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Cloning and Expression of Recombinant VP2 Capsid Protein Gene of Canine Parvovirus in *E. coli* System

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A B S T R A C T

Canine parvovirus type - 2 (CPV-2) infection is one of the most important viral diseases in dogs and wild carnivores causing severe haemorrhagic gastroenteritis in young ones. VP2 capsid protein plays an important role in determining the antigenicity and diversity of the virus. Although, several CPV variants emerged but new CPV-2a is the predominant circulating field strain of CPV in India. In the present study, new CPV-2a field strain (KLD3) isolated in cell culture was selected and the whole CPV VP2 gene was used for the expression in the *E. coli* expression system. Prokaryote expressing monocistronic DNA cassette containing open reading frame of whole CPV capsid gene (VP2) downstream of T7 promoter was synthesized. Analysis of the expression in *E. coli* cells showed the presence of capsid protein. Recombinant capsid protein showed immunoreactivity similar to the whole CPV virus antigen, when reacted with polyclonal antibodies against the whole CPV virus particles. The use of indigenously developed recombinant protein, being very economical, can be used to develop field kit. As the recombinant protein is not infectious, use of it for CPV serodiagnostic assay is considered safe.

Introduction

Canine parvovirus (CPV) infection is one of the most important viral diseases in dogs. CPV infection is characterized by nausea, enteritis, leucopenia, and myocarditis in puppies (Appel *et al.*, 1979). Canine parvovirus belongs to the genus *Protoparvovirus*, family *Parvoviridae*. The CPV virion is non-enveloped with icosahedral symmetry of 26nm diameter. It possesses a single stranded DNA genome of 5.2 kb in length. The virus has two open reading frame in its genome which encodes two non-structural (NS1 and NS2) and three structural (VP1, VP2 and VP3) proteins. VP1 contains the full length VP2 sequence plus an additional N-terminal domain. The VP2 capsid protein is a major protein and accounts for 90% of the viral capsid and is cleaved to VP3 by the host proteases.

The high rate of mutations and positive selection of the virus have led to the generation/emergence of newer CPV variants. Several CPV variants have emerged as only a few amino acid substitutions in VP2 are responsible for its antigenic properties (Parrish *et al.,* 1991). The CPV (CPV-2) variants include CPV-2a, CPV-2b, CPV-2c, new CPV-2a, new CPV-2b and all of them have spread worldwide (Hongli *et al.,* 2015). Most licensed vaccines were modified based on the original type CPV-2 (Pratelli *et al.,* 2001). The original CPV-2 was no longer found in dog population but present only in vaccine formulations (Decaro *et al.,* 2006).

VP2 protein plays an important role in determining antigenicity and host range of CPV. The emergent canine parvoviruses are characterized by specific amino acid changes in and around a raised region of the capsid termed the threefold spike. Moreover VP2 is able to self-assemble, forming virus-like particles (VLPs).

These virus like particles can be obtained by the self-assembly of one or several viral structural proteins produced in an expression system. The advantages of VLPs are their safety and high immunogenicity, and thus they could open a new frontier in diagnostics and vaccine development.

There are various diagnostic tests employed for the detection of CPV from faecal samples of dogs like haemagglutination test (Carmichael and Binn *et al.,* 1981), latex agglutination test (Veijalainen *et al.,* 1986), immunochromatographic test (IC) (Esfandiari and Klingeberne, 2000), PCR based methods (Decaro *et al.,* 2005c), Loop-mediated isothermal amplification (Mukhopadhyay *et al.,* 2012) and virus isolation and identification in cell cultures like CRFK/ MDCK/ A-72 (Kumar *et al.,* 2003; Rai *et al.,* 2004 and Hirayama *et al.,* 2005). Many diagnostic tests lack specificity and sensitivity whereas many are very laborious and time consuming. 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 (Kummitha *et al.,* 2010). Use of crude, unpurified whole virion used in the ELISA leads to high incidence of background absorbance. Further, the purification of the virion is laborious and expensive.

