การพัฒนาชุดทดสอบอินไดเรกซ์อีไลซาชนิดพีซีวีทูบีสำหรับการตรวจหาแอนติบอดีต่อเชื้อพีซีวีทู

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# DEVELOPMENT OF INDIRECT PCV2B-BASED ELISA FOR DETECTION PCV2 ANTIBODIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Medicine
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คมป่าน บัวไพจิตร : การพัฒนาชุดทดสอบอินไดเรกซ์อีไลซาชนิดพีซีวีทูบีสำหรับการตรวจหาแอนติบอดีต่อเชื้อพีซีวีทู (DEVELOPMENT OF INDIRECT PCV2B-BASED ELISA FOR DETECTION PCV2 ANTIBODIES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. น.สพ. ดร. สุพจน์ วัฒนะพันธ์ศักดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. น.สพ. ดร. พรชลิต อัศวชีพ, 79 หน้า.

การศึกษานี้เป็นการพัฒนาชุดทดสอบอินไดเรกซ์อีไลซาโดยใช้ทั้งอนุภาคไวรัสของเชื้อเซอร์โคไวรัสชนิดที่สอง (พีซีวี ไทป์ทู) ชนิดย่อยเชื้อ ซับไทป์ บี เป็นแอนติเจนพร้อมทั้งทำการตรวจสอบประสิทธิภาพในการวินิจฉัยของอีไลซาเปรียบเทียบกับวิธีมาตรฐานไอพีเอ็มเอ เริ่มจากการเตรียมแอนติเจนจากการเพาะเชื้อพีซีวีทู ซับไทป์ บี ด้วยการเลี้ยงในเซลล์เพาะเลี้ยงเอสเอสที-เอสดับเบิลยูหนึ่ง ทำเชื้อให้บริสุทธิ์ด้วยเทคนิคซูโครส กราเดียน หลังจากนั้นจึงนำแอนติเจนไปเคลือบบนผิวของอีไลซาไมโครเพลท ชนิด 96 หลุม และมีขั้นตอนการหาความเข้มข้นของแอนติเจนที่เหมาะสมเพื่อเพิ่มประสิทธิภาพโดยการใช้วิธีเช็กเกอร์บอร์ด ไตเตรชัน ผลปรากฏคือ ที่ความยาวคลื่นแสง 450 นาโนเมตร ค่าการดูดกลืนแสงที่ 0.39 ที่ใช้เป็นจุดแบ่งแยกตัวอย่างให้ผลบวกกับผลลบ ทำให้ชุดทดสอบมีความไวและความจำเพาะอยู่ที่ 92 และ 100% ตามลำดับ (จำนวนตัวอย่าง = 100) ค่ามัธยฐานของเปอร์เซ็นต์ค่าสัมประสิทธิ์ของความผันแปรจากวิธีทดสอบในเพลททดสอบเดียวกันอยู่ที่ 3.62 และ 6.21 ส่วนเพลททดสอบคนละเพลทอยู่ที่ 7.16 และ 6.34 (จำนวนตัวอย่างบวก = 10 และลบ =10 ตามลำดับ) ค่าความสอดคล้องระหว่างอีไลซาและไอพีเอ็มเออยู่ที่ 89.34% พร้อมกับค่าคัปปาที่ 0.757 (จำนวนตัวอย่าง = 657) ส่วนความสัมพันธ์ระหว่างอีไลซาและไอพีเอ็มเอให้ค่าสัมประสิทธ์สหสัมพันธ์สเปียร์แมนที่ 0.74 ($p < 0.001$) (จำนวนตัวอย่าง = 20) ดังนั้นจากผลการประเมินชุดทดสอบอินไดเรกซ์อีไลซาชนิดพีซีวีทูบี ซึ่งใช้เชื้อพีซีวีทู ซับไทป์ บี เป็นแอนติเจน พบว่าสามารถใช้ตรวจสอบแอนติบอดีต่อเชื้อพีซีวีทูได้เทียบเคียงกับการตรวจวิธีไอพีเอ็มเอ

ภาควิชา อายุรศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา อายุรศาสตร์สัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: INDIRECT PCV2B-BASED ELISA / IMMUNOPEROXIDASE MONOLAYER ASSAY / PORCINE CIRCOVIRUS TYPE 2 / WHOLE PARTICLE OF PCV2B

KOMPARN BUAPIJIT: DEVELOPMENT OF INDIRECT PCV2B-BASED ELISA FOR DETECTION PCV2 ANTIBODIES. ADVISOR: SUPHOT WATTANAPHANSAK, D.V.M., M.S., Ph.D., CO-ADVISOR: PORNCHALIT ASSAVACHEEP, D.V.M., M.Sc., Ph.D., 79 pp.

This trial was a development of an indirect enzyme-linked immunosorbent assay (ELISA) using whole particle of porcine circovirus type 2 (PCV2) subtype B as antigen and to validate these diagnostic performances. PCV2b antigen was prepared by propagation in SST-SW cell and harvested from cell cultured followed by purifying with sucrose gradient technique. The ELISA was optimized by using checker board titration. As a result, the cut-off value was set at 0.39 (450 nm) with 92% and 100% sensitivity and specificity, respectively (n = 100). Median value of %CV of intra-plate at 3.62 and 6.21 and inter-plate at 7.16 and 6.34 for negative (n = 10) and positive sample (n = 10), respectively. The observed agreement was 89.34% with 0.757 kappa value (n = 657) when compared to IPMA and correlation of ELISA and immunperoxidase monolayer assay (IPMA) resulted in Spearman correlation of coefficient (r) at 0.74 ($p < 0.001$) (n=20). Based on validation results, indirect PCV2b-based ELISA could may be used for detection of antibodies against PCV2 infection as alternative to IPMA.



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| Department: | Veterinary Medicine | Student's Signature | ..... |
| Field of Study: | Veterinary Medicine | Advisor's Signature | ..... |
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| | |
|---|---|
| 1X | 1 times concentration of solution |
| AC | accuracy |
| AEC | 3-amino-9-ethylcarbazole |
| AUC | area under curve |
| bp | base pair (s) |
| BRs | binding ratios |
| BSA | bovine serum albumin |
| Cap | capsid |
| CPE | cytopathic effect |
| CV | coefficient of variation |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid (s) |
| DSN | diagnostic sensitivity |
| DSP | diagnostic specificity |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| FBS | fetal bovine serum |
| FITC | fluorescein isothiocyanate |
| FN | false negative |
| FP | false positive |
| HRP | horse radish peroxidase |
| IFAT | indirect fluorescent antibody technique |
| IFN | interferon |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |

| | |
|---|---|
| IIF | indirect immunofluorescence assay |
| IPMA | immunoperoxidae monolayer assay |
| kb | kilobase pair (s) |
| MEM | minimum essential medium |
| min | minute (s) |
| ml | milliter (s) |
| mM | millimolar (s) |
| n | number |
| NA | neutralizing antibodies |
| NaCl | sodium chloride |
| °C | degree (s) Celsius |
| OD | optical density |
| ORF | open reading frame |
| P/N | positive/negative |
| PASC | pairwise sequence comparison |
| PBS | phosphase buffer saline |
| PBST | phosphase buffer saline with 0.05% of Tween20 |
| PCR | polymerase chain reaction |
| PCV2 | porcine circovirus type 2 |
| PCVAD | porcine circovirus associated disease |
| PD | proportionate distance |
| PDNS | porcine dermatitis and nephropathy syndrome |
| PK | porcine kidney cell |
| PMWS | post-weaning multisystemic wasting syndrome |
| PNP | proliferative and necrotizing pneumonia |
| PPV | porcine parvovirus |
| PRDC | porcine respiratory disease complex |

| | |
|---|---|
| PRRSV | porcine reproductive and respiratory syndrome virus |
| r | Spearman correlation coefficient |
| Rep | replicate |
| RNA | ribonucleic acid (s) |
| ROC | receiver operating characteristic |
| RT/PCR | reverse transcription polymerase chain reaction |
| TAE | tris-acetate-EDTA |
| $TCID_{50}$ | tissue culture infectious dose (50%) |
| TMB | 3,3′,5,5′-tetramethylbenzidine |
| TN | true negative |
| TP | true positive |
| TNE | tris-NaCl-EDTA |
| µl | microliter (s) |
| xg | unit of relative centrifugal force |
| γ | gamma |

# CHAPTER I
# INTRODUCTION

To date, porcine circovirus type 2 (PCV2) infection is one of the major diseases which has an economic impact on pig production. PCV2 is the etiology of porcine circovirus associated disease (PCVAD) causing many symptoms and clinical signs such as post weaning multi-systemic wasting syndrome (PMWS, porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC, proliferative and necrotizing pneumonia (PNP), congenital tremor and enteritis. Under field condition, co-infection of PCV2 with other diseases is commonly found. If PCV2 infects pigs alone, it will not cause significant damages to the infected pigs. However, when the PCV2 being co-infect with PRRSV, PPV and *M. Hyopneumoniae*, the outcome is more severe damaged (Ellis et al., 2003).

Considering, the loss from PCV2, especially PMWS, is unable to be accepted, many commercial vaccine products have been used to control the disease and proved to have observable efficiencies in reducing morality rate from PCV2 infection (Fachinger et al., 2008; Kixmoller et al., 2008; Opriessnig et al., 2009; Segales et al., 2009). Recently, there are four types of inactivated vaccines available in the market including two subunit vaccines (ORF2 expressed in baculovirus), whole PCV2 genome vaccine and chimeric PCV1-2 vaccine (ORF2 of PCV2 cloned into genomic backbone of PCV1) (Fenaux et al., 2003; Fenaux et al., 2004). Many evidences of vaccine efficacy were shown both in vitro and in vivo. To understand clearly how the vaccine work, the immune responses are so important for explaining this mechanism. Both humoral and cellular immunity take significant parts in protection of PCV2 infection. In experimental and filed condition, it had been found that neutralizing antibody is an important factor for clearance of PCV2 virus. The vaccinated pigs had a decline of viral DNA load in serum and less clinical sign of PCVAD when compared to unvaccinated pigs (Meerts et al., 2006; Fort et al., 2007). Interferon-γ-secreting cells (IFN-γ) is defined as cell-mediated immune mechanism against PCV2 infection. Due to the evidences, more IFN-γ and neutralizing antibodies were detected in serum according to the decline of viral

DNA load (Fort et al., 2009). Thus, neutralizing antibodies and IFN-γ are important factors for control of PCV2 infection. Normally, anti-PCV2 IgG is usually used to describe the role of immunology along with neutralizing antibodies from the specific type of ELISA (Fort et al., 2007). Nevertheless, the decline of viremia dose not apparently correlates with anti-PCV2 IgG antibodies (Opriessnig et al., 2010) but it correlates with an increase of anti-PCV2 IgM antibodies (Fort et al., 2007). Thus, the studies of humoral immunity in field condition are so important to understand more about the immune response of PCV2 infection.

Serological studies had been used to find antibodies response to PCV2 infection. Nowadays, indirect immunofluorescent assay (IIF) or indirect fluorescent antibody test (IFAT) and immunoperoxidase monolayer assay (IPMA) are used to define antibody response. However, the results are subjective and required time to grow of the virus so the technique of ELISA can be used alternatively to overcome these limitations. Moreover, ELISA can be used to understand the dynamic of immune response and level of passive immunity to predict appropriate vaccination time. To date, there are 5 subtypes of PCV2 (a, b, c, d, e). In Thailand, the first report on PCV2 was mentioned in 1998 (Tantilertcharoen et al., 1999) but according to a retrospective study, the PCV2 antigen was found in pigs in Thailand since 1993 (Banlunara et al., 2002). Moreover, the major subtype of Thai PCV2 outbreak was PCV2b with the prevalence of 83.33 % (Jantafong et al., 2011; Jittimanee et al., 2011). Therefore, it is proposed that the PCV2b subtype isolated from field can be used as antigen by coating on the ELISA test plate to detect PCV2 antibodies. Comparing to direct ELISA, indirect ELISA is more accurate due to the using of secondary antibody. Thus, the objectives of this study were to develop indirect PCV2b-based ELISA using whole purified PCV2b antigen for antibody response detection. Moreover, we would like to validate sensitivity, specificity, and diagnostic performances (agreement, repeatability, and correlation between ELISA and IPMA). At last, we expect that this ELISA shows higher sensitivity, higher specificity and higher accuracy for diagnosis of PCV2 infection in pig farms.

# CHAPTER II
# LITERATURE REVIEW

## 2.1 Background information

Presently, porcine circovirus is one of the important viral infectious disease in pig that causes economic loss in many parts of pig production all over the world. In 1974, it had been found that porcine kidney cell line (PK-15 cell) was firstly contaminated by PCV (Today known as PCV1). By morphology, characterization of PCV is a circular single strand DNA virus so that it is called porcine circovirus (Tischer et al., 1974; Tischer et al., 1982). The clinical trial was done by challenging contaminated PK-15 cell into pigs but none of them showing clinical signs (Tischer et al., 1986). After that, a wasting disease of pig had occurred in Canada first described in early 1990s and was defined as PMWS. (Harding, 1996; Clark, 1997). PCV was isolated from the infected pigs with signs of wasting syndrome. Later, the virus was inoculated into PK-15 cell culture and performed sequencing for phylogenetic tree analysis. Finally, they found differences of DNA sequences showing 70% and 76% identity to the original described by Tischer (1982) (Meehan et al., 1998; Fenaux et al., 2000). Consequently, the pathogenic strain is named porcine circovirus type 2 (PCV2) and the non-pathogenic strain is named porcine circovirus type 1 (PCV1). Because of this reason, PCV2 is mainly focused and studied in order to control and clear the infection.



