การเตรียมเซลล์ไฮบริโดมาเพื่อใช้ในการผลิตโมโนโคลนอลแอนติบอดีต่อไวรัสพีอีดี

นางสาวปานวาด ฤทธิสาร

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PREPARATION OF HYBRIDOMA CELLS FOR PORCINE EPIDEMIC DIARRHEA VIRUS MONOCLONAL ANTIBODY PRODUCTION

Miss Panwad Ritthisan

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	PREPARATION OF HYBRIDOMA CELLS FOR PORCINE EPIDEMIC DIARRHEA VIRUS MONOCLONAL ANTIBODY PRODUCTION
By	Miss Panwad Ritthisan
Field of Study	Veterinary Biosciences
Thesis Advisor	Associate Professor Meena Sarikaputi, Ph.D.
Thesis Co-Advisor	Prapruddee Piyaviriyakul, Ph.D.
	Assistant Professor Tanong Asawakarn, Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Assistant Professor Sirakarnt Dhitavat, Ph.D.)

.....Thesis Advisor

(Associate Professor Meena Sarikaputi, Ph.D.)

.....Thesis Co-Advisor

(Prapruddee Piyaviriyakul, Ph.D.)

.....Thesis Co-Advisor

(Assistant Professor Tanong Asawakarn, Ph.D.)

.....Examiner

(Suphot Wattanaphansak, Ph.D.)

.....External Examiner

(Assistant Professor Suang Rungpragayphan, Ph.D.)

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โรคพีอีดีเกิดจากเชื้อไวรัสพีอีดี ซึ่งเป็นโรคในระบบทางเดินอาหาร ลักษณะอาการของโรคคือ ท้องเสีย อาเจียน น้ำหนักลด มีการสูญเสียน้ำ และมีอัตราการตายสูงถึงร้อยละ 100 ในลูกสุกร ลักษณะอาการของโรคพีอีดีไม่สามารถจำแนกจากโรคทีจีอีได้ ดังนั้นการวินิจฉัยจึงจำเป็นต้องนำตัวอย่างส่งห้องปฏิบัติการ เพื่อตรวจหาโรคพีอีดี ซึ่งกระทำได้หลายวิธี เช่น ELISA,ปฏิกิริยาลูกโซ่โพลิเมอเรส ซึ่งเป็นวิธีที่ใช้ระยะเวลาในการตรวจนานและมีค่าใช้จ่ายสูง อีกทั้งเกษตรกรไม่สามารถทำการตรวจได้ด้วยตนเอง วัตถุประสงค์ของการศึกษาครั้งนี้ เป็นการเตรียมเซลล์ไฮบริโดมาเพื่อผลิตโมโนโคลนอล แอนติบอดี เพื่อใช้การตรวจวินิจฉัยโรคพีอีดี โดยนำหนู BALB/c จำนวนสองตัวมาฉีดด้วยไวรัสพีอีดี สายพันธุ์ K9 ในขนาด 50 ไมโครกรัมต่อตัว โดยฉีดบริเวณใต้ผิวหนังเป็นเวลาสามครั้ง ห่างกันครั้งละสามสัปดาห์ และครั้งสุดท้ายฉีดเข้าเส้นเลือดของหนู 3 วันก่อนทำการรวมเซลล์จากม้ามหนู และเซลล์ไมอิโลมาเข้าด้วยกันด้วย 50 เปอร์เซ็นต์ โพลีเอทิลีนไกลคอล ทำการคัดเลือกเฉพาะเซลล์ไฮบริโดมา โดยการเลี้ยงในอาหารเลี้ยงเซลล์ที่ประกอบด้วยไฮโปแซนธีน อะมิโนเทอรินและไธมิดีน หลังจากนั้นนำเซลล์ไฮบริโดมาที่มีความสามารถสร้างแอนติบอดีมาคัดแยกเพื่อให้ได้เป็น โมโนโคลนด้วยวิธี limiting dilution และทำการตรวจหาชนิดของอิมมูโนโกลบูลินที่สร้างได้ ในงานวิจัยครั้งนี้ สามารถเตรียมเซลล์ไฮบริโดมาได้ทั้งหมด 4 โคลน ซึ่งผลิตโมโนโคลนอลแอนติบอดีต่อไวรัสพีอีดีได้ 3 ชนิด ได้แก่ IgG1 จำนวน 2 โคลน, IgG2a และ IgG2b ชนิดละ 1 โคลน

ภาควิชา กายวิภาคศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา ชีวศาสตร์ทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: HYBRIDOMA CELL / MYELOMA CELL / MONOCLONAL ANTIBODY / PORCINE EPIDEMIC DIARRHEA VIRUS

PANWAD RITTHISAN: PREPARATION OF HYBRIDOMA CELLS FOR PORCINE EPIDEMIC DIARRHEA VIRUS MONOCLONAL ANTIBODY PRODUCTION. ADVISOR: ASSOC. PROF. MEENA SARIKAPUTI, Ph.D., CO-ADVISOR: PRAPRUDDEE PIYAVIRIYAKUL, Ph.D., ASST. PROF. TANONG ASAWAKARN, Ph.D., 71 pp.

Porcine epidemic diarrhea (PED), caused by Porcine epidemic diarrhea virus (PEDV) is an enteric disease characterized by watery diarrhea, vomiting, weight loss, dehydration, resulting in 100% mortality in suckling pigs . PED cannot be clinically distinguished from Transmissible gastroenteritis (TGE), therefore the laboratory diagnosis is necessary to identify PED. However, laboratory techniques are expensive, time-consuming and cannot be carried out in the farm. The objective of this study is to prepare hybridoma cells for monoclonal antibody production against Porcine epidemic diarrhea virus. Two BALB/c mice were immunized subcutaneously 3 times at 3 week-intervals with 50 µg purified PEDV strain K-9 per mouse. Finally, 3 days before fusion, the mice were intraveneously injected with purified PEDV. Hybridoma cells were obtained by fusion between X63Ag 8.653 myeloma cells and B- cells from mouse using 50% polyethylene glycol. Hybridoma cells are cultured in HAT selective medium and supernatant are then tested for antibodies against PEDV using ELISA. The hybridoma cells showing PED specific antibody production were subsequently subjected to limiting dilution in order to obtain monoclone. In this study,Four monoclonal antibodies against PEDV strain K-9 were produced with subclass of IgG1, IgG2a and IgG2b.

Department:	Veterinary Anatomy	Student's Signature	.....
Field of Study:	Veterinary Biosciences	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ABTS	2, 2’ diazino bis (3 ethyl) benz-thiazoline-6 sulfonic acid
BSA	bovine serum albumin
Bp	base pair
˚C	degree Celsius
E	envelope protein
ELISA	enzyme-linked immunosorbant assay
EM	electron microscopy
Fab	fragment antigen-binding
FBS	fetal bovine serum
Fc	fragment crystallizable region
HAT	hypoxanthine, aminopterin and thymidine
HGPRT	hypoxanthine guanine phosphoribosyl transferase
IFA	immunofluorescence antibody
IFT	immunofluorescence test
Ig	immunoglobulin
IHC	immunohistochemistry
ISH	*in situ* hybridization
Kb	kilo base
kDa	kilo Dalton
M	glycosylated membrane protein
MAbs	monoclonal antibodies
N	unglycosylated RNA-binding nucleocapsid protein
ORFs	open reading frames
PAbs	polyclonal antibodies
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PED	porcine epidemic diarrhea
PEDV	porcine epidemic diarrhea virus
PEG	polyethylene glycol
PRCV	porcine respiratory coronavirus
RT-PCR	reverse transcriptase polymerase chain reaction
S	spike protein
SAB	streptavidin-biotin
TGEV	transmissible gastroenteritis coronavirus
TK	thymidine kinase

Chapter I
Introduction

1.1 Importance and rationale

The swine industries are important for economy worldwide because the consumption has been much higher. However, gastrointestinal tract disease is one of the most important causes of economic loss. Porcine epidemic diarrhea (PED) is an enteric disease characterized by watery diarrhea, vomiting, weight loss, dehydration and metabolic acidosis (Kim and Chae, 2003), caused by Porcine Epidemic Diarrhea virus (PEDV). The infection of PEDV affects all age of pigs, PED caused high morbidity but low mortality in grower pigs (Olanratmanee et al., 2010). Transmission of PEDV is more by Fecal-oral transmission and contaminated equipments or feed. PEDV is a member of the Coronaviridae family (Callebaut et al., 1982) that also includes other swine viruses such as Transmissible Gastroenteritis Coronavirus (TGEV), Porcine Respiratory Coronavirus (PRCV). It is a RNA virus, positive-sense, enveloped, linear single stranded RNA virus and ranged in diameter from 70 to 190 nm (average 100 nm). Although it was first reported in England in 1971 (Wood, 1977), subsequently isolated in Hungary, Germany, Canada, it has become increasingly problematic in Asia such as China, Korea, Japan and Thailand (Pospischil A, 2002).In 1995, PEDV was first reported in Thailand (สนองศรี, 1995)

There are no specific treatments for PED. Symptomatic treatment for diarrhea is recommended. In addition, effective commercial PED vaccines are not available (Pospischil A, 2002). Therefore, good hygiene seems to be an only method to prevent a spreading of PED in the farm.

Basic clinical signs of PED are similar to those of Transmissible Gastroenteritis Coronavirus (TGEV), therefore the laboratory diagnosis is necessary for PED identification. Many techniques have been elucidated to detect of PEDV, such as enzyme-linked immunosorbant assays (ELISA) or Immunofluorescence antibody test (IFA), immunofluorescence test (IFT), immunohistochemistry (IHC) or polymerase

chain reaction (PCR) (Guscetti et al., 1998a). However, these techniques are expensive, time-consuming and cannot be carried out in the farm.

Monoclonal antibody (MAb) is an important component used in many diagnostic test kits, such as ELISA, Snap test and latex agglutination test. MAb is monospecific antibody produced by hybridoma cell obtained from fusion between plasma cells of immunized animal and myeloma cells. Plasma cell can produce specific antibody while, myeloma cell is immortal, therefore hybridoma cell can produce specific antibody and immortal. MAb has monovalent affinity and binds to specific epitope. The use of MAb can decrease an error caused by antigenic cross-reactivity or nonspecific pathogen (Kim et al., 1999).

1.2 Objective of study

To prepare monoclonal antibody from hybridoma cells against Porcine epidemic diarrhea virus.

1.3 Hypothesis

1. The hybridoma cells can be prepared for Porcine epidemic diarrhea virus.
2. Monoclonal antibodies from hybridoma cells can detect Porcine epidemic diarrhea virus.

1.4 Keywords

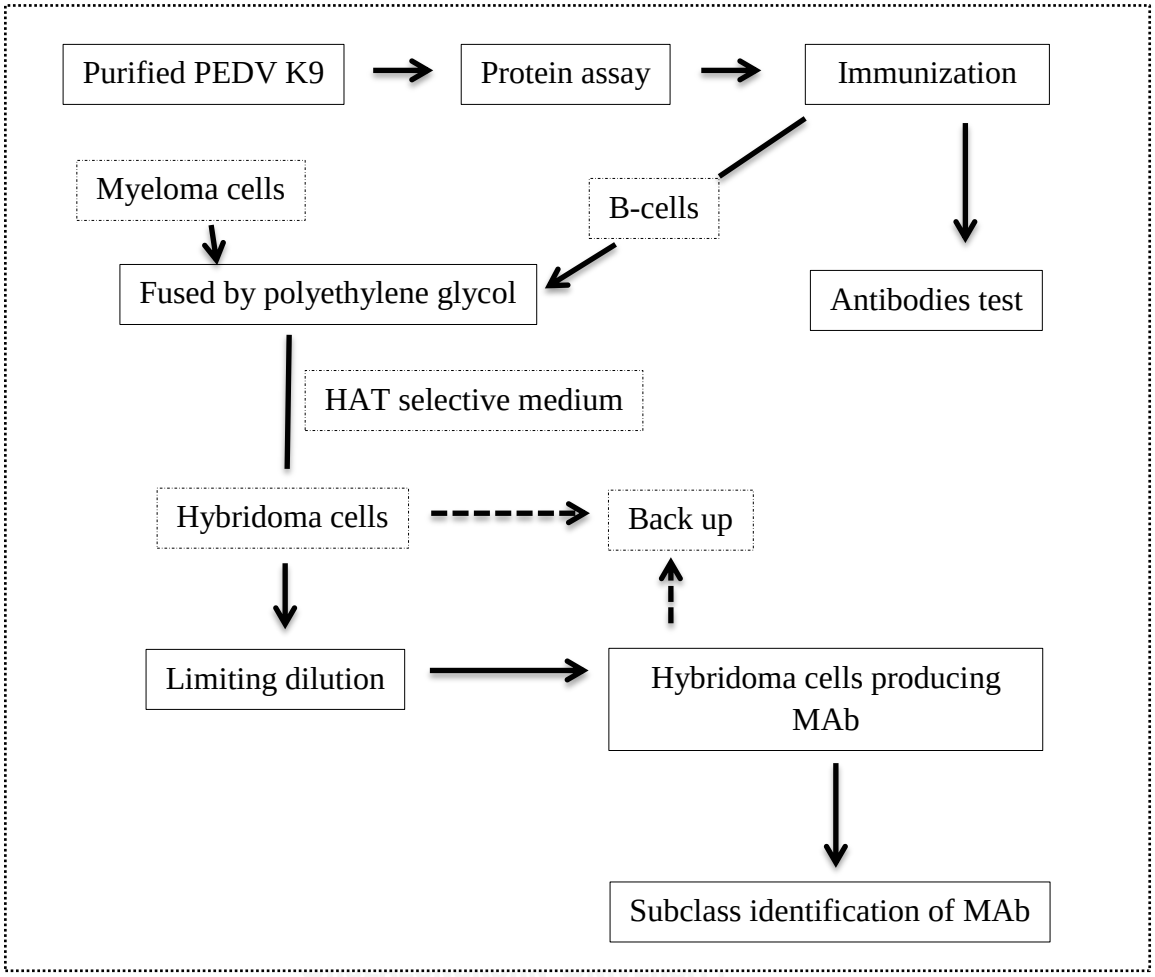
Hybridoma cell, Myeloma cell, Monoclonal antibody, PED Virus

1.5 Research merits

1. The hybridoma cells have specificity to detect Porcine epidemic diarrhea virus.
2. The Porcine epidemic diarrhea virus monoclonal antibodies can be applied for detection of PEDV antigen in swine.

