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THESIS

EXPRESSION OF RECOMBINANT VP2 PROTEIN OF CANINE PARVOVIRUS IN *ESCHERICHIA COLI*

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Siriwadee Phromnoi 2010: Expression of Recombinant VP2 Protein of Canine Parvovirus in *Escherichia coli*. Doctor of Philosophy (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Theerapol Sirinarumitr, Ph.D. 79 pages.

Canine parvovirus (CPV) appears to be endemic in almost all populations of wild and domesticated dogs. It causes serious contagious enteric disease especially in puppies. The VP2 of CPV is a major capsid protein and plays an important role in the host immune response. Twenty six isolates of CPV were obtained from infected dogs and full-length VP2 gene was amplified by PCR. Its sequences were analyzed. Nineteen isolates were characterized as CPV type 2a variants and the rest of the isolates were characterized as CPV type 2b. These results indicated that both types are currently prevalent field CPV circulating in Thailand and type 2a is the predominant genotype. Neither CPV type 2 nor type 2c was observed in this study.

In the present study, the recombinant VP2 (rVP2) protein of CPV was expressed and tested with rabbit antibody against rVP2. The whole VP2 gene was amplied by PCR using specific primers. The size of PCR product has about 1,700 bp and was ligated with plasmid pBAD202/D-TOPO to transform into *E. coli* strain TOP10. The optimum concentration of arabinose and time course for expression was 0.002% and 8 hours. By SDS-PAGE analysis, the rVP2 protein band was about 80 kDa. By Western blot analysis, the rVP2 proteins were specifically interact with rabbit anti-CPV hyperimmune serum. Each rabbit group was immunized with PBS, 300 μg of rVP2, 500 μg of rVP2 and CPV commercial vaccine. By ELISA, all immunized rabbits generated antibodies against rVP2 at the 2nd week of immunization and reached its peak within the 7th week and were stable until the end of the experiment. By ANOVA, there were significantly differences between the control group and the immunized group from the 4th week of immunization through the end of the experiment. However, there was no significantly different between the antibody titer from rabbits immunized with both recombinant protein concentrations.

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Student's signature Thesis Advisor's signature

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Siriwadee Phromnoi

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
BSA	=	Bovine serum albumin
CHO	=	Chinese hamster ovary cell
CPV	=	Canine parvovirus
CPE	=	Cytopathic effect
CRD	=	Completely randomized design
CRFK	=	Crandel feline kidney cell
DMEM	=	Dulbeco's Modified Eagle medium
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxynucleoside triphosphates
ELISA	=	Enzyme linked immunosorbent assay
FBS	=	Fetal bovine serum
FEB	=	Feline embryonic fibroblast
FPV	=	Feline panleukopenia
GLM	=	General linear model
HA	=	Hemaggluination
HI	=	Hemagglutination inhibition
HIS	=	Histidine
IFAT	=	Indirect fluorescent antibody test
IgG	=	Immunoglobulin G
MDCK	=	Madin Darby canine kidney cell
MEL	=	Murine erythroleukemia cell
NJ	=	Neighbor joining method
PCR	=	Polymerase Chain Reaction
PBS	=	Phosphate buffer saline
P.I.	=	Post immunization
OD	=	Optical Density
RNA	=	Ribonucleic acid
rVP2	=	Recombinant VP2
Sas	=	Sialic acid binding site
SDS-PAGE	=	Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis

LIST OF ABBREVIATIONS (Continued)

Tfr	=	Transferrin receptor
TMB	=	Tetra-methylbenzidine
UV	=	Ultraviolet
°A	=	Angstrom
bp	=	Base pair
°C	=	Degree Celsius
E. coli	=	*Escherichia coli*
g	=	Gram
kg	=	Kilogram
kDa	=	Kilodalton
kb	=	Kilobase
mAmp	=	Milliampere
mg	=	Milligram
ml	=	Milliliter
mM	=	Millimolar
L	=	Liter
M	=	Molar
rpm	=	Round per minute
µg	=	Microgram
µl	=	Microliter

EXPRESSION OF RECOMBINANT VP2 PROTEIN OF CANINE PARVOVIRUS IN *ESCHERICHIA COLI*

INTRODUCTION

Canine parvovirus type 2 (CPV-2) was first identified in 1978 in USA as the cause of hemorrhagic enteritis and myocarditis in dog and it has been well establish as an enteric pathogen of dogs throughout the world (Appel *et al*., 1979). It appeared to be a host range of feline panleukopenia virus (FPV) or a closely related virus of another carnivore (Parrish, 1990; Truyen *et al*., 1995). A few amino acid substitutions between CPV and FPV determine the ability for each virus to replicate in dogs and cats (Reed *et al*., 1988; Mochizuki *et al*., 1996). From 1979 to 1981, an antigenic variant CPV-2a was found. Around 1984, a second variant CPV-2b was demonstrated. These variants differ from CPV-2. However, both variants can infect, replicate and transmit between dogs and cats (Parrish, 1991a; Truyen, 1999). Recently, CPV-2c strains have been identified in parts of Europe, the America and Asia (Ikeda *et al*. 1999; Buonavoglia *et al*., 2001; Kapil *et al*., 2007; Perez *et al*., 2007; Decaro *et al*., 2007, 2009).

Canine parvovirus (CPV) is a small and non-enveloped virus containing approximate 5.2 kb of a single-stranded DNA (Parrish, 1991a). CPV are divided into several biotypes 1, 2, 2a, 2b and 2C based on the differentiation of some amino acid residue at the viral capsid protein. The genome encodes two nonstructural proteins (NS1 and NS2) and two structural proteins (VP1 and VP2). The VP2 capsid protein is a major capsid protein and plays an important role in the determination of antigenicity and host range of CPVs (Mochizuki *et al*., 1996; Truyen *et al*., 1995).

The prominent clinical signs are lethargy, vomiting, fever, and diarrhea (usually bloody). The disease can cause rapid death within 48 to 72 hours without treatment and may result in intestinal and heart damage in surviving animals. Puppies are most susceptible, but more than 80 percent of adult dogs show no symptoms

(Ettinger and Feldman, 1995). In the more common, less severe form, mortality is about 10 percent (Carter and Wise, 2006).

There are several approaches to control CPV. Effective approaches are vaccination and proper sanitation practices. The vaccines currently available are inactivated and modified-live vaccine (Truyen *et al*., 1998). Although these are effective tools to prevent, the risks of viral reservoir used for manufacturing and the problem of the viral escape during vaccine production are concerned (Turiso *et al*., 1992; Langeveld *et al*., 1994). As a result, it is important to develop alternative vaccines that lack of a complete CPV virus. The new generation vaccines that do not require the whole virus particle may have advantages over the conventional virus or escape viruses from vaccine factories.

This study focuses on the both genotypes (2a and 2b) and phylogenetic study of the CPV isolates of Thailand, and the expression of recombinant VP2 protein which is the major immunogenic protein of CPV for stimulating and neutralizing antibody. The whole VP2 sequences of 26 Thai isolates of CPV were compared with the other isolates worldwide. The genotype of Thai isolates of CPV was used to make decision for choosing the isolate for the recombinant VP2 expression. The selected isolate was used as the template for cloning whole VP2 in the *E. coli*. Subsequently, the recombinant VP2 protein was expressed and used as antigen to immunized rabbits and serum of rabbits was taken and tested for humoral immune response against CPV.

OBJECTIVES

1. To study phylogeny of CPV isolates in Thailand

2. To clone and express VP2 proteins of the selected CPV Thai isolate using *Escherichia coli*

3. To test the ability of the recombinant VP2 protein to induce antibody response in rabbits

LITERATURE REVIEW

1. **Canine parvovirus (CPV) disease**

Canine parvovirus (CPV) is a relatively new disease that appeared in the late 1970s. It was first identified in 1978 and spread around the world (Appel *et al*. 1979; Carmichael, 2005). The virus is closely related both genetically and antigenically to Feline panleukopeniavirus (FPV) Both viruses have 98% amino acid identical, differing only in two amino acids in the viral capsid protein VP2. It is also highly similar to mink enteritis, and the parvoviruses of raccoons and foxes (Parrish *et al*., 1982, 1988). However, it is possible that CPV2 is a mutant of an unidentified parvovirus (similar to feline parvovirus) of some wild carnivore (Shackelton *et al*., 2005).

1.1 Pathogenesis

Canine parvovirus is an intestinal disease. The virus has an affinity for mitotically active cells, and it is capable of replicating in a large number of tissues in neonatal animals. 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. CPV may infect the hearts of neonatal pups causing myocarditis. In older animals, the virus replicates primarily in the primary and secondary lymphoid tissues, as well as the in the replicating cells of the small intestinal crypts.

There are two forms of CPV disease which are intestinal and cardiac forms. Puppies are most susceptible, but more than 80 percent of adult dogs show no symptoms (Ettinger and Feldman, 1995). In severe case, dogs can die within 48 to 72 hours without treatment by fluids and antibiotics. In the more common case (less severe form), the mortality is about 10 percent (Carter and Wise, 2006). Certain breeds may be more susceptible to CPV2, such as Rottweilers, Doberman Pinschers,

and Pit bull terriers as well as other black and tan colored dogs (Nelson and Guillermo, 1998). Along with age and breed, other factors may increase the severity of infection such as a stressful environment, concurrent infections with bacteria, parasites, and canine coronavirus (Ettinger and Feldman, 1995). Dogs infected with parvovirus usually die from the dehydration or secondary infection rather than the virus itself.

The uterine infection of CPV can occur when a pregnant female dog is infected with CPV2. The adult may develop immunity with little or no clinical signs of disease. However, the virus may have already crossed the placenta to infect the fetus. This can lead to several abnormalities. In severe cases, the pups can be still born or born mummified. In the less severe case, the pups can be born with neurological abnormalities such as cerebella hypoplasia (Schatzberg *et al*., 2002).

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).

1.2 Clinical signs and symptoms

Infection with CPV can be silent. The symptoms of CPV infection include loss of appetite, depression, elevated fever, massive dehydration, lethargy, diarrhea and vomiting. Diarrhea may be bloody and the vomiting may be severe and repeated. The disease can cause rapid death, and may result in intestinal and heart damage in surviving animals. Dogs who successfully recover from infection develop immunity to subsequent CPV infection.

Dogs that develop the disease show symptoms of the illness within 5 to 10 days post-infection. The symptoms include lethargy, vomiting, fever, and diarrhea (usually bloody). Diarrhea and vomiting result in dehydration and secondary infections can occur. In the aspect of dehydration, the dog's electrolyte balance can

become critically affected. Because the normal intestinal lining is also compromised, blood and protein leak into the intestines leading to anemia and loss of protein. The endotoxemia may cause by escaping of endotoxins into the bloodstream. Dogs usually have a distinctive odor in the later stages of the infection. The white blood cell level falls which will further weakening the dog. Any or all of these factors can lead to shock and death (Ettinger and Feldman, 1995).

1.3 Epidemiology

When CPV first surfaced in the 1970s, it caused a wide-spread epidemic among wild and domestic canines, resulting in the death of thousands of animals (Parrish *et al*., 1988). Confirmed infections have been reported in Sweden, Germany, Italy, Brazil, 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; Kapil *et al.,* 2007). The CPV-2a replaced the former CPV-2 rapidly as many reported showed from many countries and also case report in Thailand respectively (Parrish *et al*., 1988; Sakulwira *et al*., 2001, 2003). In 2001, a new antigenic type (CPV-2c) was reported in Italy (Buonavoglia *et al.*, 2001). Then it has been detected in Europe (Decaro *et al*., 2007), Asia (Nakamura *et al*., 2004) and America (Kapil *et al.*, 2007; Perez *et al*., 2007).

CPV caused the enteritis in dogs about 61.8% in Thailand and CPV type 2a and type 2b were found as the predominant genotype (Sakulwira *et al*., 2001). In 2001, the PCR based method for the amplification of the nonstructural protein gene (NS1) and structural protein gene (VP2) to detect CPV in Thailand has been developed (Sakulwira *et al*., 2001; Sirinarumitr *et al*., 2001; Moonjit, 2006).

CPV is usually spread from dog-to-dog by direct or indirect contact with infected fecal matter. Most cases of parvovirus infection occur where dogs assemble, such as parks, dog shows, kennels, pet shops, etc. People may also contribute to the spread of CPV by carrying infected fecal matter on their shoes. CPV infection can easily occur in any dog since the virus is highly infectious and hardy, withstanding a wide range of temperatures and surviving outside the animal for months. Infected dogs may shed virus for up to 18 days after exposure without showing any symptoms of disease, which also contributes to the spread of CPV.

1.4 Viral survival in environment

CPV is stable in the broad range pH 3-9. It can be inactivated by heating to 80 °C for 40-60 minutes and it is stable at -20 °C for 1 year after this period of time it can survive at 4 °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 °C within 2-6 months appeared to low infectivity (Povey, 1988).

CPV2 continues to evolve, and the success of new strains seems to depend on extending the range of hosts affected and improved binding to its receptor, the canine transferrin receptor (Truyen, 2006). CPV2 has a high rate of evolution, possibly due to a rate of nucleotide substitution that is more like RNA viruses such as Influenzavirus A. In contrast, FPV seems to evolve only through random genetic drift (Horiuchi *et al*., 1998).

1.5 Diagnosis

History and clinical findings are the most important measure. Infected dogs may have fever, acute diarrhea, vomiting and lymph node enlargement. Mortality rate is high (Turiso *et al*., 1992; Battilani *et al*., 2002). Necropsy findings are suggestive since the dog die from suspected symptoms as mentioned, but the lesions may be confused with poisoning, histopathology characteristics consist of

cardinal hemorrhagic, intracellular inclusion bodies in cardiac myofibrils. The enteric form appearance obtained dilated intestine. Microscopic finding is the necrosis of the epithelium and dilation of crypt.

There are many ways for CPV diagnosis by detecting antigen and antibody such as virus isolation, electron microscopy (EM), immunological assays and molecular techniques.

Virus isolation is practiced using fecal sample swab which is kept in the aqueous solution such as PBS buffer and Dulbecco's Modified Eagle Medium (DMEM). There are various 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 at least 1% fetal bovine serum (FBS) in DMEM pH 7.0 under 37 °C in CO_2 supplied incubator. Harvesting of the virus should be collecting when the CPE reaches 80%. However, this method takes time about 1 week; it is time consuming (Knipe and Howley, 2001; Battilani *et al*., 2002).

Electron microscopy examination is the standard method for CPV detection which based on identification of morphology of the intact virus particle; however, the maintenance cost is high. In addition, this technique gives low degree of sensitivity and has to use skilled personnel.