## 2.2 Clinical signs and gross pathology

In early 1990s, clinical signs of porcine circovirus in which mostly relating to porcine circovirus type 2 (PCV2) were first clarified (Allan and Ellis, 2000). In 1991, PCV2 was found along with the pigs with sign of post-weaning multisystemic wasting syndrome (PMWS) in western Canada (Ellis et al., 1998). If many pathogens could found co-infection in one pigs together with PCV2, the disease of this infection would be called porcine circovirus-associated disease (PCVAD) which nominated in North America (Opriessnig et al., 2007) or porcine circovirus disease (PCVD) which nominated in Europe (Segales et al., 2005a). PCVAD is caused by many syndromes including post-

weaning multisystemic wasting syndrome (PMWS) (Harding, 1996; Clark, 1997), porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000), proliferative and necrotizing pneumonia (PNP) (Grau-Roma and Segales, 2007), Enteritis (Jensen et al., 2006), reproductive disorder (West et al., 1999) and porcine respiratory disease complex (PRDC) (Kim et al., 2003). Many studies found that only PCV2 could not trigger the severity caused by clinical signs (Ellis et al., 2003) so that PCV2 usually found co-infection with other factors (non-infectious agent or infectious agent) such as porcine reproductive and respiratory syndrome virus (PRRSv) (Harms et al., 2001), porcine parvovirus (Ellis et al., 2001), *Mycoplasma hyopneumoniae* and swine influenza virus (Pogranichniy et al., 2002) in which causing dramatic economic loss to swine producer.

**2.3 Epidemiology**

General characteristics of porcine circovirus type 2 (PCV2) are described as followings, the genome is a circular single-stranded DNA with non-envelope virus. As a result, it has resistant to many disinfectants including chlorhexidine, phenol and alcohol (Royer et al., 2001). At any rate, many disinfectants such as alkaline group, oxidizing agent and quaternary ammonium compound could inactivate this virus (Martin et al., 2008). The influence of temperature could also affect the survival of PCV2 (O'Dea et al., 2008). The transmission of the virus to other animals had been reported as evidences such as cattles, mice and humans (Tischer et al., 1995; Nayar et al., 1999) but nothing about seroconversion and lesions was found (Quintana et al., 2002). Talking about routes of transmission, PCV2 could be transmitted among pigs by both horizontal and vertical transmission. About horizontal transmission, direct contact in which oronasal route was found to be kept an eye on during experiment. The secretions including feces, saliva, urine, milk, semen and nasal discharge which containing the viral particle could be shed via pig-to-pig (Krakowka et al., 2000; Larochelle et al., 2000; Shibata et al., 2003; Park et al., 2005; Segales et al., 2005b; Shibata et al., 2006; Ha et al., 2009). About vertical transmission, trans-placental infection had to be watched for transmitting PCV2 and resulting in lesions such as myocarditis in aborted fetuses and stillborn (West et al., 1999; Park et al., 2005). Semen from boar could be the source of infection by shedding PCV2 after doing artificial

insemination (Madson et al., 2009). Thus, control strategies from knowledge of characteristics and transmission must be used to limit the transmission of PCV2.

**2.4 Genetic characterization**

Genome of PCV2 comprises 1,766-1,768 nucleotides with 4 open reading frames (ORFs). ORF1 encodes replication protein called "Rep gene", ORF2 encodes viral capsid protein called "Capsid gene", ORF3 encodes apoptotic protein called "apoptotic gene" and ORF4 which was recently discovered is not important in replication of PCV2 but can suppress caspase activity and regulate $CD4^+$ and $CD8^+$ T lymphocytes production (He et al., 2013). The stable region of viral structure is ORF1 but the variable region is ORF2 which is the major region to be selected in differentiating the PCV2 subtypes. Pairwise sequence comparison (PASC) is the method used in finding the genetic distance ($p$) in which calculated by the ratio between the number of different bases and total number of position between genomes. Due to the function of ORF2, method based on ORF2 of PCV2 is used with a cut-off value of $p$ = 0.035 (Grau-Roma et al., 2008) in which PCV2 subtypes can be separated. According to previous reasons, PCV2 subtypes can be differentiated by selecting ORF2 to do phylogenetic analysis. Up-to-date, there are 5 subtypes of PCV2 including PCV2 subtype a, b, c, d and e as previously mentioned (Zhai et al., 2011). Time table of global shift of PCV2 subtype was focused during the year of 2005 in which PCV2a mostly found in Canada and United States (Northern America) but PCV2b found in Europe and China before the year of 2005. After the year of 2005, the aspect of PCV2 subtype had globally shifted from PCV2a to PCV2b and often found together with clinical signs of PMWS (Gagnon et al., 2007; Dupont et al., 2008; Patterson and Opriessnig, 2010). Thus, PCV2b is considered as the main subtype now. In Thailand, PCV2 antigen was discovered for the first time since 1993 (Banlunara et al., 2002) and clinical signs of PMWS was found associate to PCV2 infection in the year of 1999 (Tantilertcharoen et al., 1999). Many studies had been done and demonstrated the phylogenetic tree using samples in Thailand (Jantafong et al., 2011; Jittimanee et al., 2011; Buapaichit et al., 2013). As a result, PCV2e was found to be a new subtype which usually found in China (Jantafong et al., 2011). PCV2b was found to be major subtype

with prevalence of 83.33% (Jittimanee et al., 2011). Nevertheless, recent study found that PCV2d had higher prevalence than PCV2b with 51.9% and 44.4% by order but PCV2d was previously sorted as PCV2b cluster 1C so that the major subtype still remained subtype b (Buapaichit et al., 2013).

**2.5 Vaccines**

Although the majority of PCV2 subtype is PCV2b, all available commercial vaccines are still based on derivatives of the PCV2a. Many trials show the efficiency of commercial vaccines against PCV2 infection by significant reducing mortality rate and clinical signs of PCV2 (Fachinger et al., 2008; Kixmoller et al., 2008; Opriessnig et al., 2009; Segales et al., 2009) . This mean that commercial vaccines has been accepted so as to give cross protection against PCV2b (Martelli et al., 2011). Four commercial vaccines have been used world widely and all of them are inactivated vaccines. Two vaccines including PCV Porcillis® or PCV Circumvent®( Merck Animal Health) and Ingelvac Circoflex® (Boehringer ingelheim) are based on subunit vaccine in which capsid gene (ORF2) expressed in baculovirus (Beach and Meng, 2012). Circovac®(Merial) is produced by using whole genome of PCV2 and Fostera®(Zoetis) is produced by using chimeric PCV1-2 with ORF2 gene of PCV2 cloned into genomic backbone of PCV1 (Fenaux et al., 2003; Fenaux et al., 2004). Efficacy of vaccine expressing in swine finisher period is satisfactory with evidences of vaccinated group compared to non-vaccinated group such as improved feed conversion ratio (FCR), average daily weight gain (ADWG), less signs of PMWS and decreased mortality rate.

**2.6 Immunology**

To explain about improved production parameters, the role of immune response against PCV2 needs to be understood. Humoral and cellular immune responses are important in immunology after PCV2 infection. For humoral immune response, experimental studies showed that there was a seroconversion at 10 and 28 days post-inoculation (Allan et al., 1999; Krakowka et al., 2001; Meerts et al., 2005). From the farm showing signs of PMWS, higher percentage of seroprevalence had been started at 3 months of age compared to the farm with no sign of PMWS (Rose et al.,

2002; Sibila et al., 2004). Importantly, neutralizing antibodies (NA) plays an important role in clearance of PCV2 infection. Several studies found that the viral load in serum would reduce when there was an increment of neutralizing antibodies in which the decline of the negative impacts of PCVAD and PMWS (Meerts et al., 2006; Fort et al., 2007). For cellular immune response, interferon-γ-secreting cells (IFN-γ) is important and related to the decline of level of viral load in serum like neutralizing antibodies (Fort et al., 2009). The immunity type of this cell is defined as cell-mediated immunity. Consequently, the effective PCV2 control is depended on both the neutralizing antibodies and the IFN-γ. Moreover, another important antibody is anti-PCV2 IgG antibody which often used to explain the humoral immune response after PCV2 infection compared to neutralizing antibodies. However, the relation between level of viral load in serum or viremia after PCV2 infection and anti-PCV2 IgG antibody is still confusing. Some studies had reported that both of them were not related (Opriessnig et al., 2010). By the way, anti-PCV2 IgM antibody had shown high correlation between an increase of anti-PCV2 IgM and the decline of level of viremia (Fort et al., 2007). Level of viremia was quantified and found that high level of anti-PCV2 antibodies still remained in pigs after PCV2 infection for 140 days (Opriessnig et al., 2010).

**2.7 Serology**

For monitoring the status of herd immunity, detection of antibody against PCV2 infection is very useful and reliable. In adult pigs, subclinical PCV2 infection pigs might not show any clinical signs and become carrier with persistent infection (Allan and Ellis, 2000). Several serological techniques had been developed and used for detection of antibodies to define PCV2 infection within herd. To date, indirect immunofluorescence assay (IIF) or indirect fluorescent antibody test (IFAT) (Allan et al., 1998; Allan et al., 1999), immunoperoxidase monolayer assay (IPMA) (Ellis et al., 1998) and many types of enzyme-linked immunosorbent assays (ELISA) had been developed and used. IFAT and IPMA have good sensitivity and reliable but it needs doing culture virus and optimal staining. Cross-reactivity between PCV1 and PCV2 may occur if antibodies are not specific to the region of PCV2. Therefore, a specific PCV2 monoclonal antibodies need to be used for final diagnosis of the PCV1 or PCV2 infection (Walker et al., 2000).

IFAT and IPMA are often used as reference method to detect antibodies to PCV2. Comparison of both techniques, different results may occur because of using different fixatives and methods (McNair et al., 2004). Although, IFAT and IPMA are specific to detect PCV2 infection but these techniques required expert interpreting result and growth of the virus. Therefore, ELISA is more simple, rapid and practical use in large-scale of tested animals.

Nowadays, there are many researches and commercial ELISA kits had been developed for diagnosis PCV2 infection. Many in-house ELISAs had been made and showed variable in types of coating antigen and types of ELISA. The diagnostic performances of each ELISA usually reported as diagnostic sensitivity (DSN), diagnostic specificity (DSP) and accuracy of the test (AC). Previous studies found that whole PCV2 genome-based ELISA based on cell-culture-propagated PCV2 had DSN and DSP as 99.58% and 97.14%, respectively (Walker et al., 2000). For using capsid protein of PCV2 as antigen, the results found that the recombinant baculovirus (AcMNPV.ORF2) had DSN and DSP as 90.7% and 93.2%, respectively (Nawagitgul et al., 2002) and GST-fused capsid protein had DSN and DSP as 98.2% and 94.5%, respectively. However, this technique was expensive and difficult to retrieve enough proteins for coating antigen. So, the *Escherichia coli* (*E.coli*) expression system had been used to overcome those disadvantages. The diagnostic performance of nuclear localization signal-truncated capsid protein of PCV2 from *E.coli* ELISA had DSN, DSP and AC as 95.3%, 93.9% and 95.1%, respectively compared to IFA (Shang et al., 2008). Moreover, when the diagnostic performance was compared to IPMA, it showed DSN, DSP and AC as 98.33%, 93.33% and 96.67%, respectively (Jittimanee et al., 2012). Recombinant protein ELISA was developed used antigenic domain (113-147 amino acid) of ORF2-encoded antigen and the DSN and DSP was 93.57% and 87.7%, respectively (Sun et al., 2010). Previously, most of in-house ELISAs were based on indirect ELISA technique but these assays could not differentiate the immune response between neutralizing antibody and non-neutralizing antibody.

Due to a low level of cross-reactivity of PCV1 and PCV2 (Mahe et al., 2000), most of developed ELISA techniques are based on ORF2 of PCV2 because of the immunogenicity function of this ORF2 (Truong et al., 2001). Moreover, previous studies

shown that ORF2 which encoded capsid protein (Cap) size 30 kDa had more variable nucleotides than ORF1 which encoded two replication proteins (*Rep*, *Rep'*). The recombinant protein of ORF2 showed more reaction of immune response with positive-PCV2 serum (Nawagitgul et al., 2000).

For comparison of diagnostic performances, kappa statistic and receiver operating characteristic (ROC) analysis are commonly statistic that used for diagnostic test analysis. Kappa analysis reported the level of agreement between two tests (Sim and Wright, 2005) and was used when the outcome of the test is dichotomous data. For validation of ELISA test performances, the ROC analysis is used to determine the cut-off values in which determined the true disease status of animals (infected or non-infected) (Zweig and Campbell, 1993; Gardner and Greiner, 2006). ROC analysis is reported as area under curve (AUC) and the value which provides the highest sensitivity and specificity is used to be cut-off values. The larger size of tested samples, the more accurate of cut-off values.

Several studies were conducted to determine the diagnostic performances of PCV2-ELISA tests. Three ELISA assays were compared to detect PCV2 specific antibodies after vaccination or inoculation of pigs with distinct PCV1 or PCV2 isolates. The results found that all ELISAs had area under curve (AUC) over 0.94 and all tests could detect both anti-PCV2a and PCV2b antibodies and no cross-reactivity between PCV1 and PCV2 was found. However, these ELISA systems could not distinct the subtype of PCV2 antibodies (Patterson et al., 2008). Moreover, inter-laboratory comparison was done by focusing on PCV2 IFAT and ELISA assay. Kappa analysis found that the results of IFAT among laboratories had almost perfect agreement, following by in-house ELISA and commercial available ELISA. These indicated that there was variability between results from commercial ELISA. It is possible that the variability of ELISA systems due to the different strain of coating virus antigens, specificity of antibody, cut-off value, length of PCV2-ORF2 and different techniques of technicians (Patterson et al., 2011).

# CHAPTER III

# MATERIALS AND METHODS

The methods of this study were described briefly as followings. First, viral antigen preparation was done by collecting and identifying PCV2 subtype b. Second, the indirect PCV2b-based ELISA was developed and optimized for optimal conditions of the test kit. Third, immunoperoxidase monolayer assay (IPMA) was developed for using as reference method. Finally, validation of the indirect PCV2b-based ELISA was performed to confirm a performance of the test kit. More details were described as followings.

## 3.1 Viral antigen preparation

### 3.1.1 Selection of positive sera

Positive sera (n=10) were collected from swine finisher farms in Kanchanaburi province, Thailand. Sera which were suspicious to have positive antibody against PCV2 would be selected from pigs showing clinical signs and lesions of PMWS or PDNS from natural infection (Figure 1) and confirmed with PCR.

