THE DEVELOPMENT OF ELISA KIT TO DETECT ANTIBODY RESPONSE AGAINST PORCINE EPIDEMIC DIARRHEA VIRUS FROM COLOSTRUM AND MILK

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การพัฒนาชุดทดสอบอีไลซาเพื่อใช้ในการตรวจแอนติบอดีต่อเชื้อไวรัส Porcine epidemic diarrhea จากนมน้ำเหลืองและน้ำนม

น.ส.อัญชลี ศรีจังหวัด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Field of Study	Veterinary Pathobiology
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อัญชลี ศรีจังหวัด : การพัฒนาชุดทดสอบอีไลซาเพื่อใช้ในการตรวจแอนติบอดีต่อเชื้อไวรัส Porcine epidemic diarrhea จากนมน้ำเหลืองและน้ำนม. (THE DEVELOPMENT OF ELISA KIT TO DETECT ANTIBODY RESPONSE AGAINST PORCINE EPIDEMIC DIARRHEA VIRUS FROM COLOSTRUM AND MILK) อ.ที่ปรึกษาหลัก : ผศ. น.สพ. ดร.เดชฤทธิ์ นิลอุบล

โรคพีอีดีเป็นโรคที่เกิดจากเชื้อ Porcine epidemic diarrhea virus (ไวรัสพีอีดี) ทำให้สุกรมีอาการท้องเสียและพบอัตราการตายสูงในลูกสุกรที่มีอายุต่ำกว่า 1 สัปดาห์ การระบาดของโรคส่งผลให้เกิดความเสียหายทางเศรษฐกิจโดยเฉพาะในประเทศที่มีอุตสาหกรรมการเลี้ยงสุกร วิธีการป้องกันการระบาดและติดเชื้อมีหลายวิธีโดยเฉพาะการกระตุ้นภูมิคุ้มกันในแม่สู่ลูกโดยส่งผ่านทางนมน้ำเหลืองหรือน้ำนม ดังนั้นการตรวจสอบระดับแอนติบอดีเพื่อทราบถึงสถานการณ์ในฟาร์มจึงเป็นสิ่งที่จำเป็น วิธีการที่สะดวกและรวดเร็วกว่าวิธีไวรอลนิวทราลไลเซชัน คือวิธีเอนไซม์ลิงค์อิมมูโนซอร์เบนต์แอสเสย์ (อีไลซา) การศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อพัฒนาชุดทดสอบอีไลซาเพื่อใช้ตรวจแอนติบอดีต่อเชื้อไวรัสพีอีดีจากนมน้ำเหลืองและน้ำนมสุกร โดยเตรียมแอนติเจนจากโปรตีนลูกผสมของยีนสไปค์ของเชื้อไวรัสพีอีดี ซึ่งมีบทบาทในการกระตุ้นการสร้างแอนติบอดีที่ยับยั้งเชื้อไวรัสพีอีดี และทดสอบความใช้ได้ของชุดทดสอบอีไลซาสำหรับการนำไปใช้ตรวจหาแอนติบอดีต่อเชื้อไวรัสพีอีดีจากตัวอย่างบวกจากวิธีไวรอลนิวทราลไลเซชัน 250 ตัวอย่าง และตัวอย่างลบจากวิธีไวรอลนิวทราลไลเซชัน 250 ตัวอย่าง ผลจากการศึกษาพบว่าสามารถผลิตโปรตีนลูกผสมที่ เรียกว่า เอส12 ซึ่งเป็นโปรตีนที่มีความจำเพาะต่อแอนติบอดีที่ยับยั้งเชื้อไวรัสพีอีดี และเป็นโปรตีนส่วนที่ไม่มีความหลากหลายของสายพันธุกรรม โปรตีนมีขนาด 42 กิโลดาลตัน สามารถทำโปรตีนให้บริสุทธิ์ได้ทั้งการทำบริสุทธิ์โปรตีนลูกผสมกลูตาไธโอนหรือโปรตีนลูกผสมที่มีกรดอะมิโนฮีสทิดีน โปรตีนเอส12 สามารถนำมาใช้เป็นแอนติเจนเพื่อเคลือบเพลทอีไลซาที่ความเข้มข้น 10 และ 20 ไมโครกรัมต่อมิลลิลิตร สำหรับทดสอบแอนติบอดีชนิดอิมมูโนโกลบูลิน จี และ เอ ตามลำดับ นอกจากนี้ได้ทำการเตรียมสูตรผสมที่เหมาะสมสำหรับชุดทดสอบ และทดสอบค่าความแม่นยำโดยการตรวจสอบความสามารถในการทวนซ้ำพบว่ามีค่าอยู่ในเกณฑ์ที่ยอมรับได้ มีค่าสัมประสิทธิ์ความผันแปรต่ำกว่าร้อยละ 10 และมีค่าความสามารถในการทำซ้ำสูงเมื่อเปรียบเทียบผลระหว่างห้องปฏิบัติการ การทดสอบความสัมพันธ์ระหว่างการตรวจด้วยวิธีมาตรฐานกับการตรวจด้วยชุดทดสอบได้ค่าสัมประสิทธิ์สหสัมพันธ์สูงจากทั้งการตรวจอิมมูโนโกลบูลิน จี และ เอ จึงอาจกล่าวได้ว่าค่าที่วัดได้มีความสอดคล้องกับค่าความคุ้มโรค กำหนดจุดตัดของชุดทดสอบสำหรับค่าการดูดกลืนแสงที่ 0.5 ทำให้ได้ค่าความไว ความจำเพาะและความถูกต้องของการตรวจอิมมูโนโกลบูลิน จี และ เอ เท่ากับ 96.0 95.6 95.8 และ 94.8 95.6 95.2 ตามลำดับ และสำหรับจุดตัดของสัดส่วนค่าดูดกลืนแสงของตัวอย่างต่อตัวอย่างควบคุมบวกที่ 0.4 มีค่าความไว ความจำเพาะและความถูกต้องของการตรวจอิมมูโนโกลบูลิน จี และ เอ เท่ากับ 95.2 90.8 93 และ 91.6 93.2 92.0 ตามลำดับ ชุดทดสอบอีไลซามีอายุการจัดเก็บนาน 6 เดือน นอกจากนี้จากการที่ได้ทดลองนำชุดทดสอบมาตรวจหาแอนติบอดีจากแม่สุกรที่ได้รับการป้อนลำไส้ลูกสุกรที่ติดเชื้อ ผลการศึกษาแสดงให้เห็นว่าชุดทดสอบอีไลซาสามารถนำมาใช้งานได้จริง ดังนั้นจากผลการศึกษาทั้งหมดจึงสรุปได้ว่าชุดทดสอบอีไลซานี้สามารถใช้เป็นอีกทางเลือกหนึ่งสำหรับนำไปใช้ทดสอบแอนติบอดีต่อเชื้อไวรัสพีอีดีในตัวอย่างนมน้ำเหลืองและน้ำนม

สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อนิสิต .....
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KEYWORD: ANTBODY/ COLOSTRUM/ ENZYME LINKED IMMUNOSORBENT ASSAY/ MILK/ PORCINE EPIDEMIC DIARRHEA VIRUS/ TEST KIT

Anchalee Srijangwad : THE DEVELOPMENT OF ELISA KIT TO DETECT ANTIBODY RESPONSE AGAINST PORCINE EPIDEMIC DIARRHEA VIRUS FROM COLOSTRUM AND MILK. Advisor: Asst. Prof. DACHRIT NILUBOL, D.V.M., M.Sc., Ph.D.

Porcine epidemic diarrhea is caused by porcine epidemic diarrhea virus (PEDV), which causes swine watery diarrhea and higher mortality rates in piglets less than one week of age. Outbreaks of PED affect to economic loss, especially in a swine industrial country. Many strategies were used for preventing the outbreak and infection of PEDV, especially maternal antibody stimulation in pigs and then transfer to piglet through colostrum or milk. Thus, the investigation of antibody level for the situation on the farm is required. The method which convenient and rapid than viral neutralization (VN) test is an enzyme-linked immunosorbent assay (ELISA). The purpose of this study was to develop ELISA test kits to detect antibodies to PEDV from colostrum and milk of pig by preparing antigens of recombinant protein from the spike gene of PEDV and then a validation test of the ELISA test kit was performed from 250 VN positive and 250 VN negative samples. The results of the study showed that ability to produce the protein called S12. The protein is specific to neutralizing antibodies of PEDV and produced from a conserve region of the PEDV gene. S12 protein is 42 kDa of protein size, can be purified either by purification of glutathione S-transferase (GST) gene fusion proteins or histidine-tag protein. Protein S12 can be used as antigen to coat ELISA plate at 10 µg/ml and 20 µg/ml for immunoglobulin (Ig) G and IgA investigation, respectively. In addition, suitable formulations for the test kit were prepared. The precision was verified by the repeatability test, coefficient of variation is less than 10% and has high reproducibility of inter-laboratory results. The correlation between the standardized and ELISA showed strong correlation coefficients from both IgG and IgA. Thus, it can be said that the result is consistent with protection. The cutoff point of optimal density is 0.5, DSN, DSP and accuracy of IgG and IgA were 96.0, 95.6, 95.8 and 94.8, 95.6, 95.2, respectively. As for the cutoff level of the sample to positive ratio (S/P ratio) is 0.4, DSN, DSP and accuracy of IgG and IgA were 95.2, 90.8, 93.0 and 91.6, 93.2, 92.0, respectively. Long term storage of the ELISA kit up to 6 months. In addition, the test kit was used to detect antibodies from feedback and the results show that the ELISA test kit can be used. According to all this study, it can conclude that this ELISA kit can be an alternative possibility for detecting antibody response against PEDV in colostrum and milk.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	percent
%CV	percentage of coefficient of variation
°C	degree Celsius
µg	microgram
µl	microliter
aa	amino acid
BSA	bovine serum albumin
C2H3NaO3	Sodium methyl carbonate
CV	corrective value
DI	deionized
DMSO	Dimethyl sulfoxide
DPE	days post-exposure
DPP	days post-parturition
DSN	diagnostic sensitivity
DSP	diagnostic specificity
E	envelope
ELISA	Enzyme linkage immunosorbent assay
FFN	fluorescent focus neutralization
FMIA	fluorescent microsphere immunoassays
GST	glutathione S-transferase
h	hour
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HRP	horseradish peroxidase enzyme

IgA	immunoglobulin A
IgG	immunoglobulin G
IPTG	isopropyl-ß-D-thiogalactopyranoside
kDa	kilodalton
M	membrane
mA	milliamps
min	minute
ml	milliliter
mM	millimolar
N	nucleocapsid
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Sodium hydrogen phosphate
OD	optical density
ORFs	open reading frames
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing 0.05% Tween-20
PEDV	porcine epidemic diarrhea
P/N	positive to negative ratio
PDcoV	porcine deltacoronavirus
rN	recombinant nucleocapsid protein
RNA	ribonucleic acid
ROC	receiver-operating characteristic
RT-PCR	Reverse transcription polymerase chain reaction
rS	Recombinant spike protein
S	spike

SD	standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SN	serum neutralization test
S/P	sample to positive ratio
TGEV	transmissible gastroenteritis coronavirus
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
TCID	tissue culture infective dose
VN	viral neutralization
w	with
w/o	without

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Porcine epidemic diarrhea virus (PEDV) causes porcine epidemic diarrhea in pigs. PEDV is a member of *Alphacoronavirus* in the family of *Coronaviridae*. The genome of PEDV is positive-sense, single stranded RNA. Genetic structure of PEDV contains 7 open reading frames (ORFs) encoding a viral protein, ORF1a, ORF1b, ORF3, spike (S), envelope (E), matrix or membrane (M) and nucleocapsid (N) (Song et al., 2015). PEDV was discovered in 1976 in England from fecal sample of young pig and then PEDV was detected in 1982 in Japan that was the first discovery of PEDV in Asia. PEDV outbreak has been reported in Thailand since 2007 until now (Puranaveja et al., 2009). Disease of porcine epidemic diarrhea has been divided into 2 characteristics: severe watery diarrhea with a low mortality rate, that usually occurs in pig more than 4-5 weeks of age and severe diarrhea in all ages of pig, and mortality and morbidity rate up to 100 %, especially piglet lower than 1 week of age (Pensaert and de Bouck, 1978).

PEDV transmit to other pigs through oral-nasal route. Subsequently, PEDV propagate in small intestinal and extremely of large intestine where is primary site of replication. Epithelial cells that are infected with PEDV occurred villous atrophy that effect to watery diarrhea occurrence and might be dying because of dehydration in piglets. Majority of immune responses due to PEDV infection usually is mucosal immunity, especially, immunoglobulin A (IgA) antibody secreting cells that are highly secreted in the lamina propria (de Arriba et al., 2002a). Moreover, cell mediate immunity play an important role in viral protection since the lymphocyte proliferative response in mesenteric lymph node increased after viral infection (de Arriba et al., 2002a, b; Song et al., 2007). The antibody response against PEDV found that antibody titer can be detected by viral neutralization and indirect fluorescent assays in serum at 1 – 2 weeks post inoculation.

The highest antibody titer of PEDV is founded around 4-5 weeks post inoculation (de Arriba et al., 2002b).

At present, disease of PED become endemic disease in Thailand pig farm. Many herds have experienced recurrent outbreaks of the disease at least once a year, especially in primiparous sows. The suggestion to following the outbreak and to control PEDV by planned exposure of all sows in the herd with minced intestines of PEDV-infected pigs to induce immunity were successful (Nilubol and Khatiworavage, 2012a, b). However, a pig owner cannot determine whether these replacement gilts are infected by PEDV and have immunity, because they do not have a method to verify that clearly. Although viral neutralization test is a serological test method for antibody titer detection in veterinary laboratory, it has many limitations, such as time-consuming process, expert reader, sterile technique, and several materials. An enzyme linkage immunosorbent assay (ELISA) development is a good choice for antibody detection procedure because it is convenient method and rapid test. It has been continuously improved and develop for antibody detection against PEDV. In 1990, Hofmann and Wyler tried to cultivate the virus in cell cultures and ultracentrifuge lysis cells for viral protein purification (Hofmann and Wyler, 1990). The protein was used as antigen for ELISA plate coating but found that the high nonspecific background. In 1991, van Nieuwstadt compared two types of ELISA for measuring amount of antibody. There are blocking ELISA, which measures the amount of antibody after blocked with antibodies in serum by using monoclonal type 2 (a double-antibody sandwich ELISA) and another type, fixed-cell ELISA, which measured antibody by using PEDV strain CV777 were cultured in Vero cell as antigen and used immunoglobulin G (IgG) -specific monoclonal antibody as conjugate. The results found that fixed-cell ELISA presented antibody titer higher than blocking ELISA around 5.4 times. Therefore, fixed-cell ELISA was a more suitable method than blocking ELISA. Nevertheless, this method was necessary cell culture technique that convenient method like the VN test method (van Nieuwstadt AP, 1991). After that, significant structural protein of PEDV was selected for use as antigen, including spike protein (S) and nucleocapsid protein (N) the result demonstrated that S-ELISA could be detected antibody response

PEDV in longer period than N-ELISA; however, there also was found that nonspecific background in this ELISA (Knuchel et al., 1992). The ELISA development used interested-protein that was extracted from infected-cells cytoplasm by non-ionic detergent for coating ELISA plate (Oh et al., 2005). This method was compared with serum neutralization test (SN test), the result revealed 89 percent of sensitivity, 94.5 percent of specificity and percentage of overall agreement was 84.2. The results demonstrated that this method appropriate for observation of antibody in pig herd. However, this method resulted in high background and interpretation necessary to calculate corrected value (CV) by deducting mock-infected cell value in every test. At the present ELISA development was focused on antigen specific antibodies. For example, recombinant DNA technology was emphasized production of recombinant nucleocapsid (rN) protein for ELISA plate coating. The rN protein was used as antigen that presented high sensitivity and specificity of ELISA test. Moreover, comparison with comparative method, SN test revealed 88.9 percent of correlation (Hou et al., 2007). However, structural protein of PEDV that can be stimulated neutralizing antibody and binds to a receptor on the host cells were in S protein.

Enzyme linked immunosorbent assay (ELISA) is a technique used to detect antibody or infectious agents in samples. That is rapid test and convenient test for the user because user can be performed antibody detection under non-aseptic technique and the results were read by certified instrument; therefore, ELISA technique should be developed a test kit. However, currently, there are precious few and expensive test kits ready for commercial distribution. Additionally, there is a specification of different viral strains of antigen in difference area influence to limit of detection. That is a motivation to research and develop ELISA test kits for the detection of antibodies against PEDV from the sample of infected-pigs. Moreover, the antibody values that were measured by ELISA test kit should be correlated with protective antibody because there are benefits to pig owners for conductibility of porcine epidemic diarrhea in pig farms.

Thus, the main objective of this study is to develop the ELISA test kit for antibody detection against PEDV by using strongly stimulated neutralizing epitope of S protein as

antigen. The ELISA test kit is going to develop through the process, including antigen preparation, reagent formulation, reagent component optimization, and ELISA test kit validation by comparing with VN test, standard method.

1.2 Literature Review

Genome and structure of PEDV

PED is caused by PED virus (PEDV, an enveloped, positive sense, single-stranded RNA virus belonging to genus *Alphacoronavirus*, family *Coronaviridae* and order *Nidovirales* (Kocherhans et al., 2001). The PEDV genome is approximately 28 kb in length and is composed of 7 open reading frames (ORF) (Kocherhans et al., 2001). The ORF1a and ORF1b are the two non-structural replicate genes covering the two-third of the entire genome. The other 4 ORFs code for 4 major structural proteins including spike (S), envelop (E), membrane (M) and nucleocapsid (N) proteins. ORF3 encodes for accessory protein.

Spike glycoprotein (S) is the envelope in type I glycoprotein of virion. Protein size is approximately 180-220 kDa. It can bind to the receptor binding side of host cell and stimulate neutralizing antibody, moreover the growth adaptation in cell culture and viral attenuation in field study depends on spike protein of isolation (Lee and Lee, 2013). The position of spike gene that presented hypervariability located on N-terminal of spike gene (Lee et al., 2010). Commonly, spike protein is a structural protein consisting of 2 domains, S1 and S2. The S1 domain binds to a receptor on the host cells and contains neutralizing epitopes (Bosch et al., 2003; Chang et al., 2002; Cruz et al., 2006, 2008; Sun et al., 2008). The S2 domain forms a core structure of S protein (Duarte et al., 1994).

Membrane proteins (M) are approximately 27-32 kDa, which are located on the surface of the virus. There are performs an important role in the assembly process in proliferation and maturation of the virus (Pan et al., 2012; Spaan et al., 1988; Zhang et al., 2012). Membrane proteins also play an important role in the immune response by induction of protection and mediating the course of the disease (Zhang et al., 2012).

Envelope Proteins (E) are about 7-8 kDa. It is a small structural protein located on the membrane of the virus, which can be found around the nucleus and on the surface of the infected cell. The protein will assembly with the glycoprotein membrane in the body composition, which is like other coronavirus to assembly of coronavirus-like particles; VLPs (Baudoux et al., 1998; Kuo et al., 2000).

Nucleocapsid protein (N) is approximately 55-58 kDa (Egberink et al., 1988). It arranged in the form of helical nucleotide together with the RNA genome and there is a predominant antigen which is produced in the coronavirus infected cells. Therefore, the cell becomes the primary target of the virus. The N proteins are unable to stimulate the immune response to the PEDV itself because the cell wall is surrounded the virus. N proteins are also important proteins were used in the ELISA development methods for specificity and sensitivity of antibody detection against PEDV (Hou et al., 2007).

Open reading frame 3 (ORF3) is a non-structural protein that has recently been shown to play an important role in proving the severity of the virus (Pan et al., 2012). Because it has ability of cell culture adaptation, viral attenuation and subsequence to use to study the Molecular epidemiology of PEDV vaccine strain (Temeeyasen et al., 2014). Almost of PEDV strain, ORF3 gene is high conserved gene (Jarvis et al., 2016) and use for PEDV infection diagnostic (Wang et al., 2016).

Porcine epidemic diarrhea

Porcine epidemic diarrhea (PED) is a devastating enteric disease characterized by acute severe watery diarrhea that caused by PED virus, a member of viral enteritis in pigs. In addition, the other clinical signs of PED presents vomiting, anorexia, dehydration, and weight loss (Pensaert and de Bouck, 1978). The infection of PEDV can occur in all ages of pigs, including from neonates to sows or boars. However, the severity of PED in any pigs related to the difference of age (Shibata et al., 2000). Significantly, high mortality rate was found in pigs under 7 days of age at which mortality approaches 100% following the outbreak (Chasey and Cartwright, 1978; Pensaert and de Bouck, 1978; Sun et al., 2012) that is commonly induced death from watery diarrhea and dehydration and PDEV

RNA shedding was present in earlier fecal compared to weaned-pig (Annamalai et al., 2015). The consequence of acute diarrhea is malabsorbtion because of loss of absorptive enterocyte and function disorder of the enterocyte. Degeneration of mitochondria effect to out of energy transportation that essential for absorption (Liu et al., 2015). Additionally, the high mortality in PEDV-infected piglet less than 1 week of age because extensive dehydration that resulting from not adequate of reformation of damaged intestinal epithelial cells and migration of mature enterocyte Intestinal (Jung and Saif, 2015). A gross lesions in the small intestines of the necropsies piglets after acute infection of PEDV, which were distended stomach, which containing undigested milk curd, and villus atrophy, which caused the walls of the small intestines to become thin and yellowish fluids accumulate in suckling piglets (Lee et al., 2015; Song et al., 2015). As regard, the clinical sign of PEDV-infected in older pig has a lower severity rate compared to younger pig, such as piglet older than 3 week of age, the clinical signs were not observed within 5 to 10 day post infection (Madson et al., 2014) Moreover, in certain outbreak of PEDV piglet 4-5 weeks of age do not become sick (Pensaert and Yeo, 1994). There might be the factor of the recovery rate of enterocytes of sucking pig longer than the recovery rate of enterocytes of weaning pig that is around 5-7 days and 2-3 days, respectively (Jung and Saif, 2015).

The clinical signs and pathological lesions of PEDV infection are similar to others virus, including transmissible gastroenteritis virus (TGEV), (Jung et al., 2014; Saif et al., 2012) and porcine deltacoronavirus (PDCoV) (Jung et al., 2015). Enteritis caused by a destruction of enterocytes and villous atrophy of the intestinal mucosa, the jejunum and ileum (Debouck and Pensaert, 1980; Jung et al., 2014; Saif et al., 2012). Although, clinical signs of PED very similar to TGE, it is often a slowly spread and lower mortality than TGE in piglets (Pensaert and Yeo, 1994). On the other hand, if PEDV infection occurs toward the end of the growing period, PED would be more severe disease than TGE. However, clinical signs of PEDV is indistinguishable from the others (Debouck and Pensaert, 1980). In contrast to the PDCoV-infected pig in a previous report in Lao, the mortality rate was lower than PEDV infection (Lorsirigool et al., 2016).

Epidemiology of PEDV

In Thailand, PED was first reported in 2007 (Temeeyasen et al., 2014). A subsequent study demonstrated that PED epidemics in Thailand were caused by a variant genetically distinct from CV777, a PEDV prototype isolated in Belgium (Temeeyasen et al., 2014). Its genome contains amino acid insertions and deletions in spike gene similar to a new PEDV variant responsible for PED outbreaks in China in 2011(Li et al., 2012). In 2013, the severe PEDV outbreak was reported in the naïve pig herd in the United States of America that related to PEDV Chinese strain during 2011 to 2012 (Chen et al., 2014; Huang et al., 2013). In North America, including Canada and Mexico was the first report of PEDV (Ojkic et al., 2015). At present, PED in Thailand has become endemic and continues to cause sporadic outbreaks throughout the country.

PED is an enteric disease causing high mortality in pigs during lactation length, suggesting to the need of lactogenic immunity to provide protection that is indicated by many researchers. For instance, inactivated PEDV vaccine, KNU 141112 virus strain was used to intramuscularly immunized sows. About 92% of their piglet are survivals from challenged with high virulence strain and together sows and their offspring have strong neutralizing antibody (Baek et al., 2016). Moreover, the new born piglets were protected from PEDV by recombinant S1 subunit vaccine immunization from sows (Makadiya et al., 2016). Normally, passive lactogenic immunity including IgG and IgA transfer to neonatal piglet through ingestion of colostrum and milk. The dominant immunoglobulin in colostrum is IgG, but IgA is the most predominant in milk. However, passive protection of sows against PEDV and TEGV associate to secretory of IgA in colostrum and milk (Saif et al., 1972). In addition, IgG and IgA immunoglobulin level in serum, colostrum, and milk associated with parity of sow, higher parity sow could have been produced levels of immunity and reduced the mortality rate in piglet higher than lower parity (Cabrera et al., 2012) Ackerman, 2016). Furthermore, the viral dose may be an important factor to immunize sows because of none an effective primary and booster inactivate PEDV vaccine (Langel et al., 2016).

Antibody detection against PEDV

Viral neutralization (VN) assay has been used as a standard method to detect lactogenic immunity raised specifically against PEDV, either in colostrum or milk samples. Although the VN titer can imply the protective level, the assay is laborious and requires a highly skilled researcher for viral culturing due to the difficulty of in vitro PEDV propagation. On the other hand, ELISA has been developed for convenient and rapid testing for PEDV antibody detection. However, antibody levels measured by ELISA might not be correlated with protection of which differs from the titer of VN assay.

Choosing the type of ELISA not only considered the advantage of ELISA, but also determined by available reagents together with the purpose of measuring. The advantage of indirect ELISA is high sensitivity when compared to direct ELISA because direct ELISA use one labeled antibody bound to one antigen molecule and consequently could cost-saving more than direct ELISA. The indirect ELISA can be detected maximum immunoreactivity of primary antibody because that is not labeled and can be used with different visualization marker or a variety of labeled secondary antibodies for detection (Crowther, 2000). In addition, it does not require monoclonal antibody that difficult to produce like used in sandwich ELISA.

