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Equipment-free recombinase polymerase amplification assay using body heat for visual and rapid point-of-need detection of canine parvovirus 2

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ABSTRACT

A visible and equipment-free recombinase polymerase amplification assay combined with a lateral flow strip (LFS RPA) was developed to detect canine parvovirus type 2 (CPV-2), which is the etiological agent of canine parvovirus disease. The CPV-2 LFS RPA assay was developed based on the VP2 gene and is performed in a closed fist using body heat for 15 min; the products are visible to the naked eye on the LFS within 5 min. The assay could detect CPV-2a, CPV-2b and CPV-2c, and there was no cross-reaction with the other viruses tested. Using the standard CPV-2 DNA as a template, the analytical sensitivity was 1.0×10^2 copies per reaction, which was the same result as that of a real-time PCR. The assay performance was further evaluated by testing 60 canine fecal samples, and CPV-2 DNA was detected in 46 samples (76.7%, 46/60) by LFS RPA, which was the same result as that of the real-time PCR assay and higher than that of the SNAP method (48.3%, 29/60). The novel CPV-2 LFS RPA assay is an attractive and promising tool for rapid and convenient diagnosis of CPV disease, especially cage side and in underequipped laboratories.

1. Introduction

Canine parvovirus 2 (CPV-2), a highly contagious pathogen of dogs, is a linear single-stranded DNA virus belonging to the genus *Protoparvovirus*, family *Parvoviridae*. After being infected with CPV-2, the dog mainly demonstrates clinical signs of gastroenteritis, including anorexia, lethargy, vomiting, fever and diarrhea [1,2]. Three different antigenic variants have been reported, namely, CPV-2a, CPV-2b and CPV-2c. These strains have replaced the original CPV-2 and are variously distributed worldwide [3–10]. The virus is spread through the fecal-oral route and is extremely stable in the environment [1]. Therefore, rapid and reliable diagnosis of CPV-2 infection would be of great importance to improve disease management and prevent further outbreaks, particularly within a shelter environment.

A series of gene amplification-based assays have been developed for CPV-2, such as polymerase chain reaction (PCR), nested PCR, real-time PCR, loop-mediated isothermal amplification (LAMP) and insulated isothermal PCR (iiPCR), which have played an important role in the control of CPV-2 infection [11–16]. However, due to the requirements

of an expensive thermocycler, a centralized laboratory facility and experienced technicians, implementation of the PCR assays is limited at the point-of-need (PON) diagnosis. Compared to the PCR assays, the use of isothermal technologies reduces the need for high precision instrumentation and consistent electrical power. Although the previously developed LAMP assays do not require specialized equipment, they are difficult to design as 4–6 primers are required [11,14]. A report of the iiPCR method was described its sensitive detection of CPV-2, but the assay was dependent on a specialized instrument, POCKIT™ Nucleic Acid Analyzer [16]. Furthermore, the results of the isothermal method described above are usually produced within approximately 60 min. A simple and convenient method is still needed for rapid and reliable detection of CPV-2 cage side and in laboratories without access to real-time PCR instrumentation.

As a simple, rapid and reliable isothermal DNA amplification technique, recombinase polymerase amplification (RPA) has been applied widely for the detection of different pathogens [17,18]. The RPA reaction uses enzymes called recombinases that form complexes with oligonucleotide primers and pair the primers with homologous

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Table 1
Sequences of the primers and probes for CPV-2 PCR, real-time PCR and LFS RPA assays.

Assay	Primers and probe	Sequence 5'-3'	Amplicon size (bp)	References
PCR	VP2-F	CAGGAAGATATCCAGAAGGA	583	[23]
	VP2-R	GGTGCTAGTTGATATGTAATAAACA		
real-time PCR	CPV-F	AAACAGGAATTAATACTATACTAATATATTTA	93	[11]
	CPV-R	AAATTTGACCATTGGATAAACT		
	CPV-P	FAM-TGGTCCTTAACTGCATTAATAATGTACC-BHQ1		
LFS RPA	CPV-nfo-F	CACCTACTAAGAACAGGTGATGAAITTTGCTACAG	214	This study
	CPV-nfo-R	Biotin-AGTTTGATATTTCCCATTTGAGITACACCACGTCT		
	CPV-nfo-P	FAM-CCTCAAGCTGAAGGAGGTACTAACTTTGGT		
		T(THF) TATAGGAGTTCAACAAG -C3-spacer		

