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Expression of Major Capsid Protein of Canine Parvovirus by Yeast (*Pichia pastoris*) and Efficient Purification using Arginine in Affinity Chromatography

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ABSTRACT

An immunochromatographic (IC) assay was developed for rapid detection of canine parvovirus using the monoclonal antibodies (McAbs) against canine parvovirus (CPV-2). To prepare the McAbs, gene encoding the VP2 protein of CPV-2a was expressed in a *Pichia pastoris* expression vector pPICZ-A. The recombinant VP2 was similar antigenically function to the native capsid protein as demonstrated by Western blotting using CPV-2 polyclonal antiserum. McAbs against CPV-2 were produced by fusing myeloma cell line SP2/O with spleen cells from Balb/C mice immunized with purified recombinant VP2 protein. By ELISA it was shown that the McAbs specifically recognized VP2 epitopes of CPV-2 but not those of other canine viruses such as Canine distemper virus (CDV) or canine adenovirus (CAV). An IC assay developed with the McAbs was suitable for rapid detection of canine parvovirus. Fecal samples (120) from dogs suspected of CPV-2 infection were analyzed by both haemagglutination (HA) assay and the IC assay, and 52 and 53 samples were found positive for CPV-2, respectively. Comparison between the two different assays revealed that IC assay is as sensitive as HA; the sensitivity and specificity for the IC assay is 98.6% and 98.1%, respectively.

INTRODUCTION

Canine parvovirus type 2 (CPV-2) emerged as a new pathogen causes acute hemorrhagic enteric diseases in young dogs^[1]. CPV-2 infection with its high rate of mortality in puppies has become an important disease of dogs. CPV-2 is a small non-enveloped virus with a single-stranded, negative-sense DNA genome. The genome DNA is approximately 5.2 kb^[2] encoding for two structural proteins VP1 and VP2, and two nonstructural proteins NS1 and NS2^[3]. VP2 is the major capsid protein containing the main antigenic determinants and also playing an important role in determining virus pathogenicity^[4-7]. VP2 has been shown to be useful for diagnosis and immunization against canine parvovirus disease.

Many sensitive molecular methods for detection of CPV-2 infection have been developed, including PCRs^[8,9], nested PCR^[10] and real-time PCR^[11-14]. Those are not suitable for detection by farmers. Immunological methods are less sensitive than PCR methods, but they provide a field-friendly, inexpensive way to detect and confirm causative pathogens during disease outbreaks with high accuracy in a manner (e.g., test strips) suitable for use by unskilled personnel^[15,16]. In this study, we successfully developed a simple, rapid, yet very sensitive immunochromatographic (IC) test strip for detection of CPV-2 infection by using monoclonal antibodies (McAbs) specific for the CPV-2 capsid protein prepared with recombinant VP2 protein of CPV-2.

MATERIALS AND METHODS

Virus, cells, mice and samples

The B2004 strain of canine parvovirus was from our laboratory stock, which was isolated from the stool of a sick dog in Beijing^[17]. Its genomic sequence (accession No. EF011664) indicated that B2004 is a member of a widely distributed CPV-2a subclade^[17]. FK81 (feline kidney cell line) cells from “the Clinical Central of the Agricultural Ministry” were grown in DMEM containing 10% fetal bovine serum. Sp2/0 (mice myeloma) cells were stored in our laboratory. The clean female BALB/c mice (6 weeks old) were purchased from Beijing Mofcom Technology Company of Laboratory Animal.

The 120 fecal samples were collected from 120 dogs with gastroenteritis. The swabs used to collect the samples from canine feces were inserted into the specimen tube containing 1 ml of assay diluent. Swab was washed well with the assay diluent to extract the samples. 113 of them were from one to five-month-old pups.