Therefore, use of recombinant protein as an antigen, for the detection of antibodies, proved better (Ko *et al.,* 2009). As the recombinant protein antigen is not infectious, use of it for CPV serodiagnostic assay is considered safe. Therefore, the recombinant protein based diagnostic tests can be used as alternative methods for detection of CPV infection. Recombinant VP2 protein-based indirect ELISA assay was found to be economical and more convenient (Lijun *et al.,* 2012).

New CPV-2a strain was found to be prevalent CPV strain circulating in India (Mukhopadhyay *et al.,* 2013; Mittal *et al.,* 2014), thus the development of a diagnostic tool based on the prevailing antigenic strain is the need of the hour.

Therefore, the present study was taken up to generate the recombinant VP2 capsid protein gene of CPV from a field isolate of a new CPV type-2a strain and to express it in the prokaryotic (*E.coli*) expression system. The new CPV-2a field strain (KLD3) isolated in cell culture (A-72 cell line) was selected for cloning and expression of VP2 capsid protein gene of canine parvovirus.

Indigenous development of CPV VP2 based recombinant protein has the potential to be used for developing rapid diagnostic tests and vaccines.

Materials and Methods

Virus isolate

Canine Parvovirus field strain (New CPV-2a) was used for viral DNA extraction and PCR amplification of the VP2 capsid protein gene. The new CPV-2a field strain was propagated in A-72 canine cell line for virus isolation.

Primer design and gene synthesis

Primers to amplify full length VP2 capsid protein region (1755bp) of CPV was designed based on viral genomic sequence of prototype CPV (CPV-2 strain, GenBank accession: AF204276) using the SnapGene software (version 2.5) (Table 1). Appropriate restriction enzyme sites were included at 5' end of primers to facilitate cloning of the PCR generated ORFs of viral sequences.

Construction and generation of recombinant plasmids

The VP2 capsid coding region of CPV (1755bp) was amplified from DNA of CPV field strain (New CPV-2a) using primer pair BamHI-$VP2_{For}$ & XhoI-$VP2_{Rev}$ (Table 1). Construct: VP2 gene amplified was cloned downstream of the T7 promoter (MCS) into pET45b(+) vector (Novagen) following RE digestion (using BamHI ana XhoI RE) and Ligation using T4 DNA ligase to generate a unidirectional-monocistronic prokaryotic expression cassette. The schematic strategy of the construct is given in Figure 1.

The recombinant construct (pET45b-CPV-VP2) cloned into the pET45b(+) vector was analyzed by restriction enzyme digestion using BamHI and XhoI RE enzymes. Similarly the sequence integrity is confirmed by nucleotide sequencing using the vector primer pair $T7_{for}$ and $T7_{rev}$ (Table 2) to verify the direction and correctness of their reading frame prior before expression studies. Later these recombinant plasmids (pET45b-CPV-VP2) were isolated from the transformed TOP10 *E.coli* cells.

Protein expression and isolation

The recombinant plasmid (pET45b-CPV-VP2) carrying VP2 gene of canine parvovirus was expressed in *E. coli* BL21 expression strain as per the standard protocol (Sambrook and Russell, 2001) at a final concentration of 1 mM Isopropyl-β-D-thiogalactoside (IPTG) for protein induction. The induced cells were cultured at different time interval to analyze the maximum protein expression.

Proteins were isolated from expressed *E.coli* cells by sonicating for 45 cycles involving 15 seconds of sonification and 5 seconds cooling interval in ice. Following sonication, the cell lysate (Crude lysate) were separated by centrifugation at 11,000 g for 5 minutes at 4°C and supernatant collected. The cell lysate of different time intervals were boiled with 3x SDS-PAGE sample buffer (1:10) at 100°C for 4 minutes. The processed lysates were separated by 13% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) to check for the presence of expressed protein.

