# CLONING AND EXPRESSION OF CANINE PARVOVIRUS VP2 CAPSID PROTEIN GENE

## INTRODUCTION

Canine Parvovirus (CPV) disease is highly contagious to dogs' population, more specifically to young animals since the high mortality rate of average 16-35% was reported. CPV belongs to genus *Parvovirus* of family *Parvoviridae*. The virus has the single stranded DNA. Viruses in this family usually cause the damage of dividing cells resulting in severe in gastroenteritis. At the present time these viruses are recognized as the causative agents of mammals such as mink, raccoon, fox, wolf, domestic pig, cat, dog and sporadic case in human.

CPV are divided into 6 biotypes 1, 2, 2a, 2b, 2Ca and 2Cb based on the differentiation of some amino acid residue at the viral capsid protein. The viral proteins are classified into two groups which are structural (VP1, VP2 and VP3) and nonstructural proteins (NS1 and NS2). The structural proteins are composed in viral capsid. The capsid role is protecting viral genome from host protease and stimulating antibody in animals. The VP2 protein is the major component in capsid which has the major antigenic sites.

The strategies to protect animals from CPV are much concerned as in various diseases. In general, the vaccination is the important mean to prevent the disease. At the present, all of CPV vaccines available are modified live vaccines either in monovalent or multivalent form. The obstructive aspects caused by using whole organism vaccine (e.g. modified live vaccine, killed vaccine) are indiscriminate detection between infected and vaccinated animals with the available diagnostic methods such as PCR and the disease outbreak possibility due to viral vaccine production. The subunit vaccine or peptide vaccine may overcome these problems. CPV agent detection was also reported to be advantageous. PCR based technique on VP2 gene would serve as the sensitive and useful technique for diagnosis.

The study of subunit protein production was mainly focused on the VP2 protein. This protein was considered to be the major immunogenic protein for stimulating and neutralizing antibody. Thus, the recombinant VP2 protein producing by insect cells will be useful for the further development of subunit vaccine production. The baculovirus expression system was reported to be the choice of accurate form of protein product because of the glycosylation ability of this system.

The objectives of this study are

1. To develop PCR for the detection of CPV.
2. To clone and express the VP2 gene of CPV local strain by baculovirus expression vector system.
3. To confirm the synthesis of recombinant VP2 capsid protein using mouse-antihistidine monoclonal antibody and rabbit anti-CPV polyclonal antibody.

# LITERATURE REVIEW

## Overview of Canine Parvovirus

### 1. Virus Characteristics

Canine parvovirus (CPV) belongs to family *Parvoviridae*, subfamily *Parvovirinae* and genus *Parvovirus* (Tsao *et al.*, 1991; Cortes *et al*., 1993; Langeveld *et al*., 1994). It is a nonenveloped, icosahedral, linearized single-stranded DNA virus of 5,323 nucleotides with particle size of 27 nm in diameter (Langeveld *et al*., 2001; Parrish and Kawaoka, 2005). Virus particle consists of protein and DNA (50%/50%). The virus has two open reading frames (ORF) in its genome which encode at least four proteins. The 5′end ORF encodes for the structural proteins while the 3′end ORF obtains the nonstructural protein code (Voyles 2002; Parrish and Kawaoka, 2005). The different proteins are transcribed from separate promoters and are derived by alternative splicing of RNA transcripts (Wang *et al*., 1997). The surface of CPV particle (full capsid or DNA containing) is covered by capsid which composed of the three structural proteins (VP1, VP2 and VP3).

CPV is termed autonomous virus since it has information and necessary function to replicate itself in host cells. It is closely related to Mink Enteritis virus (MEV) and Feline panleukopenia virus (FPV) which has the homology of nucleotide sequence more than 98% (Sagazio *et al*., 1998).

CPV is stable in the broad range pH (between pH 3 and 9). It can be inactivated by heating to 80 $^{0}$C for 40-60 minutes and it is stable at -20 $^{0}$C for 1 year after this period of time it can survive at 4 $^{0}$C with some decrease in infectivity. In outdoor environment or in area protected from sunlight or drying condition, it can survive about 5 months or longer. Virus surviving at 20 $^{0}$C within 2-6 months appeared to low infectivity (Povey, 1988). Though it is resistant to inactivation but some disinfectants can be used. They are sodium hypochlorite (1:32 dilution),

formalin (1:50) or glutaraldehyde, β-propriolactone, hydroxylamine and other oxidizing agents (Povey, 1988; Knipe *et al*., 2001).

## 2. Virus Replication

The virus replication takes place in the nucleus of dividing cell or mitotic cell. CPV needs host cell in the stage of cellular cycle of S phase whereas the DNA polymerase and other important components in addition to the viral NS1 polypeptide activity are needed for the CPV to synthesize the viral genetic material in the viral progeny production. The virus uses its single stranded DNA beginning readily from both 5′ end and 3′ end nucleotides sequences. They are formed the loops as the initiatives and act likewise RNA primers that needed for the second strand DNA synthesis. Then the double stranded DNA is served as the template in replication and transcription for messenger RNA (mRNA) (Knipe *et al*., 2001). The mentioned situation of replication occurs in the viral target organs, which are lymphopoietic tissue, bone marrow and the crypt epithelium of jejunum and ileum. The reservoir of virus could be found in thymus, mesenteric lymph node, spleen and ileum.

## 3. Viral Infection Mechanism

The infection mechanism involves in cell entry event and ability to propagate the progeny in nucleus of host cell. The early steps of entry into cells is attachment to the cell surface and the following step of penetration which is pH-dependent pathway by the interactions between the hydrophobic portions of the capsid proteins and the cellular membrane whereas the specific cell receptor belongs to. After that the virus might attempt to pass the nuclear pore to access the host nucleus. There was the report that without capsid deformation its small particle could pass the nuclear membrane. It required the microtubules and dynein protein in this step to move capsids within cytoplasm, facilitating transport to nucleus and cell infection. The complete cell entry effects to cell lysis because of the successful replication. The mature virions are released. In the detail, the cellular uptake the viral particles occurred by endocytosis pathway. The transferin receptor (Tfr) was reported as the

key role, which could limit the host range among the closely related parvoviruses family. For instance the CPV and FPV could bind the Tfr of the feline cells, but the FPV was not able to attach the canine cells specifically. This ability directly pointed to the successful of viral infection as some changes of receptor and viral capsid may allow binding, but appears failure in infection. Tfr and capsid are recognized to control of virus to cells interaction or host range (Agbandje *et al*., 1995; Parrish and Kawaoka 2005).

There is another receptor involving in the hemagglutination property although is non mediate infection named Sialic acid binding receptor (SAs). SAs binding site is found on the dimple of the virus capsid and specific for the N-glycolyl neuraminidic acid(NeuGC) which is found on the erythrocytes of most cats, monkeys, horses and not most of dogs. For this reason, the hemagglutination technique is used as one of the diagnosis methods to detect CPV in practice (Parrish and Kawaoka, 2005).

## 4. Virus Proteins

There are five important proteins composed for the virus. They are classified into two groups which are structural and nonstructural proteins. The structural protein is major component in viral capsid. They are stable at pH 5.5-7.5 (Weichert *et al*., 1998). The capsid role is protecting viral genome from host protease. It is composed of 60 copies of VP2 protein and small amount of VP1 and VP3, but there was the report that VP2 protein alone could assembly for the capsid (Cortes *et al*., 1993).

The VP1 protein of apparent sizes of 82.3 kDa has 748 amino acid residues. It has the complete sequence of VP2 plus the nucleotides which differs from the VP2 protein at its N-terminus. Then it has unique peptide of about 15 kDa which composes of many basic amino acids that are normally enclosed within capsid (Saliki *et al.*, 1992). It contains the conserved motif site for the cellular secreted type phospholipase A2. The known function of VP1 protein of phospholipase A2 activity is recognized for the process of host cell infection and its basic sequences of the

unique region may control the process of nuclear transport (Parrish and Kawaoka, 2005).

The VP2 protein is the major component in capsid consists of 584 amino acid residues. It has the apparent size of 65 kDa (Gupta *et al*., 2005). Its function is recognized which is important in control the viral cell receptor interaction with the transferin receptor (Tfr) and sialic acid binding site (SAs). The previous study reported that majorities of the T cell and B cell epitopes were mapped on the VP2 protein. It is also shown to have the neutralizing epitope at N-terminus (Cortes et al., 1993 and Tresnan et al., 1995). VP2 protein has the critical change to viral capsid that effect to antigenic property and host range. Amino acid substitution at VP2 residue 426 (Asp->ASN) made CPV-2a differs from CPV-2b. Natural mutation of the amino acid at the VP2 residue 300 (Gly->Asp) changed CPV-2a/2b to newest isolate of Leopard cat parvovirus (LCPV). These new biotypes were classified as CPV-2Ca and CPV-2Cb depending on the identities of either CPV-2a or CPV-2b. Therefore, differences of VP2 protein between the CPV-2 and CPV-2a /CPV-2b differed in the three coding changes in capsid protein gene at amino acid residue 87(Met->Leu), residue 300 (Ala->Gly), and residue 305 (Asp->Tyr) respectively (Truyen *et al.*, 1998).

The VP3 protein has apparent size of 63.5 kDa. The importance of VP3 is considered as the evident shown the minor amount of this protein in capsid component of complete virion. VP3 is not present in viral empty capsid (Langeveld et al., 2001). The VP3 is derived from VP2 by posttranslational proteolytic cleavage of ~ 20 amino acid residues from N-terminus (Wang *et al*., 1998; Parrish and Kawaoka, 2005). Trypsin treatment of full capsid cleaves VP2 to VP3–like protein (Cortes *et al*., 1993; Lopez et al., 1997; Langeveld *et al*., 2001).

The nonstructural protein group has two unique proteins named NS1 and NS2. The NS1 is the large protein with the size of 83 kDa. The reported function of NS1 protein is involving in viral DNA replication controlling, recognition and nicks the viral DNA during replication. It initiates replication by binding to the specific

sequence of origin of replication and maintain the replication fork. The others function is related to helicase enzyme activity which controls the DNA packaging into the viral capsid, controls cellular apoptosis; binds a number of cellular proteins. The latter nonstructural protein NS2 with size of 25 kDa is found in nucleus and cytoplasm of host. Its known function is less explained, but in many cells it influences the nuclear trafficking and viral capsid component assembly. But it does not have the major role in CPV replication cycle in neither cell line nor tested animal (Parrish and Kawaoka, 2005).

## 5. Natural Host

CPV has been shown to naturally infect wide variety of dogs and some cats including other canidae such as coyotes and Asiatic raccoon. Any breed of dog and wild Canidae can be infected with any biotypes of CPV, but Rottweiler, American Pit Bull, small Terrier, Doberman Pinschers and German shepherd are more susceptible (Flint *et al*., 2004). However, it can affect dogs of all ages. It is common in dogs less than one year of age. Susceptibility to CPV infection often coincides with the time that puppies are separated from dams as the level of protective immunity or maternal immune declined (Hoare *et al*., 1997).

## 6. Epidemiology of CPV

The first isolation of Canine Parvovirus original type 2 (CPV-2) was identified by Appel in 1978 and spread rapidly through the canine population around the world (Parrish *et al*., 1988). Confirmed infections have been reported in Sweden, Germany, Italy, Brazil, and even isolate countries like Australia and New Zealand. In 1984, the new antigenic type emerged as type 2a (CPV-2a) and type 2b (CPV-2b). Later in 1996, the latest biotypes of CPV family had been reported from the isolate of Asian small wild cat and leopard cat (*Felis bengalensis*) originated from Taiwan and Vietnam which is called the Leopard cat parvovirus (LCPV). In Europe, the endemic CPV biotype was found to be CPV-2a predominantly. In contrast, CPV- 2b was prevalence in America (Yule *et al*., 1997). The CPV-2a and CPV-2b replaced the

former CPV-2 rapidly as many reports showed from many countries and also case report in Thailand respectively (Parrish *et al.*, 1988; Sakulwira *et al.*, 2001).

CPV caused the enteritis in dogs about 61.8% in Thailand and CPV-2a and -2b were the predominant genotype found (Sakulwira *et al*., 2001). In 2001, the PCR based method for the amplification of the nonstructural protein gene (NS1) to detect CPV in Thailand has been developed (Sirinarumitr *et al.*, 2001).

## 7. Pathogenesis of CPV Disease

In general, the incubation period is 3-7 days after exposure. It depends on the dose of virus, the species susceptibility, and the ability to detect clinical signs. Virus is transmitted by fecal–oral route (Yule *et al*., 1997). It is commonly presented in feces of infected dogs. It could be also found in urine. Pathogen contact usually occurred by ingestion, licking the infected dog, via feeding utensils, hair and clothing or contaminated object with fecal material. Initiation infection occupies in tonsils then it spreads to infect cells of lymphoid tissue and intestinal epithelium. After that the viruses pass through the intestinal lumen (Yule et al., 1997; Truyen *et al*., 1998).

In dogs, clinical manifestations are studied which is individual variation among dogs. There are two main clinical syndromes: enteric and myocardial or heart forms. The enteric form is associated with sudden death and the major clinical signs are characterized by the onset of sudden depression, loss of appetite, fever (especially in puppies),vomiting and diarrhea (up to 50% of case diarrhea may be hemorrhagic), rapid dehydration, leucopenia , shock and death (in acute case). Infected dogs may not show every clinical signs, but vomiting and diarrhea are the most common signs. However some dogs may survive from enteritis and dehydration and becomes immune. But the recovery may be slow.