sequences in DNA. A single-stranded DNA-binding protein binds to the displaced DNA strand and stabilizes the resulting loop. The primer then initiates DNA amplification by a strand-displacing DNA polymerase [18]. Our laboratory had developed a real-time RPA assay based on an exo probe for the rapid detection of CPV-2, although the assay still depended on a specialized instrument, Genie III (OptiGene, West Sussex, UK) [19]. Direct visual detection of the RPA products depends on Endonuclease IV, the nfo probe and the opposing amplification primer labeled at the 5' end with biotin. The nfo probe oligonucleotide backbone includes a 5'-antigenic label (typically a FAM group), an internal abasic nucleotide analog (a tetrahydrofuran residue, or THF) and a 3'-polymerase extension blocking group (such as a C3-spacer). The nfo probe is typically 46–52 nucleotides long, at least 30 of which are placed 5' to the THF site, and at least a further 15 nucleotides are located 3' to the site. The THF residue replaces a nucleotide that would normally base-pair to the complementary sequence. The amplicons are then detected by the naked eye in a 'sandwich' assay format, such as a lateral flow strip (LFS), which uses *anti*-FAM gold conjugates and biotin-ligand molecules. A series of LFS RPA assays had been developed for the detection of porcine parvovirus (PPV), peste des petits ruminants virus (PPRV) and bovine ephemeral fever virus (BEFV) [20–22].

In this study, we developed a visual and equipment-free RPA assay for rapid, specific and sensitive detection of CPV-2, which was combined with LFS (USTAR, Hangzhou, China) and performed by incubating the reaction tubes in a closed fist using body heat.

2. Materials and methods

2.1. Virus strains and clinical samples

Canine parvovirus type 2a (CPV-2a, strain CPV-b114), canine parvovirus type 2b (CPV-2b, strain SJZ101), canine parvovirus type 2c (CPV-2c, strain HB2018/F46), canine distemper virus (CDV, strain CDV-FOX-TA), canine coronavirus (CCoV, strain ATCC VR-809), canine parainfluenza virus (CPIV, strain CPIV/A-20/8), and pseudorabies virus (PRV, strain Barth-K61) were maintained in our laboratory. Sixty fecal samples were obtained from the veterinary hospitals of the Agricultural University of Hebei and pet clinics in Shijiazhuang from May 2016 to March 2018. All the dogs were suspected of being infected with CPV-2 with diarrhea.

2.2. DNA/RNA extraction and RNA reverse transcription

The viral DNA/RNA extraction, the RNA reverse transcription, and the DNA extraction from the clinical fecal samples were all performed as described previously [19]. Sample DNA was also extracted by boiling 10 fecal samples. Briefly, the fecal samples were homogenized (10% w/v) in phosphate-buffered saline (PBS) and subsequently clarified by centrifugation at $3000 \times g$ for 10 min. Viral DNA was extracted from the supernatants of fecal homogenates by boiling for 10 min and chilling on ice. All the DNA and cDNA samples were stored at -80°C until use.

2.3. Generation of CPV-2 standard DNA

The CPV-2 standard DNA, which covers the VP2 gene of CPV-b114, was generated as described previously [19] and diluted in ten-fold serial dilutions to obtain DNA concentrations ranging from 1.0×10^6 to 1.0×10^0 copies/ μL .

2.4. RPA primers and nfo probe

Nucleotide sequence data for CPV-2 strains available in GenBank were aligned to identify the conserved regions in the VP2 gene. According to the reference sequences of different CPV-2 types (CPV-2a, accession numbers: M24003, AB054215, KF803642; CPV-2b, accession numbers: M38245, AY869724, KF803611; CPV-2c, accession numbers: FJ005196, KM236569), the primers and nfo probe were designed based on the VP2 gene of the virus. The primers and nfo probe are listed in Table 1 and were synthesized by Sangon Biotech Co. Shanghai, China.