Dot- ELISA for detection of the recombinant protein

The cell lysates were tested in Dot- ELISA. Briefly, the nitrocellulose membrane was coated as dot with the expressed cell lysate. The membrane was incubated at 37°C for 1 hr and washed 3 times with PBST [Phosphate buffered saline (PBS) containing 0.05% Tween-20]. The samples were coated with positive (A-72 cell culture viral antigen) and negative (*E.coli* cellular antigen) controls. The coated membrane was incubated and washed. Then the membrane was immersed in 1: 100

diluted rabbit anti-CPV tracing serum in blocking buffer (PBST + 5% skimmed milk powder). The membrane was washed after incubation at 37°C for 1 hr and then incubated with anti-rabbit IgG HRPO conjugate (DAKO, Germany) at 1:3000 dilutions in blocking buffer. After washing, the membrane was immersed in freshly prepared orthophenylene diamine/ hydrogen peroxide substrate for 5-10 min. The reaction was stopped using distilled water and checked for the development of brown colour spot for the immunoreactivity. The development of brown spot indicates positive reactions.

Results and Discussion

The full length VP2 gene of new CPV-2a field strain (KLD3) of a canine parvovirus was amplified by polymerase chain reaction (Figure 2) using VP2 gene specific primers as depicted earlier in Table 1. The RE digested purified PCR product (VP2 gene) was ligated into RE digested purified pET-45b(+) expression vector by uni-directional cloning under T7 promoter between BamHI and XhoI restriction site (as depicted in Figure 1). The ligated mixture was transformed into competent *E. coli* TOP10 cells and the transformed colonies were confirmed for the recombinant plasmids carrying VP2 gene by colony PCR using VP2 gene specific primer pair (Table 1).

By analytical agarose gel electrophoresis, colonies carrying gene of insert showed amplified product size approximately of 1,755bp length (Figure 3). The isolated plasmid from the colony PCR positive clones, were further confirmed by restriction enzyme digestion using BamHI and XhoI. By agarose gel electrophoresis, the clone was confirmed for the release of 1,755bp inserted VP2 gene DNA fragment from the recombinant plasmids, indicative of successful cloning (Figure 4). Finally, the specificity and orientation of the inserted gene was confirmed by sequencing using pET 45b (+) vector specific primer pair (T7 forward and T7 reverse primer). The data obtained was analyzed using pairwise BLAST. The obtained sequence showed maximum specificity with the reference strain canine parvovirus 2a available in the Genbank.

After developing the clone in *E.coli* TOP10 competent cells, the isolated recombinant plasmid was transformed into competent *E. coli* BL21 (DE3) expression strain and plated onto LB agar containing ampicillin and incubated at 37°C overnight.

Similarly the positive clones in *E.coli* BL21 cells were confirmed by colony PCR and also by restriction enzyme analysis as described earlier. The VP2 protein expression was induced by inoculating the recombinant *E.coli* BL21 colony into LB broth containing 1mM IPTG at 30°C in orbital shaker. The samples were collected at 0, 2, 4 and 6 hours post induction.

The cell lysate protein samples were analysed on 13 % SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. The expressed protein band size was found to be approximately 67KDa along with prestained protein standard molecular weight marker, which confirmed the expression of VP2 protein of CPV (Figure 5). The expressed protein bands appeared as a bright band and had compressed with other cellular protein bands.

The other bands were appeared found to be lower than the size of VP2 protein. These bands could be the products of proteolytic cleavage or degradation by host *E.coli* proteases (Park *et al.*, 2007). Expression was found to be higher after 4 and 6 hours post-induction as was evident by the intensity and thickness of the band.

