Full Length Research Paper

Development of an indirect enzyme-linked immunosorbent assay (ELISA) assay based on a recombinant truncated VP2 (tVP2) protein for the detection of canine parvovirus antibodies

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By removing the N-terminal hydrophobic sequence, truncated VP2 (tVP2) genes were cloned into the pET-32a (+) plasmid and subsequently expressed as His fusion proteins. The purified recombinant tVP2 proteins were specific to canine parvovirus (CPV), and one of them was used in an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of CPV antibodies. The minimum detection limit of this method was 1:1280. There was good agreement between tVP2-based indirect ELISA and the commercially available diagnostic kit. The results suggest that the recombinant tVP2 protein-based ELISA could be used to detect CPV antibodies.

Key words: Canine parvovirus, recombinant truncated VP2 (tVP2), enzyme-linked immunosorbent assay (ELISA), antibody detection.

INTRODUCTION

Canine parvovirus (CPV) is classified within the parvoviridae family. The CPV genome is a linear single-stranded DNA of approximately 5 kb, making it one of the smallest animal DNA viruses (Chapman and Rossmann, 1993). Dogs infected with CPV may develop acute bloody diarrhea, fever, and dehydration, and these symptoms are sometimes followed by shock and sudden death (Carman and Povey, 1985). CPV was recognized in 1978 and is now considered endemic in virtually all populations of domestic and wild canines. It causes substantial emotional hardships to the animal owner, and often causes significant suffering in infected dogs. In addition, treatment of this disease is often expensive. Therefore, it would be beneficial to develop a fast and available diagnostic method to evaluate the maternally derived antibodies and the active response to pup vaccination (Decaro and Buonavoglia, 2012).

The capsid of CPV is assembled from three viral proteins (VP1, VP2, and VP3). VP2 is the major capsid protein and consists of an eight-stranded anti-parallel β-barrel motif with four large insertions between β-strands. The insertions, called loops, contain many B-cell epitopes (Pratelli et al., 2001). Epitope-mapping experiments show that all of the epitopes generating neutralising antibodies are within VP2 (Strassheim et al., 1994). These virus-like particles could then be used as a diagnostic antigen to detect the antibodies produced by CPV infection or vaccination. The full length VP2 protein expressed in *Esche-*

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Table 1. Sequence of the primers used to amplify tVP2 segments.

Amplified region	Name	Primer sequence(5′-3′)	Size (bp)
306-397AA	VP2-306-P1	CGCGGATCCATAGGAGTTCAACAA	275
	VP2-306-P2	CCGCTCGAGTCTCTCAGGTGTTTC	
363-483AA	VP2-363-P2	TATGGATCCGGAGCGCAAACA	362

richia coli was potentially insoluble. Insoluble protein accumulates in inactive inclusion bodies. After isolation, solubilisation and refolding, inclusion bodies transform into active proteins *in vitro* (Desario et al., 2005). To improve the production and activity of recombinant proteins, soluble proteins need to be studied.

The diagnosis of CPV may be made based on characteristic internal lesions and a final diagnosis can be made by virus isolation and identification. However, these tests are very laborious and time-consuming. In recent years, the fluorescence quantitative real-time PCR assays have been developed for detection of CPV (Decaro et al., 2004), for characterisation of the antigenic type (Decaro et al., 2005) and for discrimination between vaccine and field strains (Decaro et al., 2006a, b; Decaro et al., 2007) although an ELISA test based on recombinant VP2 expressed in the baculovirus system has been recently developed (Elia et al., 2012). Although an ELISA test based on recombinant VP2 expressed in the baculovirus system has been recently developed (Elia et al., 2012). To date, the ELISA assays have been developed for antibody or antigen detection and the whole CPV virion usually acts as antigen for the detection of antibodies against CPV in the indirect ELISA assay (Bhanot et al., 2009; Iwaki and Hayashi, 2009; Kummitha et al., 2010). However, it is difficult to get the CPV virion.