There are several immunological assays such as hemagglutination (HA), Hemagglutination inhibition (HI) assay, enzyme immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and virus neutralization assay. The HA and HI assay using 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 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 cannot invade host cells (Greenwood *et al*., 1996; Sagazio *et al*., 1998).

The molecular technique for CPV detection is PCR. This method can increase amount of DNA by *in vitro* synthesis. The result is highly sensitive over other methods such as virus isolation and other immunological assays (Murphy *et al*., 1999). In addition, this technique is specific and requires DNA template in small amount.

1.6 Prevention

Prevention is the only way to ensure that a puppy or dog remains healthy since the disease is extremely virulent and contagious. The virus is extremely hardy and has been found to survive in feces and other organic material such as soil for over a year. It survives extremely cold and hot temperatures. The only household disinfectant that kills the virus is bleach (Ettinger and Feldman, 1995).

Weaning puppies can be vaccinated with a modified live virus low passage high titer vaccine at 6 weeks of age, then every 3 to 4 weeks until 15 or 16 weeks. Normally puppies are protected by passive transfer of maternal antibody (Truyen *et al.*, 1998; Oh *et al*., 2006). 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, maternal antibodies also interfere with vaccination for CPV2 and can cause vaccine failure. Thus puppies are generally vaccinated in a series of shots, extending from the earliest time that the immunity derived from the mother wears off until after that passive immunity is definitely gone (Truyen *et al*., 1998; Oh *et al*., 2006). Older puppies (16 weeks or older) are given 3 vaccinations 3 to 4 weeks apart (Nelson and Guillermo, 1998). The duration of immunity of vaccines for CPV2 has been tested for all major vaccine manufacturers in the United States and has been found to be at least three years after the initial puppy series and a booster 1 year later (Schultz, 2006).

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 bacteria 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 humoral 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 vaccines 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 (Turiso *et al*., 1992; Langeveld *et al*., 1994).

1.7 Treatment

Survival rate depends on how quickly CPV is diagnosed, the age of the animal and how aggressive the treatment is. Treatment for severe cases that are not caught early usually involves extensive hospitalization, due to the severe dehydration and damage to the intestines and bone marrow. A CPV test should be given as early as possible if CPV is suspected in order to begin early treatment and increase survival rate if the disease is found.

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).

Treatment ideally consists of crystalloid IV fluids and/or colloids, antinausea injections (antiemetics) such as metoclopramide, dolasetron, ondansetron

and prochlorperazine, and antibiotic injections such as cefoxitin, metronidazole, timentin, or enrofloxacin (Martin *et al*., 2002; Macintire, 2004). IV fluids are administered and antinausea and antibiotic injections are given subcutaneously, intramuscularly, or intravenously. The fluids are typically a mix of a sterile, balanced electrolyte solution, with an appropriate amount of B-complex vitamins, dextrose and potassium chloride. Analgesic medications such as buprenorphine are also used to counteract the intestinal discomfort caused by frequent bouts of diarrhea.

In addition to fluids given to achieve adequate dehydration, each time the puppy vomits or has diarrhea in a significant quantity; an equal amount of fluid is administered intravenously. The fluid requirements of a patient are determined by their body weight, weight changes over time, degree of dehydration at presentation and surface area. The hydration status is originally determined by assessment of clinical factors like tacky mucous membranes, concentration of the urine, sunken eyes, poor skin elasticity and blood tests (Macintire, 2004).

A blood plasma transfusion from a donor dog that has already survived CPV is sometimes used to provide passive immunity to the sick dog. Some veterinarians keep these dogs on site, or have frozen serum available. There have been no controlled studies regarding this treatment (Macintire, 2006). Additionally, fresh frozen plasma and human albumin transfusions can help replace the extreme protein losses seen in severe cases and help assure adequate tissue healing.

2. Canine parvovirus (CPV)

Canine parvovirus belongs to family *Parvoviridae*, subfamily *Parvovirinae* and genus *Parvovirus* (Cortes *et al*., 1993; Tsao *et al*., 1991). CPV2 is a non-enveloped single-stranded DNA virus. The name comes from the Latin *parvus*, meaning small, as the virus is only 20 to 26 nm in diameter. It has an icosahedral symmetry (Figure 1). The genome is approximately 5000 nucleotides long (Chapman and Rossmann, 1993; Agbandje *et al.*, 1995).

2.1 CPV genome

The genome contains two promoters directing the direction of two non-structural proteins (NS1 and NS2) in the left reading frame, or for structural proteins (VP1 and VP2) in the right reading frame (Figure 2) (Calson *et al*., 1985; Reed *et al*., 1988). The different proteins are transcribed from separate promoters and are derived by alternative splicing of RNA transcripts (Wang *et al*., 1998). 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).

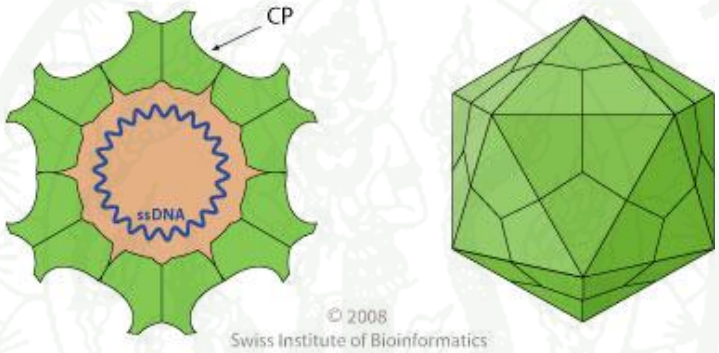

Figure 1 The characteristic of CPV genome.

Source : Swiss Institute of Bioinformatics (2009)

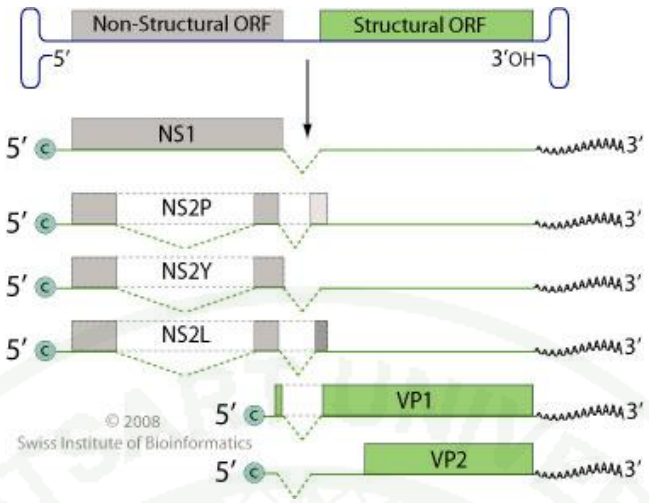

Figure 2 Schematic diagram of the CPV genome.

Source : Swiss Institute of Bioinformatics (2009)

There are five important proteins of CPV virus. They are classified into groups which are nonstructural proteins (NS1 and NS2) and structural proteins (VP1, VP2 and VP3). 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 protects 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 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 involves 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 other function is related to helicase enzyme activity which controls the DNA packing into the viral capsid, and controls cellular apoptosis. The size of the nonstructural protein NS2 is 25 kDa. It is found in nucleus and cytoplasm of host. It 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 test animal (Parrish and Kawaoka, 2005).

The structural elements of the capsid consists of eight-stranded antiparallel β-barrel with large loops between strands (Tsao *et al*., 1991). The cylindrical structure at fivefold axis of symmetry is composed of such β-barrel motifs. The connecting loops make up most of the outer surface. In addition, they form the spike like structure (22 °A long) at the threefold axes of symmetry and the cavity like depression at the twofold axis symmetry. Moreover the connecting loops form a canyon-like drpression (15 °A), surrounding the fivefold axis of symmetry (Tsao *et al*., 1991; Xie and Chapman, 1996).

The size of VP1 protein is 82.3 kDa and has 748 amino acids 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 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 (Weichert *et al*., 1998; Parrish and Kawaoka, 2005).

The VP2 protein is the major component in capsid protein. It consists of 584 amino acid residues and has size of 65 kDa (Gupta *et al.*, 2005). Its function is recognized which is important in control the viral cell receptor interaction with the transferrin 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). VP2 protein has the critical change to viral capsid that effect to antigenic property and host range.

The apparent size of VP3 protein is 63.5 kDa. The importance of VP3 is considered as the minor amount of 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).

2.2 Variants

Canine parvovirus type 2 (CPV-2) was first identified in 1978 in USA and it has been well established as an enteric pathogen of dogs throughout the world causing hemorrhagic enteritis and myocarditis (Appel *et al*., 1979). CPV has a wide host range including *Canidae, Felidae, Mustelidae and Procyonidae*, and as being stable in the environment, the virus spreads easily (Parrish, 1991a; Truyen *et al*., 1995; Kapil *et al*., 2007). A few amino acid differences between CPV and feline panleukopenia virus (FPV) determine the species specificity of these viruses (Reed *et al*., 1988, Parrish *et al*., 1991b, Mochizuki *et al*., 1996). There are two types of canine parvovirus called canine minute virus (CPV1) and CPV2. Especially, CPV2 causes the most serious disease and affects domesticated dogs and wild canids. There are variants of CPV2 called CPV-2a and CPV-2b that were identified in 1979 and 1984 respectively (Shackelton *et al*., 2005). During 1979 to 1981, an antigenic variant CPV-2a was found. Later in 1984, a second variant CPV-2b was identified. These variants differ from CPV-2. Both CPV type 2a and 2b can infect, replicate and transmit between dogs and cats and are prevalent in different countries (Appel *et al*., 1979; Reed *et al*., 1988; Parrish *et al*., 1991b; Truyen *et al*., 1995; Mochizuki *et al*., 1996). CPV type 2a is common in Germany, Italy, Korea and India, while CPV type 2b is common in America, Brazil, Taiwan and Japan (Mochizuki *et al*., 1996; Truyen *et al*., 1996; Hirayama *et al*., 2005; Chinchkar *et al*., 2006; Kapil *et al*., 2007). At present, CPV type 2c is a third variant that has been reported in several countries such as Italy, Vietnam and United States (Buonavoglia *et al*., 2001; Nakamura *et al*., 2004; Chinchkar *et al*., 2006; Decaro *et al*., 2006a, 2006b, 2009; Hong *et al*., 2007; Perez *et al*., 2007).

CPV is a small and non-enveloped virus. It possesses a single-stranded DNA genome of approximate 5.2 kb in length (Parrish *et al*., 1991b). The virus

encodes two nonstructural proteins (NS1 and NS2) and three structural proteins (VP1, VP2 and VP3). The VP2 capsid protein is a major capsid protein and plays an important role in the determination of antigenicity and host range of CPVs (Tsao *et al*., 1991; Langeveld *et al*., 1993, 2001; Truyen *et al*., 1996; Nakamura *et al*., 2004). In the VP2 gene, there are five amino acid differences at residue 87, 101, 300, 305 and 555 between CPV-2 and CPV-2a and five amino acid differences at residues 87, 101, 300, 305 and 426 between CPV-2 and CPV-2b (Nakamura *et al*., 2004; Hong *et al*., 2007). The significant difference between CPV-2a and CPV-2b is the substitution of two amino acids in the major antigenic VP2 capsid protein that is Asn-426 in 2a (Asp-426 in 2b) and Ile-555 in 2a (Val-555 in 2b) (Parrish *et al*., 1991b). Recently, CPV-2c is a new CPV mutant that has a glutamate substitution in the 426th residue of VP2 protein (Buonavoglia *et al*., 2001; Martella *et al*., 2004; Decaro *et al*., 2006b, 2009; Hong *et al*., 2007).

3. Protein expression systems

The ideal system for expression is to produce most safe, biologically active material at the lowest cost (Daniell *et al*., 2001). Present expression systems can be categorized base on type of host cell into eukaryotic and prokaryotic expression system.

3.1 Eukaryotic expression systems

Eukaryotic expression systems are frequently employed for the production of recombinant proteins for therapeutics and research tools. Useful eukaryotic expression systems include insect cells, various mammalian cells and yeast cells. Most commonly used are based on stably transfected adherent cells (CHO cell) or non-adherent cell (lymphoid cell). An efficient alternative is the infection of insect cells by recombinant baculoviruses. CHO, Sp2/0, and MEL cells are stably transfected mammalian cell lines. They give rise to production of fully glycosylated protein at variable product titers. On the other hand, COS and insect cells are the

transient expression system. They rapidly produced incomplete glycosylated and albeit biological active product (Geisse *et al*., 1996).

Mammalian cells offer the advantage over cells from lower eukaryotes in that they are more likely to possess the machinery for correct posttranslational processing, including glycosylation and secretion of viral proteins. Mammalian cell cultures are preferred choice with respect to post-translational modification. The expressed proteins are identical to those of mammalian natural system.

The use of modified mammalian cell with recombinant DNA technology has the advantage that the results in production are identical to those of mammalian. However, culturing is expensive and can only be carried out on a limited scale (Daniell *et al*., 2001). The cost is approximately 100 times that of microbial fermentation. This makes them impractical production systems for the large quantities of subunit vaccines (Streatfield and Howard, 2003).

Recently, plants have been used as recombinant biofactories to express a number of proteins including pharmaceuticals and potential vaccines (Mason *et al*., 1992; Haq *et al*., 1995; Gomez *et al*., 1998; Dozolme *et al*., 1999; Tacket and Mason, 1999; Koprowski and Yushibov, 2001). Plants have post-translational modifications similar to other higher eukaryotes and have ability to rapid scale-up and produce extremely large qualities. Using protein from plants reduces a fear of human pathogen contamination (Streatfield and Howard, 2003). However, plants have various ability to expressed protein of interest, time consume to produce stable expressed plant.

Another potential eukaryotic expression system is yeasts. They have been used as protein factories since the early 1980s for the large scale production of intracellular and extra cellular proteins of human, animal, and plant origin. Yeasts are suitable to express recombinant proteins. Firstly, yeasts offer the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic environment. Secondly, yeasts have ability to perform many eukaryote-specific post-translational modifications, such as proteolytic processing, folding, disulfide bridge

formation, and glycosylation. Thirdly, yeasts are economical comparing to the more complex eukaryotic expression system. It usually gives higher yields, and is less demanding in terms of time and effort. Nevertheless, there are disadvantages in using yeasts for expression of some heterologous proteins. It mostly related to their inability to perform certain complex post-translational modifications, such as prolyl hydroxylation and amidation, phosphorylation and glycosylation (Cereghino *et al*., 1999).

