Pakistan J. Zool., vol. 51(5), pp 1843-1852, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1843.1852

Development and Clinical Evaluation of a Direct Amplification Method to Diagnose Canine Parvovirus and Canine Distemper Viral Infections in Dogs without Nucleic Acid Extraction

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Article Information
Received 09 August 2018
Revised 04 September 2018
Accepted 12 September 2018
Available online 05 July 2019

Authors' Contribution
GNP and ZJZ conceived and designed the experiments. ZJZ, LQL and XHL performed the experiments. LQL and ZJZ developed and performed the molecular data analyses. JZ, XBG and YG analyzed the data. ZJZ and JZ wrote the paper.

Key words
Canine parvoviruses, Canine distemper virus, Without nuclear acid extraction, TaqMan real-time (RT)-PCR, Direct amplification method.

ABSTRACT

To rapidly detect canine parvoviruses (CPV) or canine distemper virus (CDV) without nuclear acid extraction, we established a direct amplification TaqMan real-time (RT)-PCR method (DARPM) to detect CPV and CDV in clinical samples. We compared this new method against real-time PCR/(RT)-PCR and it showed no cross-reactivity with other pathogens. Sensitivity testing showed the minimum detection limits of real-time (RT)-PCR were 7.44×10^1 copies·μL^{-1} (CPV) and 4.20×10^1 copies·μL^{-1} (CDV). DARPM detection of CPV with DNA showed a minimum detectable of 1.53×10^1copies μL-1, while the minimum detectable amount from the virus culture supernatant was 6.70×10^1 copies μL-1. The minimum detectable copy numbers for CDV cDNA and for the virus culture supernatant were 9.56×10^1 copies μL-1 and 7.77×10^1 copies μL-1, respectively. To validate the accuracy of DARPM, 112 clinical samples and 97 clinical samples suspected of harboring CPV and CDV were tested. DARPM showed a 100% compliance rate with ordinary PCR and colloidal gold rapid detection methodology, while the coincidence rate for DARPM and the same method with DNA added was also 100%. Therefore, DARPM detects CPV and CDV without the need for pre-PCR nuclear acid extraction. Our results show that DARPM is a specific, sensitive, fast and powerful method for detecting CPV and CDV in clinical samples.

INTRODUCTION

Canine parvovirus (CPV) and canine distemper virus (CDV) are two severe, clinically acute diseases in dogs, and both cause economic losses to the canine breeding industry. CPV (family Parvoviridae, genus Protoparvovirus), an infectious disease, is clinically characterized by severe vomiting, hemorrhagic enteritis, non suppurative myocarditis, and a marked reduction in white blood cells. CPV infections often occur in young dogs, with puppies having high mortality rates (20%–30%) and vaccine failures are problematic (Organtini *et al*., 2015). CPV was first identified in the USA in the 1970s, after which it was reported across the world in Africa, Asia, Australia, the Americas and Europe (Castanheira *et al*., 2014; Kumar and Nandi, 2010; Madias, 2012; Meers *et al*., 2007; Decaro *et al*., 2007b; Pérez *et al*., 2014; Steinel *et al*., 1998). In China, the canine CPV-2 infections first reported in 1983 rapidly spread across the whole country (Miranda and Thompson, 2016; Zhijun *et al*., 2014). Currently, more than 30 Chinese provinces (*e.g.,* Sichuan, Heilongjiang, Jilin, Liaoning, Shandong, Shenzhen, Beijing, Nanjing, and Hebei) have reported CPV infection outbreaks in dogs (Zhang *et al*., 2010; Zhao *et al*., 2015; Zhijun *et al*., 2014; Zhu *et al*., 2014). CDV (family Paramyxoviridae, genus Morbillivirus), another important viral disease in carnivores (Elia *et al*., 2006), has also been reported in many countries around the world (Bi *et al*., 2015; Kouji *et al*., 2013; Silva *et al*., 2014). CDV causes a serious

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0030-9923/2019/0005-1843 $ 9.00/0

and often fatal disease. Researchers have shown that the incidence of CDV in the global canine population has increased over the last decades (Martella *et al*., 2007). In China, the number of CDV infections also seems to have a rising trend, and many provinces (*e.g.,* Sichuan, Jiangsu, Heilongjiang and Yunnan) have reported CDV outbreaks in recent years (Bi *et al*., 2015; Li *et al*., 2014; Sun *et al*., 2009; Tan *et al*., 2011). There are some studies showed that the CDV host range is not just limited to dogs, but to monkeys, raccoon dogs and giant pandas also (Bi *et al*., 2015; Guo *et al*., 2013; Kouji *et al*., 2013; Li *et al*., 2014; Qiu *et al*., 2011; Wu *et al*., 2015).

Currently, the clinical diagnosis of CPV and CDV infections may be inconclusive because several other diseases can cause the same clinical signs in dogs (Decaro *et al*., 2007a; Kalinowski *et al*., 2012; Mihalov-Kovács *et al*., 2015; Sánchez-Cordón *et al*., 2002). Therefore, the clinical diagnosis of CPV and CDV infections is often inaccurate and requires confirmation by laboratory tests. Several diagnostic laboratory methods have been developed to detect CPV and CDV. For CPV detection, colloidal gold rapid detection (antigen capture ELISAs), hem-agglutination, virus isolation and polymerase chain reaction (PCR) methods are usually used (Streck *et al*., 2013). For CDV detection, enzyme-linked immunosorbent assays (ELISA), colloidal gold rapid detection, seroneutralization (SN) assays and virus isolation are often used (Wilkes *et al*., 2014). However, these methods have relatively low sensitivities (Streck *et al*., 2013). Among them, PCR and colloidal gold rapid detection are the most commonly used laboratory methods for detecting CPV and CDV, especially in China. However, the accuracy of the colloidal gold rapid detection method is not high and is often hampered by false results. Traditional PCR/reverse transcriptase (RT)-PCR is more complex in term of its operation, and can be negatively affected by PCR inhibitors in clinical samples and low target specificity (Wilson, 1997). Because of these drawbacks, a simple, timesaving and accurate method for detecting CPV and CDV is needed.