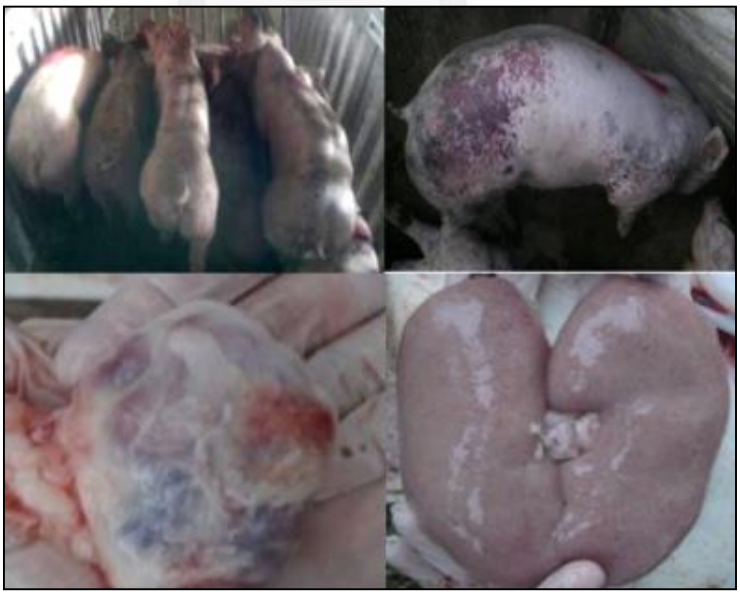

**Figure 1**. Clinical signs and lesions of PMWS with wasting pigs and enlarged superficial inguinal lymph node and PDNS with dermatitis of kidney

### 3.1.2 Extraction of Viral DNA of PCV2

Viral DNA was extracted from serum using protocol of ZR viral DNA/RNA kit™ (Zymo Research®, USA). Due to the size of PCV2 (1.7 kb), this commercial extraction kit was suitable for using. This kit was well designed for extraction DNA or RNA which was 50 bases to more than 200 kb in size from body fluids such as serum and could yield enough DNA or RNA for using in RT/PCR. The short procedure of this extraction kit was shown in Figure 2 and the protocol of this kit was shown as followings.

First, buffer was prepared by adding beta-mercaptoehanol to the viral DNA/RNA buffer to a final dilution of 0.5% (v/v) and 24 ml of 100% ethanol was added to the 6 ml of DNA/RNA wash buffer concentrate before use. Then, viral DNA/RNA buffer was added for 3 volumes to each volume of sample to get the mixture and transferred to the Zymo-Spin™IC-XL Column in a collection tube and centrifuged at 12,000 xg for 1 minute. The collection tube that containing the flow-through was discarded and then placed the column into a new collection tube.

Next, DNA/RNA Prep buffer was added for 400 µl to the column and centrifuged at 12,000 xg for 30 seconds and then discarded the flow-through. DNA/RNA Wash buffer was added for 700 µl to the column and centrifuged at 12,000 xg for 15 seconds and then discarded the flow-through. Step of adding DNA/RNA Prep buffer was repeated and DNA/RNA Wash buffer was added for 400 µl and then discarded the flow-through.

After that, Zymo-Spin™IC-XL Column was centrifuged at maximum speed for 5 minutes in the emptied collection tube to ensure complete removal of the wash buffer. The column was removed carefully from the collection tube and transferred it into a DNase/RNase-free tube and then DNase/RNase-free water was added for 10 µl directly to the column matrix and let stand for 1 minute at room temperature.

Finally, the column matrix was centrifuged at maximum speed for 30 seconds to elute DNA/RNA and stored at -70 $^{o}$C.

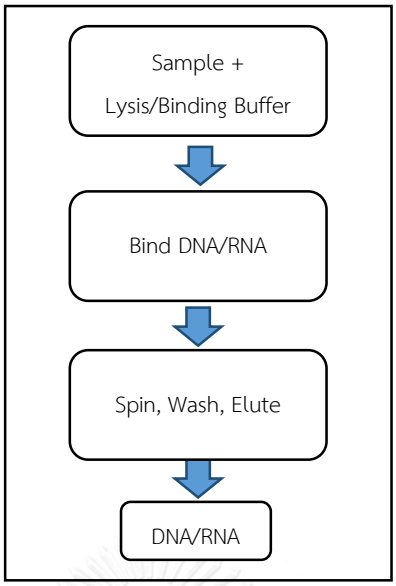


**Figure 2.** Extraction procedure for eluting nucleic acids from sample as protocol of ZR viral DNA/RNA kitTM (Zymo Research®, USA)

### 3.1.3 Identification of PCV2 Subtype b

To prove the subtype of PCV2, PCR and sequencing technique were used to do the phylogenetic analysis and confirm the subtype of PCV2 by the following protocols.

3.1.3.1 Selection of primers and concentration of mixture

Before doing phylogenetic analysis, PCR technique was done by amplifying the full length of ORF2 gene of PCV2 with specific forward and reverse primers targeting on ORF2 (F : 5'- CCA TGC CCT GAA TTT CCA TA-3', R : 5'- ACA GCG CAC TTC TTT CGT TT-3') (Takahagi et al., 2008). The mixture of PCR product was mixed with Gotaq® Green master mix ( Promega™ ) which was ready- to- use containing bacterially derived Taq DNA polymerase, Deoxynucleotides ( dNTPs) , $MgCl_2$ and reaction buffer. Then, Gotaq® Green master mix (Promega™) was mixed with prepared reagents including forward primer, reverse primer, nuclease-free water and finally with DNA template derived from extraction of viral DNA of PCV2. The concentration of mixture was set with the final volume as 50 µl (Table 1).

**Table 1**. Concentration of PCR mixture before amplifying with DNA thermal cycler

| **Reagent** | **1x (µl)** |
|---|---|
| Gotaq® Green master mix (2x) | 25 |
| 10 µM Forward primer | 4 |
| 10 µM Reverse primer | 4 |
| Nuclease-free water | 12 |
| DNA Template | 5 |
| Total | 50 |

3.1.3.2 Condition of PCR

After the mixture was ready, the condition of PCR was modified and set to get the best result (Table 2). The mixture of sample was loaded in DNA thermal cycler and compared to mixture of positive sample against PCV2 (Derived from positive sample confirmed by PCR technique), negative sample (Nuclease-free water) and the DNA ladder (Vivantis®) containing 10mM Tris-HCl (pH 8.0) and 1 mM EDTA.

**Table 2.** Condition of PCR in DNA Thermal Cycler

| **Step of PCR** | Denature | Annealing | | Extension | - |
|---|---|---|---|---|---|
| **Amount of cycle** | 1 | ← 40 → | | 1 | - |
| **Temperature (°C)** | 94 | 94 | 60 | 72 | 72 | 4 |
| **Duration (Minute)** | 2 | 0.30 | 0.30 | 1 | 7 | ∞ |

3.1.3.3 Gel Electrophoresis

All samples and DNA ladder was loaded and run in gel electrophoresis to get the PCR product of 802 bp after running and read with UV transilluminator. Gel electrophoresis and agarose gel were performed and prepared as followings.

First, TAE (50X) was diluted with distilled water to get TAE running buffer (1X). Then, TAE running buffer (1X) was mixed with agarose powder and heated with microwave for 3 minutes or until it melted to get 1.5% agarose gel. Next, nucleic acid gel stain of this study that was GelStar™ (10,000x concentrate in dimethyl sulfoxide (DMSO)) was added for 1 ml in 1.5% agarose gel. After that, 1.5% agarose gel mixed

with gel star was poured on the gel plate connecting with power supply. Then, the gel was let to dry and loaded with above mixture. The condition of power supply would be set as following conditions, voltage as 100 volts, electric current as 120 mAm and duration as 60 minutes. Finally, the gel was read under UV transilluminator and results were recorded.

3.1.3.4 Sequencing

The samples that were positive for to PCV2 (PCR product of 802 bp) were selected by submitting unpurified PCR product and primers to First BASE laboratories, Malaysia. PCR product would be purified and extracted from gel. After that, the sequencing would be performed and sequence result was sent back to researcher and for phylogenetic analysis.

3.1.3.5 Phylogenetic Analysis

The sequences of laboratory strain were compared with reference strains from GenBank to do the phylogenetic tree. Before that, assembly and reverse complement of sequence must be done by order. Then, comparison of laboratory strain and 15 reference strains (Table 3) was performed by doing the alignment using ClustalW method. Finally, phylogenetic tree was made by using MEGA 5 program with maximum likelihood and 1,000 replications of Bootstrap method.



**Table 3.** Information of 9 laboratory strains and 15 reference strains from GenBank

| Accession number/Name | Subtype | Country | Note |
|---|---|---|---|
| AY193712 | PCV1 | China | Reference |
| JQ994269 | PCV2a | Canada | Reference |
| GU049340 | PCV2a | Spain | Reference |
| AB426905 | PCV2a | Japan | Reference |
| AY146991 | PCV2a | Taiwan | Reference |
| JF682791 | PCV2a | China | Reference |
| EF565365 | PCV2b (PCV2b 1A/B) | Denmark | Reference |
| JQ866918 | PCV2b (PCV2b 1A/B) | Thailand | Reference |
| GU799576 | PCV2b (PCV2b 1A/B) | USA | Reference |

**Table 3.** Information of 9 laboratory strains and 15 reference strains from GenBank (Cont.)

| | | | |
|---|---|---|---|
| FJ644929 | PCV2d (PCV2b 1C) | China | Reference |
| GU001710 | PCV2d (PCV2b 1C) | China | Reference |
| JX679498 | PCV2d (PCV2b 1C) | China | Reference |
| EU148504 | PCV2d | Denmark | Reference |
| AY864814 | PCV2e | Thailand | Reference |
| HQ701665 | PCV2e | Thailand | Reference |
| L466.1 | - | Thailand | Laboratory |
| L466.2 | - | Thailand | Laboratory |
| L466.4 | - | Thailand | Laboratory |
| TF6 | - | Thailand | Laboratory |
| KH_1 | - | Thailand | Laboratory |
| KH_2 | - | Thailand | Laboratory |
| KH_3 | - | Thailand | Laboratory |
| KH_4 | - | Thailand | Laboratory |
| KH_5 | - | Thailand | Laboratory |

### 3.1.4 Cell Culture and Viral Inoculation

To enhance viral titer of PCV2 subtype b, viral isolation must be performed by doing subculture from primary cell, inoculation with positive serum and harvesting the virus. This process was done for several passages to gain high permissiveness to PCV2 infection and high viral titer.

3.1.4.1 Cell Culture

In this study, cell line used for doing subculture (Derived from Dr. Suphot Wattaphansak) was "SST-SW1" cell (Figure 3). The origin of this cell came from lung cell of naive piglet and was sub cultured for many times. The step of subculture had been shown as followings.

First, SST-SW1 cell was activated after it was kept in vial at -80 $^{o}$C freezer by pouring it on the flask. The cell suspension was added with growth medium or culture media prepared from 10% fetal bovine serum (FBS) and minimum essential

medium (MEM). Then, it was incubated in 37 °C incubator for 6 hours to activate the growth of the cell. SST-SW1 cell which was splitting and attaching as monolayer cell for 80-90% in the flask was subcultured and observed via inverted microscope (Figure 3). Old culture media was discarded and washed with phosphate buffer saline (PBS) 1X for 1-3 times.

Next, trypsin 1x was added (1 ml for 25 ml flask, 3 ml for 75 ml flask, 5 ml for 175 ml flask) flowing over the monolayer cell and wait for 1 minute or until the cell was rounded up in the 37 °C incubator and then discarded the trypsin and incubated again for 2 minutes. The flask was knocked or shaken to unattach monolayer cell from the flask and added culture media including 5% FBS, 1% L-glutamine, 1% amphotericin B, gentamicin and MEM to stop reaction of trypsin.

After that, the pipette was used to pipette up and down (Note. Concentration of gentamicin was calculated from formulation "volume of gentamicin (µl) = (60 x volume of media (ml) x 1,000)/100,000") and added more media (Up to volume of the flask). Then, media was poured out (cell was inside) and added more media to get concentration as 1/10 ratio.

Finally, the flask containing cell was incubated in 37 °C incubator (5% $CO_2$) for 1-2 days to get splitting cell for 50-60% (Figure 3) in the flask and infected with PCV2 in the next step.

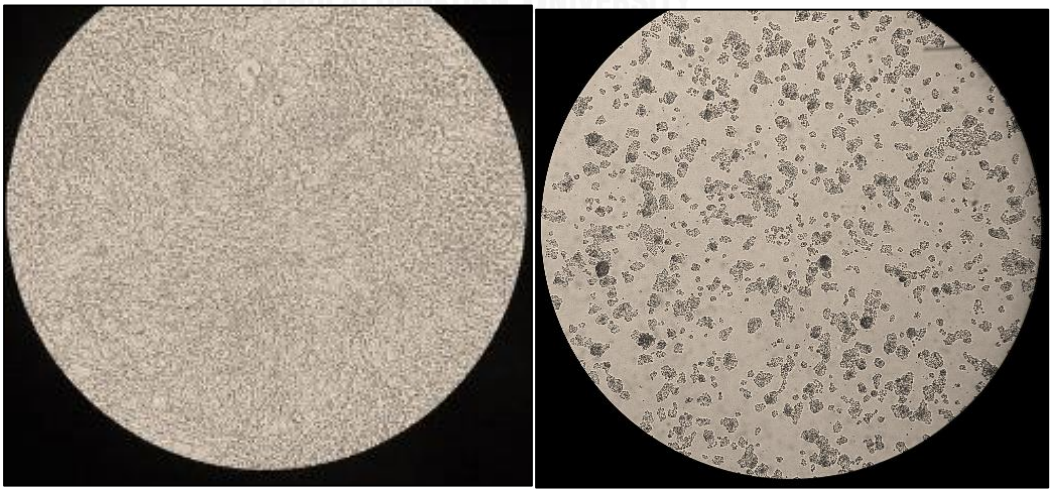

**Figure 3.** Picture from inverted microscope showing monolayer cell of SST-SW1 cell splitting for >90% (Left) and 50% (Right)

3.1.4.2 PCV2 inoculation

Positive serum was derived from the step of sequencing to confirm the subtype of PCV2. In this study, all positive samples confirmed as PCV2 subtype b were selected and tested for finding the best sample to be used as coating antigen due to the capacity of infection. The step of PCV2 inoculation were shown as followings.