In a previous study, the ELISA technique has been developed continuously for antibody detection against PEDV, the first, in 1990, Hofmann and Wyler tried to culture the PEDV in cell culture and purify them by ultracentrifugation (Hofmann and Wyler, 1990) The purified antigen was coated to the plates for ELISA, however It found that the high background from the testing. Later, researcher measured the amount of antibody by comparing two types of ELISA, the blocking ELISA, which measures the amount of antibody after blocking antibodies in serum by using 2 monoclonal antibody, a double-antibody sandwich ELISA, and another fixed-cell ELISA, which use PEDV strain CV777 from Vero cells growing as an antigen and used conjugate IgG-specific monoclonal antibody the result revealed that the antibody titer of fixed-cell ELISA were 5.4 times higher than the blocking ELISA, however the blocking ELISA is easier to perform (van Nieuwstadt and Zetstra, 1991). In 1992, the significant structural proteins of PEDV were used for

coated plates, including S protein and N protein the result demonstrates that the ability of the S-ELISA (coated with S protein) can be detected antibodies from sera of PEDV-infected pigs in the longer term than the N-ELISA (coated with N protein), but the trouble of background is remaining (Knuchel et al., 1992). The using of extracted proteins from cytoplasm of PEDV culture cell were used to prepare an indirect ELISA showed an 89.1% of sensitivity and 94.5% of specificity, and percentage of consistency 84.2 % when compared to serum neutralization test (SN). This method is suitable for screening test in a pig herd, but disadvantage of this method is a higher background that necessary to use corrected value (CV) for cut off, and all of the test samples have to be tested mock-infected cell to remove the background (Oh et al., 2005). After that, ELISA development emphasized to increase the specificity of antibodies testing. The recombinant DNA technology was employed in the production of antigens for antibody detection. As for PEDV, the recombinant nucleocapsid (N) protein was produced for ELISA plate coating and antibody detection from the samples which were confirmed PEDV- infection by reverse transcription polymerase chain reaction (RT- PCR), the result showed high sensitivity and specificity about 98% and percentage of overall agreement was 88.3% when compared to serum neutralization test (SN) (Hou et al., 2007). In addition, after PEDV outbreak in North America (NA), the recombinant NA nucleoprotein (NP) has been made for developed indirect ELISA (iELISA); a highly specific monoclonal antibody-based blocking ELISA (bELISA); fluorescent microsphere immunoassays (FMIA) and a fluorescent focus neutralization assay (FFN) to detect neutralizing antibody in serum showed high consistent to indirect fluorescent antibody (IFA) assays (Okda et al., 2015). At present, development of the ELISA test kits for antibody detection against PEDV are based on detection of neutralizing antibody and antibody result correlates with protection against subsequent challenges, anti-PEDV IgA antibodies that usually found in colostrum and milk more than in serum and fecal samples (Gerber et al., 2014; Gerber and Opriessnig, 2015).

From previous studies demonstrate that the selection of protein in PEDV for antigen preparation is very important because it is should be obtained protective

antibody. Because of s protein ability to induce the antibody, a type of protein that has potential to be developed for the screening test that the level could potentially imply for protection, therefore we focused on neutralizing epitope that located in spike gene. Lee et al. (2010) concurred that neutralizing epitope located on 1 to 697 amino acid (aa) of spike gene. However, Sun et al. (2008) asserted that neutralizing epitope situated between 636 and 789 aa, especially from 748 to 771 aa is highest immunogenic epitope against B cell. Another interesting PEDV protein is N protein. The N protein functions in viral propagation, genomic RNA formation and antibody stimulation in the early stages of viral infection (Wang and Zhang, 1999). It is a pre-dominant antigen which is genetically conserved. Thus, it is an important target for diagnostic assay. However, the comparison of recombinant spike protein (rS12), fractional of spike protein and recombinant nucleocapsid protein (rN) for IgG and IgA antibody detection against PEDV revealed that rS ELISA was higher sensitivity and specificity than rN ELISA.

1.3 Research Problem

1.3.1 How to develop the ELISA test kit for antibody detection against PEDV?

1.3.2 Is the results of IgG or IgA ELISA measurement correlate with neutralizing antibody of viral neutralization test?

1.3.3 Can we apply the ELISA test kit to use in a field?

1.4 Research Hypothesis

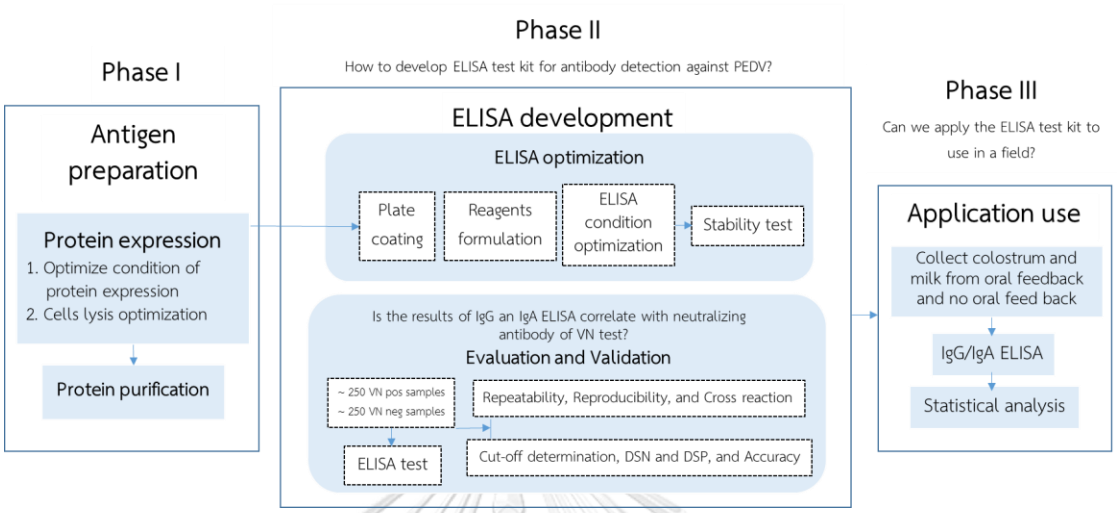
ELISA test kit can be used for testing IgG and IgA antibody of colostrum and milk from pigs.

1.5 Research Objectives

15.1 To develop ELISA test kit for antibody detection against PEDV in colostrum and milk samples of pigs

15.2 To evaluate and validate the ELISA test kit to detect antibody response against PEDV

1.6 Conceptual framework



1.7 Advantage of study

The main advantage of this study is the development of the new ELISA test kit for detecting antibody of IgG and IgA against PEDV from sow colostrum and milk. This ELISA test kit can be used as a substitute for traditional testing methods that have a lot of limitations. In addition, the results of the new method of development have also been consistent with the PEDV protection level. The antibody level which was obtained from the ELISA test kit will allow swine farmers to know the swine immunity level in a short time, that can be used to solve the problem of piglet mortality rate because of PEDV and create strategies to manage the farm correctly and properly. There are also reducing the cost of importing expensive test kits and reduce economic losses in the swine industry.

1.8 Keywords

antibody, colostrum, Enzyme linked immunosorbent assay, milk, Porcine epidemic diarrhea virus, Test kit

CHAPTER II

ANTIGEN PREPARATION

2.1 Materials and Methods

2.1.1 Protein expression

The recombinant plasmids for S12 genes, a fractional of neutralizing epitope and a conserve region in spike gene of PEDV, designated pGEX-5X-3-S12. The amplified products of rS12 designated S12- 6 Histidine (His)-tag were ligated into the prokaryotic expression vector pGEX-5X-3 (GE Healthcare, Buckinghamshire, UK) and were transformed into *E. coli* BL21 (DE3). The inserted PCR sequences were confirmed by DNA sequencing. The recombinant plasmid stock from previous study (Srijangwad et al., 2016), which resulted in the expression of proteins fused to glutathione S-transferase (GST) at their N-terminal were kept at -80°C with glycerol preservative for further study.

The transformed cells were grown in LB broth at 37°C in a shaking incubator for 3-5 h until the optical density (OD) at A_{600} reached 0.5-1. The cells were induced with 0.1, 0.5, 1 and 2 mM of isopropyl-ß-D-thiogalactopyranoside (IPTG) (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) for 2 and 4 h at 28°C and 37°C and analyzed by SDS-PAGE.

2.1.2 Protein purification

2.1.2.1 Optimization of cell lysis for recombinant fractional spike protein of PEDV and specific binding of protein

The transformed cells were grown in LB broth at 37°C in a shaking incubator for 18 h for starter culture preparation. The starter culture was diluted into LB broth (1:20) and incubated at 37°C with shaking until OD_{600} reaches 0.5-1. The rS12 protein expression by induced with 0.1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. The cells were harvested and re-suspended with a lysis buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0), and then lysed cells by stirred with 0, 1, and 5 mg/ml of lysozyme on ice

and sonicated 15 sec of burst period and 15 secs of cooling period (15x15s) compared to sonicated 15 sec of burst period and 30 secs of cooling period (15x30s) for 5 min. The rS12 protein was purified under native condition by using Protino® Ni-TED column (Macherey-Nagel, Bethlehem, PA, USA) and concentrated protein via Amicon® Pro Affinity Concentrator (Merck Millipore, USA). The protein concentration was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA)

The recombinant protein produced was tested for its binding efficacy against PEDV specific IgA in milk samples which were collected from PEDV infected and PEDV free sows. Prior to test, milk samples from both types of sows were assayed for antibody by viral neutralization test (VN) and the samples were categorized based on VN titer into high (VN titer >1:16) and low (VN titer < 1:2) VN titer. The high and low VN titer samples were used as positive and negative samples, respectively. The samples were diluted into 1:5 and 1:10 before test. The specific binding was confirmed by chemiluminescent Western blot detection using anti-his monoclonal antibody as a positive control.

The rS12 protein could be purified by affinity chromatography including His-tag protein purification and GST fusion protein purification.

2.1.2.2 His-tag protein purification

The soluble fraction was filtered through 0.45 µm membrane filter and purified protein by used Protino® Ni-TED column (Macherey-Nagel, Bethlehem, PA, USA). The column was equilibrated with 4 ml equilibria buffer under gravity. Following binding step, the solution was loaded onto the column and incubate at room temperature for 20 min. A removal of impurities and unbound proteins by washing column with 4 ml of the lysis buffer 2 times. The purified protein was eluted by incubate with 9 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) for 20 min and collect the purified protein from 1 to 9 fractions, 1 ml per tube.

As for preliminary purification was performed under native condition by using Protino® Ni-TED column (Macherey-Nagel, Bethlehem, PA, USA) with 50 ml of *E. coli* culture and obtained 5 ml of soluble protein. In the step of purification, protein samples

were collected in every 1 ml of solution, which flew through the purify column for verifying protein concentration by SDS-PAGE.

The purified protein was concentrated by using Amicon® Ultra-15 Centrifugal Filter Units (Merck Millipore, Cork, Ireland) and the protein concentration was measured by QuantiPro™ BCA Assay Kit for 0.5-30 µg/ml protein (Sigma-Aldrich, Schnelldorf, Germany) and store at -80°C until use.

2.1.2.3 GST fusion protein purification

After the protein was induced by IPTG in optimal conditions, the cells were harvested and re-suspended with 3 ml ice-cold phosphate buffered saline (PBS) per 50 ml culture. Cells were harvested by centrifugation at 3,000xg at 4°C for 10 min, discarded the supernatant. Then the cell pellet was freeze at -80°C for 1 hour and thawed cell pellet on ice and resuspended cells in 3 ml of ice-cold 1×PBS buffer per 50 ml culture. Protease inhibitor cocktail (Roche, CA, USA), 10 µl was added to the cell before there were disrupted by sonication on ice under optimal condition of cell lysis. The crude lysate was centrifuged at 12,000×g for 10 min at 4°C to separate soluble fraction from insoluble fraction. Supernatant or soluble fraction were carefully transferred to a clean and pre-chilled tube and resuspended pellet (insoluble fraction) with 3 ml of ice-cold 1×PBS buffer per 50 ml culture. Both soluble and insoluble were aliquotes 10 µl in each sample for SDS-PAGE analysis.

The soluble fraction was purified by using GST Fusion Protein Purification Kit (GenScript, New Jersey, USA). The vial of Glutathione Resin was gently shacked to completed resuspension. Two milliliters of Glutathione Resin were transferred to a disposable column and washed with 10×bed volumes of cold (4°C) PBS (20 ml). After that, the soluble fraction containing GST-fusion protein in cold PBS was applied to the equilibrated column with the flow rate at 10-15 cm per h or 0.25 cm per min. A washing step was performed after binding with all the protein solution by used 40 ml of PBS (20xbed volume) including 50 µl of protease inhibitors. GST-fusion protein was eluted with 10×bed volumes (20 ml) of freshly made 10 mM glutathione elution buffer by divided into 5 fractions for collecting (4 ml per fraction).

Each protein fraction was dialyzed by using Amicon® Ultra-15 Centrifugal Filter Units (Merck Millipore, Cork, Ireland) and the protein concentration was measured by QuantiPro™ BCA Assay Kit for 0.5-30 µg/ml protein (Sigma-Aldrich, Schnelldorf, Germany) and store at -80°C until use.

2.1.3 SDS-PAGE and western blotting analysis

The protein samples, which were lysed or purified, were mixed with equal volume of loading buffer (2X SDS Sample Buffer), boiled for 5 min and determined the amount and solubility of protein by running protein at 15 mA until protein pass through 5% stacking and 30 mA in 10% separating gel for 0.5 h. The gel was stained with Coomassie brilliant blue G 250 (Bio-Rad, Hercules California, USA) and de-stained with sterilized distilled water.

For Western blotting, the proteins were transferred from the gels to nitrocellulose membranes by semi-dry transfer apparatus (TRANS-BLOT®SD, Bio-Rad, Hercules California, USA). The membrane was incubated in blocking solution including 5% skim milk in phosphate buffered saline containing 0.05% Tween-20, 0.05% PBS-T buffer for 30 min, followed by an incubation with mouse anti-His antibody (Merck Millipore, Darmstadt, Germany) in PBS buffer containing 0.5% Tween 20 for 1.5 h. The membrane was washed with blocking solution for 10 min twice followed by distilled water for 5 min twice and then incubated in goat anti-mouse IgG conjugated with horseradish peroxidase enzyme (HRP) (Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h. The membrane was washed with distilled water for 5 min twice, followed by PBS-T buffer for 5 min and rinsed with water. A peroxidase substrate containing acetate buffer, 3-Amino-9-ethylcarbazole and H_2O_2 was used to develop the membrane or using Chemiluminescent Western blot detection using ImageQuant LAS 4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA)

2.1.4 Lot-to-lot consistency of antigen

Comparison of protein efficiency for control the quality of protein production which produced from the same raw material and same purification step in different time or different lot of antigen was performed by testing with same 86 milk samples. The IgG level from 86 milk samples were investigated in duplicate test by ELISA using three different lot of protein as antigen. The result from each lot were compared between three lots of antigen by the one-way ANOVA and Tukey post hoc test statistical analysis using SPSS software.

2.2 Results

2.2.1 Protein expression

The recombinant plasmids for S12 gene, designated pGEX-5X-3-S12 was successfully constructed. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) and rS12 protein was successfully produced (Srijangwad et al., 2016). The expression condition of rS12 proteins was optimized in different IPTG concentrations, induction times and temperature. The rS12 protein was expressed at the highest expression level following the induction with 0.5 mM IPTG for 4 h at 28°C (Figure 1A), and similar IPTG concentrations and induction time at 37°C (Figure 1B). In this study, the selected optimal condition of rS12 was the induction with 0.5 mM IPTG at 37°C for 4 h. The molecular weights of the rS12 was approximately 42 kDa.

2.2.2 Protein purification

2.2.2.1 His-tag protein purification

The purification of rS12 protein was preliminary produced prior to produce in large-scale under optimal condition by investigating from total protein in each fraction of purification step by using Protino® Ni-TED column (Macherey-Nagel, Bethlehem, PA, USA). The preliminary large-scale purifications of rS12 were no found protein in equilibrium and found a low concentration of protein in washing step, 0.15 mg/ml. In contrast to binding step was found the highest concentration of protein that could not bind

to the column, 16.82 mg/ml. The rS12 protein, specific to histidine purify column from fraction number 18 to 26 were eluted and their average concentration is 0.25 mg/ml or total protein is 2.26 mg/ml of 50 ml *E. coli* culture (Figure 2). The concentration of protein in each fraction and pattern of protein was demonstrated in Chemiluminescent Western blot detection (Figure 4)

2.2.2.2 GST-tag protein purification

The quantity of rS12 protein that purified by using GST Fusion Protein Purification Kit (GenScript, New Jersey, USA) portrayed a high concentration of protein production. In elution step of GST-tag protein purification was divided into 5 fractions, the result showed protein could bind to purify column from the first fraction to the fifth fraction. In the first fraction protein was the lowest concentration (2.5 mg/ml) and there were increased steadily from 2.5 mg/ml to 6.5 mg/ml at the second fraction. The concentration of rS12 fluctuated from the second fraction to the fifth fraction. The highest concentration of the protein was around 8.6 mg/ml, however, there are non-significant level of protein concentration from the second to the fifth fraction (Figure 3).

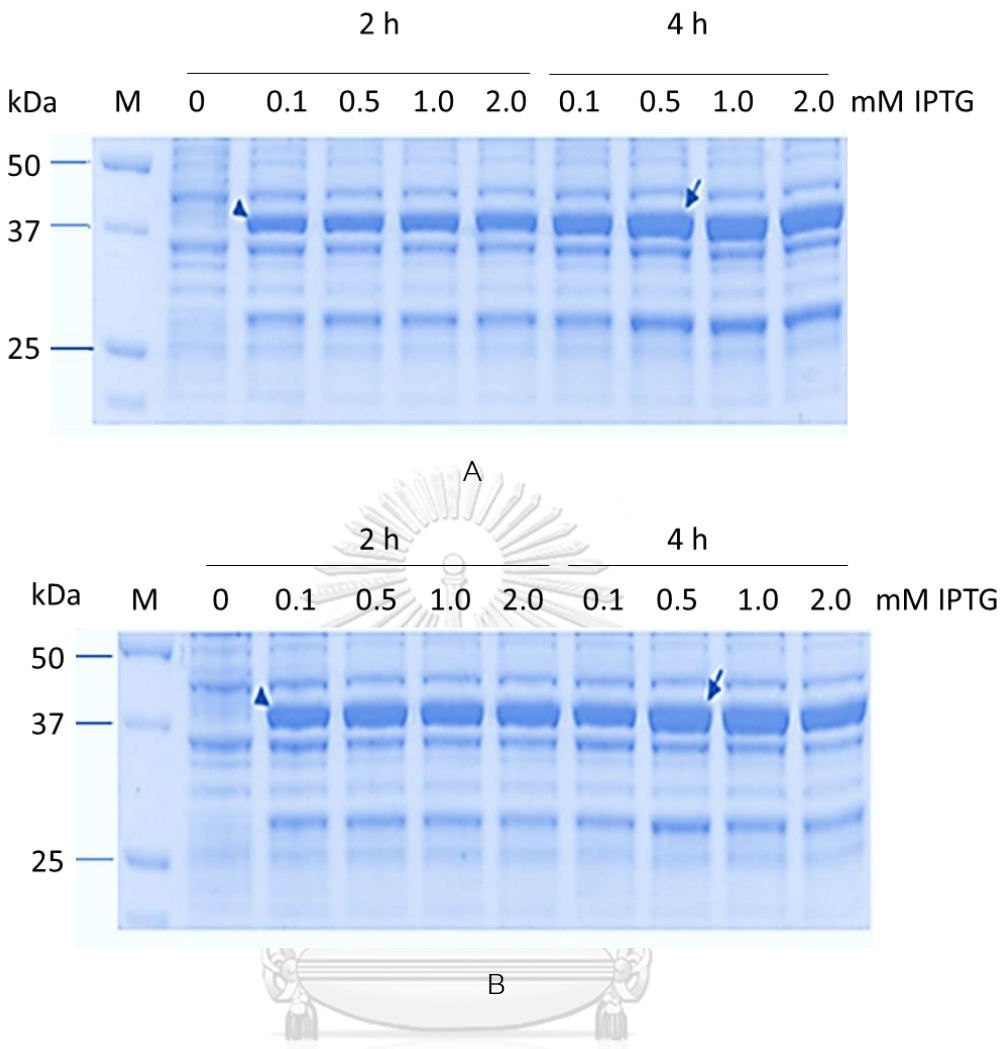


Figure 1 SDS-PAGE analysis of optimal condition for expression of rS12 protein at 28°C (A) and 37°C (B). M, protein molecular weight marker; 0, non-induced cell lysate and 0.1 – 2.0, bacteria lysate induced from 0.1 mM to 2.0 mM of IPTG for 2 h and 4 h. The expected molecular weight of rS12 was 42 kDa showing as a head arrow and an arrow showing the highest expression.

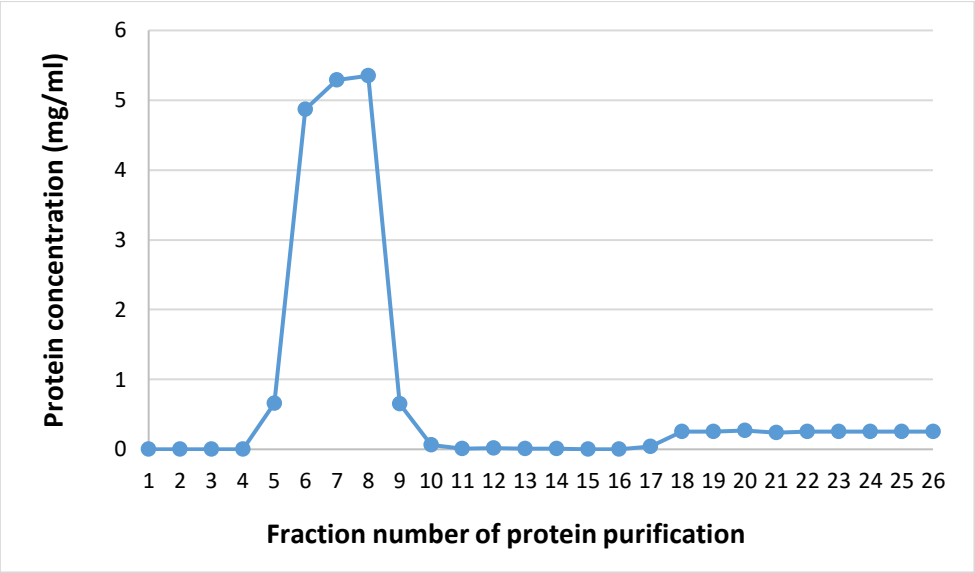


Figure 2 Total protein concentration at OD_{280} measured by Calibri microvolume spectrometer after purification by Protino® Ni-TED column (Macherey-Nagel, Bethlehem, PA, USA). The fraction number indicated sequence of sample collecting in each step of purification, 1-4 is equilibrium step, 5-10 is binding step, 11-17 is washing step and 18-26 is elution step.

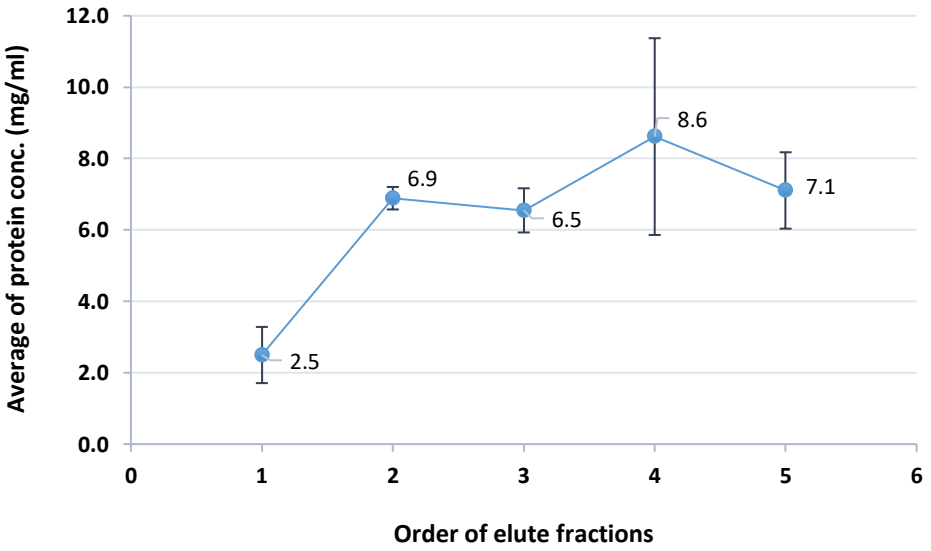


Figure 3 The concentration of rS12 protein were purified by GST-tag protein purification kit from fraction 1 to fraction 5 of elution step.

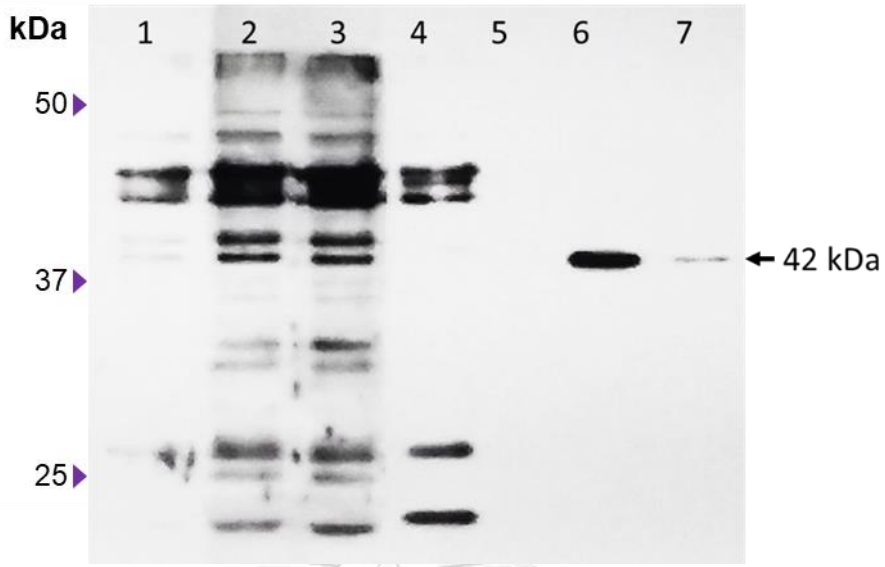


Figure 4 Chemiluminescent Western blot detection of rS12 protein purification by Protino® Ni-TED column detected with mouse anti-His antibody. Lane 1 is non-induced recombinant plasmid S12 *E. coli* BL21 cells, lane 2 is after induced recombinant plasmid S12 *E. coli* BL21 cells (crude protein), lane 3 is flow through, lane 4 is washing 1, lane 5 is washing 2, lane 6 and lane 7 are protein after purification fraction 1 and 2, respectively.

2.2.2.3 Optimal condition of cell lysis

Protein concentration which was purified by 5 mg/ml of lysozyme in cell lysis step could produce significantly higher of protein concentration than lysate cell by 0 and 1 mg/ml lysozyme in both 15x15s and 15x30s of sonication step with significant levels at $p<0.05$ and $p<0.001$, respectively (Figure 5). In cell lysis step, using 5 mg/ml lysozyme with 15x30s of sonication step could produce highest protein concentration (581 µg/ml) from 0.5 g of wet pellet cells. In addition, the cooling period in longer time is necessary for preventing cell heat during sonication time. It is found that cooling time, 30 sec in both 1 and 5 mg/ml of lysozyme can produce significant of protein concentration higher than 15 sec of cooling time (Figure 5)

Colostrum and milk samples were diluted to 1:5 and 1:10 and used for specific binding test of antibody against rS12 protein by Western blot analysis. The results showed bands of reaction at 42 kDa which was the same protein size of rS12 protein of 1:5 and

1:10 positive sample. That is similar to result of protein band were detected with mouse anti- his antibody in each protein which was purified by different condition of sonication (burst period and cooling period (Figure 6).