In recent years, TaqMan real-time PCR/reverse transcriptase (RT)-PCR has demonstrated its ability to detect viruses in clinical samples without the need for prior nucleic acid extraction, and its timesaving and easily operated (Baethgen *et al*., 2003; Bista *et al*., 2007; Cardoso *et al*., 2009; Poon *et al*., 2006; Nishimura *et al*., 2010; Victor *et al*., 2009). However, as far as we know, there are few published reports where real-time PCR has been used without nucleic acid extraction to detect CPV and CDV in clinical samples. Therefore, in this study, we established a direct amplification TaqMan real-time PCR/RT-PCR method (DARPM) to detect CPV and CDV in clinical samples, without the need of nuclear acid extraction. Our results showed that DARPM is a specific, sensitive, and timesaving method for detecting CPV and CDV infections in dogs.

MATERIALS AND METHODS

Ethics statement

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The research protocol was approved by the Research Ethics Committee of the Sichuan Agricultural University, Sichuan, China. Permission was obtained from the animal owners or managers before collecting the fecal specimens. No animals were harmed during the sampling process.

Viruses

CPV, CDV, canine adenovirus type 1 (CAV-1), canine adenovirus type 2 (CAV-2), rabies virus (RABV), and canine par influenza virus (CPIV, CPIV/A-20-8) are all stored in our laboratory. The CPV was a wild type strain and cultured in feline kidney 81 cells (F81) and Dulbecco's modified Eagle's medium (Sigma, Saint Louis, Mo, USA). The CDV was a wild type strain and cultured in Vero cells in Dulbecco's modified Eagle's medium.

Nucleic acid extraction

CPV DNA and CDV RNA were extracted from 200 µL virus cultures using a Pure Link Viral RNA/DNA Mini Kit (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions.

Real-time PCR/RT-PCR targeting the CPV NS1 *gene and* CDV N *gene*

A conserved region of the *CPV NS1* gene (nucleotide positions 1985–2096; GenBank accession No. M19296) was used to design primers and a probe for the real-time PCR assay. A conserved region of the *CDV N* gene (nucleotide positions 248–326; GenBank accession No. KJ466106) was used to design primers and a probe for the real-time RT-PCR assay (Table I).

Real-time PCR and RT-PCR detection of CPV and CDV

The individually extracted DNA and RNA samples were amplified using a Quant One Step qRT-PCR kit (TIANGEN, China). The 25 µL CPV PCR reactions contained the following: 2 × Quant One Step Probe qRT-PCR Master Mix (12.5µL), template DNA (2 µL), 10 µM forward primer (0.5 µL), 10 µM reverse primer (0.5 µL), 5 µM probe (1 µL), HotMaster Taq polymerase (1 µL), made up to 25 µL with sterile distilled water (sdH_2O). The

25 µL CDV RT-PCR reactions contained the following: 2× Quant One Step Probe qRT-PCR Master Mix (12.5 µL), template RNA (2 µL), 10 µM forward primer (0.5 µL), 10 µM reverse primer (0.5 µL), 5 µM probe (1µL), HotMaster Taq polymerase (1 µL), Quant RTase (0.4 µL), made up to 25 µL with sdH_2O.

Each CPV run consisted of incubation at 92°C for 3 min, followed by 40 cycles of denaturation at 92°C for 10 s, annealing at 55°C for 20 s, and extension at 68°C for 20 s. Fluorescence was measured at the end of each extension step. Each CDV run consisted of reverse transcription at 50°C for 30 min, inactivation of the enzyme at 92°C for 3 min, followed by 40 cycles of denaturation at 92°C for 10 s, annealing at 55°C for 20 s, and extension at 68°C for 20 s. Fluorescence was measured at the end of each extension step.

Standard curves

To create standard curves for quantifying CPV and CDV copy numbers, plasmids harboring the target sequence for the real-time PCR and the RT-PCR were generated. Briefly, DNA from the JQ996153 CPV-2 strain, and RNA from the CDV LIU strain (a dog CDV strain) (Qiu *et al*., 2011). The number of plasmid copies was calculated using the equation described by Whelan *et al*. (2003).

A standard curve was generated via successive 10-fold serial dilutions of the plasmids. The real-time PCR and RT-PCR reactions were done as described above. Amplification efficiencies were determined from the standard curves, and the threshold cycle (C_t) value was determined by the real-time PCR and RT-PCR settings.

Analytical specificity, sensitivity, and repeatability of real-time PCR and RT-PCR assays

The specificities of each of the two assays were tested with nucleic acids from six different virus strains (CPV, CDV, CAV-1, CAV-2, RABV, and CPIV) from our laboratory. Cell culture media was used for the negative controls.

The sensitivities of the reactions were tested via successive 10-fold serial dilutions of CPV and CDV recombinant plasmids, using the new real-time PCR assays. We used conventional PCR to determine the detection limits of the assays.

The plasmid standards were diluted in high, medium and low (10^{-2}, 10^{-4}, 10^{-6}) concentrations to determine the repeatability of the two different real-time PCR assays and to determine the intra-batch and inter-batch coefficient variants.

Sensitivity of DARPM and real-time PCR/RT-PCR

CPV and CDV viral nucleic acids were 10-fold diluted from the viral culture supernatants and tested by DARPM to determine the detection limits for the real time PCR/RT-PCR. The DARPM and real time PCR/RT-PCR reactions are following the 2.5 described, in DARPM, template DNA and template RNA will be replaced by CPV viral culture supernatants and CDV viral culture supernatants.

Clinical samples

Clinical samples from dogs suspected of harboring CPV or CDV (*i.e.,* dogs displaying typical clinical signs of such infections, such as acute gastroenteritis or lymphopenia, fever, purulent ocular and nasal discharge or neurological disturbance), were collected at the Sichuan Agricultural University Animal Teaching Hospital during 2015–2016. Fecal specimens were taken by a swab from the anus of dogs and homogenized in 1.0 mL phosphate buffered saline (PBS) in 1.5 mL sterilization collection tubes, after clarifying, taken 0.5 mL supernatants as CPV clinical samples. Conjunctival swabs and urine were taken from dogs suspected CDV, and be mixed together in 1.0 mL phosphate buffered saline (PBS) in 1.5 mL sterilization collection tubes, after clarifying, taken 0.5 mL supernatants as CDV clinical samples. All the samples were stored at -80°C until further analysis.

A total of 112 suspected CPV infection samples and 97 suspected CDV infection samples were collected. Nucleic acids were extracted from each sample and supernatants were collected. Colloidal gold strip, real-time PCR/RT-PCR, conventional PCR/RT-PCR and DARPM were used to detect CPV or CDV. DARPM positive samples were sequenced.

Table I.- Primer and probe sequences.