Preparation of yeast (*Pichia pastoris*) expressed recombinant VP2 protein

Construction of recombinant plasmid pPICZ-VP2 for *Pichia pastoris*

Viral genomic DNA was extracted from infected cells using Viral Genome Extraction kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. The full length VP2 gene was amplified by PCR, using two primers (Uvp2:5'CATCGAATTCAG AATAATGTCTATGAGTGATGGAGCAGTT3'(EcoR I), Lvp2: 5'GCCCGTCGACATATAATTTTCTAGGTGTAG3'(Xho I)). The PCR product was gel-purified using Qiaquick Gel Extraction Kit (Qiagen, Shanghai, China) and cloned into pPICZ-A vector (pPICZ-A Expression Kit, Invitrogen, Carlsbad, USA), according to manufacturer's protocol. Recombinant positive clones were verified by sequencing.

P. pastoris transformation and phenotype selection

All methods for *P. pastoris* (X-33) transformation and selection followed recommendation by the Easy Select *Pichia* Expression kit (Invitrogen, Carlsbad, USA). Transformants were subjected to 100 µg/mL zeocin selection on Yeast extract (1%) Peptone (2%) Dextrose (2%) Sorbitol (1M) [YPDS] agar for two to three days at 28°C. Multiple copies of the gene of interest were inserted into the yeast genome via homologous recombination, and transformants were screened for multicopy integration by streaking on YPDS agar containing 500 µg/mL and 1000 µg/mL zeocin.

Recombinant protein expression

Small scale expression trials were carried out by seeding 5 mL cultures grown in buffered glycerol-complex (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base, 0.002% biotin, 1% glycerol) for 12-16 hours at 28°C with shaking at 250 rpm. Once an OD₆₀₀ 5.0 was reached, cells were pelleted at 1500 Xg and washed once with buffered methanol-complex (BMMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base, 0.002% biotin, 0.5% methanol) before resuspension in the same medium to give an OD₆₀₀ 1.0. Methanol induction of recombinant VP2 protein expression was carried out for up to 72 hours at 28°C and 0.5% methanol was supplemented every 24 hours.

SDS-PAGE and Western blot analysis

Following methanol induction, secreted proteins were precipitated by 10% trichloroacetic acid. Protein samples were adjusted to 1x reduced sample buffer, heated at 95°C for 5 minutes and separated on 13% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) against Precision Plus protein standards (Bio-rad, Hercules California). Samples were electrophoresed in a BioRad Mini Protein II apparatus at 180 V for 45 minutes before staining with Page Blue. For western blot, the gel-resolved proteins were electrotransferred to polyvinylidene difluoride (Pall; East Hills, New York) membranes in a BioRad semi-dry transfer apparatus at 15 V for 30 minutes. Membranes were immediately blocked with 5% skim milk powder in Tris buffered saline-tween 20 (TBS-T) buffer (0.02 M Tris, 137 mM sodium chloride, 0.1% Tween20, pH 7.6) with gentle shaking for one hour at 25°C. Membranes were washed 3 × 5 minutes with TBS-T and incubated with primary antibody, washed again; binding of the primary antibody was detected with goat anti-mouse horse radish peroxidase (1:10000). The membranes were washed a final time and the HRP was activated with the SuperSignal West Pico chemiluminescent kit (PIERCE, Rockford, USA) according to the manufacturer's instructions. Membranes were wrapped in a single layer of cling film and exposed onto CL-XPosure film (Pierce, Rockford, USA) for 1-30 minutes. Protein signals were developed in GBX developer and in replenisher solution for <1 minute with agitation and excess solution was washed off with tap water. Films were then fixed in the GBX fixer and replenisher solution, and finally washed with water and allowed to dry.