Fig.1 Schematic illustration of Prokaryote expressing monocistronic DNA cassette containing open reading frame of whole CPV capsid gene (VP2) downstream of pET - 45b (+) T7 promoter (generated by SnapGene software Version 2.5)

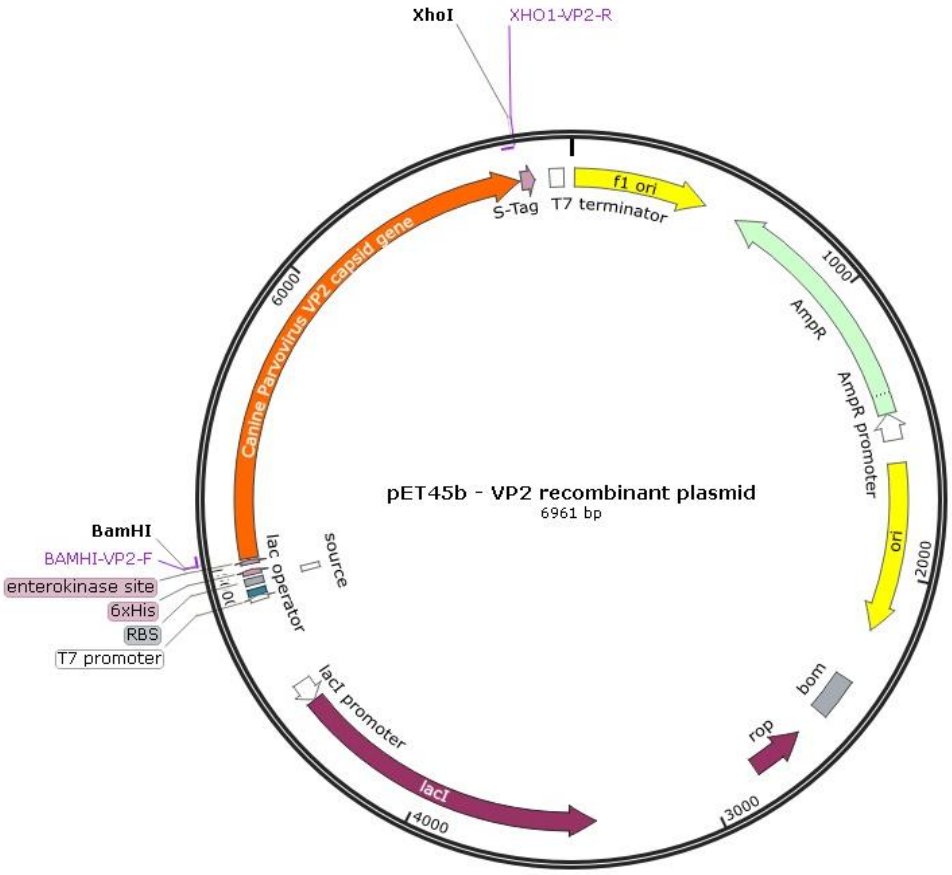

Fig.2 Amplification of CPV VP2 gene of canine parvovirus by polymerase chain reaction using BamHI-VP2$_{For}$ and XhoI-VP2$_{Rev}$ primer pair; Lane 1- DNA Molecular Weight Marker Lane 2 - Negative control, Lane 3 - Positive control (vaccine strain), Lane 4 & 5 – PCR amplified product of VP2 gene of New CPV-2a