3.2 Prokaryote expression systems

Bacterial systems can express antigens at very high levels and are suitable for expressing vaccine antigens that do not require significant post-translational modifications.

The expression of heterologous secreted proteins in bacteria has been studied both in gram positive and gram negative bacteria.

Gram-positive bacteria, for example, *Bacillus subtilis, Staphylococcus carnosus* have not achieved widespread acceptance. They excrete high levels of proteolytic enzymes that can cause extensive product degradation. In recent years, *Pseudomonas fluorescens* and *Ralstonia eutropha* have been used to produce remarkably high yields of recombinant proteins. A soluble organophosphohydrolase expressed in *R. eutropha* was high up to 10 g/L in high cell density fermentations. Although a high yield is expressed, there was little experience with this organism.

E. coli, gram negative bacteria, the number one bacterium of recombinant DNA technology, has been extensively studied as production host for heterologous proteins. Since it is very well characterized, many strategies optimizing protein expression expression and protein quality (Liljeqvist and Stahl, 1999). Conversely, the choice of bacterial strain, medium formulation, and the promoter and expression system are critical in determining protein yields. Remarkable yields of secreted proteins (in the 5–10 g/L range) have been obtained repeatedly. Such high yields are

only obtained by high cell density fermentation using finely tuned expression systems. Nonetheless, it is reasonable to expect yields of at least 0.5–0.8 g/L for the vast majority of heterologous proteins. Many mammalian proteins can now be produced routinely in secreted form with yields in the g/L scale. Disadvantages that must be concerned are efficient secretion across the inner membrane, proteolytic degradation, incorrect disulfide-bond formation and aggregation into periplasmic inclusion bodies (Georgiou and Segatori, 2005).

One potential drawback with prokaryotes as production hosts is that they are unable to carry out posttranslational modifications but other beneficial properties, and in particular the cost-efficient production systems, make bacteria the dominating hosts for production of subunit vaccine candidates.

Recombinant VP2 protein has been successfully expressed in many systems, such as in an insect cell (SF9) for baculovirus expression system (Saliki *et al*., 1992; Gilbert *et al.*, 2005) and the 10 µg protein was utilized as a vaccine in dogs (Turiso *et al*., 1992; Moonjit, 2006). The recombinant VP2 expressed in *E.coli* was applied to dogs by injecting as 100 µg DNA with and without adjuvant. The dogs injected with recombinant plasmid were protected fully from the disease (Gupta *et al.*, 2005). Consequently, *E. coli* is an interesting system to express recombinant VP2 due to the cost of expression (Park *et al*., 2007; Zeng *et al*., 2008).

Recently, *E. coli* expression system has been advance modified to solve its disadvantags. The pBAD Directional TOPO Expression system released by Invitrogen is designed to overcome the problems. The pBAD/D TOPO vector is a linear vector at a 4.4 kb in length (Figure 3). It contains pUC *ori* for maintenance in *E. coli*, and a kanamycin resistant gene to select positive transform colonies. The prominent advantages of this system are the high efficient transformation, a regulatory of protein expression, and simplified protein purification.

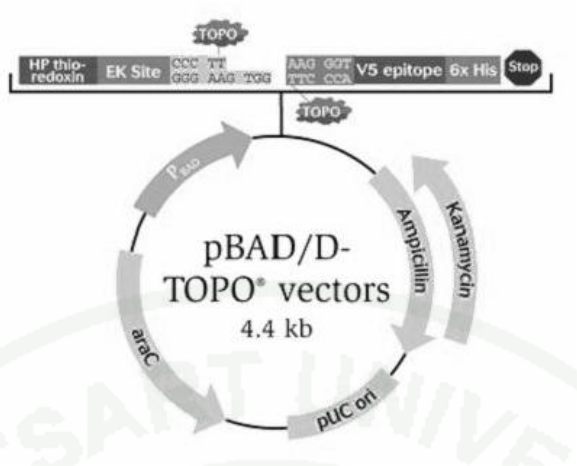

Figure 3 Showed plasmid pBAD/D TOPO map.

Source: Invitrogen (2004)

The pBAD Directional TOPO@ Expression Kits (Invitrogen) utilize a highly efficient strategy to directional1y clone a blunt-end PCR product into a vector for regulated expression and simplified protein purification in *E.coli*. Blunt-end PCR products clone directionally at greater than 90% efficiency with no ligase, post-PCR procedure, or restriction enzymes required. The system use topoisomerase I from *Vaccinia* instead of ligase. The tyrosyl residue (Tyr-274) of topoisomerase I was covalently bond to a specific duplex DNA (5'-CCCTT-) at both ends of a linear vector, pBAD20/D-TOPO®. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the PCR product, and release topoisomerase (Invitrogen, 2004).

A directional joining of double-strand DNA, the vector has been modified by adding a 4 nucleotide (GTGG) overhang sequence to the TOPO@-charged DNA. The forward primer was added with 4 complementary bases (CACC) to the 5'-end as well. The overhang in the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation.

Features of the vectors include araBAD promoter (P_{BAD}), N-terminal His-Patch thioredoxin, Directional TOPO cloning site, C-terminal fusion tags, Kanamycin

resistance gene, araC gene encoding a regulatory protein and pUC origin of replication (Figure 3) (Invitrogen, 2004).

Expression of gene of interest by the pBAD/D-TOPO$^{@}$ vectors is driven by the *araBAD* promoter. The promoter is indirectly induced by arabinose. In simply, arabinose induces araC gene to expresse. Subsequently, product from AraC gene stimulates araBAD promoter to transcripe the inserted gene. In the presence of arabinose, expression from pBAD/D-TOPO$^{@}$ vectors is induced, while only very low levels of transcription are observed in the absence of arabinose. By varying the concentration of arabinose, protein expression levels can be optimized to ensure maximum expression. Uninduced levels are repressed by growth in the presence of glucose. In addition, the tight regulation of araBAD by AraC is useful for expression of potentially toxic or essential genes.

The vector also designed to increase solubility of a recombinant protein by adding thioredoxin at the N-terminal to a recombinant protein. Thioredoxin was originally isolated from *E coli*. When used as a fusion partner, thioredoxin can increase translation efficiency and, in some cases, solubility of eukaryotic recombinant proteins expressed in *E coli*.

The directional TOPO cloning site is used for rapid and efficient directional cloning of blunt-end PCR products. The C-terminal fusion tag is used for detection and purification of recombinant fusion proteins. The kanamycin (pBAD202/D-TOPO) resistance gene is used for selection in *E.coli*. The araC gene encodes a regulatory protein for tight regulation of the P_{BAD} promoter. The pUC origin is used for maintenance in *E.coli*. In the presence of arabinose, expression of recombinant proteins encoded by pBAD vector is induced whereas only very low levels of transcription are observed from pBAD vectors in the absence of arabinose (Lee, 1980; Lee *et al*.,1987).

The pBAD/D-TOPO$^{@}$ vectors contains six histidine residues (6xHIS) at C-terminal to the inserted gene. This histidine patch has been shown to have high

affinity for divalent cations. The recombinant protein can, therefore, be easily purified on metal chelating resins (Invitrogen, 2004).

MATERIALS AND METHODS

1. Sequence analysis of VP2 gene

1.1 DNA extraction

Fecal samples were obtained from dogs that have symptoms of nausea and hemorrhagic diarrhea. The fecal samples were used for DNA extraction using phenol-chloroform extraction method as described earlier (Sambrook and Russell, 2001).

1.2 DNA amplification

For PCR, the VP2 gene was amplified using a set of primers; F (5' ACAAATGAGTGATGGAGCAGTTCAAC 3') and R (5' TTAATATAATTTTC TAGGTGCTAG 3'). The PCR mixture was composed of 10 µl of 10x buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl_2), 2 µl of 10 mM dNTPs, 4 µl of 50 mM $MgCl_2$, 1 µl of each forward and reverse primer, 0.5 µl of Taq DNA polymerase (5 U/µl) (Invitrogen^{TM}), 10 µl of template DNA and 72 µl of distilled water to give the total volume of 100 µl. The PCR condition was pre-denaturation at 94^{o}C for 5 minutes and follows by 35 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 50^{o}C for 2 min, elongation at 72^{o}C for 2 min and final extension at 72°C for another 10 minutes. The PCR products were visualized by UV illuminator after electrophoresis using 1 % agarose gel (Sambrook and Russell, 2001). The PCR products were approximately 1.7 kb. The PCR products were purified by gel purification kit (Qiagen®).

1.3 Cloning and DNA sequencing of VP2 gene

After purification of the desired DNA fragment, DNA was cloned into pGEM-T Easy vector (Promega®) and the plasmids were sent for nucleotide sequencing at Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC) Thailand.

1.4 Sequence and phylogenetic analysis

The nucleotide sequences of recombinant VP2 plasmids were translated to amino acid by Expasy translate program. The sequences were subjected to BLASTN analysis and aligned with the ClustalW program version 1.83. Phylogenetic and molecular evolutionary analyses were constructed from the VP2 gene nucleotide sequences of the CPV strains using in this study and other sequences obtained from GenBank database with MEGA version 4.0 (http://www.megasoftware.net) using the NJ method. The reliability of the phylogenetic tree obtained for the VP2 region was evaluated by running 1,000 replicates in the bootstrap test.

2. Expression of the recombinant VP2 protein

2.1 Amplification of VP2 gene

Specific primers for the amplification of a whole VP2 gene of CPV was designed using nucleotide sequences of VP2 from GenBank. For cloning of whole VP2 gene into plasmid pBAD202/D-TOPO®, the forward primer (F-VP2) sequence contained CACC sequence at 5' end was 5' CACC ATG AGT GAT GGA GCA GTT CAA C 3', which was very crucial for cloning into pBAD202/D-TOPO® and reverse primer (R-VP2) sequence was 5' ATA TAA TTT TCT AGG TGC TAG 3'. These primers gave the full-length of VP2 gene which was 1,755 bps in length.

The PCR products were used as DNA templates for cloning of VP2 into plasmid pBAD202/D-TOPO®. The PCR mixture was composed of 10 µl of 10x Pfx amplification buffer (50 mM Tris-HCl (pH 8.0), 50 mM KCl_2, a mM DTT, 0.1 mM EDTA stabilizers and 50% (v/v) glycerol), 3 µl of 10 mM dNTPs, 2 µl of 50 mM $MgSO_4$, 1 µl of each forward and reverse primer, 1 µl of Taq DNA polymerase (2.5 U/µl) (Invitrogen™), 10 µl of template DNA and 72 µl of distilled water to give the total volume of 100 µl. The PCR condition was pre-denaturation at 95°C for 5 minutes and followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, elongation at 72°C for 60 seconds and final extension at 72°C

for another 10 minutes. The PCR products were visualized by UV illuminator after electrophoresis using 1 % agarose gel (Sambrook and Russell, 2001).

2.2 Cloning of VP2 gene into plasmid pBAD202/D-TOPO®

The PCR products were purified using QIAquick® Gel Extraction Kit (Qiagen®) and subsequently ligated to plasmid pBAD202/D-TOPO® (Invitrogen®) according to the manufacturer guide line to get plasmid pBADVP2. Briefly, purified PCR products were mixed with 1 µl of salt solution (1.2 M $NaCl_2$, 60 mM $MgCl_2$), 4 µl of plasmid and 1µl of distilled water. The ligation mixture was gently mixed and incubated at room temperature for 15 minutes. Three microliters of the ligation mixture was used to transform into One shot®TOP10 competent cells (Invitrogen®). Afterward, two hundred and fifty microlitres of S.O.C. medium were added into the transformed *E. coli* and shaken at 200 rpm for an hour before was spreaded on LB agar containing 50 mg/ml of kanamycin. The present of inserted VP2 gene was determined by PCR technique (Sambrook and Russell, 2001).

2.3 Protein induction optimization

The inoculums were prepared by adding 100 µl of recombinant *E. coli* stock in 2 ml LB broth containing 50 mg/ml kanamycin and shaken at 200 rpm at 37°C for overnight. After that, one hundred microlitres of inoculums were added to each of five tubes containing 10 ml LB broth with 50 mg/ml kanamycin. The tubes were shaken for another 4-5 hours until the optical density (OD_{600}) reach ~ 0.5. Then, the optimum condition for recombinant VP2 expression was determined by adding different concentrations (2, 0.2, 0.02, 0.002 and 0.0002%) of arabinose to recombinant *E. coli* culture medium. The cultures were then sampling every two hours. Subsequently, recombinant VP2 protein was partially purified using Ni-NTA bead as described by manufacturer (Qiagen®). The recombinant VP2 proteins were kept at -80°C for further verified by SDS-PAGE. The control samples were also conducted at the same condition but no adding arabinose.

2.4 Sodium dodecyl sulfate polyacrylamide gel electrophorsis (SDS-PAGE)

One microliter of induced recombinant *E. coli* pellet were added to 80 µl of SDS sample buffer (250 mM Tris HCl, 20% glycerol, 4% SDS, and 0.006% bromophenol blue), boiled for 5 minutes and centrifuged at 5,000 rpm for 5 minutes. The supernatants were loaded into 10 % SDS-PAGE gel and electrophoresed for 90 minutes at 110 voltages. The gel was then stained with staining solution (0.01 % Coomasie Brilliant Blue, 50% methanol, 40% distilled water, and 10% acetic acid) for 10 minutes and subsequently destained with destaining solution (50% methanol, 40% distilled water, and 10% acetic acid).

2.5 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. Rabbits were immunized 3 times for 6 weeks. The rabbit hyperimmune serum against CPV was checked for the reactivity against CPV by immunoblot.

2.6 Western blot analysis

The recombinant VP2 proteins were separated in 10 % SDS-PAGE gel and electro-transferred onto nitrocellulose membrane at 400 mAmp for 300 minutes. The membrane was blocked with 5% skim milk at 4^{o}C for overnight. The membranes were then incubated with 1:500 mouse anti-histidine antibody (Sigma®) or 1:100 rabbit anti-CPV hyperimmune serum for an hour at room temperature. Subsequently, the membranes were washed and incubated with 1:3,000 goat anti-mouse IgG conjugated with horseradish peroxidase (KPL) or 1:1,500 goat anti-rabbit IgG conjugated peroxidase (Sigma®) for an hour at 37 ^{O}C. After washing, the membranes were incubated with 3 ml of 0.6 mg/ml diaminobenzidine (DAB, Sigma®) containing 0.03% H_2O_2 for 5-10 minutes at room temperature. The recombinant VP2 protein was visualized as a brown band on nitrocellulose membrane.