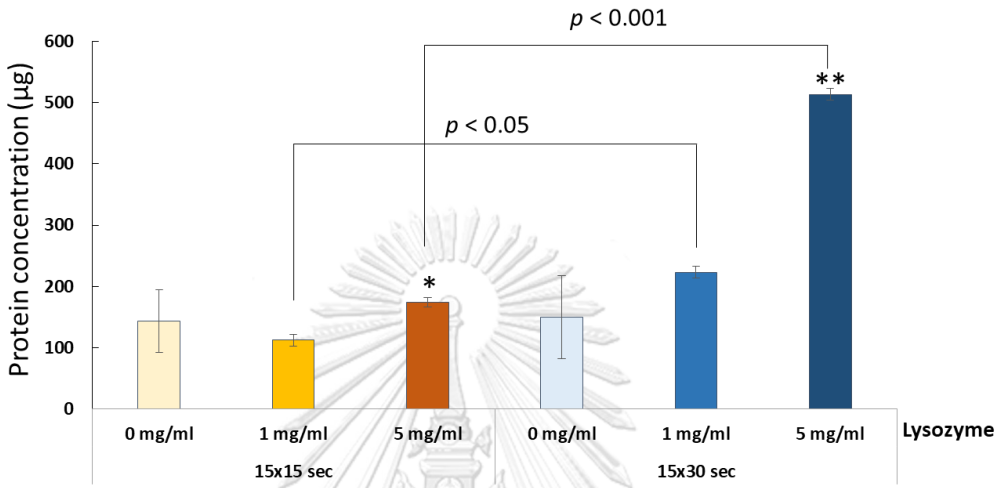


Figure 5 The concentration of rS12 proteins were purified after cell lysis with different lysozyme concentration and varied burst and cooling period of sonication. (* is p <0.05 and ** is p < 0.001).

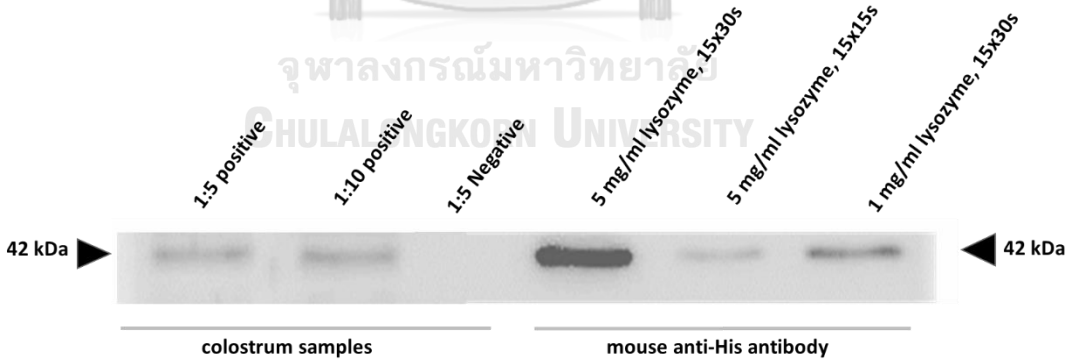


Figure 6 Western blot analysis represented specific binding of rS12 protein antigen to VN positive samples and anti-His monoclonal antibody.

2.2.2.4 Lot-to-lot consistency of antigen

The comparison of the results of IgG from three different lot of antigen showed the output of the ANOVA analysis and whether there is a statistically significant difference between three group means. The statistical analysis showed that the significance value of OD mean is 0.664 (p = 0.664) and p value of S/P ratio is 0.833, which are higher than 0.05 and, therefore, there is a statistically non-significant difference in the mean of OD and S/P ratio to complete the spreadsheet problem between the different three lot of antigen.

2.3 Discussion

Optimization of rS12 protein expression was analyses by comparing size of protein band using SDS-PAGE analysis in each condition of protein induction. The optimal condition for protein expression from our study revealed the largest protein band was occurred by the induction of 0.5 mM IPTG at 28°C for 4 h. In addition, the induction protein with 0.5 mM IPTG at 37°C for 4 h was also similar result as the previously condition. Therefore, incubation temperature for protein production in this study was settled at 37°C since this temperature was convenient for the general laboratories which usually use this temperature for incubation shaker setting. Even though some researches have tried to reduce induction temperature to avoid the risk of inclusion body formation, the result of 28°C were not different when comparing to 37°C. It is possible that the protein induction examination by using variety of lower temperature is necessary to reducing insoluble protein. On the other hand, almost protein forming to inclusion body or insoluble protein combined with 8 mM urea in lysis buffer will increase amount of solubilized protein of truncated PEDV protein production (Piao et al., 2016).

Due to rS12 was designed the component of protein by combination with affinity tags. The composition of rS12 protein consists glutathione S-transferase (GST) protein at N-terminal and C-terminal tag with six molecule of histidine (6xHis). Therefore, there are alternative method for rS12 protein purification. In addition, affinity tags are highly efficient tools for protein purification from crude extract (Lichty et al., 2005). The rS12 protein can be purified by column for histidine-tagged fusion proteins under the

principle of binding ability of histidine to immobilized metal ion on the purification column. On the other hand, since rS12 protein were constructed to pGEX expression vector, which GST gene located in N-terminal side and following by target gene, the GST-fusion proteins was produced in *E. coli* system. Then the rS12 protein can be purified by GST- fusion protein kit, fusion protein is captured by immobilized glutathione and non-captured protein were washed (Harper and Speicher, 2011).

Efficacy of rS12 protein purification under GST-tag fusion protein higher than His-tag protein. However, using GST-tag protein purification should be avoided contamination of reduced glutathione protein from elution step. The step of remove glutathione by dialysis is very important because the contamination protein will affect to corrective concentration of protein and cannot detect protein concentration by BCA protein kit.

The using of 5 mg/ml of lysozyme may be more optimal concentration for cell lysis of rS12 protein production. Although the optimal concentration of the enzyme required to bacterial lysate, strong buffer and high ionic strength in lysis buffer are also necessary to bacterial lysate and protein solubility (Structural Genomics et al., 2008). Moreover, observation ratio of protein degradation after using high concentration of lysozyme should be done in every batch number of protein purification.

Following protein purification is necessary to confirm specific binding of protein to antibody response against PEDV from colostrum and milk. According to the result of western blotting analysis could be confirmed that rS12 protein binding to colostrum and milk positive same with binding to anti-His antibody as a positive control. There may be concluded that rS12 protein is appropriate for use as antigen in antibody detection against PEDV by immunoassay.

The antigen consistency in each lot of protein production was confirmed the quality and efficiency for use as antigen in ELISA. The results of antibody detection from ELISA plate coated with different lots of antigen were not significantly difference; therefore, it could be suggested that each different lots of protein could be used as

antigen for ELISA coating base on the same quality of raw material and process of protein production.

CHAPTER III

ELISA TEST KIT DEVELOPMENT

3.1 Materials and Methods

3.1.1 ELISA condition optimization

The working condition of ELISA were optimized including, the optimal concentration of antigen and dilution of the sample by checkerboard titration (Crowther, 2000). Antigen, S12 protein was made a dilution to 80, 40, 20, 10, and 0 µg/ml protein in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.6). Each protein concentration was coated from column 1 to 5 of the ELISA plate overnight at 4°C. After coating step, the ELISA plate was blocked with 200 µl of blocking buffer in each well for 3 h at room temperature. Blocking solution was discarded and 200 µl of sample diluent was added in row A and E of ELISA plate and others row, B, C, D, F, G and H were added with 100 µl of sample diluent. The high positive and negative samples was diluted in PBS 1:10 before adding in the first row of the plate. The high positive sample was added in row A (A1-A5) 8 µl to make 1:125 dilution and two-fold serial dilution was made from well A1-A5 to D1-D5. The negative sample was conducted in the same way as the positive sample, from E1-E5 to H1-H5 and incubated at room temperature for 1 h. Following five times washing with washing buffer (0.05% PBS-T), anti-pig IgG-HRP (1: 20,000) or anti-pig IgA-HRP (1: 10,000) (Bio-Rad AbD Serotec, Kidlington, UK), which dilute in 2% BSA was added and incubated for 1 h at room temperature. After incubation, the plate was washes 4 times and added the solution of TMB, chromogenic substrate (Sigma-Aldrich, St. Louis, MO, USA). After that plates were incubated at dark place for 15 min. Finally, the reaction was stopped using 1N H_2SO_4 and the OD_{450} value was read using an ELISA plate reader (Metertech, Taipei, Taiwan).

3.1.2 ELISA plate coating

The ELISA plates were coated by 100 µl of the optimal concentration of antigen by diluting in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.6 overnight at 4°C. The plates were–blocked with 200 µl of blocking buffer, 5% skim milk and incubate for 3 h at room temperature. Following discard blocking buffer and dry plate at 37°C for 2 h, adapted from protocol for detection of IgA antibody in fecal and serum sample (Gerber and Opriessnig, 2015). The plate was covered with plastic plate sealer and store at 4°C until use.

3.1.3 Reagent formulation

The necessary ELISA kit reagent includes: sample diluent, blocking buffer, conjugate diluent, substrate, stop solution and washing buffer. The formula of reagent was prepared as follows.

3.1.3.1 Blocking buffer

Blocking buffer lwas used for ELISA plate blocking. The formulation of blocking buffer divide into 4 formulas (F) includes, F1 is 5% Skim milk dilute in PBS, F2 is 5% Skim milk with 1% sucrose, F3 is 2% BSA dilute in PBS, and F4 is 2% BSA with 1% sucrose.

3.1.3.2 Sample/conjugate diluent

The sample and conjugate diluent were used for reducing nonspecific background from the sample and for dilute anti-pig IgG-HRP and anti-pig IgA-HRP. The formulation of conjugate diluent divide into 4 formulas includes, F1 is 1% Skim milk dilute in PBS, F2 is 1% Skim milk with 0.1% sucrose, F3 is 0.1% BSA dilute in PBS, and F4 is 2% BSA with 1% sucrose.

3.1.3.3 Substrate

Substrate was used to detect horseradish peroxidase (HRP) activity. Its composition is 10% acetate buffer (pH 5.9), 3% H_2O_2 and 3, 3', 5, 5'-tetramethylbenzidine (TMB) was used to develop blue color.

3.1.3.4 Stop solution

Stop solution in this study is 1 N sulfuric acid (1 N H_2S0_4) which was used for stopping reaction of ELISA before reading a result.

3.1.3.5 Washing buffer

Preparation of washing buffer was mixed PBS with 0.05% Tween-20. The pH was adjusted in range 7.0-7.4.

All reagents including, sample diluent, blocking buffer, conjugate diluent and substrate was kept at 4°C until use except wash buffer and stop solution was kept at room temperature.

3.1.4 Stability test of reagents and ELISA kit

After reagent formulation, the reagents were tested the shelf life or stability by comparison of level of IgG or IgA (OD_{450}) against PEDV by testing with stock of reagents at 4°C and new prepared reagents. One high positive sample (VN titer> 1:32), one low positive (VN titer = 1:8) and one negative sample (VN titer < 1:2) was used to test stability of the reagents once a month until appear changing of reagent or OD_{450} pattern.

The stability test of ELISA test kit was conducted after selecting optimal reagent, and then each reagent was formulated to more concentration which sufficient volume and appropriate for containers (Appendix). All reagents and coated plate were stored in 4°C and used to investigate IgG and IgA of high positive, low positive and negative sample once a month.

3.1.5 Evaluation and validation of ELISA

3.1.5.1 Samples preparation

Two hundred and fifty of colostrum and milk samples was collected from PEDV-positive farm and other 250 colostrum and milk samples were collected from PEDV-negative farm. All of samples was confirmed positive and negative neutralizing antibody by viral neutralization assay. Colostrum samples were collected from non-infected and PEDV-infected multiparous sows within 3 h post parturition. Colostrum and milk samples

were centrifuged at 12,000 rpm for 20 min at 4°C. The lipid layer was removed, and the middle layer was collected (Figure 7). Samples were stored in -20°C for further analysis.

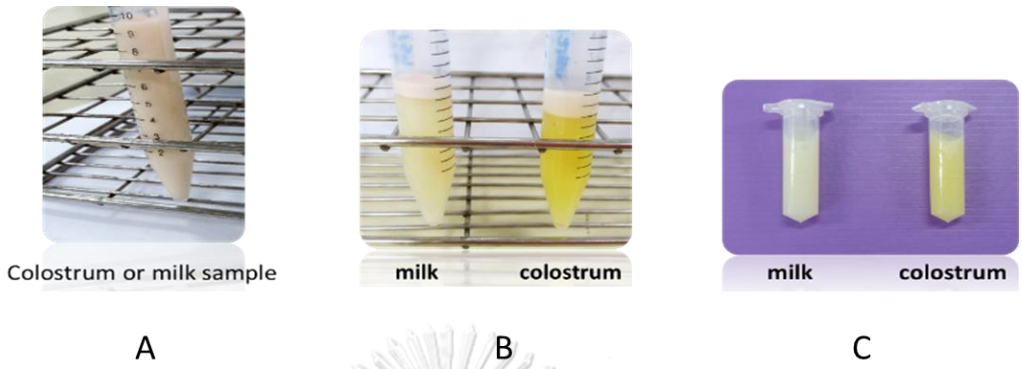


Figure 7 Procedure of colostrum and milk sample preparation. A is the sample after collected from pig, B is the samples after centrifugation and C is the samples after collected at the middle layer.

3.1.5.2 Viral neutralization (VN) assay

Colostrum samples were heat-inactivated at 56°C for 30 min before use for viral neutralization. To perform the assay, the samples were two-fold serially diluted in maintenance medium (MM). Each dilution of samples was mixed with an equal volume of PEDV diluted to contain 10^2 $TCID_{50}$/0.05 ml. The samples/virus mixtures were incubated at 37°C in 5% CO_2 incubator for 1 h. The mixtures were added in duplicate to the wells of a 96-well microtiter plate containing 48-h-old confluent Vero cell monolayer and incubated at 37°C in 5% CO_2 incubator. The cytopathic effect was observed every day until 7 days post inoculation. The neutralizing titer were expressed as the highest sample dilution which no evidenced of cytopathic effect (100% normal cell). The viral concentration (10^2 $TCID_{50}$/0.05 ml) were also verified by back titration test.

3.1.5.3 Enzyme linkage immunosorbent assay (ELISA)

The PEDV specific antibody from colostrum samples were assayed by ELISA using recombinant S12 (rS12) protein as an antigen. In brief, ELISA plate (Thermo Fisher Scientific, Roskilde, Denmark) was coated with optimal concentrations of recombinant spike protein in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.6 for IgG ELISA or IgA ELISA and

hold overnight at 4°C. The plate was blocked with blocking solution and incubated for 3 h at room temperature. After blocking ELISA plate was dried and keep at 4°C until use. The colostrum and milk samples were diluted with sample diluent in optimal dilution and then there were pipetted 100 µl/sample to ELISA plate and incubated at room temperature for 1 h. Following five times washing with washing buffer (0.05% PBS-T), prior to incubate with anti-pig IgG-HRP (1:20,000) or anti-pig IgA-HRP (1:10,000) (Bio-Rad AbD Serotec, Kidlington, UK) for at room temperature for 1 h. Subsequently, the ELISA plates were washed 4 times with washing buffer prior to incubation with 3, 3', 5, 5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, St. Louis, Missouri USA) at room temperature for 15 min. The reaction was stopped using 1 N H_2SO_4, and the optical density at 450 nm (OD_{450}) value was read using an ELISA plate reader (AccuReader, Metertech, Taipei, Taiwan). The OD results were converted to sample-to-positive (S/P) ratio using the equation as follows and the cut-off value of IgG and IgA were determined.

3.1.5.4 Evaluation of repeatability and reproducibility test

Repeatability test can divide into two processes, the first is within plate repeatability (intra-assay repeatability) the experiment was performed in the same plate and another is between plate repeatability (inter-assay repeatability) which was performed in different plates at different times. In trial of within plate repeatability, one plate ELISA containing triplicates ten of VN positive samples and ten of VN negative samples were used. As for between plate repeatability was evaluated in 5 different plates of triplicate ten colostrum or milk samples in different days.

An inter-laboratory test of the study was conducted by testing ten samples of the colostrum or milk samples which known VN titer were used for IgG and IgA investigation by ELISA from two operators in difference laboratory. The ELISA test kit and the samples were tested in laboratory of Veterinary Microbiology, the Faculty of Veterinary Science, Chulalongkorn University (lab A) and compared to the results from Veterinary of Microbiology laboratory the Faculty of Agriculture, University of Miyazaki (Lab B). The sample was tested in triplicates test both IgG and IgA ELISA. After repeatability and

reproducibility test, measured OD value of IgG and IgA. Thereafter, mean, standard deviation (SD), and percentage of coefficient of variation (%CV) of OD and S/P ratio were calculated as follow equations.

$$\text{S/P ratio} = \frac{\text{OD of sample} - \text{OD of negative control sample}}{\text{OD of positive control sample} - \text{OD of negative control sample}}$$

$$\%\,\text{CV} = \frac{\text{SD}}{\text{Mean}} \times 100$$

3.1.5.5 Cross reaction

The testing of specificity of ELISA test kit was examined by positive serum against other swine coronavirus members include porcine deltacoronavirus (PDCoV) and transmissible gastroenteritis coronavirus (TGEV). The samples were tested in triplicate together with PEDV positive serum and PEDV negative serum, and then the mean of S/P ratio of each sample was calculated. If the S/P ratio of the sample less than the cut-off value of ELISA test kit the result can be interpreted no-cross reactivity between the others.

3.1.5.6 Cut-off determination

Two hundred and fifty of colostrum and milk samples from PEDV-positive farm and other 250 colostrum and milk samples from PEDV-negative farm was investigated both IgG and IgA against PEDV by ELISA. The mean of S/P ratio of each sample from ELISA was calculated and compared to result of VN test (standard method) from the same sample group. Selection of positive-negative cut-off value was determined by the maximized point of the diagnostic sensitivity (DSN) and diagnostic specificity (DSP) that perform on a receiver-operating characteristic (ROC) analysis by IBM SPSS Statistics 22 software.

3.1.5.7 Diagnostic sensitivity (DSN), diagnostic specificity (DSP) and accuracy test

The results of IgG and IgA ELISA from 500 samples was determined to positive or negative value that depend on cutoff value and compare with the result of VN test. The consideration of VN positive titer were considered at 1:4 or 2^2 (Oh et al., 2005). DSN and DSP and accuracy between IgG and IgA, and VN titers was calculated using Microsoft excel as equation below.

$$DSN = \frac{\text{True positive}}{\text{True positive + False negative}} \text{ X } 100$$

$$DSP = \frac{\text{True negative}}{\text{True negative + False positive}} \text{ X } 100$$

$$\text{Accuracy} = \frac{\text{True positive + True negative}}{\text{True positive + True negative + False positive+ False negative}} \text{X } 100$$

Remark: The True positive is the number of positive samples from both ELISA and VN

The True negative is the number of negative samples from both ELISA and VN

The False positive is the number of positive samples from ELISA, but negative VN

The False negative is the number of negative samples from ELISA, but positive VN

3.1.5.8 Correlation of ELISA test of IgG and IgA comparing to VN test

An optical density (OD) level of IgG and IgA and a sample to positive ratio (S/P) of IgG and IgA from 250 VN positives samples and 250 VN negative samples were analyzed the correlation with VN titer which use as a standard method. The Spearman correlation were examined by IBM SPSS statistic 22 software.

3.2 Results

3.2.1 ELISA condition optimization

The optimal concentration of antigen and sample dilution in IgG and IgA ELISA were optimized by using checkerboard. In the IgG and IgA ELISA test, diluted 1:250 sample showed low nonspecific background and highest positive to negative ratio (P/N) at 10 μg/ml and 20 μg/ml of antigen concentration in IgG and IgA test, respectively. The result of the study reveals that the optimal concentration of antigen of IgG (Figure 8) and IgA (Figure 9) is 10 μg/ml and 20 μg/ml, respectively and optimal dilution of sample is 1:250.

3.2.2 Stability test of reagent

The result of blocking buffer and sample/conjugate diluent when comparing between using of 1% BSA and 5% skim milk were included with (w) and without (w/o) 1% sucrose, the reagent was formulated by 5% skim milk at 1 month found the changing of precipitation appearance in sample and conjugate diluent in both with and without sucrose. On the other hand, the reagent was composed with 1%BSA were not found precipitation at 1-2 months.

The stability of IgG or IgA ELISA against PEDV by testing with stock of reagents at 4°C and new prepared reagents which was composed of BSA and sucrose with one high positive sample (VN titer> 1:32 and OD_{450} > 0.7), one low positive (VN titer = 1:8 and OD_{450} = 0.5 - 0.7) and one negative sample (VN titer < 1:2 and OD_{450} <0.1).

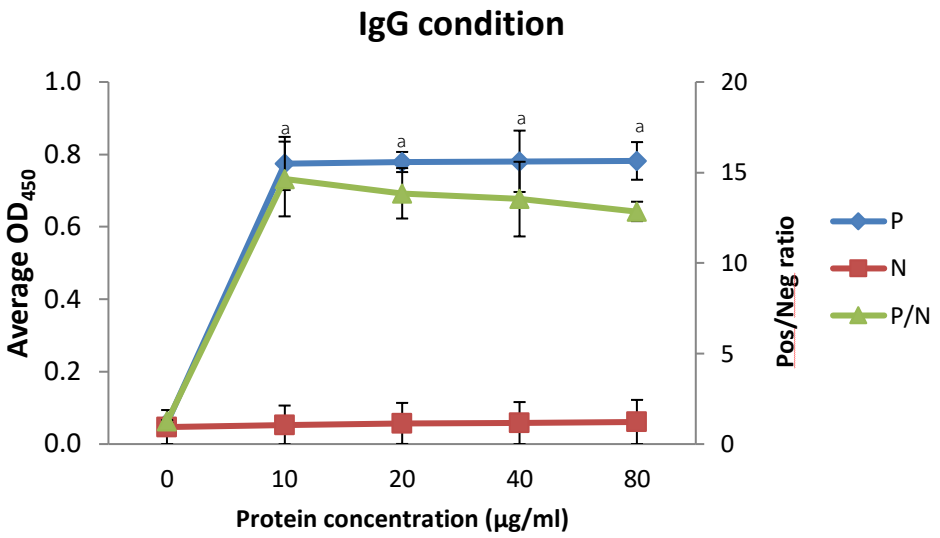


Figure 8 Optimization of IgG ELISA condition between difference protein concentrations with positive and negative sample (diluted 1:250) and anti-pig IgA HRP (diluted 1:15,000). Different letters in superscript indicate statistically significant differences ($p \leq 0.05$) between P/N ratio at each concentration of protein.

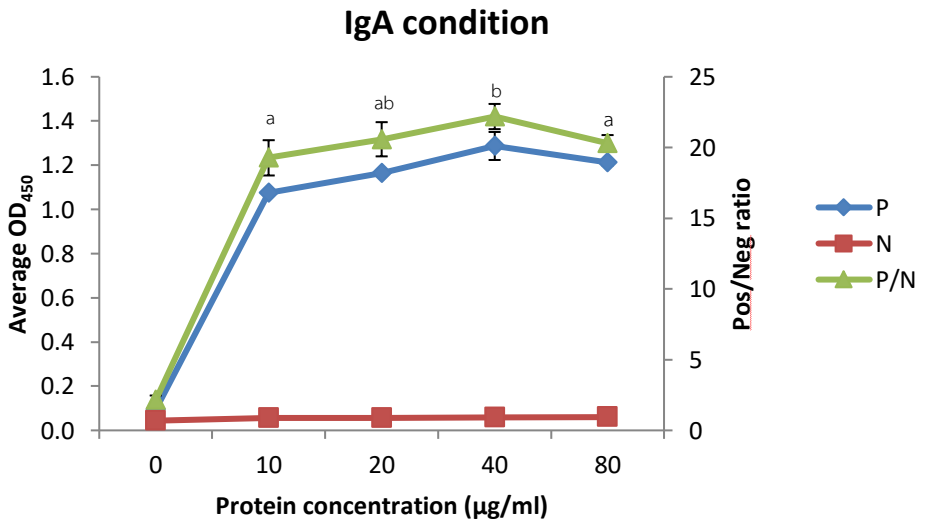
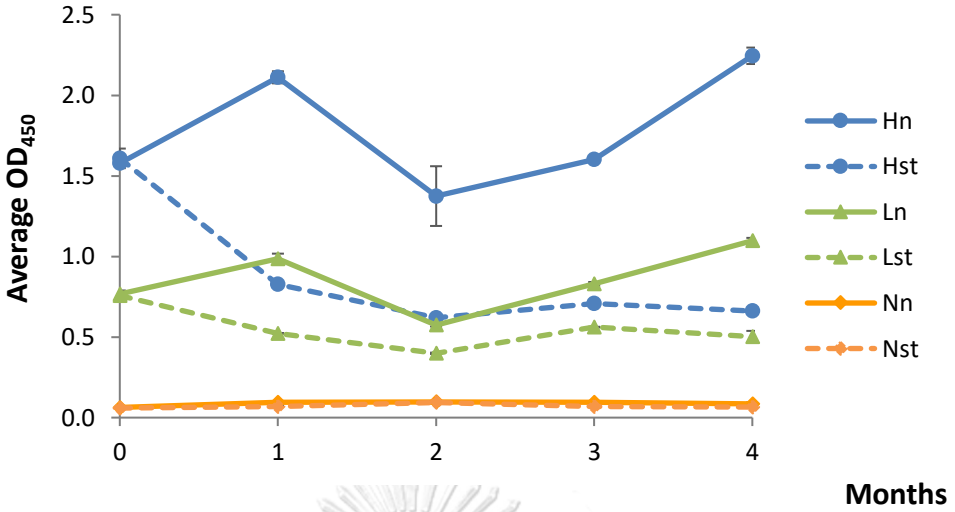


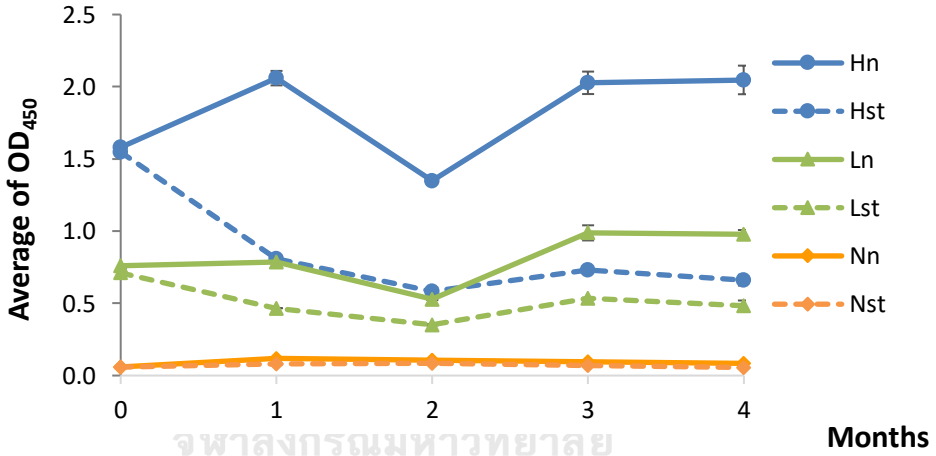
Figure 9 Optimization of IgA ELISA condition between difference protein concentrations with positive and negative sample (diluted 1:250) and anti-pig IgA HRP (diluted 1:15,000). Different letters in superscript indicate statistically significant differences ($p \leq 0.05$) between P/N ratio at each concentration of protein.

The Figure 10A and 10B showed similarity of OD level of IgG in high positive, low positive, and negative sample in both reagent w sucrose and w/o sucrose. The OD level of high positive sample which prepared new reagent before an ELISA test was more than 1.3 (thick line) and fluctuated until 4 months. In contrast to the ELISA test with stock reagent (dash line), the OD value were not different at 0 months or starting date, however the OD value decreased after 1 month, OD 0.826 and 0.788 in w and w/o stabilizer, respectively and founded consistency of OD until 4 months. In low positive sample was tested with reagent from stock showed slightly decreased of OD when comparing to new reagent from 0.7 to 0.5 and 0.4 of reagent with and without sucrose, respectively. In contrast to OD of negative sample showed low OD (0.059-0.094) in ELISA test with sucrose included reagent similar to ELISA test with reagent without sucrose (OD, 0.056-0.085).

