We did not detect amplification products with the genome of FHV, FECV, FeSFV, and FIV (Fig. 8).

In addition to considering rapid and convenient detection and lower cost than other methods, sensitivity is another important factor for the evaluation of a constructed method. We compared the sensitivity results using the RPA-LFD method and PCR assay. The RPA-LFD method was able to detect 10^2 copies of the plasmid template. The sensitivity of RPA assay was one order of magnitude higher than that of traditional PCR, which was observed to be only 10^3 copies of the plasmid template (Fig. 7). In addition, the RPA-LFD showed a positive rate that was higher than that of the PCR detection (Table 2).

The RPA reaction mixtures are provided in vacuum-sealed pouches, which can be kept at room temperature for several days. As an indicator system, the LFD strips provided results within 5 min and are completely portable, while agarose gel electrophoresis requires the relatively non-portable electrophoresis apparatus.

Nonetheless, there are some limitations for the use of LFD-RPA detection method. The first, and perhaps most important, step is to design efficient primers. We designed five primer pairs for a wide range of screening (Fig. 4), and then two probes of primer pair 2 were selected for optimizing. The biggest challenge is to avoid duplexes between primer pairs and probe that might give false positive results and reduce amplification efficiency, further reducing the sensitivity of the primers. Non-specific amplification phenomena also occurred in some cases but could be ameliorated or even removed by primer selection. There present method has not shown serious contamination issues, as seen in LAMP. The first probe design showed a terrible amplification result, but non-specific amplification and false positives were eliminated by optimizing the probe sequence, such as by mutation of one to three bases. All of the detection processes have avoided the dependence on laboratory conditions, but preparation of suspicious examples and virus genome extraction still require laboratory apparatus. Numerous laboratories have developed their own extraction methods, but all depend on centrifugation, as do commercially available kits for nucleic acid extraction (Bag and Saha, 2016; Ling and Zhu, 2019; Zhang and Li, 2019). Nonetheless, RPA-LFD has greatly reduced the reliance on precision instruments like PCR instruments and electrophoresis systems, bringing field detection closer to reality.

In a word, compared with PCR, RPA-LFD can reduce costs of detection and achieve higher sensitivity. Another end-point detection format, real-time recombinase polymerase amplification (RT-RPA) assay, employs a device to collect a fluorescence signal. Several RPA detection methods have been developed so far; in addition to animal virus, plant virus, fungi, bacteria, mycoplasma, and parasite RPA detection methods have been developed in recent years (Cui and Zhao, 2018; Karakkat and Hockemeyer, 2018; Zeng and Luo, 2019; Zhao and Hou, 2018; Hu and Zhong, 2019). Although the RPA technology has not been promoted on the market, some reports have verified its capabilities and its potential to eclipse PCR for further revolutions the life sciences (Li and Macdonald, 2019). With the development of RPA-LFD, it is possible to achieve field detection.

5. Summary

An FPV RPA-LFD method based on nfo probe has been successfully developed for the detection of FPV. The assay can be completed within 15 min at 38 °C, with high sensitivity and specificity. The portability of the RPA-LFD system means that it can be used at quarantine stations, ports, or sites of outbreaks. Greatly reduced need for professional staff and sophisticated instruments makes field detection more convenient and feasible than existing diagnostic methods. The effective and rapid RPA assay developed in this study would be highly useful in the control of FPV, especially in resource-limited settings.

Acknowledgments

This work is supported by The National Key R & D Program of China (2017YFD0502300, 2017YFD0502200), and by the National Natural Science Foundation of China (31772739, 31572515).

References

Agbandje, M., McKenna, R., et al., 1993. Structure determination of feline panleukopenia virus empty particles. Proteins 16 (2), 155–171.

Allison, A.B., Harbison, C.E., et al., 2012. Role of multiple hosts in the cross-species transmission and emergence of a pandemic parvovirus. J. Virol. 86 (2), 865–872.

Bag, S., Saha, B., et al., 2016. An Improved Method for High Quality Metagenomics DNA Extraction from Human and Environmental Samples. Sci. Rep. 6, 26775.

Cohen, D., Yohn, D.S., et al., 1961. The relationship of a feline virus isolated by Bolin to feline panleukopenia, kidney cell degenerating virus, and 2 feline respiratory viruses. Am. J. Vet. Res. 22, 637–643.

Cui, J., Zhao, Y., et al., 2018. Detection of Babesia gibsoni in dogs by combining recombinase polymerase amplification (RPA) with lateral flow (LF) dipstick. Parasitol. Res. 117 (12), 3945–3951.

Daher, R.K., Stewart, G., et al., 2016. Recombinase Polymerase Amplification for Diagnostic Applications. Clin. Chem. 62 (7), 947–958.

Decaro, N., Desario, C., et al., 2008. Specific identification of feline panleukopenia virus and its rapid differentiation from canine parvoviruses using minor groove binder probes. J. Virol. Methods 147 (1), 67–71.