3. Animal Immunization

3.1 Recombinant VP2 protein production

The overnight growth of recombinant *E. coli* was inoculated into 1 liter of LB medium at a concentration of 1: 100 and shaken at 200 rpm at 37^{o}C for 3 hours. Next, the cells were induced with 0.002% arabinose and shaken for another 8 hours. Finally, the induced recombinant *E. coli* was harvested by centrifugation at 6,000 rpm for 5 minutes. The cell pellet was stored at -20^{o}C and lysed before used.

3.2 Protein purification

The induced recombinant *E. coli* was lysed with lysis buffer pH 8.00 (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M urea), vigorously shaken and incubated for 2 hours at 4 ^{O}C. The lysed cells were sonicated for 10 minutes until the cell slurry changed from turbid to translucent. The cell debris was then separated by centrifugation at 5,000 rpm. The recombinant VP2 protein was purified using Ni-NTA bead as described by manufacturer (Qiagen®). Briefly, Ni-NTA beads were first equilibrated with 3 volume of lysis buffer B at room temperature for 10 minutes. Buffer was then removed by centrifugation. An equal volume of cell lysate was added to Ni-NTA beads, shaken on ice at 200 rpm for 2 hours and centrifuged at 2,000 rpm for 2 minutes. The beads were washed with 3 volume of washing buffer C, mixed well by hand shaking, and centrifuged at 2,000 rpm for 2 minutes. The bead was then washed with 2 volume of washing buffer D. These washing steps were repeated for 5 times. The recombinant VP2 protein was eluted from beads by 1 volume of elute buffer E. This step was repeated for 5 times. The purified recombinant VP2 was verified by 10% SDS-PAGE. Western blot analysis was performed to test the ability of recombinant VP2 to interact with rabbit hyper-immune serum against CPV as described before (Sambrook and Russell, 2001). The purified protein was concentrated using acetone precipitating method. Briefly, two volumes of acetone were added to the protein, gently mixed and allowed the protein to sediment at -80 ^{O}C for overnight. Afterward, the mixture was centrifuged at 13,000 rpm for 10 minutes.

The pellet was allowed to air dry and stored at -20 ^{O}C before used. Protein quantification was performed with spectrophotometer by using standard curve of various BSA concentrations to determine the concentration of total protein.

3.3 Rabbit immunization

Twenty male New Zealand white rabbits (2-3 months, approximately 3 kgs) were separately reared. They were randomly divided into four groups of five animals each. Group A was immunized with 500 μl of protein from non-transformed *E. coli*. Group B and group C were immunized with purified recombinant VP2 300 and 500 μg/dose, respectively. Each dosage of purified recombinant VP2 protein was vigorously mixed with the equal volume of adjuvant for first dose and boosting. Group D was immunized with commercial CPV modified live vaccine. Each group was immunized at week 0, 2, 4 and 6 of the experiment. The rabbits were bled at week 0, 2, 4, 6, and 8 for post immunization (P.I.). The sera from immunized rabbits were assayed for anti-VP2 antibody detection.

3.4 Serology test

3.4.1 Cell culture and virus preparation

The feline kidney (CRFK) cells (ATCC® CCL 94). were cultured in Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS). Then, the Canine parvovirus strain Cornell strain 780916-80 (ATCC® VR-953) was propagated in 75-cm^2 plastic flasks using 50-60 % monolayer CRFK cells. Cell cultures were inoculated with virus within 2 hours of seeding and then changed medium with 2% FBS instead of medium with 10% FBS. These infected cells were incubated for 4 to 6 days at 37 °C, 5% CO_2. Cultures were frozen and thawed three times and were clarified by low-speed centrifugation. Supernatant portions were combined and assayed for hemagglutination activity and were stored at -80 °C.

3.4.2 Serum preparation for hemegglutination (HA) test

Porcine erythrocytes were used routinely for HA test. Porcine's blood samples were collected in Alsever's solution, using care to clotting, and were stored at 4 °C for at least 24 hours, but not more than 7 days, before use. Erythrocytes were washed three times in PBS. Washed porcine red blood cells (RBC) was used within 3 days of their preparation and discarded if hemolysis was evident. The washed erythrocytes were measured concentration of RBC by using hematocrit and diluted RBC with PBS to final concentration 0.5%. for use in test

3.4.3 Hemagglutination test

Tests were performed in 96-well plastic V-plates. Serial two-fold dilutions (50 µl) were prepared from each immunized rabbit serum. An equal volume of 0.5% ice-cold porcine RBC suspension was added to each well and plates were incubated at 4 °C. Tests were read after the porcine RBC controls had settled completely, usually after 1 to 2 hours.

3.4.4 Anti-VP2 antibody detection by Enzyme linked immunosorbent assay (ELISA)

Rabbit sera were tested for antibodies against recombinant VP2 protein by ELISA technique and data were analyzed in term of mean optical intensity (OD value). Indirect ELISA was performed to determine the antibody titer. The microplates (MaxiSorp, Nunc.) were coated with CPV culture diluted (1:2000) coating buffer to get the final intensity appropriate for detection by ELISA reader. The plate was incubated overnight at 4^{O}C. The 100 µl of sera were added into each well and incubated at 37 ^{O}C for 1 hour. The excess sera were washed out and added 100 µl of 1:3000 goat anti rabbit IgG conjugated with horseradish peroxidase (Sigma®) into each well and incubated at 37 ^{O}C for 1 hour before washed out. Finally, 100 µl of Tetra-methylbenzidine (TMB) substrate (Sure Blue, KPL) was added to each well for 5 minutes. The reaction was then stopped by adding 100 µl of 0.1 N

H_2SO_4. The reaction intensity was measured by the ELISA plate reader at the absorbance of 620.

3.5 Data analysis

The effect of each vaccine was compared to the control group by measurement of the level of antibody against recombinant VP2 antigen and determined by analysis of variance (ANOVA) on completely randomized design (CRD). Means of each group's antibody were compared and significant differences between groups of immunization by ANOVA when $p < 0.05$. All analyses were carried out using the SAS program.

RESULTS

1. Sequence analysis of VP2 gene

The 3 commercial CPV vaccines and total of 26 fecal samples collected during 2003-2004 and 2008-2009 were used in this study. The locations of sample collection were shown in Table 1. These samples were amplified and sequenced using VP2 specific primers. The PCR products were approximately 1.7 kb in length which contained regions spanning the amino acids 426 and 555 in VP2 gene for the differentiation of CPV type 2a and 2b.

The VP2 gene sequences and deduced amino acid sequences of these isolates were compared with each other as well as with the CPV VP2 sequences available in the GenBank (Table 1). Phylogenetic tree was constructed using the full-length VP2 gene nucleotide sequences of the CPV strains using in this study and other sequences obtained from GenBank database (Figure 4).

The prevalence of CPV type was shown in Table 3. In 2003, we found equal number of CPV type 2a and 2b. In 2004, CPV type 2a was the predominant type. In 2008, we found 4 isolates of CPV type 2a and 3 isolates of CPV type 2b. In 2009, we found only CPV type 2a. According to these results, type 2a was the predominant type of CPV that has circulated in the Bangkok Metropolitan Area and Central part of Thailand.

The variant, CPV type 2a, differed from the original type 2 strain with amino acid changes at residue 87 (Met→Leu), 101 (Ile→Thr), 300 (Ala →Gly), 305 (Asp→Tyr), and 555 (Val→Ile) in the gene encoding the VP2 capsid protein (Parrish *et al*., 1991b; Mochizuki *et al*., 1996; Truyen *et al*., 1996). In this study, the first four changes were observed in all 19 isolates, but none of the sequenced isolates had a Val→Ile substitution at residue 555. CPV type 2b was reported to have two substitutions at residue 426 (Asn→Asp) and 555 (Ile→Val), when compared with type 2a in the VP2 amino acid sequence (Parrish, 1991a; Parrish *et al*., 1991b). Seven of the isolates described in this study had the 426 (Asn→Asp) substitution and all of

the sequenced isolates had Val at residue 555 (Table 2). However, no CPV type 2 was found in this study.

According to the phylogenetic analysis, the Thai isolates of CPV were clustered closely together and to Asian isolates. The phylogenetic tree of CPV type 2a of Thai isolates were clustered close to the Korean isolate DH426 (EF599096) and Taiwanese isolate CPV-T37 (U72698). The isolates of CPV type 2b were closely related to the isolates from India IIL P19 (DQ182622) and IIL P25 (DQ182625), Vietnam HCM-23 (AB120723), Japan Sho-nan (AB128923), Korea K031 (EU009206) and USA CPV-436 (AY742955) and CPV-193 (AY742932) (Figure 4). The amino acids sequences alignments of Thai isolates of CPV type 2a and type 2b showed 100% homology with its own type (data not shown). There was no indication of a specific Thailand parvovirus lineage. However, the Thai isolates of CPV may be evolved from the European types because there were no significant amino acids changes from the European types (Figure 5). Interestingly, neither commercial vaccines using in this study have type 2a. According to the sequencing results, two imported commercial vaccines (VAC_M and VAC_P) were the CPV type 2 and the remaining (VAC_S) was the CPV type 2b (Table 2). They were also clustered in the different group from the field isolates of Thailand in this study and closed to a Germany isolate ChowChow (AJ002927) that has been characterized as CPV type 2 (Figure 4).

Table 1 Canine parvovirus isolates from Thailand and GenBank accession numbers of other CPVs obtained from the GenBank.

Isolate	Origin	GenBank accession no.	Type
HCM-23	Vietnam	AB120723	2b
V139	Vietnam	AB054222	2c(a)
HNI4_1	Vietnam	AB120727	2c
CPV-T37	Taiwan	U72698	2a
LCPV-T1	Taiwan	AB054214	2a
DH426	Korea	EF599096	2a
Pome	Korea	EF599098	2a variant
K031	Korea	EU009206	2b
RPPV	China	DQ354068	2a
Sho-nan	Japan	AB128923	2b
97-008	Japan	AB115504	2b
IIL P19	India	DQ182622	2a
IIL P25	India	DQ182625	2b
CPV-193	USA	AY742932	2b
CPV-436	USA	AY742955	2b
FPV-b	USA	M38246	FPV
PVACAP	USA	M24005	Raccoon
U6	Germany	AY742935	2a
ChowChow	Germany	AJ002927	2
U51	Germany	AY742942	2c
CPV-677	Italy	AF306448	2a
CPV 56/00	Italy	AY380577	2c
VAC_M*	-	GU212790	This study
VAC_S*	-	GU212792	
VAC_P*	-	GU212791	
KU1_08 (2008)	Bangkok, Thailand	FJ869122	
KU3_08 (2008)	Suphanburi, Thailand	FJ869123	
KU4_08 (2008)	Bangkok, Thailand	FJ869124	
KU5_04 (2004)	Phatum Thani, Thailand	FJ869125	
KU5_08 (2008)	Phatum Thani, Thailand	FJ869126	
KU9_04 (2004)	Nonthaburi, Thailand	FJ869127	
KU11_04 (2004)	Suphanburi, Thailand	FJ869128	
KU12_04 (2004)	Ayutthaya, Thailand	FJ869129	
KU13_04 (2004)	Bangkok, Thailand	FJ869130	
KU18_04 (2004)	Bangkok, Thailand	FJ869131	
KU19_04 (2004)	Bangkok, Thailand	FJ869132	
KU21_04 (2004)	Bangkok, Thailand	FJ869133	
KU23_03 (2003)	Bangkok, Thailand	FJ869134	
KU39_03 (2003)	Bangkok, Thailand	FJ869135	
KU46_03 (2003)	Nonthaburi, Thailand	FJ869136	
KU52_03 (2003)	Suphanburi, Thailand	FJ869137	
KU53_03 (2003)	Nonthaburi, Thailand	FJ869138	
KU66_03 (2003)	Phatum Thani, Thailand	FJ869139	
KU13_08 (2008)	Ayutthaya, Thailand	GQ379042	
KU14_08 (2008)	Bangkok, Thailand	GQ379043	
KU143_09 (2009)	Ayutthaya, Thailand	GQ379044	
KU146_09 (2009)	Ayutthaya, Thailand	GQ379045	
KU531_09 (2009)	Nonthaburi, Thailand	GQ379046	
KU616_09 (2009)	Bangkok , Thailand	GQ379047	
KU739_09 (2009)	Bangkok, Thailand	GQ379048	
KU18_08 (2008)	Bangkok, Thailand	GQ379049	

* (VAC_M = Primodog; VAC_S = Quantum; VAC_P = Vanguard)

Table 2 Comparison of amino acid sequences of canine parvovirus isolates reported in this study

	Residues													
Isolate	87	101	297	300	305	324	413	418	426	435	440	463	555	type
KU9_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU11_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU12_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU13_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU14_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU18_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU19_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU21_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU23_03	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU52_03	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU53_05	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU143_09	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU146_09	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU5_08	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU531_09	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU616_09	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU739_09	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU13_08	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2a**
KU18_08	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2a**
KU1_08	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
KU3_08	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
KU4_08	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
KU5_04	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
KU39_03	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
KU46_03	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
KU66_03	**L**	**T**	**A**	**G**	**Y**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**
VAC_M	**M**	**I**	**S**	**A**	**D**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2**
VAC_P	**M**	**I**	**S**	**A**	**D**	**Y**	**D**	**I**	**N**	**P**	**T**	**V**	**V**	**2**
VAC_S	**M**	**I**	**S**	**A**	**D**	**Y**	**D**	**I**	**D**	**P**	**T**	**V**	**V**	**2b**

Table 3 Showed province and number of CPV type isolated in each year.

year/type	2a	total	2b	total
2003	KU23(BK)*, 52(SB), 53(NB)	3	KU39(BK), 46(NB),	3
2004	KU9(NB), 11(SB), 12(AY), 13(BK), 18(BK), 19(BK), 21(BK)	7	66(PT) KU5(PT)	1
2008	KU5(PT), 13(AY), 14(BK), 18(BK)	4	KU1(BK), 3(SB), 4(BK)	3
2009	KU143(AY), 146(AY), 531(NB), 616(BK), 739(BK)	5	-	-

* (BK = Bangkok; SB = Suphanburi; NB = Nonthaburi; AY = Ayutthaya; PT = Pathum Thani)