2.5. CPV-2 LFS RPA assay

CPV-2 LFS RPA reactions were performed in a 50 μL volume containing 29.5 μL of rehydration buffer and 2.5 μL of magnesium acetate (280 mM) from the TwistAmp™ nfo kit (TwistDX, Cambridge, UK). Other components included 420 nM each RPA primers (CPV-nfo-F and CPV-nfo-R), 120 nM nfo probe (CPV-nfo-P), and 1 μL of viral DNA or 5 μL of sample DNA. All reagents except for the viral template and magnesium acetate were prepared in a master mix, which was distributed into each 0.2 mL freeze-dried reaction tube containing a dried enzyme pellet. One microliter of viral DNA or 5 μL of sample DNA was added to the reaction tubes, and magnesium acetate was pipetted into the tubes subsequently. The tubes were vortexed briefly, spun down once again and immediately incubated in a different technician's closed fist at room temperature. The RPA was performed using body heat for 5, 10, 15 and 20 min, and an LFS (USTAR, Hangzhou, China) was used to detect the amplicons that were dual-labeled with FAM and biotin. For each RPA reaction, 10 μL of product was added to the sample pad of the strip, and the strip was then placed in a well of a 96-well plate containing 100 μL of running buffer and incubated in an upright position. The final result was read visually after incubation for 5 min at room temperature. A testing sample was considered positive when both the test line and the control line were visible and considered negative when only the control line was visible. The assay was considered invalid when the control line was not visible.

2.6. Analytical specificity and sensitivity analysis

Ten nanograms of viral DNA or cDNA were used as template in the analytical specificity analysis of the CPV-2 LFS RPA assay. The assay was evaluated against a panel of viruses considered to be dangerous to dogs: CPV-2a, CPV-2b, CPV-2c, CDV, CCoV, CPIV and PRV. Three independent reactions were performed by three different technicians.

The tenfold serial dilutions of standard CPV-2 DNA were used as the

standard DNA for the CPV-2 LFS RPA assay. One microliter of each dilution was amplified by the LFS RPA to determine the limit of detection (LOD) of the assay. Three independent reactions were performed by three different technicians.

2.7. Real-time PCR

A real-time PCR assay specific for CPV-2 was performed on an ABI 7500 instrument as described previously with some modifications [12]. Premix Ex Taq™ (Takara, Dalian, China) was used, and the reaction was performed as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 32 s.

2.8. Validation with clinical samples

Sample DNA extracted from 60 fecal samples with a commercial kit were tested by the CPV-2 LFS RPA, and the results were compared with those obtained by a real-time PCR assay described previously [12]. For the CPV-2-positive samples, PCR amplification was carried out using VP2-F and VP2-R [23]. The PCR products were sequenced by a commercial company (Sangon Biotech Co. Shanghai, China). The sequences were compared with the VP2 sequences of the reference strains of CPV obtained from GenBank. Comparisons of nucleotide and deduced amino acid sequences were made using DNASTAR software, and the CPV-2 antigenic types were identified.

Sample DNA extracted by boiling was also tested by the CPV-2 LFS RPA, and the results were compared with those obtained using the DNA extracted with the commercial kit.

All the fecal samples were also tested by the SNAP method with a commercial kit (IDEXX Laboratories, Westbrook, USA) following the manufacturer's instructions.

3. Results

3.1. Optimization of the reaction time

To determine the optimal reaction time for the CPV-2 LFS RPA reaction, the reaction tubes were incubated in three different technicians' closed fists for 5, 10, 15 and 20 min using 1.0×10^4 copies of the standard DNA as template. As shown in Fig. 1, no amplified products were observed in the reactions incubated for 5 min, and weakly amplified products were observed after a 10-min incubation. When the incubation time was increased to 15 min or more, the assay performance was improved, and there were no clear differences between the products observed after 15 and 20 min incubations. Similar results were observed in three independent reactions. Therefore, the optimal incubation time for CPV-2 LFS RPA assay was set at 15 min.