Demeter, Z., Gal, J., et al., 2009. Feline parvovirus infection in an Asian palm civet (Paradoxurus hermaphroditus). Vet. Rec. 164 (7), 213–216.

Digangi, B.A., Gray, L.K., et al., 2011. Detection of protective antibody titers against feline panleukopenia virus, feline herpesvirus-1, and feline calicivirus in shelter cats using a point-of-care ELISA. J. Feline Med. Surg. 13 (12), 912–918.

Esfandiari, J., Klingeborn, B., 2000. A comparative study of a new rapid and one-step test for the detection of parvovirus in faeces from dogs, cats and mink. J. Vet. Med. B Infect. Dis. Vet. Public Health 47 (2), 145–153.

Fei-Fei, D., Yong-Feng, Z., et al., 2017. Molecular characterization of feline panleukopenia virus isolated from mink and its pathogenesis in mink. Vet. Microbiol. 205, 92–98.

Hu, S., Zhong, H., et al., 2019. Rapid and visual detection of Group B streptococcus using recombinase polymerase amplification combined with lateral flow strips. Diagn. Microbiol. Infect. Dis. 93 (1), 9–13.

Ikeda, Y., Miyazawa, T., et al., 1998. New quantitative methods for detection of feline parvovirus (FPV) and virus neutralizing antibody against FPV using a feline T lymphoid cell line. J. Vet. Med. Sci. 60 (8), 973–974.

Karakkat, B.B., Hockemeyer, K., et al., 2018. Detection of root-infecting fungi on cool-season turfgrasses using loop-mediated isothermal amplification and recombinase polymerase amplification. J. Microbiol. Methods 151, 90–98.

Lane, E.P., Brettschneider, H., et al., 2016. Feline panleukopaenia virus in captive non-domestic felids in South Africa. Onderstepoort J. Vet. Res. 83 (1), a1099.

Li, J., Macdonald, J., et al., 2019. Review: a comprehensive summary of a decade development of the recombinase polymerase amplification. Analyst 144 (1), 31–67.

Ling, Y., Zhu, Y., et al., 2019. Rapid method for detection of Staphylococcus aureus in feces. J. Biomed. Nanotechnol. 15 (6), 1290–1298.

Nan, H., Yoo, D.J., et al., 2013. Fast parallel detection of feline panleukopenia virus DNA by multi-channel microchip electrophoresis with programmed step electric field strength. J. Sep. Sci. 36 (2), 350–355.

Parrish, C.R., 1999. Host range relationships and the evolution of canine parvovirus. Vet. Microbiol. 69 (1-2), 29–40.

Schunck, B., Kraft, W., et al., 1995a. A simple touch-down polymerase chain reaction for the detection of canine parvovirus and feline panleukopenia virus in feces. J. Virol. Methods 55 (3), 427–433.

Schunck, B., Kraft, W., et al., 1995b. A simple touch-down polymerase chain reaction for the detection of canine parvovirus and feline panleukopenia virus in feces. J. Virol. Methods 55 (3), 427–433.

Shin, A., Lee, M., et al., 2012. On-line capillary electrophoresis for enhanced detection sensitivity of feline panleukopenia virus. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 909, 22–25.

Siegl, G., Bates, R.C., et al., 1985. Characteristics and taxonomy of parvoviridae. Intervirology 23 (2), 61–73.

Steinel, A., Parrish, C.R., et al., 2001. Parvovirus infections in wild carnivores. J. Wildl. Dis. 37 (3), 594–607.

Stuetzer, B., Hartmann, K., 2014. Feline parvovirus infection and associated diseases. Vet. J. 201 (2), 150–155.

Truyen, U., Parrish, C.R., 1992. Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: distinct host cell tropisms of each virus in vitro and in vivo. J. Virol. 66 (9), 5399–5408.

Truyen, U., Parrish, C.R., 2013. Feline panleukopenia virus: its interesting evolution and current problems in immunoprophylaxis against a serious pathogen. Vet. Microbiol. 165 (1-2), 29–32.

Veijalainen, P.M., Neuvonen, E., et al., 1986. Latex agglutination test for detecting feline panleukopenia virus, canine parvovirus, and parvoviruses of fur animals. J. Clin. Microbiol. 23 (3), 556–559.

Zeng, R., Luo, J., et al., 2019. Rapid detection of Cucumber green mottle mosaic virus by reverse transcription recombinase polymerase amplification. Mol. Cell. Probes 43, 84–85.

Zhang, M., Li, L., et al., 2019. Adsorption of DNA by using polydopamine modified magnetic nanoparticles based on solid-phase extraction. Anal. Biochem. 19, 30061–30062.

Zhao, G., Hou, P., et al., 2018. Development of a recombinase polymerase amplification combined with a lateral flow dipstick assay for rapid detection of the Mycoplasma bovis. BMC Vet. Res. 14 (1), 412.