Expression and purification of recombinant his-tagged VP2 in large scale

Yeast transformants were grown overnight in BMGY medium to OD₆₀₀ 5.0 at 28°C to generate biomass, then the yeast cells were washed before 0.5% methanol induction in BMMY medium at 20°C for 48 hours for secreted expression. At the completion of protein expression, secreted his-tagged VP2 was concentrated from BMMY medium by 70% ammonium sulfate precipitation. Protein pellets were collected by centrifugation at 20,000 g and 4°C for 30 minutes and then gently resuspended in PBS by a combination of soaking and pipetting. VP2 rich samples were dialysed against chromatography equilibration buffer to remove

excess ammonium sulfate, His Select affinity purification was carried out according to manufacturer's instructions. Pure his-tagged VP2 eluted from the column was buffer exchanged with PBS and concentrated using an Amicon stirred cell 8050 fitted with a 3K MWCO membrane (Pal I). VP2 purification was monitored by SDS-PAGE. The concentration of protein samples in PBS was determined using the BCA Protein Estimation kit (Pierce, Rockford, USA) following the manufacturer's instruction.

Preparation of monoclonal antibodies

BALB/C mice were immunized with the purified recombinant his-tagged VP2 proteins in complete Freund's adjuvant. Aliquots of 200 µg of purified protein were injected intraperitoneally 30 and 60 days later. Mice were euthanized 5 days later and spleens were removed. Spleen cells (6×10^8) from immunized mice were fused with SP-2/O cells (6×10^7). After fusion the cells were diluted in the HAT (hypoxanthine/aminopterin/thymidine) medium and distributed in 0.1 ml aliquots to the wells of 96 well microtitre plates. Hybridoma clones that produced antigen-specific antibody were screened by ELISA. Antibody-secreting tumours were produced by subcutaneous inoculation of 3-5 month-old Balb/c mice with approximately 5×10^6 hybrid cells. Animals were tail-bled when tumours became palpable and exsanguinated between 3-5 days later. The tumours were removed, minced and frozen in liquid N₂. The ascites were collected and centrifuged with 20000 g for 15 minutes. The McAbs in the conditioned medium were purified using a protein G-agarose column according to the manufacturer's instructions (Roche). The purity of monoclonal antibody was checked by SDS-PAGE. The antibodies were dialyzed against phosphate buffer (PB: 10 mM phosphate buffer, pH 7.3), and the concentration was adjusted to 1 mg/ml.

Preparation and trial of IC test strip

IC test strip was prepared as **Figure 1**. First, one McAb was conjugated to colloidal gold (diameter=10 nm) and sprayed onto a glass fiber pad at 2 µg/cm before being dried at 40°C overnight. Secondly, another McAb (at 0.5-1 mg/ml) was sprayed onto the nitrocellulose membrane at a position that would become the capture test line (T) near the middle of the membrane (oriented left to right). Goat anti-mouse IgG antibody (0.8 mg/ml) was sprayed onto the same nitrocellulose membrane at the position that would become the strip capture control line (C). The primed membrane was then dried at 20°C overnight. For membrane assembly, the dried glass fiber pad containing McAb conjugated with colloidal gold was attached to the primed nitrocellulose membrane approximately 5 mm to the left (downstream) of the T line and upstream of a sample pad attached at the extreme left of the primed membrane. At the extreme right of the primed membrane, upstream from the C line, an absorption pad was attached for collection of excess sample liquid and to promote sample flow over the membrane from the point of application. The completed membrane assembly was then cut into 4.5 mm wide strips that were housed individually in plastic cassettes that were stored desiccated in plastic bags until use.