The aim of this study was to extract the recombinant truncated proteins and develop a recombinant tVP2 protein-based ELISA. Compared with the CPV-ELISA, the protein-based ELISA assay is more economical and more convenient.

MATERIALS AND METHODS

Virus strains, serum samples and other biochemical reagents

Canine parvovirus was isolated from the faecal samples of infected dogs. The isolated strain was identified as new CPV-2a (Wang et al., 2011). 80 canine of 3 to 6 months age serum samples were used in this study, 46 serum samples were from immunity canine, and 34 of them from unimmunity canine. To evaluate the specificity of the ELISA, serum samples testing negative for rabies virus (RV), canine distemper virus (CDV) and *E. coli* were used. All the test serum above were collected and preserved at -80°C until use. Restriction endonucleases, polymerase, and DNA and protein weight markers were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian,

China). The plasmid pET-32a (+) was obtained from Novagen (Darmstadt, Germany). Mouse anti-his tag monoclonal antibody and HRP-labelled goat anti-mouse antibody was purchased from Sigma (St. Louis, Missouri, USA). HRP-Protein A was purchased from Boster bio-engineering Co., Ltd. (Wuhan, China). Prestained protein ladder was purchased from Fermentas International Inc. (Burlington, Canada). Ni-NTA His bind resin was obtained from Invitrogen (Carlsbad, California, USA). AxyPrep body fluid viral DNA/RNA miniprep kit was purchased from AXYGEN Scientific Co. (Central Avenue Union City, California, USA). CPV antibody ELISA kit was purchased from Rapidbio (RB) Co. (USA).

Plasmid construction

Genomic DNA was extracted from the cell-cultured CPV with the AXYGEN kit and was used as a template to amplify two truncated VP2 fragments by PCR. The sense strand primer included a *Bam*H I restriction site, and the antisense strand primer included a *Xho*I restriction site (Table 1). The templates were denatured at 94°C for 2 min, followed by 30 PCR amplification cycles (45 s at 94°C, 45 s at 66°C, and 72°C for 2 min) and a final extension at 72°C for 10 min. The PCR product and plasmid pET-32a (+) were both digested with *Bam*HI and *Xho*I, and then ligated with T4 DNA ligase. The constructs were transformed into *E. coli*, and transformed bacteria were identified with restriction enzyme digestion. Additional sequencing confirmed transformation.

Expression and purification of truncated VP2 proteins

The expression of the two truncated VP2 genes was carried out according to conventional protocols. In brief, pET-VP2-275 and pET - VP2-362 were transformed into *E. coli* BL21, which were cultured at 28°C in LB medium. The transformed bacteria were induced by adding IPTG at a final concentration of 1 mM for 5 h. After the cells were harvested by centrifugation at 4,000 × *g* at 4°C for 20 min, the pellet was suspended in 20 ml of PBS and then lysed by sonication in an ice water bath. The suspension was then centrifuged at 9,000 × *g* at 4°C for 30 min. The supernatant and pellets were subjected to SDS-PAGE, respectively, and visualised using Coomassie Brilliant Blue. Recombinant proteins were purified with Ni-NTA His bind resin.

Western blot analysis

A western blot was performed according to standard procedures. Protein samples were separated by SDS-PAGE with 15% gel before electrophoretic transfer to two nitrocellulose membranes. The nitrocellulose membranes were then blocked overnight at 4℃ with 10% skimmed milk in PBST. The membranes were washed three times in 10 ml of PBS containing 0.05% Tween-20 (PBST) for

10 min each. Membranes were then incubated with mouse anti-his tag monoclonal antibody and CPV-positive canine serum at 37℃ for 60 min, respectively. The membranes were then washed and incubated for 60 min with HRP-labelled goat anti-mouse antibody and horseradish peroxidase-conjugated protein-A. After additional washing, immunoreactive proteins were visualised with 3, 3'-diaminobenzidine (DAB).