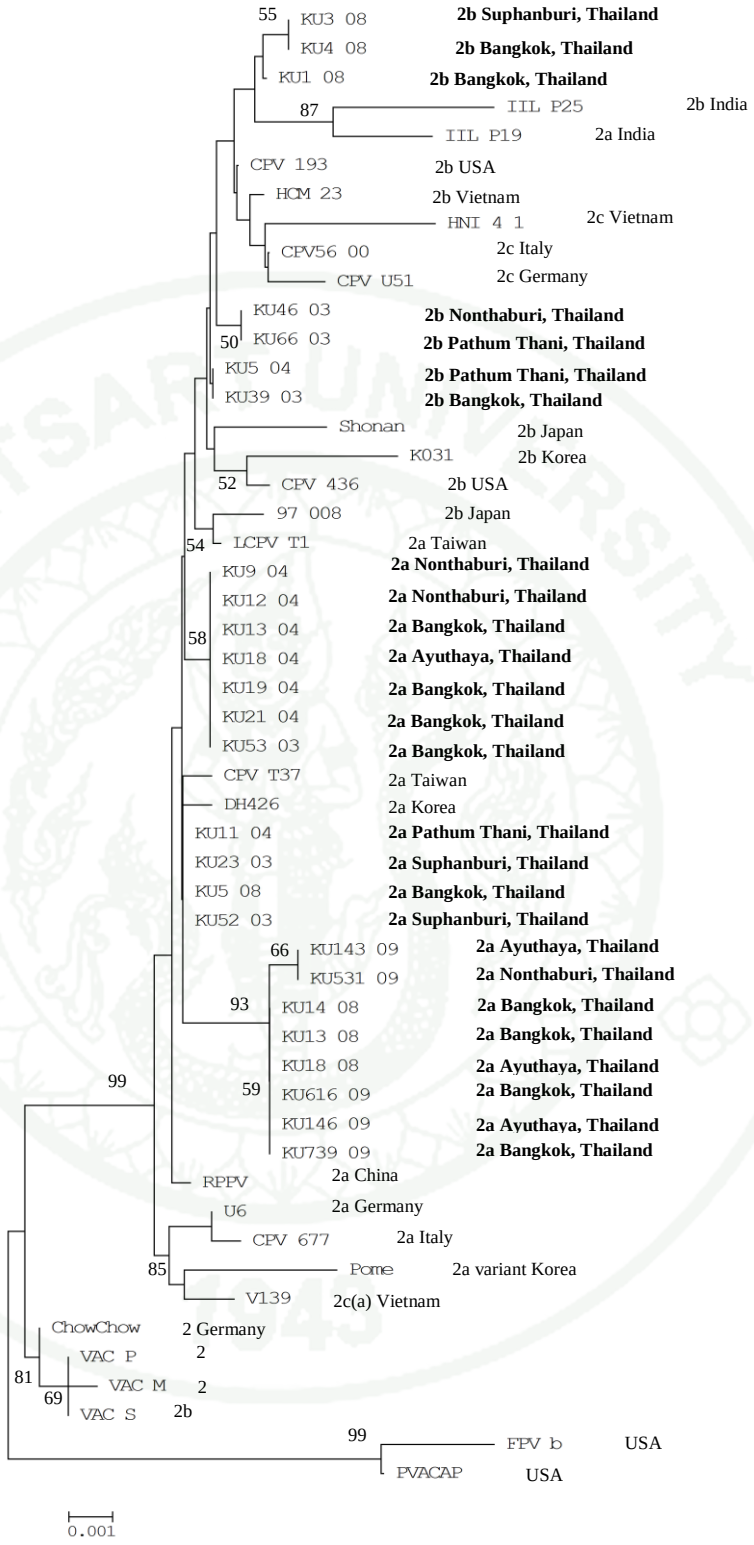

Figure 4 Comparison of the nucleotide sequences of the VP2 gene of CPV isolates obtained from dogs in Thailand and other sequences obtained from the GenBank database using the neighbor-joining method and bootstrap analysis performed with 1000 trials. Drawn using MEGA version.

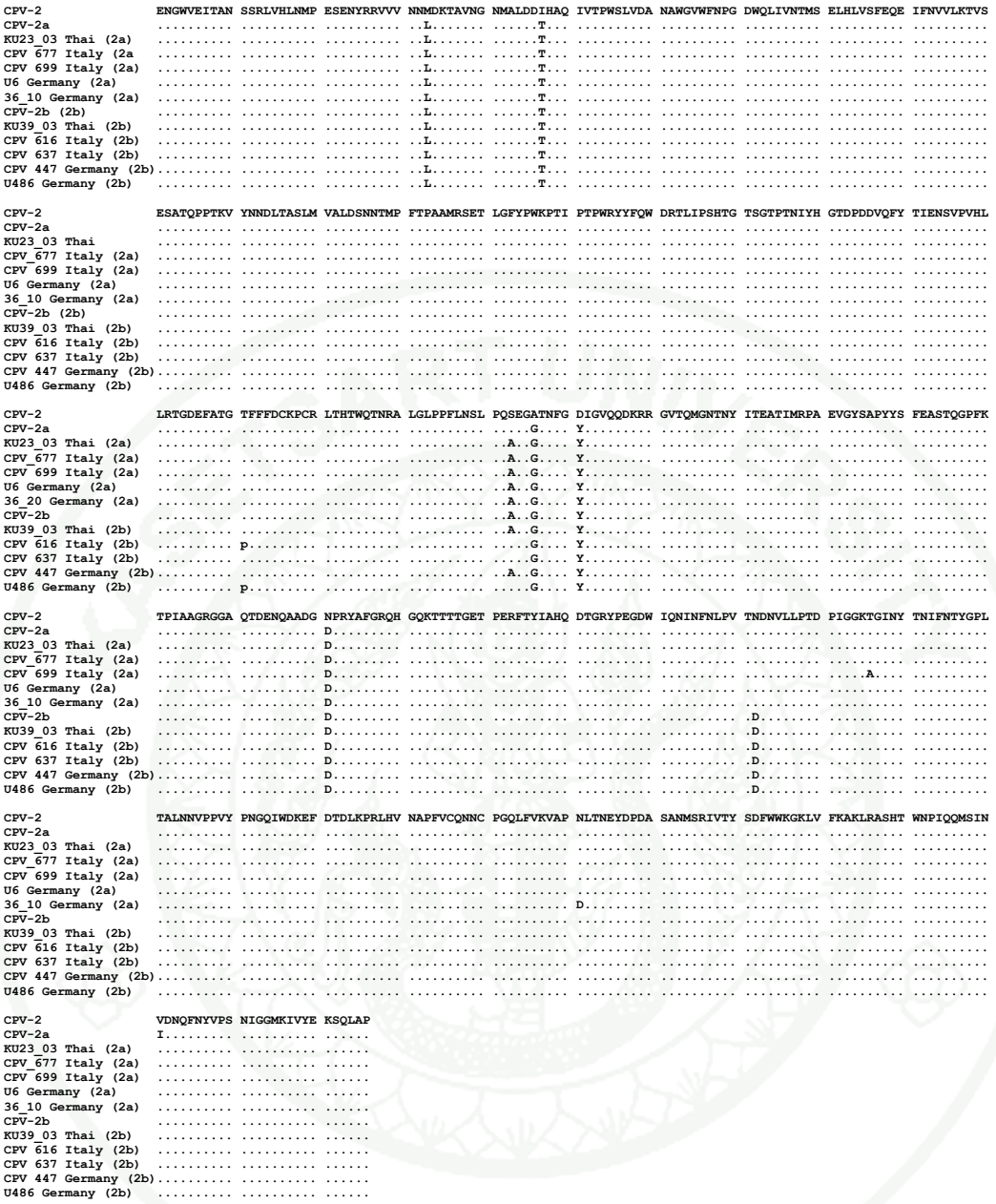

Figure 5 Amino acid alignment of a VP2 fragment of representative of Thai isolates CPV-2a (CPV23_03) and CPV-2b (CPV39_03) with reference strains CPV-2 (M23255), CPV-2a (M24003) and CPV-2b (M74849) and European isolates.

2. Expression of recombinant VP2 protein

2.1 Cloning of VP2 gene into TOP10

The full-length VP2 gene of selected CPV isolate type 2a could be successfully amplified and the PCR products were approximately 1,700 bp (Figure 6). The VP2 gene was cloned in to pBAD/D-TOPO plasmid before being transformed into *E. coli* strain TOP10. The size of PCR products of the positive clones was approximately 1,900 bps of VP2 gene when visualized by electrophoresis technique (Figure 7). The present of the VP2 gene in the recombinant plasmid containing was confirmed by sequencing and submitted in GenBank database accession no. FJ869126.

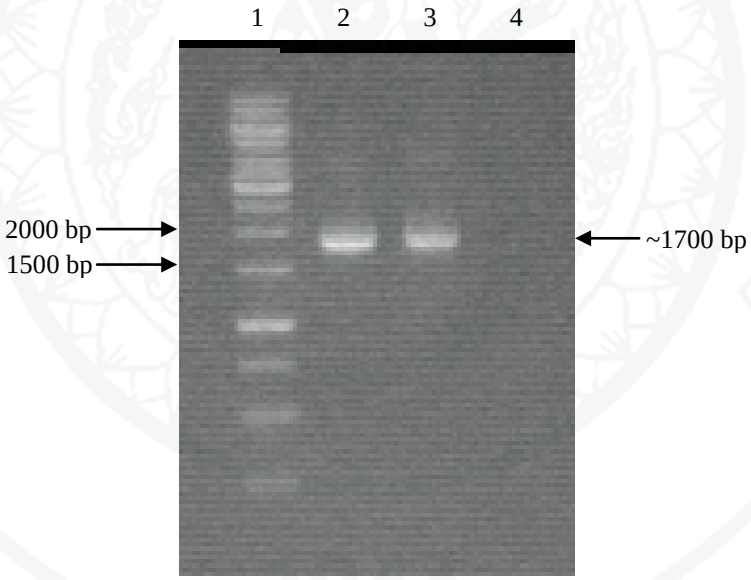

Figure 6 PCR product of the VP2 gene. Lane 1: 1 kb DNA marker; lane 2-3: VP2 gene; lane 4: negative control.

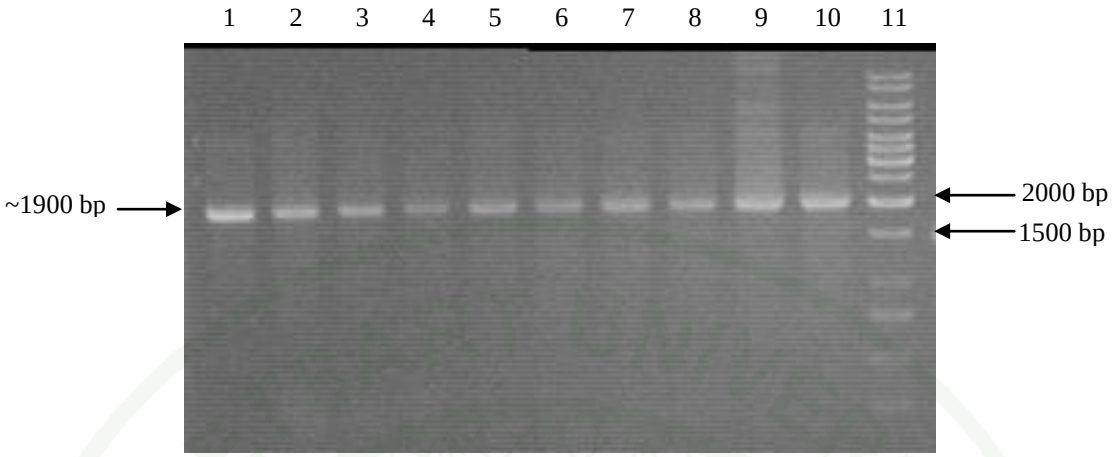

Figure 7 The recombinant VP2 plasmids. Lane 1-10: recombinant VP2 plasmid; lane 11: 1 kb DNA marker.

2.2 Optimization of protein induction

The recombinant VP2 protein was expressed *in vitro* using the pBAD expression vector and the *E. coli* strain TOP10 expression system. A series of expression conditions were carried out under different arabinose concentration and time course. Five different arabinose concentrations (2%, 0.2%, 0.02%, 0.002% and 0.0002%) were tested for the optimization of protein induction. At 0.2%, 0.02% and 0.002% concentration of arabinose gave approximately the same amount of the recombinant VP2 (Figure 8). For optimal time-course determination, samples were collected every 2 hours after adding 0.002% arabinose. The recombinant VP2 protein was detected at 2, 4, 6, and 8 hours post induction (h.p.i.) and peaked at 8 h.p.i (Figure 9). Therefore, 0.002% of arabinose and 8 hours induction time were selected for the recombinant VP2 production. The recombinant protein was expressed as an insoluble protein. A molecular weight of recombinant VP2 protein was approximately 80 kDa on 10% polyacrylamide (Figure 8-9). The purified VP2 protein by Ni-NTA migrates at 80 kDa band (Figure 10).

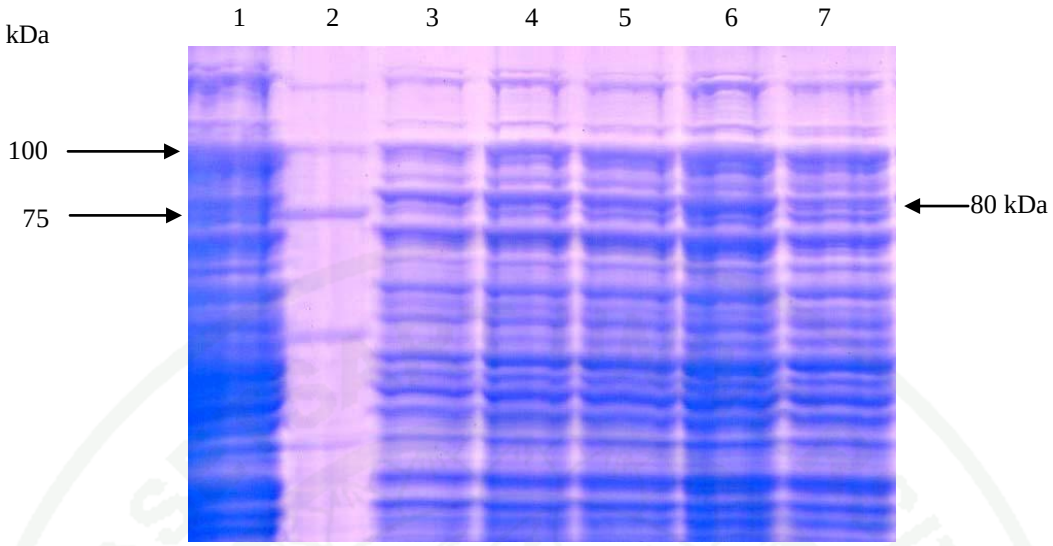

Figure 8 10% SDS-PAGE of the arabinose concentration study for recombinant VP2 protein induction. Lane 1: Wild type protein; lane 2: protein marker; lane 3, 4, 5, 6, 7: 2, 0.2, 0.02, 0.002 and 0.0002% arabinose, respectively.