Primer/Probe	Sequence 5′-3′	Nucleotide position
CPV forward primer	GAAGGTATAAATTCACCAGGTTGC	2077–2096
CPV reverse primer	GTGCAAGGTCCACTACGTCC	1985–2009
CPV probe	FAM-AGACACAAGCGGCAAGCAATCCTC-BHQ1	2020–2044
CDV forward primer	AGGTCTCGACTATTGGATAGACTT	326–347
CDV reverse primer	CGAACAAGGAGAGGATACTGAT	248–271
CDV probe	FAM- ATTGGTTGGTGATCCGAAAATCAAC-BHQ1	277–301

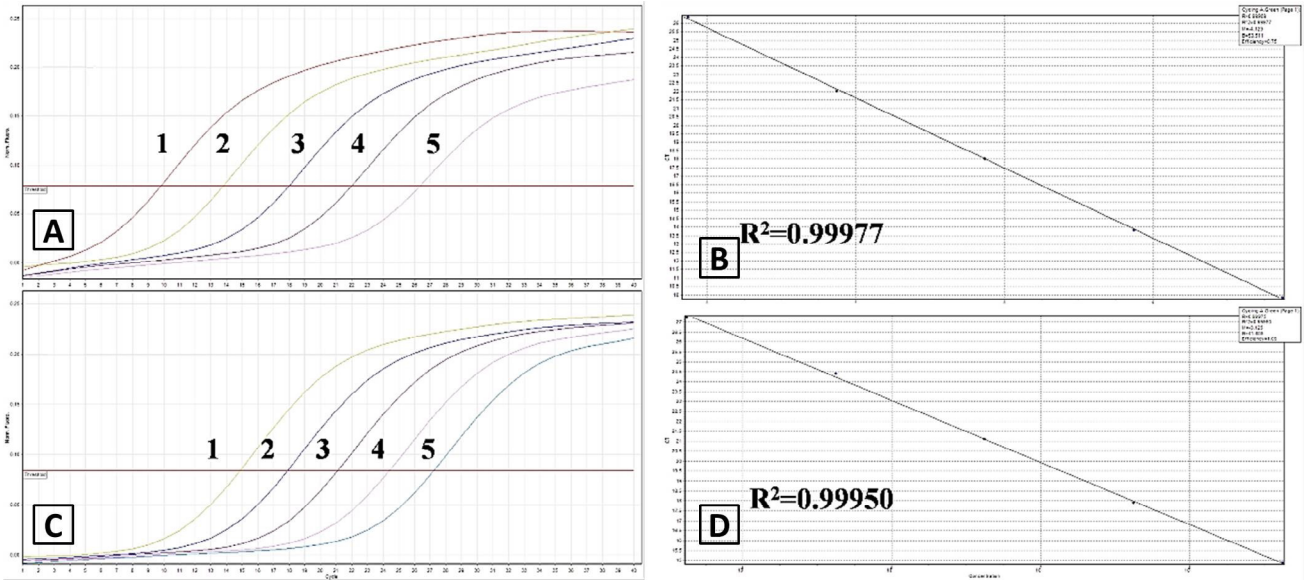

Fig. 1. Amplification curves (A, CPV; C, CDV) and standard curves (B, CPV; D, CDV) for CPV and CDV. Ten-fold serial dilutions of the CPV plasmid standard and the CDV plasmid standard were used for amplification, as indicated on the X-axis, and the corresponding cycle threshold (CT) values are presented on the Y-axis. A: 1–5: Equal dilutions of the CPV plasmid 10^{-1}–10^{-5}; C: 1-5: equal dilutions of the CDV plasmid 10^{-1}–10^{-5}.

RESULTS

Plasmid standards

The extracted CPV DNA and the CDV reverse-transcribed RNA were PCR amplified using virus-specific primers. After amplification and DNA sequencing, the recombinant plasmids were successfully constructed (Supplementary Fig. S1). The concentration of the CPV recombinant plasmid was 7.44×10^{9} copies·μL^{-1}, and the concentration of CDV recombinant plasmid was 4.20×10^{8} copies·μL^{-1}.

Standard curves for Real-time PCR/RT-PCR

Figure 1 shows the kinetic curves and standard curves for CPV and CDV produced by real-time PCR/RT-PCR amplification of the 10-fold serial dilutions of the recombinant plasmids containing known concentrations of CPV and CDV. The initial template nucleic acid concentration and C_t value showed a good linear relationship for each virus, and the correlation coefficient (R^2) for both CPV and CDV was 0.999.

Specificity of Real-time PCR/RT-PCR

Nucleic acids from CDV, CAV-1, CAV-2, CPIV, RABV and CPV standard plasmids were tested by real-time PCR using CPV-F and CPV-R primers. As expected, only CPV plasmid standards were amplified successfully. To test the specificity of the CDV primers, nucleic acids from CPV, CAV-1, CAV-2, CPIV, RABV virus culture medium and the CDV plasmid standard were checked by real-time PCR/RT-PCR. The result showed that only the CDV plasmid standard was successfully amplified. Non-CDV nucleic acids, including CPV, CAV-1, CAV-2, CPIV and RABV were not amplified (Supplementary Fig. S2).

Sensitivity of real-time PCR/RT-PCR

CPV and CDV plasmids were serially diluted ten times. Our results showed that the real-time PCR detected the CPV template with a dilution equal to 10^{8}, and the minimum detectable copy number was 7.44×10^{1} copies·μL^{-1}. The CDV template was detectable with a dilution factor of 10^{7}, and with a minimum detectable copy number of 4.20×10^{1} (Fig. 2). Conventional PCR detected a minimum of 7.44×10^{3} copies of the CPV standard, and ordinary reverse transcriptase PCR detected a minimum of 4.20×10^{4} copies of the CDV standard (Supplementary Fig. S3).

Repeatability of real-time PCR/RT-PCR

Three different concentrations of CPV (7.44×10^{7} copies·μL^{-1}, 7.44×10^{5} copies·μL^{-1}, 7.44×10^{3} copies·μL^{-1}) and CDV (4.2×10^{6} copies·μL^{-1}, 4.2×10^{4} copies·μL^{-1}, 4.2×10^{2} copies·μL^{-1}) plasmids were amplified by real-time PCR/RT-PCR in triplicate. The intra-batch coefficient variant (CV) for CPV was 0.09% to 1.3%, and the inter-batch CV was 0.57% to 0.94%. The intra-batch CV for

CDV was 0.62% to 1.11%, while the inter-batch CV was 0.45% to 1.02% (Supplementary Table I). These results confirm the reproducibility of the new Real-time (RT)-PCR assay.

