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Short communication

An insulated isothermal PCR method on a field-deployable device for rapid and sensitive detection of canine parvovirus type 2 at points of need

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ABSTRACT

Canine parvovirus type 2 (CPV-2), including subtypes 2a, 2b and 2c, causes an acute enteric disease in both domestic and wild animals. Rapid and sensitive diagnosis aids effective disease management at points of need (PON). A commercially available, field-deployable and user-friendly system, designed with insulated isothermal PCR (iiPCR) technology, displays excellent sensitivity and specificity for nucleic acid detection. An iiPCR method was developed for on-site detection of all circulating CPV-2 strains. Limit of detection was determined using plasmid DNA. CPV-2a, 2b and 2c strains, a feline panleukopenia virus (FPV) strain, and nine canine pathogens were tested to evaluate assay specificity. Reaction sensitivity and performance were compared with an in-house real-time PCR using serial dilutions of a CPV-2b strain and 100 canine fecal clinical samples collected from 2010 to 2014, respectively. The 95% limit of detection of the iiPCR method was 13 copies of standard DNA and detection limits for CPV-2b DNA were equivalent for iiPCR and real-time PCR. The iiPCR reaction detected CPV-2a, 2b and 2c and FPV. Non-targeted pathogens were not detected. Test results of real-time PCR and iiPCR from 99 fecal samples agreed with each other, while one real-time PCR-positive sample tested negative by iiPCR. Therefore, excellent agreement ($k = 0.98$) with sensitivity of 98.41% and specificity of 100% in detecting CPV-2 in feces was found between the two methods. In conclusion, the iiPCR system has potential to serve as a useful tool for rapid and accurate PON, molecular detection of CPV-2.

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Canine parvovirus type 2 (CPV 2) is a non-enveloped, single stranded DNA virus belonging to the species Carnivore protoparvovirus 1, which also includes feline parvovirus (feline panleukopenia virus), mink enteritis virus, and raccoon parvovirus 118-A. This species, with associated viruses, is a member of the genus *Protoparvovirus*, subfamily *Parvovirinae*, family *Parvoviridae* (Cotmore et al., 2014). CPV-2 has been reported to be a highly contagious pathogen of domestic dogs and other members of the family *Canidae* worldwide (reviewed by Desario et al., 2005). Unlike other DNA viruses, CPV-2 evolved rapidly and current genetic variants include CPV-2a, CPV-2b, and CPV-2c strains (Buonavoglia et al., 2000; Clegg et al., 2011). The currently circulating strains can also infect domestic cats and can cause severe disease and death in cats, just as feline panleukopenia does. CPV-2 infection causes myocarditis in the fetuses of dogs infected in late gestation and also in infected neonates lacking maternally derived antibodies (Carpenter et al., 1980; Hayes et al., 1979). The most common problem in dogs between weaning and 6 months is enteritis (Houston et al., 1996; Parrish et al., 1982; Pollock and Coyne, 1993), with clinical signs of lethargy, anorexia, vomiting and hemorrhagic diarrhea. Parvovirus also causes leukopenia, with lymphopenia and neutropenia due to infection and damage to the bone marrow (Houston et al., 1996). Mortality rates may be high in puppies (Pollock and Coyne, 1993).

The virus is spread through the fecal-oral route. Fomites containing virus-laden fecal material are a common source of infection because dogs shed billions of virions or more per gram of feces during the height of infection and CPV-2 is highly stable in the environment for many months (Decaro et al., 2005b). CPV-2 is shed in the feces of infected dogs within 4–5 days prior to the onset of clinical symptoms, throughout the period of illness, and up to several

weeks after clinical recovery (Decaro et al., 2005a). Cats can also shed CPV-2 and may have in-apparent infection. Such cats would also be a source of infection for in-contact dogs.