3.2. Analytical specificity and sensitivity

Using 10 ng of viral DNA and cDNA as template, the results showed that only CPV-2a, CPV-2b and CPV-2c were detected by the LFS RPA, while the other viruses were not detected (Fig. 2). No cross-detections were observed. Three independent reactions were repeated, and similar results were observed, demonstrating the high specificity and repeatability of the assay.

The LFS RPA was performed using a dilution range from 1.0×10^6 to 1.0×10^0 copies/ μ L of the standard CPV-2 DNA as template. As shown in Fig. 3, the LOD of the LFS RPA was 1.0×10^2 copies per reaction, which was the same as real-time PCR. The CPV-2 LFS RPA assay was performed three times by three different technicians, and results similar to those described above were obtained.

3.3. Evaluation of LFS RPA with clinical samples

For the 60 canine fecal samples, 46 samples were CPV-2 DNA-

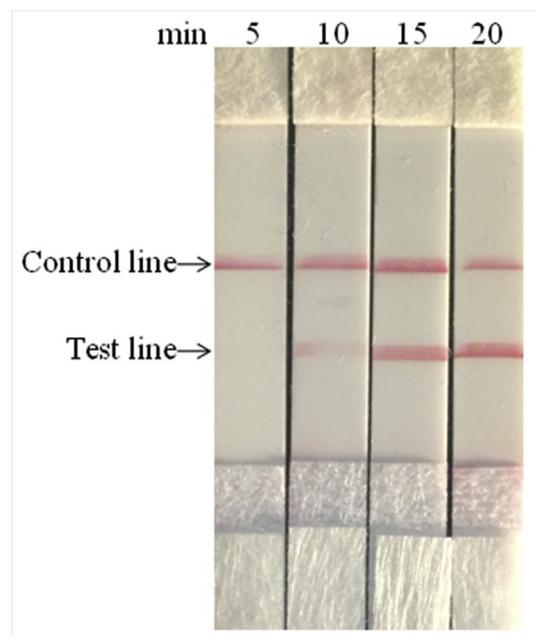


Fig. 1. Determination of the CPV-2 LFS RPA reaction time. The CPV LFS RPA assay was performed by incubating the reaction tubes in a closed fist for different times as shown. The test line was visible when the amplification time is longer than 10 min, and there was no significant difference in visibility between the 15 min and 20 min incubation time.

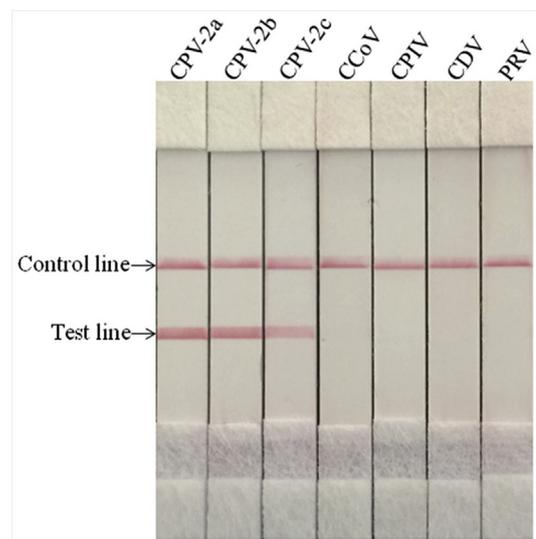


Fig. 2. Analytical specificity of the CPV-2 LFS RPA assay. Reactions tubes were incubated in a closed fist for 15 min using 10 ng of viral DNA or cDNA as template. The results showed LFS RPA only amplified CPV-2a, CPV-2b and CPV-2c DNA but not the other viruses tested. CPV-2a: canine parvovirus type 2a; CPV-2b: canine parvovirus type 2b; CPV-2c: canine parvovirus type 2c; CDV: canine distemper virus; CCoV: canine coronavirus; CPiV: canine parainfluenza virus; PRV: pseudorabies virus.