The formulated reagent was included with and without 1% sucrose when comparing between new reagent and stock reagent in each period, there were a fluctuation of OD in H+ and L+ between 0 – 4 months. However, in individual sample gave similar result of OD, H+ sample showed OD value from 1.349 to 2.245, L+ sample showed OD value from 0.529 to 0.978, and N- sample showed OD value from 0.060-0.119 (Figure 11A). As for using of stock reagent in each month was founded the reagent which included with sucrose non-effect to OD value changing when comparing to without sucrose reagent. On the other hand, after one-month stock of reagent effected to OD value investigation of IgG and remain stable until 4 mounts. The OD value of H+ sample decreased steadily from 1.5 to 0.8, L+ sample, OD value decreased slightly from 0.7 to 0.4, and N – sample OD value were remained constant from 0 to 4 months (Figure 11B).



A



B

Figure 10 Stability test of ELISA for IgG antibody detection when using new (n) and stock of reagent (st) which were included with (A) and without (B) 1% sucrose as stabilizer in blocking buffer and sample and conjugated diluent were test with high positive (H), low positive (L), and negative (N) samples for 4 months.

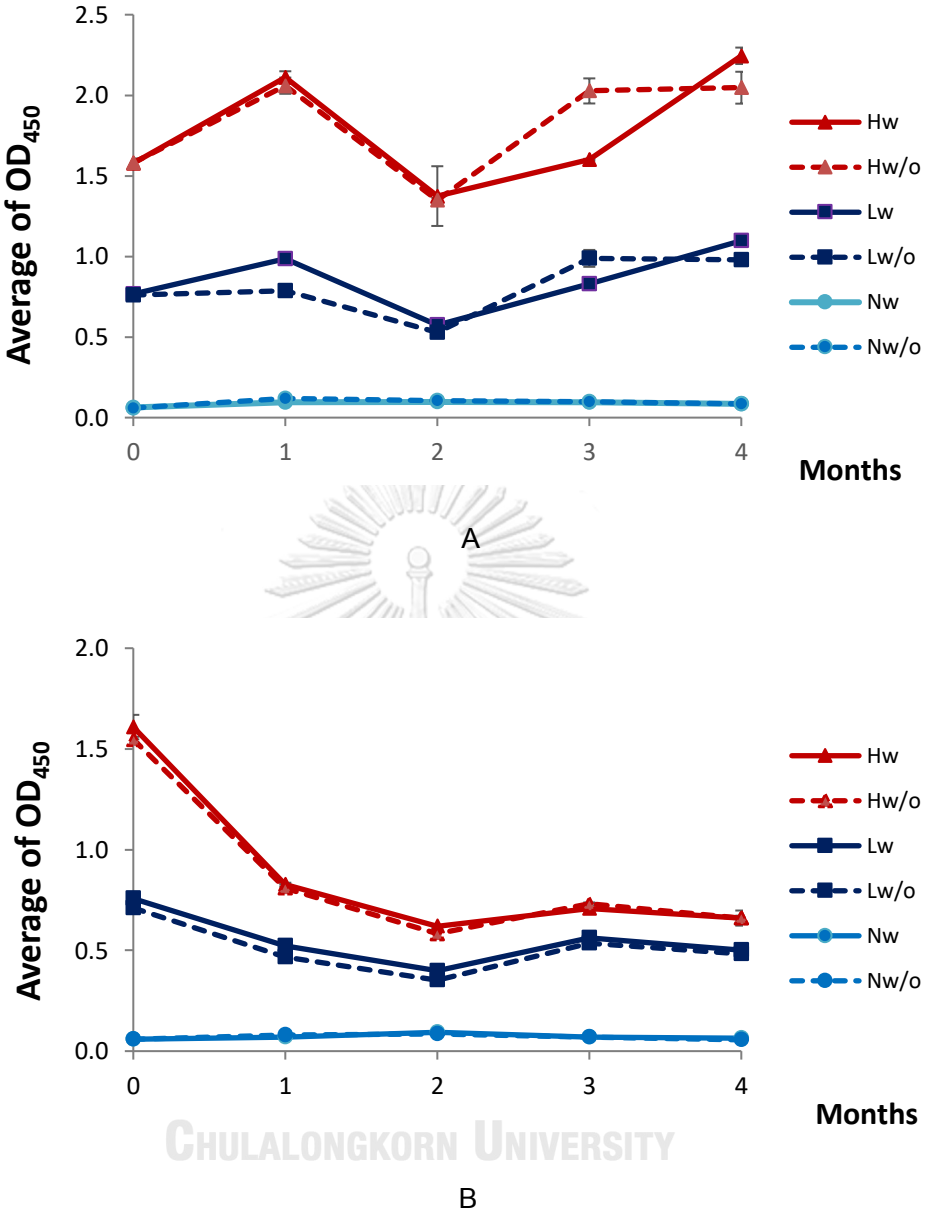


Figure 11 Stability test of ELISA for IgG antibody detection which were included with (w) and without (w/o) 1% sucrose as stabilizer in blocking buffer and sample and conjugated diluent in new reagent (A) and stock reagent (B) were test with high positive (H), low positive (L), and negative (N) samples for 4 months.

The IgA investigation by ELISA test with reagent included with BSA and sucrose (Figure 12A), the result of OD from new reagent illustrated rather stable of OD value in all samples (H+, L+, and N-). In contrast to stock reagent, there declined dramatically of OD, more than 2 times from 1.4 to 0.6 of H+ samples and from 0.8 to 0.1 of L+ samples, after that, at 2, 3, and 4 months almost stable OD. In negative sample, OD stays the same from starting until 4 months in both with and without sucrose in reagent.

The result of an IgA test with the reagent without sucrose showed a trend of OD value in each sample and each time similar to test with reagent was included with sucrose. The OD value of H+ and L+ at 1, 2, 3, and 4 months were tested with new reagent that presented in thick line higher than stock reagent (dash line) except the OD value of N- sample almost the same value in new and stock reagent (Figure 12B).

The comparison of stability test of reagent for IgA ELISA test between with and without sucrose reagent, the Figure 13A presented almost same OD when used new reagent in individual samples (H+, L+, and N-) except at 3 and 4 mounts were different OD value of L+ sample. On the other hand, when used stock reagent which kept at $4^{O}C$ at each period found the OD level decreased at 1 month in both H+ and L+ sample. The OD of H+ and L+ sample dramatically decreased from 1.3 to 0.6 and from 0.9 to 0.2, respectively. The using of stock reagent without sucrose showed rise moderately from 1 month (~0.2) to 3 months (~0.6) that is similar to using new reagent without sucrose slowly raised of OD at 1 month, which was lower than H+ OD. As for N- sample, OD value was almost consistent amount between 0.1-0.2 (Figure 13B).

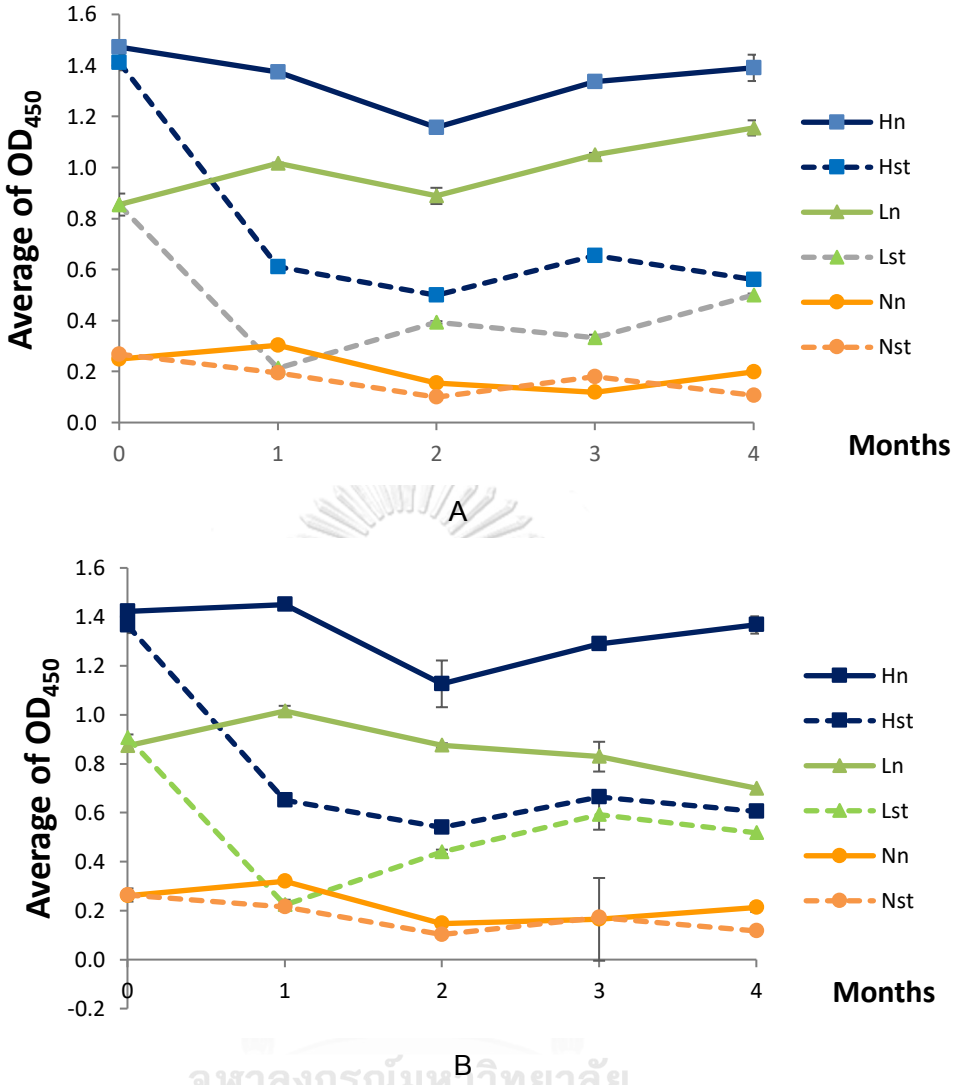


Figure 12 Stability test of ELISA for IgA antibody detection when using new (n) and stock of reagent (st) which were included with (A) and without (B) 1% sucrose as stabilizer in blocking buffer and sample and conjugated diluent were test with high positive (H), low positive (L), and negative (N) samples for 4 months.

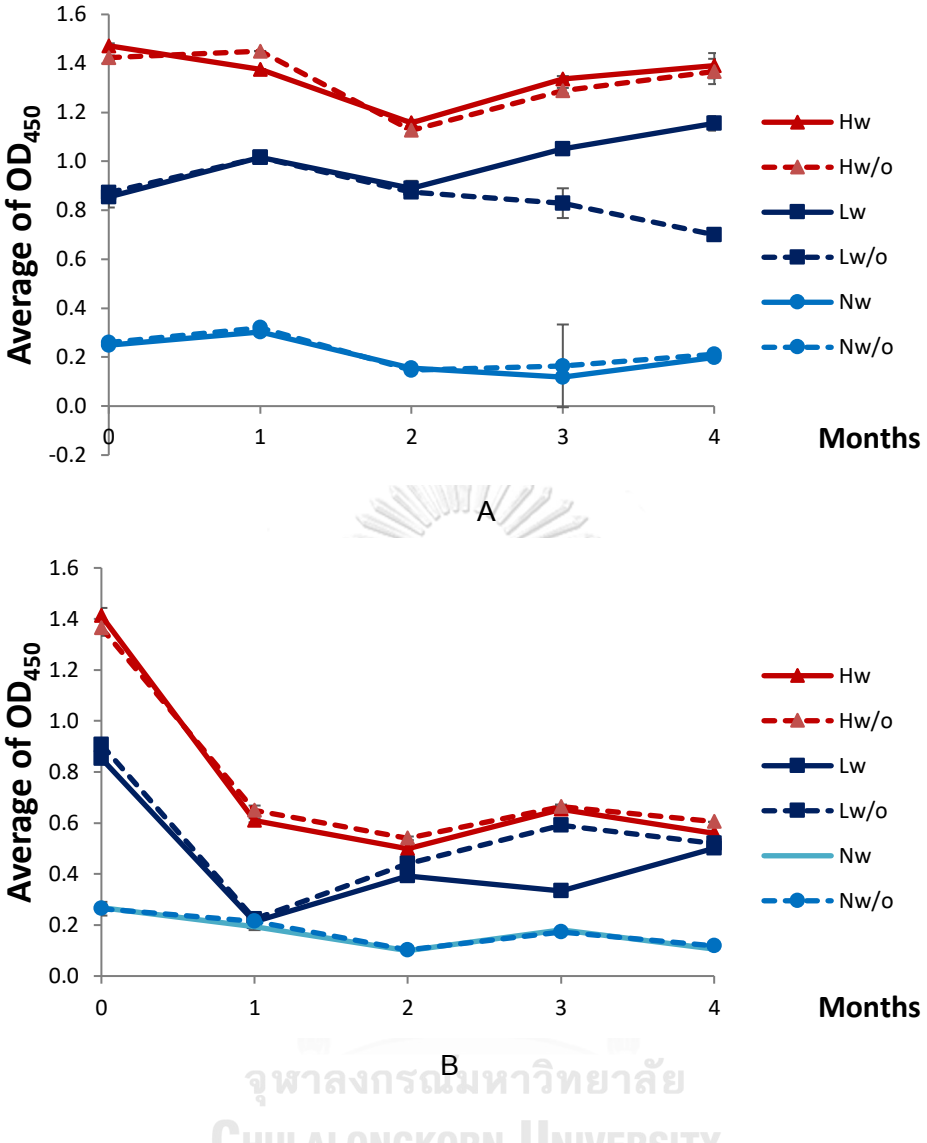


Figure 13 Stability test of ELISA for IgA antibody detection which were included with (w) and without (w/o) 1% sucrose as stabilizer in blocking buffer and sample and conjugated diluent in new reagent (A) and stock reagent (B) were test with high positive (H), low positive (L), and negative (N) samples for 4 months.

In consideration of the cause which effected to decreasing of OD at 1 month was presented in Figure 14. The different reagent was used for OD investigation on a positive sample by ELISA. The result of optical density (OD) of IgG after 1 month by comparison of difference reagent condition in an ELISA test showed low level of OD when used all reagents (*st*) from stock at 4^{O}C that is like an OD level of condition which using

substrate from stock (*n+substrate (st)* and *n+sample dil. (st)+ substrate II*). In contrast to the result which conducted by all reagents from new preparation before used (*n*), all new reagents with a stock of sample diluent and 3% H_2O_2 immediately mixed substrate (*n + sample dil. (st) + substrate I*), and all new reagents with a stock of conjugate diluent (*n + conj dil. (st)*).

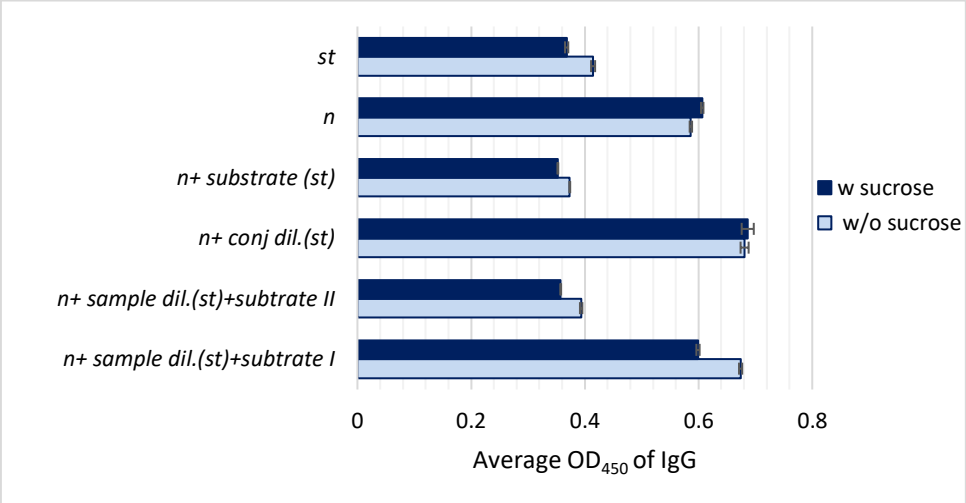


Figure 14 Optical density results of PEDV-specific IgG antibody detection by ELISA in positive VN colostrum. The ELISA were run in different reagents at 1 month.
(w sucrose = sample/conjugate diluent were included with 1% sucrose, w/o sucrose = sample/conjugate diluent were non-included with 1% sucrose, *st* = all reagents from stock at $4^{O}C$, *n* = all reagents from new preparation before used, *n + substrate (st)* = using all new reagents excepted substrate was used from stock, *n + conj dil. (st)* = using all new reagents excepted conjugate diluent was used from stock, *n + sample dil. (st) + substrate I* = using all new reagents excepted sample diluent was used from stock and substrate was mixed with 3%H_2O_2 immediately, *n + sample dil. (st) + Substrate II* = using all new reagents excepted sample diluent and substrate from stock)

Although the result of OD in both IgG and IgA were not shown an evidence that supported the effect of sucrose, the reagents were included with sucrose which used as stabilizer is important for long term keeping. Therefore, the increasing concentration of

stabilizer might be necessary for increase stability of the reagents especially, blocking buffer, sample diluent, and conjugate diluent.

The increasing of sucrose concentration from 1% became to 10% was conducted together with 10 % goat serum addition, and then non-specific background were investigated from OD of positive sample by ELISA test which coated with rS12 and BSA as a control antigen (Xiao and Isaacs, 2012).

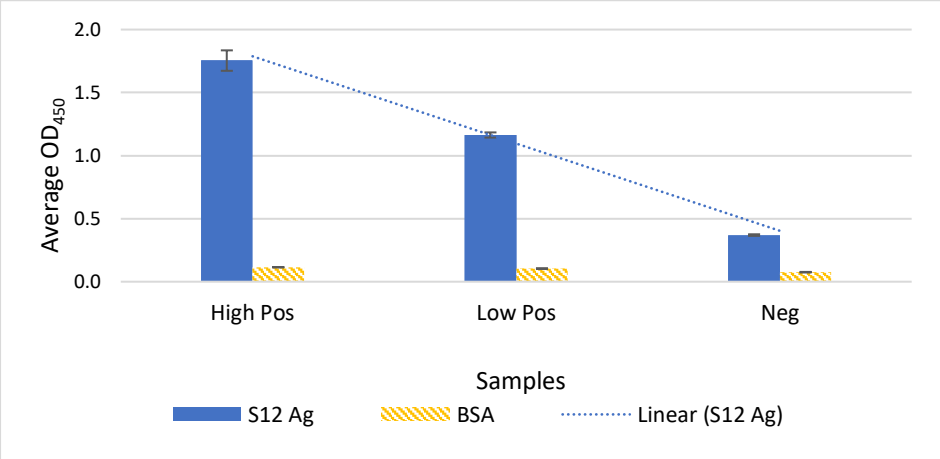
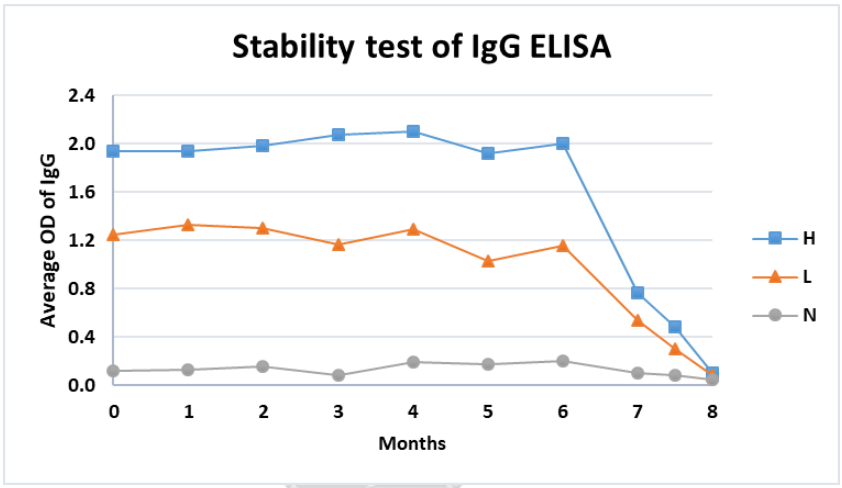


Figure 15 Investigation of non-specific background after using solution of 2% BSA and 10% goat serum as sample diluent and conjugate diluent in rS12 ELISA coating comparing to equal concentration of BSA for coating.

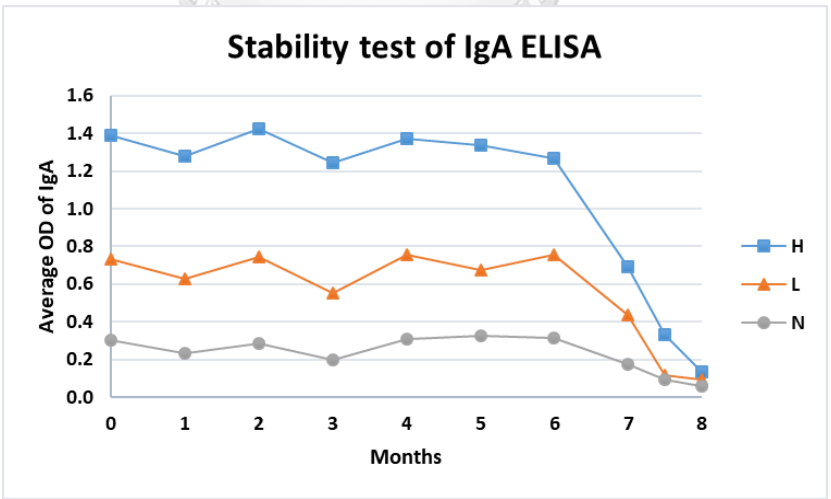
3.2.3 Stability test of ELISA kit

The shelf life of ELISA test kit which contained with ELISA plate were coated with rS12 protein as antigen and blocked with blocking buffer, sample and conjugate diluent (2X), the 2nd antibody (anti-pig IgG or IgA HRP) (100X), substrate solution A, substrate solution B, stop solution and washing buffer (20X), was examined with high positive, low positive and negative sample. The stability of ELISA when collecting the data every month from ELISA kit preparing date until eighth month, found that the OD value were investigated from preparing time to sixth month of stock reagent and ELISA plate rather stable individual sample. After sixth month, OD value of IgG and IgA decreased rapidly until eighth month. At seven and a half month the OD value of IgG of the low

positive sample was equal to OD negative sample, and all measured values are on the same level as the OD value of a negative sample in the eighth month. The stability of OD value within 6 months showed range of sample means of high positive, low positive and negative sample of IgG ELISA is 1.917 to 2.107, 1.033 to 1.332, and 0.084-0.201, respectively (Figure 16A) and for IgA ELISA is 1.277 to 1.479, 0.550 to 0.757, and 0.200 to 0.323 (Figure 16B).



A



B

Figure 16 The stability test of IgG (A) and IgA (B) ELISA kit detect by stock of reagent keep at 4°C with high positive (H), low positive (L) and negative (N) sample against PEDV for 8 months.

3.2.4 Evaluation and validation

3.2.4.1 Repeatability

Within plate repeatability test of ELISA showed low level of the percentage of coefficient of variation (%CV), which less than 10% in both OD and S/P ratio of positive and negative sample observation (Table 1). In contrast to the repeatability test in different plate presented low level of %CV of OD, while in S/P ratio showed more wide range of %CV than in OD value in both IgG and IgA (Table 2).

Table 1 Range of mean, range of SD and range of CV (%) from triplicate test of 10 positive and 10 negative samples in same plate (within plate repeatability) by IgG ELISA.

Parameters	OD		S/P ratio	
	Positive samples	Negative samples	Positive samples	Negative samples
Mean	0.822-1.611	0.062-0.134	0.479-1.000	-0.024-0.026
SD	0.019-0.049	0.002-0.009	0.012-0.033	0.002-0.009
CV (%)	1.47-4.95	1.56-8.89	1.57-5.50	<0.000

Table 2 Range of mean, range of SD and range of CV (%) from 10 milk samples tested in 5 different plates at different days (between plate repeatability).

Parameters	IgG ELISA		IgA ELISA	
	OD	S/P ratio	OD	S/P ratio
Mean	0.060-1.654	0.070-1.557	0.066-1.810	-0.075-0.803
SD	0.005-0.089	0.022-0.123	0.005-0.079	0.007-0.049
CV (%)	2.36 - 9.75	-31.54-12.82	0.99-7.62	-31.65-13.79

3.2.4.2 Reproducibility or Inter-laboratory test

The inter-laboratory test of this ELISA kit presented perfected agreement between two laboratories that showed in plotted graph in Figure 17. The results of IgG and IgA of ELISA test were presented R-squared (R^2) value higher than 0.9 that is

demonstrated ELISA kit had highly reproducibility results. Although, OD value from lab B lower than OD value of lab A, all samples from ELISA test in both laboratories correlated with the result from VN (Appendix).

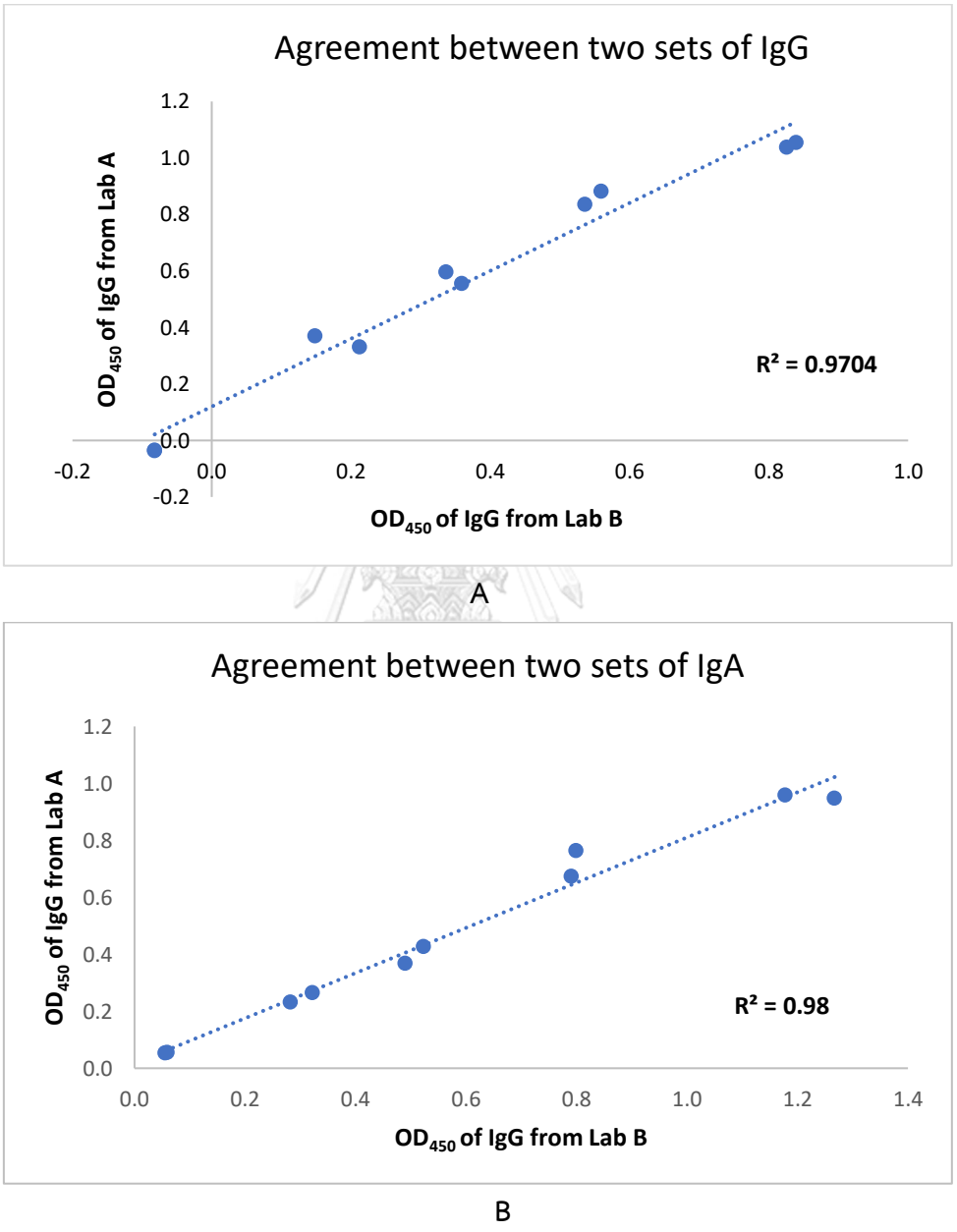


Figure 17 Agreement between two sets of OD of IgG (A) and OD of IgA (B) from different laboratory A (Lab A) and (Lab B) in ELISA test.