First, old media was discarded and washed with PBS 3 times. Then, the cell was inoculated with positive serum and then let to be adsorbed in 37 $^{o}$C incubator for 1 hour. The maintenance media was prepared including 2% FBS, 1% L-Glutamine, 1% Amphotericin B, Gentamicin and MEM (Concentration of Gentamicin was the same as culture step) and then added to the flask. Next, the flask was incubated in 37 $^{o}$C incubator for 5-7 days and kept looking the infection of PCV2 via inverted microscope during this step. Finally, the infection must be more than 80% to harvest the PCV2 culture and check the infection by using indirect fluorescent antibody technique (IFAT) (Figure 4).

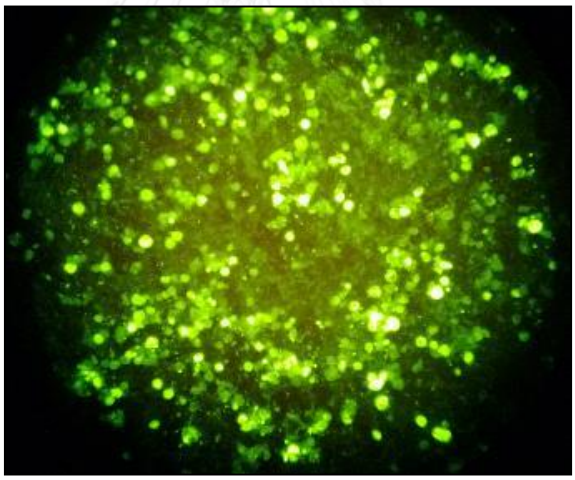

**Figure 4.** The infection of SST-SW1 cell inoculated with positive serum against PCV2 and checked infection with indirect fluorescent antibody technique (IFAT).

3.1.4.3 Indirect Fluorescent Antibody Technique (IFAT)

IFAT was used to classify the capacity of PCV2 infection and in this study using scoring system showing in Table 4. Infected cell from positive sample would produce apple-green color glowing with fluorescence microscope. Modified IFAT was performed by following steps.

First, the slide and moisture chamber were prepared (Figure 5). One drop of sample was transferred to the slide for only 1 drop with pipette and marked the sample under the slide with permanent marker (Figure 5) and incubated in 37 °C incubator for 45 minutes or until it dried.

Then, the slide was rehydrated with distilled water and incubated in 37 °C incubator for 5 minutes and then poured the liquid out. Then, PCV2 monoclonal antibody ( anti- pig IgG which derived from Sigma- Aldrich®) was added with concentration as 1:1000 in PBS and then incubated in 37 °C incubator for 1 hour in moisture chamber (Figure 5).

Next, the liquid was poured out and washed with PBS for 3 times. After that, secondary antibody (fluorescein isothiocyanate (FITC) anti-mouse IgG) was added with concentration as 1:200 in PBS and then incubated in 37 °C incubator for 1 hour in moisture chamber (Figure 5).

Finally, the slide was washed with PBS 3 times and covered the slide with cover glass and then read and record the result with fluorescence microscope with blue-white light.

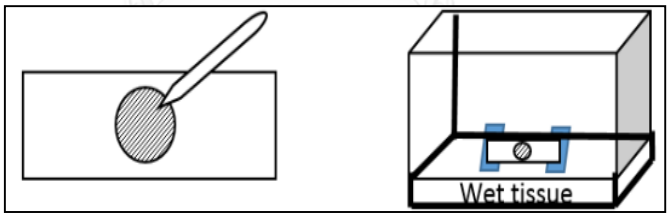


**Figure 5.** Preparation of the slide and moisture chamber using in IFAT

**Table 4**. Scoring system of glowing infected cell with apple-green color by IFAT

| Level | Interpretation |
|---|---|
| 0 | No infection |
| +1 | Mild infection |
| +2 | Mild-moderate infection |
| +3 | Moderate infection |
| +4 | Moderate-severe infection |
| +5 | Severe infection |

3.1.4.4 Reinfection

The step of PCV2 inoculation was repeated but exchanging the positive serum to PCV2 culture and performing PCV2 isolation for several passages to gain high viral titer and permissiveness of PCV2 infection. Finally, PCV2 culture was submitted for sequencing again to confirm the subtype of PCV2.

**3.1.5 Viral Titration**

Selection of the optimal positive sample of PCV2 subtype b must depend on the level of infection by IFAT and viral titer by viral titration technique. In this study, viral titration technique of tissue cell culture was performed by followings.

First, virus stock was thawed (If needed) and the infection media containing 2% FBS and MEM was prepared and transferred for 180 µl to every wells of 96-well culture plate (U-bottom shape). PCV2 stock was transferred for 20 µl to the first column with row A-D (Only 1 strain) and performed serially 10-fold dilution from first column to eleventh column by using 8 channel multipipette (concentration as $10^{-1}$ to $10^{-11}$ by order). No virus added in twelfth column (used as negative control) (Figure 6).

Then, 96-well culture plate (flat-bottom shape) was prepared containing SST-SW1 cell for inoculation that prepared from cell culture step but must be split for 90-100 %. The old media was discarded from the plate and washed with PBS 2 times.

After that, each viral dilution was transferred for 50 µl to corresponding wells in the SST-SW1 cell plate and absorpted with virus for 1 hour in 37 $^{o}$C incubator. Then, the inoculums were removed and washed with PBS 1 time. More infection media was added for 100 µl to each well and incubated in 37 $^{o}$C incubator.

Finally, viral cytopathic effect (CPE) was kept observing for 3-5 days via inverted microscope until the cell of negative control died. The result was recorded and calculated the titer of stock virus by using Reed & Muench method (Reported as tissue culture infectious dose ($TCID_{50}$/volume of stock virus)).

3.1.5.1 *Formulation*: Proportionate distance(PD) = A-50/A-B

$\log TCID_{50} = (\log C) - (PD \times \log X)$

$1TCID_{50}$/Volume (Stock virus) = Dilution $10^{\log TCID_{50}}$

A = % accumulate response (First>50%)

B = % accumulate response (First<50%)

C = Dilution of virus giving “A”

X = Dilution factor

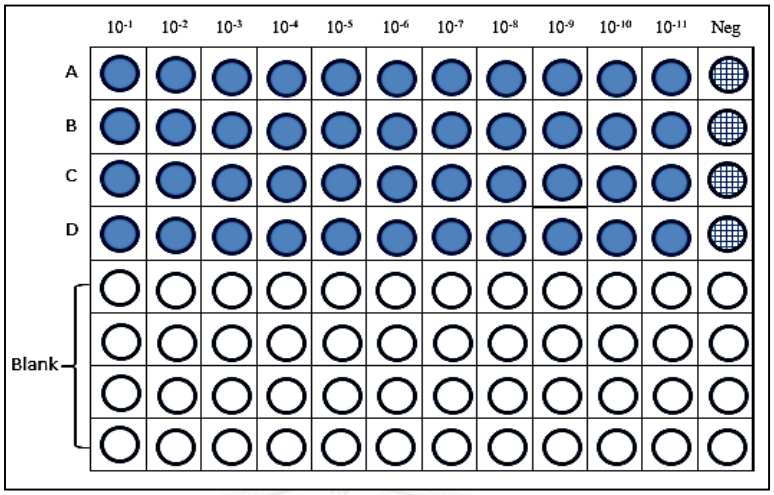


**Figure 6**. 10-fold Serial dilution from column 1 to 11 and negative control in column 12 of 96-well culture plate (U-bottom shape) with 4 replicates (rows A to D).

### 3.1.6 Collection of PCV2b Antigen

After getting the optimal positive samples of PCV2 subtype b by viral titration technique, PCV2b virus antigen would be harvested from SST-SW1 cell inoculated with PCV2 positive serum. The steps of harvest and collect PCV2 antigen were shown as followings.

First, cells with PCV2 subtype b were frozen to break the cells in -20 °C freezer and thawed 3 times to get the viral suspension and centrifuged at speed of 8,000 xg 4 °C for 20 minutes for collecting the supernatant.

Then, the supernatant was transferred for 24 ml to 6 centrifuge tubes (4 ml per tube) of ultra-high speed centrifuge (Fixed angle rotor with 6 centrifuge tube) and centrifuged at 100,000 xg, 4 °C for 2 hours and then collected the supernatant to centrifuge again with 200,000 xg, 4 °C for overnight.

Next, the supernatant was discarded and collected the viral pellet (From 6 tubes) and resuspended with TNE buffer (Figure 7). TNE buffer was prepared (pH 7.2) including Tris 20mM, NaCl 100 mM and EDTA 2mM and adjusted the pH of TNE buffer

with HCl. Finally, the viral pellet was resuspended with 3.6 ml of TNE buffer showing as figure 7.

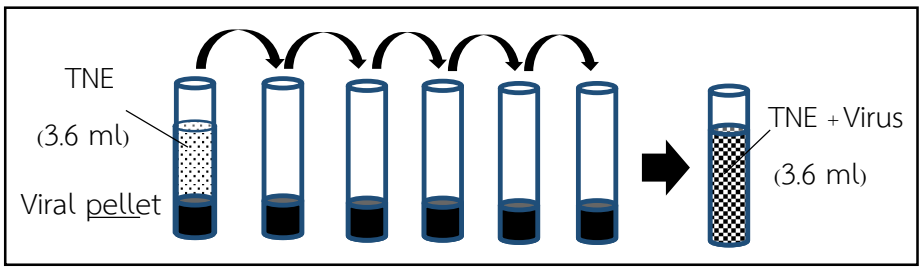


**Figure 7**. Viral pellet was resuspended with TNE buffer (pH 7.2) from first tube to the sixth tube and finally collected with centrifuge tube.

### 3.1.7 Purifying of PCV2b Antigen

After resuspending the viral pellet with TNE buffer, sucrose gradient technique (Figure 8, 9) was used to purify PCV2 antigen and get high intensity for coating on ELISA plate. Purifying steps were shown as followings.

First, sucrose gradient technique was performed with sucrose concentration at 10, 20, 40 and 60 % (600 µl per level of concentration) in one tube (Figure 8). The gradient tube was topped up with 600 µl of viral pellet which resuspended with TNE buffer (Figure 9).

Then, previous steps of getting sucrose concentration gradient and topping up with TNE buffer containing viral pellet were reversed for getting 6 gradient sucrose tubes and all tubes were centrifuged with ultra-high speed centrifuge at 200,000 xg, 4 $^{o}$C for 16 hours and then collected and pooled each level with centrifuge tube (Figure 9).

Next, sucrose dilution was performed to remove the sucrose by transferring 1.8 ml of each tube to new tube and added 2.2 ml TNE buffer to both tubes with total volume as 4 ml per tube and centrifuged with ultra-high speed centrifuge at 200,000 xg, 4 $^{o}$C for overnight and discarded supernatant and collected the pellet.

After that, the pellet was resuspened of each tube with 4 ml of TNE buffer with the protocol from previous step but got 2 tubes become 1 tube of each level of

sucrose concentration and then centrifuged with ultra-high speed centrifuge at 200,000 xg, 4 °C for overnight and discarded supernatant and collected the pellet.

Finally, the pellet was resuspened of each tube with 4 ml of TNE buffer and collected them as pure PCV2b antigen of each level of sucrose concentration.

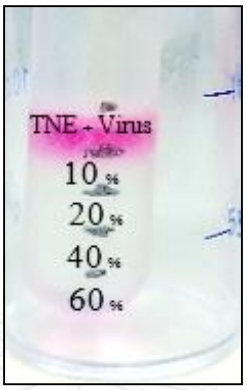


**Figure 8.** Sucrose gradient technique with varying sucrose concentration at 10, 20 ,40 and 60 % and topped up with viral pellet resuspended by TNE buffer.

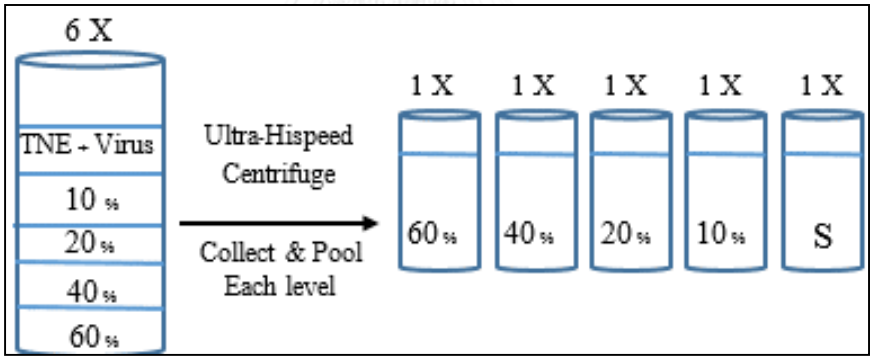


**Figure 9**. Each level of sucrose concentration was collected and pooled including 10, 20, 40 and 60 %. The tube "S" referred to collection from a top level of sucrose gradient tube (TNE + virus) after performing sucrose gradient technique.

### 3.1.8 Quantification of PCV2b Antigen

Finally, the last step of viral antigen preparation was antigen quantification performed by Bradford dye-binding method (Bradford, 1976) to select the best candidate of PCV2b antigen with highest intensity and amount for using as coating antigen. The quantification of PCV2b antigen, which referred to concentration of

protein, was determined using protocol from Bio-Rad protein assay. In this study, bovine serum albumin (BSA) was mainly used to do the standard curve comparing with sample and this assay could be adapted from standard concentration to low concentration (less than 25 µg/ml). The protocol of Bio-Rad protein assay was shown as followings.

First, dye reagent was prepared by diluting 10 ml of concentrated dye reagent with 40 ml of distilled water that was deionized and then filtered through Whatman #1 filter to remove particles and kept in 4 °C.

Then, stock solution of BSA standard was prepared for 2 mg/ml by putting 20 mg of lyophilized BSA standard in 10 ml of water and mixing until dissolved and then kept in 4 °C. Next, serial dilutions of BSA standard was prepared as table 5 and figure 10.