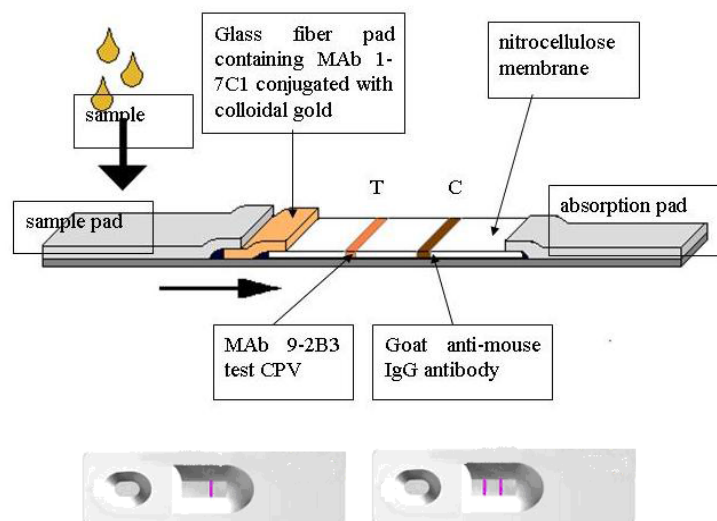


Figure 1. a. Sketch map of preparation of IC test strip; b. The presence of only one band within the result window indicates a negative result; c. The presence of two color bands ("T" and "C") within the result window indicates a positive result.

The swabs used to collect the samples from canine feces were inserted into the specimen tube containing 1 ml of assay diluent. Swab was washed well with the assay diluent to extract the samples. Four drops of a sample were applied into the sample hole using the disposable dropper. For best result, 5 diarrhea feces in the farm were tested. Test results were inspected at 5-10 minutes. The presence of only one band within the result window indicated a negative result (**Figure 1b**). The presence of two color bands ("T" and "C") within the result window, no matter which band appears first, indicated a positive result (**Figure 1c**).

Hemagglutination (HA) assay

Two-fold dilutions of the clarified fecal samples were made in PBS (pH7.2) starting from a 1:2 dilution. Tests were carried out in 96 well V-plates, using 0.8% pig erythrocytes in PBS. The results were read after 4 hrs at 4°C.

RESULTS

Expression and analysis of recombinant VP2

Recombinant VP2 protein expression was analyzed by SDS-PAGE. As expected, the molecular weight of the protein was approximately 68 KD (**Figure 2a**). Western blot analysis using anti-His McAbs and polyclonal serum against CPV-2 (gift by Prof. Sun) confirmed the authenticity of the protein band (**Figures 2b and 2c**). Thus the his-tagged VP2 was successfully expressed as judged by appropriate molecular size.

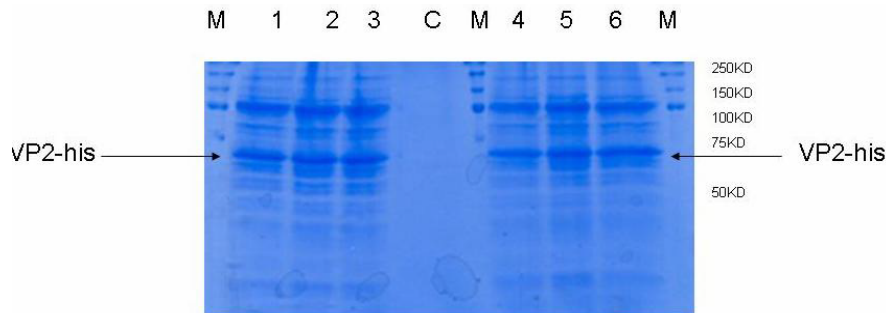


Figure 2a. SDS-PAGE analysis of recombinant his-tagged VP2 expressed in *P. pastoris* X-33 following methanol induction. Secreted proteins were precipitated by 10% trichloroacetic acid. Lane M: protein molecular weight marker; lane C: blank; lane 1, 4: methanol induction 24 hrs; lane 2,5: methanol induction 48 hrs; lane3,6: methanol induction 72 hrs.

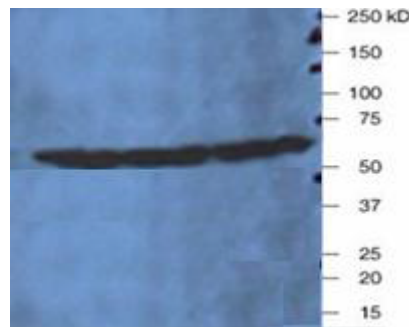


Figure 2b. Western blot analysis of recombinant VP2 using McAb against his-tag.