The less common myocardial form is myocarditis (inflammation of the heart). It is associated with congestive heart failure and there is slower onset of clinical signs of depression, dyspnea, cough and ascites (Truyen *et al.*, 1998). This form of CPV is

usually seen in puppies at age of 12 –16 weeks. The multiplication of CPV is in myocardium (the heart muscle cells). In new born puppy, viruses attack myocardium and cause myocarditis. The quantity of virus particles shed is the highest during the first two weeks of exposure. It remains infectious in the environment for weeks or month in cold and dry condition (Yule *et al*., 1997). Disease is often asymptomatic in older dogs or in puppies that receive a low virus dose. The puppy commonly appears normal, and then gasps, mucous membranes turn pale and cyanotic. Death occurring is about two hours due to acute non-suparative myocarditis (Pratelli *et al*., 2001).

Mortality rate caused by CPV of young animals whose age from 3-16 weeks could be 20%-100% depending on the proper treatments. In general, the mortality is reported to be 16-35% when the prevention of opportunistic bacteria infection is administered (Langeveld *et al*., 1992).

## 8. Prevention and Treatment

Normally puppies are protected by passive transfer of maternal antibody (Truyen *et al*., 1998). It has resulted in the virtual disappearance of myocarditis, so that maternal antibody is the major concern preventing in young animals (Langeveld *et al*., 2001). However, problem can occur to puppies from vaccinated or naturally exposed dams become susceptible when the maternally derived antibody declined below the protective level, but still interfere with the CPV immunization (Hoare *et al*., 1997).

The best method to protect the dogs against CPV is vaccination with either inactivated virus or modified-live virus. Modified–live and inactivated vaccines for CPV are available in either monovalent or multivalent vaccine which contain canine distemper, canine parvovirus, canine adenovirus, leptospira bacterin and inactivated rabies virus (Truyen *et al*., 1998). It produces both humoral and cell-mediated immunity and provides prolonged immune, but it is less stable. Inactivated vaccine is more stable and produces mainly humeral immunity, but it requires booster for several times (Povey; 1988; Murphy *et. al*., 1999). The use of synthetic or subunit

vaccines might overcome the limitation over the live vaccine and provide suitable alternatives to conventional vaccine as the subunit protein vaccine could still have the ability of protection against viral infection, elimination of viral reservoirs used for manufacturing and moreover it has advantage in avoidance of the possible outbreak due to the viral escape during vaccine production (Lopez DE TURISO *et al*., 1992; Langeveld *et al*., 1994).

The other prevention program is to clean the kennel, feed bowl and other objects before admission the new puppies whose age less than 3 months or unvaccinated dogs to the contaminated area, disinfectant as mentioned above must be used. Quarantine the new introduced dogs and isolation of the infected dogs should be usually done due to the highly infectious nature of the disease.

Treatment primarily consists of supportive nursing care, no food or water is provided for a day. Water requirement is met by providing intravenous or subcutaneous electrolyte. Intravenous of sugar solutions and B-vitamins may be used during hospitalization. When the secondary bacterial infection is concerned, the antibiotics prescription must be done (Murphy *et. al.*, 1999)

## 9. Laboratory Diagnostic for Canine Parvovirus

History and clinical findings is the most important measure. Infected dogs may have fever, vomiting, acute diarrhea and lymph node enlargement. Mortality rate of dogs caused by CPV is high (Lopez DE TURISO *et al*., 1992; Battilani *et al*; 2002). However, there are several diseases that may have similar clinical signs such as canine coronaviral enteritis (CCV), hemorrhagic gastroenteritis (HGE), parasitic infection (nematode) cocidia and obstructive lesions etc. (Sagazio *et al*., 1998). Necropsy findings are suggestive since the dog die from suspected symptoms as mentioned, but the lesions caused by CPV may be confused with poisoning, histopathology characteristics consist of cardinal hemorrhagic ,intracellular inclusion bodies in cardiac myofibrils (in myocarditis case). The enteric form appearance

obtained dilated intestine. Microscopic finding is the necrosis of the epithelium and dilation of crypt.

Detection of CPV antigen and antibody is the definitive diagnosis for CPV infection which can be done in several ways such as electron microscopy, virus isolation, immunological assays and molecular techniques. Direct EM examination of feces using negative staining is the standard method for parvoviruses detection which is based on identification of morphological virus particle intact, but it is very expensive as the equipment and maintenance cost are high by electron microscopy. The disadvantages are low degree of sensitivity and skilled personnel (Schunck *et al*., 1995). This method is successful during high excretion of virus using positive staining (Povey; 1988). Fecal sample should be collected when the animal shows some clinical signs.

Virus Isolation is practiced by fecal sample swab collecting kept in the aqueous solution such as PBS buffer and Dulbecco's Modified Eagle Medium (DMEM). There are several cell lines that can be used to isolate CPV such as Canine A-72, Crandel feline Kidney cell (CRFK), Feline embryonic fibroblast(FEB) and Madin Darby Canine Kidney cell line(MDCK). They require minimum 1% fetal bovine serum (FBS) in commercial Dulbecco's Modified eagle medium (DMEM) pH. 7 under 37°C in $CO_2$ supplied incubator. CPV-2 could be isolated in canine cells, but is excluded feline. While the CPV-2a isolate gains ability to propagate in feline cells, but only isolate CPV-2a from dogs can grow in feline and canine cell lines. The CPE of CPV could be observed (Knipe *et al*., 2001). Harvesting of the virus should be done when the CPE reaches 80 %. This method is time consuming, because it takes about 1 week (Battilani *et al*., 2002).

There are several techniques such as enzyme linked immunosorbent assay (ELISA), indirect Fluorescent Antibody Test (IFAT), Hemagglutination (HA), Hemagglutination Inhibition (HI) assay and virus neutralization assay. For HA and HI assay the Rhesus monkey erythrocyte or porcine red blood cell of dilution 1:32 in fecal emulsions is considered significant. The test is carried out at 4 °C and the sera

must be pretreated with kaolin and porcine erythrocyte to remove the nonspecific background under the broad range of pH (pH 6.0-8.0). For virus neutralization assay, the paired sera are used in this test and the specific antibody would neutralize viral biological function then it can not invade host cell (Greenwood *et al*., 1996; Sagazio *et al*., 1998).

Polymerase chain reaction (PCR) is the technique to increase amount of DNA by in vitro synthesis. The result is highly sensitive over other methods. It is specific and required DNA template from sample even in small amount. PCR is the rapid method compared with the virus isolation and others immunological assays (Murphy *et al*., 1999).

## Baculoviruses as Expression Vectors for Recombinant Protein Production

Baculoviruses are DNA-containing viruses that infect insects or other invertebrates. They are double - stranded, circular, supercoiled DNA molecules in rod-shaped capsid. More than 500 Baculoviruses are isolated based on hosts of origin; most of them are from Lepidopteran family (Jarvis *et al*., 1996). Baculoviruses have been evaluated as biological pesticides, but their efficacy limited as they killed insects too slowly. The genetic engineering methods were developed and made it possible to produced recombinant baculoviruses as eukaryotic expression vectors for foreign protein production and more effective pesticide (Jarvis *et al*., 1996). Two of common isolates used in foreign gene expression are Autograph California multiple nuclear polyhedrosis virus (*AcMNPV*) and Bombyx mori (silkworm) nuclear polyhedrosis virus (*BmNPV*) Wild - type baculoviruses have both lytic and occluded life cycles independent developing throughout three phases of virus replication as following.

1. Early Phase or Virus synthesis phase

Viruses used infected cell for viral DNA replication (Jarvis *et al*., 1996). There are many steps included as attachment, penetration, uncoating, early viral gene expression. This step occurs 0.5 to 6 hour after infection (Ghosh *et al*., 2002.).

2. Late phase or viral structural phase

Late genes that code for replication of viral DNA and assembly of virus are expressed. (6-12 hours after infection). The cell produces extracellular virus (EV) or budded virus (BV). It contains plasma membrane envelope and glycoprotein (gp) 64, which is necessary for, endocytosis. At 18-36 hours the EV is released (Ghosh *et al.,* 2002.).

3. Very late phase or viral occlusion protein phase

The polyhedrin and p 10 genes are expressed, and the occluded virus (OB) or polyhedra occlusion bodies are formed and host cell was lyzed between 24 - 96 hours after infection (Jarvish *et al*., 1996 ; Ghosh *et al*., 2002).

The general approach used to baculovirus expression vector systems present by replacing the polyhedrin protein coding region with the foreign gene (gene of interest) (Ghosh *et al*., 2002.). Using the polyhedrin and p10 promoters, which are strong and provide high levels of transcription during the very late phase of infection. The resulting recombinant virus can infect cultured lepidopteran insect cells or larvae and express the foreign gene under the control of these promoters (Jarvish *et al*., 1996). Baculovirus expression vector system was reported about its safety, non pathogenic to mammals. It was also explained about the posttranslational modification which resulted in accurate form of produced protein.

# MATERIALS AND METHODS

## 1. Cell line and Virus

Canine A-72 cells were grown to 70-80% monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Fecal sample from a CPV-suspected dog was collected at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen campus. Fecal sample was dissolved in DMEM, spin for 13,000 round per minute for 15 min at 4°C, filtrated using 0.45µm syringe filter and inoculated to A-72 cells. The viruses were harvested at 120 hours post inoculation when the CPE reached approximately 80%. CPV was passaged for 3 times. Infected cells were used to extract DNA using phenol-chloroform extraction method (Sambrook and Russell, 1998).

## 2. Polymerase Chain Reaction for Diagnostic of CPV

The twenty collected samples of dog performed diarrhea or CPV suspected cases which were then found to be ten positive cases by commercial test kit (Animal Genetic®) based on ELISA technique which the test membrane was coated with the monoclonal antibody against CPV. All of the twenty samples of field fecal specimens from CPV-suspected dogs were then used for DNA extraction (Sambrook and Russell, 1998). The extracted DNA samples were used as template for performing Polymerase chain reaction (PCR) technique**.** Fecal suspension in either Phosphate Buffer Saline solution (PBS) or DMEM was extracted using phenol-chloroform extraction method A set of primer pair named VP2-400 bp was designed to be started from nucleotide 1,351 of VP2 gene to the stop codon sequence. The size of amplicon was approximately 400 bp in size. The sequence of the forward primer was 5′-TAT GGT CCT TTA ACT GCA TTA AA -3′ , and the reverse primer of 5′-TTA-ATA TAA TTT TCT AGG TGC TAG -3′. The PCR condition was pre-denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min and the final extension at 72°C for 10min**.**

The PCR mixture was composed of 1X PCR buffer, 3 mM dNTPs, 2.5 mM $MgCl_2$, and 1 pmol of each forward and reverses primer, 1.0 U of Taq DNA polymerase (Invitrogen®) and 10 µl of DNA templates to give the total volume of 100 µl. The PCR was done using Primus96 $^{plus}$ thermalcycler (Hybaid). PCR products were checked using 2% agarose gel electrophoresis at 100 volts 30 mins, staining with ethidium bromide and visualized under UV illumination (Spectroline).

**3. Polymerase Chain Reaction for Cloning VP2 Gene**

DNA of a CPV field isolate was used as the template for performing PCR technique. Two sets of primer pairs specific for the VP2 gene were designed using DNASIS program based on reported nucleotide sequence of GenBank. The first set of primer pair gave the first two-third of VP2 gene, which was approximately 1 kb in size. The sequence of forward primer of the first set was 5′-GGG-GGA-TCC-ACC-ATG-AGT-GAT-GGA-GCA-GTT-CAA-C-3′ (containing *BamH*I cleavage site), and reverse primer of 5′-TTA-TTG-TGT-CGA-CGC-CTC-AAA-AGA-3′ (containing *Sal*I cleavage site). The PCR conditions were pre-denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30 sec and extension at 72°C for 1min 30sec and the final extension at 72°C for 20 min**.**

The second set of primer pair was used to amplify the last one-third of VP2 gene which was 700 bp in size. The sequence of the forward primer of the second set was 5′-CCA-CCA-TGG-CGT-CGA-CAC-AAG-GGC-CA- 3′ (containing *Sal***I** cleavage site), and the reverse primer of 5′-GGG-TCT-AGA-TTA-ATA-TAA-TTT-TCT-AGG-TGC-TAG-3′(containing *Xba*I cleavage site). The PCR conditions were pre-denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min and additional final extension at 72°C for 15 min. The PCR mixture for PCR reaction was composed of 1X PCR buffer, 3 mM dNTPs, 2.5 mM $MgCl_2$, and 1 pmol of each forward and reverses primer, 1.0 U of Taq DNA polymerase (Invitrogen®) and 10 µl of DNA template to give the total volume of 100 µl. The PCR was done using

Primus96 $^{plus}$ thermalcycler (Hybaid). PCR products were checked using 1% agarose gel electrophoresis at 100 volts 30 mins, staining with ethidium bromide and visualized under UV illumination (Spectroline).

**4. Construction of Transfer Vector**

PCR products were purified using QIA quick gel extraction kit (QIAGEN®) and ligated to plasmid pGEM®-T Easy (Promega) as manufacturer described. The ligation reaction was used to transform E.coli strain JM109 (Gibco®). The transformed E.coli was grown in Lauria Bertani medium (LB) which was the selective media containing100 µg/ml ampicilin, 0.5mM IPTG and 80µg/ml X-Gal. The positive clone was checked by white-blue colony screening, the PCR assay and restriction enzyme digestion. Then the positive colony was scaled up and used for plasmid isolation. The plasmid pGEM®-T Easy circle map is showed in appendix figure 2.