1.6 Thesis framework

The experiment was performed as following diagram.



Chapter II

Literature review

2.1 Porcine epidemic diarrhea virus

2.1.1 Characteristics of PEDV

Porcine epidemic diarrhea (PED) is an enteric disease of swine caused by the porcine epidemic diarrhea virus (PEDV). The disease was clinical characterized by vomiting, severe enteritis, water diarrhea, dehydration. In suckling pig, the disease can be acute with high mortality of up to 100% while in growing pig, the disease is mild with high morbidity but low death loss, resulting in economic losses to the swine industry.

The PEDV is a member of the family Coronaviridae, subfamily Coronavirinae , order Nidovirales, genus Alphacoronavirus (Pensaert and De Bouck, 1978). It is a single-stranded RNA virus, positive-sense, enveloped, range in diameter from 70 to 190 nm (average 100 nm). The structure of PEDV gene is comprised of genes coded for glycosylated peplomer (S) protein, envelope (E) protein, glycosylated membrane (M) protein and unglycosylated RNA-binding nucleocapsid (N) protein (Chen et al., 2008). The virus is stable between pH 5.0 and 9.0 at 4˚C and between pH 6.5 and 7.5 at 37˚C.

The PEDV and the transmissible gastroenteritis virus (TGEV) belong to the group 1 of the family Coronaviridae as shown in Table1, caused an enteric disease that was similar and difficult to distinguish clinically. Both viruses cause destruction of villus within jejunum and ileum thus reducing the absorptive surface, with loss nutrients and electrolytes resulting in dehydration(Debouck and Pensaert, 1980). Specific laboratory diagnostic test is necessary to identify the viruses (Jung et al., 2003).

PEDV was first reported in the Europe in the early 1970 and was first isolated in 1977. The epizootic outbreaks are distributing in several Europe and Asia countries

including England, Hungary, Korea, Thailand and other countries (Figure 1)(Puranaveja et al., 2009). The PED outbreaks are ongoing to United stated in 2013 (Huang et al., 2013)

Table 1 Classification and diseases caused by Coronaviruses

Group	Virus member	Host	Disease
Group 1	- Canine coronavirus (CCV)	Dog	Enteritis
	- Feline enteric coronavirus (FECV)	Cat	Enteritis
	- Feline infectious peritonitis (FIPV)	Cat	Peritonitis
	- Human coronavirus 229E (HCV-229E)	Human	Respiratory disease
	-Porcine epidemic diarrhea virus (PEDV)	**Pig**	**Enteritis**
	-Transmissible gastroenteritis virus (TGEV)	Pig	Enteritis
	- Porcine respiratory coronavirus (PRCV)	Pig	Respiratory disease
Group 2	- Murine hepatitis virus (MHV)	Mouse	Hepatitis, enteritis, encephalomyelitis
	- Rat coronavirus (Rt CoV)	Rat	Respiratory disease
	- Sialodacryodenitis virus (SADV)	Rat	Respiratory disease
	- Bovine coronavirus (BCV)	Cattle	Enteritis
	- Human coronavirus OC43 (HCV-OC43)	Human	Respiratory disease
	-Porcine haemagglutinating encephalomyelitis virus (HEV)	Pig	Encephalomyelitis
Group 3	- Infectious bronchitis virus (IBV)	Chicken	Respiratory disease, nephritis
	- Turkey coronavirus (TCV)	Turkey	Enteritis

2.1.2 Location of outbreaks

Starting in 1971s, Porcine epidemic diarrhea was first described as a devastating enteric disease in feeder and growing pigs in United Kingdom (Wood, 1977). During the 1978s and 1990s, the PEDV was identified in Belgium (Pensaert and De Bouck, 1978), with a following results 14 percent of sow serum and 50 percent of fattening pigs were positive antibodies of PEDV. In 1990, PED was founded as the cause of watery diarrhea in Spain, the PEDV antibody was detected in 55 percent of breeding farm and on one farm the virus became enzootic in sows (Carvajal et al., 1995).In 1993, an acute outbreak of PED was distributed in Netherlands with an epidemic diarrhea severe persistent in fattening pigs and pregnant sows but absent in suckling pigs (Pijpers et al., 1993). In The Czech Republic, an acute outbreak of PED in old weaner was described by Smid and others (Smíd et al., 1992). In 1995, 92 fecal samples from nursing piglets on 19 farms in Hungary were positive for PEDV (Nagy et al., 1995). In Italy, an epidemic of diarrhea in all ages of pigs, was reported in 2006s (Martelli et al., 2008). However, outbreaks of PED in Europe are rare while outbreaks of PED in Asia are often more acute and high mortality of which resembles TGE outbreaks.

In Asia, the situation of PED developed differently. The disease was first reported in Japan as from 1983 (Takahashi et al., 1983). The mortality ranging from 30 to 100% in nursing piglets .The virus has subsequently been reported in Korea, China, Thailand, Philippines and Vietnam. PED in Korea was described in 2000 and widely prevalent in suckling pigs, much more similar to of TGE (Chae et al., 2000). In 2005, PEDV was isolated in China and caused acute outbreaks over 10 provinces in southern china and over 1,000,000 piglets died. 100% of piglets after birth was illness and 80%-100% was death (Jinghui and Yijing, 2005). In 1995, PEDV was first reported In Trung province of Thailand (สนองศรี, 1995) but the disease did not spread to other farm. During the late 2007, the several outbreaks of PED were reported throughout the country with 8 provinces in Thailand had positive antibodies of PEDV and 100% of newborn piglets were subsequently lost. Phylogenetic analysis of Thai PEDV strain

revealed a close relationship with Chinese strain (Puranaveja et al., 2009). In the United stated, the first PED identified in June 2013, starting from the state of Ohio where PEDV has rapidly spread throughout the country, resulting in high morality in nursing piglets from 17 states (Wang et al., 2014). In addition, USA PEDV strains showed similarity with Chinese strains.

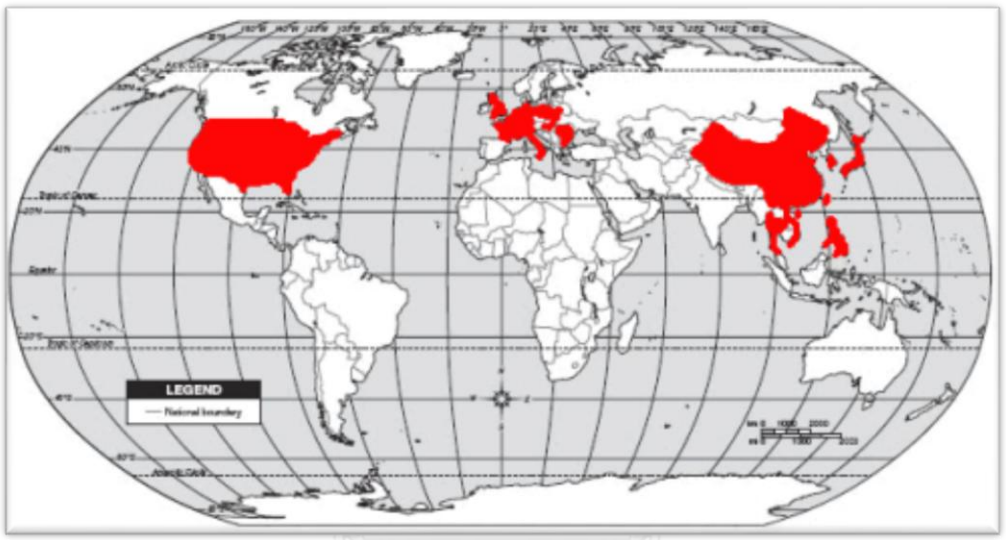


Figure 1 Location of PEDV outbreaks in several Europe and Asia countries including Korea, China, Thailand, Philippines, Vietnam and ongoing to United states.

2.1.3 Molecular and genetic

PEDV has linear plus-single stranded RNA genome with 5'cap and 3'polydenylate tail. The virus is enveloped virus, length of genome was found to be 28 kb (excluding the polydenylate tail). The structure of gene contained seven open reading frames (ORFs) that encode for four structural proteins including glycosylated peplomer (S) protein (150-220 kDa), envelope (E) protein (7 kDa), glycosylated membrane (M) protein (20-30 kDa) and unglycosylated RNA-binding nucleocapsid (N) protein (58 kDa) and three open reading frames (ORFs) for non- structural proteins such as ORF 1a, ORF 1b and ORF3, as shown in Figure 2 (Lee et al., 2010).

The polymerase gene is composed of 2 ORFs (ORF 1a and ORF 1b), the ORF 1a gene encodes for three proteases and one growth factor while the ORF 1b express one polymerase domain, one helicase domain and one meal ion binding domain (Kocherhans et al., 2001).

The ORF3 is located between S protein and E, M, N protein. It encodes an accessory proteins of which the sequences are different between other coronaviruses. The ORF3 is thought to be accounted for virulence.

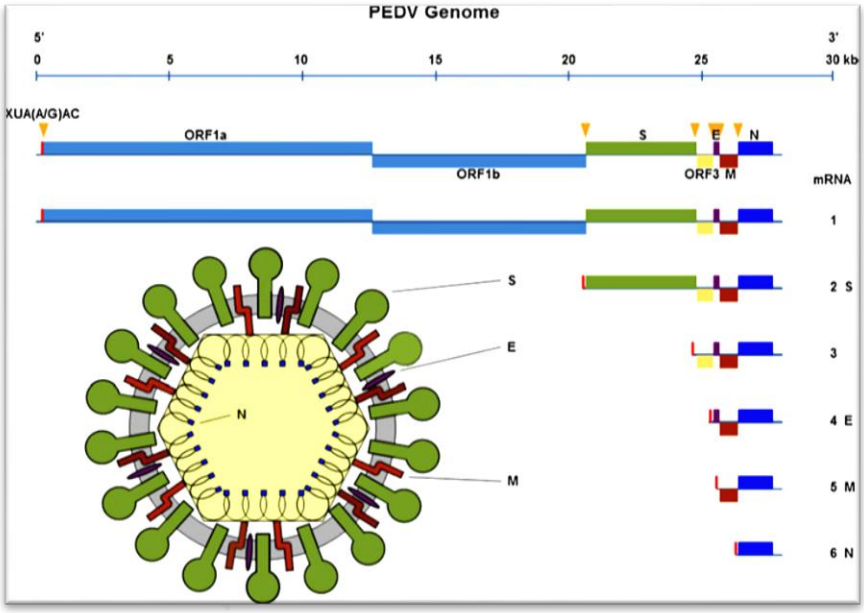


Figure 2 Structure of PEDV genome containing seven open reading frames (ORFs) which encode for four structural proteins (S, E, M and N protein) and 3 non-structural proteins (ORF 1a, ORF 1b and ORF3).

Spike protein or S protein consists of 1383 amino acid of 150-220 kDa. It belongs to type 1 of glycoprotein and is composed of signals peptide, neutralizing epitopes, transmembrane domain and short cytoplasmic domain. Spike protein is a protein on viral surface, interacting with host cell receptors and mediates a viral entry into host cell by membrane and associates with attenuation *in vivo* and adaptation *in vitro*. The S protein can be divided into N-terminal subunit (S1) and

membrane-anchored subunit (S2) domains (Bosch et al., 2003).The S protein of PEDV is comprised of S1 domain necessary for receptor-binding (Lee et al., 2011).

Unglycosylated RNA-binding nucleocapsid protein or N protein, 150-220 kDa, has a structure of helical nucleocapsid and genome associated with phosphoprotein. N protein is important for regulation of RNA synthesis, transcription, virus budding and may be induction of cell-mediated immunity.

Glycosylated membrane protein or M protein is a triple-spanning structure consisting of glycoprotein with a long carboxy-terminal domain (C-terminal) inside and a short amino-terminal domain (N-terminal) outside. M protein is necessary for viral assembly process. (Song and Park, 2012).

Envelope protein or E protein is a small protein linked to viral envelope and plays a role in virus assembly in a pre-Golgi compartment before progressing to the Golgi apparatus and release on the host cell (Xu et al., 2013).

2.1.4 Phylogenetic

Phylogenetic analyses based on the S genes, M genes and ORF3 genes have been used to classify PEDV.

PEDV can be divided into 3 groups base on phylogenetic analysis of partial S genes, G1, G2 and G3. PEDV in group G1 can be further classified into 4 subgroups, G1-1, G1-2, G1-3 and G1-4. As shown in Table 2.(Temeeyasen et al., 2014).

Phylogenic analysis of complete M gene can result in separation of PEDV into 3 groups, G1, G2 and G3. (Puranaveja et al., 2009) while the analysis of ORF3 genes generated 3 groups of PEDV which are G1, G2 and G3, as shown in Table 2 (Temeeyasen et al., 2014)

Table 2 Phylogenetic analyses based on the S genes, M genes and ORF3 genes

Group/ Gene	S	M	ORF3
G1	Chinese strain, Korea strain, Thai strain, Europe strain	Thai strain, Chinese strain	Thai strain , Chinese strain Korean strains
G2	Korea strain	Korean strains, Japanese strain, Chinese strain ,Thai strain	Chinese strain, Korea strain, Belgium strain
G3	Korea strain	Russian strain, Chinese strain	Chinese strain

2.1.5 Transmission

The spread of PED can be achieved by oral-foecal transmission. Clinical signs of can be observed on day 4-5 after being infected. The virus can also contaminate persons, fomites and equipment. After PED outbreak, PEDV may disappear or become enzootic in farms (Pospischil A, 2002).

2.1.6 Zoonotic Potential

PEDV only causes disease in swine and has not affected the meat or meat products. PED is not transmissible to human and other species. Antibodies to the virus have not been found in other animal species (Pospischil A, 2002).