**Table 5**. Serial dilutions of the BSA standard

| **Tube (Final concentration of BSA in the cuvette)** | **Dilutions** |
|---|---|
| 1 (12 µg/ml) | 210 µl of stock + 6,790 µl $H_2O$ |
| 2 (10 µg/ml) | 5,000 µl from tube 1 + 1,000 µl $H_2O$ |
| 3 (8 µg/ml) | 4,000 µl from tube 2 + 1,000 µl $H_2O$ |
| 4 (6 µg/ml) | 3,000 µl from tube 3 + 1,000 µl $H_2O$ |
| 5 (4 µg/ml) | 2,000 µl from tube 4 + 1,000 µl $H_2O$ |
| 6 (2 µg/ml) | 1,000 µl from tube 5 + 1,000 µl $H_2O$ |

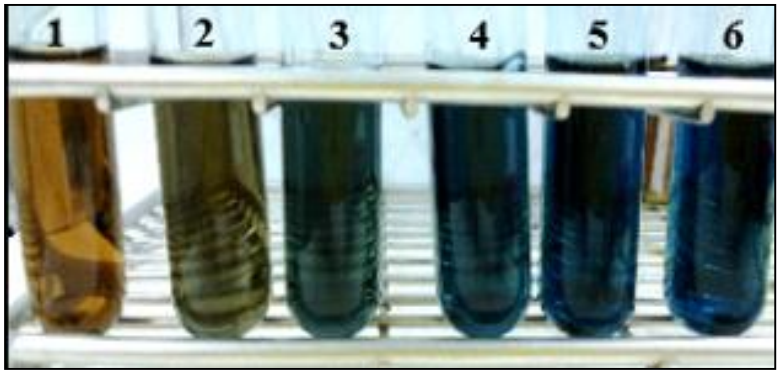


**Figure 10**. Serial dilutions of the BSA standard from tube 1 to tube 6

After that, one Eppendorf tube for each solution that needed to be quantified was prepared (BSA dilutions, samples and water for the blank). Protein solutions were normally assayed in duplicate. The details of preparation of each solution including standard of each dilution was put for 200 µl, water for the blank was put for 200 µl and the samples that was an extract was put for 25 µl added with water for 175 µl (Final volume as 200 µl). Then, dye reagent was added for 800 µl in each tube and then vortexed. The tubes were incubated in the dark at room temperature for 15 minutes and each solution was transferred to the cuvette and measured the absorbance at 595 nm (Figure 11).

Finally, amount of the proteins was calculated by comparison with the standard curve derived from BSA standard solution.

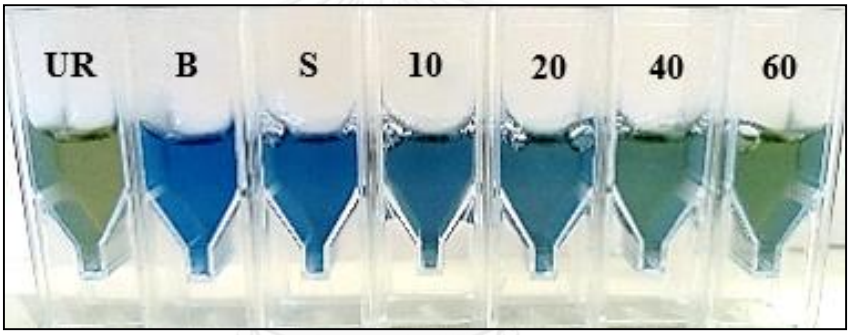


**Figure 11.** Protein solutions of samples after adding dye reagent including "UR" as virus resuspended with TNE, "B" as blank, "S" as top level of sucrose gradient tube after performing sucrose gradient technique and "10 -60" as percent of sucrose concentration.

### 3.2 Development of ELISA

#### 3.2.1 ELISA Procedure

Since the limitation of direct assay, indirect assay was widely used to detect immunoglobulin binding to antigen because of using secondary antibodies (antispecies antiserum) labeled with enzyme (conjugate). This assay was more specific and flexible in selecting antispecies antiserum due to specificities of conjugate (Crowther, 2000). The procedure was done as followings (Figure 12).

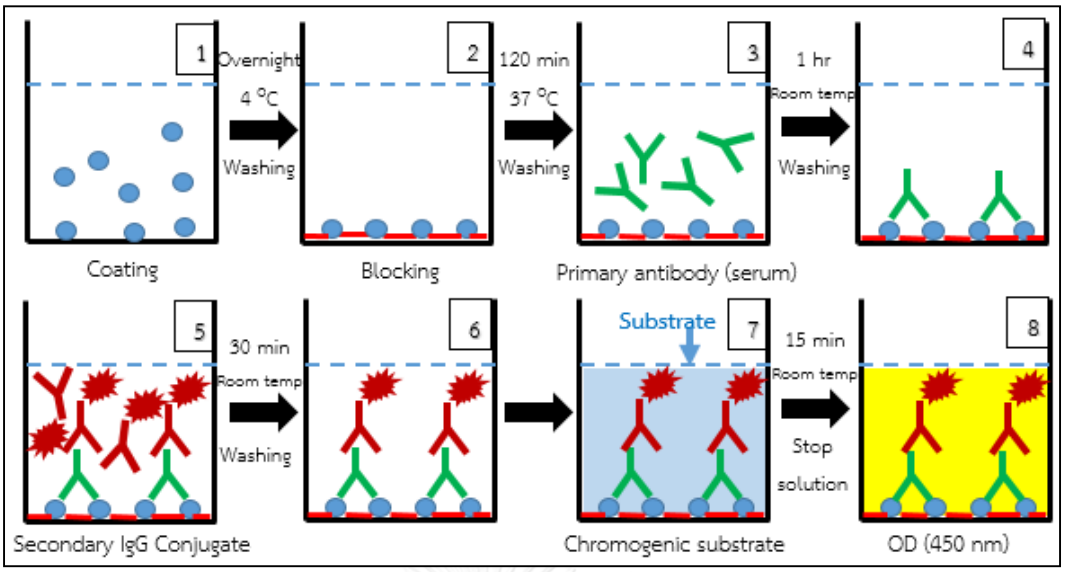


**Figure 12.** ELISA procedure of indirect PCV2b-based ELISA

First, each well of 96-well plate (ELISA plate) was coated with 100 µl of PCV2b antigen in 0.05M bicarbonate-carbonate buffer (pH 9.6) for overnight at 4 °C and then 2 times washing with PBS 0.05% Tween20 (PBST).

Then, each well of the plate was blocked with 200 µl of 5% skimmed milk (blocking buffer) in PBST to reduce error from non-specific background and incubated at 37 °C for 2 hours following by 3 times washing.

Then, diluted serum in blocking buffer was added for 100 µl into each well and incubated at room temperature for 1 hour following by 4 times washing.

Next, HRP-labeled goat anti swine IgG (Secondary antibody labeled with conjugate; HRP = Horse Radish Peroxidase) derived from KPL (Kirkegaard & Perry Laboratories, Inc.) in blocking buffer was added for 100 µl into each well and incubated at room temperature for 30 minutes following by 3 times washing.

After that, colorimetric reaction was developed by adding 100 µl of chromogenic substrate (SureBlue™ TMB Microwell Peroxidase Substrate; TMB = 3,3′,5,5′-Tetramethylbenzidine) derived from KPL (Kirkegaard & Perry Laboratories, Inc.) into each well at room temperature for 15 minutes.

Finally, stop solution (2M $H_2SO_4$) was added for 100 µl into each well for reading by ELISA reader with optical density at 450 nm ($OD_{450}$) and recorded the result.

### 3.2.2 Optimization of ELISA

To get the optimal procedure of ELISA, condition of antigen concentration, serum and conjugate were adjusted. Checker board titration technique was used in this study based on "The ELISA guidebook" (Crowther, 2000). The protocol of optimization of indirect PCV2b-based ELISA was shown as followings.

3.2.2.1 Checker Board Titration

This technique was used to get the best condition of ELISA resulted in high plateau, good end point, highest $OD_{450}$ ratio (P/N value) and minimal OD background. Three variables had been focused in this study including concentration of coating antigen, conjugate and primary antibodies (Serum). The titration could be performed only two variables in one assay by using the ELISA procedure from above. Checker board titration was done as followings.

3.2.2.1.1 Antigen Titration Dilution of positive and negative sera was used with constant conjugate as recommended level of manufacture protocol. Conjugate was set dilution at 1/2000 adding to every wells, positive and negative sera were performed serial 2-fold dilution from 1:30-1:1920 (Row A-G) and coating antigen was performed serial 2-fold dilution from 1:50-1: 51,200 (Column 1-11). Column 12 and row H were received only diluent (Figure 13).

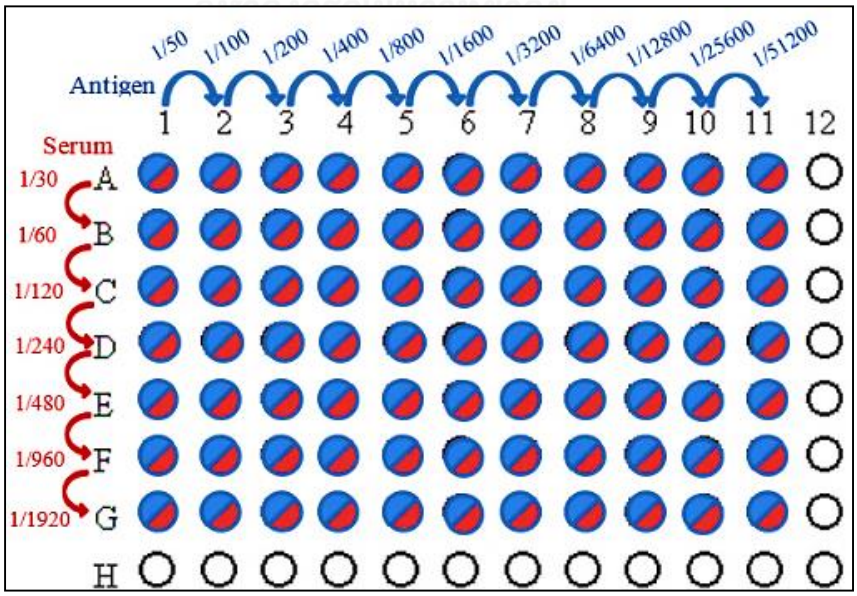


**Figure 13**. Serial 2-fold dilution of coating antigen and sera. Single dilution of conjugate was set as 1:2000.

3.2.2.1.2 Sera and Conjugate Titration From the step of antigen titration, dilution range of positive serum might not be enough to titrate the endpoint of antibodies. Thus, dilution of positive and negative sera was set and performed serial 2-fold dilution from 1:30-1: 30,720 (Column 1-11). Since the dilution of conjugate was fixed at the step antigen titration from the recommended level of manufacture protocol. However, nonspecific background might occur and decrease the sensitivity of the test so that conjugate dilution was set and performed serial 2-fold dilution from 1:500 – 1: 32,000 (Row A-G). Column 12 and row H were received only diluent. Antigen dilution was set at optimal dilution from step of antigen titration (Figure 14).

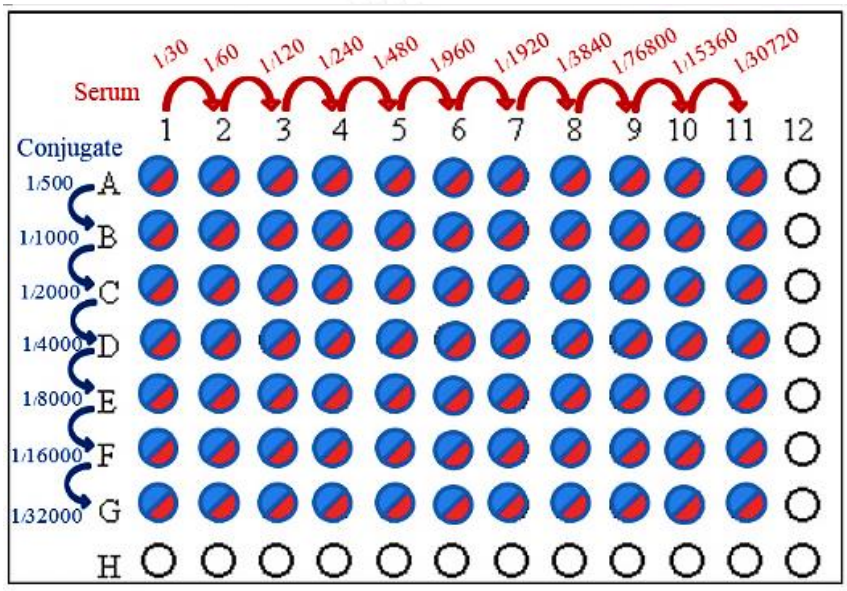


**Figure 14.** Serial 2-fold dilution of sera and conjugate. Single dilution of antigen was derived from antigen titration step.

3.2.2.1.3 Binding Ratios (BRs). To get a clear view of determining optimal conditions for performing ELISA procedure, binding ratios was used by using ration between OD value of positive and negative serum (P/N value) from the step of sera and conjugate titration at same dilution.

### 3.3 Immunoperoxidase Monolayer Assay (IPMA)

In this study, a modified immunoperoxidase monolayer assay (IPMA) was performed as previous study (McNair et al., 2004) to use as a gold standard of detecting

antibodies of PCV2 compared with indirect PCV2b-based ELISA. The materials and methods of cell culture and inoculation of IPMA were applied as same as protocol above. IPMA technique was done as followings.

First, 96-well plate was cultured with SST-SW1 cell and incubated for 24 hours before inoculation. Then, inoculation with high titer of PCV2b pure culture (the same strain as above) was absorbed and the infected plates would be kept in 5% $CO_2$, 37 $^{o}$C incubator for 4 days. As a reason of preservation (kept for using later), the plate was fixed by cold (1:1) acetone: methanol with 300 µl each well for 30 seconds. After that, the fixed solution was poured out and the plate was stored in -20 $^{o}$C freezer to preserve until use.

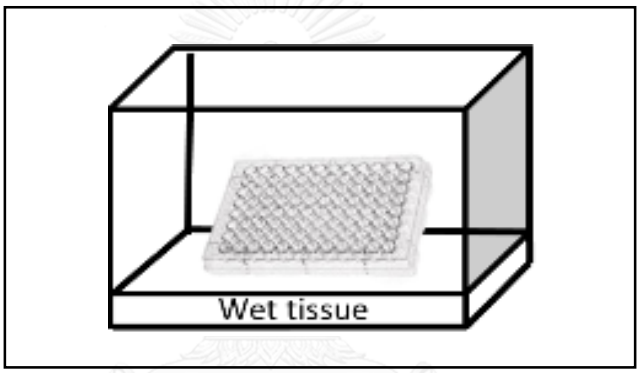


**Figure 15.** Moisture chamber with cultured plate inside

For staining assay, the infected plates were rehydrated with distilled water at 37 $^{o}$C in moisture chamber for 40 minutes (Figure 15). Each diluted pig serum (sample) was added for 100 µl at 1:60 in PBST with 5% skimmed milk to each well and incubated at 37 $^{o}$C in moisture chamber for 45 minutes and then washed with PBS 3 times.