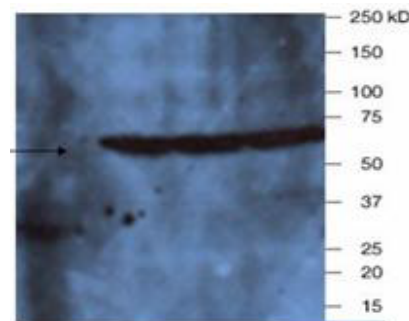


Figure 2c. Western blot analysis of recombinant VP2 using specific McAb against CPV.

Large scale expression and purification protocols for his-tagged VP2 were optimized. The recombinant VP2 was expressed in YP with 0.5% methanol (pH 8) for 48 hrs at 28°C with 250 rpm shaking. The secreted his-tagged VP2 was concentrated from BMMY medium by 70% ammonium sulfate precipitation followed by His Select affinity purification (**Figure 2d**). High yield of recombinant VP2 (3 mg/L) were obtained.

Preparation of monoclonal antibodies

In order to generate mouse monoclonal antibodies that recognize VP2, we used purified his-tagged VP2 as immunogen. Antigen-specific antibodies were screened repeatedly by ELISA, resulting in 27 antibodies and 11 positive strains of hybridoma cell line by their strong immunoreactivity against CPV-2.

Four IgG type subclasses of positive hybridoma cell line were identified from the 11 positive strains selected (**Table 1**). The four corresponding antibodies were named 1 (1-7A7), 3 (1-7C1), 23 (8-3A4), and 26 (9-2B3). The McAbs (IgG) produced by the 4 strains of cell lines were purified by G-protein from ascites, and antibody titers in hybridoma cell supernatant or ascites were determined (**Table 2**). SDS-PAGE analysis showed that antibodies were highly purified (**Figure 3**).

Indirect ELISA test showed that 4 McAbs reacted specifically only with supernatants of VP2 or CPV infected cells, not with that of Canine distemper virus (CDV), canine adenovirus (CAV), SP2-20-infected cells and oligomeric histidine.

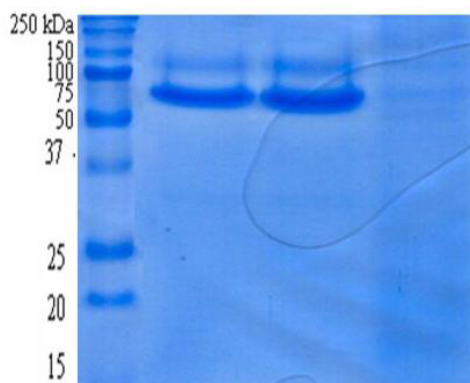


Figure 2d. SDS-PAGE analysis of purified recombinant VP2 using histidine affinity column. Lane1: protein marker; lane2: eluted his-tagged VP2; lane3: protein after dialysis; lane 4: column flow.

Table 1. Detection of antibody titers in hybridoma cell supernatant.

Monoclonal antibody	subclass	Antibody titer of culture supernatant	Antibody titer of accites
1 1-7A7	IgG2b	1: 256	1: 51200
3 1-7C1	IgG1	1: 512	1: 204800
23 8-3A4	IgG2b	1: 256	1: 51200
26 9-2B3	IgG1	1: 512	1: 204800

Table 2. The test result by HA and IC.