Sensitivities of the DARPM and real-time PCR assays

The sensitivities of DARPM and real-time PCR were evaluated. 2μL nucleic acids were detected by real-time PCR, and a 2μL cultured virus supernatant was used for DARPM detection. The concentrations of CPV nucleic acid and the CPV virus culture supernatant were 1.39×10^{9} copies·μL^{-1} and 7.11×10^{9} copies·μL^{-1}, respectively. Comparing these results with the standard curve, the minimum detection dilution ratios of the CPV nucleic acids and virus culture supernatant were 10^{8} each (Fig. 3). The minimum DNA concentration detected by real-time PCR amounted to 1.53×10^{1} copies·μL^{-1}, while the minimum detectable amount using DARPM with the virus culture supernatant was 6.70×10^{1}.

The concentrations of the CDV nucleic acids and the CDV virus culture supernatant were 6.23×10^{8} copies·μL^{-1} and 2.28×10^{6} copies·μL^{-1}, respectively. Comparing these values with the standard curves, the minimum detection dilution ratios of the CDV nucleic acids and virus culture supernatants were 10^{7} and 10^{5}, respectively (Fig. 4). The minimum detectable cDNA and virus culture supernatant values were 9.56×10^{1} copies/μL^{-1} and 7.77×10^{1} copies/μL^{-1}, respectively.

Clinical test samples

Samples (209 in total; 112 feces samples from suspected CPV and 97 urine samples from suspected CDV infections) were tested using rapid colloidal gold test strips, conventional PCR/RT-PCR, real-time PCR/RT-PCR and DARPM. Nucleic acids from the CPV samples were detected by conventional PCR and real-time PCR. Rapid colloidal gold test strips and DARPM were used to detect CPV in viral supernatants, without nucleic acid extraction. The test results for CPV and CDV samples are shown in Supplementary Tables I and II.

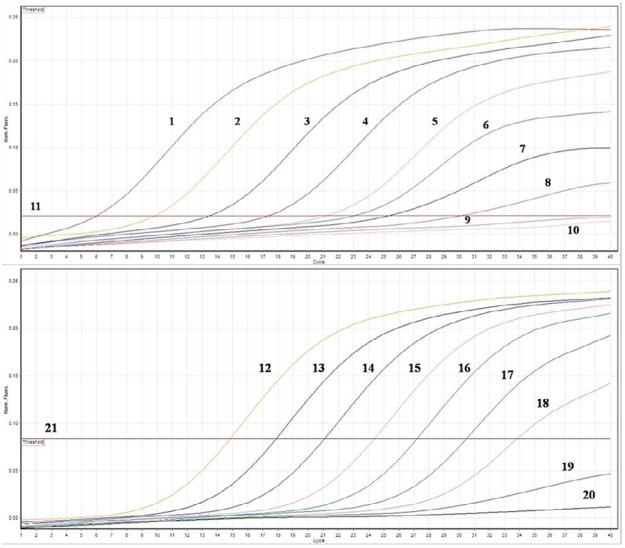

Fig. 2. Sensitivity of the real-time PCR/RT-PCR for the detection of CPV and CDV plasmid standards. Serial 10-fold plasmid dilutions (CPV: 7.44×10^{8} copies·μL^{-1} to 7.44×10^{0} copies·μL^{-1}; CDV: 4.20×10^{7} copies·μL^{-1} to 4.20×10^{0} copies·μL-1) were plotted against the threshold cycle (Ct) values. A minimum of 7.44×10^{1} copies of CPV (A) and 4.20×10^{1} copies of CDV (B) were detected. 1–9: Equal dilutions of the CPV plasmid standard at 10^{1}–10^{9}; 12-19: equal dilutions of the CDV plasmid standard at 10^{1}–10^{8}; 10, 20: negative control; 11, 21: threshold line.

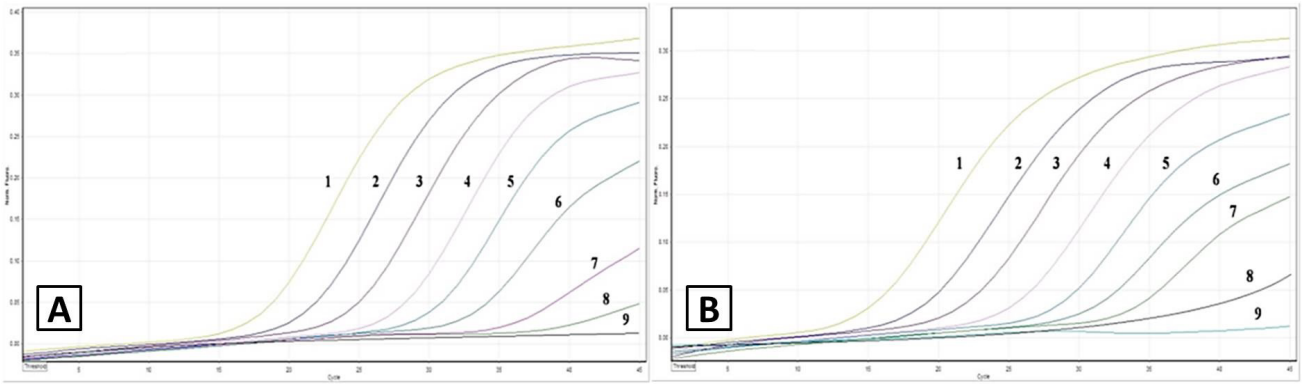

Fig. 3. Sensitivity tests for real-time PCR and DARPM. Serial 10-fold dilutions of CPV DNA and CPV virus culture supernatant (CPV DNA: 1.39×10^{8} copies·μL^{-1} to 1.39×10^{0} copies·μL^{-1}, CPV virus culture supernatant: 7.11×10^{8} copies·μL^{-1} to 7.11×10^{0} copies·μL-1) were detected. A minimum of 1.53×10^{1} copies of CPV DNA and 6.70×10^{1} copies of CPV virus culture supernatant were detected. A: 1–9: Equal dilutions of CPV DNA at 10^{-1}–10^{-9}; B: 1–9: CPV virus culture supernatant at 10^{-1}–10^{-9}.