Subsequently, the inserts in plasmid pGEM®-T Easy were cut, purified and ligated with plasmid pFastBac™Htb (Invitrogen) to give plasmid either pFastBac-VP2-5′end or pFastBac-VP2-3′end. The ligation reaction mixture was used to transform E.coli strain DH5-α(Gibco®) competent cell. The transformed E.coli was grown in LB selective media containing kanamycin (50 µg /ml), gentamicin (7 µg/ml), and tetracycline (10 µg/ml). The restriction enzyme digestions of recombinant plasmid pFastBac-VP2-3′end were done by *Sal*I and *Xba*I then the insert of 700 bp was purified and ligated to purified plasmid pFastBac-VP2-5′end linearized with *Sal*I to give plasmid pFastBac-VP2 containing the insert size of 1.7 kb (full length VP2). The positive clones were checked again using the *BamH*I and *Xba*I restriction enzymes to be assured the presence of clone that obtained recombinant plasmid with the full length VP2 gene. Thus it was scaled up and sequenced. The sequence of full length VP2 was aligned with VP2 of CPV, which is available on GenBank. The purified recombinant plasmids pFastBac-VP2 and pFastBac-VP2-5′end were later

used to transform E. coli strain DH10-Bac (Invitrogen®). The plasmid pFastBac™ Htb circle map is showed in Appendix figure 3.

**5. Insect Cell Transfect ion and Recombinant Virus Amplification**

SF 21 cells were grown in SF900II medium (Invitgrogen®) supplemented with 4% FBS and 10% antibiotics at 27°C. The wild type baculovirus DNA and the recombinant baculovirus DNA containing 1 kb and 1.7 kb of VP2 gene were used to transfect SF 21 (*Spodoptera frugiperda*) cells separately using Cellfectin (Invitgrogen®). The inoculum was overlaid for at least an hour at room temperature. After that the inoculum was removed and new medium of SF900II (Gibco®) with 4% FBS was added. At 72 hours of transfection, the wild type baculovirus and recombinant virus were harvested and checked by PCR reaction using either forward primer of vector (M13) and the gene specific reverse primers in case of recombinant virus detection or using vector forward and reverse primers of M13 for wild type baculovirus.

For viral amplification, the supernatant from SF 21 infected cells was collected then used as inoculums for the next passages. The proper multiplicity of infection (MOI) of 0.01 to 0.1 was needed for viral amplification. The amplified viruses were harvested at 48 hours post inoculation and were used to determine the viral titer. Two million of SF21 cells per milliliter were used for inoculation with ten-fold dilution of recombinant baculovirus. The inoculum was overlaid for an hour and the new solid media containing 2% methyl cellulose in SF900II with 4%FBS was used to substitute the old media. Seven to ten days post-inoculation, infected-cells were fixed with 1% formalin solution and stained with 0.1% methylene blue. The clear spots were counted. The inoculum required is according to the following formula.

$$\text{Inoculum required} = \frac{\text{desired MOI (pfu/cell)} \times \text{total number of cells}}{\text{Titer of viral inoculum (pfu/ml)}}$$

## 6. Recombinant Protein Expression and Detection

Hi Five ™ cells *(Trichoplusia ni*) grown in Express Five serum-free medium (Invitrogen®) supplemented with 9% L-glutamine and 10% antibiotics were used to produce recombinant protein. The appropriate MOI of 2-5 was needed for VP2 protein expression (Weichert *et al.,* 1998; Yuan *et. al.,* 2000). The recombinant baculoviruses containing first 2/3 of VP2 gene and full-length VP2 gene were used to produce recombinant truncated VP2 and whole VP2 proteins. The recombinant proteins were harvested at 72 hours post inoculation. Cells were harvested by thoroughly pipetting and centrifuged at 500g for 5 minutes. The cell pellets were washed using 1ml. of phosphate buffer saline and the suspension was centrifuged. The pellet of 5 µl was lyzed by either 10% Sodium dodecyl sulfate (SDS) or 8M Urea as denaturing agent for crude protein preparation. The cell lysate was discarded after centrifugation and the supernatant is considered as crude protein. Comparison of recombinant truncated VP2 and whole VP2 proteins with wild type baculovirus produced protein were checked by 10% polyacrylamide gel electrophoresis, dot blot and western blot analysis.

## 7. Preparation of Polyclonal Antibody against CPV

The polyclonal antibody against CPV was prepared by immunized rabbit with 1 ml of Canine Parvovirus modified-lived vaccine (Intervet). Rabbits were immunized 3 times for 2 weeks apart. The rabbit hyperimmune serum against CPV was checked for the reactivity against CPV by immunodot using commercial vaccine.

## 8. Immunodot Analysis

The crude extracted protein of total volume 3 µl was dotted on nitrocellulose membrane and incubated with either rabbit anti CPV hyperimmune sera (1:250) or mouse anti-histidine IgG monoclonal antibody (1:3000) for 2 hrs at room temperature. Subsequently, the membrane was incubated with either goat anti-rabbit IgG (1:500) or goat anti-mouse IgG conjugated with peroxidase (1:500) for 1 hr at room temperature.

The membrane was finally incubated with diaminobenzidine solution (Sigma®) containing 1% $H_2O_2$ for 5-10 mins at room temperature.

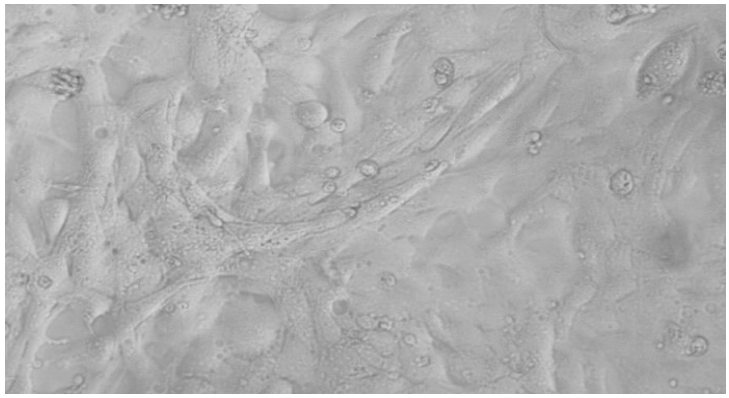
**9. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis**

Ten microliters of crude extracted was analyzed using 10 % SDS-PAGE and stained with Coomasie brilliant blue. Electrophoresis was run at constant 100 volt/cm for 60mins. For Western blot analysis, proteins on SDS-PAGE were transferred onto nitrocellulose membrane under condition of constant 400 mAmp for 5 hours in prepared cold transfer buffer. Nitrocellulose membranes were incubated overnight in 5% skim milk in PBS+0.5%Tween 20 at 4°C. Subsequently, the membranes were incubated with either rabbit anti-CPV hyperimmune sera (1:100) or mouse anti-histidine IgG monoclonal antibody (1:3,000) for 2 hours at room temperature. The nitrocellulose membrane was incubated with either goat anti rabbit IgG (1:300) or goat anti-mouse IgG (1:500) conjugated with peroxidase for 1 hour at room temperature. The membrane was then incubated with diaminobenzidine solution (Sigma®) containing 1% $H_2O_2$ for 5-10 mins.

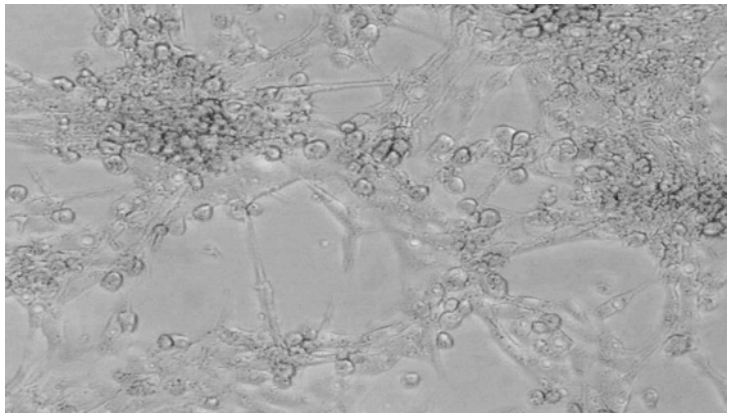
# RESULTS

## 1. Virus Isolation

The A-72 cells showed cytopathic effects at 120 hour post-inoculation (h.p.i.), whereas the control A-72 cells did not. The normal A-72 cells at 120 h.p.i. had fibroblast shape (Figure 1(a)). On the other hand, the inoculated cells were round up and slough off the surface (Figure 1(b)). In order to confirm the presence of CPV in A-72 cells, the PCR assay was conducted and the PCR results showed the positive results from the 2nd and 3rd passage of A-72 cells. A-72 cell that showed cytopathic effect were the positive by PCR and the negative control cells were negative.



**(a)**



**(b)**

**Figure 1 (a)** The non infected A-72 cell line at 120 hr. under 400X of light field microscope. and **(b)** The CPE performance of CPV infected A-72 cells of the 3rd passage at120 hr. under 400X of light field microscope

**2. Polymerase Chain Reaction for Diagnostic of CPV**

The amplicon of 400 bp was detected from 10 out of the 20 samples of CPV-suspected or diarrhea cases which were approximately 50% from the total cases and 100% from the ten proved positive cases by Elisa test kit. The PCR product was showed in figure 2.

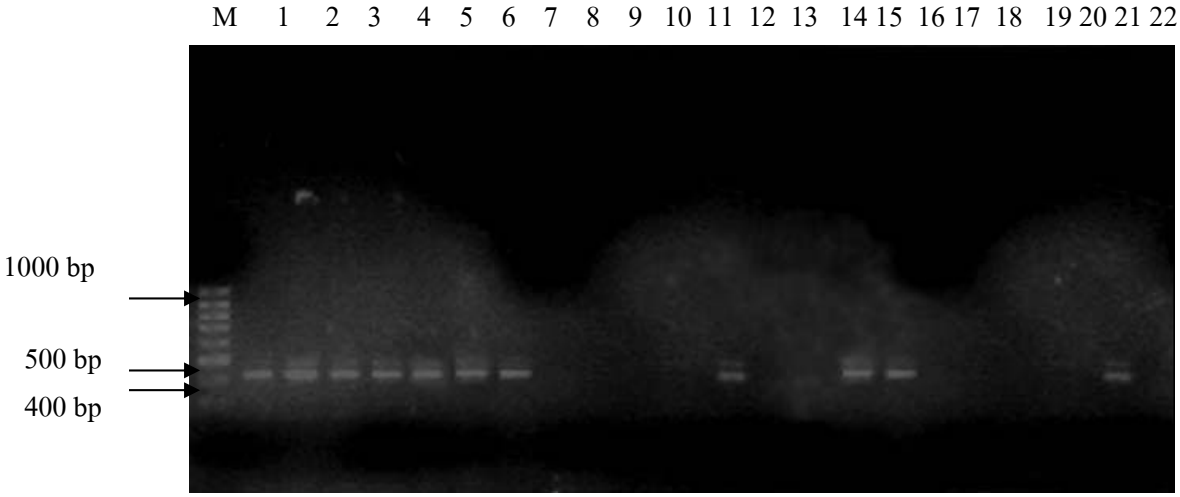



**Figure 2** Analysis of PCR products of 400 bp fragment of VP2 gene using 2% agarose gel electrophoresis. A 5 μl of PCR mixture was loaded onto each lane of agarose gel. Lane M = DNA marker, Lane 1 = PCR product of 400 bp of positive control, lane 2-21=PCR products of field samples, Lane 22=negative control

**3. Cloning of VP2 Gene.**

The strategy for cloning VP2 gene in this study was shown as figure 3. The amplified products of 1 Kb (the first two-third of VP2 gene) and 700bp fragment (the last one-third of VP2 gene) showed the expected size of products in figure 4 and 5 Both fragments successfully cloned to pFastBac™ Htb. The recombinant pFastBac-VP2 and the recombinant pFastBac-VP2-5′end were then proved by PCR and restriction enzymes digestion which showed correspondence in figure 6. The nucleotide sequence of recombinant VP2 gene was shown in figure 7. The nucleotide and deduced amino acid sequences of recombinant VP2 gene was used to compare to the available sequences of other biotypes. The CPV-KPS of Kamphaengsaen isolate had the 100% homology to both nucleotide and amino acid sequences of CPV-2b (figure 8). Comparison CPV-KPS isolate with CPV-2a found a distinguished amino acid at residue 426.