Procedures for the developed ELISA

The 96-well microtiter plates were coated with 100 µL of a dilution of purified proteins in sodium bicarbonate buffer (pH 9.6) and were incubated at 4℃ overnight. The optimal antigen concentration and the optimal serum dilution were determined by titration of a coating antigen sample with a gradient dilution (5, 10, 20 and 40 µg/mL) and an antiserum sample with gradient dilution (1:20, 1:40, 1:80, 1:160). Plates were incubated with 100 µl of blocking solution (10% foetal calf serum in PBST) for 120 min at 37°C, then 100 µl of positive and negative diluted serum, respectively were added. The plates were kept at 37°C for 60 min; 100 µl of diluted PBS (1:1000, 1:2000, 1:3000, 1:4000) and the secondary antibody HRP-labelled protein A was added, and the plates were incubated at 37℃ for 45 min. Each time, the plates were washed three times with PBST before incubation. Then, 100 µl/well of 3, 3', 5, 5'-tetramethyl-benzidine (TMB) was added. After incubating at 37°C for 5 min, the reaction was stopped by the addition of 50 µl of 2 mol/L H_2SO_4. The optical density (OD) was measured at 450 nm using a Thermo Multiskan MK3 Model Plate Reader.

Specificity and sensitivity of the developed ELISA

The specificity of the ELISA was evaluated with RV, CDV, and *E. coli* lysate positive serum. Meanwhile, the CPV-positive serum and CPV-negative serum control groups were also designed. The 96-well microtiter plates were coated with an optimal concentration of purified proteins in sodium bicarbonate buffer (pH 9.6), and the serial diluted serum (1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560) were added. Additional operating procedures were followed according to those described above.

The coincidence rate between the developed ELISA and CPV-ELISA

The clinical serum samples were tested with the developed ELISA and the CPV-ELISA. The CPV-ELISA was used according to the manufacturer's instructions. Therefore, the coincidence rates were calculated from the developed ELISA and CPV-ELISA.

RESULTS

Expression of the recombinant CPV proteins

To obtain the fusion protein, two truncated VP2 genes were expressed by using E. coli BL21 and plasmid vector pET-32a (+). We tested a series of expression conditions that differed with respect to induction time, IPTG concentration, and induction temperature. As expected, transformants grew fastest at 28℃. The optimum cell density for pET-VP2-275 and pET-VP2-362 induction

reached 0.6 at OD600, and a time course and IPTG concentration study established that optimal protein expression occurred 5 h after induction with 1 mM IPTG. The molecular weight of the target proteins was approximately 30 and 33 ku, which were similar to the theoretical value (the molecular weight of the VP2-275 and VP2-362 are 9 and 12 ku, while the His-tag is 21 ku). The recombinant bacterium produced higher quantities of the fusion protein pET-VP2-275 and pET-VP2-362 than pET-32a -VP2 (Figure 1A). The target protein appeared in the supernatant of the induced pET-VP2-275 and pET-VP2-362 after sonication (Figure 1B).

Ni-NTA His Bind resin was applied to purify the fusion protein. The fusion proteins were eluted by 10 mL of denaturing elution buffer (imidazole in PBS). Samples were collected at different elution times. A single band was detected by SDS-PAGE. The pET-VP2-275 protein was purified with 200 mM imidazole, and the pET-VP2-362 protein was purified with 300 mM imidazole (Figure 1C).

Western blot analysis

To confirm the identity of His-tagged VP2-275 and VP2-362, the purified fusion proteins were subjected to a western blot assay with mouse anti-his tag monoclonal antibody. The results show that the fusion proteins had epitopes with His-tagged VP2-275 and VP2-362 (Figure 2A). The polyclonal antibodies recognised VP2-275 and VP2-362, and the band had the appropriate molecular weight. The immunoblot of these membranes using anti-CPV antibodies showed that the fusion protein had epitopes with CPV (Figure 2B).