3.2.4.3 Cross reaction

The positive serum of TGEV, PDCoV and PEDV were investigated antibody of IgG by ELISA. The results of study found that S/P ratio of TGEV positive serum and PDCoV positive serum were not showed cross reaction in ELISA. The level of S/P ratio of TGEV positive serum and PDCoV positive serum less than cut-off value (S/P ratio < 0.4). In contrast to S/P ratio of PEDV positive serum was showed S/P ratio and OD value higher than cut-off level, S/P ratio > 0.4 and OD > 0.5 (data not showed).

3.2.4.4 Cut-off determination

ROC analysis determined cut off level of OD value and S/P ratio in both IgG and IgA are the same in this ELISA kit. The cut off level of OD is 0.5 and cut off level of S/P ratio is 0.4, that is convenient for detect antibody and interpret the result of IgG and IgA in the same kit. Diagnostic sensitivity, diagnostic specificity and accuracy of individual test were calculating under cut off level which choose from ROC analyses can demonstrated effective of ELISA kit. In addition, an effective of test kit was described from area under curve (AUC) approach to 1 in ROC that could be found in this study (Hanley and McNeil, 1982, Hajian-Tilaki, 2013). The AUC of OD IgG, OD IgA, S/P ratio IgG, and S/P ratio IgA is 0.994, 0.977, 0.973, and 0.954, respectively (Figure 18).

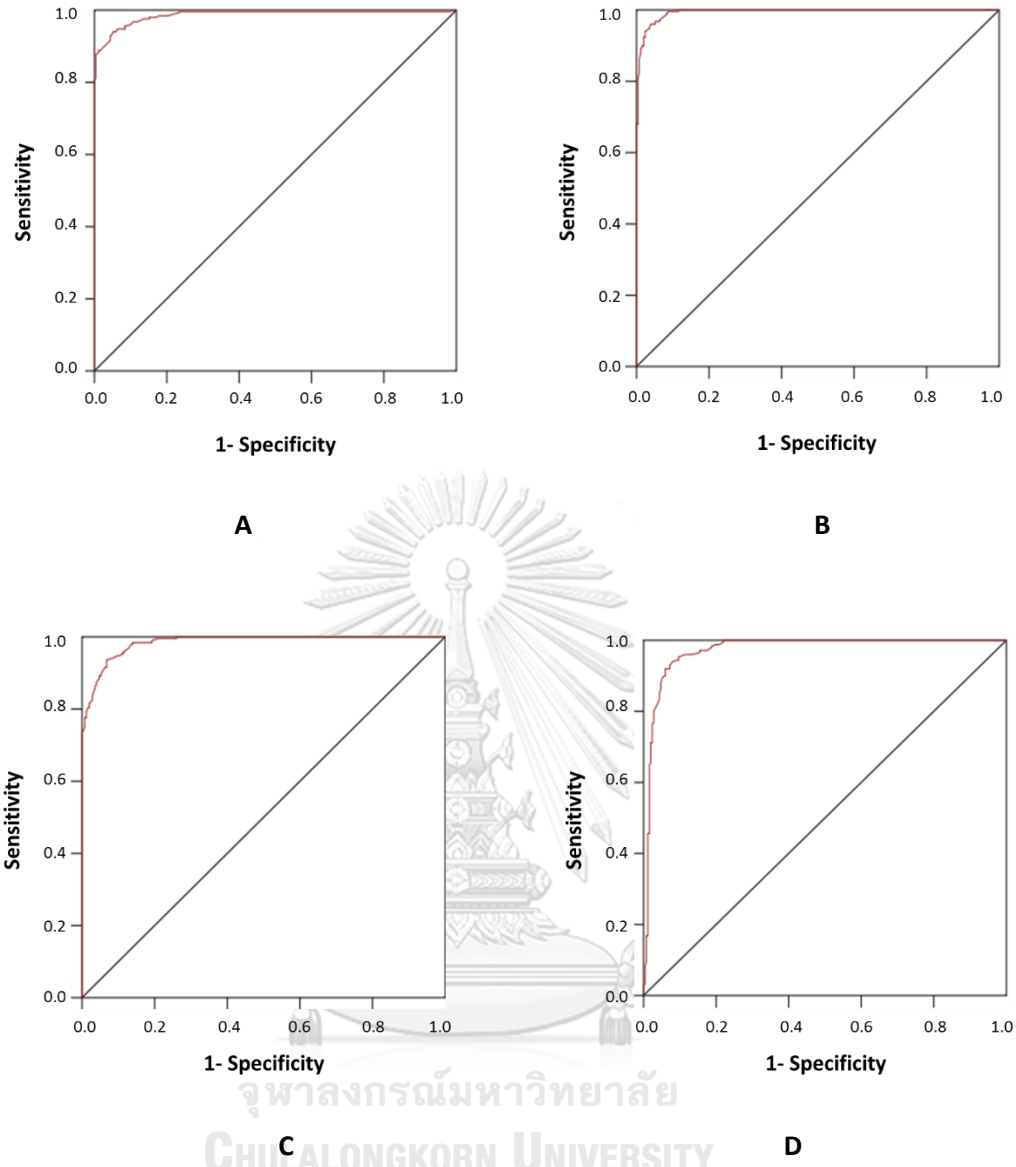


Figure 18 ROC curve of OD IgA (A) and IgG (B) and ROC curve of S/P ratio of IgA (C) and IgG (D).

3.2.4.5 Diagnostic sensitivity, diagnostic specificity and accuracy

Following cut off point determination by ROC curve via IBM SPSS statistic 22 software, the statically analysis of DSN, DSP and accuracy was performed. Under three decimal cut-off point, found that DSN, DSP and accuracy of OD value of IgG higher than OD value of IgA. In contrast to S/P ratio, only the DSN of IgG was higher than IgA, DSP of IgG lower than IgA and non-different level in accuracy test (Table 3). On the other hand, when determined with optimal cut off level from ROC, the OD value of IgG ELISA showed DSN higher than OD value of IgA, an equal level of DSP, and similarity of accuracy test. For S/P ratio, although, DSN of IgG higher than IgA, DSP of IgG lower than IgA. However, in S/P ratio of IgG and IgA the similarity of accuracy was showed when consider under one decimal cut-off point (Table 4). Subsequently, individual test both OD value and S/P ratio were presented by Scatterplot of OD value, which analyzed via GraphPad Prism 6 software. There was highly significant difference between VN positive and VN negative ($p < 0.001$) (Figure 19 and Figure 20).

3.2.4.6 Correlation coefficient

The measurement of correlation coefficient (r) was analyzed the strength and direction of a linear relationship on a scatterplot of two interest variable. The analysis of correlation between VN titer of colostrum and milk sample with OD value and S/P ratio of IgG and IgA ELISA showed a strong positive linear relationship between VN titer and ELISA. Sort by the highest to the lowest of correlation in this study is OD value of IgG (r= 0.80), OD value of IgA (r= 0.74), S/P ratio of IgG (r= 0.71) and S/P ratio of IgA (r= 0.70), respectively. The linear regression equation of each type of immunoglobulin and value measurement including, OD value of IgG, OD value of IgA, S/P ratio of IgG and S/P ratio of IgA were presented in figure of each study (Figure 21 and Figure 22)

Table 3 Percentage of DSN, DSP and Accuracy of IgG and IgA under cut- off determination by ROC curve of OD value and S/P ratio.

Correctly Identification	OD		S/P ratio	
	IgG	IgA	IgG	IgA
Cut off	0.518	0.500	0.432	0.373
DSN (%)	96.0	94.8	94.0	93.6
DSP (%)	96.4	95.6	92.8	93.2
Accuracy (%)	96.2	95.2	93.4	93.4

DSN, Diagnostic sensitivity

DSP, Diagnostic specificity

Table 4 Percentage of DSN, DSP and Accuracy of IgG and IgA under cut- off determination by ROC curve of OD value and S/P ratio in one decimal cut-off point.

Correctly Identification	OD		S/P ratio	
	IgG	IgA	IgG	IgA
Cut off	0.5*	0.5*	0.4*	0.4*
DSN (%)	96.0	94.8	95.2	91.6
DSP (%)	95.6	95.6	90.8	93.2
Accuracy (%)	95.8	95.2	93.0	92.4

DSN, Diagnostic sensitivity, DSP, Diagnostic specificity, * cut-off value after change to one decimal

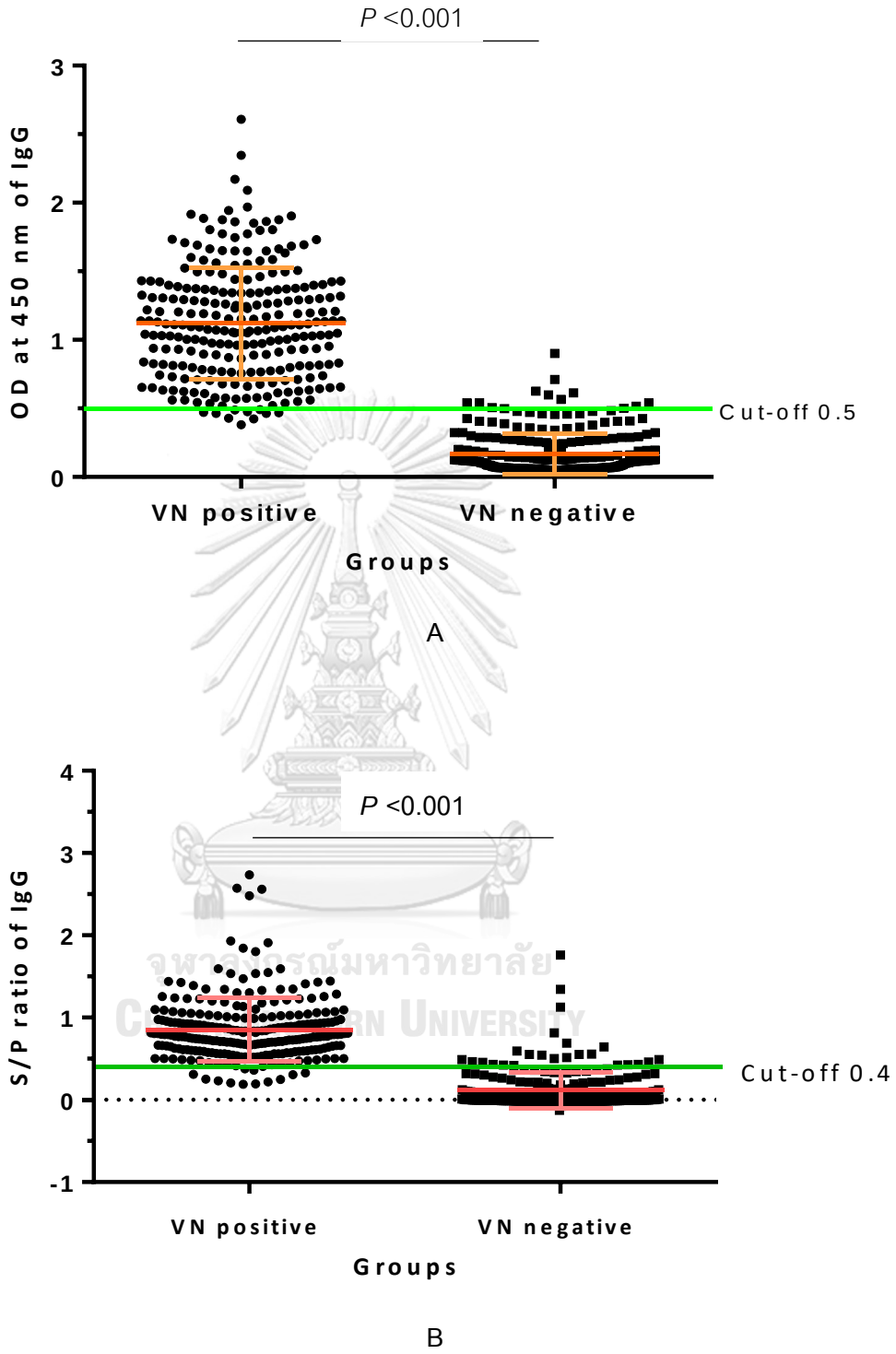


Figure 19 Scatterplot of OD value (A) and S/P ratio (B) from VN positive and VN negative sample of IgG ELISA. Green line indicates cut-off level of OD value as 0.5 and cut-off level of S/P ratio as 0.4.

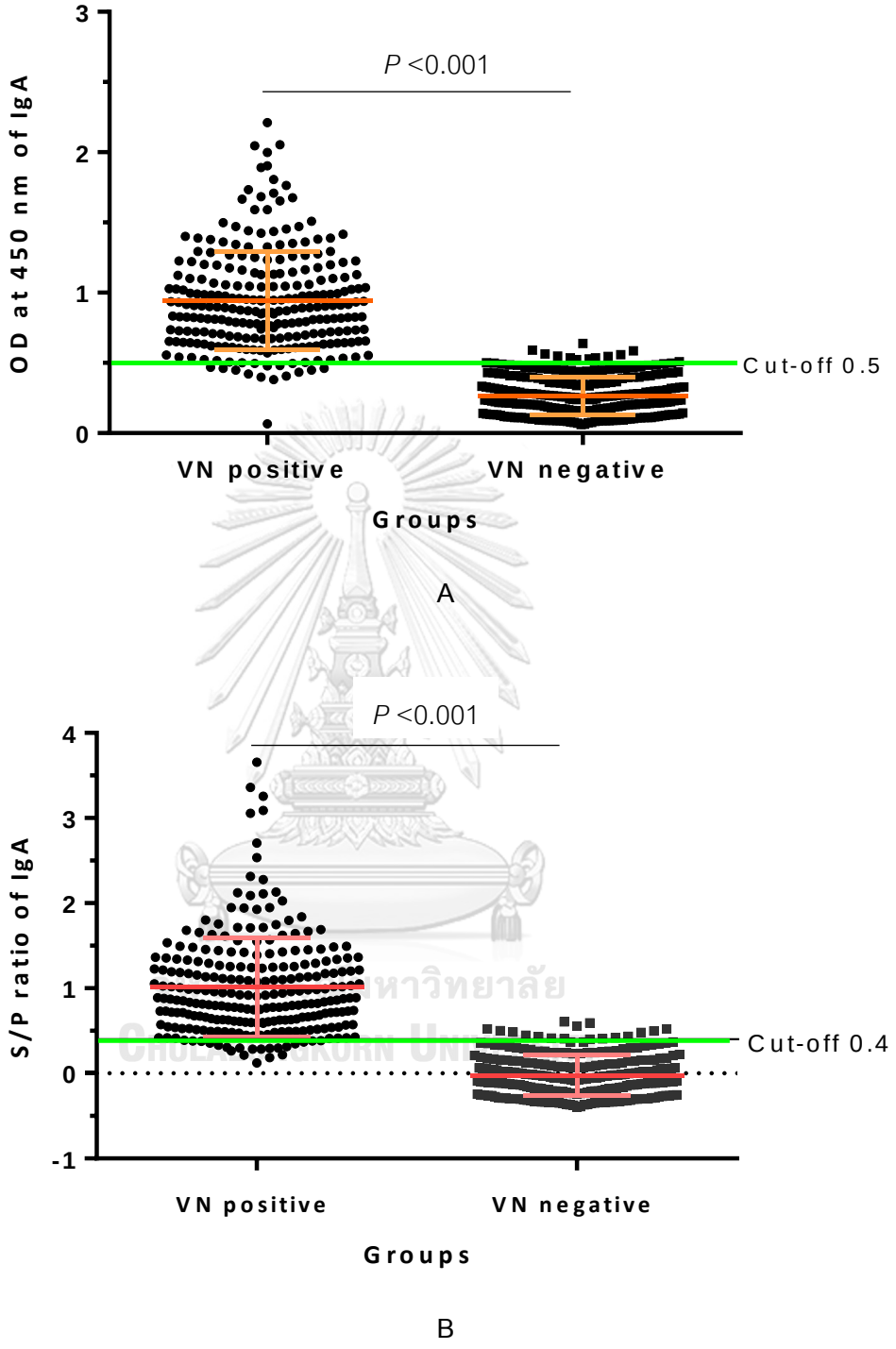
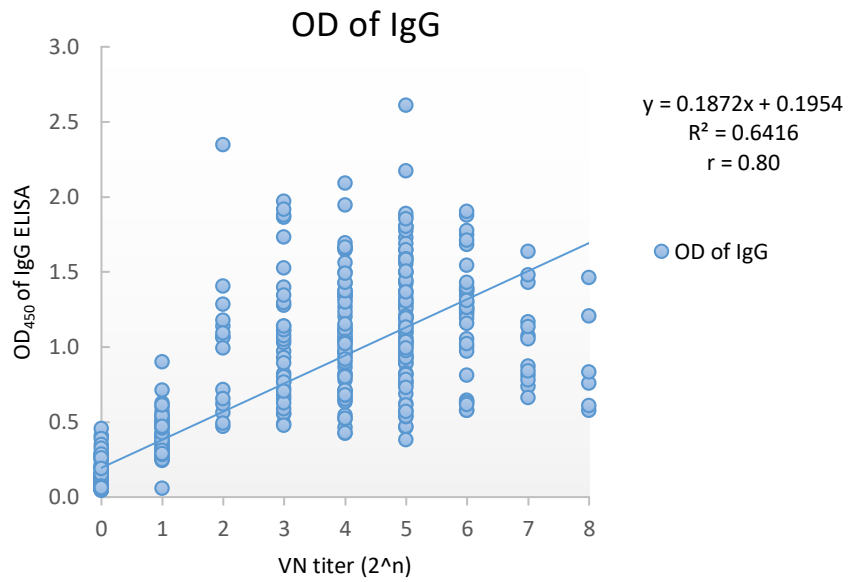
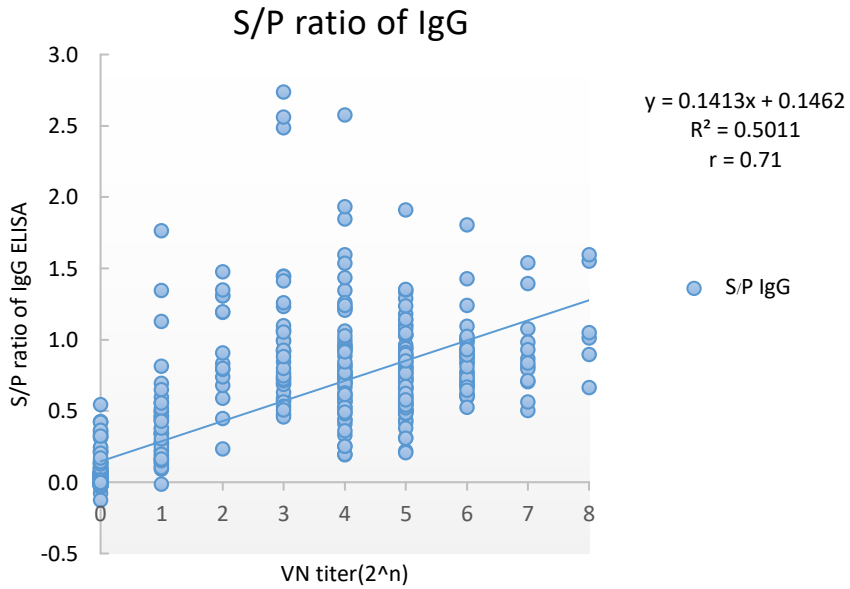


Figure 20 Scatterplot of OD value (A) and S/P ratio (B) from VN positive and VN negative sample of IgA ELISA. Green line indicates cut-off level of OD value as 0.5 and cut-off level of S/P ratio as 0.4.

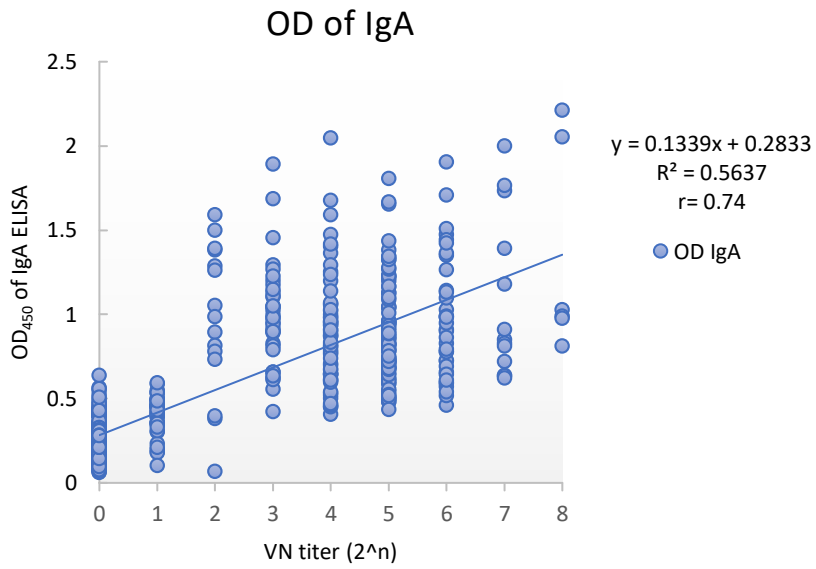


A

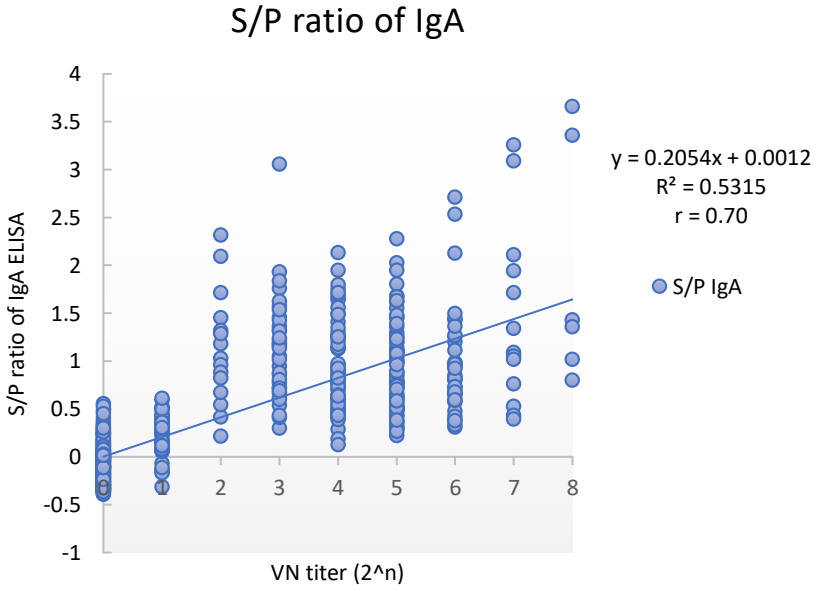


B

Figure 21 Scatter plots showing the correlation between VN titer against PEDV and OD values of IgG (A) and between VN titer and S/P ratio of IgG (B)



A



B

Figure 22 Scatter plots showing the correlation between VN titer against PEDV and OD values of IgA (A) and between VN titer and S/P ratio of IgA (B)

3.3 Discussion

The reagent and blocking solution preparation were composed with skim milk and bovine serum albumin (BSA). The result of using skim milk in composition aggregated after 1 month of stock reagent that was demonstrate that the stability of BSA better than skim milk. The results were consistent with properties of albumins, there were a good stabilizer for other solubilized protein. It was stable in low concentration of ammonium sulfate, precipitated in the lowing pH, and it was coagulated by heat (Lewis, 1993). Moreover, BSA is usually used as a blocking agent to prevent non-specific binding in ELISA plate. Nevertheless, the different composition of BSA might be influence to reactant of negative control; therefore, it should test non-specific background with negative control (Xiao and Isaacs, 2012). In this study, there were no critical effects on different composition in BSA when used as blocking buffer. For the samples and conjugate diluents, compositions of reagents were included with goat serum to block non-specific binding from secondary antibody producing from goat.

Another effect on reaction of ELISA test after 1 month of stock reagent preparation is substrate. The composition of substrate was used in this study containing 10% acetate buffer (pH 5.9), 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 3% H_2O_2. Normally, Hydrogen peroxide is an oxidizing agent that can be oxidized non-enzymatically with TMB solution and cause to degrade with non-enzymatic reaction (Bally and Gribnau, 1989). Therefore, stock substrate preparation should be divided into two stock solutions such research of a stable and highly sensitive TMB-based substrate reagent for ELISA (Frey et al., 2000), they prepared substrate solution by separation of the TMB solution and hydrogen peroxide with potassium citrate in different polyethylene tubes in the dark place. In addition, after separated preparation of substrate reagent, will prolong shelf life more than six months.

In term of evaluation and validation test of ELISA kit include repeatability, reproducibility, correlation, diagnostic sensitivity, diagnostic specificity and accuracy. Normally, the accepted value of %CV for precision test should not exceed 10% in intra-assay and not exceed 15% in inter-assay; however, 20%-30% was acceptable (Jacobson,

1998). Therefore, we concluded that the results of this ELISA kit were acceptable of repeatability in both intra- assay and inter-assay. Moreover, the comparison between laboratories of ELISA kit showed R-squared (R^2) was greater than 0.9 which can prove ELISA kit has highly reproducibility result.

In case of correlation coefficient, there was strong positive correlation of antibody detection between ELISA kit and VN test method which could conclude that the antibody response using this ELISA kit correlated with protection of PEDV in samples. Since the investigation of neutralizing antibody by viral neutralization test can demonstrate the protection of pig against PEDV. In the study, we can show that the correlation of IgG higher than IgA because the sample selection was conducted under result of neutralization assay (VN test) which sample came from various sources. If the samples came from intra-muscular injection vaccine could induce more IgG than IgA, especially in colostrum sample; whereas, naive sows were orally vaccinated with high virulent PEDV might be able to induce high titer of IgA in colostrum and milk similar with TGEV (Bohl et al., 1972).