Because CPV-2 is highly contagious and can cause severe disease and death, a sensitive and specific diagnostic test is essential to help detect small amounts of parvovirus shed in the stool from dogs in acute stages of infection, during recovery from the disease, or from dogs that are subclinical shedders. These dogs are a source of infection for contact animals in animal shelters, veterinary hospitals, or in breeding facilities, and are an important source of environmental contamination (Gordon and Angrick, 1986). Rapid identification and isolation of CPV-2-positive animals at these stages of infection could help timely implementation of proper supportive treatment of the patient and reduce CPV-2 spread among the population.

Some diagnostic tests lack the sensitivity to detect CPV-2 in stool samples, despite clinical signs and postmortem findings that are supportive of a CPV-2 diagnosis (Parrish et al., 1982; Studdert et al., 1983). The most commonly used in-house SNAP test (IDEXX; Westbrook, MA, USA) for CPV-2 diagnosis can only detect CPV-2 in samples with >10^5 virion equivalents per milligram of feces (Decaro et al., 2010). A sensitive test would help confirm a CPV-2 diagnosis based on clinical signs, in the absence of a positive in-house ELISA result (Decaro et al., 2010). Situations like this, in some cases with high virus titers, have happened with significant frequency, so the recommended approach for parvovirus diagnosis is to use the in-house assay first, followed by testing with a more sensitive molecular method (such as PCR) in questionable cases (Decaro et al., 2010). Nested PCR (Hirasawa et al., 1994) and real-time PCR (qPCR) assays (Decaro et al., 2005b) have been shown to be highly sensitive tests. However, application of nested and qPCR assays has been limited to professional laboratories due to their requirement of experienced technicians and costly equipment.

An on-site highly sensitive molecular test for CPV-2 diagnosis would enable appropriate quarantine and disease management of infected dogs, besides helping cut down sample shipping and processing costs and shorten test turnaround time. The newly developed convection PCR method, termed insulated isothermal PCR (iiPCR) (Tsai et al., 2012), provides this capability. It utilizes a hydrolysis probe (similar to Taq-man based qPCR) and runs in a commercially available device, POCKIT[TM] Nucleic Acid Analyzer (POCKIT[TM]; GeneReach USA, Lexington, MA, USA). The reaction mixture is automatically passed sequentially through different temperature zones in a capillary tube that is placed within the device to complete the 3 stages (denaturation, annealing, and extension) required for PCR. Optical signals produced by probe hydrolysis during the reaction are converted to S/N ratios ($signal_{after}/signal_{before}$) by a data processing module and automatically reported as positive/negative results on a display screen. The reaction takes approximately 1 h to complete and is user-friendly enough to be run by animal shelter personnel or clinical technical staff. The device can be operated with a rechargeable battery or car battery. The small size (28 cm × 25 cm × 8.5 cm, W × D × H), light weight (2.1 kg), and ruggedness enable the POCKIT[TM] iiPCR device to be field deployable. Excellent sensitivity and specificity of iiPCR systems in detecting target pathogens from clinical samples have

Table 2
Analytical sensitivity analysis of CPV-2 iiPCR using plasmid DNA.

Copies/reaction	Total	Positive	Rate (%)	Avg.	SD
NTC	20	0	0	0.98	0.03
100	10	10	100	4.04	0.36
50	20	20	100	2.86	0.73
10	20	15	75	3.9	0.77
5	10	8	80	2.98	0.96

NTC, no template control.
95% hit rate was 10.725 by Probit analysis.

been reported for different DNA and RNA viruses, such as white spot syndrome virus in shrimp, equine influenza virus in horses, and canine distemper virus in dogs (Balasuriya, 2014; Balasuriya et al., 2014; Tsai et al., 2014; Wilkes et al., 2014).