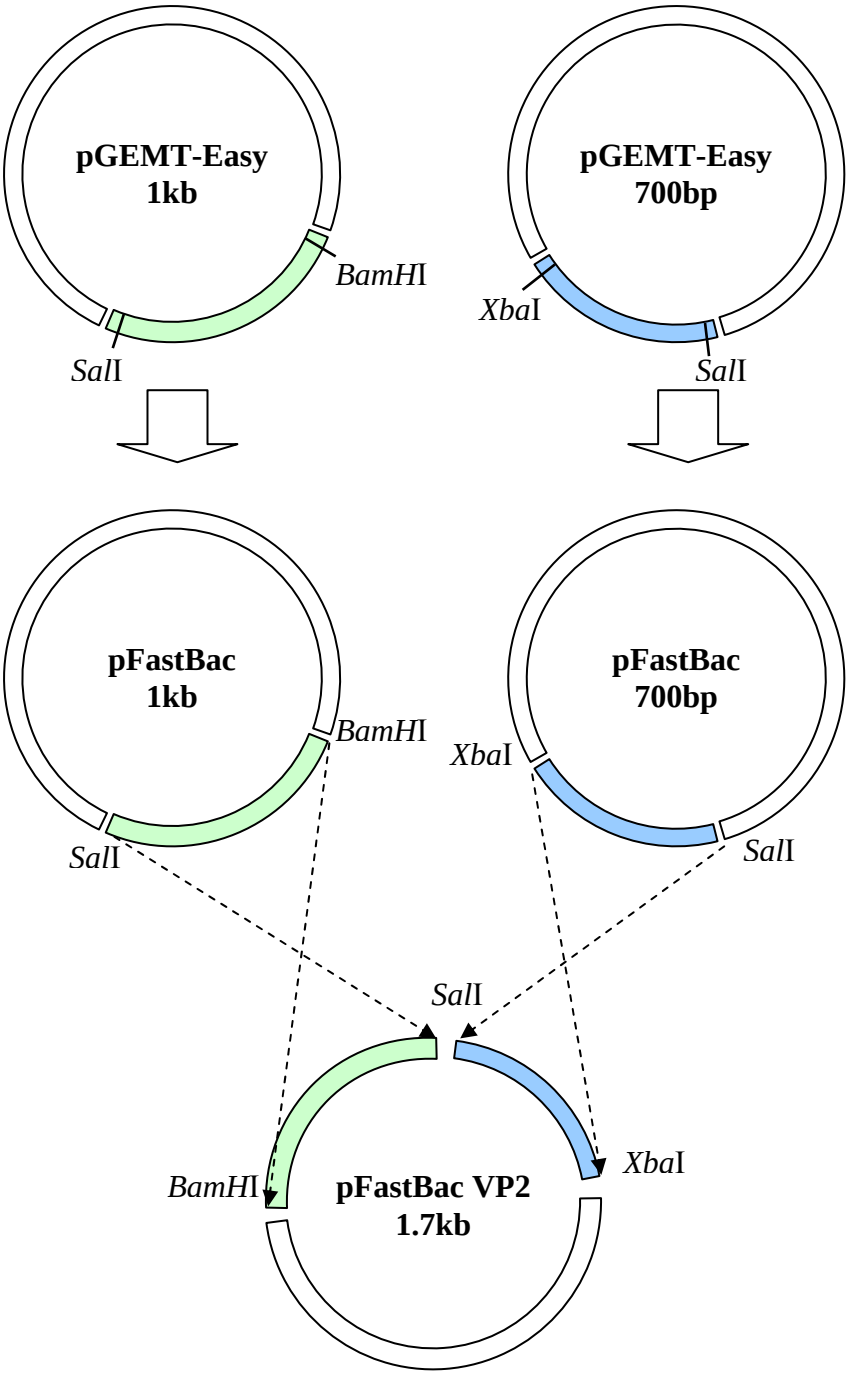



**Figure 3** The schematic of the cloning steps of CPV VP2 gene in this study.

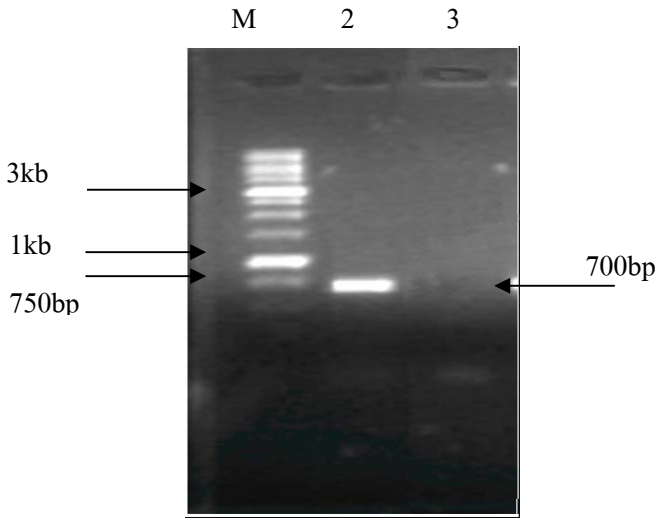



**Figure 4** Analysis of PCR products of the 3′end of VP2 gene using 1% agarose gel electrophoresis. A 5µl of PCR mixture was loaded onto each lane of agarose gel. Lane M = DNA marker, Lane 2 = PCR product of 700 bp VP2, Lane 3=negative control

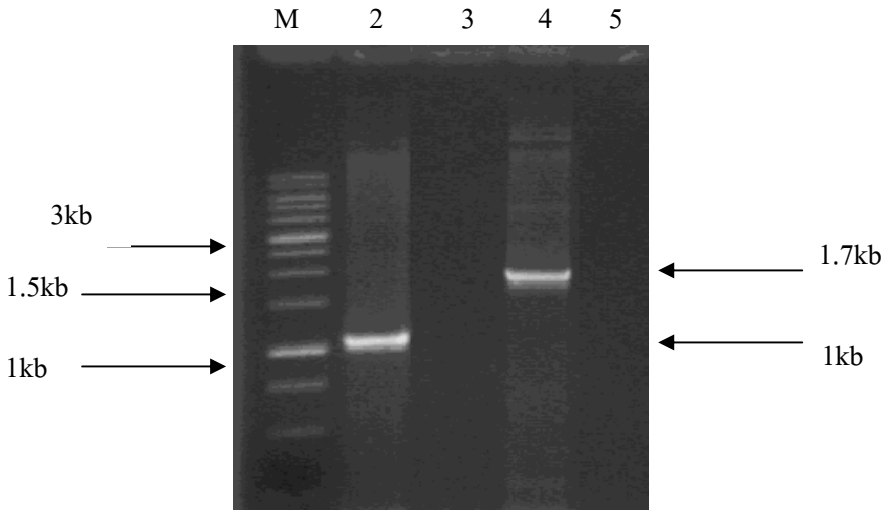



**Figure 5** Analysis of PCR products of VP2 gene using 1.5% agarose gel electrophoresis. A 5 µl of PCR mixture was loaded onto each lane of agarose gel. Lane M = DNA marker, Lane 2 = PCR product of ~1kb of VP2, Lane 3=negative control of 1Kb reaction, Lane 4 = PCR products of whole VP2 gene and lane 5=negative control.

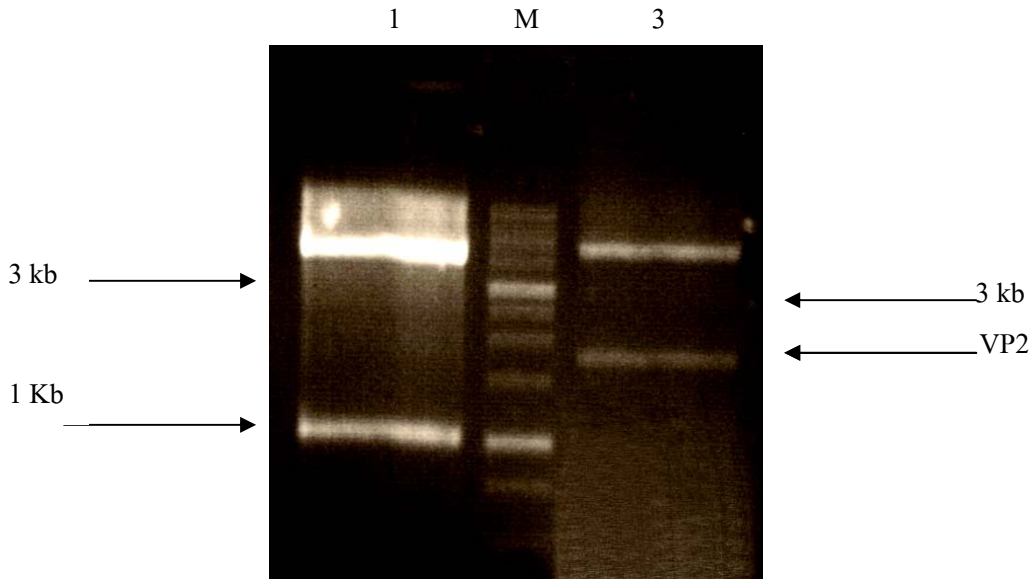



**Figure 6** Analysis of restriction enzyme digestion of recombinant pFastBac™Htb-VP2 and recombinant pFastBac™Htb 1 kb using 1.5% agarose gel electrophoresis. Forty microliters of mixture was loaded on lane 1 and lane 3. Five microliters of DNA marker was loaded onto lane M. Lane 1= recombinant pFastBac™Htb 1kb digested by *Sal*I and *BamH*I, Lane 3= recombinant pFastBac™Htb VP2 cleaved by BamHI and *Xba*I.

```
CPV-KPS         ATGAGTGATGGAGCAGTTCAACCAGACGGTGGTCAGCCTGCTGTCAGAAATGAAAGAGCT 60
CPV-2a          ATGAGTGATGGAGCAGTTCAACCAGACGGTGGTCAGCCTGCTGTCAGAAATGAAAGAGCT 60
CPV-2b          ATGAGTGATGGAGCAGTTCAACCAGACGGTGGTCAACCTGCTGTCAGAAATGAAAGAGCT 60
                ************************************ ************************

CPV-KPS         ACAGGATCTGGGAACGGGTCTGGAGGCGGGGGTGGTGGTGGTTCTGGGGGTGTGGGGATT 120
CPV-2a          ACAGGATCTGGGAACGGGTCTGGAGGCGGGGGTGGTGGTGGTTCTGGGGGTGTGGGGATT 120
CPV-2b          ACAGGATCTGGGAACGGGTCTGGAGGCGGGGGTGGTGGTGGTTCTGGGGGTGTGGGGATT 120
                ************************************************************

CPV-KPS         TCTACGGGTACTTTCAATAATCAGACGGAATTTAAATTTTTGGAAAACGGATGGGTGGAA 180
CPV-2a          TCTACGGGTACTTTCAATAATCAGACAGAATTTAAATTTTTGGAAAACGGATGGGTGGAA 180
CPV-2b          TCTACGGGTACTTTCAATAATCAGACGGAATTTAAATTTTTGGAAAACGGATGGGTGGAA 180
                ************************* ******************************

CPV-KPS         ATCACAGCAAACTCAAGCAGACTTGTACATTTAAATATGCCAGAAAGTGAAAATTATAGA 240
CPV-2a          ATCACAGCAAACTCAAGCAGACTTGTACATTTAAATATGCCAGAAAGTGAAAATTATAGA 240
CPV-2b          ATCACAGCAAACTCAAGCAGACTTGTACATTTAAATATGCCAGAAAGTGAAAATTATAGA 240
                ************************************************************

CPV-KPS         AGAGTGGTTGTAAATAATTTGGATAAAACTGCAGTTAACGGAAACATGGCTTTAGATGAT 300
CPV-2a          AGAGTGGTTGTAAATAATTTGGATAAAACTGCAGTTAACGGAAACATGGCTTTAGATGAT 300
CPV-2b          AGAGTGGTTGTAAATAATTTGGATAAAACTGCAGTTAACGGAAACATGGCTTTAGATGAT 300
                ************************************************************

CPV-KPS         ACTCATGCACAAATTGTAACACCTTGGTCATTGGTTGATGCAAATGCTTGGGGAGTTTGG 360
CPV-2a          ACTCATGCACAAATTGTAACACCTTGGTCATTGGTTGATGCAAATGCTTGGGGAGTTTGG 360
CPV-2b          ACTCATGCACAAATTGTAACACCTTGGTCATTGGTTGATGCAAATGCTTGGGGAGTTTGG 360
                ************************************************************

CPV-KPS         TTTAATCCAGGAGATTGGCAACTAATTGTTAATACTATGAGTGAGTTGCATTTAGTTAGT 420
CPV-2a          TTTAATCCAGGAGATTGGCAACTAATTGTTAATACTATGAGTGAGTTGCATTTAGTTAGT 420
CPV-2b          TTTAATCCAGGAGATTGGCAACTAATTGTTAATACTATGAGTGAGTTGCATTTAGTTAGT 420
                ************************************************************

CPV-KPS         TTTGAACAAGAAATTTTTAATGTTGTTTTAAAGACTGTTTCAGAATCTGCTACTCAGCCA 480
CPV-2a          TTTGAACAAGAAATTTTTAATGTTGTTTTAAAGACTGTTTCAGAATCTGCTACTCAGCCA 480
CPV-2b          TTTGAACAAGAAATTTTTAATGTTGTTTTAAAGACTGTTTCAGAATCTGCTACTCAGCCA 480
                ************************************************************

CPV-KPS         CCAACTAAAGTTTATAATAATGATTTAACTGCATCATTGATGGTTGCATTAGATAGCAAT 540
CPV-2a          CCAACTAAAGTTTATAATAATGATTTAACTGCATCATTGATGGTTGCATTAGATAGCAAT 540
CPV-2b          CCAACTAAAGTTTATAATAATGATTTAACTGCATCATTGATGGTTGCATTAGATAGTAAT 540
                ************************************************************ ***

CPV-KPS         AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
CPV-2a          AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
CPV-2b          AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
                ************************************************************

CPV-KPS         AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
CPV-2a          AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
CPV-2b          AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
                ************************************************************

CPV-KPS         TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
CPV-2a          TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
CPV-2b          TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
                ************************************************************

CPV-KPS         GTTCAATTTTATACTATTGAAAATTCTGTGCCAGTACACTTACTAAGGACAGGTGATGAA 780
CPV-2a          GTTCAATTTTATACTATTGAAAATTCTGTGCCAGTACACTTACTAAGGACAGGTGATGAA 780
CPV-2b          GTTCAATTTTATACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
                ********************************************* ************

CPV-KPS         TTTGCTACAGGAACATTTTTTTTTTGATTGTAAACCATGTAGACTAACACATACATGGCAA 840
CPV-2a          TTTGCTACAGGAACATTTTTTTTTTGATTGTAAACCATGTAGACTAACACATACATGGCAA 840
CPV-2b          TTTGCTACAGGAACATTTTTTTTTTGATTGTAAACCATGTAGACTAACACATACATGGCAA 840
                ************************************************************
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**Figure 7** Nucleotide sequences alignment of VP2 gene of CPV Kamphaengsaen isolate (top line) compared to other biotypes available on GenBank;CPV-2a (Accession number AB054217)and CPV-2b (Accession number AB054218) using ClustalW program. * = identical nucleotide sequence found