2.1.7 Clinical presentation

Clinical presentations caused by PEDV is highly vary and also dependent on previous exposure, immunological and outbreaks status of the farm. The principle

clinical signs are watery diarrhea which may be flocculent, vomiting, severe enteritis, weight loss, dehydration and metabolic acidosis. Clinical signs of PEDV are similar in all ages of pigs infected, starting as early as within 12-24 hours after infection or 2-4 days at the herd level.

In suckling pigs, PED can result in 80-100% death loss. The high mortality in piglets might be due to 1) intestinal villi of suckling pigs are longer than older pigs, more surface of enterocytes were infected of PEDV. 2) replacement rate of enterocytes are slowly compared to older pigs 3) the colon of suckling pigs have less ability to reabsorb water, nutritions and electrolytes than older pigs.

Clinical sign of PED are similar to TGE, however, PED may spread more slowly than TGE and recover within 7-10 days(Pospischil et al., 2002).

2.1.8 Pathology

Infected of enterocytes of small intestine can be observed as early as 12 to 18 hours post inoculation and maximum reached within 24 and 36 hours post inoculation. The stomachs are empty because vomiting. The intestines are light yellow water-like content and thin walled.

After virus infected enterocytes, the virus was expresses viral protein in enterocytes and increased replication of mRNA within 12–24 hours post inoculation. PEDV cause a release of epithelial cells from the intestinal villus together with billions of virus particles to infect more cells (Pensaert and Callebaut, 1994). The virus damages enterocytes, resulting in decrease of absorptive surface. The damage of intestinal surface leads to loss of nutrients and electrolytes resulting in watery diarrhea, dehydration and death (Kim and Chae, 2003).

2.1.9 Diagnosis

Due to similarity to TGE, PED cannot be diagnosed by clinical signs alone. Many laboratory tests have been used to identify PEDV including immunohistochemistry (IHC), immunofluorescence test (IFT), electron microscopy (EM), indirect fluorescent antibodies (IFA), reverse transcriptase polymerase chain

reaction (RT-PCR) and enzyme-linked immunosorbant assay (ELISA) (Pospischil et al., 2002).

However, These techniques are time-consuming, expensive, low sensitivity, low specificity and limited to be used only in laboratory (Song and Park, 2012).

2.1.10 Treatment and prevention

The specific treatments of PED are not available. Good hygienes are recommended for PED prevention.

PEDV is stable at pH 5.0 and 9.0 at 4˚C and between pH 6.5 to 7.5 at 37˚C. PEDV loses infectivity when heated to 60˚C for 30 minutes. The virus can survive in fresh feces at 4˚C for 1 week, in dry feces at 25˚C for 1 week, wet feed at 25˚C for 4 week, in slurry at 25˚C for 2 week and drinking water at 25˚C for 1 week.

PEDV is inactivated by chloroform, sodium carbonate (4% anhydrous or 10% crystalline), 2% sodium hydroxide, 1% formalin, phosphoric acid, ionic detergents and non-ionic detergents (Pospischil et al., 2002).

2.1.11 Vaccine

Maternal antibodies via colostrum can help suckling pigs in protection of PEDV until 13 days old. The time protect of immunity depends on titer of mother(Song and Park, 2012).

Several PED vaccines are currently available in South Korea, Japan and China. Types of vaccines are killed or attenuated. At present, the use of vaccine is not quite popular among swine industry, due to the PEDV strain is different from wild type PEDV. The failure of vaccine can also be proven in secondary outbreaks.

2.2 Methods for diagnosis of PED

2.2.1 ELISA for detection of antibodies against PEDV

The detection of PEDV by ELISA was first reported by Hofmann and Wyler, 1990. ELISA is commercially available in Asia and Europe, however it was found with low sensitivity at detection of infected pig in the USA. The ELISA has some disadvantage due to (1) PEDV N protein is conserved and mostly used to coat ELISA plate, However it becomes more variable at longer period of infected time while S protein can provide higher sensitivity and specificity M protein is cross-reacted with other virus (Knuchel et al., 1992). (2) Sensitivity and specificity of ELISA depends upon quality of antibodies. Monoclonal antibodies can result is high specificity (Rodak et al., 2005). (3) ELISA was occasionally present negative results(Guscetti et al., 1998b).

2.2.2 RT-PCR for detection of PEDV

Reverse transcriptase polymerase chain reaction (RT-PCR) consists of single and multiplex RT-PCR. This technique is convenient, rapid and sensitive for detection of PEDV. Multiplex real time RT-PCR detects N gene of PEDV which is highly conserved, resulting in no cross-reaction with TGEV and other diarrhea causing virus such as rotavirus, reovirus and enterovirus. However, the efficiency of multiplex real-time RT-PCR is highest level on day 1 after PEDV infection and decreased until death (Kim et al., 2007). The sensitivity of RT-PCR depended on conditions of RNA extraction and RT-PCR protocol as there are many factors in specimen inhibiting enzyme reactions (Ishikawa et al., 1997).

2.2.3 *In Situ* Hybridization (ISH) for detection of PEDV

In Situ Hybridization is a technique for localizing specific of nucleic acid targets within a histologic section. *In situ* hybridization requires DNA probes complementary with conserved region of PEDV gene for localizing PEDV infected site in swine intestine (Kim and Chae, 2000). The hybridization signal has been detected in the enterocyte of jejunum and ileum but not those of cecum, colon and rectum.

Part of intestine showing positive for PEDV are vary depends on age of pig, duration of infection and strain of virus. The disadvantages of ISH include (1) radioactive labels are short half-life probe. (2) Biotin probe provides non-specific reaction and (3) Formalin fixation can denature antigens (Jung and Chae, 2005).

2.2.4 Immunohistochemistry for detection of PEDV

Immunohistochemistry (IHC) is method to detect antigens in cells of a tissue section by the interaction of target antigens with specific antibodies tagged with visible label on biological tissues. The intestinal of PEDV infected pig was investigated by the streptavidin-biotin (SAB) technique and the primary antibody used was rabbit anti-PEDV. The small intestine, the virus was observed in the crypts, lamina propria and payer's patches of jejunum and ileum greater than duodenum. The site of PEDV replication was clearly different from that of TGEV (Sueyoshi et al., 1995). However, Immunohistochemistry require sample of intestinal of dead pigs.

2.2.5 Electron microscopy (EM) for detection of PEDV

Electron microscopy (EM) is a technique for visualizing virus particle in sample. The PEDV was observed at amplification of 19,000X - 25,000X in cytoplasmic vacuoles or microvilli, as shown in Figure 3. EM can be used to measure size of viral particle of 70-140 nm. However, EM requires an experienced person and specific immune serum to identify viral particle(Martelli et al., 2008).

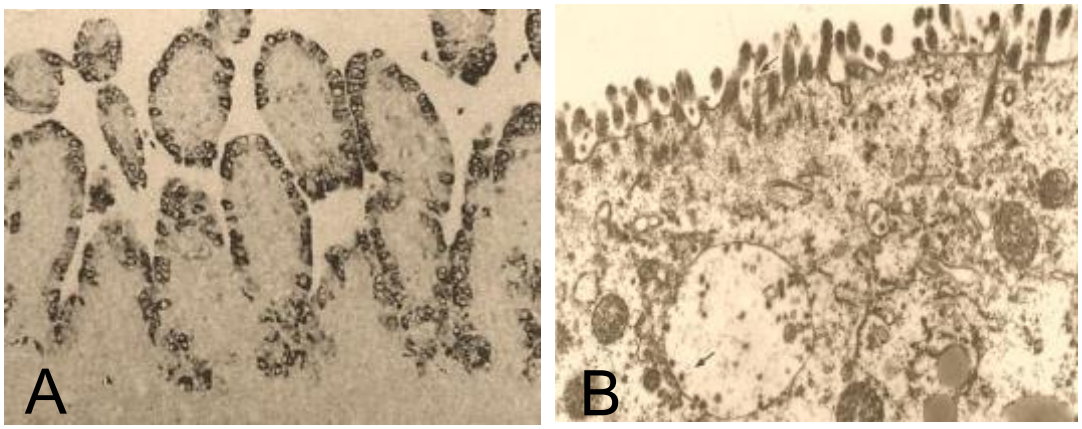


Figure 3 A: PEDV located in intestinal mucosal cells on villi of jejunum, detected by immunohistochemistry, B: PEDV located in enterocytes of jejunum, detected by transmission electron microscope. (Sueyoshi et al., 1995).

2.3 Monoclonal antibodies

2.3.1 Antibody Structure and Function

Antibodies belong to a group of glycoprotein secreted by specialized B lymphocytes known as plasma cells. Antibodies react specifically with antigens, which are responsible for production of specific antibodies. The antibody-antigen complexes are eliminated through phagocytosis by macrophages.

An antibody is a Y shape molecule, as shown in Figure 4. Each Y molecule (~150 kD) contains four polypeptides. Two identical copies of a polypeptides called heavy chain (~55 kD) and light chain (~25 kD), both of which are linked by disulfide bonds and noncovalent bonds. The light and heavy chains are associated to form an antigen-binding site at the tip of the arms. The light chains consist of one variable and one constant region while heavy chains consist of one variable and three constant regions. The Y shaped molecule can be cleaved by papain at hinge region, generating Fab and Fc fragments as shown in Figure 4 (Lipman et al., 2005).

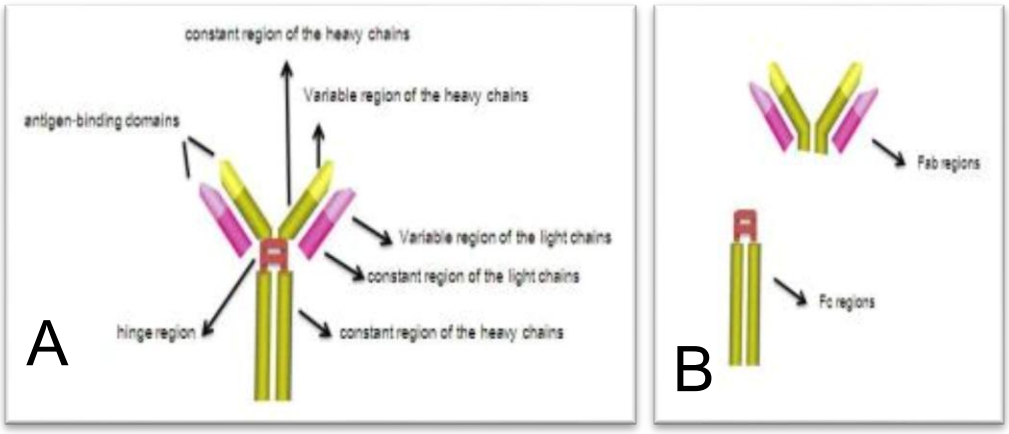
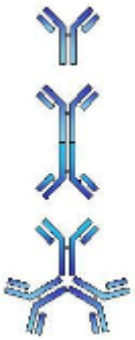


Figure 4 A: Structure of antibody molecule, Immunoglobulin, B: Immunoglobulin after being cleaved by papain.

In mammal, antibodies are divided into five class of immunoglobulin (Ig) , including IgG, IgM, IgA, IgD, and IgE, due to type of heavy chains and number of Y units per immunoglobulin, as shown in Table 3

Table 3 Class, function and properties of antibody.

Characteristics	**IgG**	**IgM**	**IgA**	**IgE**	**IgD**
Light chain	K or λ	K or λ	K or λ	K or λ	K or λ
Heavy chain	γ	μ	α	ε	δ
Concentration in serum	8-16 mg/ml	0.5-2 mg/ml	1-4 mg/ml	10-400 mg/ml	0-0.4 mg/ml
Function	Secondary response	Primary response	Protects mucous membranes	Protects against	
Valence	2	10	2,4 or 6	2	2
Structure					

The roles of antibodies are two steps (1) when arm of antibodies binding with epitope on an antigen at antigen-binding domains (Fab), making each antibody molecule forming antibody-antigen complexes and antigens is removed through phagocytosis by macrophages. (2) Constant region of the heavy chains (Fc) generates biological effector functions such as activation of natural killer cell, classical complement pathway and phagocytosis.

2.3.2 Polyclonal antibodies

Polyclonal antibody (PAb) is antiserum produced by numerous B lymphocytes of animal. PAb is mixture of different antibodies with high sensitivity but low specificity. PAb can be produced only *in vivo*, meaning that the method requires animal. Rabbits are frequently animal of choice for PAb production. Immunization starts with injection of antigen subcutaneously and the boosters are carried out for 3-8 months. Blood is collected from ear of rabbit and serum is obtained by centrifugation. Purification of PAb can be achieved by ammonium sulfate precipitation followed by ion exchange chromatography.

2.3.3 Monoclonal antibodies

In 1975, Kohler and Milstein (Köhler and Milstein, 1975) developed a technique that antibody-producing cells can grow eternally in cell culture. In this technique, plasma cells isolated from immunized mice were fused with myeloma cells, resulting in hybridoma cells. The antibody produced from one hybridoma cell is monoclonal antibody. MAb has high specificity against epitope of antigen (Harlow 1988).

2.3.4 Myeloma

Myeloma is a cancer of plasma cells which can multiply without control. The myeloma cells can be induced in a few strains of mouse and rats. In 1972, myeloma was first isolated from BALB/C mice after injecting mineral oil intraperitoneally (Potter, 1972). Myeloma cells used in the production of MAb have neither antibody production nor some specific enzymes such as HGPRT and TK.

In order to obtain myeloma cells lacking HGPRT and TK, myeloma cells should be cultured in special medium containing 8-azaguanine and bromodeoxyuridine. The mutant cells are able to survive via *de novo* pathway (Eichmann, 2005).