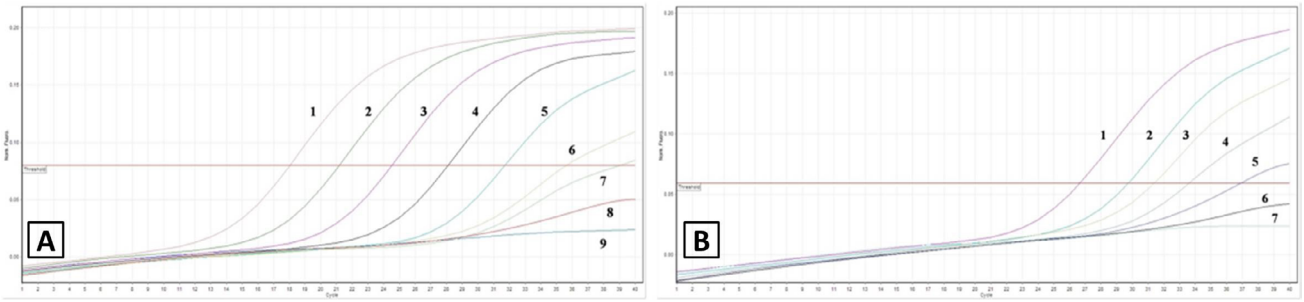

Fig. 4. Sensitivity tests for real-time RT-PCR and DARPM. Serial 10-fold dilutions of CDV cDNA and CDV virus culture supernatant (CDV cDNA: 6.23×10^{7} copies·μL^{-1} to 6.23×10^{0} copies·μL^{-1}, CDV virus culture supernatant: 2.28×10^{5} copies·μL^{-1} to 2.28×10^{0} copies·μL^{-1}) were detected. A minimum of 9.56×10^{1} copies of CDV cDNA and 7.77×10^{1} copies of CDV virus culture supernatant were detected. A: 1-8: Equal dilutions of CDV cDNA at 10^{-1}–10^{-8}, 9: negative control; B: 1–6: CDV virus culture supernatant at 10^{-1}–10^{-6}, 7: negative control.

Table II.- Comparison of CPV and CDV detection methods (colloidal gold strip, conventional PCR/RT-PCR, real-time PCR/RT-PCR and DARPM) in clinical samples.

	DARPM	Positive	Negative	Total
CPV				
Colloidal gold strip	Positive	49	0	49
	Negative	29	34	63
	Total	78	34	112
Ordinary PCR	Positive	64	0	64
	Negative	14	34	38
	Total	78	34	112
Real-time PCR	Positive	78	0	78
	Negative	0	34	34
	Total	78	34	112
CDV				
Colloidal gold strip	Positive	31	0	31
	Negative	29	37	66
	Total	60	37	97
Ordinary RT-PCR	Positive	43	0	43
	Negative	17	37	54
	Total	60	37	97
Real-time RT-PCR	Positive	60	0	60
	Negative	0	37	37
	Total	60	37	97

The real-time PCR results (Table II) showed that 78/112 samples were CPV-positive (C_t values, 20.19 to 35.83), and the detection limits for the nucleic acids were 2.21×10^{2} to 2.62×10^{7} copies·μL^{-1}. Positive results for the colloidal gold detection strip, PCR, real-time PCR and DARPM were 49/112, 64/112, 78/112 and 78/112, respectively. The real-time RT-PCR detection results (Table II) showed that 60/97 samples were positive for CDV (C_t, 25.75 to 35.64), with the copy numbers of the nucleic acids detected being 9.30×10^{1} to 3.12×10^{7} copies·μL^{-1}. The colloidal gold detection strip, RT-PCR, real-time RT-PCR and DARPM results showed that 31/97, 43/97, 60/97 and 60/97samples were positive, respectively. Real-time (RT-) PCR and DARPM had higher positivity rates for the samples suspected of harboring CPV and CDV. Sequencing results also confirmed that DARPM positive results are accurate.

To evaluate the reliability of DARPM for clinical CPV and CDV detection, its performance was compared with colloidal gold detection, PCR/RT-PCR and real-time PCR/RT-PCR. Of the 112 suspected CPV infection samples, 49 were positive by colloidal gold, 64 were positive by PCR and 78 were positive by real-time PCR and DARPM. Among the 63 samples that were colloidal gold-negative, only 48 were PCR-negative. Only 34 of the 63 negative samples were negative by DARPM. The same numbers of positive and negative samples were obtained by DARPM and real-time PCR for the 112 suspected CPV samples. These findings show that colloidal gold and standard PCR methods readily generate false-negative results. Among the 97 suspected CDV samples, all 31 colloidal gold-positive samples were positive by DARPM. The 43 RT-PCR-positive samples were also positive by DARPM. The positive detection results for real-time RT-PCR and DARPM match perfectly with the 97 suspected CDV infection samples, 60 samples were detected positive by both methods. Among the 66 samples that were colloidal gold-negative, 54 were negative by RT-PCR, but only 37 were negative by DARPM. Therefore, 37 samples were negative by DARPM and real-time RT-PCR. In summary, of the 112 CPV and 97 CDV samples suspected of harboring such viruses, DARPM and real-time PCR/RT-PCR had higher detection rates (69.64% for CPV and

61.86% for CDV) than the rapid colloidal gold test strip and conventional PCR/RT-PCR.

DISCUSSION

CPV and CDV cause high mortality diseases in dogs. Currently, PCR/RT-PCR and the colloidal gold rapid detection kit are the preferred methods for CPV and CDV detection in China. However, traditional PCR/RT-PCR requires multiple steps and has poor species specificity (Wilson 1997; Dildar *et al*., 2018); while colloidal gold rapid detection can easily generate false results (Uwatoko *et al*., 1995). Real-time PCR/RT-PCR is an attractive alternative to conventional PCR/RT-PCR for determining viral loads and has low inter- and intra-assay variability (Mackay *et al*., 2002). The analytical sensitivity of real-time PCR/RT-PCR is higher than that of traditional virus culture, immunofluorescence, conventional single-round PCR/RT-PCR and nested PCR/RT-PCR. TaqMan real-time PCR/RT-PCR has been widely used to detect virus infections, such as canine coronavirus (Francino *et al*., 2006), feline parvoviruses (Streck *et al*., 2013), CPV and CDV (Decaro *et al*., 2005; Wilkes *et al*., 2014).