Based on cut off level determined by ROC curve could be able to evaluate and validate ELISA kit in percentage of DSN, DSP and OD of IgG accuracy whether is more or equal from the validation result of OD of IgA. Meanwhile, the evaluation and validation in parameters as described above in this study were similar to S/P ratio, except the percentage of DSP of IgA was higher than DSP of IgG. It might be indicated that the interpretation from S/P ratio using this ELISA kit was highly accurate, especially in the no IgA antibody against PEDV of pig sample which could check from true negative value. On the other hand, the ability of IgG investigation via ELISA kit has high correctly to identify the positive samples.

In conclusion, this ELISA kit has precision and high correlation comparing with standard method. Therefore, it might be concluded that new ELISA kit can be used as a substitute for traditional testing method. In addition, the results of this ELISA kit can specify protection of PEDV in the piglets which was delivers from that colostrum and milk.

CHAPTER IV

APPLICATION USE OF ELISA KIT

The ELISA test kit was applied to investigate lactogenic immunity following oral exposure to porcine epidemic diarrhea virus.

4.1 Materials and methods

4.1.1 Group of study

In application use of ELISA, the sample were collected from tree different treatment group in different area. First group is PEDV free herd (Neg group). The second group, gilts were orally administered with PEDV-infected intestines at 24 weeks of age or single feedback group (FB-A group). Last group were double oral feedback group, gilts were orally administered with PEDV-infected intestines at 24 weeks of age and 4 weeks prior to farrowing (FB-B group). Both feedback group were confirmed PEDV infection following feedback by RT-PCR. Blood was collected at -14, -7 and 0 days post-exposure (DPE) for sera preparation and then assayed for the presence of PEDV-specific antibody using IgG/IgA ELISA, and VN assay. Colostrum was collected within 3 h, post parturition, and milk samples was collected at 7, 14, and 21 days post-parturition (DPP) and assayed for the presence of PEDV-specific antibody using IgG/IgA ELISA, and VN assay.

4.1.2 Sample preparation

Blood samples were centrifuge at 2,500 rpm for 10 min and collected the serum sample. Colostrum and milk samples was centrifuged to separate the lipid layer at 12,000 rpm for 20 minutes at 4°C. The middle layer was collected for antibody detection. The collected samples were heat-inactivated at 56°C for 30 minutes before use for VN assay.

4.1.3 Lactogenic immunity assay

The colostrum and milk were measured antibody of IgG and IgA levels against PEDV by this IgG/IgA ELISA. Moreover, the same sample was evaluated neutralizing titer by VN test that described below. In every testing was interpreted the result by comparing to positive and negative control. The antibody response in colostrum and milk was represented as sample-to-positive (S/P) ratios and was discussed with cut-off value.

4.1.3.1 Viral neutralization assay (VN)

Heat inactivated serum, colostrum and milk samples were two-fold serially diluted from 1:4 to 1:512. Each dilution of samples was mixed with an equal volume of PEDV diluted to contain 100 $TCID_{50}$/0.1 ml. The samples/virus mixtures were incubated at 37°C in 5% CO_2 incubator for 1 hour. The mixtures were added in duplicate to the wells of a 96-well microtiter plate containing a 48-hour-old confluent Vero cell monolayer and were incubated at 37°C in a 5% CO_2 incubator. The cytopathic effect was observed every day until 7 days post inoculation. The neutralizing titer were expressed as the highest sample dilution which no evidenced of cytopathic effect (100% normal cell). The viral concentration (10^2 $TCID_{50}$/0.05 ml) were also verified by back titration test.

4.1.3.2 Enzyme-linked immunosorbent assay (IgG/IgA ELISA)

Microtiter 96-well plates (Nunc MaxiSorp™, Nalge Nunc International, Rochester, New York, USA) were coated with recombinant spike protein 20 µg/ml or 50 µg/ml for IgG ELISA or IgA ELISA, respectively [1] and held overnight at 4°C in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.6. The wells were blocked with 5 % (w/v) skim milk in PBS and incubated for 3 hours at room temperature. The wells were incubated with 500-folded diluted colostrum and milk at room temperature for 1 hour. Following five washes with washing buffer (0.5% PBS-T), anti-pig IgG-HRP or IgA-HRP (AbD Serotec, Kidlington, United Kingdom) was added and incubated for 1 hour at 37°C, and then plates were washed four times with washing buffer prior to incubation with 3, 3', 5, 5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, St. Louis, Missouri USA) at room temperature for 15 min. The reaction was stopped using 1 N H_2SO_4,

and the optical density at 450 nm (OD_{450}) value was read using an ELISA plate reader (AccuReader, Metertech, Taipei, Taiwan). Negative and positive serum, colostrum and milk samples from a herd free of PED and a herd with PED outbreak were used as assay controls. The antibody response was represented as sample-to-positive (S/P) ratios calculated as: S/P ratio = (sample OD- blank control OD)/ (positive OD- blank control OD). For IgG ELISA, the S/P ratios of >0.4 were considered antibody positive, and <0.4 as negative. About IgA ELISA, the S/P ratios of >0.4 were considered antibody positive, and <0.4 as negative.

4.1.4 Statistical analysis

The statistical analyses were performed using SPSS Statistics V22.0 (IBM Corporation, Armonk, NY, USA). Data was analyzed using analysis of variance (ANOVA) to determine if there are any significant difference between treatment groups. If the *p*-value for the ANOVA is ≤ 0.05, the differences between treatment groups was evaluated by pairwise comparisons using least significant differences at the $p \leq 0.05$ rejection level.

4.2 Results

4.2.1 Antibody response in sera

Following the primary exposure at 24 weeks of age, pigs in the Neg group had no detectable antibody in their serum as measured by IgG/IgA ELISA and the VN assay throughout the study (Figure 23B). In contrast, the antibody responses in the serum of pigs of the FB group increased at 14, 28 and 56 DPE compared to -7 DPE. However, the levels of IgG and IgA antibodies were below or close to the cutoff levels (Figure 23A). The VN titer in serum was relatively low and observed in only 30 and 42% of the gilts in the FB group (single infection) at 14 (0.6) and 28 DPE (0.8), respectively. However, the VN titer increased slightly at 56 DPE (1.5), and VN antibodies were detected in all gilts. The levels of IgG/IgA ELISA in serum of the present study were relatively low.

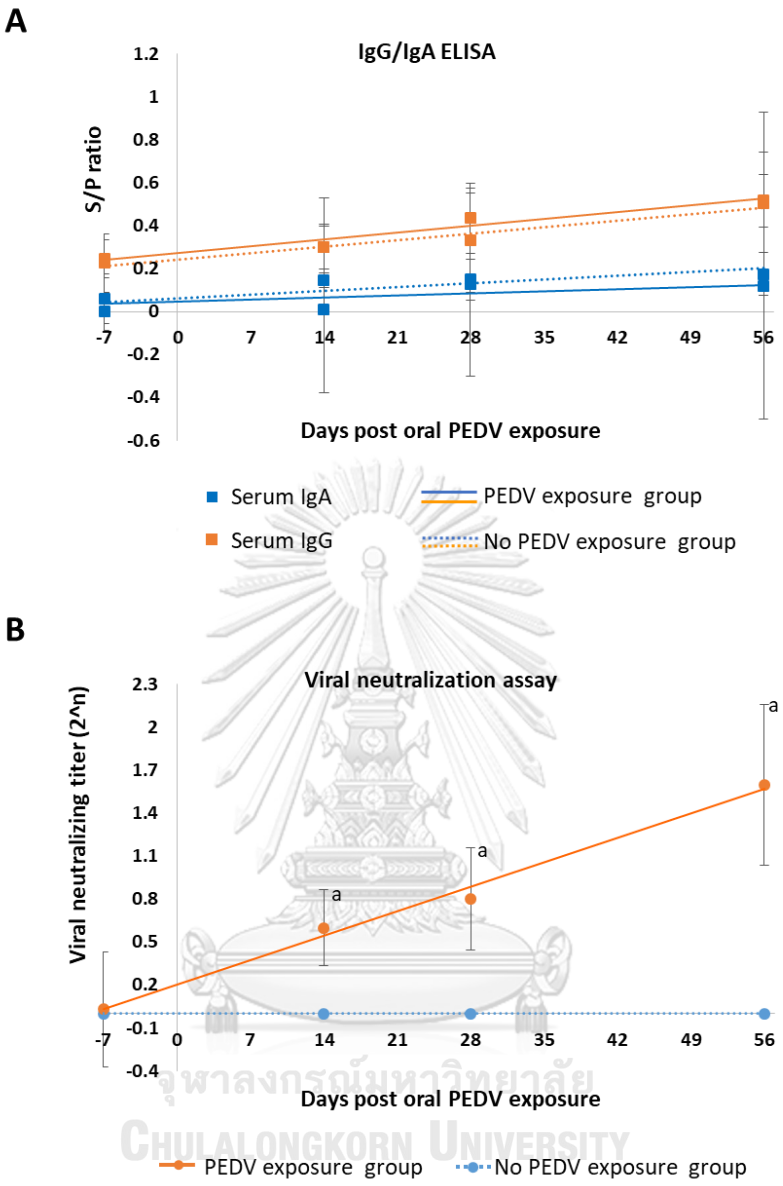


Figure 23 Immune response as measured by IgG/IgA ELISA and viral neutralization assay in serum samples of sows following in primary oral PEDV exposure. A) Antibody response as measured by IgG and IgA ELISA. B) Serum virus-neutralizing antibody titer). Variation is expressed as the standard deviation. Different letters in superscript indicate statistically significant differences ($p < 0.05$) between groups at each time point.

4.2.2 Antibody response in colostrum and milk

Following parturition, antibody titers in colostrum and milk samples as measured by IgG/IgA ELISA and VN assay were not detected in the Neg group throughout the lactation period. In contrast, the FB-A and FB-B groups both had a detectable level of neutralizing antibody in colostrum and milk samples throughout the lactation period. The neutralizing titers in colostrum and milk samples of both the FB-A and FB-B groups were significantly higher than that of the Neg group (Figure 24A). The FB-B group had significantly higher neutralizing antibody titers than those of the FB-A group throughout the lactation period. In contrast to neutralizing antibody, the antibody response as measured by IgG and IgA ELISA was detected in colostrum samples of both the FB-A and FB-B groups. The levels of IgG and IgA in the colostrum samples did not differ, but they were significantly higher than those of the non-orally exposed group. The FB-B group had a significantly higher level of IgG as measured by ELISA compared to that of the FB-A group. In contrast to the antibody response in colostrum, the antibody response as measured by IgG ELISA was not detected in the milk samples of any of the three groups at 7, 14, and 21 DPP. The IgG levels in milk samples did not differ between the three groups and were below 0.2, which is considered a negative status. The results show that an IgG response was observed in colostrum only after farrowing and decreased thereafter to an undetectable or low level in milk samples (Figure 24B). Following the decline of IgG antibody in colostrum, PEDV-specific IgA antibody subsequently predominated and remained consistently in the milk samples. In colostrum samples, the antibody response as measured by IgA ELISA was detected in both the FB-A and FB-B groups, and the IgA level in the FB-B group was higher than in the FB-A group. In milk samples, the IgA level of the FB-A group declined and was barely detectable at 7, 14, and 21 DPP. The level of IgA in milk samples of the FB-A group was below or close to 0.4, which is considered a negative status. In contrast, the FB-B group had significantly higher levels of IgA than that of the FB-A group on 7, 14 and 21 DPP (Figure 24C). The results of the study suggest that, following oral PEDV exposure, the antibody response in milk samples switches toward the production of IgA, and the secondary oral exposure at prepartum provides an

anamnestic response, as indicated by the significantly higher level of IgA compared to pigs with no secondary exposure.

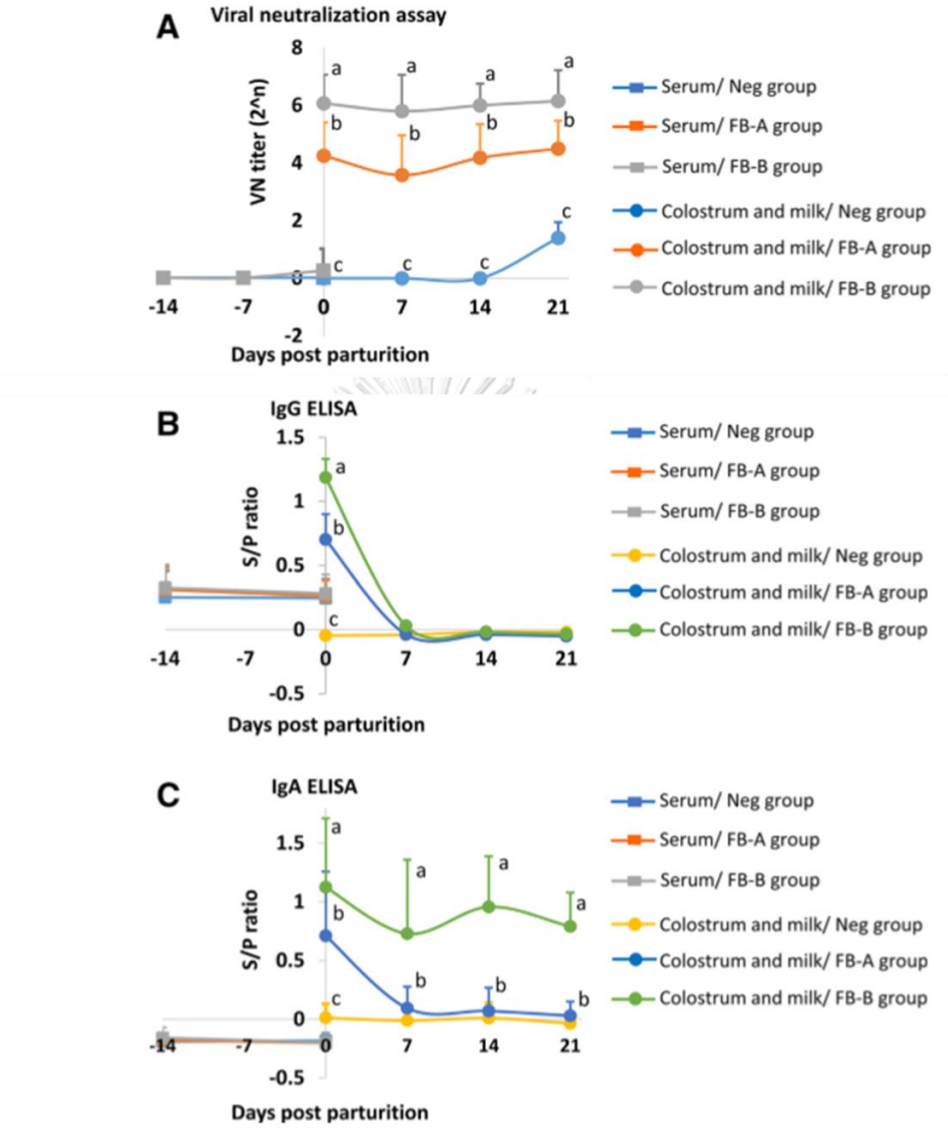


Figure 24 Immune response as measured by IgG/IgA ELISA and virusneutralization assay in serum, colostrum (0 DPP) and milk (7, 14 and 21 DPP) samples of sows following secondary oral PEDV exposure. A) Virus-neutralizing antibody titer. B) Antibody response as measured by IgG ELISA. C) Antibody response as measured by IgA ELISA. Variation is expressed as the standard deviation. Different letters in superscript indicate statistically significant differences ($p<0.05$) between groups at each time point.

4.3 Discussion

In this study, the antibody response in colostrum and milk samples from the gilts after parturition were different results with the response in serum samples. It might be explain that the gilts orally exposed with PEDV exhibited antibody responses IgA more than IgG, especially in milk samples. Unfortunately, piglets were not challenged to measure the protective efficacy of the lactogenic immunity. However, if the lactogenic immunity of PEDV is assessed based on the level of IgA, the increased IgA level in the study following oral exposure can indicate protection against PEDV. The predominance of IgA in milk samples is consistent with previous studies on the lactogenic immunity and milk antibodies to TGEV and PEDV in sows (Langel et al., 2016; Lanza et al., 1995) suggesting that oral PEDV exposure induces immunity similar to that of natural infection. It is noteworthy that although the levels of lactogenic immunity as measured by IgG/IgA ELISA were at or close to the negative cutoff level and were similar to those of the negative control group. The VN titer in milk samples remained at a detectable level. Surprisingly, the results of measurement of IgG via ELISA and VN assays in the milk samples of the FB-A group showed low IgG response with ELISA, but high levels of VN, which normally the serum VN titer and Level of IgG should be high coherently. The assay had previously been validated and published elsewhere (Srijangwad et al., 2016). We repeated the tests and obtained similar results. In addition, we had been getting similar results in our other field investigations. The cause of this result might be the nature of the immune response against oral infection or IgA response or Influence of hormones. In addition, whereas the spike protein is cleaved into S1 and S2 subunits, in the ELISA assay used in the present study, the plate was coated with a truncated S1 spike protein subunit. There are at least four identified virus-neutralizing epitopes on the spike protein (Temeeyasen et al., 2014). The truncated S1 gene region might not cover all four epitopes, which could potentially result in contradictory results between the ELISA and VN assay.

In conclusion, the single oral PEDV infection during the gilt acclimatization period results in a slightly increased serum antibody response as measured by IgG ELISA

and the VN assay, but not by IgA ELISA. The results suggest that oral PEDV exposure induces an antibody response in serum, mainly toward the production of IgG rather than IgA, and this increased antibody production correlates with the neutralizing activity. An antibody response in the single-infection group was later observed in colostrum and milk, but the level was relatively low. The single infection during the gilt acclimatization period or at 24 weeks of age, which was approximately 6 months prior to parturition, might not have provided sufficient levels of protection to piglets during lactation. The double infection at prepartum is suggested to increase the antibody level in colostrum and milk, as shown by the results of the present study. The repeated oral feedback (double infection) at the late stage of gestation provides a booster effect, as demonstrated by the significantly higher level of antibody in colostrum and milk as measured by IgA ELISA and VN titration in the group FB-B (double infection) compared with that of the FB-A group (single infection). Further investigations including lactogenic immunity and protection of piglets against virus challenge are urgently needed to provide a successful vaccination protocol.

CHAPTER V

GENERAL DISSCUSSION AND CONCLUSION

Development of ELISA test kits needs to consider the appropriate method for each type of inspection. It is well known that there are four systems of ELISA: direct, indirect, sanswich and competition or inhibition (Crowther, 2000). Choosing the right approach takes into account the various factors and many aspects, especially the availability or potential of the manufacturer that mean the reagent in our laboratory. Moreover, it should also consider for the purpose of the assay (antibody or antigen detection) and the purpose of development (only for internal use or distributing to other laboratories or commercial test kit). In this study, we have previously performed protein efficiency tests and found that rS12 protein can be used as an antigen for effective detection of antibody against PEDV (Srijangwad et al., 2016). We selected indirect ELISA method because we can produce antigens, which specific to neutralizing antibody in our laboratory and due to other systems have more limitation of antibody species. It is described as follows, when using the direct sandwish ELISA system, antibodies specific to the antigen can be used as conjugates and the antigen must have at least two different antigenic sites for bind to antibody from the samples and conjugate and it is usually suitable for antigen detection (Stanker and Hnasko, 2015). In addition, the single antibodies preparation is limited to specific antigens, can not be used for different types of antigens. In indirect sandwhish ELISA, the sample antibody for coating must be different species to detecting antibody and can not bind to the anti species conjugate. As for indirect competition ELISA, the pre-titrated antibody mixed with anti species conjugate which come from different species and can not cross reaction (Crowther, 2000).

As mentioned above, the selection of the antigen is the key of the ELISA test kit development. The purpose of the ELISA test kit is for detecting the antibody which can

neutralize PEDV. Therefore, the recombinant protein was chosen from conserve region, locate on neutralizing epitope of the PEDV genes (Srijangwad et al., 2017) and the position of the gene is rarely changed or stable when compared to various strains of PEDV in different area. In previous study, spike protein of PEDV was accepted for use as antigen in antibody detection by ELISA. All of the result showed high specificity and sensitivity (Gerber et al., 2014; Gerber and Opriessnig, 2015) and high correlation when comparing to standard method. Esspecially S1 domain of spike protein which majority region of neutralizing epitope are located on (Gerber et al., 2014). However, the rS12 protein were expressed under *E. coli* system that can happen problem of protein misfolding and inclusion body. Possibility of misfolding of protein is increasing of a strong promoter and high concentration of inducer using. These environmental stresses affect to protein structure loss. The two other misfolding conformations are a newly synthesized chain to correct conformation failure and premature termination of protein translation process. The most factor to protein misfolding is folding helper, chaperones, can help to preventing from the interactive solvents and other, and can be folding catalysts. Traditionally, misfolding and inclusion body prevention can be achieved by using weak promoter, low concentration of inducer and low temperature of protein expression, reduce transcription and translation rate and interactive force of hydrophobic (Baneyx and Mujacic, 2004).

The procedure which complicated and needs to be carefully considered, as the antigen preparation is ELISA optimization. The process of ELISA optimization need to optimized all of material were used in ELISA kit. The ELISA plate should pass random inspection and have a good feature (Gibbs, 2001). All reagents including, blocking buffer, sample diluent, conjugate diluent, substrate, stop solution, and washing buffer are necessary to check stability and must not affect to background occurrence. Especially, blocking agent, not only could be reduced non-specific binding and preserve biomolecules bind to well surface (Gibbs, 2001) but also do not bind to ELISA reactants (Xiao and Isaacs, 2012). The determining the optimum concentration of the components to obtain a suitable formula for the antigen and antibody reaction is very important. This

experiment adapted formula from others and guide book recomendation (Crowther, 2000; Gerber and Opriessnig, 2015), due to the optimal condition of ELISA always differenced in each type of ELISA. The formula of reagents in this ELISA kit was revealed in Appendix.

According to the references from several ELISA development researches and guide books about validation test of ELISA kit, it is representing reliability of test kit. However, not only the tests are accurate, the selection of the sample also should sampling by cover multilevel of the antibodies in equal number. The sample selection of this study is collected from many sources and different immunization route or vaccine program. The sample are not precisely grouping, only divided into positive and negative VN group for validation test. The majority of sample ratio is lack of high number of high VN titer, that has led to the correlation between ELISA and VN assay trend to lower than it should be.

An important process of antibody investigation is sample preparation. Since colostrum and milk compositions contain fat (~7-8%), lactose (~4%), minerral and protein; casein and whey protein (Ig, serum akbumin and lactofferrin) from 19% by volume of milk (Hurley, 2012). The composition of the colostrum and milk in each pig is different. Therefore, the sample preparation should be avoided fat and other proteins contamination.

Another limitation of validation test is that if pigs are stimulated with a different group of PEDV or different nucleotide on neutralizing epitope PEDV, the result of VN titer may in erroneous antibody levels when use another PEDV strain, do not match with the PEDV strains trigger the antibody for neutralizing assay. This is one limitation that make the results of sensitivity and specificity in validation tests lower than using same PEDV strains which similarly report of S1 protein of G2 prototype U.S. strain ELISA that use same strain of PEDV for standard method comparing to ELISA. They showed that ELISA obtained 100% of specificity and 99 % of sensitivity of IgA in colostrum (Gerber et al., 2014). On the other hand, It is possible that if the samples are collected from vaccinated

pig, stimulate with vaccine strain, then they are examined against the original strains in standardize method may decrease sensitivity and specificity of ELISA.

In terms of usage, the test kit also has limitations for use in operating rooms, in rooms with no overheating, or in temperature control room and with the necessary equipment. In addition, the lab worker must be proficient in the use of laboratory equipment.

In conclusion this ELISA test kit can be used for antibody detection against PEDV from colostrum and milk sample of pig replace the original method, viral neutralization test. However, the interpretation will be performed in a straightforward manner and indicative of OD values and S/ P ratio, and specimens with or without antibodies to PDEV can be identified from the samples. Lab worker can not claim whether the animal have immunity against PEDV. The consideration will depend on the farm veterinarian, as there are other factors affecting the results.

REFERENCES

Annamalai T, Saif, LJ, Lu, Z and Jung, K 2015. Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs. Vet Immunol Immunopathol 168(3-4): 193-202.

Baek PS, Choi HW, Lee S, Yoon IJ, Lee YJ, Lee du S, Lee S and Lee C 2016. Efficacy of an inactivated genotype 2b porcine epidemic diarrhea virus vaccine in neonatal piglets. Vet Immunol Immunopathol 174: 45-49.

Bally RW and Gribnau TCJ 1989. Some Aspects of the Chromogen 33^5,5'-Tetramethylbenzidine äs Hydrogen Donor in a Horseradish Peroxidase Assay. J Clin Chem Clin Biochem. 27(10): 791-796.

Baneyx F and Mujacic M 2004. Recombinant protein folding and misfolding in Escherichia coli. Nat Biotechnol 22(11): 1399-1408.

Baudoux P, Carrat C, Besnardeau L, Charley B and Laude H, 1998. Coronavirus Pseudoparticles Formed with Recombinant M and E Proteins Induce Alpha Interferon Synthesis by Leukocytes Journal of virology 72(11): 8636–8643.

Bohl EH, Gupta RK, Olquin MV and Saif LJ 1972. Antibody responses in serum, colostrum, and milk of swine after infection or vaccination with transmissible gastroenteritis virus. Infect Immun 6(3): 289-301.

Bosch BJ, van der Zee R, de Haan CAM, and Rottier PJM 2003. The Coronavirus Spike Protein Is a Class I Virus Fusion Protein: Structural and Functional Characterization of the Fusion Core Complex. Journal of Virology 77(16): 8801-8811.

Cabrera RA, Lin X, Campbell JM, Moeser AJ and Odle J 2012. Influence of birth order, birth weight, colostrum and serum immunoglobulin G on neonatal piglet survival. J Anim Sci Biotechnol 3(1): 42.

Chang SH, Bae JL, Kang TJ, Kim J, Chung GH, Lim CW, Laude H, Yang MS and Jang YS 2002. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. Mol Cells 14(2): 295-299.

Chasey D, and Cartwright SF 1978. Virus-like particles associated with porcine epidemic diarrhoea. Res Vet Sci 25(2): 255-256.

Chen, Q., Li, G., Stasko, J., Thomas, J.T., Stensland, W.R., Pillatzki, A.E., 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol 52(1): 234-243.

Crowther JR 2000. The ELISA guidebook. Methods Mol Biol 149: III-IV, 1-413.

Cruz DJ, Kim CJ and Shin HJ 2006. Phage-displayed peptides having antigenic similarities with porcine epidemic diarrhea virus (PEDV) neutralizing epitopes. Virology 354(1): 28-34.

Cruz DJ, Kim CJ and Shin HJ 2008. The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize Porcine epidemic diarrhea virus. Virus Res 132(1-2): 192-196.

de Arriba ML, Carvajal A, Pozo J and Rubio P 2002a. Isotype-specific antibody-secreting cells in systemic and mucosal associated lymphoid tissues and antibody responses in serum of conventional pigs inoculated with PEDV. Vet Immunol Immunopathol 84(1-2): 1-16.

de Arriba ML, Carvajal A, Pozo J and Rubio P 2002b. Mucosal and systemic isotype-specific antibody responses and protection in conventional pigs exposed to virulent or attenuated porcine epidemic diarrhoea virus. Vet Immunol Immunopathol 85(1-2): 85-97.