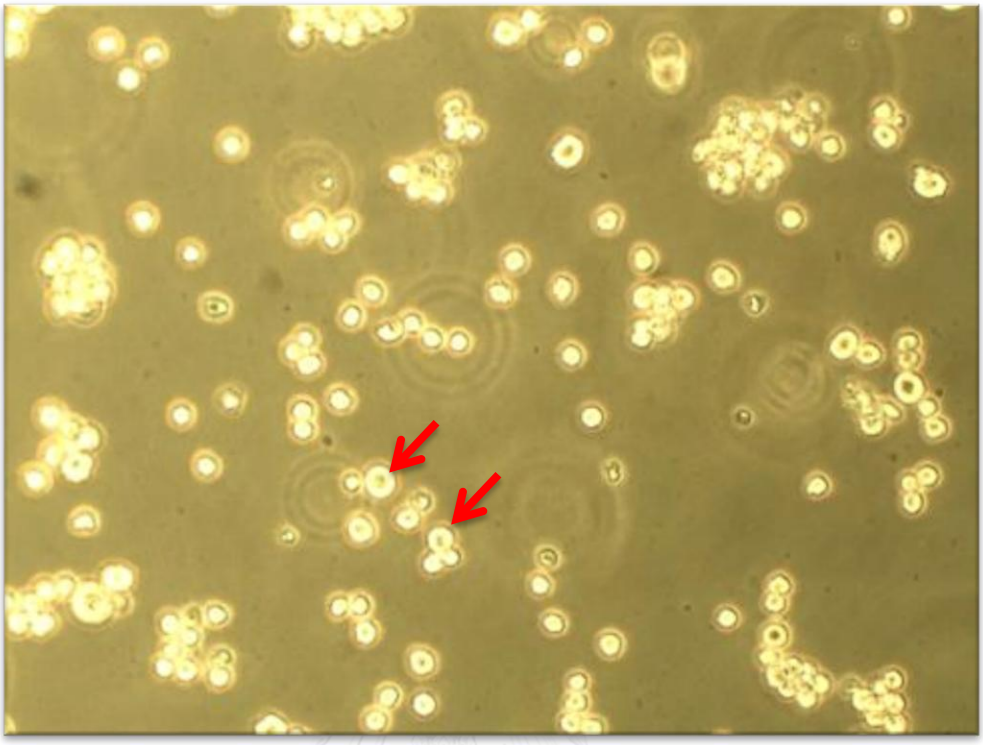


Figure 5 Myeloma cells strain X63Ag8.653, at passage 23, cultured in RPMI1640 medium containing HEPES and L-glutamine, supplemented with 20% fetal bovine serum, incubated at 37 ºC 5% CO_2.

Table 4 Myeloma cell lines used as fusion parents (Ed H and David L., 1988).

Cell line	Reference	Derived from	Chains expressed	Secreting
Mouse Line				
P3-X63Ag8	Kohler and Milstein (1975)	P3K	Gramma1 ,kappa	IgG1
X63Ag8.653	Kearney et al. (1976)	P3-X63Ag8	None	No
Sp2/0-Ag14	Kohler and Milstein (1976) Shulman et al. (1978)	P3-X63Ag8 x BALB/C None	None	No
FO	De St.Groth and Scheidegger (1980)	Sp2/)-Ag14	None	No
NSI/1-ag4-1	Kohler et al. (1976)	P3-X63Ag8	Kappa	No
NSO/1	Galfre and Milstein (1981)	NSI/1-Ag4-1	None	No
FOX-NY	Taggart and Samloff (1984)	NSI/1-Ag4-1	Kappa	No

2.3.5 Hybridoma

Hybridoma cell is a somatic cell hybrid created by fusion between plasma cell and myeloma cell. The resulting hybridoma cells can be produce antibody and survive *in vitro* forever. Antibody produced from one hybridoma cell is so called monoclonal antibody (Harlow 1988).

2.3.6 Polyethylene glycol (PEG)

In 1975, Polyethylene glycol (PEG) was first used to fuse the mammalians cells by Pontecoro (Pontecorvo, 1975). PEG is quick and manageable to fusion mammalians cells. PEG at the concentration of more than 35% can induce cell agglutination and cell-to-cell contact, leading to subsequent cell fusion which generates single cell of two or more nuclei (JACOBSON, 1983). Fused cells can have abnormal chromosome number, either odd or even number, resulting in the failure of cell division during mitosis.

2.3.7 Hybridoma selection

The selection of hybridoma cell is achieved by using selective medium, HAT, containing hypoxanthine, aminopterin and thymidine.

Normal animal cells can synthesize nucleotide by *de novo* pathway and *salvage* pathway. Aminopterin blocks the activation of tetrahydrofolate, resulting in the inhibition of purine synthesis by *de novo* pathway. Aminopterin treated cells can use only *salvage* pathway to synthesize purine. The myeloma deficient in $HGPRT^-$ and TK^- cannot survive via *salvage* pathway while B lymphocytes can use *salvage* pathway for purine synthesis. However, B lymphocytes are immortalized. In HAT medium, unfused myeloma cells and unfused B cells eventually die. Only hybridoma cells can survive and grow in HAT medium due to the immortal property and purine synthesis via via *salvage* pathway (figure 6) (Harlow 1988).

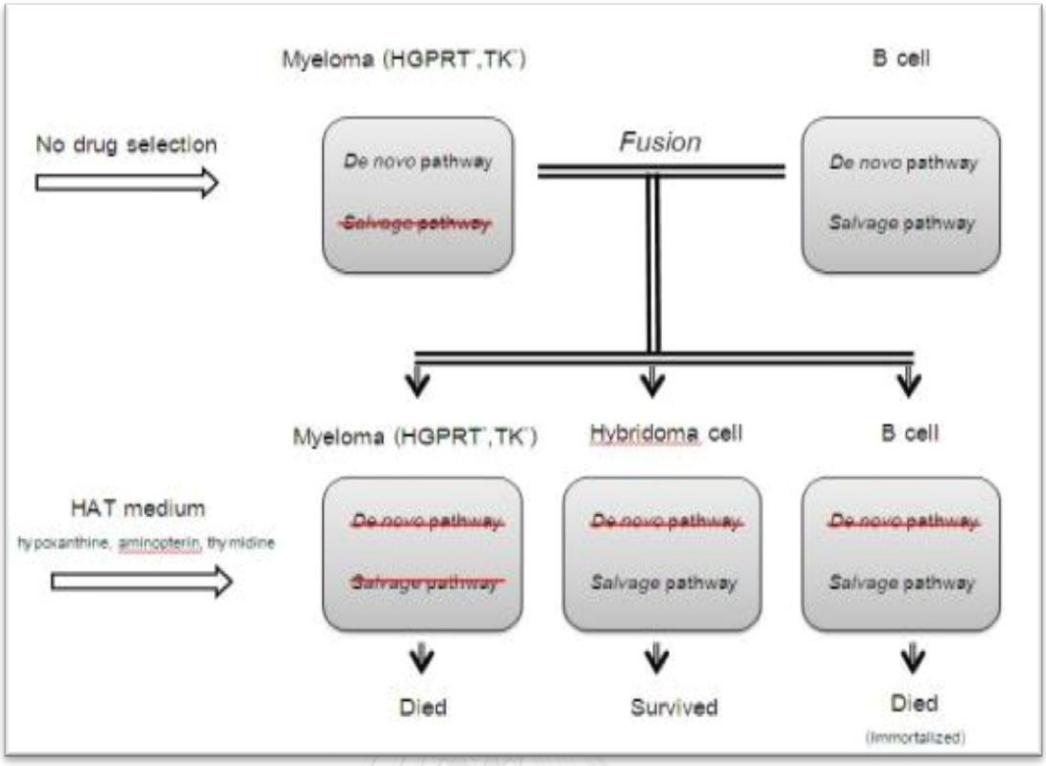


Figure 6 Hybridoma cells in selective medium: HAT containing hypoxanthine, aminopterin and thymidine.

Chapter III

Materials and Methods

Chemicals

All chemicals and culture reagents were purchased from Sigma-Aldrich (St Louis, Mo, USA), JR Scientific (Woodland, CA, USA) and Invitrogen Life Technologies (Carlsbad, CA, USA). Myeloma cells strain X63Ag8.653 was kindly obtained from Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University. PEDV strain K-9 and monoclonal antibody against PEDV was gifts from Dr.Suphot Wattanphansak.

3.1 Purification of PEDV strain K-9 vaccine

One bottle of PEDV strain K-9 vaccine from Korea was dissolved in 5 ml of Phosphate buffered saline (PBS) and subjected to centrifugation at 200000xg at 4˚C for 1 hour. The pellet was harvested, resuspended in PBS and re-centrifuged at the same conditions. Finally the pellet containing virus was collected, resuspended in 1ml of PBS. The virus was determined by RT- PCR using PEDV specific primers.

3.2 PEDV RNA extraction

Total viral RNA was extracted by using ZR viral DNA/RNA kit™. The process was performed according to the manufacturer's recommendation. Briefly, 600 µl of viral DNA/RNA buffer was added to 200 µl of PEDV suspension. The mixture was centrifuged at 12,000 rpm for 1 min using spin column. After adding 400 µl of DNA/RNA Prep Buffer to the column, centrifugation at 12,000 rpm for 30 sec was carried out. After washing twice with 700 µl and 400 µl DNA/RNA wash buffer, followed by centrifuging at 12,000 rpm for 15 sec, PEDV RNA was eluted with 20 µl DNase/RNase-free water. The isolated PEDV RNA was stored in -20˚C until used.

3.3 The one step RT-PCR

20 µl of isolated PEDV RNA was assayed in 25 µl reaction tube containing AccessQuick™ Master Mix, 100unit AMV Reverse Transcriptase, 3 µmol Forward primer (5'-TTCTGAGTCACGAACAGCCA-3') and Reverse primer (5'-CATATGCAGCCTGCTCTGAA-3')(Park et al., 2007). The thermal cycling consisted of Reverse Transcription at 48 ˚C for 45 min, initial denaturation at 95 ˚C of for 2 min, followed by 30 cycles of polymerase chain reaction (PCR) (denaturing at 94 ˚C for 30 sec, annealing at 57 ˚C for 1 min and chain elongating at 72 ˚C for 1 min) and final chain polymerization at 72˚C for 5 min. The PCR product was kept at -20˚C.

3.4 Agarose gel electrophoresis

1.5% agarose gel in TAE (Tris-Acetate-EDTA; electrophoresis buffer) containing ethidium bromide was prepared. 4 µl of PCR product from one step RT-PCR of PEDV was applied together with 3 µl of DNA ladder of 100 bp standard (Fermentas, life science, PA, USA). DNA electrophoresis was done at constant voltage of 120 volt for 35 min. The DNA bands were elucidated using UV illuminator.

3.5 Determination of PEDV protein concentration

PEDV protein concentration was determined by absorbance at 280 nm using UV-visible spectrophotometer (Layne, 1957). The PEDV suspended in PBS was diluted at 1:100 with PBS and subjected to spectrophotometer at 280 nm using PBS as blank. The PEDV protein concentration was calculated as follows;

$$\text{Protein concentration (mg/ml)} = \frac{\mathbf{A280}}{\mathbf{1.5}} \times 100$$

3.6 Immunization with purified PEDV

Two BALB/c mice of 7 weeks old were subcutaneously immunized with purified PEDV. The immunization was done 3 times at 3 weeks intervals. Briefly, 0.3 ml of PBS containing 100 µg of PEDV protein in complete Freund's adjuvant was injected per mouse at the first immunization while the same amount of protein with

incomplete Freund's adjuvant was used at the second and the third immunization. Mouse serum containing antibodies against PEDV was measured after the third immunization using enzyme-linked immunosorbent assay (ELISA). Finally, 3 days before fusion, the mice were intravenously injected with 50 µg of purified PEDV without adjuvant. The mice were euthanatized, the spleens were collected and prepared for fusion.

3.7 Preparation of Myeloma cells

The myeloma cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Mo, USA) containing HEPES and L-glutamine, supplemented with 20% fetal bovine serum (JR Scientific, CA, USA) and antibiotic-antifungal agents (Invitrogen Life Technologies, CA, USA) at 37 ºC 5% CO_2 . In order to obtain myeloma cells without HGPRT enzyme, the myeloma cells were cultured in the same medium with the addition of $6.6x\ 10^{-3}$ M of 8-azaguanine for 2 months. The myeloma cells growth was observed everyday using inverted microscope (ZEISS Axiovert 40 CFL). The myeloma cells obtained after being cultured with 8-azaguanine were used for cell fusion.

3.8 Preparation of spleen cells

After being euthanatized, the spleen was collected from immunized mouse with aseptic technique. The splenocytes were washed twice with sterile PBS buffer. The cell pellet collected after centrifugation at 1,000 rpm for 5 min at room temperature was counted then subjected to cell fusion.

3.9 Determination of viable cells.

The cells were diluted at 1:100 with PBS. 10 µl of 10% trypan blue was added to 90 µl of diluted cells and the viable cells was determined using hemocytometer. The cell concentration is then calculated as follows:

$$\text{Viable cell concentration (cells/ml)} = \frac{\text{number of non-stained cell} \times 10^4 \times \text{dilution factor}}{\text{number of square being counted}}$$

3.10 Cell fusion

$1x10^7$ of 8-azaguanine treated myeloma cells was added to $5x10^7$ of splenocytes obtained from immunized mouse. The cell fusion was performed in 50 ml sterile centrifuge tube containing 1 ml of warm 50% polyethylene glycol (PEG-4000) at 37 ºC for 1 min. after fusion, 10 ml of RPMI 1640 was gentlely added and the tube was centrifuged at 1,000 rpm for 5 min at room temperature. The pellet was harvested and resuspended in RPMI 1640 containing HEPES and L-glutamine, supplemented with 20% fetal bovine serum. The cells were cultured in 100 mm sterile culture dish at 37 ºC 5% CO_2 for 18 hours.

3.11 Selection of hybridoma cells

After being incubated at 37 ºC 5% CO_2 for 18 hours, the fused cells were further cultured in HAT selective medium containing RPMI 1640 HEPES, L-glutamine, 20% fetal bovine serum and Hypoxanthine-Aminopterin-Thymidine (HAT) using 96 well culture plate. The hybridoma cells were then transferred to 96 well culture plate containing Hypoxanthine-Thymidine (HT) medium and kept for 14days. Finally, the supernatant from each well was collected and sent for antibodies detection by ELISA.