Then, anti-pig IgG peroxidase conjugate was added for 100 µl (derived from Sigma-Aldrich®) diluted at 1:1000 in PBST with 5% skimmed milk to each well and incubated at 37 $^{o}$C in moisture chamber for 45 minutes and then washed with PBS 3 times.

Next, freshly prepared chromogen solution was added for 100 µl (prepared from 9.5 ml of acetate buffer, 8 µl of $H_2O_2$ and 600 µl of AEC solution (3-amino-9-ethylcarbazole; AEC powder + 2.5 ml of N, N-dimethylformamide)) to each well and incubated the plates for 20 minutes and washed with water.

Finally, the result was read by using inverted microscope and recorded.

## 3.3 Validation of ELISA

### 3.3.1 Criteria for Selecting Standard Serum

Standard serum used in this study were selected by following criteria, PCV2-positive standard serum for using in this indirect PCV2b-based ELISA and IPMA were selected from natural infected pigs and PCV2-negative standard serum were selected from pigs in herd at age lower than 5 weeks with no history of PCV2 vaccination or infection. Both sera were confirmed by PCR and IPMA and used for performing checker board titration technique of ELISA. Moreover, they were used for positive and negative standard sera of ELISA and IPMA.

### 3.3.2 Cut-Off Determination

Cut-off determination of PCV2b-based ELISA contained 100 field sera composed of 50 sera from positive-PCV2 pigs (IPMA positive and pigs showed clinical signs of PMWS or PDNS) and 50 sera from negative-PCV2 pigs (IPMA negative and sera obtained from a herd at age lower than 8 weeks with no history of PCV2 vaccination and outbreak). Receiver Operating Characteristic (ROC) analysis was used to define positive-negative cut-off with optical density (OD) values of ELISA for finding highest diagnostic sensitivity (DSN), diagnostic specificity (DSP), and accuracy (AC). DSN was calculated from the proportion of IPMA-positive samples giving positive result of ELISA, DSP was calculated from the proportion of IPMA-negative samples giving negative results of ELISA and AC was calculated from followings:

$$AC = (TP+TN)/\text{Total number of samples} \times 100$$

when * $DSN = TP/(TP+FN)$

* $DSP = TN/(TN+FP)$

TP=True positive, TN=True negative, FP=False positive, FN=False negative

Moreover, positive and negative predictive values and also the prevalence were calculated to ensure the diagnostic performances from followings:

$$\text{Positive predictive value (PPV)} = TP / \text{All positive}$$

$$\text{Negative predictive value (NPV)} = TN / \text{All negative}$$

$$\text{Prevalence} = \text{Positive from IPMA} / \text{Total number of samples} \times 100$$

### 3.3.3 Repeatability

Repeatability tests contained 20 field sera (10 positive, 10 negative). Intra-plate (within-plate) and inter-plate (between-run) were used for finding repeatability of PCV2b-based ELISA by using 3 replicates per serum of each protocol and mean OD ratio, standard deviation (SD), percent of covariance (%CV) were calculated (Figure 16).

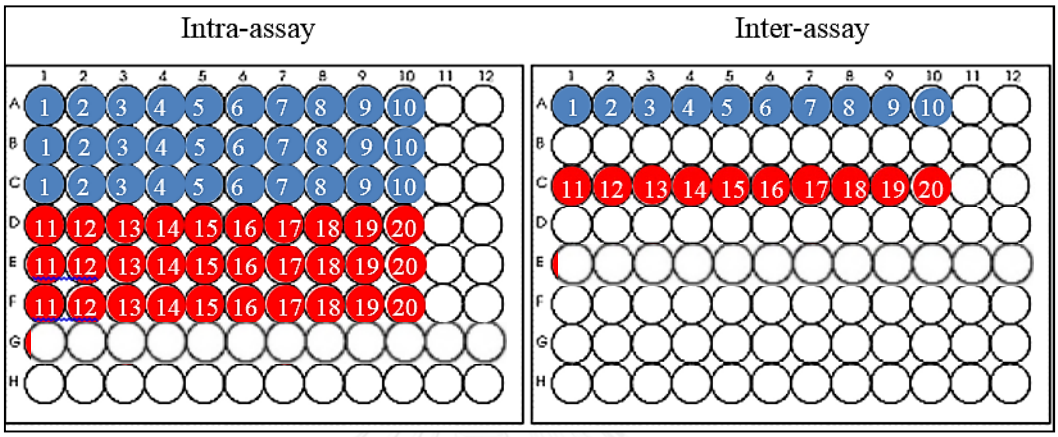


**Figure 16**. Repeatability test of intra-assay (Left) and inter-assay (Right). Sample 1-10 (blue color) referred to negative sera and sample 11-20 (red color) referred to positive sera (3 replicates per serum). Intra-assay was done for only 1 plate but inter-assay was done for 3 plates.

### 3.3.4 Comparative Agreement

Comparative agreement test contained 657 field sera (Unknown status). Positive-negative results from cut-off value of PCV2b-based ELISA and given antibody titer of IPMA were compared to find level of agreement between 2 tests. All materials and methods were done as same as optimal procedure of both tests.

### 3.3.5 Correlation Coefficient Test

For finding correlation between PCV2b-based ELISA and IPMA, 20 PCV2-positive sera (PCR positive) were used by comparing antibody titer of IPMA ($log_{10}$) and OD values of ELISA. All positive sera were performed serial 2-fold dilution ranged from 1:30 – 1:1920 in both tests. By the way, other materials and methods were done as same as optimal procedure of both tests. End point of antibody titer (last dilution of serum which still positive) of each serum was recorded together with OD values of ELISA at given dilution.

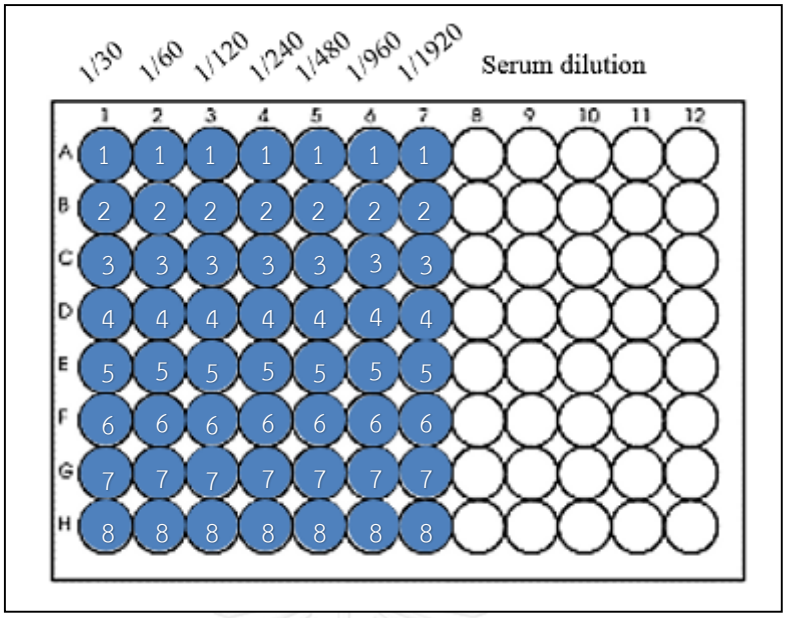


**Figure 17.** Twenty sera (8 sera per plate) were performed serial 2-fold dilution of serum for both assays (IPMA and ELISA) ranged from 1:30-1:1920.

### 3.3.6 Statistical Analysis

For data analysis, MedCalc® software was used. Comparative agreement of IPMA and ELISA was determined by Kappa statistic. Correlation between IPMA (Antibody titers) and ELISA (OD values) was determined by Spearman correlation coefficient test. Percent of coefficient of variation (%CV) was analyzed by Microsoft Excel® software.

# CHAPTER IV

# RESULTS

## 4.1 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

The result of PCR products which were positive to PCV2 at 802 bp was shown in Figure 18. The samples were derived from selection of clinical signs of PCV2 and compared to positive and negative samples. 9 of 10 Samples that were positive against PCV2 by performing PCR were sent to do sequencing and phylogenetic analysis.

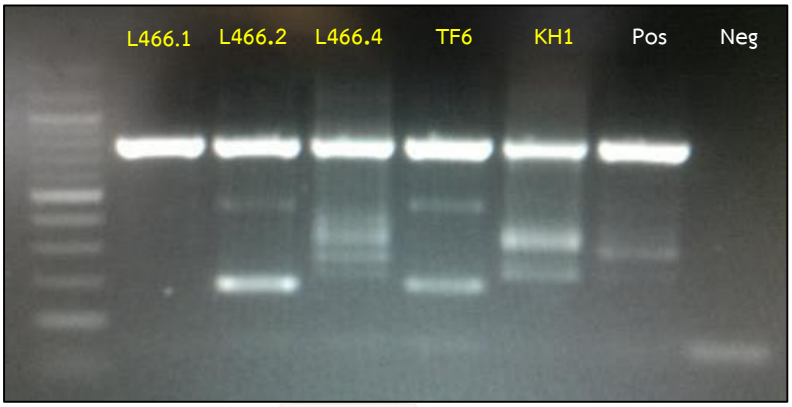


**Figure 18.** Result of positive PCR product at 802 bp

## 4.2 Sequencing and Phylogenetic Analysis

After performing sequencing and phylogenetic analysis, 5 of 9 laboratory strains were found to be subtype b of PCV2 and other 4 laboratory strains were subtype d of PCV2. The results were shown as Table 6 and Figure 19.

**Table 6.** Results of phylogenetic analysis showing subtypes of PCV2

| Accession number/Name | Subtype (cluster) | Country | Note |
|---|---|---|---|
| L466.1 | PCV2b (PCV2b 1A/B) | Thailand | Laboratory |
| L466.2 | PCV2b (PCV2b 1A/B) | Thailand | Laboratory |
| L466.4 | PCV2b (PCV2b 1A/B) | Thailand | Laboratory |
| TF6 | PCV2b (PCV2b 1A/B) | Thailand | Laboratory |
| KH_1 | PCV2b (PCV2b 1A/B) | Thailand | Laboratory |
| KH_2 | PCV2d (PCV2b 1C) | Thailand | Laboratory |
| KH_3 | PCV2d (PCV2b 1C) | Thailand | Laboratory |

**Table 6.** Results of phylogenetic analysis showing subtypes of PCV2 (Cont.)

| | | | |
|---|---|---|---|
| KH_4 | PCV2d (PCV2b 1C) | Thailand | Laboratory |
| KH_5 | PCV2d (PCV2b 1C) | Thailand | Laboratory |

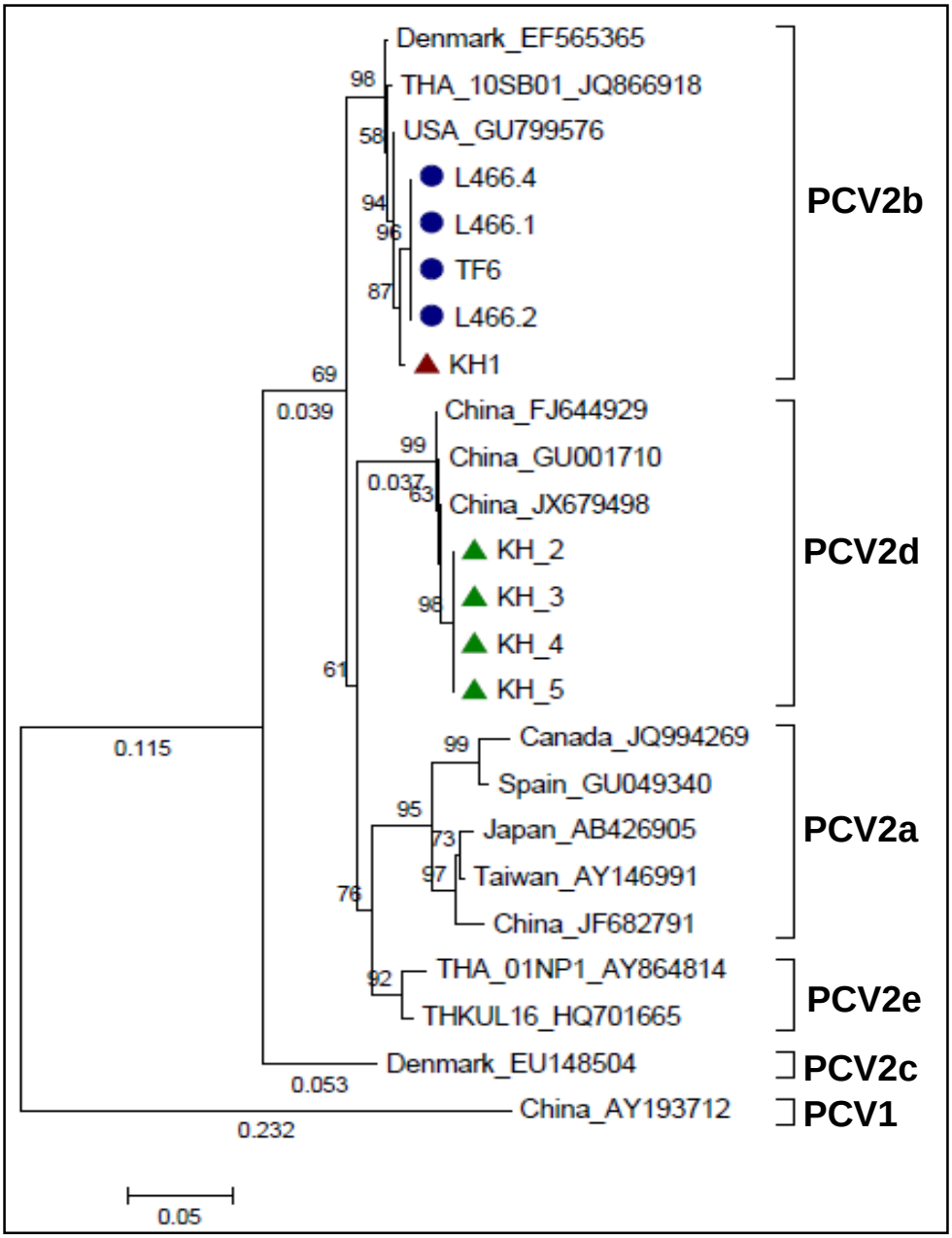


**Figure 19.** Phylogenetic tree analysis of overall 24 strains including 9 laboratory strains and 15 reference strains from GenBank

### 4.3 PCV2 Inoculation (IFAT)

Five positive samples confirmed as PCV2 subtype b were chosen and inoculated in SST-SW1 cell. Indirect fluorescent antibody technique (IFAT) was used and the result was shown as Table 7 and Figure 20. Level of PCV2 infection was ranged from +1 to +5 (mild to severe infection) so that L466.2 was selected with +5 severity.