```
CPV-KPS         ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
CPV-2a          ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
CPV-2b          ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
                ************************************************************

CPV-KPS         ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
CPV-2a          ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
CPV-2b          ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
                ************************************************************

CPV-KPS         AATACAAACTATATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
CPV-2a          AATACAAACTATATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
CPV-2b          AATACAAACTATATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
                ************************************************************

CPV-KPS         CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
CPV-2a          CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
CPV-2b          CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
                ************************************************************

CPV-KPS         CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140
CPV-2a          CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140
CPV-2b          CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140
                ************************************************************

CPV-KPS         GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200
CPV-2a          GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200
CPV-2b          GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200
                ************************************************************

CPV-KPS         ATAGCACATCAAGATACAGGAAGATATCCAGAAGGAGATTGGATTCAAAATATTAACTTT 1260
CPV-2a          ATAGCACATCAAGATACAGGAAGATATCCAGAAGGAGATTGGATTCAAAATATTAACTTT 1260
CPV-2b          ATAGCACATCAAGATACAGGAAGATATCCAGAAGGAGATTGGATTCAAAATATTAACTTT 1260
                ************************************************************

CPV-KPS         AACCTTCCTGTAACAAATGATAATGTATTGCTACCAACAGATCCAATTGGAGGTAAAACA 1320
CPV-2a          AACCTTCCTGTAACAGATGATAATGTATTGCTACCAACAGATCCAATTGGAGGTAAAACA 1320
CPV-2b          AACCTTCCTGTAACAAATGATAATGTATTGCTACCAACAGATCCAATTGGAGGTAAAACA 1320
                *************** ********************************************

CPV-KPS         GGAATTAACTATACTAATATATTTAATACTTATGGTCCTTTAACTGCATTAAATAATGTA 1380
CPV-2a          GGAATTAACTATACTAATATATTTAATACTTATGGTCCTTTAACTGCATTAAATAATGTA 1380
CPV-2b          GGAATTAACTATACTAATATATTTAATACTTATGGTCCTTTAACTGCATTAAATAATGTA 1380
                ************************************************************

CPV-KPS         CCACCAGTTTATCCAAATGGTCAAATTTGGGATAAAGAATTTGATACTGACTTAAAACCA 1440
CPV-2a          CCACCAGTTTATCCAAATGGTCAAATTTGGGATAAAGAATTTGATACTGACTTAAAACCA 1440
CPV-2b          CCACCAGTTTATCCAAATGGTCAAATTTGGGATAAAGAATTTGATACTGACTTAAAACCA 1440
                ************************************************************

CPV-KPS         AGACTTCATGTAAATGCACCATTTGTTTGTCAAAATAATTGTCCTGGTCAATTATTTGTA 1500
CPV-2a          AGACTTCATGTAAATGCACCATTTGTTTGTCAAAATAATTGTCCTGGTCAATTATTTGTA 1500
CPV-2b          AGACTTCATGTAAATGCACCATTTGTTTGTCAAAATAATTGTCCTGGTCAATTATTTGTA 1500
                ************************************************************

CPV-KPS         AAAGTTGCGCCTAATTTAACAAATGAATATGATCCTGATGCATCTGCTAATATGTCAAGA 1560
CPV-2a          AAAGTTGCGCCTAATTTAACAAATGAATATGATCCTGATGCATCTGCTAATATGTCAAGA 1560
CPV-2b          AAAGTTGCGCCTAATTTAACAAATGAATATGATCCTGATGCATCTGCTAATATGTCAAGA 1560
                ************************************************************

CPV-KPS         ATTGTAACTTACTCAGATTTTTGGTGGAAAGGTAAATTAGTATTTAAAGCTAAACTAAGA 1620
CPV-2a          ATTGTAACTTACTCAGATTTTTGGTGGAAAGGTAAATTAGTATTTAAAGCTAAACTAAGA 1620
CPV-2b          ATTGTAACTTACTCAGATTTTTGGTGGAAAGGTAAATTAGTATTTAAAGCTAAACTAAGA 1620
                ************************************************************

CPV-KPS         GCCTCTCATACTTGGAATCCAATTCAGCAAATGAGTATTAATGTAGATAACCAATTTAAC 1680
CPV-2a          GCCTCTCATACTTGGAATCCAATTCAACAAATGAGTATTAATGTAGATAACCAATTTAAC 1680
CPV-2b          GCCTCTCATACTTGGAATCCAATTCAGCAAATGAGTATTAATGTAGATAACCAATTTAAC 1680
                ************************* **********************************

CPV-KPS         TATGTACCAAGTAATATTGGAGGTATGAAGATTGTATATGAAAAATCTCAACTAGCACCT 1740
CPV-2a          TATGTACCAAGTAATATTGGAGGTATGAAAATTGTATATGAAAAATCTCAACTAGCACCT 1740
CPV-2b          TATGTACCAAGTAATATTGGAGGTATGAAGATTGTATATGAAAAATCTCAACTAGCACCT 1740
                ***************************** ****************************

CPV-KPS         AGAAAATTATATTAA 1755
CPV-2a          AGAAAATTATATTAA 1755
CPV-2b          AGAAAATTATATTAA 1755
                ***************
```

**Figure 7** (cont'd)

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CPV-KPS         MSDGAVQPDGGQPAVRNERATGSGNGSGGGGGGGSGGVGISTGTFNNQTEFKFLENGWVE 60
CPV-2a          MSDGAVQPDGGQPAVRNERATGSGNGSGGGGGGGSGGVGISTGTFNNQTEFKFLENGWVE 60
CPV-2b          MSDGAVQPDGGQPAVRNERATGSGNGSGGGGGGGSGGVGISTGTFNNQTEFKFLENGWVE 60
                ************************************************************

CPV-KPS         ITANSSRLVHLNMPESENYRRVVVNNLDKTAVNGNMALDDTHAQIVTPWSLVDANAWGVW 120
CPV-2a          ITANSSRLVHLNMPESENYRRVVVNNLDKTAVNGNMALDDTHAQIVTPWSLVDANAWGVW 120
CPV-2b          ITANSSRLVHLNMPESENYRRVVVNNLDKTAVNGNMALDDTHAQIVTPWSLVDANAWGVW 120
                ************************************************************

CPV-KPS         FNPGDWQLIVNTMSELHLVSFEQEIFNVVLKTVSESATQPPTKVYNNDLTASLMVALDSN 180
CPV-2a          FNPGDWQLIVNTMSELHLVSFEQEIFNVVLKTVSESATQPPTKVYNNDLTASLMVALDSN 180
CPV-2b          FNPGDWQLIVNTMSELHLVSFEQEIFNVVLKTVSESATQPPTKVYNNDLTASLMVALDSN 180
                ************************************************************

CPV-KPS         NTMPFTPAAMRSETLGFYPWKPTIPTPWRYYFQWDRTLIPSHTGTSGTPTNIYHGTDPDD 240
CPV-2a          NTMPFTPAAMRSETLGFYPWKPTIPTPWRYYFQWDRTLIPSHTGTSGTPTNIYHGTDPDD 240
CPV-2b          NTMPFTPAAMRSETLGFYPWKPTIPTPWRYYFQWDRTLIPSHTGTSGTPTNIYHGTDPDD 240
                ************************************************************

CPV-KPS         VQFYTIENSVPVHLLRTGDEFATGTFFFDCKPCRLTHTWQTNRALGLPPFLNSLPQAEGG 300
CPV-2a          VQFYTIENSVPVHLLRTGDEFATGTFFFDCKPCRLTHTWQTNRALGLPPFLNSLPQAEGG 300
CPV-2b          VQFYTIENSVPVHLLRTGDEFATGTFFFDCKPCRLTHTWQTNRALGLPPFLNSLPQAEGG 300
                ************************************************************

CPV-KPS         TNFGYIGVQQDKRRGVTQMGNTNYITEATIMRPAEVGYSAPYYSFEASTQGPFKTPIAAG 360
CPV-2a          TNFGYIGVQQDKRRGVTQMGNTNYITEATIMRPAEVGYSAPYYSFEASTQGPFKTPIAAG 360
CPV-2b          TNFGYIGVQQDKRRGVTQMGNTNYITEATIMRPAEVGYSAPYYSFEASTQGPFKTPIAAG 360
                ************************************************************

CPV-KPS         RGGAQTDENQAADGDPRYAFGRQHGQKTTTTGETPERFTYIAHQDTGRYPEGDWIQNINF 420
CPV-2a          RGGAQTDENQAADGDPRYAFGRQHGQKTTTTGETPERFTYIAHQDTGRYPEGDWIQNINF 420
CPV-2b          RGGAQTDENQAADGDPRYAFGRQHGQKTTTTGETPERFTYIAHQDTGRYPEGDWIQNINF 420
                ************************************************************

CPV-KPS         NLPVTNDNVLLPTDPIGGKTGINYTNIFNTYGPLTALNNVPPVYPNGQIWDKEFDTDLKP 480
CPV-2a          NLPVTDDNVLLPTDPIGGKTGINYTNIFNTYGPLTALNNVPPVYPNGQIWDKEFDTDLKP 480
CPV-2b          NLPVTNDNVLLPTDPIGGKTGINYTNIFNTYGPLTALNNVPPVYPNGQIWDKEFDTDLKP 480
                *****:******************************************************

CPV-KPS         RLHVNAPFVCQNNCPGQLFVKVAPNLTNEYDPDASANMSRIVTYSDFWWKGKLVFKAKLR 540
CPV-2a          RLHVNAPFVCQNNCPGQLFVKVAPNLTNEYDPDASANMSRIVTYSDFWWKGKLVFKAKLR 540
CPV-2b          RLHVNAPFVCQNNCPGQLFVKVAPNLTNEYDPDASANMSRIVTYSDFWWKGKLVFKAKLR 540
                ************************************************************

CPV-KPS         ASHTWNPIQQMSINVDNQFNYVPSNIGGMKIVYEKSQLAPRKLYX 585
CPV-2a          ASHTWNPIQQMSINVDNQFNYVPSNIGGMKIVYEKSQLAPRKLYX 585
CPV-2b          ASHTWNPIQQMSINVDNQFNYVPSNIGGMKIVYEKSQLAPRKLYX 585
                ********************************************
```
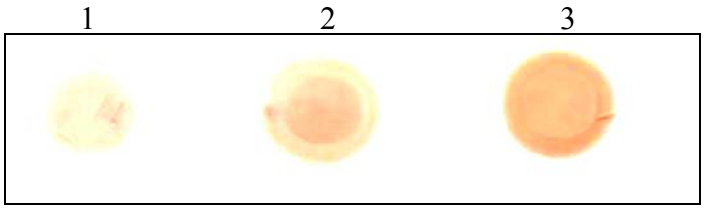
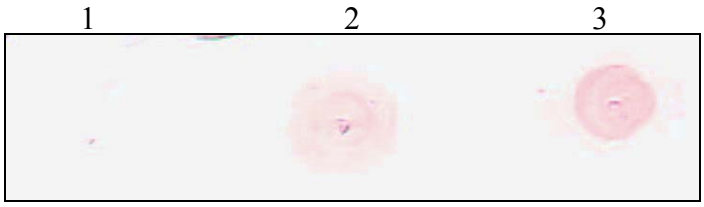

**Figure 8** Amino acid sequence alignment of the VP2 protein of CPV Kamphaengsaen isolate (CPV in the top line) and other biotypes available on GenBank; CPV-2a (protein identification number BAB21023.1) and CPV-2b (BAB21024.1) using ClustalW program. * = identical amino acid

**3. Detection of Recombinant Protein**

Dot blot analysis of crude protein extracted from recombinant baculovirus infected High Five™ cells after 72 h.p.i. using rabbit hyperimmune serum against Canine Parvovirus and monoclonal antibody against histidine showed positive results of expressed 1 Kb part of VP2 and complete VP2 protein figure 9(a) and figure 9(b). SDS-PAGE (figure 10) gave a distinct band of approximately 45 kDa and 68 kDa of VP2 proteins respectively which were approximately the sizes of target protein (Battilani *et.al* 2002). Western blot analysis of the crude protein extracted from baculovirus infected High Five™ cells using monoclonal antibody against histidine was also positive for VP2 protein (Figure 11).




**(a)**




**(b)**

**Figure 9** Dot blot analysis of crude protein extracted from wild type baculovirus infected High Five™ cells (1), expressed of 1 kb of VP2 infected High Five™ cells (2 ), the expressed whole VP2 (3) using rabbit hyperimmune serum against CPV (a) and monoclonal antibody against histidine (b).