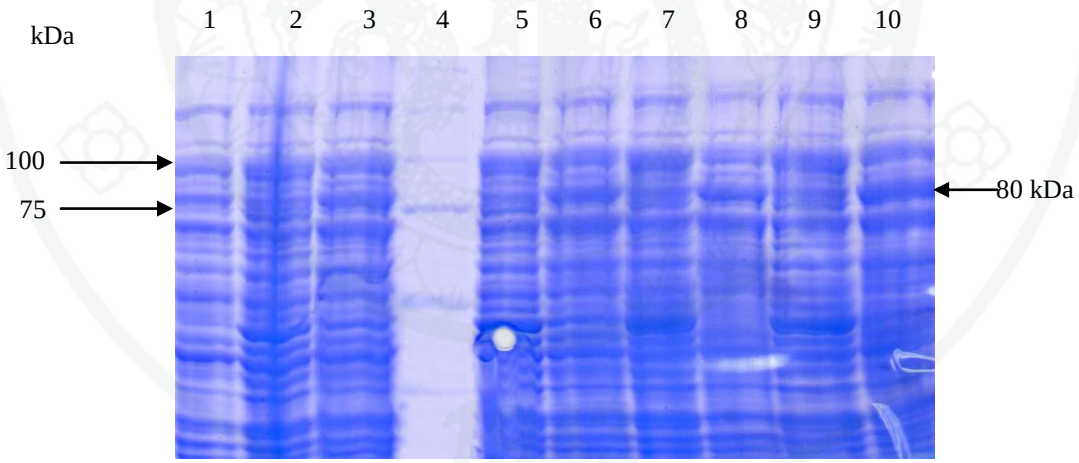

Figure 9 10% SDS-PAGE of the time course study for recombinant VP2 protein induction. Lane 2, 5, 7, 9: Wild type protein at 2, 4, 6, 8 hours; lane 1, 3, 6, 8, 10: recombinant VP2 protein at 0, 2, 4, 6, 8 hours, respectively; lane 4: protein marker.

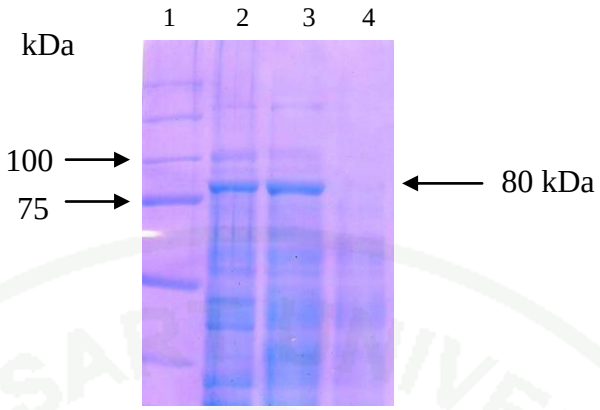

Figure 10 10% SDS-PAGE analysis of the purified recombinant VP2 protein. Lane 1 protein marker; lane 2 crude rVP2 protein; lane 3: purified rVP2 protein; lane 4: purified wide type protein.

2.3 Dot blot and Western blot analysis

By Dot blot analysis, the crude protein showed interaction with mouse anti-histidine IgG monoclonal antibody and rabbit anti-CPV polyclonal antibody (Figure 11A and 12A). The crude and purified recombinant VP2 proteins were electro-transferred onto nitrocellulose membrane, subsequently the recombinant VP2 proteins were incubated with rabbit anti-CPV hyperimmune serum or mouse anti-histidine monoclonal antibody. For crude protein and purified, the band that detected by mouse anti-histidine monoclonal antibody had molecular weight approximately 80 kDa. Protein of non-transformed *E. coli* showed negative result (Figure 11B and Figure 13A). In contrast, the crude and purified recombinant VP2 protein that detected by rabbit anti-CPV hyperimmune serum gave a specific band of approximately 80 kDa (Figure 12B and Figure 13B).

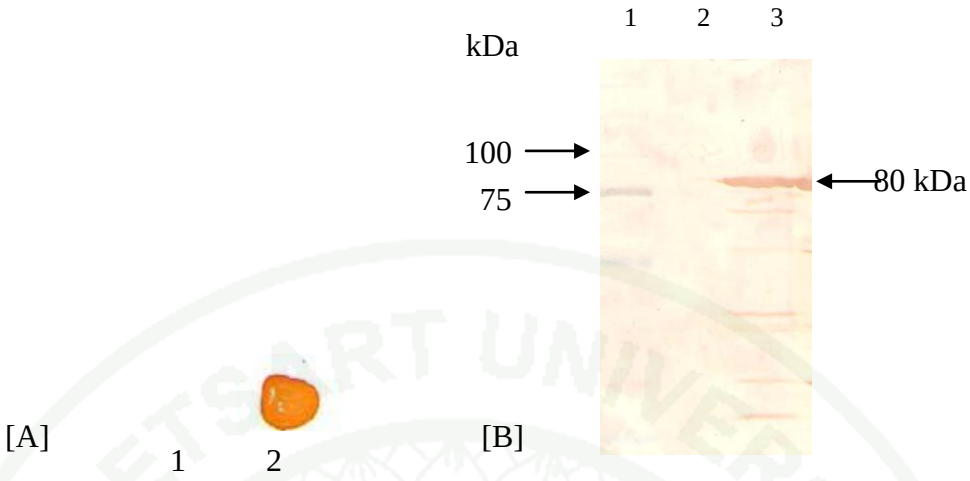

Figure 11 Dot blot and Western blot analysis of recombinant VP2 protein. Determination of immunogenicity of rVP2 protein using mouse anti-histidine monoclonal antibody. (A) Dot blot analysis. 1: Wild type protein; 2: crude rVP2 protein. (B) Western blot analysis. Lane 1: prestain protein marker; lane 2: Wild type protein; lane 3: rVP2 protein.

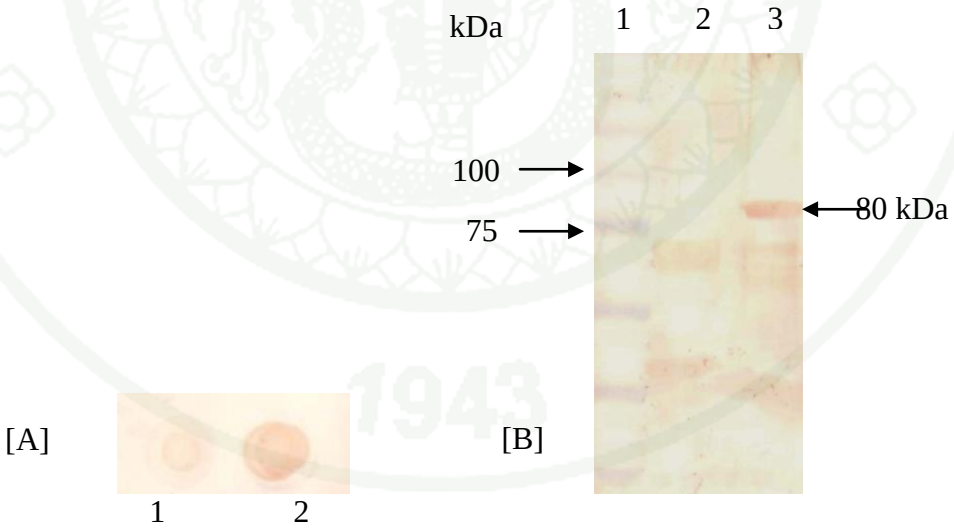

Figure 12 Dot blot and Western blot analysis of recombinant VP2 protein. Determination of immunogenicity of rVP2 protein by using rabbit anti-CPV polyclonal antibody. (A) Dot blot analysis. 1: Wild type protein. 2: crude rVP2 protein. (B) Western blot analysis. Lane 1: prestain protein marker; lane 2: Wild type protein; lane 3: rVP2 protein.

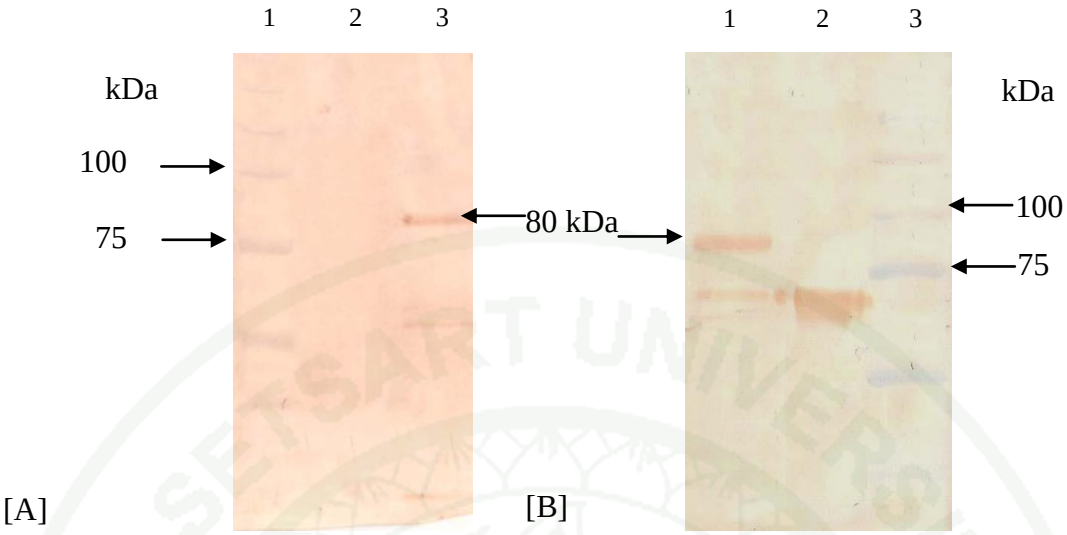

Figure 13 Western blot analysis of purified recombinant VP2 protein. Determination of immunogenicity of rVP2 protein. (A) Using mouse anti-histidine monoclonal antibody. Lane 1: molecular prestain protein marker; lane 2: Wild type protein; lane 3: purified rVP2 protein. (B) Using rabbit anti-CPV polyclonal antibody; lane 1: purified rVP2 protein; lane 2: Wild type protein; lane 3: prestain protein marker.

3. Animal Immunization

The purified recombinant VP2 protein was mixed with the same volume of adjuvant to get a homogenous mixture that had the concentration of 300 µg (group B) and 500 µg (group C) per 500 µl. Assessment of immunogenicity of recombinant VP2 protein was conducted in rabbits. Rabbits were subjected to be immunized and tested for levels of antibodies against recombinant VP2 protein by ELISA and the data were analyzed before immunizations in term of mean optical density (OD). The mean of reaction intensity of antibody was 0.448 (group A), 0.448 (group B), 0.438 (group C) and 0.446 (group D) with no significant differences among groups. In treatment groups, the levels of antibodies against recombinant VP2 protein and commercial modified live vaccine were gradually increasing at the second weeks after immunization and the levels are maintained throughout experiment (Figure 14). The

antibodies of treatment groups were significantly higher than the control group from week 2 to 8. The mean OD value between group B and C were not significantly different (Table 4). However, when compared with group D that immunized with commercial modified live vaccine, the levels of antibodies against recombinant VP2 protein (group B and C) were rapidly increasing at the second weeks after immunization and the levels are maintained throughout experiment (Figure 14). The antibodies of vaccine groups were significantly higher than the recombinant protein groups from week 2 to 8 week (Table 4).

Table 4 Statistic analysis of antibody responses against recombinant VP2 and commercial modified live CPV vaccine compared to the control group using ANOVA.

Week (s) of serum collection	Mean±SE (n=20)				*p*
	Group A	Group B	Group C	Group D	
0	0.448±0.005^{A}	0.448±0.004^{A}	0.438±0.002^{A}	0.446±0.002^{A}	0.1722
2	0.45±0.003^{A}	0.631±0.036^{B}	0.586±0.024^{B}	1.406±0.136^{C}	<0.001
4	0.452±0.002^{A}	0.71±0.043^{B}	0.678±0.035^{B}	1.86±0.249^{C}	<0.001
6	0.458±0.002^{A}	0.819±0.059^{B}	0.77±0.028^{B}	2.13±0.148^{C}	<0.001
8	0.45±0.005^{A}	0.979±0.061^{B}	0.957±0.029^{B}	2.34±0.151^{C}	<0.001

Note: Different superscript in the same row indicated significant differences between groups.

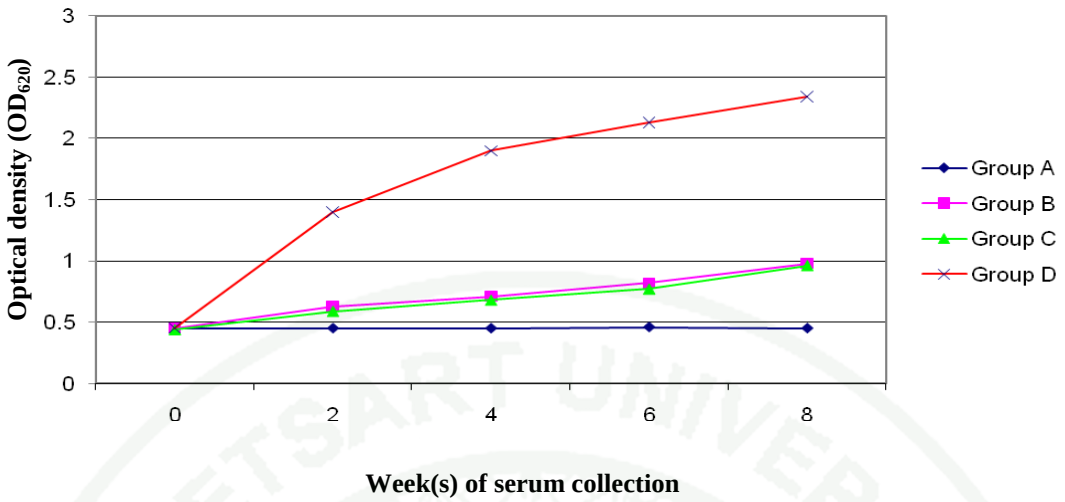

Figure 14 General Linear Model (GLM) of mean OD value of rabbit that immunized with recombinant VP2, modified live CPV vaccine and control group. General Linear Model (GLM) of mean value of optical density of antibodies from sera of rabbit immunized with PBS was group A, recombinant VP2 300 μg was group B, and recombinant VP2 500 μg was group C by indirect ELISA.