In recent years, TaqMan real-time PCR/RT-PCR has demonstrated its abilities for detecting viruses without prior nucleic acid extraction from clinical samples, making it a rapid and straightforward method to use. Nakamichi *et al*. (2011) used a real-time PCR assay to detect the John Cunningham polyomavirus without nucleic acid extraction. Nie *et al*. (2012) used a direct reverse transcription loop-mediated isothermal amplification method without RNA extraction to detect human enterovirus. Vuong *et al*. (2016) used TaqMan real-time PCR without nucleic acid extraction to detect bacterial meningitis pathogens. These three studies all show that using real-time PCR/RT-PCR to detect pathogens without prior nucleic acid extraction of the sample is feasible for clinical samples.

In common with previous studies that have sought to avoid nucleic acid extraction of samples, the DARPM assay we developed to detect CPV and CDV infection in dogs requires no nuclear acid extraction step. Our study has shown that real-time (RT-)PCR had high sensitivity, specificity and repeatability. The sensitivity between DARPM and real-time (RT)-PCR using virus culture supernatant and nucleic acids to detect CPV or CDV revealed the minimum detectable CPV DNA number to be 1.53×10^{1} copies·μL^{-1} and virus culture supernatant minimum detectable amount was 6.70×10^{1} copies·μL^{-1}, the minimum detectable CDV cDNA amount and virus culture supernatant minimum detectable amount were 9.56×10^{1} copies·μL^{-1} and 7.77×10^{1} copies·μL^{-1}, respectively. In our study, we found that DARPM had good detection limit when compared with others studies using nucleic acid extraction method to detect CDV and CPV. In previous studies, Streck *et al*. (2013) established an updated TaqMan real-time PCR for CPV diagnose, the detection limitation was 1×10^{1} copies of the viral genome per μL after nucleic acid extraction. Elia *et al.* (2006) established a real-time RT-PCR method to detect CDV and the detection limitation was up to1×10^{2} copies per μL after nucleic acid extraction. These results suggest that the DARPM is feasible method for quantification of CPV and CDV genome. The minimum detectable amount of CDV and CPV by DARPM and real-time PCR/RT-PCR are both $< 1\times10^{2}$ copies·μL^{-1}.

We further used DARPM and three common methods to investigate whether 112 clinical samples harbored CPV and 97 harbored CDV. Our results showed DARPM had a higher positive detection rate for CPV (78/112, 69.64%) and CDV (60/97, 61.86%) than colloidal gold (49/112 and 31/97, 43.75% and 31.96%) and (RT-) PCR (64/112 and 43/97, 57.14% and 44.32%). DARPM and real-time (RT-) PCR had the same detection rates, sensitivities and accuracies. These results indicated that DARPM can be directly used in clinical detection for CPV and CDV. This is the first study to use sample supernatants directly without extracting nucleic acids to detect CDV and CPV by TaqMan real-time PCR/RT-PCR.

CONCLUSION

In conclusion, we developed and evaluated DARPM as a method to detect CDV and CPV pathogens in clinical samples directly without the need for nucleic acid extraction. DARPM showed a consistent 100% positivity rate with ordinary PCR and colloidal gold rapid detection, and the coincidence rate for DARPM and the nucleic acid method was 100%. Sensitivity and specificity tests showed the newly established method could potentially be used for testing clinical samples suspected to contain CDV and CPV. Because the detection process does not need to use extracted nucleic acid from samples, it is will be easy to employ as a clinical diagnostic method.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31272620), the Key Project of the Education Department in Sichuan, China (13ZA0263) and the Chengdu Giant Panda Breeding Research Foundation (CPF2015-4).

Supplementary material

There is supplementary material associated with

this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1843.1852

Statement of conflict of interest

The authors declare no conflict of interest.

REFERENCES

Baethgen, L.F., Moraes, C., Weidlich, L., Rios, S., Kmetzsch, C.I., Silva, M.S., Rossetti, M.L. and Zaha, A., 2003. Direct-test PCR for detection of meningococcal DNA and its serogroup characterization: standardization and adaptation for use in a public health laboratory. *J. med. Microbiol*., **52**: 793-799. https://doi.org/10.1099/jmm.0.05192-0

Bi, Z., Wang, Y., Wang, X. and Xia, X., 2015. Phylogenetic analysis of canine distemper virus in domestic dogs in Nanjing, China. *Arch. Virol*., **160**: 523-527. https://doi.org/10.1007/s00705-014-2293-y

Bista, B., Ishwad, C., Manna, P., Randhawa, P., Gupta, G., Adhikari, M., Tyagi, R., Gasper, G. and Vats, A., 2007. Development of a loop-mediated isothermal amplification assay for rapid detection of BK virus. *J. clin. Microbiol*., **45**: 1581-1587. https://doi.org/10.1128/JCM.01024-06

Cardoso, M.A., Cardoso, R.F., Hirata, R.D.C., Hirata, M.H., Leite, C.Q.F., Santos, A.C.B., Siqueira, V.L.D., Okano, W., Rocha, N.S. and Lonardoni, M.V.C., 2009. Direct detection of *Mycobacterium bovis* in bovine lymph nodes by PCR. *Zoon. Publ. Hlth*., **56**: 465-470. https://doi.org/10.1111/j.1863-2378.2008.01199.x

Castanheira, P., Duarte, A., Gil, S., Cartaxeiro, C., Malta, M., Vieira, S. and Tavares, L., 2014. Molecular and serological surveillance of canine enteric viruses in stray dogs from Vila do Maio, Cape Verde. *BMC Vet. Res*., **10**: 91-91. https://doi.org/10.1186/1746-6148-10-91

Decaro, N., Elia, G., Martella, V., Desario, C., Campolo, M., Trani, L.D., Tarsitano, E., Tempesta, M. and Buonavoglia, C., 2005. A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. *Vet. Microbiol*., **105**: 19-28. https://doi.org/10.1016/j.vetmic.2004.09.018

Decaro, N., Martella, V., Elia, G., Campolo, M., Desario, C., Cirone, F., Tempesta, M. and Buonavoglia, C., 2007a. Molecular characterisation of the virulent canine coronavirus CB/05 strain. *Virus Res*., **125**: 54-60. https://doi.org/10.1016/j.virusres.2006.12.006

Decaro, N., Desario, C., Addie, D.D., Martella, V., Vieira, M.J., Elia, G., Zicola, A., Davis, C., Thompson, G., Thiry, E., Truyen, U. and Buonavoglia, C., 2007b. Molecular Epidemiology of canine parvovirus, Europe. *Emerg. Infect. Dis*., **13**: 1222-1224. https://doi.org/10.3201/eid1308.070505