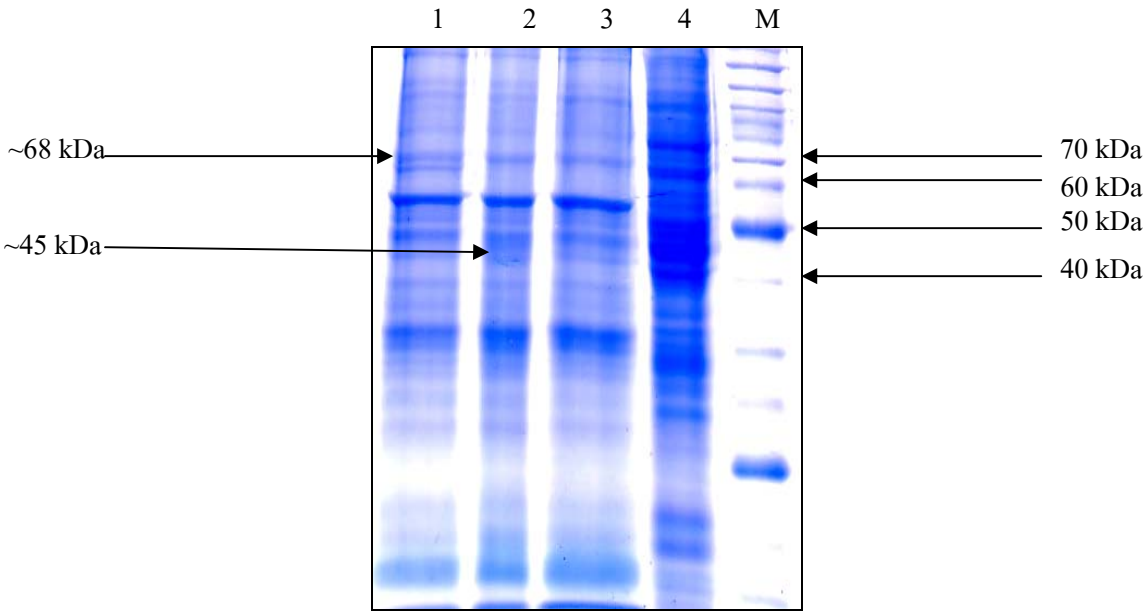



**Figure 10** 10% SDS-PAGE analysis of crude protein from VP2 recombinant baculovirus infected High Five™ cells (1), expressed of 1 kb (2), wild type infected cells (3), normal High Five™ cells (4) and protein standard (Lane M)

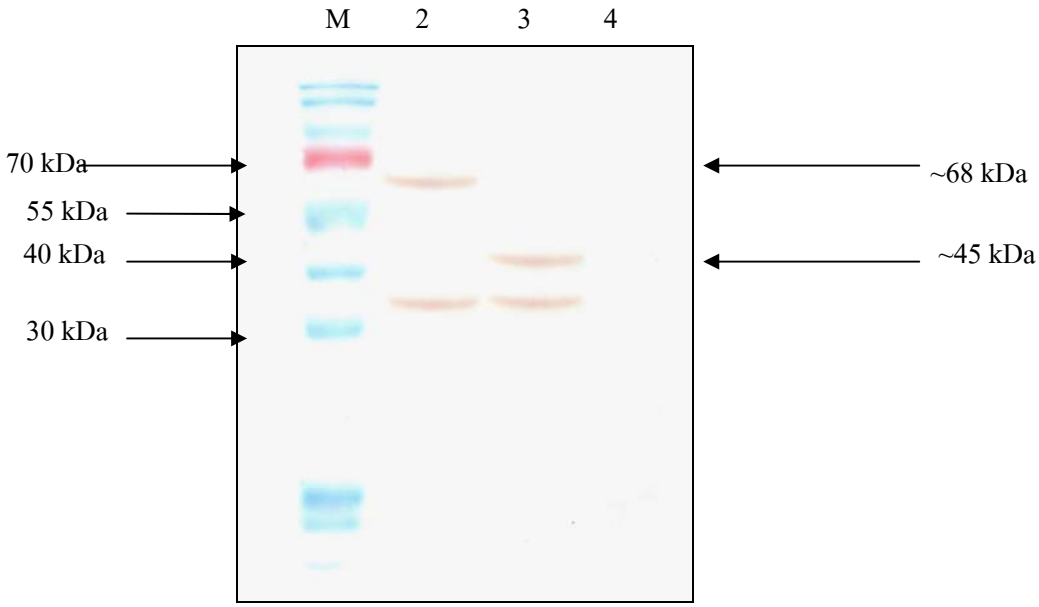



**Figure 11** Western blot analysis of crude protein from recombinant baculovirus VP2 infected cells(Lane 2), recombinant baculovirus with 1Kb of VP2 (lane 3) and wild type infected High Five™ cells (Lane 4) using monoclonal antibody against histidine. Lane M was the protein standard marker.

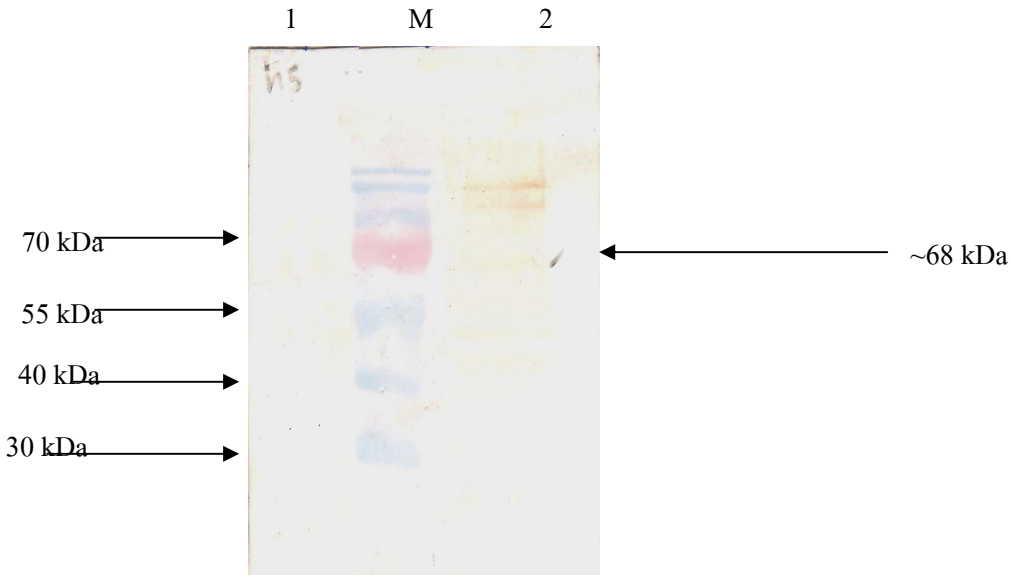



**Figure 12** Western blot analysis of crude protein from wild type infected High Five™ cells (Lane1) and recombinant baculovirus VP2 infected cells (Lane 2), and Lane M was the protein standard marker using rabbit hyperimmune serum against Canine Parvovirus.

# DISCUSSION

## Diagnostic of CPV

Diagnosis of CPV is usually based on the integrated information obtained from the history taking, clinical signs and altogether with one of the following techniques which are hemagglutination (HA), hemagglutination inhibition(HI), serological test or immunological methods such as ELISA, virus isolation , direct electron microscopy and polymerase chain reaction. However the gold standard of CPV detection is direct electron microscopy which is needed for the skilled person, expensive equipment and high cost for maintenance while ELISA for CPV is commercially used as test kit (Schunck *et al*., 1995; Knipe *et al*., 2001; Battilani *et al*.,2002). Serological technique that widely used for diagnosis could be used in CPV detection, but it may be interfered with neutralizing antibody. Besides HA followed by HI assay is routinely applied, but it is typically found the nonspecific agglutinin from the other content in fecal material. Time of viremia and antibody level also affected to the detection of CPV agent by the mentioned immunological techniques which is caused by antibody disruption since the produced antibody would neutralize the agent to be false negative for the CPV agent detection (Knipe *et al*., 2001). The viral isolation method is served as one of definitive measure although it is time consuming depending on viral titer or concentration of excreted virus (Greenwood *et al*., 1996; Sagazio *et al*., 1998).

In this study the virus isolation and PCR assay were demonstrated, virus isolation result found to induce CPE in canine A-72 cell line which conducted that CPV-KPS presence of CPE as had been described similar to the previous report (Murphy *et al*., 1999). The viral stock obtained from positive case of CPV by viral isolation method would be essential for future study toward reactivity of antibody against recombinant protein, because it is required to the viral titer determination. Afterwards the twenty fecal samples from diarrhea cases were screened by commercial test kit derived from ELISA technique. Then the PCR assay was conducted and gave the result at 100% from ten positive cases caused of CPV. It was

presented that CPV to be causative agent by the 50% from total of twenty collected diarrhea cases which the result was related to reported enteritis cases conducted in Thailand by Sakulwira *et al*., 2001 which was found to be 61.8%(34 out of 55 cases). The studies conducted by Mochizuki *et al.*, in 1995 with PCR performed the result of 37.3% positive sample tests (22 out of 59 cases). Due to the high percentage of CPV from diarrhea cases found in this report and other researches might be pointed to CPV to be the major cause. This result revealed that the other negative cases derived from the diarrhea symptoms may caused by other pathogens as had been reported previously. The other causative agents of diarrhea in dogs may include parasite, bacteria and other viruses such as corona virus, adenovirus and paramyxovirus and etc., (Sakulwira *et al*., 2001, Sirinarumitr *et al*., 2001). This report and previous researches indicated that diagnostic for CPV is precisely concerned for enteritis case in dogs (Hoare *et al*., 1997; Parrish *et al*., 1988; Sakulwira *et al*., 2001). Thus the PCR based technique for the detection of CPV in this study as the above mentioned result conducted that it is useful for detection of CPV DNA from fecal specimens. It might prove advantageous for routine application for diagnostic laboratory.

**Cloning of VP2 Gene**

The nucleotide sequence of VP2 gene of CPV in this study was found to be 1,755 bp as the same size of reported Genbank data base that coded for the authentic VP2 protein of 584 amino acids. The complete VP2 gene sequence was also as the same size with the previous submitted report (Ikeda *et al*., 2000). The PCR and restriction enzyme digestion were conducted to confirm the correctness of the gene obtained code for VP2 gene. According to the nucleotide sequence alignment of the CPV-KPS showed the homology of 100% to CPV-2b biotype and the difference of an amino acid at residue 426 to CPV-2a found from reported nucleotide sequence in Genbank data base. The compared alignment result also found that the amino acid residue 1 to residue 20 which were reported to be neutralizing epitope for stimulating of neutralizing antibody against CPV was still conserved(Langeveld *et al.,* 2001). These result confirmed that the nucleotide sequence of cloned VP2 gene was correct. Recombinant VP2 gene was found to be extended by the 79 of nucleotide sequences

from the pFastBac™Htb transfer vector which included the six histidine tagged nucleotide sequences.

**Recombinant Protein Expression Systems**

There were the reports of many expression systems. For instance protein expression in E. coli which considered as the quick process, simplicity, well known genetics and high yield of recombinant protein expression. Although this system has been well developed, but its obstacle listed that E. coli produced protein lacked of eukaryotic post translational modifications (Sorensen and Mortensen, 2005). Thus the undesired point of this system is insoluble form of expressed protein that caught in occlusion body of cells. It is noted that this system not compatible with those protein antigen whereas the protein modification is required.

Yeast and mammalian systems of expressions that have been reported for eukaryotic expression which could be used to produce many kinds of protein conferred the ability of the posttranslational modification, but no report for the VP2 protein of CPV was found. Yeast is one of useful system that is suited the large scale for industrial protein expression. The prior published report upon yeast for protein production In vitro generated the essential product of human hepatitis B vaccine. In general inappropriate form of produced protein such as absence of carbohydrate to the protein that could lead the problem affected to reduce activity or lost of immunogenic property. These could be a problem caused by yeast system whereas over glycosylation found (Pissarra, 2000). The other useful mammalian cells system provides the advantage over lower eukaryotes, because of its ability to produce protein with correct form.

There is alternative expression tool derived to plant system which various kinds of plant were available such as tobacco and potato. This system was based on the chloroplast genetic engineering. It was also given the appropriately folded and postranslationally produced protein including multi-gene engineering in a single transformation event. Transgene occurrence found via the maternal inheritability.

Edible vaccine or oral delivery antigen is the valuable representative for plant system (Vijay and Henry 2004).

The baculovirus expression system or insect cell system had been wildly used for many kinds of eukaryotic proteins derived from the silk industry knowledge. Replacement of the polyhedrin gene by foreign genes has been the basis of this system (Pratelli *et al*., 2001). It has been used to produce a variety of parvovirus specific proteins. Among these parvoviruses, virus- like particles have been successfully produced for human parvovirus B19, adeno-associated virus, porcine parvovirus, aleutian mink disease parvovirus and *etc*., (Vijay and Henry 2004). Since it was reported that expressed protein performed correct folding and modified to be similar with the authentic protein (Singh *et al*., 2005). This system was preferred of the published information of high yield and accurate form of produced recombinant protein as the posttranslational modification for instance glycosylation and phosphorelation abilities. In this study the baculovirus was chosen to express recombinant protein because of the mentioned advantages and its simplicity. However, the drawback of this system was reported about the generation of defective interfering viruses, which causes sharp drop in serial passages of baculoviruses in cultured insect cells. This situation can occur to both wild type and recombinant baculoviruses (Krell, 1996). Upon using this system, it has previously been demonstrated by Saliki *et.al*, in 1992 that virus like particles comprised of recombinant FPV-CPV VP2 protein produced by baculovirus could be used as the alternative vaccine which could rise the antibody against CPV while this study was focused on the major antigenic protein VP2 of CPV field isolate.

**Recombinant VP2 Protein Expression in Insect Cells**

In the present study, the expression in baculovirus system of recombinant VP2 capsid protein of CPV which including the addition of approximately 3 kDa from histidine tagged peptide was shown as a band of 68 kDa in SDS-PAGE and western blotting. The expressed recombinant protein of the first two third of VP2 gene was shown as approximately 45 kDa. The molecular weight of VP2 protein in this study

after removal of the histidine tag molecule weight was then found quite similar in size to the size of produced protein in insect cell(SF21) conducted by Saliki *et al* in 1992. Result was compared to the reported authentic protein found nearly the same size (Gupta *et al*., 2005). This indicated that the produced protein is the correct VP2 protein revealed to the successful expression. These recombinant proteins were tagged with six histidine at their amino terminus. Recombinant proteins carrying six histidine residues could be further purified using the nickel ions resin that have been immobilized by covalently attached nitrilotriacetic acid (Fency *et al.,*1996).