DISCUSSIONS

1. Phylogenetic analysis of VP2 gene

The significant difference between CPV-2a and CPV-2b is the substitution of two amino acids in the major antigenic VP2 capsid protein that is Asn-426 in 2a (Asp-426 in 2b) and Ile-555 in 2a (Val-555 in 2b) (Parrish *et al*., 1991). Interestingly, all of the CPV type 2a of Thai isolates had Val at residue 555 instead of Ile. Since there was a A→G transition at nucleotide 1,663 that caused the residue change at 555 (ATA:Ile→GTA:Val) (Table 2). The presence of Val at residue 555 in CPV type 2a has also been shown previously for Taiwanese, Indian, Japanese, Korean, US, German and Italian isolates (Appendix A Figure 1) and it was proposed that this variation may be the transitional stage from 2a to 2b. In addition, the CPV type 2a and type 2b might also have evolved independently of each other from the original CPV type 2 (Wang *et al*., 2005; Chinchkar *et al*., 2006; Kang *et al*., 2008).

The Indian isolates (IIL P19 type 2a and IIL P25 type 2b) were clustered together in the type 2b because both isolates had the amino acid substitution at residue 135 (Val→Ile) which was not found in other isolates of this study (Appendix A Figure 1). This amino acid substitution brought IIL P19 type 2a clustered with IIL P25 type 2b which isolated from the same country. Interestingly, Vietnamese isolate V139 type 2c(a) were clustered together with classical type 2a strains in this study. The Vietnamese isolate V139 type 2c(a) using in this dendrogram had Asp at residue 300. CPV Asp-300 variant was referred to as CPV2c(a) or CPV 2c(b) on the basis of the amino acid encountered at residue 426 (Asn or Asp) of the VP2 protein (Nakamura *et al*., 2004). The Glu-426 mutant was referred to as true antigenic variant CPV type 2c, whereas the Asp-300 mutant should be regarded as mutants of CPV type 2a and CPV type 2b (Ikeda *et al*., 1999; Kang *et al*., 2008). The presence of Asp at residue 300 has also been shown previously for Korean isolate Pome CPV type 2a variant (Kang *et al*., 2008) (Appendix A Figure 1). However, the Vietnamese isolate V139 does not have glutamate substitution at residue 426th which is the marker for

type 2c. Therefore, Vietnamese isolate V139 should be considered as type 2a rather than type 2c.

Jeoung *et al*. (2008) reported that there were unique mutations of VP2 gene at amino acid Gln7His, Ser297Ala, Ile324Tyr, Asp413Asn, Ile418Thr, Pro435Ser, Thr440Ala and Ile463Val in Korean isolates. According to this report, CPV 2a was subdivided into CPV 2a-I to CPV 2a-V subcluster and CPV 2b was subdivided into CPV 2b-I and CPV 2b-II subcluster, based on the amino acid substitution of the VP2 gene (Moon *et al*., 2008). When comparing the CPV 2a of Thai isolates with Korean isolates, the Thai isolates could be placed in subcluster CPV 2a-V of Korea because they had amino acid substitution at position Ser297Ala and Ile324Tyr (Table 2). The CPV 2a-V was also found in other countries including Italy, Vietnam, Taiwan and USA (Chang *et al*. 1996; Ikeda *et al*., 2000; Shackelton *et al*., 2005; Pereira *et al*., 2007). CPV type 2b of Thai isolates can be placed in subcluster 2b-I of Korean isolates because of amino acid substitution at position Ser297Ala (Table 2). The amino acid substitution at position 297 (Ser→Ala) was consistently found as previously reports (Truten *et al*., 2000; Battilani *et al*., 2001). This mutation has been seen in both CPV type-2a and -2b in various parts of the world including Asia (Ikeda *et al*., 2000; Nakamura *et al*., 2004; Wang *et al*., 2005; Chinchkar *et al*., 2006; Doki *et al*., 2006; Jeoung *et al*., 2008; Kang *et al*., 2008), Europe (Buonavoglia *et al*., 2001; Battilani *et al*., 2001, 2002; Truyen *et al*., 1996, 2000; Truyen, 1999, 2006) and America (Parrish *et al*., 1991; Kapil *et al*., 2007; Perez *et al*., 2007) (Appendix A Figure 1).

2. Expression of VP2 gene

The 1,700 bps PCR product of VP2 gene was successfully cloned in plasmid pBAD202/D-TOPO® and sequenced. According to the pBAD202/D-TOPO® expression system, the concentration of arabinose and the induction time can influence the amount of the expression of recombinant protein. In this experiment, the optimum concentration of arabinose and the optimum induction time were 0.002% and 8 hours, respectively. The molecular weight of VP2 protein was calculated by

summation of its amino acids (Adams, 2003) which was 65 kDa. The calculated molecular weight of recombinant VP2 protein expressed using recombinant *E. coli* was 80 kDa due to the extra molecular weights of thioredoxin and 6xhistidine included in the plasmid pBAD 202/D-TOPO. By SDS-PAGE, the recombinant VP2 produced from recombinant *E. coli* showed prominent bands at 80 kDa. By Western blot analysis, when we detected the total and purified recombinant VP2 protein with the rabbit anti-CPV hyperimmune serum we also found 80 kDa band (Figure 10B and 11B). The result in this study differed from the previous report that they found other bands lower than the size of VP2 protein (Park *et al*, 2007). These bands could be cross reacted *E. coli* protein or proteolytic cleavage or degradation by host protease or the VP2 may contain sequences which cause premature degradation of the gene products in *E.coli* (Paradiso *et al*., 1982, 1984; Tullis *et al*., 1992; Park *et al*, 2007). However, these proved that the recombinant VP2 protein had epitopes that specifically reacted with rabbit anti-CPV hyperimmune serum.

From the problem of proteolytic cleavage and degradation, the eukaryotic expression system is another way to improve efficiency of recombinant VP2 protein for subunit vaccine production instead of bacterial system. Saliki *et al*. (1992) and Turiso *et al*. (1992) also used the baculovirus expression system to produce CPV VP2 protein in insect cells for use as a potential vaccine antigen. They found that expressed VP2 protein reacted with anti-CPV monoclonal antibodies at the same size in CPV-infected cells about 65 kDa and had only one band on the Western blot analysis.

3. Animal immunization

In the current study, rabbits were selected as a model to test ability of recombinant VP2 to induce antibody response. The rabbits were divided into groups and each group contained at least three rabbits to reduce error from variation of individual (Halow and Lane, 1988). A wide dose range of antigen per does (50-1000 µg/animal) for rabbits was recommended by Halow and Lane (1988). In this study, five rabbits in each group were immunized with two dosage concentrations of 300

μg/dose for group B and 500 μg/dose for group C produced antibodies to recombinant VP2 protein. The ability of recombinant VP2 protein for the induction of antibody was investigated. The reaction intensity of serum between group B and group C was not significantly different when statistical analysis using ANOVA. However, they had been significantly different from those of the control group since the second week of the trial. The 300μg/dose (in group B) and 500 μg/dose (in group C) gave the same antibody response may due to these two doses were in the same range of antigen to stimulate the same level of antibody. The study of Turiso *et al*. (1992) found that a dose of 10 μg of VP2 with Quil A and alumina was able to elicit a good protective response in dogs. In addition, Zeng *et al*. (2008) reported that 50 μg purified VP2/dose produced high titers of specific antibodies responses that were elicited after injection in rabbits.

Compared to recombinant VP2 protein and commercial modified live CPV vaccine, at week 5 the commercial CPV vaccine caused threefold higher CPV specific antibodies response than recombinant VP2 protein. In the previous study that compared between inactivated vaccine and recombinant VP2 protein, they found that at week 5 the commercial vaccine had twofold higher CPV specific antibodies response than recombinant VP2 protein (Zeng *et al*., 2008). From these results, although the subunit vaccine induces low immune response when compared with the conventional virus vaccine. Nevertheless, subunit vaccine can eliminate risks associated with the production, risks for contamination with toxic compounds, risks of reversion to virulent genetypes or incomplete inactivation of whole-cell vaccines (Liljevist and Stahl, 1999). In addition, subunit vaccine can give high doses to pass maternal immunity in puppy which live attenuated vaccine cannot since it may cause disease in animal.

From these results, we not only know that vary doses of protein antigen which may necessary to produce antibody response, but also depends on other factors such as correct folding of antigen, efficiency of adjuvant and type of host.

The choice of adjuvant is also crucial in vaccine development. Since subunit vaccine was less immunogenic than traditional vaccine (Tizard, 2000; Aucouturier *et al*., 2001; Hagan *et al*., 2001). The effectiveness of subunit vaccines is increased by giving them in adjuvant. It is necessary to selected adjuvant for animal species as well as economic cost (Singh and Hogan, 2003). However, the potential may be increase or the dose may be reduced when the protein used with appropriated adjuvant. They may be mineral oil emulsion or aluminum hydroxide with additional compounds for immunopotentiation. In present experiment, Dilavia (Interrvet) adjuvant was selected and it can help promote antibody response in rabbits. Turiso *et al*. (1992) found that the highest antibody titers of dogs were obtained when Quil A alone or in combination with Alumina included with low dose of VP2 antigen. These similar to another study of Langeveld *et al*. (1993) reported that aluminum hydroxide/Quil A was shown to be very effective for inducing neutralizing anti-CPV antibodies in dogs.

Each production host offers numerous advantages for the recombinant antigen to be produced, but there are also limitations, which have to be considered when choosing a host. *E.coli* has been extensively studied as production host for heterologous proteins. Since it is very well characters. However, one potential drawback with prokaryotes as production hosts is that they are unable to carry out post-translational modification. The protein loss or modification during expression in *E.coli* resembles the structural shift described in previous reports that parvoviral capsids showed a tendency to be cleaved into a number of fragments in vivo infection (Paradiso *et al*., 1982, 1984; Tullis *et al*., 1992). Therefore, yeast, baculovirus and plant expression system are other ways to produce protein antigen correctly conformation and same real pathogen (Langeveld *et al*., 2001; Gilbert *et al*., 2005; Guppa *et al.*, 2005).

CONCLUSION

Two types of CPV, CPV type 2a variant and CPV type 2b, were identified from 26 fecal samples examined in this study. All CPV-2a isolates had substitution of Val at residue 555, which has been previously reported in Taiwan, India and Korea (Wang *et al*., 2005; Chinchkar *et al*., 2006; Kang *et al*., 2008). Our results provide additional data on CPV isolates which follow the same evolution as observed in other Asian countries except Vietnam. Since there is no indication of separate lineage, monitoring of further isolates to detect genetic and antigenic changes for CPV in Thailand will be needed.

The VP2 gene of CPV was cloned into plasmid pBAD202/D-TOPO. A recombinant VP2 protein was successfully expressed in *E. coli* expression system. The optimum concentration of arabinose and induction period for the expression of recombinant VP2 protein was 0.002% and 8 hours. The recombinant VP2 protein at 80 kDa band interacted specifically with rabbit anti-CPV polyclonal antibodies and mouse anti-histidine IgG monoclonal antibody.

The recombinant VP2 protein at doses 300 μg/injection and 500 μg/injection was capable to stimulate antibodies response which could detect by ELISA technique. It indicated that recombinant VP2 expressed from TOPVP2 has a potential to be a subunit vaccine. There was no difference between the two treatments groups. However, the commercial modified live vaccine induced much higher titer than the recombinant VP2 protein. Therefore, it requires further time to improve its efficacy.

Base on these results, our studies had established a procedure to produce immunogenic recombinant VP2 protein of CPV using prokaryotic expression system. The recombinant protein could be considered an interesting alternative for potential production of a subunit vaccine against CPV.

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APPENDICES

Appendix A

Chemical reagents and substances

Appendix A

Chemical Reagents and Substances

1. Buffers for Protein Purification (Qiagen)

Lysis buffer B (1 litre):

100 mM NaH_2PO_4; 13.8 g NaH_2PO_4 H_2O (MW 137.99 g/mol)

10 mM Tris Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 8.0 using NaOH

Washing buffer C (1 litre):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris-Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 6.3 using HCI.

Elution buffer D (1 litre):

100 mM NaH_2PO_4;13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris.Cl; 1.2 g Tris base (MW 121.1 g/mol)

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 5.9 using HCl.

Elution buffer E (1 litre):

100 mM NaH_2PO_4; 13.8 g NaH_2PO_4 H_20 (MW 137.99 g/mol)

10 mM Tris-Cl 1.2 g Tris base (MW 121.1 g/mol

8 M urea; 480.5 g (MW 60.06 g/mol)

Adjust pH to 4.5 using HCl.

Remark: The pH of buffer should be adjusted immediately prior to use. Do not autoclave.