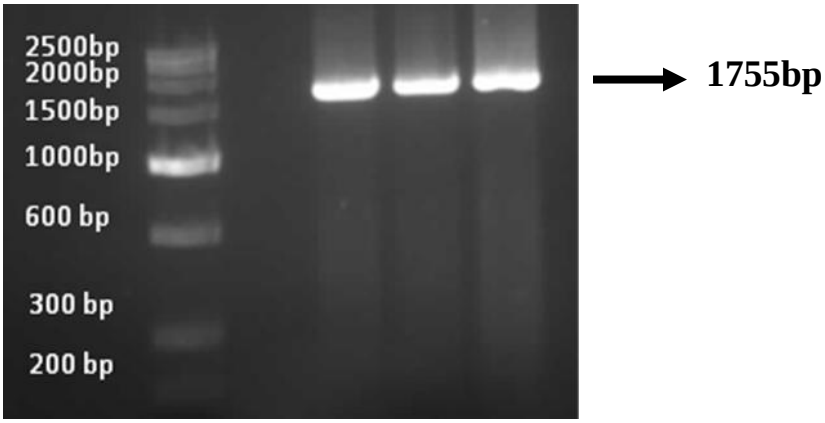

Fig.3 Colony PCR amplification of recombinant *E. coli* TOP 10 colonies using BamHI-VP2$_{For}$ and XhoI-VP2$_{Rev}$ primer pair; Lane 1 - DNA Molecular Weight Marker, Lane 2, 3, 4 – Recombinant *E. coli* colonies carrying pET45b-VP2 plasmid, Lane 5 - Negative control (Fecal sample from healthy dog), Lane 6 - Positive control (CPV Vaccine)

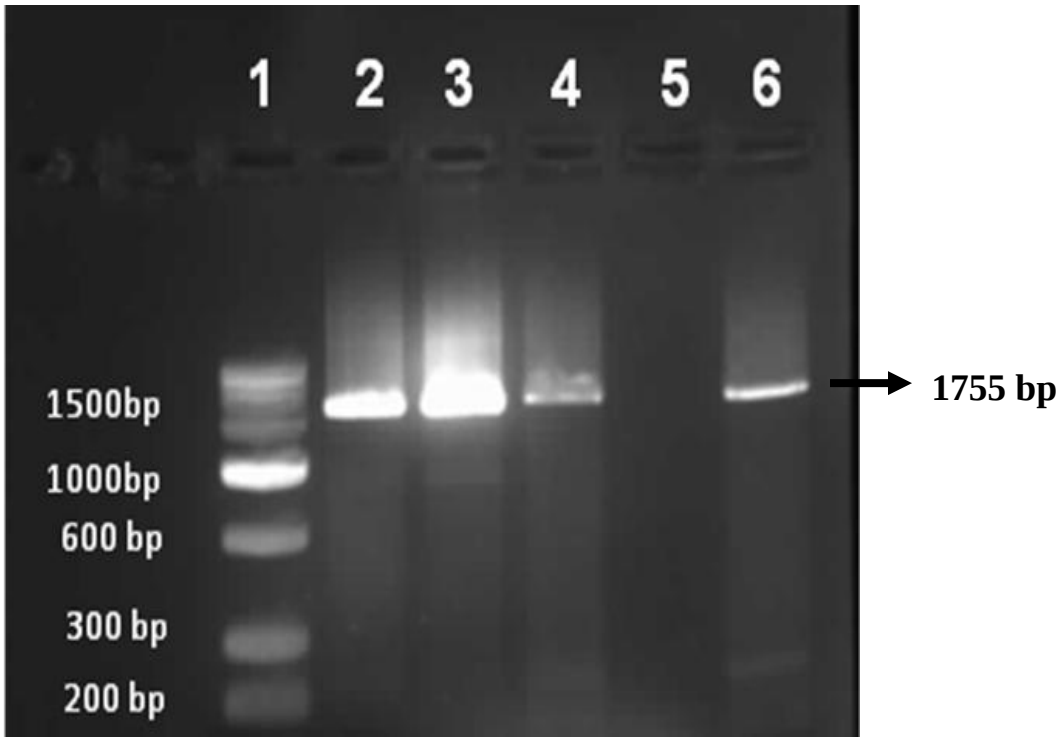

Fig.4 Confirmation of recombinant plasmid (pET45b-VP2) by Restriction Enzyme digestion using BamHI and XhoI. Lane 1, 2, 3 – RE digested recombinant plasmid, Lane 4 – RE digested pET-45b (+) vector, Lane 5 – DNA Molecular Weight Marker