Test result (n=120)		
	positive	negative
HA	52	68
immuno-chromatographic test	53	67

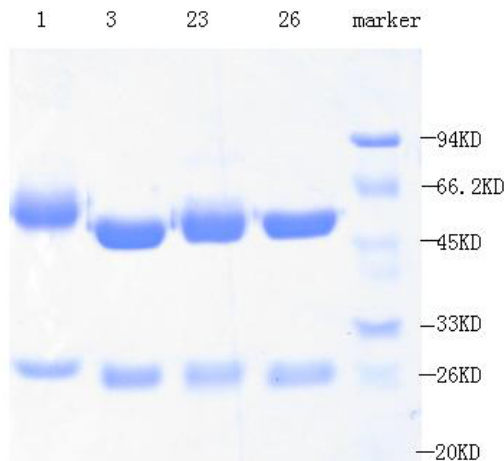


Figure 3. SDS-PAGE of purification result of ascites monoclonal antibodies in mice. M. Protein molecule weight Marker; Purified ascites monoclonal antibodies (1,3,23,26)

Test strip optimization

In final optimization for a strip test, McAb 1-7C1 (12 µg/ml) conjugated colloidal gold was sprayed onto the glass fiber pad of each strip at 5 µl/cm. For the test (T) and control (C) lines, McAb 9-2B3 (0.5 mg/ml) and Goat anti-mouse IgG antibody (0.8 mg/ml) were sprayed at 3 µl/cm onto nitrocellulose membrane respectively. These concentrations gave the highest intensity and sensitivity for diluted, infected samples without false positive for uninfected samples.

Specificity and sensitivity of the strip test

When samples prepared from feces containing CDV, CAV and subjected to the strip test, only a single band at C was observed, indicating no cross-reaction with other viruses sometimes concurrently infecting canines with CPV-2.

120 fecal samples from dogs were tested by both the IC assay and HA. The results show that 52 and 53 samples were found positive for CPV-2, respectively; and the sensitivity and specificity of the IC assay were 98.6% and 98.1%, respectively (**Table 1**).

DISCUSSION

A rapid detection of CPV infection is especially important in kennels and shelters in order to isolate infected dogs and to prevent secondary infections of susceptible contact animals. Tests detecting viral antigens by means of antibody-based methods are suitable for CPV diagnosis in the veterinary practice^[15, 18, 19]. An IC (IC) test that is easier to perform and provides results more quickly (in about five minutes) should be useful for CPV detection.

In the present study, a recombinant VP2 similar antigenetically to the native capsid protein was expressed to produce McAbs against canine parvovirus. From those McAbs an IC test was prepared for rapid detecting of canine parvovirus. Fecal samples (120) from dogs suspected of CPV2 infection were analyzed parallel by both haemagglutination assay and the IC assay, 52 and 53 samples were found positive for CPV2 respectively. Comparison between the results of two different assays revealed that the IC assay was as sensitive as HA, and sensitivity and specificity were 98.6% and 98.1%, respectively.

Advantage of IC assay over HA is the easiness for use. Although HA test on feces is specific and sensitive^[20, 21], it cannot be carried out at the farm setting because the requirement of fresh erythrocytes. A simpler HA protocol, designated as slide agglutination test, has been proposed to detect all CPV variants in fecal and intestinal samples, but this test does not seem to overcome the limitations of the classical methods^[22].

High specificity and low sensitivity of the antigen-detection based assays have been recognized by recent study^[23] that compared the performances of three different commercially available kits: antibody-based tests for rapid detection of CPV antigens, PCR based detection, and immunoelectron microscopy. The study showed that the relative sensitivity of IC test did not exceed 50% of that by the nucleic acid-based methods; whereas the specificity IC was 100%^[14].

Park et al.^[16] expressed a His-tagged VP2 of Canine Parvovirus (CPV) VP2 in *E. coli*, and a polyclonal antisera raised in rabbit against *E. coli* expressed recombinant VP2 protein were compared with a commercial monoclonal antibody in testing of numerous feces of diarrhoeic dogs in clinics by immuno-dot blot assays. The polyclonal antisera were found to be more rapid, sensitive, but less specific than the monoclonal antibody.

Despite the lower sensitivity compared to PCR-based techniques, the IC strip test is much more convenient because it can be used by farmers at the pond side without special equipment.

CONCLUSION

An IC strip test for CPV-2 was developed using MAbs specific for VP2 of the CPV capsid protein. The detection sensitivity of this strip test was similar to that of HA. The simplicity and specificity of the strip test would make it suitable for confirmation of CPV infections in animals at the early stage of disease outbreaks.