Although the distinct band of the whole VP2 protein was found both in SDS-PAGE and western blotting. The extra bands found at the target protein lane and the other nonspecific band about more than 30 kDa in size was found at the same line of that expressed the first two third of VP2 gene by western blotting using monoclonal antibody against histidine. This result revealed that the whole VP2 gene and the first two third of VP2 protein including the target and unknown proteins which tagged with six histidine were expressed. The unknown protein may be from either the incomplete post translational modification or truncated form by protease of the first two third of VP2 protein. Theses proteins specifically bound to monoclonal antibody against histidine confirmed with immunodot analysis.

Dot blotting was used to determine structure correctness of recombinant proteins whereas the less time and amount of antibody consumption are needed compared to western blotting. It is therefore can not give the result of size of estimated proteins, but western blot analysis can provide this information. The recombinant VP2 protein was shown to have the specific interaction antibody against histidine in both assays, but using antibody against CPV found specific result only with dot blotting. This result indicated that the recombinant VP2 performed the correct structure.

The result performed the product band density which was thinner than the standard marker by SDS-PAGE demonstrated unfavorable yield in this study. This result might reveal the low yield of expressed recombinant proteins. Due to the

product band density The SDS-PAGE and western blot analysis conducted result with the previous report of similar study proposed by Saliki *et.al*., in 1992 reported that SF 21 cell was used for recombinant protein expression in comparison to High Five™cell in this study. The protein at amount of 1.4 μg/cm$^2$ from10$^6$ cells was harvested. The result of VP2 recombinant protein harvested from the crude protein was also low. Weichert *et.al*, in 1998 reported previously toward using this selected system to produce VP2 protein of CPV found that the MOI required in between 2-5. Derived to the plaque assay in this study it performed the MOI of about 0.1 for recombinant baculovirus amplification and 1 for the protein expression (Data not shown). In the detail the MOI for amplification was optimized, but the MOI for expression needed to be corresponded to the previous report. The attempt to find out the technique to increase MOI for protein expression was suggested. One of the processes was to increase recombinant baculovirus titer. For this reason, it might have the strong impact to the level of protein production as the higher level of yield should be achieved toward the additional MOI (Weichert *et al.,* 1998; Yuan *et. al.,* 2000).

# CONCLUSION

In this study, the PCR technique using the specific primer pair has been developed and optimized. It has proven for rapid detection of CPV DNA by the presence of the DNA of virus in fecal specimens. It can be a useful and reliable technique to diagnose the cause of sickness in dogs whereas the diarrhea or gastroenteritis symptom found. Further study of CPV may be required PCR with this specific primer and DNA sequencing in order to differentiate the strain of agent and to follow the spread of the latest reported biotype CPV-2C which has been found in the leopard cat and Asian small wild cat. During recent year the personal contact to Sirinarumitr for the situation of CPV in Thailand found some evidence that this newest biotype might gain the new host range.

The VP2 gene of CPV Thailand's isolate of dog in the present study was found to be similar to CPV-2b. It was successfully cloned. In this study, the recombinant of the first two third of VP2 and whole VP2 proteins were successfully expressed. The amino acid comparison showed the difference at the amino acid residue 426 from the biotype 2a. Additional the sequence of the VP2 protein of 20 residues from its N-terminus which had been reported as the neutralizing epitope was still found conserved. The further step that should be done is the attempt to increase the high yield of recombinant protein production. For the produced protein would be useful for the immunological feature.

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# APPENDIX

# Appendix A

## Chemical Reagents and Substances

### 1. Bacterial media and solution

- Luria Bertani (LB) medium (broth)
  - 10 g/litre tryptone
  - 5 g/litre yeast extract
  - 10 g/litre NaCI
- LB agar
  - LB medium containing 15 g/litre agar
- Ampicillin stock solution
  - 100 mg/ml in $H_2O$, sterile filter, store in aliquots at -20 °C
- Kanamycin stock solution
  - 25 mg/ml in $H_2O$, sterile filter, store in aliquots at -20 °C
- IPTG (1 M) 238 mg/ml in $H_2O$, sterile filter, store in aliquots at -20 °C

### 2. Plasmid isolation reagents

- Solution I
  - 25 mM Tris-HC1 pH 8.0
  - 10 mM EDTA
  - 50 mM glucose
- Solution II
  - 0.2 N NaOH
  - 1% SDS
- Solution Ill
  - 60 ml of 5 M Potassium acetate
  - 11.5 ml of glacial acetic acid
  - Distilled water 28.5 ml

3 M Sodium acetate pH 5.2

99.5% ethanol

- Tris-EDTA (TE pH 8.0)

10 mM Tris HCI

1 mM EDTA

adjust pH to 8.0

- Phenol / chloroform / isoamyl alcohol (25:25:1) total volume 204 ml

100 ml TE-saturated phenol

100 ml chloroform

4 ml isoamyl alcohol

**3. Buffer for agarose gel electrophoresis**

- 20X TAE buffer pH 8.3 (1 litre)

0.8 M Tris HCl ; 96.9 g

0.4 M sodium acetate; 32.8 g of NaOAc-$3H_2O$

0.04 M $Na_2EDTA$; 14.9 g

Adjust pH with glacial acetic acid to pH 8.3 and bring to 1 litre with distilled water.

- 10X loading buffer / dye

20% glycerol

0.01% bromphenol blue

add TE to final volume

- 5 mg/ml ethidium bromide (EtBr)

500 mg EtBr

add distilled water to 100 ml

**4. Buffer for DNA extraction from agarose gel**

- lysis buffer pH 7.0 (100 ml)

4M guanidine Thiocyanate; 47.28 g

50mM Tris HCl ; 0.6055 g

20mM EDTA; 0.80894 9

adjust pH to 7.0

- Washing buffer pH 7.0 (100 ml)

50% ethanol . 50 ml

200mM NaCI ; 1.1688 g

10mM EDTA; 0.40497 g

50mM Tris HCl; 0.6055 g

adjust pH to 7.0

**5. SDS-PAGE reagents**

- 2x SDS-PAGE sample buffer

2.5 ml of 4xTris HCl / SDS, pH 6.8 (250 mM Tris HCl)

2.0 ml of glycerol (20% glycerol)

0.4 g of SDS or 4 ml of 1 0%SDS (4% SDS)

0.2 ml of beta- mercaptoethanol (2% of 2-ME)

0.006% bromophonol blue

- 4x Tris HCl / SDS pH 6.8, buffer for stacking gel (250 ml)

0.5M Tris HCl ; 15 g Tris HCl

0.4% SDS; 1 g SDS

200 ml distilled water

Adjust pH using concentrated HCl , then add distilled water to 250 ml total volume

- 4x Tris HCl / SDS pH 8.8. buffer for separating gel (500 ml)

1.5M Tris HCl; 91 g Tris Base

0.4% SDS; 2 g SDS

400 ml distilled water

Adjust pH using concentrated HCI then add distilled water to 500 ml total volume

- 30% Acrylamide / Bis-acrylamide (bis-acrylamide acrylamide =1:36) to prepare 513.5 ml of solution:

150 g acrylamide

4.1 g Bis-acrylamide

Add distilled water to 513.5 ml sterilize by filter and store at 4°C

- 10% Ammonium persulfate

100 mg ammonium persulfate

1 ml distilled water

- TEMED (N,N,N',N'-tetramethylethylenediamine) store protected from light at 40°C

- Glycine buffer

192 mM glycine

25 mM Tris base

0.1% SDS

- Coomasie Brilliant Blue stain (2 litres)

2 g Coomasie brilliant blue powder

1 litre methanol

200 ml acetic acid

800 ml distilled water

Stir for minimum 2 hours and filter through Whatman filter disc

- Destainning solution (100 ml)

225 ml methanol

10 ml acetic acid

225 ml distilled water

## 6. Immunoblotting reagents

- PBS buffer, pH 7.4 (1 litre)

8.0 g NaCI

0.2 g $K_2H_2PO_4$

0.2 g KCl

1.15 g $Na_2HPO_4$

Distilled water adjust to 1000 ml

- PBS-Tween buffer

add to final concentration of 0.5% Tween 20 (Sigma®)

- Blocking agent

5% skim milk in PBS- 0.5 %Tween buffer

- Serum diluting agent

2% skim milk in PBS- 0.5 %Tween buffer

- DAB (Sigma®) substrate

6 mg of DAB

10 μl of $H_2O_2$

990 μl of sterilize water

- Transfer buffer (1 litre)

25mM Tris; 3 g Tris base

190mM glycine; 14.4 g glycine

20% methanol ; 200 ml conc. methanol (water adjust to 1,000 ml)

# Appendix B

## The standard methods

### 1. Preparation of Ultra-competent cells for transformation (Inoue et al., 1990)

1. Culture cells on LB agar plate at 37 °C overnight. In case of M15 strain *E. coli* LB agar contains 25 μg/ml kanamycin.
2. Pick up a large colony and culture in 1 ml of LB broth at 37°C overnight with vigorous shaking (~ 250 rpm).
3. From 500 μl of overnight culture, subculture to 100 ml of SOB medium containing 25μg/ml kanamycin, incubate at 37°C until OD600 is 0.4 - 0.8 (approximately 3 - 4 hrs).
4. Store the culture on ice for 10 mins.
5. Centrifuge at 4 °C, for 10 mins at 3,000 rpm, discards the supernatant.
6. Gently resuspend the pellet in 33 ml of ice-cold TB and store on ice for additional 10mins
7. Centrifuge at 4°C, for 10 mins at 3,000 rpm, discards the supernatant.
8. Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 μl)
9. Aliquot the cell to micro tube each 200 μl and store at -70°C until use for transformation.

### 2. Transformation

1. Thaw the competent cell on ice, for 30 mins.
2. Mix the constructed plasmid from ligation to the competent cell, stand on ice for 30 mins.
3. Heat shock the cell at 42°C for 90 sec in the heat block, and immediately place tube on ice for 5 mins.
4. Add the L.B. broth 900 μl and incubate with shaking at 37°C for 1 hr.
5. Centrifuge the culture at 6,000 rpm for 1 min.

6. Spread the cells on the prewarmed LB plate containing 100 μg/ml ampicillin and 7 μg/ml Gentamycin air dry plate, and incubate for 12-36 hrs at 37°C

7. The recovery clone of *E. coli* with recombinant plasmid was determined by PCR assay, restriction endonuclease, and DNA sequencing.

**3. Ligation (adapted from Sambrook et al, 1989)**

The following reaction conditions are for ligation of DNA inserts with cohesive ends to DNA vectors with complementary cohesive ends to produce circular recombinant molecules. A molar ratio of 5:1 insert: vector was used.

1. Add the following component to the 0.5 ml micro tube

10x T4 ligase buffer 2 μl

vector DNA : insert DNA (1:5)

autoclaved distilled water to 20 μl

2. Add 1.0 μl (0.1 unit) of T4 DNA ligase (Promega®, Inc.). Mix gently and then briefly spin down to bring the content to the bottom of the tube.

3. Incubate at 4°C, 48 hrs.

4. The ligation reaction was used in transformation to competent cell of *E. coli* 7 μl each reaction.

**4. Method for plasmid isolation (adapted from Sambrook *et at.,* 1989)**

1. From overnight cultures of *E. coli* in LB broth, pour 1.5 ml into 1.5 ml micro tube.
2. Centrifuge for 5 mins, at 13,000 rpm and carefully aspirate off the medium.
3. Add 100 μl of P1 buffer or TE pH.8 buffer and resuspend by vortexing until the suspension become homogenous.
4. Add 200 μl of P2 buffer and gently inverse the tube up side down. The cells should lyse and turn clear and viscous.
5. Let it stand 3- 5 mins and then add 150 μl of cold P3 buffer. Mix again by inversion until a white clot of protein/SDS form. Centrifuge the mixture at 13,000 rpm for 10 mins. Keep the supernatant by transfer all of the aqueous phase to the new

micro tube and discard the precipitin. Carefully done and avoid proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.Add DNA phenol 150 µl and chloroform 150 µl and shake for 5 mins to extract the plasmid.

6. Centrifuge the sample at 13,000 rpm for 5 mins to separate the phases.

7. Transfer the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface.

8. Add 2-3 volume of absolute ethanol or 1 volume of isopropanol to precipitate the nucleic acid. Invert gently up side down and keep in – 80°C for 40 mins

9. Centrifuge at 13,000 rpm for 10 - 15 mins. Carefully decant the supernatant.

10. Wash the DNA pellet with 75% ethanol twice. Centrifuge at 13.000 rpm for 5 mins. Decant the supernatant, and air drying the DNA pellet

**5. Restriction endonuclease assay**

This procedure is used for cutting the target DNA with one enzyme (Single digestion method).

1. Isolate the plasmid from *E. coli* or prepared target DNA.

2.Add the following components to 1.5 ml micro tube;

Target DNA 6 µl (in TE & distilled water), the volume depends on the concentration of DNA. 3µ l of 10X buffer (buffer code depending on enzyme used as manufacturer described e.g. buffer E,D of Promega®, Inc or multicore buffer in case of double digestion) 0.5µl of Bovine serum albumin(BSA) 1µ l of restriction enzyme and add distilled water to desired volume (30 µl)

3. Incubate at 37°C for 3 hrs to complete enzyme reaction.

4. After the 1st enzyme digestion the mixture of the step 3 is purified before the 2nd digestion by QIAGEN quick gel extraction kit as the method following and the purified plasmid is eluted with 30 µl distilled water.

5. When complete the two enzymes digestion. Purify the target DNA by agarose gel electrophoresis and DNA extraction from agarose gel again.