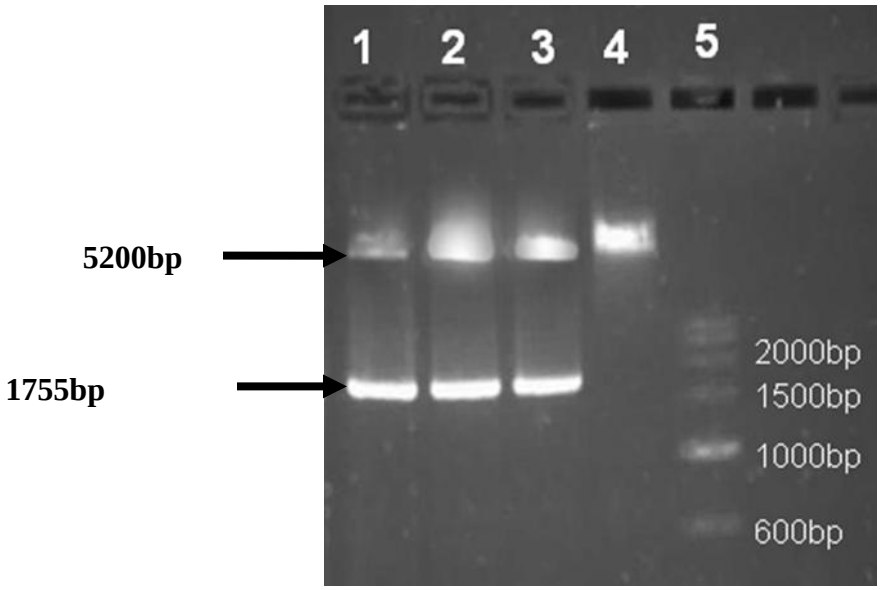

Fig.5 SDS - Polyacrylamide gel electrophoresis showing the expression of recombinant VP2 protein at different time intervals. Lane 1, 2, 3, 4 - Cell lysate of expressed VP2 capsid protein collected at 0, 2, 4, 6 hour post induction respectively, Lane 5 - Pre-stained protein marker (170-25 kDa)