Dildar, H.K.,. Shan, L., Muhammad, S.K., Shoaib, A.P., Nasir, R., Muhammad, N., Fahmida, P. and Yongjie, L., 2018. Identification and genetic evolution analysis of one strain of H3N2 canine influenza virus isolated from Nanjing, China. *Pakistan J. Zool.,* **50**: 817-824. http://dx.doi.org/10.17582/journal.pjz/2018.50.3.817.824

Elia, G., Decaro, N., Martella, V., Cirone, F., Lucente, M.S., Lorusso, E., Trani, L.D. and Buonavoglia, C., 2006. Detection of canine distemper virus in dogs by real-time RT-PCR. *J. Virol. Meth.*, **136**: 171-176. https://doi.org/10.1016/j.jviromet.2006.05.004

Francino, O., Altet, L., Nchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Nchez, A. and Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. *Vet. Parasitol*., **137**: 214-221. https://doi.org/10.1016/j.vetpar.2006.01.011

Guo, L., Yang, S.L., Wang, C.D., Hou, R., Chen, S.J., Yang, X.N., Liu, J., Pan, H.B., Hao, Z.X. and Zhang, M.L., 2013. Phylogenetic analysis of the haemagglutinin gene of canine distemper virus strains detected from giant panda and raccoon dogs in China. *Virol. J*., **10**: 1-7. https://doi.org/10.1186/1743-422X-10-109

Kalinowski, M., Adaszek, L., Miłoszowska, P., Skrzypczak, M., Zietekbarszcz, A., Kutrzuba, J., Gradzki, Z. and Winiarczyk, S., 2012. Molecular analysis of a fragment of gene E1B 19K of canine adenovirus 2 (CAV-2) isolated from dogs with symptoms of cough. *Polish J. Vet. Sci*., **15**: 425-430. https://doi.org/10.2478/v10181-012-0066-7

Kouji, S., Noriyo, N., Yasushi, A., Fumio, S., Yuriko, S., Naoko, I.Y., Tadaki, S., Shuetsu, F., Tetsuya, M. and Tomoki, Y., 2013. Lethal canine distemper virus outbreak in cynomolgus monkeys in Japan in 2008. *J. Virol*., **87**: 1105-1114. https://doi.org/10.1128/JVI.02419-12

Kumar, M. and Nandi, S., 2010. Molecular typing of canine parvovirus variants by polymerase chain reaction and restriction enzyme analysis. *Transbound. Emerg. Dis*., **57**: 458-463. https://doi.org/10.1111/j.1865-1682.2010.01167.x

Li, W., Li, T., Liu, Y., Gao, Y., Yang, S., Feng, N., Sun, H., Wang, S., Wang, L. and Bu, Z., 2014. Genetic characterization of an isolate of canine distemper

virus from a Tibetan Mastiff in China. *Virus Genes*, **49**: 45-57. https://doi.org/10.1007/s11262-014-1062-z

Mackay, I.M., Arden, K.E. and Nitsche, A., 2002. Real-time PCR in virology. *Nucl. Acids Res*., **30**: 1292-1305. https://doi.org/10.1093/nar/30.6.1292

Madias, J.E., 2012. Effects of canine parvovirus strain variations on diagnostic test results and clinical management of enteritis in dogs. *J. Am. Vet. med. Assoc*., **241**: 66-72. https://doi.org/10.2460/javma.241.1.66

Martella, V., Elia, G., Lucente, M.S., Decaro, N., Lorusso, E., Banyai, K., Blixenkrone-Miller, M., Lan, N.T., Yamaguchi, R. and Cirone, F., 2007. Genotyping canine distemper virus (CDV) by a hemi-nested multiplex PCR provides a rapid approach for investigation of CDV outbreaks. *Vet. Microbiol*., **122**: 32-42. https://doi.org/10.1016/j.vetmic.2007.01.005

Meers, J., Kyaw-Tanner, M., Bensink, Z. and Zwijnenberg, R., 2007. Genetic analysis of canine parvovirus from dogs in Australia. *Austral. Vet. J*., **85**: 392-396. https://doi.org/10.1111/j.1751-0813.2007.00206.x

Mihalov-Kov, C.S.E., Gell, Á., Marton, S., Farkas, S.L., Feh, R.E., Oldal, M., Jakab, F., Martella, V. and Nyai, K., 2015. Candidate new rotavirus species in sheltered dogs, Hungary. *Emerg. Infect. Dis*., **21**: 660-663. https://doi.org/10.3201/eid2104.141370

Miranda, C. and Thompson, G., 2016. Canine parvovirus: The worldwide occurrence of antigenic variants. *J. Gen. Virol*., **97**: 2043-2057. https://doi.org/10.1099/jgv.0.000540

Nakamichi, K., Kurane, I. and Saijo, M., 2011. Evaluation of a quantitative real-time PCR assay for the detection of JC polyomavirus DNA in cerebrospinal fluid without nucleic acid extraction. *Japanese J. Infect. Dis*., **64**: 211-216.

Nie, K., Qi, S., Zhang, Y., Luo, L., Xie, Y., Yang, M., Zhang, Y., Li, J., Shen, H. and Li, Q., 2012. Evaluation of a direct reverse transcription loop-mediated isothermal amplification method without RNA extraction for the detection of human enterovirus 71 subgenotype C4 in nasopharyngeal swab specimens. *PLoS One*, **7**: 0052486. https://doi.org/10.1371/journal.pone.0052486

Nishimura, N., Nakayama, H., Yoshizumi, S., Miyoshi, M., Tonoike, H., Shirasaki, Y., Kojima, K. and Ishida, S., 2010. Detection of noroviruses in fecal specimens by direct RT-PCR without RNA purification. *J. Virol. Meth.*, **163**: 282-286. https://doi.org/10.1016/j.jviromet.2009.10.011

Organtini, L.J., Allison, A.B., Tiit, L., Parrish, C.R. and Susan, H., 2015. Global displacement of canine parvovirus by a host-adapted variant: structural comparison between pandemic viruses with distinct host ranges. *J. Virol*., **89**: 1909-1912. https://doi.org/10.1128/JVI.02611-14

Perez, R., Calleros, L., Marandino, A., Sarute, N., Iraola, G., Grecco, S., Blanc, H., Vignuzzi, M., Isakov, O. and Shomron, N., 2014. Phylogenetic and genome-wide deep-sequencing analyses of canine parvovirus reveal co-infection with field variants and emergence of a recent recombinant strain. *PLoS One*, **9**: e111779. https://doi.org/10.1371/journal.pone.0111779