Debouck P and Pensaert M 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV777. American journal of veterinary research 41(2): 219-223.

Duarte M, Tobler K, Bridgen A, Rasschaert D, Ackermann, M and Laude H 1994. Sequence analysis of the porcine epidemic diarrhea virus genome between the nucleocapsid and spike protein genes reveals a polymorphic ORF. Virology 198(2): 466-476.

Egberink HF, Ederveen J, Callebaut P and Horzinek MC 1988. Characterization of the structural proteins of porcine epizootic diarrhea virus, strain CV777. Am J Vet Res 49(8): 1320-1324.

Frey A, Meckelein B, Externest D and Schmidt MA 2000. A stable and highly sensitive 3,3',5,5'-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays. J Immunol Methods 233(1-2): 47-56.

Gerber PF, Gong Q, Huang YW, Wang C, Holtkamp D and Opriessnig T 2014. Detection of antibodies against porcine epidemic diarrhea virus in serum and colostrum by indirect ELISA. Vet J 202(1): 33-36.

Gerber PF and Opriessnig T 2015. Detection of immunoglobulin (Ig) A antibodies against porcine epidemic diarrhea virus (PEDV) in fecal and serum samples. MethodsX 2: 368-373.

Gibbs J 2001. Immobilization Principles – Selecting the Surface, ELISA technical bulletin No. 1 (Kennebunk, Maine, labcluster.com), 1-8.

Harper S and Speicher DW 2011. Purification of proteins fused to glutathione S-transferase. Methods Mol Biol 681: 259-280.

Hofmann M and Wyler R 1990. Enzyme-linked immunosorbent assay for the detection of porcine epidemic diarrhea coronavirus antibodies in swine sera. Vet Microbiol 21(3): 263-273.

Hou XL, Yu LY and Liu J 2007. Development and evaluation of enzyme-linked immunosorbent assay based on recombinant nucleocapsid protein for detection of porcine epidemic diarrhea (PEDV) antibodies. Vet Microbiol 123(1-3): 86-92.

Huang YW, Dickerman AW, Piñeyro P, Li L, Fang L and Kiehne R 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio 4(5):e00737-13. doi: 10.1128/mBio.00737-13.

Jacobson RH 1998. Validation of serological assays for diagnosis of infectious diseases Rev sci tech Off int Epiz. 17(2): 469-486.

Jarvis MC, Lam HC, Zhang Y, Wang L, Hesse RA, Hause BM, Vlasova AN, Wang Q, Zhang J, Nelson MI, Murtaugh MP and Marthaler D 2016. Genomic and evolutionary inferences between American and global strains of porcine epidemic diarrhea virus. Prev Vet Med. 123: 178-184.

Jung K, Hu H, Eyerly B, Lu Z, Chepngeno J and Saif LJ 2015. Pathogenicity of 2 porcine deltacoronavirus strains in gnotobiotic pigs. Emerg Infect Dis 21(4): 650-654.

Jung K and Saif LJ 2015. Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. Vet J 204(4): 134-143.

Jung K, Wang Q, Scheuer KA, Lu Z, Zhang Y and Saif LJ 2014. Pathology of US porcine epidemic diarrhea virus strain PC21A in gnotobiotic pigs. Emerg Infect Dis 20(4): 662-665.

Knuchel M, Ackermann M, Muller HK and Kihm U 1992. An ELISA for detection of antibodies against porcine epidemic diarrhoea virus (PEDV) based on the specific solubility of the viral surface glycoprotein. Vet Microbiol 32(2): 117-134.

Kocherhans R, Bridgen A, Ackermann M and Tobler K 2001. Completion of the porcine epidemic diarrhoea coronavirus (PEDV) genome sequence. Virus Genes 23(2), 137-144.

Kuo L, Godeke GJ, Raamsman MJB, Masters PS and Rottier PJM 2000. Retargeting of Coronavirus by Substitution of the Spike Glycoprotein Ectodomain: Crossing the Host Cell Species Barrier. J Virol 74(3): 1393-1406.

Langel SN, Paim FC, Lager KM, Vlasova AN and Saif LJ 2016. Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Historical and current concepts. Virus Res 226: 93-107.

Lanza I, Shoup DI and Saif LJ 1995. Lactogenic immunity and milk antibody isotypes to transmissible gastroenteritis virus in sows exposed to porcine respiratory coronavirus during pregnancy. Am J Vet Res 56(6): 739-748.

Lee DK, Park CK, Kim SH and Lee C 2010. Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea. Virus Res 149(2): 175-182.

Lee S, Kim Y and Lee C 2015. Isolation and characterization of a Korean porcine epidemic diarrhea virus strain KNU-141112. Virus Res 208.

Lee YN and Lee C 2013. Complete genome sequence of a novel porcine parainfluenza virus 5 isolate in Korea. Arch Virol 158(8): 1765-1772.

Lewis RJ 1993. Hawley's Condensed Chemical Dictionary 12th Edition. Van Nostrand Reinhold Co., New York.

Li W, Li H, Liu Y, Pan Y, Deng F, Song Y, Tang X and He Q 2012. New variants of porcine epidemic diarrhea virus, China, 2011. Emerg Infect Dis 18(8): 1350-1353.

Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ and Tan S 2005. Comparison of affinity tags for protein purification. Protein Expr Purif 41(1): 98-105.

Liu C, Tang J, Ma Y, Liang X, Yang Y, Peng G, Qi Q, Jiang S, Li J, Du L and Li F 2015. Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. J Virol 89(11), 6121-6125.

Lorsirigool A, Saeng-Chuto K, Temeeyasen G, Madapong A, Tripipat T, Wegner M, Tuntituvanont A, Intrakamhaeng M and Nilubol D 2016. The first detection and full-length genome sequence of porcine deltacoronavirus isolated in Lao PDR. Arch Virol 161(10): 2909-2911.

Madson DM, Magstadt DR, Arruda PH, Hoang H, Sun D, Bower LP 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol 174(1-2): 60-68.

Makadiya N, Brownlie R, van den Hurk J, Berube N, Allan B, Gerdts V and Zakhartchouk A 2016. S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. Virol J 13: 57.

Nilubol D and Khatiworavage C 2012a. Comparitive efficacy of oral administration of minced piglet intestine vs intramusculor vaccination in the control porcine epidemic diarrhea in gilts. In: International Pig Veterinary Congress, Jeju, Korea, p381.

Nilubol D and Khatiworavage C 2012b. Control of porcine epidemic diarrhea virus through intestinal feedback and parity segregation system: field cases. In: International Pig Veterinary Congress, Jeju, Korea, p222.

Oh JS, Song DS, Yang JS, Song JY, Moon HJ, Kim TY and Park BK 2005. Comparison of an enzyme-linked immunosorbent assay with serum neutralization test for serodiagnosis of porcine epidemic diarrhea virus infection. J Vet Sci 6(): 349-352.

Ojkic D, Hazlett M, Fairles J Marom A, Slavic D and Maxie G 2015. The first case of porcine epidemic diarrhea in Canada. Can Vet J 56(2): 149-152.

Okda F, Liu X, Singrey A, Clement T, Nelson J, Christopher-Hennings J, Nelson EA and Lawson S 2015. Development of an indirect ELISA, blocking ELISA, fluorescent microsphere immunoassay and fluorescent focus neutralization assay for serologic evaluation of exposure to North American strains of Porcine Epidemic Diarrhea Virus. BMC Vet Res 11: 180.

Pan Y, Tian X, Li W, Zhou Q, Wang D, Bi Y, Chen F and Song Y 2012. Isolation and characterization of a variant porcine epidemic diarrhea virus in China. Virol Jl 9:195.

Pensaert M and Yeo S 1994. Porcine epidemic diarrhoea. STRAW, BE; ZIMMERMAN, JJ; D'ALLAIRE, S.

Pensaert MB and de Bouck P 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch Virol 58(3): 243-247.

Piao DC, Lee YS, Bok JD, Cho CS, Hong ZS, Kang SK and Choi YJ 2016. Production of soluble truncated spike protein of porcine epidemic diarrhea virus from inclusion bodies of Escherichia coli through refolding. Protein Expr Purif. 126: 77-83.

Puranaveja S, Poolperm P, Lertwatcharasarakul P, Kesdaengsakonwut S, Boonsoongnern A and Urairong K 2009. Chinese-like strain of porcine epidemic diarrhea virus. Thailand Emerg Infect Dis 15(7)1112-1115.

Saif L, Pensaert M, Sestak K, Yeo S and Jung K 2012. Coronaviruses, p 501–524. Disease of swine 35.

Saif LJ, Bohl EH and Gupta RK 1972. Isolation of porcine immunoglobulins and determination of the immunoglobulin classes of transmissible gastroenteritis viral antibodies. Infect Immun 6(4): 600-609.

Shibata I, Tsuda T, Mori M, Ono M, Sueyoshi M and Uruno K 2000. Isolation of porcine epidemic diarrhea virus in porcine cell cultures and experimental infection of pigs of different ages. Vet Microbiol 72(3-4): 173-182.

Song D, Moon H and Kang B 2015. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. Clin Exp Vaccine Res 4, 166-176.

Song DS, Oh JS, Kang BK, Yang JS, Moon HJ and Yoo HS 2007. Oral efficacy of Vero cell attenuated porcine epidemic diarrhea virus DR13 strain. Research in veterinary science 82(1):134-140.

Spaan W, Cavanagh D and Horzinek MC 1988. Coronaviruses: Structure and Genome Expression. Journal of General Virology 69: 2939-2952.

Srijangwad A, Nilubol D, Chongcharoen W, Phoolcharoen W, Chuanasa T and Tantituvanont A 2016. Production of spike and nucleocapsid recombinant proteins of porcine epidemic diarrhea virus for antibody detection by ELISA. Asian Journal of Pharmaceutical Sciences 11(1): 85-86.

Srijangwad A, Stott CJ, Temeeyasen G, Senasuthum R, Chongcharoen W, Tantituvanont A and Nilubol D 2017. Immune response of gilts to single and double infection with porcine epidemic diarrhea virus. Arch Virol 162(7): 2029-2034.

Structural Genomics C, China Structural Genomics C, Northeast Structural Genomics C, Graslund S, Nordlund P, Weigelt J, Hallberg BM, Bray J, Gileadi O, Knapp S, Oppermann U, Arrowsmith C, Hui R, Ming J, dhe-Paganon S, Park HW, Savchenko A, Yee A, Edwards A, Vincentelli R, Cambillau C, Kim R, Kim SH, Rao Z, Shi Y, Terwilliger TC, Kim CY, Hung LW, Waldo GS, Peleg Y, Albeck S, Unger T, Dym O, Prilusky J, Sussman JL, Stevens RC, Lesley SA, Wilson IA, Joachimiak A, Collart F, Dementieva I, Donnelly MI, Eschenfeldt WH, Kim Y, Stols L, Wu R, Zhou M, Burley SK, Emtage JS, Sauder JM, Thompson D, Bain K, Luz J, Gheyi T, Zhang F, Atwell S, Almo SC, Bonanno JB, Fiser A, Swaminathan S, Studier FW, Chance MR, Sali A, Acton TB, Xiao R, Zhao L, Ma LC, Hunt JF, Tong L, Cunningham K, Inouye M, Anderson S, Janjua H, Shastry R, Ho CK, Wang D, Wang H, Jiang M, Montelione GT, Stuart DI, Owens RJ, Daenke S, Schutz A, Heinemann U, Yokoyama S, Bussow K and Gunsalus KC 2008. Protein production and purification. Nat Methods 5: 135-146.

Sun D, Feng L, Shi H, Chen J, Cui X, Chen H, Liu S, Tong Y, Wang Y and Tong G 2008. Identification of two novel B cell epitopes on porcine epidemic diarrhea virus spike protein. Vet Microbiol 131(1-2): 73-81.

Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK and Song CX 2012. Outbreak of porcine epidemic diarrhea in suckling piglets. China Emerg Infect Dis 18(1): 161-163.

Temeeyasen G, Srijangwad A, Tripipat T, Tipsombatboon P, Piriyapongsa J, Phoolcharoen, W., Chuanasa, T., Tantituvanont, A., Nilubol, D., 2014. Genetic diversity of ORF3 and spike genes of porcine epidemic diarrhea virus in Thailand. Infect Genet Evol 21: 205-213.

van Nieuwstadt AP and Zetstra T 1991. Use of two enzyme-linked immunosorbent assays to monitor antibody responses in swine with experimentally induced infection with porcine epidemic diarrhea virus. American journal of veterinary research 52(7): 1044-1050.

Wang E, Guo D, Li C, Wei S, Wang Z, Liu Q, Zhang B, Kong F, Feng L and Sun D 2016. Molecular Characterization of the ORF3 and S1 Genes of Porcine Epidemic Diarrhea Virus Non S-INDEL Strains in Seven Regions of China, 2015. PLoS One 11(8), e0160561.

Wang Y and Zhang X 1999. The nucleocapsid protein of coronavirus mouse hepatitis virus interacts with the cellular heterogeneous nuclear ribonucleoprotein A1 in vitro and in vivo. Virology 265(1): 96-109.

Xiao Y and Isaacs SN 2012. Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA)--not all BSAs are alike. J Immunol Methods 384(1-2): 148-151.

Zhang Z, Chen J, Shi H, Chen X, Shi D, Feng L and Yang B 2012. Identification of a conserved linear B-cell epitope in the M protein of porcine epidemic diarrhea virus. Virol J 9: 225.

APPENDIX

Chemicals and reagents preparation

Media for cell culture and bacterial culture

1. Maintenance medium (MM)

Minimum Essential Media (MEM) (GibcoTM, USA)		9.6 g
Sodium bicarbonate ($NaHCO_3$)		2.2 g
Distill water	1000 ml	
Filtrate with 0.45 µM		
Streptomycin Sulfate and Penicillin G Sodium		1.0 %
Tryptose Phosphate Broth (TPB)		0.3 %
Keep at 4°C		

2. Growth medium (GM)

Maintenance medium (MM)	90 ml
Fetal bovine serum	10 ml
Keep at 4°C	

3. Medium for PEDV

Maintenance medium (MM)	100 ml
Trypsin EDTA	40 µl

4. LB broth

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Distill water	1 L
Sterile at 121 °C for 15 min and keep at room temperature	

5. LB agar

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Agar powder	17 g
Distill water	1 L

Sterile at 121 °C for 15 min, pour plate and put at room temperature until become solid agar

Reagent for GST-fusion protein purification

1. 1M Tris-HCl, pH 7 (100 ml)
   - Dissolved 12.114 g Tris in 80 ml of DI
   - Adjust pH with HCl to pH 7
   - Add DI to 100 ml
2. 1M Sodium chloride, NaCl (500 ml)
   - Dissolved 14.61 g NaCl to 500 ml with DI
3. 1M Sodium acetate, $C_2H_3NaO_2$ (100 ml)
   - Dissolved 8.2035 g $C_2H_3NaO_2$ to 100 ml with DI
4. 50 mM Tris-HCl, pH 8 (50 ml)
   - Diluted 2.5 ml of 1M Tris-HCl in DI 40 ml
   - Adjust pH to pH 8
   - Add DI to 50 ml
5. 0.1 M Tris-HCl + 0.5 M NaCl, pH 8.5
   - 50 ml of Tris-HCl
   - 250 ml of 1M NaCl
   - Adjust pH to pH 8.5
6. 0.1 M Sodium acetate + 0.5 M NaCl, pH 4.5
   - 250 ml of 1M NaCl
   - 50 ml of 1M Sodium acetate
   - Adjust pH to pH 4.5

Reagent stock of S27 IgG/IgA PEDV ELISA test kit (for 5 plates)

1. Phosphate buffer saline (20X), (20X PBS)
   1.1 dissolve NaCl 80 g + KCl 2 g + Na_2HPO_4 14.4 g + KH_2PO_4 2.4 g in distilled water up to 500 ml
   1.2 adjust pH to 7.0-7.4

2. Wash buffer (20X), 55 ml*
   2.1 54.5 ml of 20X PBS mix with 550 µl Tween-20
   2.2 store at room temperature

3. Sample /conjugate diluent (2X), 80 ml*
   3.1 dilute 20X Wash buffer with distilled water to 2X Wash buffer 62 ml (20X Wash buffer 3.2 ml + DI water 60.8 ml)
   3.2 dissolve 3.2 g of BSA in 64 ml of 2X Wash buffer
   3.3 add 16 ml of heat-inactivated goat serum
   3.4 mix well

4. Conjugate, IgG (150X), 500 µl*
   4.1 dilute goat anti-pig IgG: HRP (AbD Serotec, USA) in PBS 1:100
   4.2 aliquot 50 µl/tube in amber plastic tube
   4.3 store at $4^{O}C$

5. Conjugate, IgA (150X), 500 µl*
   5.1 dilute goat anti-pig IgA: HRP (AbD Serotec, USA) in PBS 1:100
   5.2 aliquot 50 µl/tube in amber plastic tube
   5.3 store at $4^{O}C$

6. Substrate solution A, 50 ml*
   6.1 Mix 45 ml of distilled water, 5 ml of acetate-citric acid, and 62.5 µl of 3% H_2O_2
   6.2 keep in amber plastic bottle

6.3 store at 4^{O}C

7. TMB solution or substrate solution B, 500 µl*

7.1 dissolve 0.101 g of 3, 3', 5, 5'-Tetramethylbenzidine in Dimethyl sulfoxide (DMSO), 10 ml

7.2 aliquot 1 ml into amber plastic tube

7.3 store at 4^{O}C

8. Acetate-citric buffer

8.1 prepare 1 M Sodium acetate

Dissolve 8.203 g of Sodium acetate to 100 ml in distilled water

8.2 prepare 1 M Citric acid

Dissolve 1.92 g of citric acid to 10 ml in distilled water

8.3 mix 100 ml of 1 M Sodium acetate with 1.5 ml of 1 M citric acid

8.4 adjust pH with citric acid to pH 5.9

8.5 store at room temperature (RT)

9. 3% H_2O_2

9.1 Dilute 35 µl of 30% H2O2 with 315 µl of distilled water

9.2 aliquot 70 µl into amber plastic tube

9.3 store at 4 ^{O}C

10. Stop solution, 30 ml*

Prepare 1 N Sulfuric acid (1 N H_2SO_4) by dilute conc. H_2SO_4 10 ml with distilled water 340 ml

* is a component of ELISA kit

Reagents for ELISA plate coating

1. Coating buffer (0.1 M Carbonate-Bicarbonate Buffer, pH 9.6)
   1.1 Prepare solution A: Bicarbonate buffer by dissolve 420.05 mg $NaHCO_3$ in 50 ml dH_2O.
   1.2 Prepare solution B: Carbonate buffer by dissolve 529.95 mg Na_2CO_3 in 50 ml dH_2O.
   1.3 Prepare the needed quantity of coating buffer by mixing A and B in a 70: 30 ratio (7 ml A and 3 ml B)
   1.4 adjust pH to pH 9.6
2. Blocking buffer
   2.1 dissolve 2.7 g of BSA in 1X wash buffer 90 ml to make 3% BSA in PBST
   2.2 Add 10 ml of heat-inactivated goat serum
   2.3 mix well

Figure 25 Packaging design of ELISA kit.

Table 5 Raw data of cell lysis optimization.

Sonication Time	Lysozyme concentration	Avg of total protein conc. (mg/ml)	SD of total protein conc. (mg/ml)
15x15 sec	0 mg/ml	143.759	51.387
	1 mg/ml	112.392	9.434
	5 mg/ml	174.113	8.264
15x30 sec	0 mg/ml	150.006	67.639
	1 mg/ml	222.807	9.530
	5 mg/ml	513.333	9.866

Table 6 Antibody titer, OD value and S/P ratio of samples in VN positive group

No.	VN titer ($1:2^n$)	IgG		IgA		No.	VN titer ($1:2^n$)	IgG		IgA	
		OD	S/P ratio	OD	S/P ratio			OD	S/P ratio	OD	S/P ratio
1	8	0.578	1.549	1.028	1.427	51	6	1.308	0.926	0.610	0.376
2	8	0.757	0.894	0.810	1.017	52	6	0.970	0.522	0.793	0.594
3	8	0.831	1.009	0.990	1.356	53	6	1.020	1.237	0.983	0.919
4	8	1.205	1.595	2.212	3.657	54	6	0.631	0.664	1.904	2.124
5	8	0.609	0.662	2.054	3.360	55	6	0.616	0.642	1.423	1.495
6	8	1.462	1.045	0.975	0.800	56	5	0.578	0.613	0.588	0.599
7	7	1.075	1.391	1.178	1.710	57	5	0.464	0.435	0.818	1.032
8	7	0.871	1.072	0.848	1.089	58	5	0.562	0.588	0.856	1.104
9	7	1.168	1.537	1.998	3.254	59	5	0.938	1.177	1.162	1.680
10	7	0.811	0.978	0.828	1.051	60	5	0.889	1.100	0.796	0.991
11	7	0.738	0.864	1.390	2.109	61	5	0.912	1.136	1.046	1.461
12	7	0.779	0.928	0.810	1.017	62	5	2.172	1.097	0.815	1.136
13	7	1.635	0.811	0.911	1.340	63	5	1.027	0.487	0.616	0.713
14	7	1.429	0.701	0.637	0.757	64	5	1.646	0.817	0.478	0.419
15	7	1.052	0.500	1.734	3.091	65	5	1.864	0.933	0.960	1.445
16	7	1.135	0.792	0.721	0.527	66	5	1.887	0.945	0.870	1.253
17	7	0.838	0.561	0.633	0.433	67	5	1.601	0.793	0.617	0.715
18	7	1.481	0.833	0.622	0.391	68	5	0.891	0.414	0.676	0.840
19	7	0.662	0.709	1.765	1.942	69	5	2.610	1.330	1.233	2.026
20	6	0.575	0.609	0.722	0.851	70	5	1.730	0.861	0.789	1.081
21	6	0.633	0.700	1.028	1.427	71	5	0.470	0.218	1.197	1.949
22	6	0.577	0.612	0.908	1.202	72	5	1.310	0.730	0.826	0.633
23	6	0.810	0.977	1.708	2.708	73	5	0.623	0.311	0.848	0.659
24	6	0.645	0.718	0.572	0.569	74	5	1.035	0.562	0.823	0.629
25	6	1.229	0.594	0.725	0.945	75	5	1.184	0.653	0.729	0.518
26	6	1.683	0.836	0.461	0.383	76	5	0.931	0.499	0.949	0.779
27	6	1.244	0.602	0.591	0.660	77	5	1.800	1.028	0.593	0.356
28	6	1.877	0.940	1.472	2.534	78	5	1.192	0.658	0.651	0.425
29	6	1.389	0.680	0.597	0.672	79	5	1.355	0.757	0.946	0.776
30	6	1.747	0.870	0.657	0.800	80	5	1.325	0.739	1.222	1.103
31	6	1.370	0.766	1.350	1.255	81	5	1.690	0.961	1.382	1.293
32	6	1.253	0.695	1.508	1.443	82	5	1.005	0.544	0.737	0.527
33	6	1.383	0.774	0.986	0.823	83	5	1.576	0.891	1.129	0.993
34	6	1.543	0.871	0.691	0.473	84	5	0.954	0.513	0.940	0.768
35	6	1.904	1.091	1.361	1.268	85	5	0.934	0.500	0.602	0.367
36	6	1.218	0.674	0.644	0.417	86	5	1.096	0.599	0.541	1.802
37	6	1.342	0.749	1.441	1.363	87	5	1.424	0.799	0.723	0.511
38	6	1.774	1.012	0.901	0.722	88	5	1.209	0.668	1.653	1.615
39	6	1.292	0.913	0.517	0.308	89	5	1.374	0.769	1.217	1.097
40	6	1.054	0.728	0.988	0.814	90	5	1.495	0.842	0.497	1.671
41	6	1.709	1.802	0.780	0.732	91	5	0.817	0.429	0.961	0.793
42	6	1.375	1.426	0.948	0.985	92	5	1.030	0.559	0.712	0.498
43	6	0.998	1.000	0.826	0.640	93	5	1.438	0.807	1.037	0.884
44	6	1.193	0.836	0.540	0.333	94	5	1.803	1.030	0.728	0.517
45	6	1.155	0.807	0.778	0.589	95	5	1.096	0.599	1.436	1.357
46	6	1.302	0.921	0.861	0.678	96	5	0.929	0.497	1.124	0.987
47	6	1.264	0.892	1.096	0.930	97	5	1.648	0.935	1.225	1.107
48	6	1.399	0.997	1.142	0.980	98	5	1.063	0.579	0.903	1.192
49	6	1.262	0.890	1.265	1.112	99	5	1.004	0.543	0.709	0.494
50	6	1.430	1.021	1.130	0.967	100	5	1.198	0.661	0.953	0.784