In this study, taking advantage of the iiPCR system with the field-deployable POCKIT[TM] device, a reaction was developed to detect CPV-2 at points of need (PON). The primer/probe set was designed to target a highly conserved region within the *VP2* genes of CPV-2 strains available in GenBank, based on the recommended principles for iiPCR (http://www.iipcr.com/eweb/uploadfile/20130522114104277.pdf). The probe was labeled with a fluorescent reporter dye (6-carboxyfluorescein, FAM) at the 5′ end and a minor groove binder group (MGB) with a non-fluorescent quencher at the 3′ end (Applied Biosystems, Foster City, CA, USA). The optimized iiPCR included 0.5 μM forward primer, 0.5 μM reverse primer, 0.05 μM probe (Table 1), 26 U of Taq DNA polymerase (BioMi, Taichung, Taiwan), and 1× Uni-ii HS Buffer (GeneReach USA) and generated an amplicon of 111 bp. The primers, probe, dNTP, and Taq were lyophilized and rehydrated with 50 μl of reconstitution buffer (GeneReach USA) before use. After mixing with 5 μl of sample nucleic acid, 50 μl of the mixture was transferred to a capillary R-tube[TM] (GeneReach USA). The tube was spun briefly in a Cubee[TM] mini centrifuge (GeneReach USA) and placed into the commercial iiPCR device for reaction. A lyophilized positive control (plasmid DNA), provided with the kit, was reconstituted and used for each run, and water was used as a negative control for each run. Serial dilutions of a standard plasmid DNA, which contained the target sequence, were tested in 10 or 20 repeats to evaluate detection limit of the iiPCR. Limit of detection 95% was determined to be 13 copies of standard DNA by probit analysis (Table 2).

No gold standard method exists currently to detect CPV-2 in fecal samples. To evaluate the performance of iiPCR-POCKIT[TM] reagent in detecting CPV-2 in fecal samples, side-by-side comparison with that of the in-house qPCR routinely used at University of Tennessee Veterinary Medical Center (UTVMC) was performed. Fecal samples were prepared by mixing with a 1:1 volume of PBS and supernatant was used for DNA extraction using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Primers and probe of the CPV-2 qPCR were designed with Primer 3 (Rozen and Skaletsky, 2000) to target a 158-bp fragment in a highly conserved region of the *NS1* gene (Table 1). Five μl of DNA samples were run in 25-μl total volume reactions using Premix Ex Taq Probe qPCR (Clontech, Mountain View, CA, USA)

Table 1
Primer and probe sequences used in CPV-2 iiPCR and qPCR.

Reaction	Primer/probe name	Sequence (5′ to 3′)	Nucleotide location[a]	Target gene
CPV-2 iiPCR	CPV F2	GTTAACGGAAACATGGCTTTAGATGA	3052–3077	*VP2*
	CPV R1	TAGTTGCCAATCTCCTGGATTAAAC	3138–3162	
	CPV probe	FAM-CCCCAAGCATTTGCATCA-MGB-NFQ	3114–3131	
CPV-2 qPCR	Parvo forward	GACGACAGCACAGGAAACAA	1062–1081	
	Parvo reverse	CTGGTTGTGCCATCATTTCA	1200–1219	*NS1*
	Parvo probe	FAM-TCACCTGAAGACTGGATGATGTTACAACCA-BHQ1	1159–1188	

[a] Based on GenBank Accession number JQ268284.

Table 3
Detection limit comparison between CPV-2 iiPCR and qPCR.

Dilutions	iiPCR (result (S/N))			qPCR		
−5	+(4.12)	+(4.52)	+(4.00)	31.53	32.91	31.93
−6	+(2.40)	+(3.49)	+(3.36)	35.82	35.5	34.2
−7	+(1.71)	+(1.75)	+(2.00)	38.96	39.57	ND
−8	−(0.99)	−(1.00)	−(0.98)	ND	ND	ND

ND, not detected.

containing 200 nM of probe and 300 nM of each primer. Samples were amplified in a Smart Cycler II (Cepheid, Sunnyvale, CA, USA) with an activation step for the hot start Taq polymerase at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Analytical sensitivity of the qPCR was determined to be 10 copies of standard plasmid DNA (data not shown). The qPCR had a linear dynamic range between 10^8 and 10^1 copies, with a slope of −3.4, a y-intercept of around 39, and a mean coefficient linearity (R^2) of 0.998. The reaction was validated internally to be more sensitive than electron microscopic detection of parvovirus and a commercially available ELISA (IDEXX) (data not shown).