positive, and 14 samples were negative by the LFS RPA assay, which were the same results as those obtained by real-time PCR. A sample of the CPV-2 LFS RPA results is shown in Fig. 4. Further analysis demonstrated that the two assays had a diagnostic agreement of 100% (Table 2). The CPV-2 LFS RPA positive samples had real-time RT-PCR Ct values ranging from 13.56 to 37.09, indicating the wide applicability of the developed assay. It took less than 20 min for the LFS RPA assay to obtain all the positive results, while real-time PCR took much longer (approximately 60 min). The CPV-2 antigenic types were identified

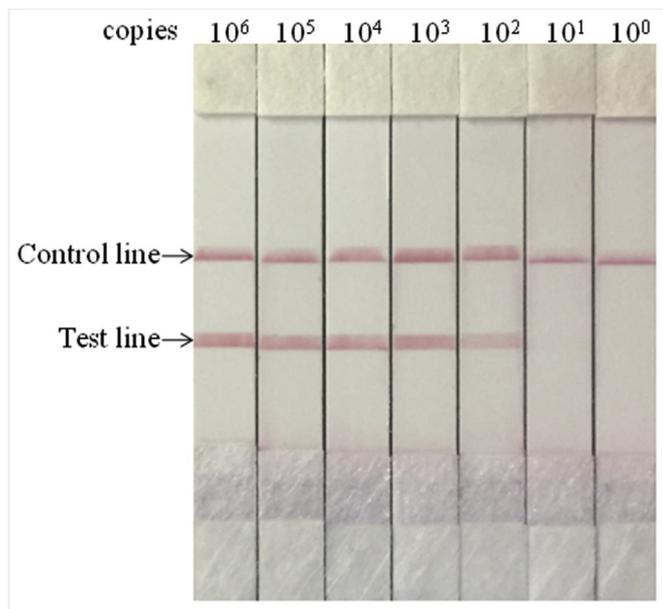


Fig. 3. Analytical sensitivity of the CPV-2 LFS RPA assay. The sensitivity of the CPV LFS RPA assay was performed using a dilution range of 1.0×10^6 – 1.0×10^0 copies of the standard CPV-2a DNA. The LOD of the assay was 1.0×10^2 copies per reaction.

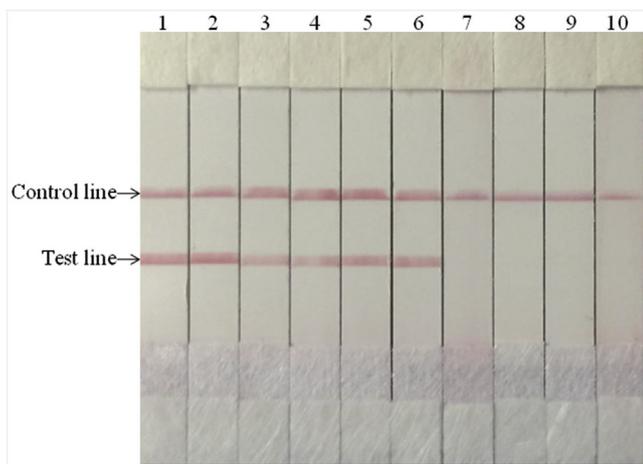


Fig. 4. Detection of CPV-2 in the clinical samples by LFS RPA. Ten samples were selected randomly from the clinical samples. Lines 1–4 are samples that were CPV-2 DNA-positive by the LFS RPA, real-time PCR and SNAP assays; lines 5–6 are samples that were CPV-2 DNA-positive by the LFS RPA and real-time PCR assays but negative by the SNAP assay; and lines 7–10 are samples that were CPV-2 DNA-negative by all three assays.

successfully for 37 positive samples, and the predominant type was CPV-2a (86.5%, 32/37) (Table 2).

The relative sensitivity of the CPV-2 LFS RPA assay was further evaluated by comparing the results with those of the SNAP test. The data showed that the SNAP test was able to detect the CPV-2 antigen in 29/60 (48.3%) of the analyzed samples and the Ct values ranged from 13.56 to 30.46 (Table 2). The relative sensitivity of the SNAP test was 63.0% (29/46) compared to that of the LFS RPA.