3.12 Determination of antibody by ELISA

Polystyrene ELISA plate were coated with purified PEDV (250 ng/well) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 overnight at 4˚C. The plates were washed with PBS for 3 times and block with 2% bovine serum albumin (BSA) (VWR, Radnor Corporate Center, Radnor, PA) for 1 hour at room temperature. After being washed with 0.05% Tweens-PBS for 3times, 50 µl of supernatant from each plate containing hybridoma cells were added and incubated for 1 hour at room temperature. The plate was washed with 0.05% Tween-PBS for 3 times and incubated with 50µl of 1:3000 diluted Goat anti- Mouse IgG (H+L) labeled with horseradish peroxidase (SouthernBiotech, Birmingham, AL, USA) for 1 hour at room temperature. Finally, 100

µl of 2, 2' diazino bis (3 ethyl) benz-thiazoline-6 sulfonic acid (ABTS) (KPL, Gaithersburg, MD, USA) the concentration of 0.3 g/L in a citric acid buffer, containing 0.01% of H_2O_2 was added and the color developed after incubation for 30 min at room temperature in the dark place. The intensity of the color in each well was measured by ELISA reader (Synergy HT) at 405 nm.

3.13 Preparation of feeder cells

In order to perform limiting dilution step, the feeder cells has to be prepared to promote the growth of the single hybridoma cell. The thymus of 1 week-old young mouse was collected with aseptic techniques. The thymocytes were prepared and washed twice with sterile PBS buffer. The cells collected after centrifugation at 1,000 rpm for 5 min were resuspended in RPMI 1640 containing HEPES and L-glutamine, supplemented with 20% fetal bovine serum and kept at 37 ºC 5% CO_2 for 18 hours before use.

3.14 Limiting dilution

After wells of hybridoma cells containing antibody have been identified. The hybridoma cells were count and subjected to limiting dilution procedure. In brief, the hybridoma cells were harvested after centrifugation at 1,000 rpm for 5 min at room temperature, counted and diluted to a cell concentration of 10 cells/ml medium. 100 µl of diluted hybridoma cells were seeded to each of 96 well culture plates and incubated at 37 ºC 5% CO_2 for14 days. On the second day of incubation, 50 µl of feeder cells (10^4 cell/ml) were added to each well of hybridoma. After 14 days, the supernatant of each well was collected and sent for antibody detection. The limiting dilution was repeated to ensure an achievement of monoclone.

3.15 Determination of antibody subclass by ELISA

ELISA were performed as previously mentioned except using 50µl of Goat anti- Mouse IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 labeled with horseradish peroxidase (SouthernBiotech, Birmingham, AL, USA) per well as secondary antibody.

Chapter IV

Results

4.1 Preparation and purification of PEDV strain K9 vaccine

After centrifugation at 200,000 xg for 1 hour at 4˚C, the presence of PEDV in the pellet was confirmed by RT-PCR using PEDV specific primers and agarose gel electrophoresis. The result was shown in Fig 7. The 700 bp PCR product of PEDV from the 1st centrifuged and 2nd centrifuged were shown in lane 2 and lane 3 respectively, indicating the presence of PEDV in the pellet.

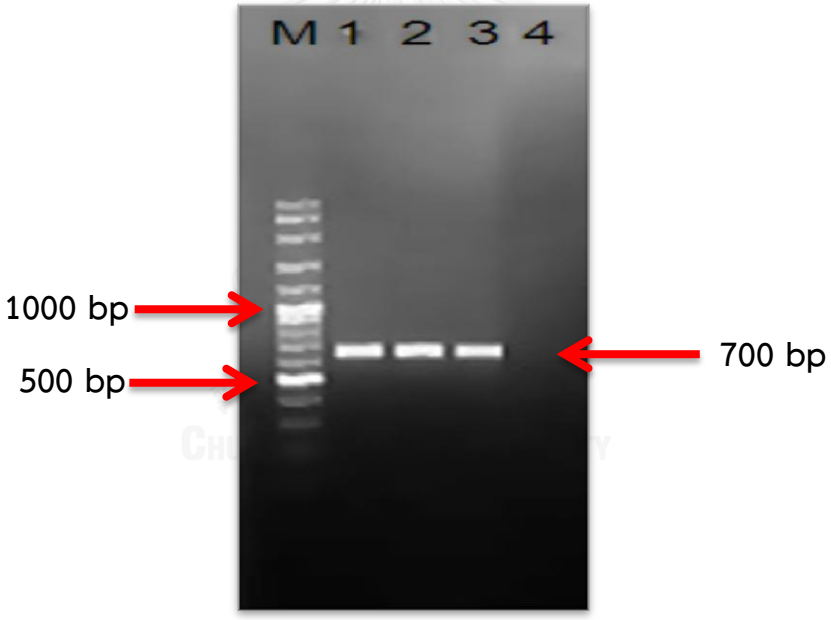


Figure 7 The PCR products of 700 bp of PEDV were obtained from 1st and 2nd of ultracentrifugation (200,000 xg for 1 hour at 4˚C). Lane M; 100bp marker, lane 1 ; positive control, lane 2 ; PCR products from pellet obtained from 1st ultracentrifugation, lane 3 ; PCR products from pellet obtained from 2nd ultracentrifugation and lane 4; negative control.

4.2 Immunization with purified PEDV.

Two weeks of after 3nd immunization with purified PEDV in complete Freund's adjuvant, the blood collection and serum preparation from mouse were performed and the antibody against PEDV was diluted using ELISA and the results are shown in the table 5

	OD
Positive control	**0.381**
Negative control	**0.093**

Table 5 Absorbance at 405 nm of diluted (1:10) serum from immunized mouse, containing PEDV specific antibody (A=0.381) served as positive control compared to A=0.093 of the negative control using PBS instead of diluted serum.

4.3 Cell fusion and hybridoma cells preparation

After cell fusion and cultured in selective HAT medium, unfused myelomas and B cells are gradually disappeared due to cell death. Only the hybridoma cells can survive and rapidly grow in the culture. The growth of cells can be observed using inverted microscope at different times after fusion as shown in fig 8-10.

Figure 8 Myeloma cells, plasma cells and fused cells in RPMI1640 medium, on day 1 after fusion under light microscope (100X).

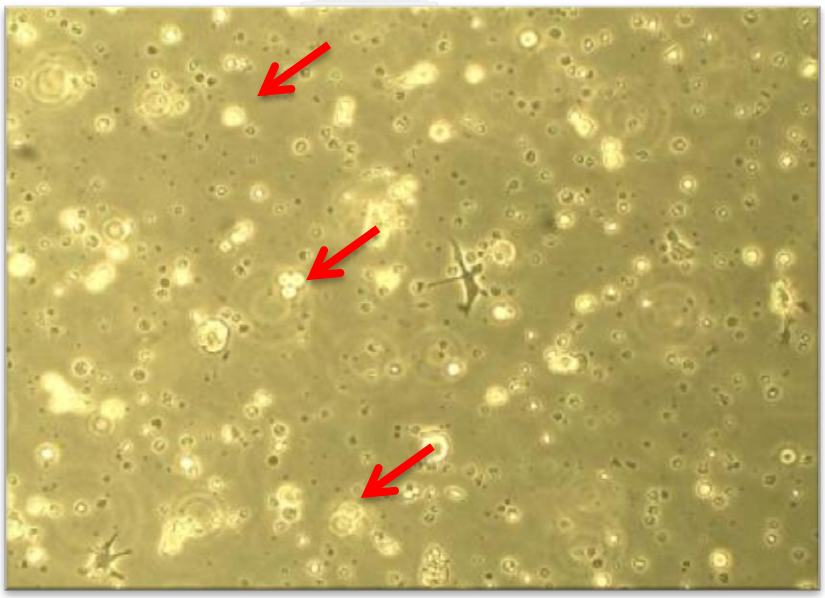


Figure 9 Myeloma cells, plasma cells and fused cells in HAT medium on day 12 after fusion under light microscope (100X). The arrows indicated healthy hybridoma cells. Dead cells comprising of unfused cells were seen among the hybridoma cells.

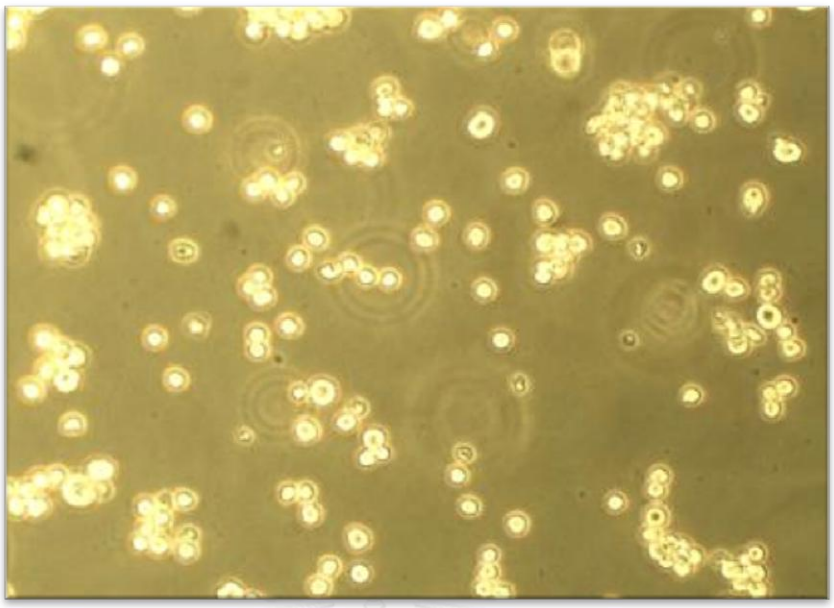


Figure 10 Hybridoma cells in HT medium on day 23 after fusion, under at 100X light microscope.

4.4 Determination of antibody by ELISA

The antibodies produced by hybridoma cells were determined on 15 days after culturing in HT medium using ELISA. The absorbance at wavelange of 405 nm of 2-3 times above negative control defined positive. The absorbance values of all wells tested for antibodies was shown in appendix A. The percentage of well containing hybridoma cells and hybridoma cells were determined and the result was shown in table 6.

	Hybridoma cells	Antibody- producing hybridoma cells
1st mice (A1)	100 % (864/864)	1.15 % (10/864)
2nd mice (A2)	100 % (672/672)	0.89 % (6/672)

Table 6 Percentage of wells containing hybridoma cells and those containing antibody producing hybridoma cells from mouse A1 and A2.

4.5 Limiting dilution

After fusion, 10 wells and 6 wells of antibody producing hybridoma cells were obtained from mouse A1 and A2 respectively. According to the growth rate of hybridoma cells, finally, only 2 wells of hybriddoma cells were selected, namely, A1/A2 and D3. The first limiting dilution of both wells generated 9 culture plates of 96 wells. The growth of hybridoma cells were observed every day as shown in the figure 11-15.

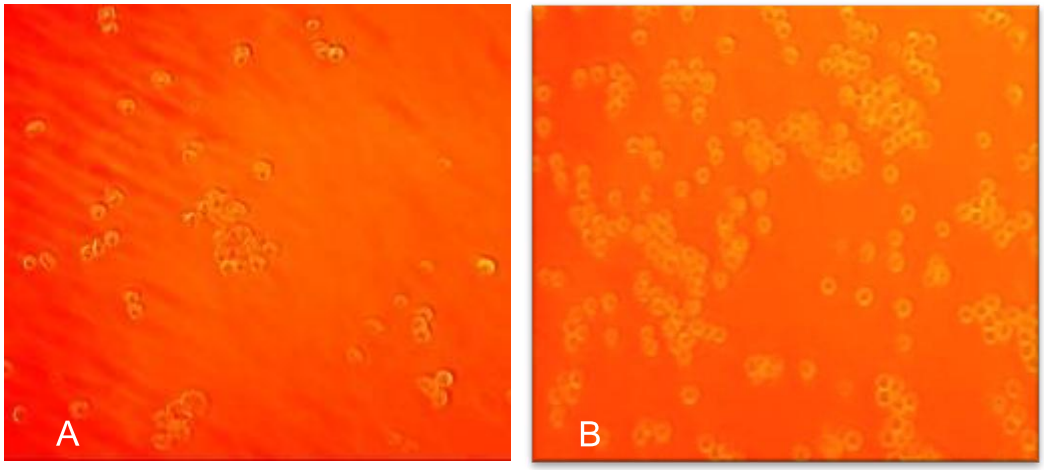


Figure 11 A: Hybridoma cells "A1/A2" clone in culture medium, B: Hybridoma cells "D3" clone in culture medium.

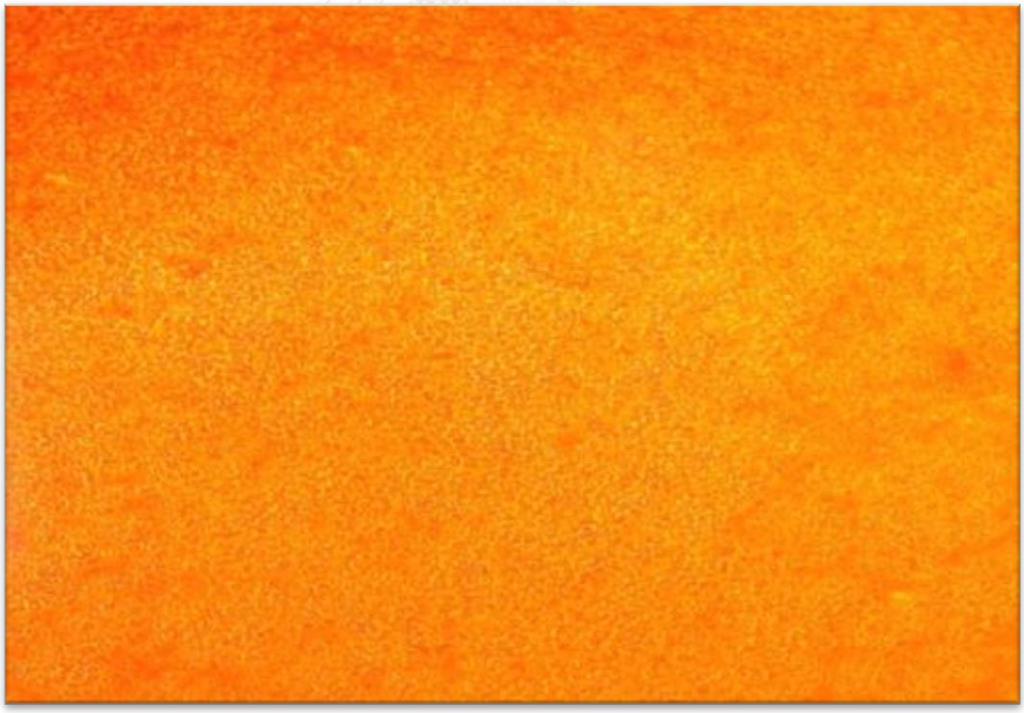


Figure 12 Thymus feeder cells cultured in RPMI1640 medium before transferred to hybridoma cell at limiting dilution step.