**Table 7.** Result of PCV2 positive samples by using IFAT and scoring system

| Samples | Results |
|---|---|
| L466.1 | +4 |
| L466.2 | +5 |
| L466.4 | +3 |
| TF6 | +1 |
| KH1 | +2 |

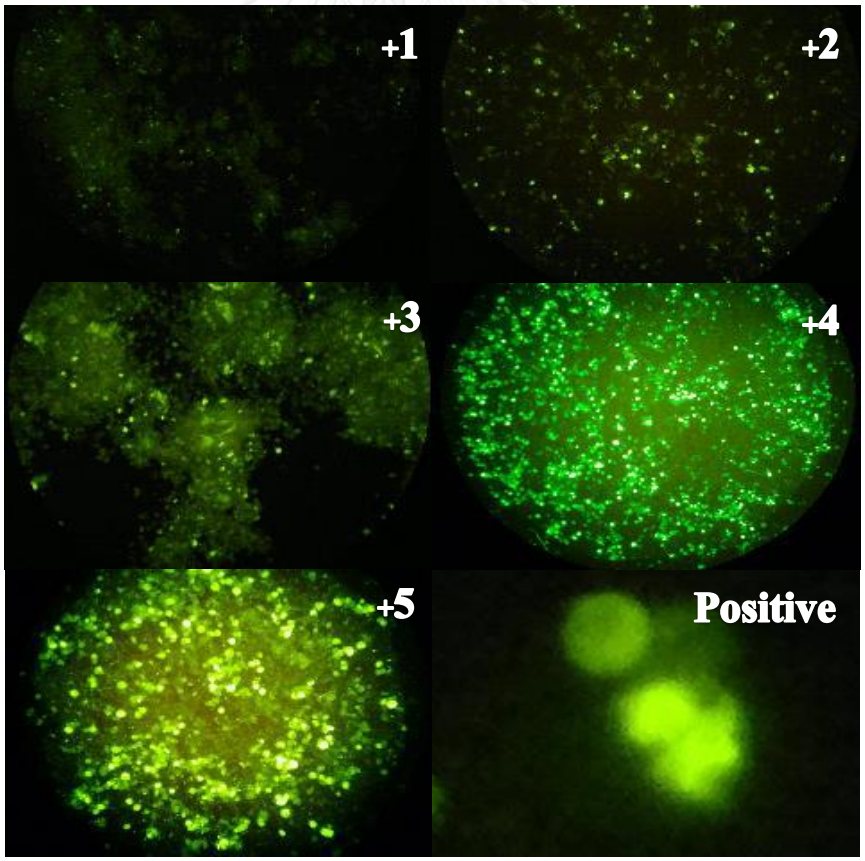


**Figure 20.** Degree of apple-green color glowing of infected cells with fluorescence microscope

### 4.4 Viral Titration

Positive sample named "L466.2" was chosen due to highest level of infection (IFAT). Chosen sample was selected to titrate and calculate by Reed & Muench method to know the concentration of stock virus. The result was shown as Figure 21.

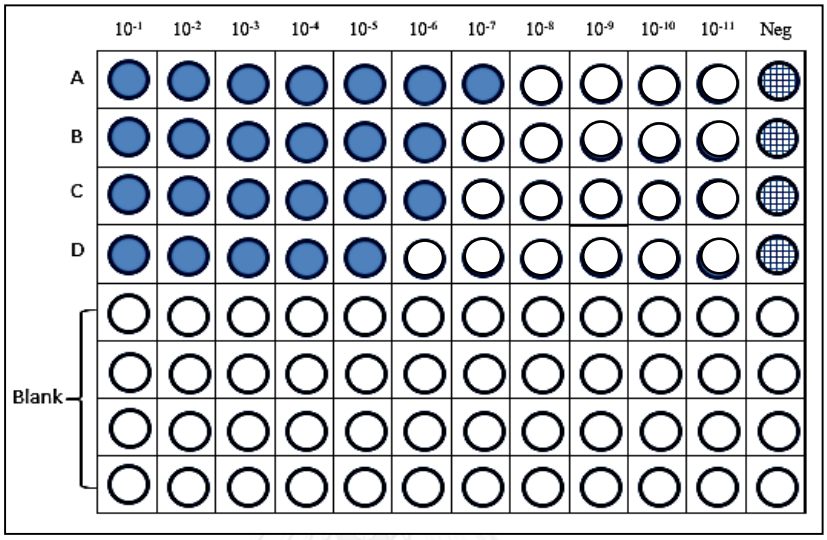


**Figure 21.** Result of viral titration showing CPE of each well

**Table 8.** Result of viral titration showing CPE and calculation of viral concentration

| Dilution of virus | Observed results | | | | Accumulation results | | | |
|---|---|---|---|---|---|---|---|---|
| | CPE | No CPE | Ratio | % | CPE | No CPE | Ratio | % |
| 10-5 | 4 | 0 | 4/4 | 100 | 7 | 0 | 7/7 | 100 |
| 10-6 | 3 | 1 | 3/4 | 80 | 4 | 1 | 4/5 | 80 |
| 10-7 | 1 | 3 | 1/4 | 20 | 1 | 4 | 1/5 | 20 |
| 10-8 | 0 | 4 | 0/4 | 0 | 0 | 7 | 0/7 | 0 |

***Calculation:*** Proportionate distance (PD) = A-50/A-B

$= (80-50) / (80-20) = 0.5$

$\log TCID_{50} = (\log C) - (PD \times \log X)$

$= (-6) - (0.5 \times \log 10) = -6.5$

$1TCID_{50}/\text{Volume (Stock virus)} = \text{Dilution } 10^{\log TCID_{50}}$

$TCID_{50} = 10^{6.5} \times 50\ \mu l = 5 \times 10^{7.5}$

Concentration of Stock virus = $5 \times 10^{7.5}$ $TCID_{50}$/ml

### 4.5 Quantification of PCV2b antigen

Bradford dye-binding method was used by comparing absorbance of samples with standard curve of BSA (Figure 22). Concentration of protein/coating antigen were shown in Table 9. Sample "40" was selected due to highest concentration (1.87 µg/ml).

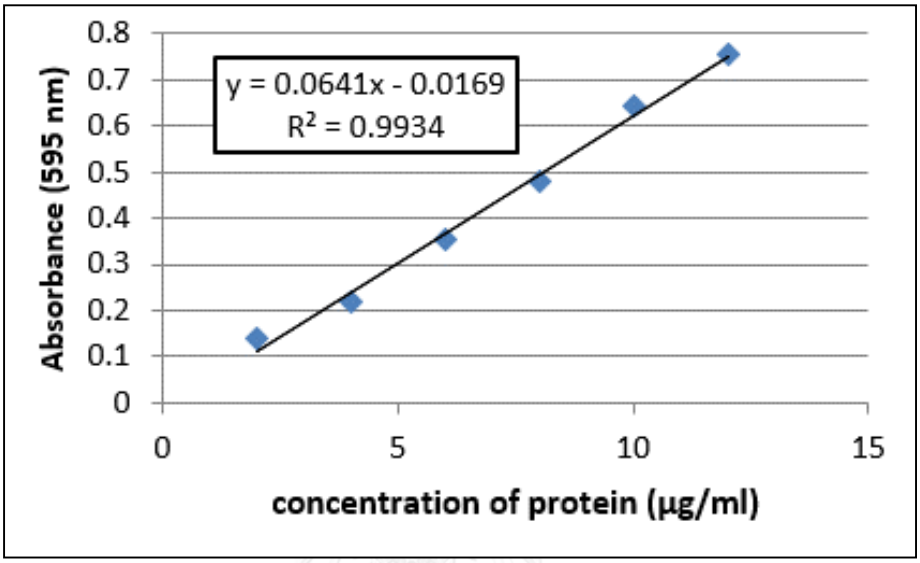


**Figure 22.** Standard curve showing absorbance of BSA of each dilution

**Table 9.** Concentration of protein (coating antigen) of each sample

| X (µg/ml) | Y (Abs) | Sample | Abs | Protein (µg/ml) |
|---|---|---|---|---|
| 2 | 0.138 | UR | 1.242 | 19.64 |
| 4 | 0.218 | S | 0 | 0.26 |
| 6 | 0.355 | 10 | 0.047 | 1.00 |
| 8 | 0.481 | 20 | 0.069 | 1.34 |
| 10 | 0.643 | 40 | 0.103 | 1.87 |
| 12 | 0.755 | 60 | 0.071 | 1.37 |

### 4.6 Optimization of ELISA

Checker board titration technique was performed with antigen titration, sera and conjugate titration and binding ratios. The results were shown in Table 10-14. The yellow color indicated that there was a color development (OD>0.2).

**Table 10**. OD value of antigen titration of PCV2 positive serum

| Ag / Serum | 50 | 100 | 200 | 400 | 800 | 1600 | 3200 | 6400 | 12800 | 25600 | 51200 | - |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30 | 3.95 | 3.93 | 3.85 | 3.50 | 2.66 | 1.99 | 1.57 | 1.45 | 1.34 | 1.30 | 1.29 | 1.33 |
| 60 | 3.99 | 3.90 | 3.73 | 2.69 | 2.04 | 1.42 | 1.17 | 1.03 | 0.97 | 0.98 | 0.92 | 0.97 |
| 120 | 3.96 | 3.73 | 2.94 | 1.95 | 1.30 | 0.92 | 0.77 | 0.67 | 0.60 | 0.60 | 0.60 | 0.66 |
| 240 | 3.90 | 3.19 | 2.34 | 1.40 | 0.95 | 0.62 | 0.53 | 0.45 | 0.41 | 0.42 | 0.42 | 0.42 |
| 480 | 3.65 | 2.44 | 1.56 | 0.96 | 0.60 | 0.40 | 0.34 | 0.29 | 0.27 | 0.26 | 0.26 | 0.28 |
| 960 | 3.10 | 1.86 | 1.15 | 0.65 | 0.41 | 0.28 | 0.22 | 0.19 | 0.18 | 0.17 | 0.17 | 0.19 |
| 1920 | 2.32 | 1.31 | 0.82 | 0.49 | 0.31 | 0.22 | 0.18 | 0.15 | 0.14 | 0.14 | 0.12 | 0.18 |
| - | 0.08 | 0.07 | 0.07 | 0.09 | 0.06 | 0.08 | 0.06 | 0.06 | 0.07 | 0.06 | 0.06 | 0.05 |

(Ag: 1:50-1:51200, Serum: 1:30-1:1920)

**Table 11.** OD value of antigen titration of PCV2 negative serum

| Ag / Serum | 50 | 100 | 200 | 400 | 800 | 1600 | 3200 | 6400 | 12800 | 25600 | 51200 | - |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30 | 0.20 | 0.17 | 0.18 | 0.17 | 0.18 | 0.19 | 0.18 | 0.17 | 0.17 | 0.17 | 0.17 | 0.21 |
| 60 | 0.16 | 0.12 | 0.13 | 0.14 | 0.13 | 0.14 | 0.13 | 0.12 | 0.15 | 0.14 | 0.13 | 0.15 |
| 120 | 0.15 | 0.15 | 0.13 | 0.14 | 0.13 | 0.14 | 0.14 | 0.13 | 0.14 | 0.13 | 0.11 | 0.14 |
| 240 | 0.11 | 0.10 | 0.09 | 0.09 | 0.09 | 0.09 | 0.08 | 0.09 | 0.09 | 0.10 | 0.09 | 0.11 |
| 480 | 0.13 | 0.12 | 0.11 | 0.11 | 0.11 | 0.10 | 0.12 | 0.11 | 0.11 | 0.11 | 0.13 | 0.19 |
| 960 | 0.18 | 0.16 | 0.14 | 0.15 | 0.16 | 0.15 | 0.17 | 0.16 | 0.16 | 0.16 | 0.15 | 0.24 |
| 1920 | 0.12 | 0.10 | 0.09 | 0.10 | 0.09 | 0.11 | 0.10 | 0.10 | 0.11 | 0.11 | 0.11 | 0.12 |
| - | 0.14 | 0.11 | 0.10 | 0.12 | 0.10 | 0.11 | 0.10 | 0.12 | 0.12 | 0.11 | 0.12 | 0.13 |

(Ag: 1:50-1:51200, Serum: 1:30-1:1920)