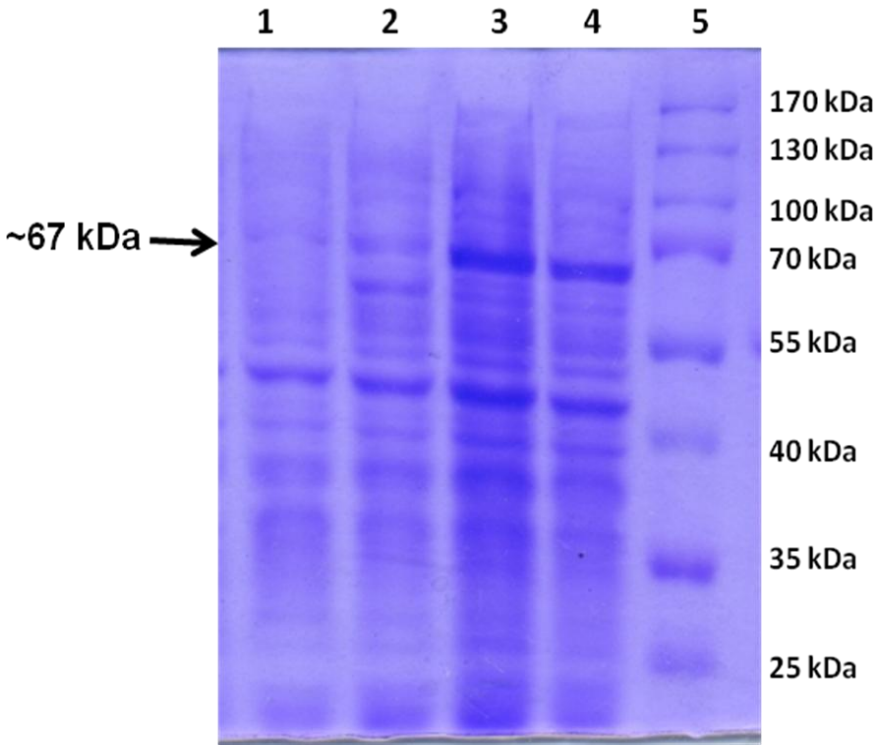

Fig.6 Dot-ELISA using polyclonal antibodies against the whole CPV virus particles raised in Rabbit. The result showing distinct brown spots in Test sample (T) along with Positive control (P) and Cell control (C). Positive Control (P): New CPV-2a Field strain virus, Test Sample (T): Cell lysate of expressed VP2 capsid protein (expressed @ 4 hrs post induction), Competent cells (C): Cell lysate of *E.coli* BL21 Strain.

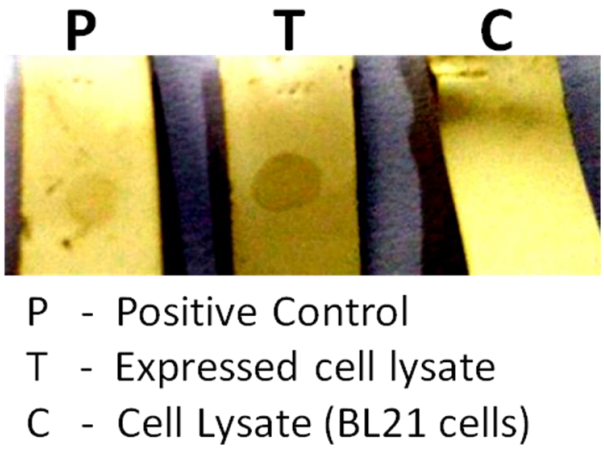

Table.1 Oligonucleotide primers designed for amplification of full length VP2 gene of canine parvovirus

PRIMER	TARGET GENE	PRIMER SEQUENCE (5'→3'DIRECTION)	RESTRICTION SITE	AMPLICON SIZE
BamHI-VP2$_{For}$	**CPV VP2 capsid protein gene**	5'-TG*GGATCC*GATGAGTGATGGAGCAGTTCAA-3' 30mer	BamHI	1,755bp
XhoI-VP2$_{Rev}$		5'TG*CTCGAG*TTAGTATAATTTTCTAGGTGCTAGTTGAGAT TTTT-3' 43mer	Xho I	

Table.2 pET45b(+) vector binding primers

PRIMER	TARGET GENE	PRIMER SEQUENCE (5'→3'DIRECTION)
T7$_{for}$	**T7 promoter of pET45b(+) vector**	5`TAATACGACTCACTATAGGG-3'
T7$_{rev}$	**T7 terminator of pET45b(+) vector**	5'-GCTAGTTATTGCTCAGCGG-3'

By Dot-ELISA the crude recombinant VP2 capsid protein of new CPV-2a showed immunoreactivity with the anti-CPV antibody raised in rabbits. The crude recombinant CPV VP2 protein were coated on nitrocellulose membrane subsequently the sample were incubated with anti-CPV hyperimmune serum raised in rabbits the appearance of dot in the test sample and the Positive control showed immunoreactivity (Figure 6).

In conclusion, the recombinant CPV VP2 capsid protein was expressed in the *E.coli* prokaryotic expression system. The expressed VP2 CPV capsid protein showed immunoreactivity with the anti-CPV hyperimmune serum raised in rabbits by DOT-ELISA. The expressed CPV VP2 capsid protein can be used as an antigen in the development of diagnostic kit such as indirect-ELISA / immunochromatography against field strain of CPV infection. The currently available diagnostic tests lack specificity and sensitivity and also laborious and time consuming. In contrast, this recombinant protein can be easily produced within short span and is less expensive. As the recombinant protein as antigen is not infectious, use of it for CPV serodiagnostic assay is considered safe. Recombinant protein based diagnostic tests can also be used for screening maternally derived CPV antibodies in puppies, sero-surveillance studies and for measuring post vaccination titre.

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