Poon, L.L., Wong, B.W., Ma, E.H., Chan, K.H., Chow, LM., Abeyewickreme, W., Tangpukdee, N., Yuen, K.Y., Guan, Y., Looareesuwan, S. and Peiris, J.S., 2006. Sensitive and inexpensive molecular test for falciparum malaria: Detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin. Chem*., **52**: 303-306. https://doi.org/10.1373/clinchem.2005.057901

Qiu, W., Zheng, Y., Zhang, S., Fan, Q., Liu, H., Zhang, F., Wang, W., Liao, G. and Hu, R., 2011. Canine distemper outbreak in rhesus monkeys, China. *Emerg. Infect. Dis*., **17**: 1541-1543. https://doi.org/10.3201/eid1708.101153

Sanchez-Cordon, P.J., Salguero, F.J., Nunez, A., Gomez-Villamandos, J.C. and Carrasco, L., 2002. Glomerulonephritis associated with simultaneous canine adenovirus-1 and dirofilaria immitis infection in a dog. *J. Vet. Med. B: Infect. Dis. Vet. Publ. Hlth*., **49**: 235-239. https://doi.org/10.1046/j.1439-0450.2002.00554.x

Silva, A.P., Bodnar, L., Headley, S.A., Alfieri, A.F. and Alfieri, A.A., 2014. Molecular detection of canine distemper virus (CDV), canine adenovirus A type 1 and 2 (CAdV-1 and CAdV-2), and canine parvovirus type 2 (CPV-2) in the urine of naturally infected dogs. *Sem. Cienc. Agrar*., **35**: 1970-1977. https://doi.org/10.5433/1679-0359.2014v35n6p3231

Steinel, A., Venter, E.H., Van, V.M., Parrish, C.R. and Truyen, U., 1998. Antigenic and genetic analysis of canine parvoviruses in southern Africa. *Onderstepoort J. Vet. Res*., **65**: 239-242.

Streck, A.F., Ruster, D., Truyen, U. and Homeier, T., 2013. An updated TaqMan real-time PCR for canine and feline parvoviruses. *J. Virol. Meth.*, **193**: 6-8. https://doi.org/10.1016/j.jviromet.2013.04.025

Sun, Z., Li, A., Ye, H., Shi, Y., Hu, Z. and Lin, Z., 2009. Natural infection with canine distemper virus in hand-feeding Rhesus monkeys in China. *Vet. Microbiol*., **141**: 374-378. https://doi.org/10.1016/j.vetmic.2009.09.024

Tan, B., Wen, Y.J., Wang, F.X., Zhang, S.Q., Wang, X.D., Hu, J.X., Shi, X.C., Yang, B.C., Chen, L.Z. and Cheng, S.P., 2011. Pathogenesis and phylogenetic analyses of canine distemper virus strain ZJ7 isolate from domestic dogs in China. *Virol. J*., **8**: 1-9. https://doi.org/10.1186/1743-422X-8-520

Uwatoko, K., Sunairi, M., Nakajima, M. and Yamaura, K., 1995. Rapid method utilizing the polymerase chain reaction for detection of canine parvovirus in feces of diarrheic dogs. *Vet. Microbiol*., **43**: 315-323. https://doi.org/10.1016/0378-1135(94)00102-3

Victor, S.T., Lezhava, A., Ishidao, T., Endo, R., Mitani, Y., Kawaoka, Y. and Hayashizaki, Y., 2009. Isothermal single nucleotide polymorphism genotyping and direct PCR from whole blood using a novel whole-blood lysis buffer. *Mol. Diagn. Ther*., **13**: 383-387. https://doi.org/10.1007/BF03256344

Vuong, J., Collard, J.M., Whaley, M.J., Bassira, I., Seidou, I., Diarra, S., Ouedraogo, R.T., Kambir, D., Taylor, Jr. T.H. and Sacchi, C., 2016. Development of real-time PCR methods for the detection of bacterial meningitis pathogens without DNA extraction. *PLoS One*, **11**: 0147765. https://doi.org/10.1371/journal.pone.0147765

Whelan, J.A., Russell, N.B. and Whelan, M.A., 2003. A method for the absolute quantification of cDNA using real-time PCR. *J. Immunol. Meth*., **278**: 261-269. https://doi.org/10.1016/S0022-1759(03)00223-0

Wilkes, R.P., Sanchez, E., Riley, M.C. and Kennedy, M.A., 2014. Real-time reverse transcription polymerase chain reaction method for detection of Canine distemper virus modified live vaccine shedding for differentiation from infection with wild-type strains. *J. Vet. Diagn. Investig. Offic. Publ. Am. Assoc. Vet. Lab. Diagnost. Inc*., **26**: 27-34. https://doi.org/10.1177/1040638713517232

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. environ. Microbiol*., **63**: 3741-3751.

Wu, L., Sun, Y., Xu, C., Li, C. and Xia, C., 2015. Molecular cloning and sequence analysis of hemagglutinin gene of a novel strain canine distemper virus. *Int. J. appl. Res. Vet. Med*., **13**: 36-41.

Zhang, R., Yang, S., Zhang, W., Zhang, T., Xie, Z., Feng, H., Wang, S. and Xia, X., 2010. Phylogenetic analysis of the VP2 gene of canine parvoviruses circulating in China. *Virus Genes*, **40**: 397-402. https://doi.org/10.1007/s11262-010-0466-7

Zhao, H., Wang, J., Jiang, Y., Cheng, Y., Lin, P., Zhu, H., Han, G., Yi, L., Zhang, S. and Guo, L., 2015. Typing of canine parvovirus strains circulating in north-east China. *Transbound. Emerg. Dis*., **64**: 495-503. https://doi.org/10.1111/tbed.12390

Zhijun, Z., Luqi, L., Juan, Z., Xiaoyang, X., Xuefeng, C., Xuehan, L., Ziyao, Z., Zhihua, R., Liuhong, S. and Yi, G., 2014. First isolation of new canine parvovirus 2a from Tibetan mastiff and global analysis of the full-length VP2 gene of canine parvoviruses 2 in China. *Int. J. mol. Sci*., **15**: 12166-12187. https://doi.org/10.3390/ijms150712166

Zhu, Y., Huang, Y., Wang, Y., Chen, K., Niu, X., Luo, Y. and Guo, X., 2014. Genome sequence of a canine parvovirus strain, CPV-s5, prevalent in Southern China. *Genome Announc*., **2**: e01141-13. https://doi.org/10.1128/genomeA.01141-13