Figure 13 Hybridoma cells and feeder cells in RPMI1640 medium on day 1 after limiting dilution.

Figure 14 Hybridoma cells and feeder cells in RPMI1640 medium on day 7 after limiting dilution.

Figure 15 Hybridoma cells and feeder cells in RPMI1640 medium on day 14 after limiting dilution.

The percentage of antibody in each well with determined and the results shown in appendix A.

According to the absorbance values, 12 wells from A1/A2 well and 12 wells from D3 well were selected and subjected to second limiting dilution. After second limiting dilution, 3 wells from A1/A2 and 5 wells from D3 with high absorbance values were obtained (data were shown in appendix A). All of 8 antibody positive wells were finally sent to subclass identification.

4.6 Subclass identification of monoclonal antibodies.

The subclass of monoclonal antibodies were determined by ELISA using secondary antibody goat anti- Mouse IgG_1 IgG_{2a}, IgG_{2b} and IgG_3 conjugate with HRP. 4/8 clones of hybridoma with high level of antibody production were generated, namely, “P1D3L2”, “P2D3L2”, “P3AL1”, “P5AL1”, as shown in Figure. 17-20.

Figure 16 Hybridoma clone “P1D3L2” produces MAb which belongs to subclass IgG_{2a}.

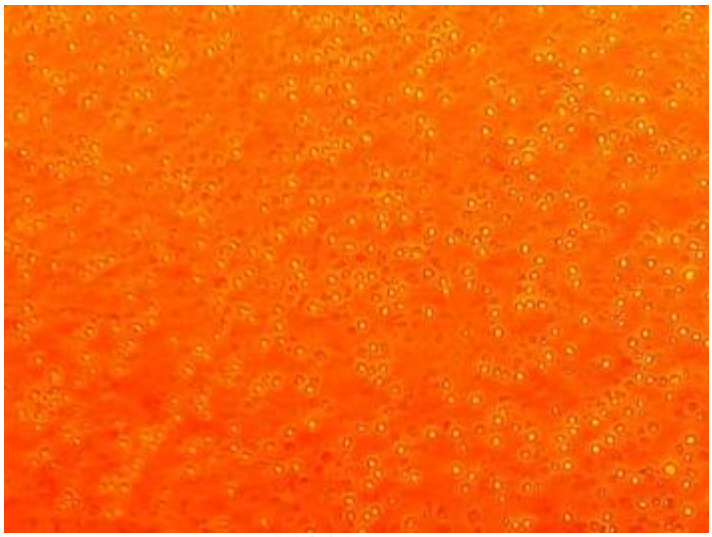


Figure 17 Hybridoma cells "P2D3L2" produces MAb which belongs to subclass IgG_1.

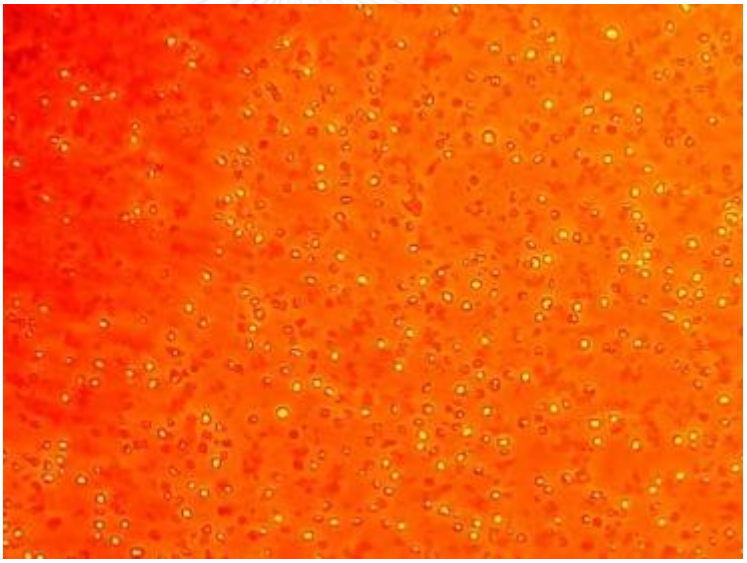


Figure 18 Hybridoma cells "P3AL1" produces MAb which belongs to subclass IgG_1.

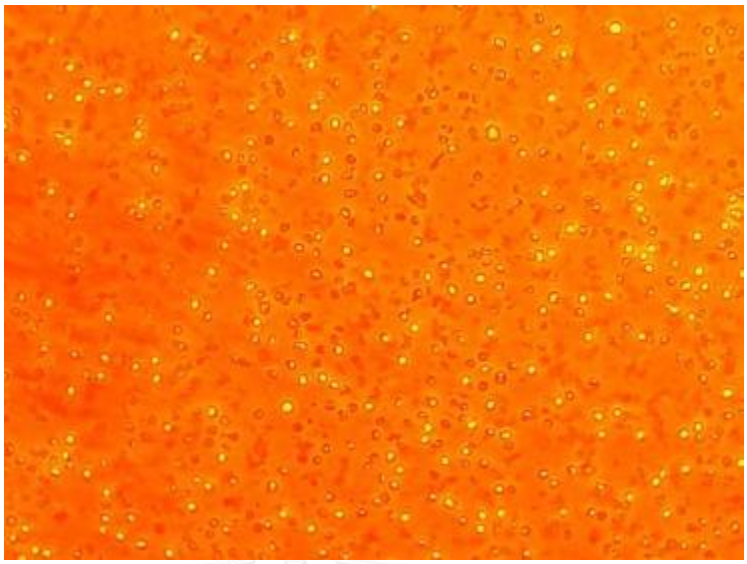


Figure 19 Hybridoma cells "P5AL1" produces MAb which belongs to subclass IgG_{2b}.

The antibodies produced belong to 3 subclass, IgG_1 ("P2D3L2", "P3AL1"), IgG_{2a} ("P1D3L2") and IgG_{2b} ("P5AL1").

Chapter V
Discussion

5.1 Preparation and purification of PEDV strain K9 vaccine

There are many techniques to purify virus such as density gradient centrifugation (Ali and Roossinck, 2007), specific interaction based affinity chromatography (Hu et al., 2010), size exclusion ultra-centrifugal filter (Hensgen et al., 2010) and charge base ion-exchange chromatography (Czermak et al., 2008). However, only Sucrose density gradient centrifugation has been used for PEDV purification (Rodak et al., 1999). Unfortunately sucrose density gradient centrifugation remains many limitations such as sucrose is toxic to cell. In addition, variation in viral yield can be obtained with the loss of more than 90 % (Hutornojs et al.).

In the present study, the ultracentrifugation at 200000xg was chosen to purify PEDV, instead of sucrose gradient centrifugation. In the preliminary study, the sucrose gradient centrifugation was tried to purify PEDV, however, the particle loss of virus was resulted (data not shown). In addition to avoid cellular toxicity caused by sucrose, the virus K-9 vaccine was also in limited supply, leading to the use of ultracentrifugation as a method of purification.

5.2 Preparation of hybridoma cells for PEDV monoclonal antibodies production

The immunization is an important step in the production of antibodies. In general BALB/c mice are the most commonly used in the production of monoclonal antibody due to genetic similarity in the group of mice (Harlow 1988).

The cell fusion performed in this study was randomly somatic cell fusion using 50% polyethylene glycol (PEG), generating the fuse cells comprising of various chromosome numbers. Furthermore, cells with abnormal chromosome number usually are unstable and eventually losing their chromosome.

PEG is the most widely used substance for cell fusion, especially, with a molecular weight between 1000 to 6000 dalton. Roos et al., 1983 Showed that more

than 90% of cells were fused with 50% PEG of 1500 dalton. However, the size of PEG and the fusion time can be varied depending on cell types (Yang and Shen, 2006).

In the present study, with 50% PEG of 4000 dalton, 100% wells containing fused cells could be achieved. The high percentage of fused cell might be due to the culture of fused cells in enriched medium RPMI1604 for 18 hours after fusion.
In order to obtain monoclonal antibody, the antibody producing cell or hybridoma must be isolated to be 1 hybridoma cell per culture well by the method named limiting dilution. The single hybridoma cell could not survive due to an insufficient amount of growth factors in the medium. The addition of thymocyte feeder cells could provide growth factors and suitable culture condition for growth of single hybridoma cells.

Moreover, in thus study, it was shown that the thymocyte feeder cells can be useful not only in the limiting dilution step, but they can be used as rescue protocol for promotion of growth in culture containing low number of hybridoma cells.

Four monoclonal antibodies were produced in this study. The MAbs belong to IgG1, IgG2a and IgG2b subclasses. The differecnes between subclasses of IgG are number of disulfide bond located at the hinge region of IgG molecule. Competitive inhibition assay is needed to be performed in the furture in order to identify the antigen binding site of each monoclonal antibody.

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APPENDIX

APPENDIX A

1. Result of antibody test of hybridoma cells from mice A1 were produced antibody against PEDV after fusion.

plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.108	0.13	0.138	0.129	0.127	0.129	0.132	0.126	0.199	0.121	0.123	0.133
B	0.113	0.129	0.13	0.132	0.132	0.124	0.124	0.134	0.124	0.165	0.134	0.133
C	0.104	0.129	0.133	0.13	0.13	0.128	0.323	0.115	0.127	0.344	0.13	0.128
D	0.113	0.129	0.133	0.127	0.132	0.145	0.127	0.122	0.129	0.126	0.244	0.132
E	0.101	0.13	0.131	0.134	0.126	0.141	0.129	0.125	0.131	0.157	0.181	0.133
F	0.108	0.126	0.127	0.133	0.128	0.109	0.128	0.116	0.126	0.129	0.133	0.132
G	0.101	0.115	0.125	0.128	0.129	0.127	0.127	0.118	0.136	0.127	0.147	0.134
H	0.109	0.13	0.13	0.131	0.13	0.136	0.128	0.122	0.126	0.135	0.15	0.129

plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.116	0.134	0.114	0.114	0.149	0.111	0.121	0.115	0.113	0.111	0.113	0.127
B	0.113	0.111	0.114	0.112	0.116	0.11	0.114	0.112	0.115	0.114	0.113	0.112
C	0.118	0.115	0.112	0.111	0.11	0.109	0.112	0.11	0.111	0.114	0.112	0.113
D	0.115	0.111	0.111	0.114	0.113	0.112	0.113	0.116	0.113	0.116	0.116	0.119
E	0.114	0.113	0.112	0.112	0.113	0.111	0.111	0.115	0.11	0.116	0.115	0.112
F	0.114	0.112	0.112	0.113	0.11	0.122	0.113	0.115	0.109	0.116	0.113	0.125
G	0.117	0.113	0.116	0.125	0.114	0.113	0.113	0.109	0.113	0.118	0.114	0.12
H	0.118	0.116	0.117	0.115	0.119	0.114	0.113	0.113	0.124	0.117	0.115	0.115

plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.122	0.122	0.117	0.122	0.043	0.121	0.122	0.117	0.187	0.114	0.162	0.121
B	0.121	0.122	0.117	0.121	0.04	0.117	0.115	0.118	0.169	0.118	0.116	0.118
C	0.122	0.119	0.118	0.121	0.054	0.115	0.115	0.116	0.17	0.115	0.121	0.119
D	0.112	0.119	0.123	0.12	0.041	0.116	0.115	0.113	0.17	0.119	0.12	0.12
E	0.122	0.119	0.117	0.115	0.04	0.116	0.117	0.114	0.168	0.121	0.121	0.116
F	0.12	0.119	0.118	0.122	0.044	0.119	0.118	0.116	0.168	0.119	0.118	0.122
G	0.122	0.12	0.117	0.119	0.045	0.119	0.114	0.114	0.17	0.118	0.12	0.119
H	0.153	0.122	0.119	0.125	0.096	0.041	0.102	0.119	0.159	0.118	0.118	0.119

plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	0.123	0.124	0.124	0.12	0.122	0.12	0.12	0.119	0.119	0.114	0.12	0.116
B	0.124	0.122	0.123	0.12	0.12	0.12	0.144	0.118	0.121	0.121	0.116	0.123
C	0.123	0.12	0.126	0.117	0.114	0.105	0.102	0.111	0.114	0.114	0.12	0.119
D	0.123	0.123	0.125	0.118	0.118	0.113	0.115	0.117	0.121	0.12	0.128	0.119
E	0.128	0.122	0.123	0.116	0.118	0.12	0.119	0.121	0.118	0.125	0.121	0.12
F	0.122	0.122	0.125	0.118	0.119	0.12	0.123	0.115	0.117	0.123	0.125	0.122
G	0.123	0.1241	0.124	0.12	0.12	0.118	0.122	0.12	0.122	0.125	0.125	0.12
H	0.123	0.124	0.125	0.109	0.121	0.123	0.124	0.121	0.125	0.121	0.124	0.123

plate 5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.132	0.132	0.131	0.128	0.126	0.128	0.125	0.122	0.123	0.124	0.123	0.121
B	0.129	0.131	0.127	0.126	0.128	0.127	0.125	0.123	0.123	0.125	0.123	0.12
C	0.132	0.135	0.127	0.128	0.128	0.126	0.126	0.124	0.12	0.12	0.125	0.124
D	0.13	0.132	0.128	0.129	0.128	0.129	0.126	0.131	0.126	0.125	0.128	0.125
E	0.141	0.129	0.125	0.125	0.125	0.122	0.126	0.125	0.126	0.127	0.126	0.128
F	0.129	0.131	0.13	0.13	0.123	0.128	0.126	0.125	0.124	0.126	0.13	0.126
G	0.133	0.132	0.128	0.127	0.128	0.132	0.131	0.125	0.126	0.128	0.128	0.137
H	0.133	0.134	0.322	0.13	0.13	0.127	0.128	0.128	0.125	0.153	0.129	0.128