Analytical sensitivity of CPV-2 iiPCR in detecting viral DNA was compared to that of the reference qPCR using serial dilutions of DNA extracted from a CPV-2b clinical isolate (provided by Dr. Edward Dubovi, Cornell University, Ithaca, NY, USA) run in triplicate. Limits of detection of iiPCR and qPCR were reached at 10^7- and 10^6-fold dilution, respectively, indicating that the established reaction had sensitivity equivalent to, if not better than, that of the qPCR (Table 3). Furthermore, to assess the specificity of the CPV-2 iiPCR, one subtype 2a isolate (provided by Dr. Jeremiah T. Saliki, Athens Veterinary Diagnostic Laboratory, University of Georgia, Athens, GA, USA), four subtype 2b, and three 2c samples, identified previously by sequencing analysis, were tested. Positive results were obtained from all eight samples, demonstrating that the reagent could detect all current CPV subtypes. The iiPCR reagent could also detect feline panleukopenia virus (FPV), a close relative of CPV-2. Furthermore, an exclusivity panel including nine important canine pathogens (canine adenovirus 2, canine herpesvirus, canine parainfluenza virus, canine enteric coronavirus, canine distemper virus, *Salmonella enterica* serovars *Typhimuruim* and *Dublin*, *Clostridium perfringens* with *tox A* gene, and *Cryptosporidium canis*) were analyzed. All of them generated negative results with iiPCR, indicating excellent specificity of the established assay.

Performance of the established system for detecting CPV2 in feces of animals with diarrhea was evaluated by comparing test results of iiPCR and the reference qPCR. Nucleic acid extracts of fecal samples from dogs with clinical signs suggestive of CPV-2 infection at UTVMC from 2010 to 2014 were tested in parallel. Among the 100 clinical samples, 62 were CPV-2 positive by the qPCR assay, generating Ct values between 10.01 and 39.19. The iiPCR did not detect one of the qPCR-positive samples. The discrepancy between the qPCR and iiPCR result may be due to the very low amount of virus in the sample (Ct = 38.00) and was likely detection of vaccine shedding by the qPCR method. All 38 qPCR-negative samples also tested negative by iiPCR.

Taken together, excellent agreement (k = 0.98) was found between iiPCR and qPCR, with sensitivity of 98.41% (confidence index = 93.36–100%) and specificity of 100% (confidence index = 93.18–100%), indicating that the iiPCR system detected CPV-2 from clinical samples with sensitivity and specificity comparable to those of the qPCR.

In conclusion, the results of the current study indicate that the performance of the CPV-2 iiPCR was equivalent to that of the reference qPCR. A positive iiPCR result is consistent with detection of CPV-2 viral DNA in the sample. On the contrary, a negative iiPCR result indicates that no viral DNA or an extremely low level of the viral DNA (below assay detection limit) is present in the sample. Based on the sensitivity of the test, similar to qPCR assays, this assay can detect low amounts of modified-live vaccine virus shedding and this should be taken into consideration in recently vaccinated animals.

The reagent was demonstrated to work in a lyophilized format, which allowed it to be shipped and stored without refrigeration to facilitate PON application. For resource-limited settings, both manual nucleic acid extraction method (PetNAD™ Co-prep, GeneReach USA) and field-deployable automatic nucleic acid extraction device (Taco™ mini, GeneReach USA) are available to work with the CPV-2 iiPCR/POCKIT™ system. Including the nucleic acid extraction step, which takes about 30 min for either method, the time required from samples to results is around 1.5 h. In conclusion, performed in a portable POCKIT™ device, the established iiPCR reagent has potential to serve as a useful tool for PON molecular diagnosis and surveillance of CPV-2, a threat to animals, especially puppies in animal shelters.

Conflict of interest

Acknowledgements

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