Optimisation procedure of developed ELISA

The developed ELISA tests were operated with pET-VP2-275 protein and pET-VP2-362 protein in the same conditions, respectively. The results show that antigenicity of pET-VP2-362 protein was better than the pET-VP2-275 protein. So, the pET-VP2-362 protein was used as a coating antigen to establish the indirect ELISA. The coating antigen concentration, serum dilution and HRP-Protein A, corresponding to the highest P/N value (OD test sample /OD negative control), were regarded as an optimal condition. The optimal protein concentration, the serum dilution and the secondary antibody HRP-Protein A dilution were found by checkerboard titration at 10 µg/ml, 1:40 and 1:2000, respectively.

Specificity and sensitivity of the developed ELISA

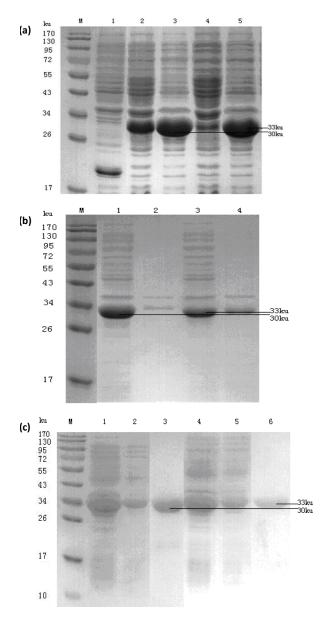By testing antibodies against CPV, RV, CDV and *E. coli*

Figure 1. (A) SDS-PAGE analysis of recombinant proteins. Lane M, High molecular weight protein marker; lane 1, induced blank vector; lane 2, uninduced bacteria of pET-VP2-275; lane 3, induced bacteria of pET-VP2-275; lane 4, uninduced bacteria of pET-VP2-362; lane 5, induced bacteria of pET-VP2-362. (B) Soluble analysis of recombinant proteins with SDS-GAGE. Lane M, high molecular weight protein marker; lane 1, supernatant of induced pET-VP2-275 after sonication; lane 2, pellet of induced pET-VP2-275 after sonication; lane 3, supernatant of induced pET-VP2-362 after sonication; lane 4, pellet of induced pET-VP2-362 after sonication. (C) SDS-PAGE analysis of the purified recombinant protein. Lane M, high molecular weight protein marker; lane 1, whole bacterium of induced pET-VP2-275; lane 2, pET-VP2-275 bacteria sample after sonication; lane 3, purified protein pET-VP2-275; lane 4, whole bacterium of induced pET-VP2-362; lane 5, pET-VP2-362 bacteria sample after sonication; lane 6, purified protein pET-VP2-362.
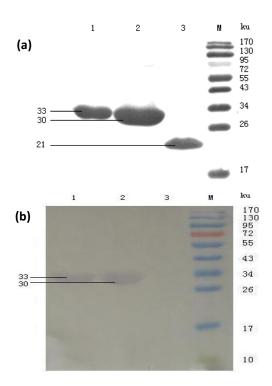

Figure 2. (A) Western blot analysis with anti-his tag McAb. Lane 1, purified protein pET-VP2-362; lane 2, purified protein pET-VP2-275; lane 3, induced blank vector; lane M, high molecular weight protein marker. (B) Western blot analysis with anti-CPV PcAb. lane 1, purified protein pET-VP2-362; lane 2, purified protein pET-VP2-275; lane 3, induced vector; lane M, high molecular weight protein marker.

greater than 2.1 was considered as positive, and the P/N value of the other samples was less than 2.1. The results indicate that the developed ELISA had a high specificity for the CPV-positive serum and there was no cross-reaction with antiserum against RV, CDV and *E. coli* lysate. After evaluating the sensitivity of a panel of diluted serum, a minimum detection limit of 1:1280 (P/N = 2.729) was obtained according to the P/N value (Table 2).