plate 6	1	2	3	4	5	6	7	8	9	10	11	12
A	0.117	0.12	0.121	0.12	0.119	0.12	0.117	0.12	0.122	0.144	0.117	0.133
B	0.124	0.114	0.12	0.12	0.121	0.122	0.116	0.114	0.118	0.119	0.118	0.116
C	0.122	0.113	0.123	0.12	0.119	0.118	0.119	0.126	0.116	0.116	0.118	0.116
D	0.122	0.114	0.121	0.123	0.119	0.123	0.117	0.121	0.117	0.12	0.121	0.119
E	0.124	0.112	0.121	0.121	0.119	0.119	0.118	0.119	0.116	0.12	0.118	0.127
F	0.122	0.121	0.12	0.121	0.118	0.12	0.122	0.113	0.116	0.119	0.122	0.121
G	0.124	0.115	0.126	0.118	0.119	0.131	0.122	0.119	0.119	0.123	0.121	0.118
H	0.128	0.117	0.122	0.121	0.121	0.125	0.122	0.121	0.122	0.123	0.12	0.121

plate 7	1	2	3	4	5	6	7	8	9	10	11	12
A	0.114	0.119	0.119	0.117	0.12	0.115	0.115	0.084	0.115	0.114	0.118	0.117
B	0.113	0.117	0.117	0.136	0.126	0.108	0.117	0.113	0.117	0.117	0.127	0.119
C	0.117	0.114	0.208	0.293	0.281	0.116	0.11	0.108	0.134	0.129	0.117	0.121
D	0.114	0.117	0.123	0.23	0.114	0.115	0.121	0.116	0.184	0.115	0.12	0.125
E	0.115	0.117	0.119	0.121	0.118	0.109	0.112	0.119	0.117	0.121	0.119	0.122
F	0.114	0.118	0.118	0.117	0.114	0.114	0.117	0.111	0.116	0.119	0.121	0.116
G	0.114	0.118	0.118	0.116	0.114	0.116	0.121	0.118	0.117	0.124	0.123	0.12
H	0.123	0.126	0.12	0.121	0.121	0.121	0.12	0.119	0.121	0.12	0.125	0.133

plate 8	1	2	3	4	5	6	7	8	9	10	11	12
A	0.119	0.118	0.124	0.124	0.052	0.119	0.125	0.125	0.123	0.121	0.121	0.121
B	0.119	0.117	0.121	0.125	0.119	0.121	0.119	0.12	0.119	0.122	0.121	0.121
C	0.119	0.115	0.121	0.117	0.112	0.116	0.115	0.141	0.154	0.117	0.121	0.119
D	0.112	0.119	0.118	0.115	0.112	0.116	0.115	0.28	0.152	0.123	0.122	0.124
E	0.116	0.119	0.118	0.119	0.116	0.119	0.121	0.137	0.134	0.124	0.123	0.126
F	0.121	0.118	0.12	0.123	0.117	0.124	0.121	0.117	0.114	0.122	0.125	0.117
G	0.122	0.119	0.124	0.123	0.119	0.119	0.127	0.12	0.117	0.126	0.124	0.124
H	0.12	0.126	0.126	0.125	0.124	0.13	0.129	0.128	0.123	0.125	0.131	0.126

plate 9	1	2	3	4	5	6	7	8	9	10	11	12
A	0.127	0.127	0.127	0.125	0.125	0.13	0.133	0.129	0.126	0.124	0.132	0.104
B	0.126	0.126	0.128	0.126	0.13	0.128	0.123	0.128	0.123	0.128	0.139	0.122
C	0.202	0.13	0.126	0.126	0.129	0.128	0.126	0.125	0.122	0.123	0.14	0.107
D	0.138	0.127	0.128	0.125	0.126	0.118	0.117	0.123	0.124	0.124	0.139	0.108
E	0.128	0.126	0.126	0.128	0.126	0.117	0.125	0.125	0.124	0.127	0.126	0.117
F	0.127	0.125	0.126	0.126	0.125	0.122	0.13	0.124	0.125	0.125	0.139	0.108
G	0.128	0.127	0.131	0.124	0.127	0.126	0.125	0.128	0.124	0.129	0.124	0.139
H	0.133	0.127	0.128	0.127	0.126	0.131	0.128	0.126	0.132	0.124	0.156	0.162

2. Result of antibody test of hybridoma cells from mice A2 were produced antibody against PEDV after fusion.

plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.107	0.121	0.127	0.134	0.103	0.106	0.111	0.136	0.14	0.139	0.136	0.106
B	0.12	0.122	0.185	0.154	0.118	0.112	0.119	0.125	0.128	0.165	0.128	0.125
C	0.201	0.124	0.14	0.158	0.115	0.112	0.11	0.115	0.147	0.129	0.134	0.124
D	0.188	0.153	0.275	0.182	0.161	0.179	0.125	0.155	0.167	0.154	0.178	0.15
E	0.134	0.169	0.159	0.197	0.114	0.13	0.125	0.144	0.126	0.18	0.146	0.149
F	0.102	0.099	0.107	0.109	0.105	0.108	0.106	0.107	0.131	0.131	0.115	0.121
G	0.107	0.101	0.095	0.092	0.102	0.101	0.106	0.096	0.124	0.126	0.12	0.122
H	0.12	0.161	0.101	0.096	0.114	0.112	0.112	0.118	0.135	0.125	0.126	0.27

plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.096	0.117	0.114	0.11	0.104	0.109	0.115	0.13	0.119	0.115	0.125	0.13
B	0.098	0.104	0.117	0.13	0.099	0.103	0.107	0.116	0.111	0.118	0.124	0.131
C	0.173	0.123	0.114	0.162	0.105	0.15	0.108	0.12	0.113	0.123	0.128	0.134
D	0.108	0.117	0.126	0.112	0.105	0.112	0.112	0.125	0.116	0.113	0.128	0.128
E	0.102	0.1	0.099	0.111	0.102	0.102	0.106	0.105	0.11	0.128	0.114	0.13
F	0.101	0.099	0.095	0.115	0.116	0.145	0.11	0.102	0.102	0.107	0.115	0.12
G	0.105	0.103	0.115	0.099	0.099	0.111	0.11	0.145	0.106	0.119	0.111	0.117
H	0.104	0.128	0.1	0.104	0.098	0.104	0.104	0.111	0.108	0.115	0.123	0.113

plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.101	0.098	0.1	0.108	0.099	0.096	0.103	0.11	0.106	0.12	0.133	0.124
B	0.125	0.108	0.099	0.104	0.096	0.096	0.092	0.106	0.097	0.113	0.114	0.119
C	0.171	0.102	0.107	0.111	0.102	0.101	0.096	0.102	0.104	0.109	0.119	0.12
D	0.119	0.118	0.115	0.122	0.106	0.101	0.101	0.109	0.115	0.116	0.123	0.123
E	0.108	0.106	0.102	0.148	0.103	0.099	0.101	0.118	0.105	0.108	0.134	0.107
F	0.097	0.094	0.103	0.104	0.103	0.099	0.106	0.111	0.098	0.107	0.106	0.105
G	0.099	0.089	0.12	0.096	0.095	0.094	0.096	0.1	0.098	0.105	0.102	0.103
H	0.095	0.098	0.101	0.113	0.096	0.092	0.098	0.107	0.104	0.112	0.117	0.106

Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	0.104	0.1	0.103	0.043	0.104	0.102	0.106	0.123	0.107	0.108	0.106	0.122
B	0.106	0.095	0.096	0.043	0.095	0.096	0.1	0.121	0.104	0.112	0.106	0.114
C	0.193	0.11	0.091	0.044	0.099	0.108	0.104	0.118	0.108	0.107	0.107	0.117
D	0.094	0.094	0.079	0.044	0.099	0.099	0.098	0.115	0.108	0.108	0.111	0.13
E	0.103	0.098	0.095	0.047	0.127	0.109	0.103	0.104	0.11	0.105	0.102	0.12
F	0.099	0.095	0.094	0.044	0.092	0.098	0.105	0.117	0.094	0.101	0.093	0.112
G	0.102	0.099	0.098	0.045	0.098	0.097	0.098	0.111	0.109	0.117	0.104	0.121
H	0.09	0.096	0.092	0.043	0.101	0.102	0.105	0.111	0.109	0.111	0.127	0.124

plate 5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.094	0.096	0.099	0.101	0.097	0.099	0.114	0.1	0.12	0.124	0.124	0.056
B	0.098	0.099	0.098	0.104	0.102	0.097	0.103	0.111	0.108	0.108	0.107	0.123
C	0.192	0.11	0.096	0.111	0.099	0.106	0.104	0.097	0.108	0.117	0.112	0.124
D	0.097	0.096	0.101	0.105	0.095	0.095	0.097	0.102	0.104	0.102	0.111	0.146
E	0.1	0.095	0.107	0.099	0.095	0.093	0.096	0.099	0.099	0.098	0.113	0.134
F	0.101	0.109	0.095	0.118	0.088	0.088	0.093	0.099	0.093	0.108	0.102	0.124
G	0.095	0.092	0.103	0.114	0.094	0.091	0.097	0.093	0.097	0.106	0.102	0.122
H	0.096	0.095	0.093	0.102	0.094	0.091	0.099	0.103	0.097	0.098	0.1	0.128

plate 6	1	2	3	4	5	6	7	8	9	10	11	12
A	0.112	0.158	0.109	0.104	0.11	0.108	0.117	0.142	0.126	0.1	0.111	0.131
B	0.11	0.142	0.125	0.159	0.101	0.123	0.101	0.123	0.125	0.137	0.113	0.115
C	0.118	0.052	0.141	0.118	0.101	0.12	0.103	0.12	0.124	0.136	0.119	0.124
D	0.114	0.108	0.099	0.108	0.134	0.111	0.101	0.116	0.119	0.119	0.111	0.117
E	0.114	0.098	0.1	0.11	0.101	0.115	0.108	0.106	0.108	0.116	0.127	0.113
F	0.125	0.131	0.106	0.092	0.098	0.11	0.104	0.099	0.101	0.106	0.108	0.103
G	0.098	0.101	0.098	0.092	0.103	0.1	0.098	0.098	0.108	0.107	0.1	0.102
H	0.102	0.094	0.098	0.096	0.1	0.104	0.102	0.108	0.109	0.106	0.1	0.114

plate 7	1	2	3	4	5	6	7	8	9	10	11	12
A	0.142	0.118	0.116	0.096	0.109	0.164	0.108	0.101	0.114	0.12	0.144	0.17
B	0.111	0.11	0.113	0.111	0.127	0.157	0.107	0.112	0.118	0.126	0.146	0.155
C	0.122	0.112	0.117	0.117	0.143	0.193	0.131	0.14	0.124	0.121	0.157	0.136
D	0.137	0.128	0.134	0.136	0.147	0.165	0.145	0.132	0.123	0.125	0.15	0.134
E	0.121	0.126	0.111	0.11	0.128	0.173	0.122	0.121	0.135	0.127	0.175	0.149
F	0.121	0.11	0.109	0.1	0.228	0.382	0.126	0.114	0.155	0.139	0.128	0.139
G	0.126	0.106	0.116	0.102	0.113	0.155	0.125	0.109	0.125	0.263	0.197	0.13
H	0.112	0.12	0.127	0.109	0.126	0.143	0.118	0.112	0.138	0.133	0.134	0.131

3. Result of antibody test of hybridoma cells from A1/A2 were produced antibody against PEDV from limiting dilution 1.

plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.083	0.071	0.074	0.068	0.072	0.068	0.072	0.07	0.093	0.07	0.075	0.073
B	0.074	0.089	0.086	0.076	0.079	0.07	0.072	0.068	0.076	0.076	0.076	0.071
C	0.072	0.08	0.083	0.071	0.074	0.072	0.074	0.072	0.072	0.072	0.075	0.072
D	0.075	0.078	0.077	0.075	0.078	0.076	0.073	0.067	0.076	0.072	0.074	0.072
E	0.071	0.071	0.075	0.081	0.077	0.077	0.073	0.07	0.072	0.081	0.074	0.072
F	0.082	0.071	0.07	0.073	0.073	0.069	0.073	0.066	0.073	0.073	0.079	0.071
G	0.103	0.073	0.072	0.106	0.072	0.07	0.077	0.069	0.081	0.074	0.079	0.072
H	0.075	0.073	0.089	0.071	0.071	0.071	0.07	0.077	0.073	0.071	0.078	0.072

plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.085	0.068	0.072	0.072	0.074	0.067	0.081	0.073	0.082	0.072	0.072	0.069
B	0.075	0.068	0.072	0.071	0.075	0.08	0.079	0.071	0.076	0.074	0.073	0.075
C	0.069	0.067	0.071	0.081	0.072	0.226	0.074	0.07	0.086	0.073	0.178	0.071
D	0.072	0.077	0.077	0.07	0.073	0.073	0.068	0.071	0.076	0.074	0.075	0.078
E	0.075	0.072	0.075	0.067	0.07	0.069	0.071	0.08	0.116	0.062	0.075	0.072
F	0.079	0.072	0.069	0.069	0.073	0.075	0.122	0.077	0.145	0.074	0.079	0.072
G	0.08	0.071	0.076	0.192	0.065	0.06	0.12	0.076	0.14	0.075	0.111	0.073
H	0.074	0.07	0.075	0.072	0.073	0.073	0.072	0.185	0.073	0.074	0.075	0.071

plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.087	0.067	0.069	0.067	0.069	0.066	0.073	0.067	0.072	0.068	0.071	0.072
B	0.07	0.076	0.075	0.067	0.068	0.066	0.107	0.068	0.069	0.071	0.071	0.069
C	0.068	0.065	0.067	0.067	0.076	0.111	0.247	0.064	0.068	0.067	0.07	0.067
D	0.076	0.07	0.073	0.101	0.177	0.188	0.158	0.078	0.071	0.078	0.07	0.069
E	0.084	0.07	0.075	0.067	0.078	0.201	0.068	0.067	0.071	0.067	0.08	0.071
F	0.088	0.067	0.077	0.066	0.072	0.133	0.106	0.134	0.068	0.066	0.069	0.068
G	0.072	0.067	0.068	0.065	0.068	0.085	0.14	0.087	0.068	0.068	0.069	0.069
H	0.073	0.067	0.069	0.068	0.068	0.068	0.068	0.077	0.07	0.067	0.069	0.069

plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	0.08	0.069	0.073	0.068	0.07	0.067	0.076	0.068	0.071	0.068	0.072	0.07
B	0.07	0.103	0.205	0.157	0.147	0.138	0.149	0.153	0.181	0.165	0.128	0.069
C	0.095	0.197	0.155	0.176	0.18	0.194	0.176	0.155	0.173	0.189	0.272	0.069
D	0.096	0.188	0.21	0.186	0.066	0.178	0.233	0.191	0.227	0.142	0.171	0.07
E	0.088	0.159	0.127	0.101	0.205	0.169	0.246	0.135	0.242	0.143	0.104	0.068
F	0.072	0.146	0.207	0.164	0.122	0.157	0.17	0.14	0.151	0.151	0.156	0.07
G	0.078	0.069	0.118	0.19	0.168	0.159	0.162	0.136	0.162	0.154	0.159	0.069
H	0.131	0.082	0.07	0.069	0.07	0.069	0.071	0.068	0.076	0.067	0.069	0.065

plate 5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.095	0.069	0.074	0.079	0.078	0.072	0.079	0.075	0.075	0.073	0.079	0.084
B	0.074	0.07	0.078	0.072	0.073	0.07	0.077	0.076	0.078	0.077	0.078	0.077
C	0.076	0.071	0.079	0.071	0.072	0.071	0.072	0.077	0.075	0.074	0.076	0.075
D	0.081	0.078	0.084	0.071	0.071	0.07	0.075	0.07	0.074	0.078	0.075	0.074
E	0.101	0.08	0.075	0.068	0.074	0.073	0.071	0.071	0.074	0.084	0.082	0.072
F	0.081	0.07	0.074	0.072	0.07	0.069	0.074	0.07	0.074	0.071	0.075	0.07
G	0.099	0.086	0.08	0.07	0.175	0.069	0.073	0.073	0.075	0.072	0.071	0.075
H	0.102	0.072	0.078	0.077	0.074	0.071	0.085	0.074	0.079	0.074	0.076	0.083

4. Result of antibody test of hybridoma cells from D3 were produced antibody against PEDV from limiting dilution 1.

plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.51	1.12	0.699	0.058	0.846	0.66	0.068	0.729	0.722	0.693	0.786	0.76
B	0.707	0.944	0.837	0.886	0.753	0.667	0.574	0.738	0.523	1.024	0.479	0.819
C	0.721	1.164	0.961	0.875	1.175	0.853	0.401	0.63	0.489	0.568	0.426	0.842
D	0.66	1.023	0.776	0.865	0.839	0.718	0.556	0.481	0.578	0.721	0.657	0.619
E	0.777	1.042	1.024	1.037	1.5	1.092	0.855	0.885	0.505	0.654	0.318	0.908
F	0.905	0.855	1.067	1.281	1.023	1.152	0.852	0.96	0.679	0.453	0.841	0.829
G	1.015	1.218	0.877	1.354	1.232	1.293	0.81	0.841	0.676	0.667	0.63	0.86
H	1.084	1.574	1.061	1.346	0.875	1.277	0.993	1.038	0.69	0.964	0.729	1.225

plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.569	0.606	0.502	0.666	0.646	0.5	0.548	0.617	0.656	0.626	0.679	0.581
B	0.631	0.617	0.723	0.628	0.499	0.465	0.518	0.71	0.652	0.578	0.546	0.73
C	0.725	0.642	0.587	0.584	0.485	0.538	0.418	0.414	0.608	0.655	0.637	0.729
D	0.818	0.637	0.529	0.681	0.453	0.496	0.435	0.466	0.509	0.57	0.593	0.537
E	0.531	0.746	0.562	0.616	0.444	0.648	0.445	0.508	0.633	0.562	0.627	0.765
F	0.676	0.67	0.529	0.52	0.561	0.649	0.777	0.817	0.504	0.7	0.608	0.824
G	0.559	0.889	0.594	0.634	0.691	0.57	0.484	0.599	0.523	0.618	0.566	0.574
H	0.569	0.687	0.728	0.767	0.619	0.527	0.526	0.489	0.622	0.634	0.637	0.599

plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.672	0.635	0.592	0.564	0.604	0.619	0.513	0.554	0.569	0.514	0.561	0.565
B	0.652	0.707	0.718	0.533	0.618	0.481	0.688	0.447	0.439	0.426	0.45	0.307
C	0.74	0.815	0.72	0.49	0.754	0.478	0.537	0.424	0.553	0.42	0.523	0.382
D	0.842	0.802	0.764	0.672	0.609	0.462	0.507	0.458	0.518	0.455	0.617	0.339
E	0.781	0.685	0.686	0.558	0.569	0.491	0.497	0.473	0.506	0.393	0.417	0.396
F	0.829	0.736	0.89	0.599	0.954	0.503	0.529	0.412	0.401	0.396	0.452	0.34
G	0.807	0.735	0.84	0.607	0.994	0.606	0.471	0.53	0.536	0.451	0.432	0.355
H	0.963	0.876	0.898	0.828	0.905	0.723	0.537	0.517	0.524	0.581	0.456	0.467

plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	0.359	0.391	0.322	0.312	0.413	0.44	0.478	0.391	0.369	0.417	0.383	0.595
B	0.328	0.301	0.308	0.286	0.392	0.291	0.504	0.39	0.284	0.381	0.324	0.296
C	0.351	0.309	0.349	0.272	0.424	0.405	0.473	0.427	0.246	0.326	0.394	0.327
D	0.378	0.335	0.294	0.289	0.408	0.459	0.472	0.487	0.294	0.324	0.391	0.311
E	0.337	0.346	0.293	0.276	0.466	0.296	0.49	0.323	0.357	0.387	0.358	0.373
F	0.441	0.412	0.559	0.299	0.797	0.434	0.546	0.519	0.417	0.468	0.417	0.374
G	0.401	0.405	0.531	0.455	0.577	0.462	0.601	0.512	0.466	0.393	0.458	0.42
H	0.77	0.448	0.556	0.601	0.553	0.622	0.649	0.704	0.587	0.506	0.67	0.545

5. Result of production of hybridoma from A1/A2 and D3 secreting antibody highly specific with PEDV.

	1	2	3
A	0.794	0.621	0.472
B	0.39	0.43	0.374
C	0.484	0.468	0.582
D	0.562	0.605	0.683
E	0.567	0.91	0.95
F	1.006	0.849	0.926
G	0.187	0.405	0.37
H	0.265	0.429	0.568

6. Result of antibody test of hybridoma cells from A1/A2 and D3 were produced

P1D3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.23	0.157	0.247	0.186	0.268	0.215	0.291	0.246	0.221	0.159	0.131	0.141
B	0.152	0.169	0.216	0.223	0.406	0.181	0.219	0.159	0.206	0.184	0.153	0.144
C	0.314	0.198	0.227	0.25	0.259	0.269	0.239	0.177	0.24	0.2	0.185	0.146
D	0.308	0.174	0.202	0.211	0.218	0.23	0.253	0.239	0.237	0.201	0.17	0.203
E	0.21	0.333	0.247	0.261	0.265	0.202	0.241	0.218	0.263	0.19	0.174	0.181
F	0.318	0.239	0.396	0.492	0.31	0.191	0.249	0.194	0.292	0.202	0.174	0.147
G	0.244	0.207	0.501	0.207	0.292	0.327	0.234	0.203	0.261	0.206	0.16	0.196
H	0.195	0.33	0.29	0.196	0.274	0.283	0.373	0.301	0.189	0.188	0.173	0.235

P2D3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.248	0.187	0.339	0.193	0.273	0.233	0.24	0.227	0.256	0.197	0.299	0.233
B	0.239	0.146	0.29	0.184	0.277	0.216	0.232	0.202	0.311	0.244	0.233	0.21
C	0.311	0.203	0.315	0.291	0.255	0.206	0.257	0.205	0.318	0.27	0.228	0.239
D	0.354	0.233	0.375	0.34	0.298	0.274	0.324	0.274	0.27	0.284	0.29	0.272
E	0.316	0.27	0.295	0.31	0.307	0.21	0.24	0.18	0.338	0.278	0.194	0.287
F	0.341	0.188	0.501	0.234	0.29	0.238	0.304	0.204	0.3	0.274	0.264	0.243
G	0.567	0.213	0.302	0.237	0.293	0.23	0.246	0.158	0.275	0.253	0.276	0.244
H	0.297	0.242	0.244	0.331	0.33	0.26	0.281	0.219	0.308	0.352	0.283	0.367

P3A	1	2	3	4	5	6	7	8	9	10	11	12
A	0.226	0.167	0.188	0.216	0.198	0.259	0.258	0.224	0.314	0.243	0.23	0.224
B	0.224	0.168	0.196	0.194	0.291	0.271	0.243	0.195	0.293	0.204	0.243	0.199
C	0.229	0.193	0.268	0.227	0.342	0.303	0.238	0.196	0.35	0.275	0.254	0.19
D	0.333	0.207	0.237	0.261	0.305	0.212	0.222	0.221	0.251	0.178	0.166	0.188
E	0.313	0.24	0.268	0.286	0.275	0.22	0.227	0.165	0.289	0.205	0.187	0.217
F	0.22	0.23	0.276	0.2	0.338	0.221	0.234	0.161	0.207	0.163	0.212	0.171
G	0.228	0.242	0.296	0.192	0.302	0.212	0.206	0.155	0.237	0.166	0.178	0.183
H	0.276	0.316	0.326	0.189	0.272	0.21	0.214	0.19	0.275	0.486	0.211	0.371

P4A	1	2	3	4	5	6	7	8	9	10	11	12
A	0.167	0.264	0.302	0.202	0.208	0.195	0.244	0.209	0.261	0.172	0.192	0.174
B	0.207	0.163	0.127	0.151	0.144	0.136	0.173	0.145	0.203	0.164	0.172	0.183
C	0.222	0.229	0.216	0.28	0.364	0.163	0.182	0.137	0.213	0.136	0.162	0.178
D	0.151	0.308	0.196	0.398	0.269	0.231	0.34	0.164	0.283	0.144	0.287	0.177
E	0.179	0.223	0.249	0.15	0.277	0.179	0.271	0.157	0.185	0.146	0.151	0.146
F	0.145	0.2	0.149	0.17	0.221	0.254	0.262	0.125	0.311	0.15	0.162	0.164
G	0.117	0.163	0.253	0.13	0.508	0.195	0.264	0.144	0.224	0.164	0.165	0.198
H	0.214	0.329	0.154	0.133	0.205	0.353	0.325	0.175	0.357	0.172	0.183	0.266

P5A	1	2	3	4	5	6	7	8	9	10	11	12
A	0.142	0.13	0.152	0.127	0.142	0.133	0.129	0.161	0.214	0.2	0.199	0.176
B	0.152	0.139	0.385	0.132	0.129	0.123	0.238	0.143	0.146	0.166	0.212	0.322
C	0.167	0.158	0.131	0.127	0.135	0.149	0.133	0.146	0.152	0.134	0.242	0.284
D	0.183	0.162	0.153	0.115	0.135	0.116	0.129	0.128	0.137	0.135	0.143	0.136
E	0.442	0.172	0.169	0.126	0.212	0.135	0.137	0.13	0.148	0.168	0.142	0.164
F	0.194	0.159	0.172	0.166	0.16	0.128	0.124	0.114	0.135	0.128	0.195	0.265
G	0.253	0.165	0.162	0.166	0.208	0.161	0.133	0.133	0.192	0.142	0.154	0.187
H	0.259	0.218	0.179	0.159	0.227	0.153	0.241	0.183	0.224	0.148	0.229	0.279

7. Result of subclass of hybridoma cells.

	P1D3L1	P1D3L2	P1D3L3	P2D3L1	P2D3L2	P3AL1	P4AL1	P5AL1
IgG_1	0.367	0.182	0.177	0.152	0.223	0.231	0.216	0.145
IgG_{2a}	0.271	0.429	0.182	0.181	0.19	0.167	0.21	0.15
IgG_{2b}	0.139	0.191	0.156	0.141	0.144	0.189	0.198	0.217
IgG_3	0.121	0.096	0.118	0.133	0.124	0.113	0.159	0.146

APPENDIX B

Poster presentation

1. Ritthisan P., Asawakarn T., Wathanaponsark S., Piyaviriyakul P. and Sarikaputi M. A New purification technique for porcine epidemic diarrhea virus isolation. The 13th Chulalongkorn university veterinary conference 2014, Thailand. 12-14 May 2014.

Publication

1. Preparation of hybridoma cells for porcine epidemic diarrhea virus monoclonal antibody production. (manuscript in preparation)
2. Modified purification technique for PEDV isolation. (manuscript in preparation).

VITA

Miss Panwad Ritthisan was born on December 21th, 1988 in Rachaburi,Thailand. In 2006, she graduated from Ratchaburi in Mathematics-Science program with the grade point average of 3.17 from Benjamarachutit. In 2011, she was graduated with Bachelor degree of Science program (Second Class Honors) in Biology from the Faculty of Science, Silpakorn University, Nakhon pathom, Thailand. She worked as research assistant at R&D, quality and control in Pharmacy and Science Technology, Faculty of Pharmacy, Silpakorn University for 6 months before she got scholarship from Chulalongkorn University for study in Master program at Biochemistry Unit, Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. She has been the Student President in Primary Education, Thai Dance Club President in high school. She got the student of the year in 2000 from sheriff and, in 2004, from the Provincial Governor. Recently, she was a volunteer of The Thai Red Cross. Miss Panwad is interested in Immunology and Molecular biology. Her research in Bachelor's degree is "Using DNA barcoding to identify medicinal plant species within Plumbago for doing the DNA barcode to be the database" and her research in Master degree is "Preparation of hybridoma cells for Porcine epidemic diarrhea virus monoclonal antibody production".