No.	VN titer ($1:2^n$)	IgG		IgA		No.	VN titer ($1:2^n$)	IgG		IgA	
		OD	S/P ratio	OD	S/P ratio			OD	S/P ratio	OD	S/P ratio
101	5	1.294	0.720	1.042	0.890	151	4	1.266	0.703	1.676	1.643
102	5	1.268	0.704	1.340	1.243	152	4	0.423	0.189	1.056	0.906
103	5	1.498	1.073	1.266	1.113	153	4	1.041	0.566	0.450	1.799
104	5	0.827	0.552	0.687	0.491	154	4	1.089	0.595	0.808	0.612
105	5	1.286	0.909	0.741	0.549	155	4	0.780	0.407	0.641	0.413
106	5	1.052	0.727	0.504	0.294	156	4	1.652	0.938	1.019	0.862
107	5	1.031	0.711	0.688	0.492	157	4	1.233	0.682	0.820	0.626
108	5	0.766	0.505	0.640	0.440	158	4	0.651	0.328	0.521	1.408
109	5	1.132	0.789	0.554	0.348	159	4	1.342	0.749	0.501	0.494
110	5	1.443	1.031	0.481	0.607	160	4	0.429	0.193	0.644	0.417
111	5	1.252	0.883	0.524	0.316	161	4	1.377	0.770	1.251	1.138
112	5	0.815	0.543	1.165	1.004	162	4	1.561	0.882	0.997	0.836
113	5	1.109	0.771	0.685	0.489	163	4	1.664	0.945	0.979	0.815
114	5	1.773	1.287	1.088	1.195	164	4	1.110	0.607	0.533	0.285
115	5	0.764	0.503	0.865	0.860	165	4	1.344	0.750	1.068	0.920
116	5	1.152	0.805	0.673	0.571	166	4	0.936	0.502	0.707	0.492
117	5	0.743	0.712	1.325	1.552	167	4	1.056	0.575	0.950	0.780
118	5	1.036	1.043	0.823	0.797	168	4	0.520	0.248	0.948	0.778
119	5	0.536	0.478	0.710	0.627	169	4	0.968	0.521	0.616	0.384
120	5	0.538	0.481	1.807	2.277	170	4	0.965	0.660	0.668	0.750
121	5	0.686	0.648	1.277	1.480	171	4	1.362	0.968	2.045	1.950
122	5	1.306	1.348	1.008	1.075	172	4	1.111	0.773	1.361	1.215
123	5	1.804	1.909	0.780	0.732	173	4	0.677	0.436	1.137	0.974
124	5	1.205	1.233	0.885	0.890	174	4	1.346	0.955	0.406	1.344
125	5	0.784	0.519	0.931	0.753	175	4	1.694	1.226	0.877	0.695
126	5	0.382	0.206	0.507	0.298	176	4	1.664	1.202	0.894	0.713
127	5	1.192	0.836	0.677	0.480	177	4	1.319	0.934	0.909	0.729
128	5	1.586	1.142	0.495	0.285	178	4	2.091	1.535	0.786	0.597
129	5	1.852	1.348	0.434	0.219	179	4	1.073	0.743	0.647	0.448
130	5	0.976	0.668	0.720	0.507	180	4	1.299	0.919	0.646	0.447
131	5	0.731	0.377	1.325	1.226	181	4	1.005	0.690	0.764	0.574
132	5	1.367	0.764	0.915	0.739	182	4	1.139	0.625	0.698	0.481
133	5	1.132	0.621	1.667	1.632	183	4	0.704	0.360	0.676	0.455
134	5	0.614	0.306	0.887	0.705	184	4	1.424	0.799	0.809	0.613
135	5	1.553	0.878	0.783	0.582	185	4	0.974	0.525	0.835	0.644
136	5	1.582	0.895	1.100	0.958	186	4	0.896	0.477	0.449	0.185
137	5	1.506	0.849	0.516	0.265	187	4	1.116	0.611	1.027	0.872
138	5	0.994	0.537	0.751	0.379	188	4	0.523	0.250	0.738	0.529
139	5	0.570	0.574	1.343	1.390	189	4	1.372	0.767	0.460	0.922
140	4	0.822	0.995	1.402	2.132	190	4	0.918	0.491	0.959	0.125
141	4	0.962	1.214	0.870	1.130	191	4	0.712	1.344	0.540	0.542
142	4	0.708	0.817	0.604	0.629	192	4	0.812	1.023	1.294	1.688
143	4	0.463	0.433	0.958	1.296	193	4	0.679	0.841	0.597	0.629
144	4	0.651	0.728	0.868	1.126	194	4	1.944	2.574	1.472	1.559
145	4	0.543	0.559	0.936	1.254	195	4	1.034	1.258	0.469	1.174
146	4	0.863	1.059	1.198	1.748	196	4	1.431	1.844	1.416	1.486
147	4	0.635	0.703	0.774	0.949	197	4	1.021	1.239	0.608	0.428
148	4	1.204	1.593	0.934	1.250	198	4	1.489	1.929	1.591	1.715
149	4	1.648	0.818	0.983	1.494	199	4	0.799	0.912	0.907	0.819
150	4	1.496	0.737	1.060	1.657	200	4	1.152	1.432	1.235	1.249

No.	VN titer ($1:2^n$)	IgG		IgA		No.	VN titer ($1:2^n$)	IgG		IgA	
		OD	S/P ratio	OD	S/P ratio			OD	S/P ratio	OD	S/P ratio
201	3	0.675	0.765	0.916	1.217	226	3	1.140	0.795	1.177	1.017
202	3	0.724	0.842	0.900	1.186	227	3	1.525	1.094	0.980	0.806
203	3	0.654	0.732	0.556	0.539	228	3	1.734	1.257	0.655	0.430
204	3	0.489	0.474	0.892	1.171	229	3	1.344	2.736	1.048	1.314
205	3	0.551	0.571	0.994	1.363	230	3	1.877	2.482	0.655	0.717
206	3	0.560	0.585	0.828	1.051	231	3	0.705	0.877	0.634	0.685
207	3	0.821	0.994	1.202	1.755	232	3	1.115	1.438	1.147	1.134
208	3	0.775	0.922	0.622	0.663	233	3	1.138	1.412	1.454	1.535
209	3	0.971	1.228	1.892	3.055	234	3	1.917	2.560	1.226	1.237
210	3	0.586	0.626	1.044	1.458	235	3	0.894	1.052	1.685	1.838
211	3	0.627	0.690	1.102	1.567	236	2	0.562	0.588	0.894	1.175
212	3	0.476	0.454	1.294	1.928	237	2	0.717	0.831	1.380	2.090
213	3	0.770	0.914	0.976	1.330	238	2	0.619	0.678	1.498	2.313
214	3	1.108	1.443	1.132	1.623	239	2	0.471	0.446	0.816	1.028
215	3	1.969	0.989	0.950	1.423	240	2	0.656	0.736	0.780	0.960
216	3	1.399	0.685	0.421	0.298	241	2	2.346	1.190	0.381	0.213
217	3	1.278	0.710	0.942	0.771	242	2	1.405	0.787	0.398	0.414
218	3	1.030	0.559	0.898	0.719	243	2	1.283	0.906	1.051	0.882
219	3	1.294	0.720	0.810	0.614	244	2	1.140	0.795	0.733	0.540
220	3	1.863	1.066	1.268	1.158	245	2	0.491	0.231	0.986	0.823
221	3	1.050	1.059	1.108	0.943	246	2	1.065	1.304	1.390	1.452
222	3	0.930	0.923	1.023	0.852	247	2	1.179	1.472	1.287	1.317
223	3	0.799	0.531	0.789	0.600	248	2	1.065	1.304	0.067	0.673
224	3	0.764	0.503	1.046	0.876	249	2	0.991	1.195	1.260	1.281
225	3	1.074	0.744	0.612	0.410	250	2	1.093	1.345	1.591	1.715

Table 7 Antibody titer, OD value and S/P ratio of samples in VN negative group

No.	VN titer ($1:2^n$)	IgG		IgA		No.	VN titer ($1:2^n$)	IgG		IgA	
		OD	S/P ratio	OD	S/P ratio			OD	S/P ratio	OD	S/P ratio
1	1	0.500	0.491	0.466	0.369	51	0	0.105	-0.127	0.525	0.479
2	1	0.302	0.182	0.502	0.437	52	0	0.409	0.349	0.132	-0.260
3	1	0.425	0.374	0.451	0.076	53	0	0.455	0.421	0.160	-0.207
4	1	0.483	0.465	0.362	0.173	54	0	0.269	0.130	0.283	0.024
5	1	0.506	0.501	0.198	-0.136	55	0	0.388	0.316	0.440	0.064
6	1	0.266	0.125	0.528	0.486	56	0	0.071	-0.001	0.399	0.243
7	1	0.323	0.214	0.458	0.354	57	0	0.069	-0.004	0.565	0.556
8	1	0.398	0.332	0.491	0.416	58	0	0.113	0.059	0.297	0.050
9	1	0.480	0.460	0.450	0.075	59	0	0.066	-0.008	0.260	-0.019
10	1	0.405	0.343	0.449	0.337	60	0	0.058	-0.020	0.362	0.173
11	1	0.323	0.214	0.536	0.501	61	0	0.057	-0.021	0.302	0.060
12	1	0.541	0.556	0.449	0.337	62	0	0.068	-0.006	0.111	-0.299
13	1	0.462	0.432	0.466	0.369	63	0	0.103	0.044	0.132	-0.260
14	1	0.462	0.432	0.408	0.260	64	0	0.069	-0.004	0.421	0.284
15	1	0.289	0.161	0.498	0.429	65	0	0.059	-0.018	0.272	0.003
16	1	0.253	0.105	0.356	0.162	66	0	0.062	-0.014	0.216	-0.102
17	1	0.363	0.277	0.392	0.230	67	0	0.058	-0.019	0.248	-0.042
18	1	0.402	0.338	0.389	0.224	68	0	0.105	0.047	0.549	0.524
19	1	0.273	0.136	0.485	0.405	69	0	0.062	-0.013	0.320	0.093
20	1	0.478	0.457	0.185	-0.160	70	0	0.091	0.028	0.253	-0.033
21	1	0.499	0.490	0.302	0.060	71	0	0.063	-0.013	0.291	0.040
22	1	0.405	0.343	0.352	0.154	72	0	0.073	0.002	0.323	0.212
23	1	0.363	0.277	0.586	0.595	73	0	0.067	-0.007	0.396	0.236
24	1	0.242	0.088	0.410	0.264	74	0	0.086	0.020	0.441	0.321
25	1	0.288	0.160	0.232	-0.072	75	0	0.083	0.016	0.281	0.021
26	1	0.355	0.264	0.485	0.405	76	0	0.069	-0.004	0.132	-0.261
27	1	0.358	0.269	0.428	0.051	77	0	0.069	-0.004	0.293	0.043
28	1	0.340	0.241	0.347	0.145	78	0	0.076	0.006	0.160	-0.207
29	1	0.540	0.554	0.493	0.420	79	0	0.059	-0.018	0.146	-0.234
30	1	0.566	0.595	0.547	0.522	80	0	0.064	-0.011	0.353	0.155
31	1	0.379	0.302	0.462	0.362	81	0	0.085	0.019	0.305	0.065
32	1	0.250	0.100	0.535	0.499	82	0	0.088	0.023	0.218	-0.099
33	1	0.627	0.690	0.408	0.260	83	0	0.080	0.012	0.398	0.018
34	1	0.427	0.377	0.308	0.072	84	0	0.080	0.012	0.265	-0.009
35	1	0.516	0.516	0.435	0.311	85	0	0.053	-0.025	0.335	0.122
36	1	0.598	0.645	0.460	0.358	86	0	0.056	-0.021	0.398	0.240
37	1	0.459	0.427	0.457	0.082	87	0	0.067	-0.006	0.423	0.288
38	1	0.311	0.196	0.420	0.282	88	0	0.063	-0.011	0.182	-0.166
39	1	0.280	0.147	0.354	0.157	89	0	0.067	-0.006	0.346	0.143
40	1	0.288	0.160	0.330	0.112	90	0	0.064	-0.011	0.254	-0.030
41	1	0.540	0.554	0.592	0.606	91	0	0.053	-0.026	0.248	-0.041
42	1	0.060	-0.016	0.430	0.301	92	0	0.065	-0.009	0.292	0.040
43	1	0.712	1.344	0.484	0.111	93	0	0.064	-0.011	0.638	0.022
44	1	0.471	0.813	0.178	-0.173	94	0	0.071	0.000	0.483	0.401
45	1	0.613	1.126	0.102	-0.316	95	0	0.059	-0.017	0.299	0.055
46	1	0.901	1.760	0.210	-0.113	96	0	0.062	-0.013	0.260	-0.020
47	0	0.136	-0.078	0.338	0.128	97	0	0.066	-0.008	0.320	0.093
48	0	0.276	0.141	0.263	-0.013	98	0	0.072	0.001	0.399	0.243
49	0	0.275	0.139	0.356	0.162	99	0	0.069	-0.003	0.558	0.191
50	0	0.182	-0.006	0.454	0.347	100	0	0.113	0.059	0.215	-0.105

No.	VN titer ($1:2^n$)	IgG		IgA		No.	VN titer ($1:2^n$)	IgG		IgA	
		OD	S/P ratio	OD	S/P ratio			OD	S/P ratio	OD	S/P ratio
101	0	0.066	-0.008	0.382	0.211	151	0	0.054	-0.020	0.112	-0.298
102	0	0.058	-0.019	0.349	0.149	152	0	0.050	-0.026	0.117	-0.288
103	0	0.057	-0.021	0.259	-0.022	153	0	0.060	-0.011	0.112	-0.298
104	0	0.068	-0.005	0.466	0.369	154	0	0.049	-0.028	0.471	0.259
105	0	0.102	0.044	0.198	-0.137	155	0	0.049	-0.028	0.233	-0.070
106	0	0.069	-0.004	0.238	-0.061	156	0	0.060	-0.011	0.211	-0.111
107	0	0.059	-0.017	0.256	-0.027	157	0	0.135	0.101	0.116	-0.290
108	0	0.062	-0.013	0.236	-0.064	158	0	0.131	0.064	0.109	-0.303
109	0	0.058	-0.018	0.359	0.167	159	0	0.123	0.046	0.104	-0.313
110	0	0.116	0.064	0.166	-0.197	160	0	0.158	0.123	0.095	-0.330
111	0	0.062	-0.013	0.335	0.121	161	0	0.126	0.053	0.101	-0.318
112	0	0.092	0.029	0.225	-0.086	162	0	0.123	0.046	0.088	-0.343
113	0	0.063	-0.011	0.213	-0.107	163	0	0.162	0.132	0.094	-0.331
114	0	0.074	0.004	0.205	-0.122	164	0	0.142	0.088	0.098	-0.324
115	0	0.066	-0.007	0.447	0.332	165	0	0.348	0.542	0.085	-0.348
116	0	0.086	0.021	0.208	-0.117	166	0	0.212	0.242	0.156	-0.215
117	0	0.065	-0.004	0.243	-0.051	167	0	0.243	0.311	0.091	-0.337
118	0	0.065	-0.004	0.498	0.243	168	0	0.137	0.077	0.117	-0.288
119	0	0.054	-0.020	0.193	-0.146	169	0	0.158	0.123	0.328	0.109
120	0	0.058	-0.014	0.209	-0.116	170	0	0.116	0.031	0.194	-0.143
121	0	0.073	0.009	0.277	0.013	171	0	0.147	0.099	0.062	-0.392
122	0	0.057	-0.015	0.242	-0.054	172	0	0.294	0.423	0.118	-0.286
123	0	0.057	-0.016	0.204	-0.125	173	0	0.100	-0.004	0.125	-0.273
124	0	0.058	-0.014	0.260	-0.020	174	0	0.256	0.339	0.127	-0.269
125	0	0.059	-0.012	0.346	0.143	175	0	0.111	0.020	0.089	-0.341
126	0	0.054	-0.020	0.422	0.044	176	0	0.105	0.007	0.101	-0.318
127	0	0.063	-0.007	0.266	-0.008	177	0	0.099	-0.007	0.093	-0.333
128	0	0.063	-0.007	0.282	0.022	178	0	0.201	0.218	0.146	-0.234
129	0	0.050	-0.026	0.300	0.056	179	0	0.119	0.037	0.159	-0.209
130	0	0.052	-0.023	0.216	-0.102	180	0	0.116	0.031	0.161	-0.205
131	0	0.045	-0.034	0.269	-0.003	181	0	0.103	0.002	0.263	-0.013
132	0	0.065	-0.003	0.430	0.300	182	0	0.136	0.075	0.428	0.298
133	0	0.059	-0.012	0.222	-0.090	183	0	0.195	0.205	0.109	-0.303
134	0	0.052	-0.023	0.242	-0.054	184	0	0.114	0.026	0.111	-0.299
135	0	0.053	-0.021	0.198	-0.137	185	0	0.145	0.095	0.092	-0.335
136	0	0.046	-0.032	0.278	0.014	186	0	0.112	0.022	0.137	-0.250
137	0	0.046	-0.032	0.268	-0.005	187	0	0.108	0.013	0.151	-0.224
138	0	0.054	-0.020	0.214	-0.106	188	0	0.121	0.042	0.133	-0.258
139	0	0.062	-0.008	0.186	-0.159	189	0	0.126	0.053	0.175	-0.179
140	0	0.062	-0.008	0.228	-0.079	190	0	0.126	0.053	0.147	-0.232
141	0	0.062	-0.008	0.401	0.246	191	0	0.116	0.031	0.137	-0.250
142	0	0.080	0.020	0.235	-0.067	192	0	0.113	0.024	0.178	-0.173
143	0	0.062	-0.008	0.407	0.257	193	0	0.129	0.059	0.170	-0.188
144	0	0.056	-0.017	0.367	0.182	194	0	0.118	0.035	0.085	-0.348
145	0	0.047	-0.030	0.074	-0.368	195	0	0.123	0.046	0.089	-0.341
146	0	0.060	-0.011	0.323	0.100	196	0	0.122	0.044	0.091	-0.337
147	0	0.050	-0.026	0.084	-0.351	197	0	0.133	0.068	0.240	-0.056
148	0	0.055	-0.018	0.111	-0.299	198	0	0.120	0.040	0.178	-0.173
149	0	0.049	-0.027	0.079	-0.360	199	0	0.136	0.075	0.089	-0.341
150	0	0.053	-0.022	0.110	-0.301	200	0	0.124	0.048	0.131	-0.262

No.	VN titer ($1:2^n$)	IgG		IgA		No.	VN titer ($1:2^n$)	IgG		IgA	
		OD	S/P ratio	OD	S/P ratio			OD	S/P ratio	OD	S/P ratio
201	0	0.138	0.079	0.257	-0.024	226	0	0.131	0.064	0.313	0.081
202	0	0.129	0.059	0.111	-0.299	227	0	0.130	0.062	0.203	-0.126
203	0	0.126	0.053	0.118	-0.286	228	0	0.120	0.040	0.240	-0.056
204	0	0.124	0.048	0.124	-0.275	229	0	0.121	0.042	0.200	-0.132
205	0	0.125	0.051	0.174	-0.181	230	0	0.119	0.037	0.140	-0.245
206	0	0.124	0.048	0.084	-0.350	231	0	0.120	0.040	0.308	0.072
207	0	0.127	0.055	0.133	-0.258	232	0	0.266	0.327	0.159	-0.209
208	0	0.130	0.062	0.072	-0.373	233	0	0.126	0.103	0.151	-0.224
209	0	0.126	0.053	0.170	-0.188	234	0	0.196	0.215	0.206	-0.121
210	0	0.126	0.053	0.090	-0.339	235	0	0.289	0.364	0.272	0.004
211	0	0.113	0.024	0.089	-0.341	236	0	0.323	0.419	0.204	-0.124
212	0	0.118	0.035	0.091	-0.337	237	0	0.259	0.317	0.095	-0.330
213	0	0.123	0.046	0.508	0.448	238	0	0.158	0.154	0.243	-0.052
214	0	0.117	0.033	0.223	-0.089	239	0	0.196	0.215	0.188	-0.154
215	0	0.136	0.075	0.326	0.105	240	0	0.121	0.095	0.254	-0.030
216	0	0.127	0.055	0.063	-0.390	241	0	0.287	0.361	0.148	-0.230
217	0	0.135	0.073	0.161	-0.205	242	0	0.214	0.244	0.274	0.008
218	0	0.134	0.070	0.075	-0.367	243	0	0.188	0.202	0.282	0.023
219	0	0.128	0.057	0.076	-0.365	244	0	0.141	0.127	0.170	-0.188
220	0	0.119	0.037	0.105	-0.311	245	0	0.261	0.320	0.143	-0.239
221	0	0.116	0.031	0.173	-0.183	246	0	0.151	0.142	0.210	-0.113
222	0	0.112	0.022	0.274	0.008	247	0	0.072	0.015	0.214	-0.105
223	0	0.131	0.064	0.282	0.023	248	0	0.065	0.000	0.220	-0.094
224	0	0.145	0.095	0.220	-0.094	249	0	0.063	-0.003	0.210	-0.113
225	0	0.129	0.059	0.210	-0.113	250	0	0.188	0.168	0.278	0.015

Table 8 Antibody titer of IgG against three different lot of S12 antigen of PEDV, including OD value and S/P ratio from antigen lot A, B and C.

Pig number	Optimal density (OD at 450 nm)			S/P ratio		
	Antigen lot A	Antigen lot B	Antigen lot C	Antigen lot A	Antigen lot B	Antigen lot C
1	1.835	1.75	1.783	2.055	1.713	1.999
2	1.83	1.872	1.823	2.048	1.869	2.056
3	0.773	0.772	0.738	0.545	0.468	0.497
4	1.802	1.73	1.837	2.009	1.688	2.076
5	1.351	1.37	1.399	1.367	1.229	1.447
6	1.42	1.445	1.283	1.465	1.325	1.280
7	1.129	1.326	1.367	1.051	1.173	1.401
8	1.079	1.26	1.21	0.980	1.089	1.175
9	2.027	1.817	1.902	2.329	1.799	2.170
10	1.504	1.528	1.478	1.585	1.431	1.560
11	1.573	1.597	1.488	1.683	1.518	1.575
12	0.244	0.251	0.243	-0.208	-0.196	-0.214
13	1.13	1.14	1.123	1.053	0.936	1.050
14	0.244	0.258	0.32	-0.208	-0.187	-0.103
15	0.27	0.291	0.201	-0.171	-0.145	-0.274
16	0.324	0.363	0.348	-0.094	-0.054	-0.063
17	0.492	0.518	0.499	0.145	0.144	0.154
18	0.594	0.582	0.477	0.290	0.225	0.122
19	0.662	0.659	0.567	0.387	0.324	0.251
20	0.469	0.468	0.39	0.112	0.080	-0.003
21	0.582	0.532	0.601	0.273	0.162	0.300
22	0.425	0.485	0.488	0.050	0.102	0.138
23	0.769	0.891	0.762	0.539	0.619	0.532
24	1.628	1.413	1.392	1.761	1.284	1.437
25	1.528	1.603	1.655	1.619	1.526	1.815
26	1.544	1.582	1.532	1.642	1.499	1.638
27	1.19	1.173	1.199	1.138	0.978	1.159
28	1.528	1.59	1.562	1.619	1.510	1.681
29	1.818	1.73	1.881	2.031	1.688	2.139
30	0.892	1.105	0.87	0.714	0.892	0.687
31	1.09	1.214	1.209	0.996	1.031	1.174
32	1.278	1.358	1.31	1.263	1.214	1.319
33	1.12	1.307	1.129	1.038	1.149	1.059
34	0.69	0.69	0.981	0.427	0.363	0.846
35	1.899	1.957	1.792	2.147	1.977	2.011
36	1.851	1.962	1.932	2.078	1.983	2.213
37	1.940	2.017	1.911	2.205	2.054	2.182

Pig number	Optimal density (OD at 450 nm)			S/P ratio		
	Antigen lot A	Antigen lot B	Antigen lot C	Antigen lot A	Antigen lot B	Antigen lot C
38	1.073	1.178	1	0.972	0.985	0.874
39	0.964	1.144	1.101	0.817	0.941	1.019
40	1.564	1.821	1.67	1.670	1.804	1.836
41	1.415	1.732	1.309	1.458	1.690	1.318
42	1.8	1.935	1.892	2.006	1.949	2.155
43	0.218	0.418	0.268	-0.245	0.017	-0.178
44	0.903	1.664	0.999	0.730	1.604	0.872
45	1.531	1.652	1.492	1.623	1.589	1.580
46	1.792	1.743	1.636	1.994	1.704	1.787
47	1.093	1.19	1.088	1.000	1.000	1.000
48	0.39	0.405	0.392	0.000	0.000	0.000
49	0.146	0.199	0.136	0.022	-0.059	-0.005
50	0.108	0.341	0.108	-0.003	0.038	-0.023
51	0.177	0.309	0.118	0.043	0.016	-0.017
52	0.13	0.19	0.28	0.012	-0.065	0.088
53	0.146	0.28	0.134	0.022	-0.003	-0.006
54	0.203	0.193	0.12	0.060	-0.063	-0.016
55	0.397	0.153	0.244	0.188	-0.090	0.065
56	0.203	0.212	0.201	0.060	-0.050	0.037
57	0.134	0.302	0.321	0.015	0.012	0.115
58	0.125	0.188	0.219	0.009	-0.066	0.049
59	0.142	0.215	0.166	0.020	-0.048	0.014
60	0.135	0.234	0.311	0.015	-0.035	0.108
61	0.199	0.283	0.352	0.058	-0.001	0.135
62	0.164	0.208	0.228	0.034	-0.053	0.054
63	0.273	0.222	0.281	0.106	-0.043	0.089
64	0.202	0.24	0.392	0.060	-0.031	0.161
65	0.096	0.165	0.049	-0.011	-0.082	-0.061
66	0.139	0.338	0.329	0.018	0.036	0.120
67	0.151	0.206	0.374	0.026	-0.054	0.149
68	0.149	0.263	0.219	0.024	-0.015	0.049
69	0.159	0.198	0.382	0.031	-0.060	0.154
70	0.225	0.241	0.219	0.075	-0.030	0.049
71	1.119	1.948	1.9	0.666	1.140	1.137
72	2.369	2.195	2.538	1.493	1.309	1.550
73	0.856	1.427	0.994	0.492	0.783	0.550
74	0.116	0.277	0.126	0.003	-0.005	-0.012
75	0.152	0.297	0.399	0.026	0.008	0.165
76	0.134	0.278	0.422	0.015	-0.005	0.180
77	0.178	0.249	0.092	0.044	-0.025	-0.034

Pig number	Optimal density (OD at 450 nm)			S/P ratio		
	Antigen lot A	Antigen lot B	Antigen lot C	Antigen lot A	Antigen lot B	Antigen lot C
78	0.201	0.273	0.217	0.059	-0.008	0.047
79	0.176	0.255	0.234	0.042	-0.021	0.058
80	0.198	0.217	0.289	0.057	-0.047	0.094
81	0.151	0.287	0.261	0.026	0.001	0.076
82	0.145	0.231	0.389	0.022	-0.037	0.159
83	0.19	0.496	0.211	0.052	0.145	0.043
84	0.18	0.615	0.29	0.045	0.226	0.094
85	1.624	1.744	1.689	1.000	1.000	1.000
86	0.112	0.285	0.144	0.000	0.000	0.000

Area Under the Curve of ROC

Area Under the Curve

Test Result Variable(S): score of OD IgA

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.977	.006	.000	.965	.988

The test result variable(S): score of OD IgA has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Area Under the Curve

Test Result Variable(S): score of OD IgG

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.994	.002	.000	.990	.998

The test result variable(S): score of OD IgA has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Area Under the Curve

Test Result Variable(S): score of S/P IgA

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.954	.008	.000	.938	.970

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Area Under the Curve

Test Result Variable(S): score of S/P IgG

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.973	.007	.000	.958	.987

The test result variable(S): score of S/P IgG has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

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PUBLICATION	1. Srijangwad A, Stott CJ, Temeeyasen G, Senasuthum R, Chongcharoen W, Tantituvanont A, Nilubol D. 2017. Immune response of gilts to single and double infection with porcine epidemic diarrhea virus. Arch Virol. 162(7):2029-2034. 2. Srijangwad A, Nilubol D, Chongcharoen W, Phoolcharoen W, Chuanasa T, Tantituvanon A, Production of spike and nucleocapsid recombinant proteins of porcine epidemic diarrhea virus for antibody detection by ELISA, Asian Journal of Pharmaceutical Sciences 11(1): 85-86. http://dx.doi.org/doi: 10.1016/j.ajps.2015.11.114. 3. Srijangwad A, Sawattrakool K, Stott CJ, Temeeyasen G, Tripipat T, Chongcharoen W, Tantituvanont A, Carr J, Nilubol D. 2017. Lactogenic Immunity Following Oral Exposure with Porcine Epidemic Diarrhea Virus. Thai J Vet Med Suppl. 46. 393-394. 4. Srijangwad A, Rattanapisit K, Jermsutjarit P, Tantituvanont A, Nilubol D. 2017. Optimization of cell lysis for recombinant fractional spike protein of porcine epidemic diarrhea virus and specific binding of protein. Thai J Vet Med Suppl. 2017.

47: 83-84.

5. Srijangwad A, Jermsutjarit P, Tantituvanont A, Luckanakul J, Nilubol D. 2018. Immunoassay precision of S27 PEDV IgG/IgA ELISA kit for antibody detection against porcine epidemic diarrhea virus in colostrum and milk. The 17th Chulalongkorn University Veterinary Conference (CUVC2018). (Poster presentation)

AWARD RECEIVED

Gold Medal Award for 28th International Invention, Innovation & Technology Exhibition (ITEX2017) on the 11–13th May 2017 in Kuala Lumpur, Malaysia.