The coincidence rate between the developed ELISA and CPV-ELISA

Using the developed ELISA and the CPV-ELISA, the serum samples (n = 80) were tested. The developed ELISA showed a 96.25% (43+34/80) coincidence rate with the CPV-ELISA (Table 3).

DISCUSSION

CPV is now considered endemic in virtually all popula-

Table 2. Sensitivity of the developed ELISA.

Dilution	1:80	1:160	1:320	1:640	1:1280	1:2560
Positive	0.621±0.004	0.365±0.022	0.295±0.006	0.246±0.006	0.185±0.005	0.116±0.003
Negative	0.089±0.003	0.087±0.006	0.076±0.005	0.070±0.005	0.066±0.005	0.068±0.004
P/N value	6.958±0.156	4.182±0.102	3.866±0.149	3.533±0.143	2.796±0.152	1.704±0.069

Table 3. Coincidence rate between the developed ELISA and CPV-ELISA.

Developed ELISA	CPV-ELISA		Total
	Positive	Negative	
Positive	43	1	44
Negative	2	34	36
Total	45	35	80

tions of domestic and wild canines and has been reported to account for up to 26% of the mortality among all viral diseases of dogs Meunier et al., 1985; Walter and Kirchhoff, 1995). The VP2 protein is highly conserved among CPV and it can be used as a candidate diagnostic antigen because of its specificity (Shackelton et al., 2005). In this study, two truncated VP2 genes of CPV were expressed in prokaryotic expression systems. The recombinant proteins were soluble while the full-length protein was not. In addition, the soluble protein had a higher yield than the full-length protein. The purification of expressed soluble proteins by Ni-NTA His Bind resin was able to ensure that the unique components of the proteins were preserved. Purification also decreased the incidence of non-specific binding.

Furthermore, the purified proteins were not dialysed by gradient to reduce the concentration of urea, which was added at the time of dissolving inclusion bodies. In addition, the *E. coli* system was used to produce the proteins. Many advantages of *E. coli* have ensured that it remains a valuable organism for the high-level production of recombinant proteins (Gold, 1990; Chao et al., 2002). To enhance the specificity and sensitivity of the ELISA, the optimal antigen concentration of pET-VP2-362 proteins, the serum dilution and secondary antibody HRP-Protein A dilution were 10 μg/mL, 1:40 and 1:2000, respectively. To obtain the optimal coating buffer, 0.1, 0.05 and 0.01 M bicarbonate/carbonate buffer (pH 9.6) were screened. The results indicate that 0.05 M bicarbonate/carbonate buffer (pH 9.6) was the best coating buffer. Related reports in the literature state that the protein-based ELISA can be used to distinguish antibodies induced by the wild-type virus strain infection from those induced by vaccinated animals (McElroy et al., 2009; Idrissi et al., 1999; Kit et al., 1990).

Unpurified whole virion may be used in the ELISA, which could lead to a high incidence of background ab-

sorbance. In addition, the purification of the virion is laborious and expensive. Therefore, many researchers choose the recombinant protein, which has advantages over the whole virion as an antigen for the detection of antibodies (Ko et al., 2009; Chu et al., 2009). Firstly, the recombinant protein antigen is not infectious, so one can use the CPV serodiagnostic assay rapidly and safely, without the use of special virus-treated facilities. Secondly, the recombinant protein antigen can be easily produced in a general laboratory without any limitations. Particularly, with the help of the recombinant antigen, the specificity of the ELISA was greatly improved. This enhanced specificity is especially important for CPV immune detection. In developing countries, true-positive results are often missed due to inappropriate serum transport and storage conditions, which are one of the most common reasons for unspecific results on the ELISA (Escribano et al., 1989).

This study demonstrated that truncated tVP2 proteins were expressed successfully and the recombinant VP2 protein-based ELISA could be used to detect CPV antibodies. The developed ELISA, replacing the CPV-ELISA, is a simple and economical method that may exert a strong influence on immune detection in CPV-infected areas of the world.

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