2. 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

3. lmmunoblotting 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

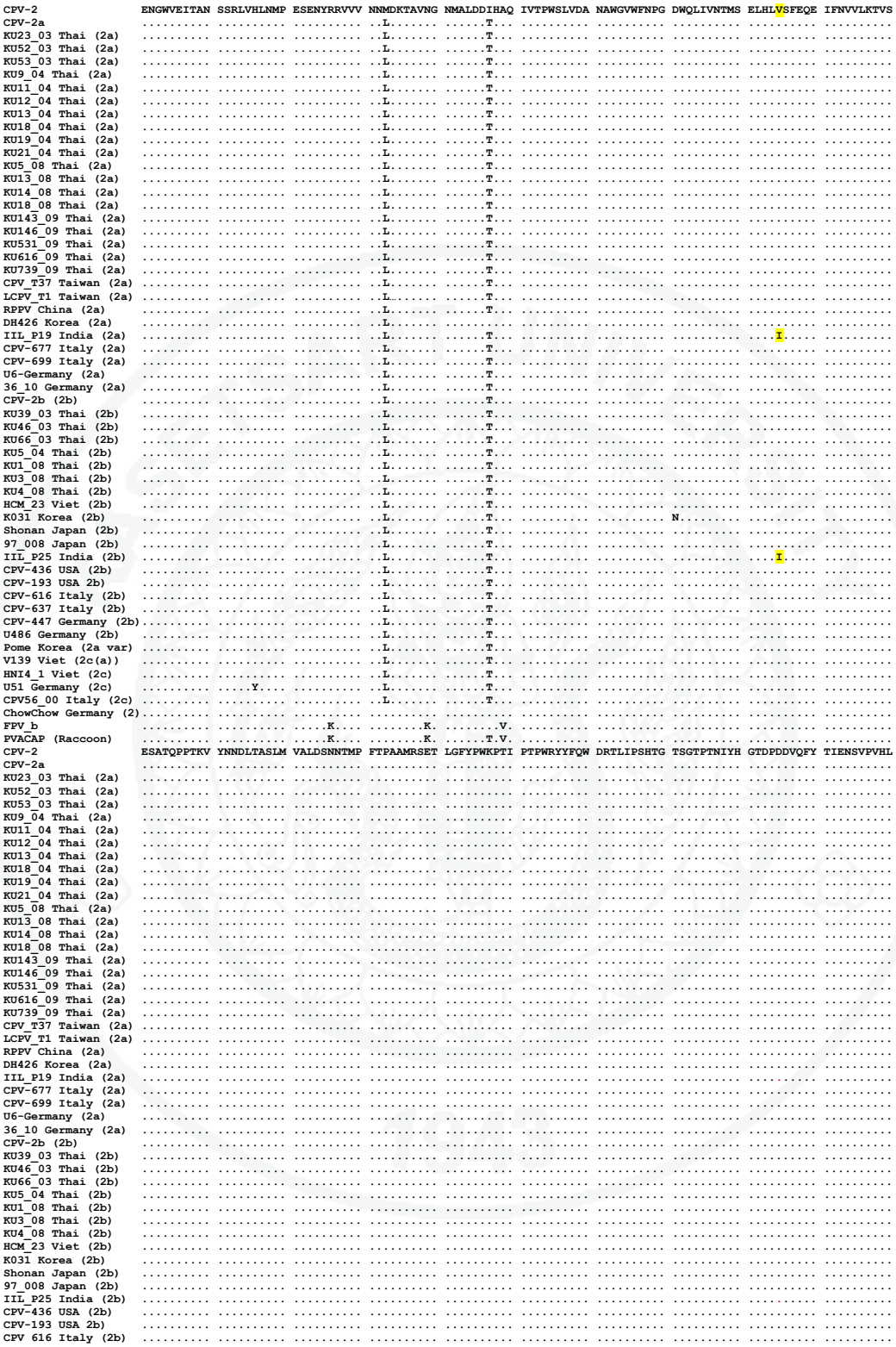
990 μl of sterilize water

- Transfer buffer (1 litre)

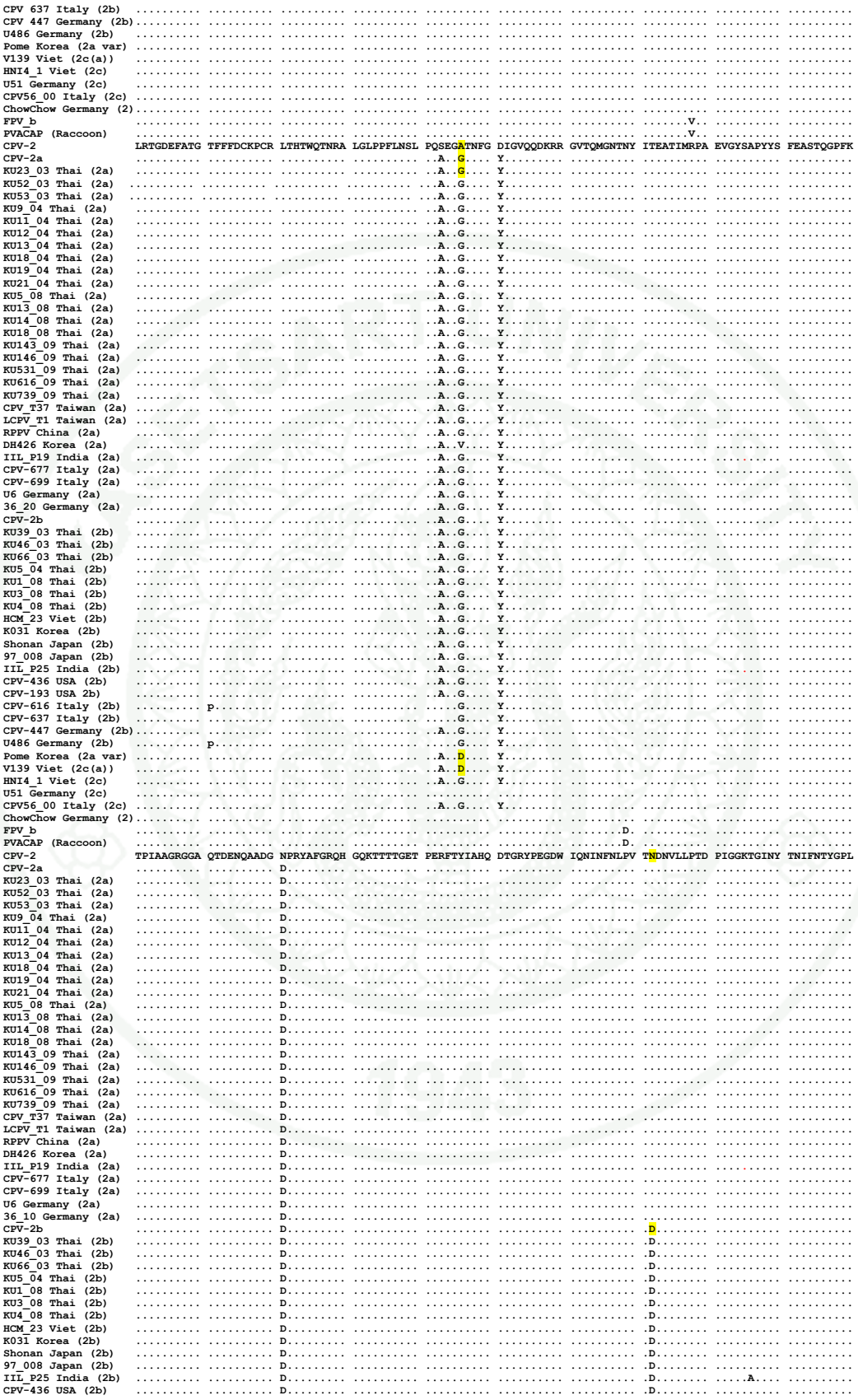
25mM Tris; 3 g Tris base

190mM glycine; 14.4 g glycine

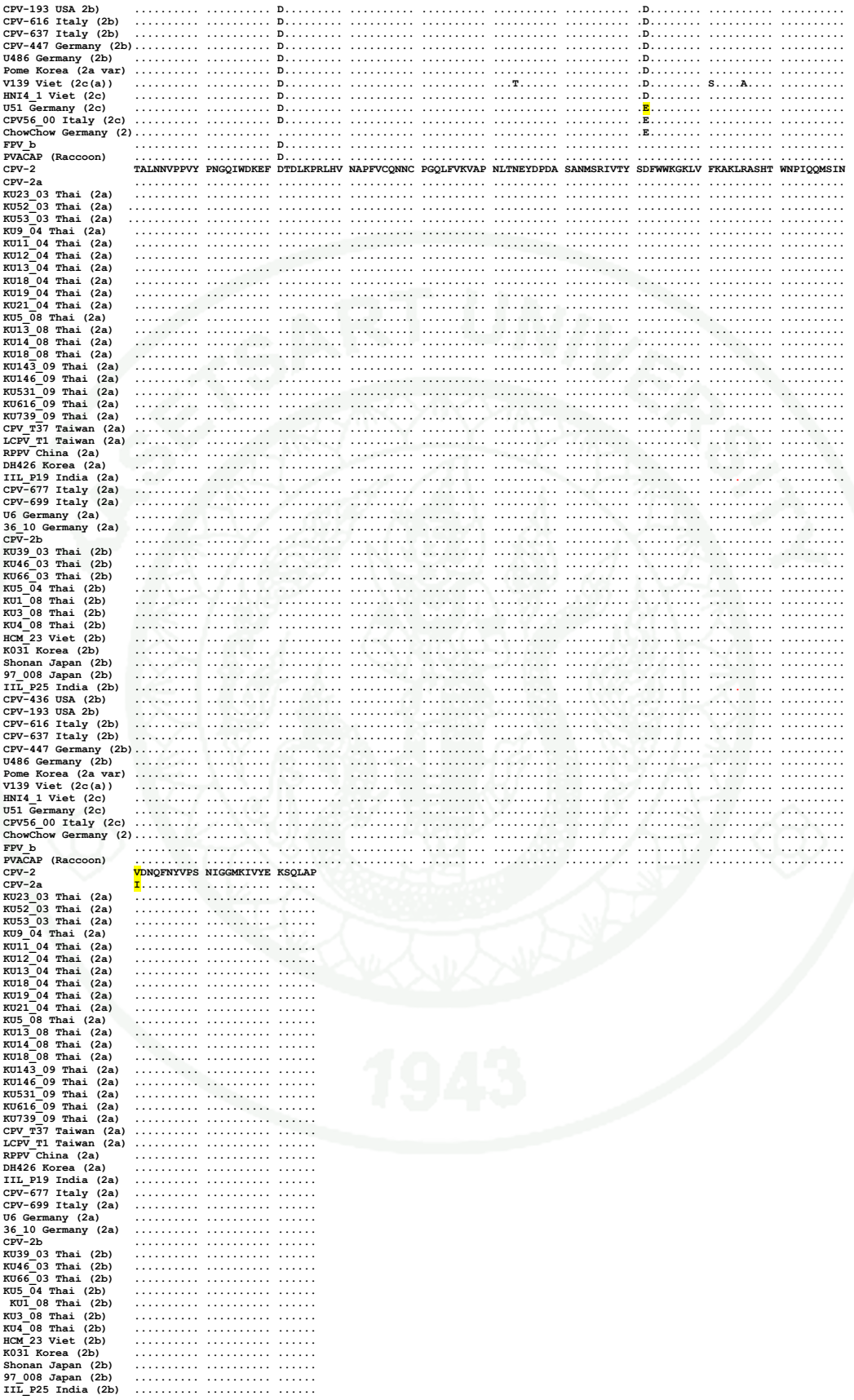
20% methanol ; 200 ml conc. methanol (water adjust to 1,000 ml)



Appendix Figure A1 Amino acid alignment of a VP2 fragment of representative of Thai isolates CPV-2a and CPV-2b with reference strains CPV-2 (M23255), CPV-2a (M24003) and CPV-2b (M74849) and other country isolates.



Appendix Figure A1 (Continued)



Appendix Figure A1 (Continued)

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CPV-436 USA (2b)     .......... .......... ......
CPV-193 USA 2b)      .......... .......... ......
CPV-616 Italy (2b)   .......... .......... ......
CPV-637 Italy (2b)   .......... .......... ......
CPV-447 Germany (2b).......... .......... ......
U486 Germany (2b)    .......... .......... ......
Pome Korea (2a var)  .......... .......... ......
V139 Viet (2c(a))    .......... .......... ......
HNI4_1 Viet (2c)     .......... .......... ......
U51 Germany (2c)     .......... .......... ......
CPV56_00 Italy (2c)  .......... .......... ......
ChowChow Germany (2).......... .......... ......
FPV_b                .......... .......... ......
PVACAP (Raccoon)     .......... .......... ......
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Appendix Figure A1 (Continued)

Appendix B

The standard methods

Appendix B

The standard methods

1. Preparation of *E. coli* competent cells for transformation

1.1 Culture *E. coli* strain DH5α cells on LB agar plate at 37 °C overnight.

1.2 Pick up a large colony and culture in 1 ml of LB broth at 37°C overnight with vigorous shaking (~ 250 rpm).

1.3 From 500 µl of overnight culture, subculture to 100 ml of SOB medium containing 25 µg/ml kanamycin, incubate at 37°C until OD_{600} is 0.4 - 0.8 (approximately 3 - 4 hrs).

1.4 Store the culture on ice for 10 minutes.

1.5 Centrifuge at 4 °C, for 10 minutes at 3,000 rpm, discard the supernatant.

1.6 Gently resuspend the pellet in 33 ml of ice-cold TB and store on ice for additional 10 minutes.

1.7 Centrifuge at 4°C, for 10 minutes at 3,000 rpm, discard the supernatant.

1.8 Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 ul).

1.9 Aliquot the cell to eppendorf tube each 200 µl and store at -70°C until use for transformation.

2. QIA quick gel extraction kit protocol

2.1 Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2.2 Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg/100 µl).

2.3 Incubate at 50°C for 10 min. To help dissolve gel, mix by vortexing the tube every 2-3 minutes during incubation.

2.4 After the gel slice has dissolved completely, check that color of the mixture is yellow.

2.5 Add 1 gel volume of isopropanol to the sample and mix.

2.6 Place a QIAquick spin column in a provided 2 ml collection tube.

2.7 Apply the sample to the QIAquick column, and centrifuge for 1 minute.

2.8 Discard flow-through and place QIAquick column back in the same collection tube.

2.9 Add 0.75 ml of PE buffer to QIAquick colume and centrifuge for 1 minute.

2.10 Discard flow-through and place QIAquick column an additional 1 minute at 13,000 rpm.

2.11 Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

2.12 To elute DNA, add 50 ul of EB buffer (10 mM Tris-HCl, pH 8.5) to the center of QIAquick column membrane, let the column stand for 1 minute, and centrifuge for 1 minute.

3. Phenol-Chloroform extraction of RNA and ethanol precipitation (Sambrook and Russell, 2001)

3.1 RNA was extracted from 100 µl of viral stock that mixed with 500 µl of denature solution and 50 µl of 2M NaAc, was shaken for 5-10 minutes.

3.2 Add RNA phenol 150 µl and chloroform 150 µl was shaken for 5 minutes.

3.3 Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.

3.4 Remove about 90% 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 a second time with same procedure.

3.5 Repeat 2-4 again.

3.6 Remove about 90% of the upper, aqueous layer to a clean tube. Add isopropanol 550 µl and 0.5 µl of glycogen (20ng/ml), invert gently up side down and keep in – 80°C for 40 minutes.

3.7 Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.

3.8 To wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Prepare the gel by the recipe as follow for 2 gels

- Seperating 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 TrisHCI/SDS pH 6.8	1.25 ml
30% Acryl/0.8%bis-Acrylamide	0.65 ml
10% APS	25 µl
TEMED	5 µl

4.1 If not already in electrophoresis sample buffer, add an equal volume of 2×sample buffer to all samples and boil for 5 minutes.

4.2 Apply 20-25 µl (1 - 10µg total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel.

4.3 Run electrophoresis (100 volt, 70 minutes, constant ampere).

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