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REFERENCES

1. Thomson GW and Gagnon AN. Canine gastroenteritis associated with a parvovirus-like agent 1978; *Can Vet J* 19: 346.
2. Reed AP, et al. Nucleotide sequence and genome organization of canine parvovirus. *J Virol* 1988; 62: 266-276.
3. Parrish CR. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. *Virology* 1991; 183: 195-205.
4. Horiuchi M, et al. Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses. *J Gen Virol* 1994; 75: 1319-1328.
5. Llamas-Saiz AL, et al. Structural analysis of a mutation in canine parvovirus which controls antigenicity and host range. *Virology* 1996; 225: 65-71.
6. Parker JS and Parrish CR. Canine parvovirus host range is determined by the specific conformation of an additional region of the capsid. *J Virol* 1997; 71: 9214-9222.
7. Strassheim ML, et al. Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. *Virology*. 1994; 198: 175-184.
8. Cho HS, et al. Detection of canine parvovirus in fecal samples using loop-mediated isothermal amplification. *J Vet Diagn Invest* 2006; 18: 81-84.
9. Senda M, et al. Detection by PCR of wild-type canine parvovirus which contaminates dog vaccines. *J Clin Microbiol* 1995; 33: 110-113.

10. Hirasawa T, et al. Sensitive detection of canine parvovirus DNA by the nested polymerase chain reaction. *Vet Microbiol* 1994; 41: 135-145.
11. Decaro N, et al. A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. *Vet Microbiol* 2005; 105: 19-28.
12. Elia G, et al. Detection of infectious canine parvovirus type 2 by mRNA real-time RT-PCR. *J Virol Methods* 2007; 146: 202-208.
13. Kumar M and Nandi S. 2010. Development of a SYBR Green based real-time PCR assay for detection and quantitation of canine parvovirus in faecal samples. *J Virol Methods* 2010; 169: 198-201.
14. , Almberg ES, et al. Use of real-time PCR to detect canine parvovirus in feces of free-ranging wolves. *J Wildl Dis* 2012; 48: 473-476.
15. Lacheretz A, et al. Diagnosis of canine parvovirus by rapid immunomigration on a membrane. *Vet Rec* 2003; 152: 48-50.
16. Park JS, et al. Immunodetection of Canine Parvovirus (CPV) in clinical samples by polyclonal antisera against CPV-VP2 protein expressed in *Escherichia coli* as an antigen. *J Virol Methods* 2007; 146: 281-287.
17. Ying H, et al. Expression or subcellular targeting of virus capsid proteins with cloning genome of a canine parvovirus from China. *Res Vet Sci* 2009; 87: 239-241.
18. Kumar M, et al. Development of a polyclonal antibody-based AC-ELISA and its comparison with PCR for diagnosis of canine parvovirus infection. *Viol Sin* 2010; 25: 352-360.
19. Mildbrand MM, et al. Rapid detection of canine parvovirus in feces using monoclonal antibodies and enzyme-linked immunosorbent assay. *Am J Vet Res* 1984; 45: 2281-2284.
20. Carmichael LE, et al. Hemagglutination by canine parvovirus: serologic studies and diagnostic applications. *Am J Vet Res* 1980; 41: 784-791.
21. Mathys A, et al. Hemagglutination with formalin-fixed erythrocytes for detection of canine parvovirus. *Am J Vet Res* 1983; 44: 150-151.
22. Marulappa SY and Kapil S. Simple tests for rapid detection of canine parvovirus antigen and canine parvovirus-specific antibodies. *Clin Vaccine Immunol* 2009; 16: 127-131.
23. Schmitz S, et al. Comparison of three rapid commercial Canine parvovirus antigen detection tests with electron microscopy and polymerase chain reaction. *J Vet Diagn Invest* 2009; 21: 344-345.