## 6. QIA quick gel extraction kit protocol

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl)
3. Incubate at 50°C for 10 mins. To help dissolve gel, mix by vortexing the tube every 2-3 mins during incubation.
4. After the gel slice has dissolve completely, check that color of the mixture is yellow
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. To wash, add 0.75 ml of PE buffer to QIAquick column and centrifuge for 1 min.
10. Discard flow-through and place QIAquick column an additional 1 min at 13,000 rpm.
11. Place QIAquick column into a clean 1.5 ml micro centrifuge tube.
12. To elute DNA, add either distilled water or 50 µl of EB buffer (10 mM Tris-HCl, pH 8.5) to the center of QIAquick column membrane, let the column stand for 1 min, and centrifuge at high speed for 1 min.

## 7. Phenol-Chloroform extraction of DNA and ethanol precipitation (adapted from Sambrook and Russell, 2001)

1. DNA was extracted from 100 µl of either fecal specimen diluted in TE buffer pH.8 or supernatant of infected cell culture that it was mixed with 500 µl of denature solution, was shaken for 5-10 mins
2. Add DNA phenol 150 µl and chloroform 150 µl was shaken for 5 mins.
3. Centrifuge the sample at 13,000 rpm for 5 mins to separate the phases.

4. Transfer of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted for second time with same procedure.
5. Repeat step 2-4 again.
6. Transfer the majority of the upper, aqueous layer to a clean tube; add 2-3 volume of absolute ethanol or 1 volume of isopropanol to precipitate the nucleic acid. Invert gently up side down and keep in – 80°C for 40 mins
7. Centrifuge at 13,000 rpm for 10 - 15 mins. Carefully decant the supernatant.
8. Wash the DNA pellet with 75% ethanol twice. Centrifuge at 13.000 rpm for 5 mins. Decant the supernatant, and air drying the DNA pellet.

**8. Agarose gel electrophoresis**

1. Prepare an agarose gel, according to recipes list e.g. 1% gel in 1XTAE buffer, by combining the agarose with buffer in the Erlenmeyer flask and wrapped with clear pored plastic (low gel temperature agarose may also be used) and melt the agarose for 3 mins by microwave oven. Check the gel temperature before pouring the gel onto plate, the desired temperature is about 55-60 °C.
2. Pour the gel onto a taped plate with casting combs in place. Allow 20 - 30 mins for solidification.
3. Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1x TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.
4. Add at least one- tenth volume of 5x agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophoresis the gel at 50 - 100 V/cm until the required separation has been achieved.
5. Incubate the agarose gel in EtBr tank for 15-20 mins.
6. Visualize the DNA fragments on a long wave UV light box.

**9. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (Cochet *et al*., 1998)**

Prepare the gel by the recipe as follow for 2 gels

**Separating gel** (12%)

| | |
|---|---|
| $H_2O$ | 2.8 ml |
| 4x Tris HCI/SDS pH 8.8. | 2.0 ml |
| 30% Acry/0.8%bis-Acrylamide | 3.2 ml |
| 10% APS | 26.6 µl |
| TEMED | 5.3 µl |

**Stacking gel**

| | |
|---|---|
| $H_2O$ | 3.05 ml |
| 4x Tris-HCl/SDS pH 6.8 | 1.25 ml |
| 30% Acryl/0.8%bis-Acryl | 0.65 ml |
| 10% APS | 25 µl |
| TEMED | 5 µl |

If not already in electrophoresis sample buffer, add an equal volume of 2X sample buffer to all samples and boil for 5 mins. Apply 20-25 µl (1 - 10µg total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel. Run electrophoresis (100 volt, 70 mins, constant ampere)

## 10. Western transfer and blotting (Cochet *et al.*, 1998)

1. Cut the nitrocellulose membrane with sharp blade to the expected size
2. Soak the gel (from SDS-PAGE), the nitrocellulose membrane, the Whatman papers, and sponges in cool transfer buffer for 10 mins.
3. Set the transfer apparatus and then transfer proteins from gel to nitrocellulose membrane at constant 400 mAmp for 6 hrs in transfer buffer (25mM Tris base, 190mM Glycine, and 20% Methanol). The transfer set should be placed in ice box to control the temperature in avoiding of loss of buffer quality from heat.
4. Remove the blot from the transfer apparatus immediately place into blocking buffer (5% skim milk in 1x PBS+ 0.5 % Tween 20) and incubate for 1 hr at room temperature with gently agitation (optional 2 hrs at 37°C /overnight at 4°C).

5. Dilute the primary antibody (rabbit anti Canine parvovirus hyper immune sera absorb for 3 hrs at 37°C with supernatant of virus wild type (1:50) or mouse IgG anti-histidine monoclonal antibody (1:3,000). Decant the blocking buffer from the blot, add the primary antibody solution, and incubate with agitation for 90 mins at room temperature.

6. Wash 3 times with agitation in PBS-0.5% Tween for 5 - 10 mins each.

7. Dilute the secondary antibody with 2% skim milk in PBS+0.5% Tween (goat anti rabbit IgG (1:500) or goat anti-mouse IgG (1:500) conjugated with peroxidase). Decant the primary antibody from the blot, add the secondary antibody solution, and incubate with agitation for 45-60 mins at room temperature

8. Decant the secondary antibody solution. Wash 3 times with agitation in PBS + 0.5 % Tween for 5 - 10 mins each.

9. Add DAB substrate and incubate for 5 - 15 mins.

10. To stop enzyme-substrate reaction, place the blot in distilled water.

## 11. Nucleotide base abbreviations used in nucleic acid sequences

| | |
|---|---|
| A | adenosine |
| C | cytidine |
| G | guanine |
| T | thymidine |
| U | uridine |
| R | G A (purine) |
| Y | T C (pyrimidine) |
| M | A C (amino) |
| S | G C (strong bond) |
| W | A T (weak bond) |
| B | G T C |

# APPENDIX FIGURE

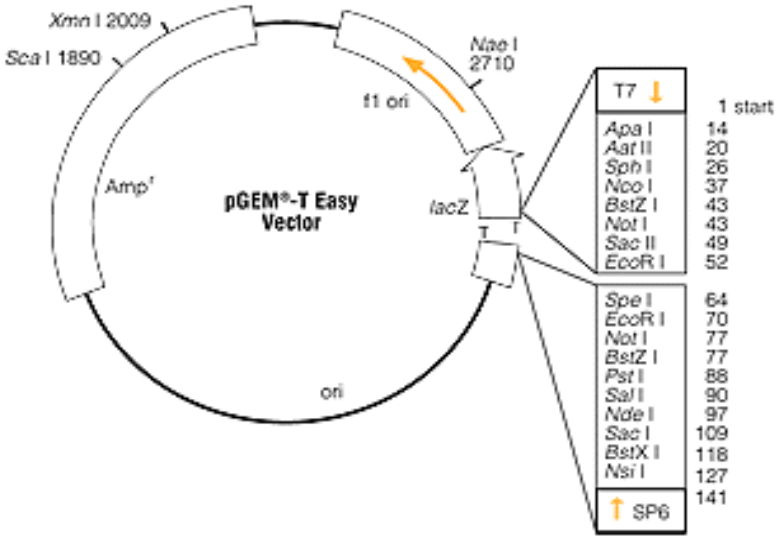


**Figure 1** The pGEM-T Easy plasmid (Invitrogen®) circle map and sequence reference points

**Source**: Invitrogen®

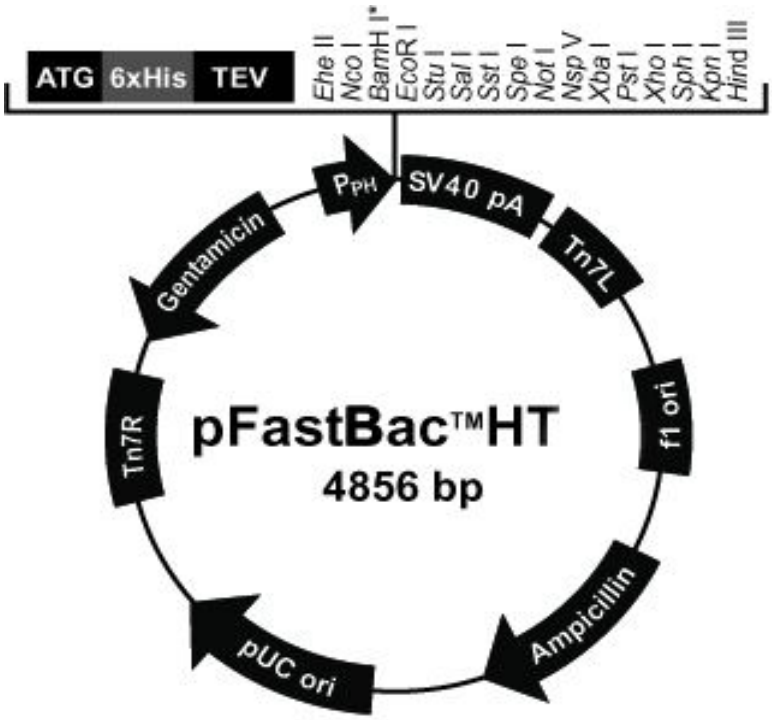


**Figure 2 The** pFastBac$^{TM}$ Htb plasmid (Invitrogen$^{®}$) circle map and sequence reference points

**Source**: Invitrogen$^{®}$

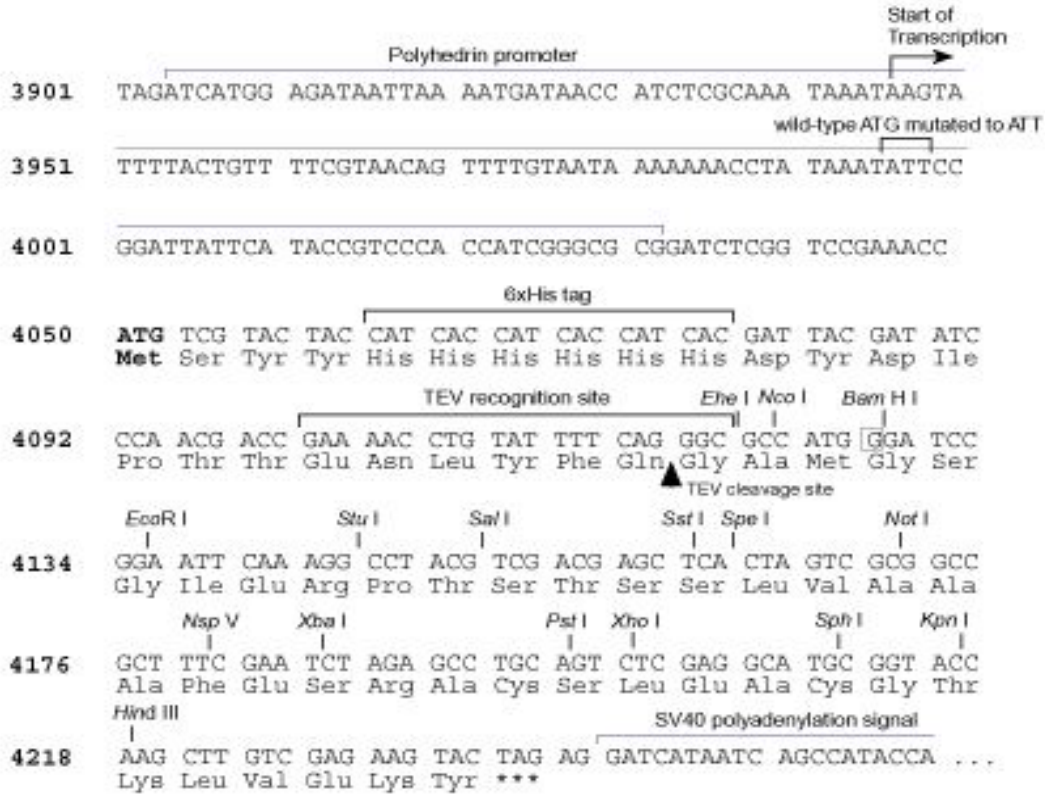


**Figure 3** The nucleotide sequence of a baculovirus promoter and multiple cloning sites of pFastBac™ Htb plasmid (Invitrogen®).

**Source**: Invitrogen®

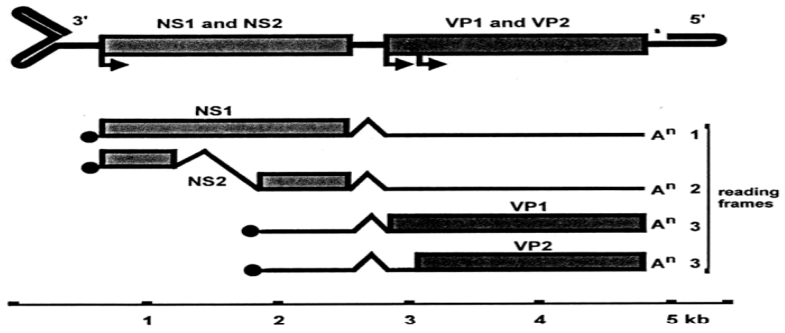


**Figure 4 Canine** parvovirus genome and transcription

**Source:** Murphy et. al., 1999

# CURRICULUM VITAE

**NAME** : Mrs. Pattra Moonjit

**BIRTH DATE** : Feburary, 23, 1971

**BIRTH PLACE** : Ratchaburi, Thailand

**EDUCATION** : **YEAR INSTITUTION DEGREE/DIPLOMA**

: 1993 Kasetsart Univ. Bsc.Agriculture (Animal Husbandry)

**POSITION/TITLE** : Scientist

**WORK PLACE** : Department of Animal Medicine Production and Farm Resource, Faculty of Veterinary, Kasetsart Univ.

**SCHOLARSHIP/AWARDS:** _