To evaluate the potential cage-side applicability of the CPV-2 LFS RPA assay, 5 CPV-2 DNA-positive fecal samples (with Ct values of 17.29, 23.00, 27.76, 31.91 and 35.40) were selected and tested. The results showed that CPV-2 DNA could be detected in the above 5 samples by the LFS RPA assay either using the DNA extracted with the commercial kit or using the DNA obtained by boiling (Fig. 5). The

Table 2

Comparison of CPV-2 LFS RPA assay with real-time PCR and SNAP assays on the clinical canine fecal samples.

Sample No.	LFS RPA	real-time PCR(Ct)	SNAP	Antigenic type
F1	+	14.54	+	2a
F2	+	16.53	+	2a
F3	-	>40	-	-
F4	+	32.47	-	2a
F5	+	19.92	+	2b
F6	+	27.47	+	2a
F7	-	>40	-	-
F8	+	27.94	+	2a
F9	+	37.09	-	unidentified
F10	+	17.29	+	2a
F11	+	34.44	-	2a
F12	+	19.28	+	2a
F13	-	>40	-	-
F14	+	35.69	-	unidentified
F15	+	27.65	-	2a
F16	+	23.14	+	2b
F17	+	27.76	+	2a
F18	+	32.99	-	2a
F19	+	23.00	+	2a
F20	-	>40	-	-
F21	-	>40	-	-
F22	+	30.46	+	2a
F23	+	29.79	+	2a
F24	+	36.40	-	unidentified
F25	+	20.03	+	2a
F26	+	17.63	+	2a
F27	-	>40	-	-
F28	+	36.16	-	unidentified
F29	+	28.05	+	2a
F30	+	18.47	+	2a
F31	+	28.05	-	2a
F32	+	35.40	-	unidentified
F33	-	>40	-	-
F34	+	20.03	+	2a
F35	-	>40	-	-
F36	+	33.91	-	unidentified
F37	-	>40	-	-
F38	+	17.63	+	2a
F39	+	31.91	-	unidentified
F40	+	19.11	+	2a
F41	+	13.56	+	2a
F42	+	15.32	+	2a
F43	+	17.25	+	2b
F44	+	15.36	+	2a
F45	-	>40	-	-
F46	+	15.88	+	2c
F47	+	27.42	-	2a
F48	-	>40	-	-
F49	+	27.18	+	2a
F50	+	25.01	+	2a
F51	-	>40	-	-
F52	-	>40	-	-
F53	+	34.58	-	unidentified
F54	+	23.22	+	2c
F55	+	32.24	-	2a
F56	+	30.08	+	2a
F57	+	29.35	-	2a
F58	-	>40	-	-
F59	+	35.12	-	unidentified
F60	+	23.15	+	2a

+ : positive; - : negative.

above data demonstrated that the developed CPV-2 LFS RPA could be used as a promising cage-side diagnostic tool.

4. Discussion

This study describes a visible, equipment-free LFS RPA assay with high sensitivity and specificity for rapid detection of CPV-2. The CPV LFS RPA reaction tubes were held in a closed fist for 15 min, and the results were inspected directly with the naked eye within 5 min. As

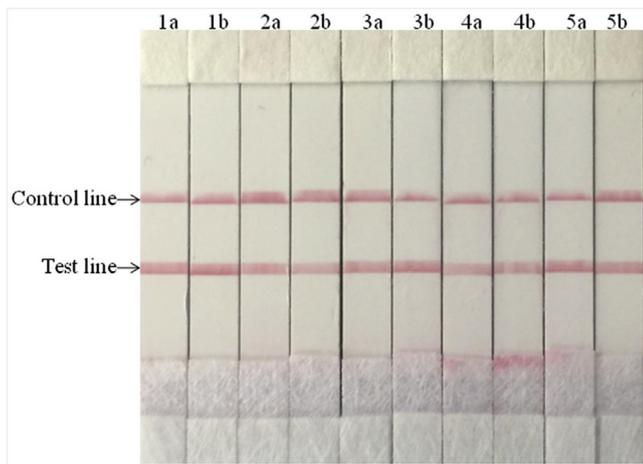


Fig. 5. Comparison of CPV-2 detection results using DNA extracted by a commercial kit or by boiling. The developed CPV-2 LFS RPA assay performed well using the DNA extracted by simply boiling. Lines 1–5 show samples that were CPV-2 DNA-positive by real-time PCR with different Ct values. a, DNA extracted by the commercial kit; b, DNA extracted by boiling.

