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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

Libing Liu[a,c,1], Jianchang Wang[a,c,1], Yunyun Geng[d], Jinfeng Wang[a,c], Ruiwen Li[b], Ruihan Shi[a,c], Wanzhe Yuan[b,*]

[a] Center of Inspection and Quarantine, Hebei Entry-Exit Inspection and Quarantine Bureau, Shijiazhuang 050051, China
[b] College of Veterinary Medicine, Agricultural University of Hebei, Baoding 071001, China
[c] Hebei Academy of Science and Technology for Inspection and Quarantine, Shijiazhuang 050051, China
[d] College of Life Sciences, Hebei Normal University, Shijiazhuang 050024, China

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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

* Corresponding author. College of Veterinary Medicine, Agricultural University of Hebei, No. 38 Lingyusi Street, Baoding, Hebei 071001, People's Republic of China.
E-mail address: yuanwanzhe2015@126.com (W. Yuan).
[1] These authors contributed equally to this work.

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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	AAACAGGAATTAACTATACTAATATATTTA	93	[11]
	CPV-R	AAATTTGACCATTTGGATAAACT		
	CPV-P	FAM- TGGTCCTTTAACTGCATTAAATAATGTACC -BHQ1		
LFS RPA	CPV-nfo-F	CACTTACTAAGAACAGGTGATGAATTTGCTACAG	214	This study
	CPV-nfo-R	Biotin-AGTTTGTATTTCCCATTTGAGTTACACCACGTCT		
	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 × 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 TwistAmpTM 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