were most of the molecular methods for detection of CPV-2, such as PCR [15], nested PCR [13], real-time PCR [12], iPCR [16], and LAMP [11,14], the assay was developed based on the VP2 gene. Although the point mutations in the VP2 protein have been associated with CPV types, the full consideration of the CPV variants were taken into account when designing the primers and probe, and the highly conserved region of the VP2 gene was set as the template. Through the developed novel LFS RPA assay, three different antigenic types of CPV-2 can be detected specifically.

RPA operates at a wide range of temperatures and does not require the reaction temperature to be precisely controlled [18]. TwistDx recommends an incubation temperature of 37 °C (the temperature of the human body), and other studies have shown that RPA retains reliable functionality between 31 °C and 43 °C [24,25] and even between 30 °C and 45 °C [24,25]. Our studies also showed that RPA could work well for detection of PCV2 between 34 °C and 42 °C [26]. Normal human body temperature (36.1–37 °C) is within the above temperature range, and several RPA assays have been developed to perform the reaction using body heat by either holding the reaction tube in the axilla or in closed fists [27,28]. Most of the established LFS RPA assays were performed by running the reactions in water baths or heating blocks [20–22,25,28]. In this study, the CPV-2 LFS RPA assay was performed by holding the reaction tubes in a closed fist, which is the feature of the assay. The temperature in a closed fist was monitored via a type-K insulated thermocouple (PN: 5TC-TT-K-36-36, Omega Engineering, Norwalk, CT). The wire thermocouple probe was held tightly in the closed fist, and the temperature was measured and recorded every second. The assays were performed by three different technicians in the laboratory, office and field with an ambient temperature of 18.4 °C, 27.0 °C and 29.6 °C, respectively, and the average temperature in the closed fists was 37.4 °C ± 0.4, 36.9 °C ± 1.0 and 35.7 °C ± 0.7, respectively. The assays were further performed in cold rooms, which were approximately 4 °C and 10 °C. The temperature in the closed fist did not reach above 32 °C, and the LFS RPA assay did not work as well as before.

The LOD of the CPV-2 LFS RPA was the same as that of the CPV-2 real-time PCR assay [29] and 10 times higher than that of the CPV-2 real-time RPA [12,19], while the latter assays depend on specialized instruments. In the evaluation of the performance of the assays on the clinical samples, the diagnostic agreement was 100% between the CPV-2 LFS RPA and real-time PCR, demonstrating excellent agreement between the two assays. Nevertheless, the LFS RPA assay showed distinct advantages in terms of detection time and equipment requirements. For

CPV-2 detection, it is often sufficient to simply boil the fecal samples before running diagnostic PCR [30]. With the sample DNA extracted by simply boiling used as template, the developed LFS RPA assay detected CPV-2 successfully, and the results were the same as those obtained using the DNA extracted with the commercial kit. The above results are encouraging, but the assay must be validated by analysis of a larger number of CPV-2-positive clinical samples using DNA extracted by boiling.

The commercial SNAP test for rapid detection of CPV-2 antigen is commonly used for cage-side diagnosis and can be completed in approximately 8 min, but it was less sensitive than PCR-based methods and the RPA assays [14,19,30–32]. The detection target of the SNAP test is the CPV-2 antigen, and there is no amplification of the detection target during the test. However, in the LFS RPA, the detection target is CPV-2 genomic DNA. The virus-specific gene is first amplified exponentially by RPA, and then the amplicons are tested by the LFS. In the process, the detection signal is magnified hundreds of thousands of times.

In conclusion, a rapid, visible and equipment-free method using body heat has been developed successfully for cage-side diagnosis of canine parvovirus disease. The good analytical specificity and sensitivity and easy sample-to-answer protocol make the LFS RPA ideal for the rapid and reliable detection of CPV-2 in an under-equipped laboratory and at the PON diagnosis, especially in resource-limited settings.

Conflicts of interest

The authors declare that they have no competing interests.

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