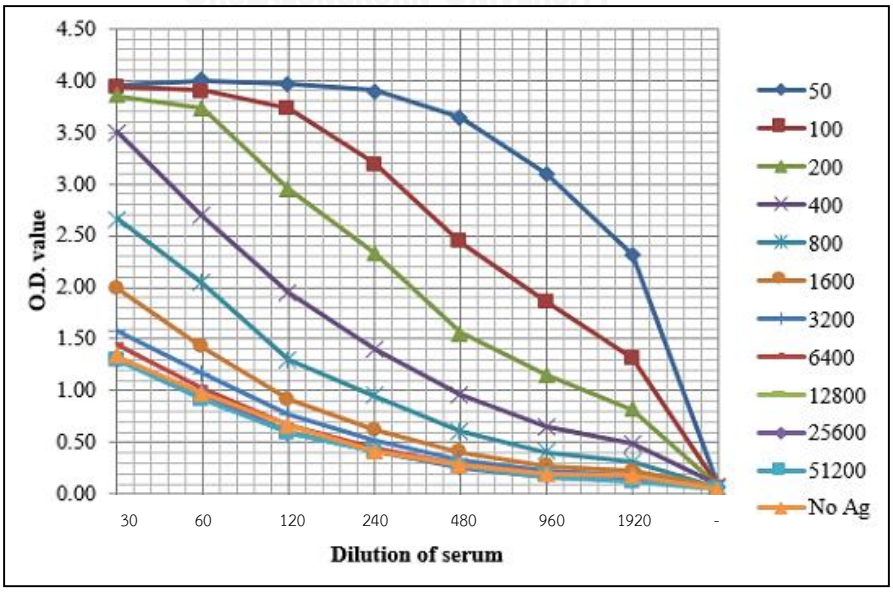


**Figure 23**.Graph relating OD value with dilution of antigen against serum

**Table 12**. OD value of sera and conjugate titration of PCV2 positive serum

| Serum / Conjugate | 30 | 60 | 120 | 240 | 480 | 960 | 1920 | 3840 | 7680 | 15360 | 30720 | - |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 500 | 3.96 | 3.90 | 3.97 | 3.88 | 3.74 | 3.26 | 2.26 | 1.46 | 0.64 | 0.42 | 0.28 | 0.20 |
| 1000 | 3.94 | 3.89 | 3.74 | 3.49 | 2.64 | 1.91 | 1.28 | 0.74 | 0.34 | 0.25 | 0.16 | 0.16 |
| 2000 | 3.79 | 3.53 | 3.03 | 2.51 | 1.63 | 1.10 | 0.71 | 0.44 | 0.19 | 0.15 | 0.11 | 0.11 |
| 4000 | 2.57 | 2.34 | 1.79 | 1.45 | 0.92 | 0.62 | 0.40 | 0.25 | 0.12 | 0.09 | 0.07 | 0.06 |
| 8000 | 1.55 | 1.34 | 1.09 | 0.78 | 0.53 | 0.34 | 0.22 | 0.15 | 0.08 | 0.07 | 0.06 | 0.06 |
| 16000 | 0.95 | 0.80 | 0.64 | 0.46 | 0.30 | 0.21 | 0.14 | 0.10 | 0.07 | 0.06 | 0.06 | 0.05 |
| 32000 | 0.54 | 0.46 | 0.35 | 0.26 | 0.18 | 0.13 | 0.10 | 0.08 | 0.06 | 0.05 | 0.05 | 0.05 |
| - | 0.32 | 0.27 | 0.21 | 0.16 | 0.12 | 0.09 | 0.07 | 0.06 | 0.05 | 0.05 | 0.05 | 0.05 |

(Serum: 1:30-1:30720, Conjugate: 1:500-1:32000)

**Table 13.** OD value of sera and conjugate titration of PCV2 negative serum

| Serum / Conjugate | 30 | 60 | 120 | 240 | 480 | 960 | 1920 | 3840 | 7680 | 15360 | 30720 | - |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 500 | 0.17 | 0.14 | 0.11 | 0.10 | 0.09 | 0.10 | 0.09 | 0.10 | 0.10 | 0.11 | 0.10 | 0.23 |
| 1000 | 0.13 | 0.12 | 0.09 | 0.10 | 0.08 | 0.10 | 0.11 | 0.10 | 0.09 | 0.14 | 0.09 | 0.11 |
| 2000 | 0.11 | 0.08 | 0.07 | 0.06 | 0.06 | 0.06 | 0.06 | 0.07 | 0.07 | 0.08 | 0.07 | 0.09 |
| 4000 | 0.08 | 0.06 | 0.06 | 0.05 | 0.06 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| 8000 | 0.06 | 0.06 | 0.06 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| 16000 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| 32000 | 0.05 | 0.05 | 0.05 | 0.07 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| - | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |

(Serum: 1:30-1:30720, Conjugate: 1:500-1:32000)

**Table 14**. Binding ratios were calculated from OD value of Table 12 (positive) divided by OD value of table 13 (negative) with same dilution.

| Serum / Conjugate | 30 | 60 | 120 | 240 | 480 | 960 | 1920 | 3840 | 7680 | 15360 | 30720 | - |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 500 | 22.8 | 28.9 | 37.6 | 40.7 | 41.9 | 31.3 | 24.3 | 14.5 | 6.6 | 3.7 | 2.8 | 0.9 |
| 1000 | 30.3 | 32.1 | 43.4 | 35.5 | 31.5 | 19.2 | 11.6 | 7.1 | 3.7 | 1.8 | 1.8 | 1.5 |
| 2000 | 36.1 | 43.7 | 41.0 | 40.9 | 25.6 | 17.3 | 10.9 | 6.1 | 2.6 | 1.9 | 1.5 | 1.2 |
| 4000 | 30.8 | 38.1 | 31.0 | 28.9 | 15.7 | 12.5 | 8.3 | 4.9 | 2.5 | 1.8 | 1.5 | 1.2 |
| 8000 | 24.0 | 24.0 | 19.3 | 15.7 | 9.8 | 6.9 | 4.6 | 3.1 | 1.7 | 1.4 | 1.2 | 1.1 |
| 16000 | 17.5 | 16.0 | 13.1 | 9.3 | 6.1 | 4.2 | 2.7 | 1.9 | 1.3 | 1.3 | 1.2 | 1.1 |
| 32000 | 10.7 | 9.3 | 7.2 | 3.7 | 3.8 | 2.7 | 2.2 | 1.6 | 1.2 | 1.1 | 1.1 | 1.2 |
| - | 6.3 | 5.5 | 4.5 | 3.4 | 2.5 | 1.8 | 1.5 | 1.3 | 1.2 | 1.1 | 1.0 | 1.0 |

(Serum: 1:30-1:30720, Conjugate: 1:500-1:32000)

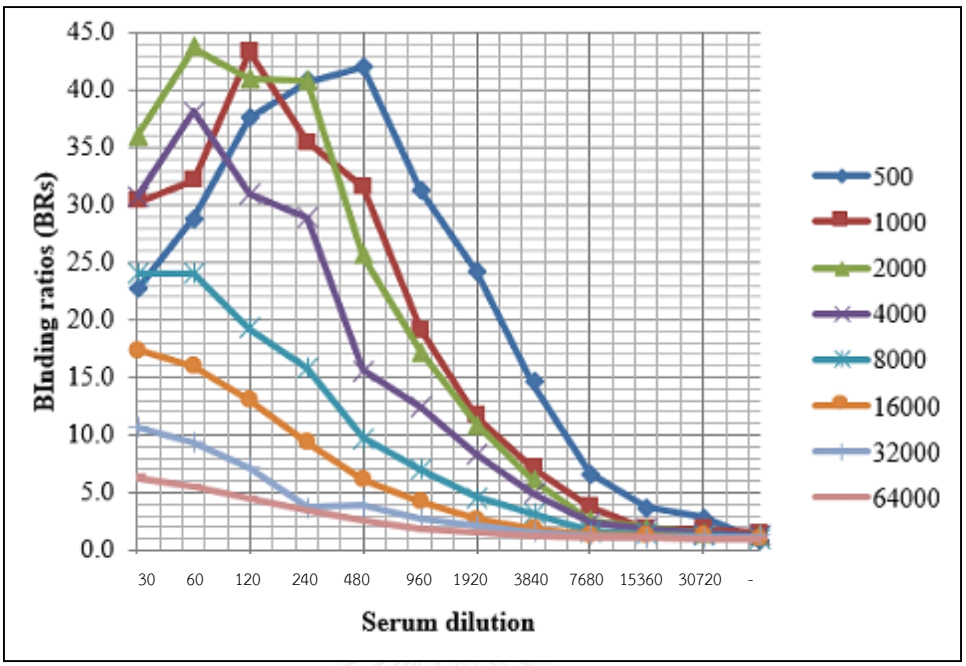


**Figure 24.** Graph relating binding ratios with dilution of conjugate against serum

## 4.7 Immunoperoxidase Monolayer Assay (IPMA)

There was a color development from clear to red color in which positive sera were added to corresponding wells (Figure 25). PCV2 infected cells were shown in Figure 26.

**Figure 25.** Color development of infected SST-SW1 cell from nothing to red color (Positive serum)

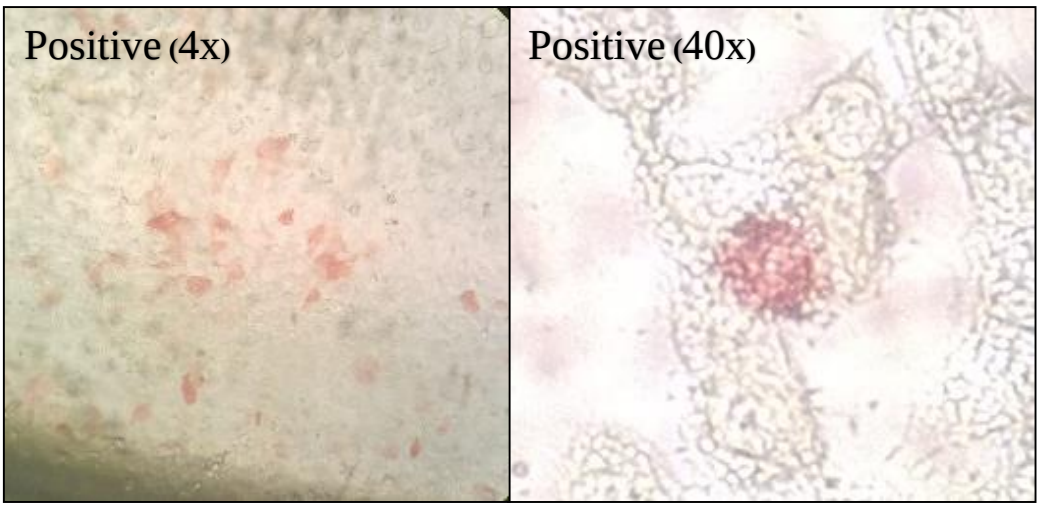


**Figure 26.** PCV2 infected SST-SW1 cells in which positive serum was added. A clear view of picture used magnification of inverted microscope at 40x (Left) and 4x (Right).

### 4.8 Validation of Indirect ELISA

#### 4.8.1 ROC Analysis and Cut-Off Determination

From total of 100 tested sera, 46 sera were positive and 54 sera were negative by this ELISA. The area under the curve (AUC) from ROC analysis was 0.994 ± 0.00414 SE ($p < 0.0001$) (Figure 27, Table 15). The optimal cut-off value was set at 0.39 ($OD_{450}$) with diagnostic sensitivity, specificity and accuracy of 92%, 100%, 96% respectively (Table 16). According to cut-off value, OD value higher than 0.39 was classified as positive against PCV2 antibodies (Figure 28). Positive and negative predictive values were calculated as 100% and 92.59%, respectively and prevalence of the disease was 50%.

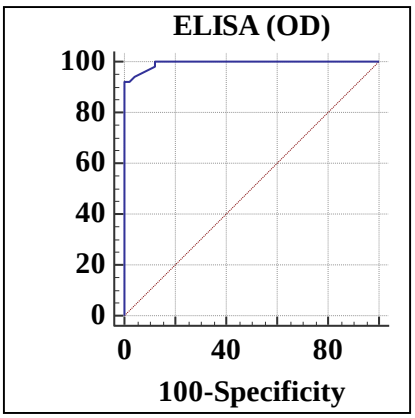


**Figure 27.** Area under curve (AUC) from ROC analysis of ELISA

**Table 15.** Area under the ROC curve (AUC)

| **Area under the ROC curve (AUC)** | **0.994** |
|---|---|
| Standard Error [a] | 0.00414 |
| 95% Confidence interval [b] | 0.952 to 1.000 |
| z statistic | 119.327 |
| Significance level P (Area=0.5) | <0.0001 |

[a] DeLong et al., 1988

[b] Binomial exact

**Table 16.** Criterion values and coordinates of the ROC curve

| Criterion | Sensitivity | 95% CI | Specificity | 95% CI | +LR | -LR |
|---|---|---|---|---|---|---|
| ≥0.14 | 100.00 | 92.9 - 100.0 | 0.00 | 0.0 - 7.1 | 1.00 | |
| >0.31 | 100.00 | 92.9 - 100.0 | 88.00 | 75.7 - 95.5 | 8.33 | 0.00 |
| >0.32 | 98.00 | 89.4 - 99.9 | 88.00 | 75.7 - 95.5 | 8.17 | 0.023 |
| >0.35 | 94.00 | 83.5 - 98.7 | 96.00 | 86.3 - 99.5 | 23.50 | 0.063 |
| >0.37 | 92.00 | 80.8 - 97.8 | 98.00 | 89.4 - 99.9 | 46.00 | 0.082 |
| >0.39 | 92.00 | 80.8 - 97.8 | 100.00 | 92.9 - 100.0 | | 0.080 |
| >1.69 | 0.00 | 0.0 - 7.1 | 100.00 | 92.9 - 100.0 | | 1.00 |

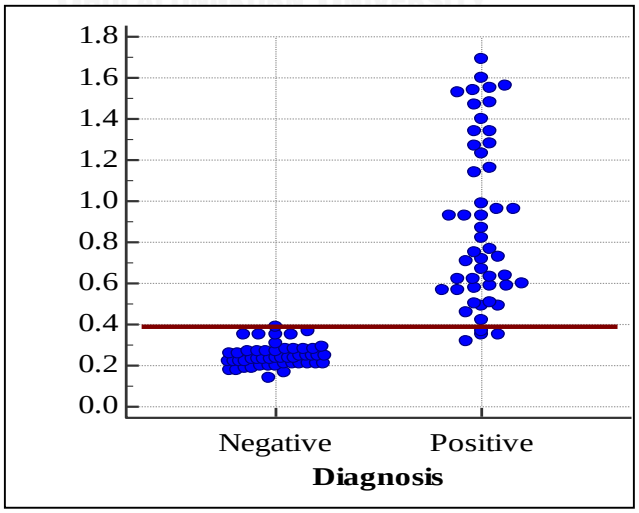


**Figure 28**. Distribution of ELISA OD values from 50 PCV2-negative sera and 50 PCV2-positive sera. Cut-off OD value was set at 0.39.

### 4.9 Repeatability Test

Repeatability test showed %CV of intra-plate of negative samples (n=10) ranged from 0.47-11.27% with median value of 3.62% and positive samples (n=10) ranged from 2.9-10.58 % with median value of 6.21%. For %CV of inter-plate of negative samples (n=10) ranged from 1.01-23% with median value of 7.16% and positive samples ranged from 2.27-19.62% with median value of 6.34%. Data were shown in Table 17-20.

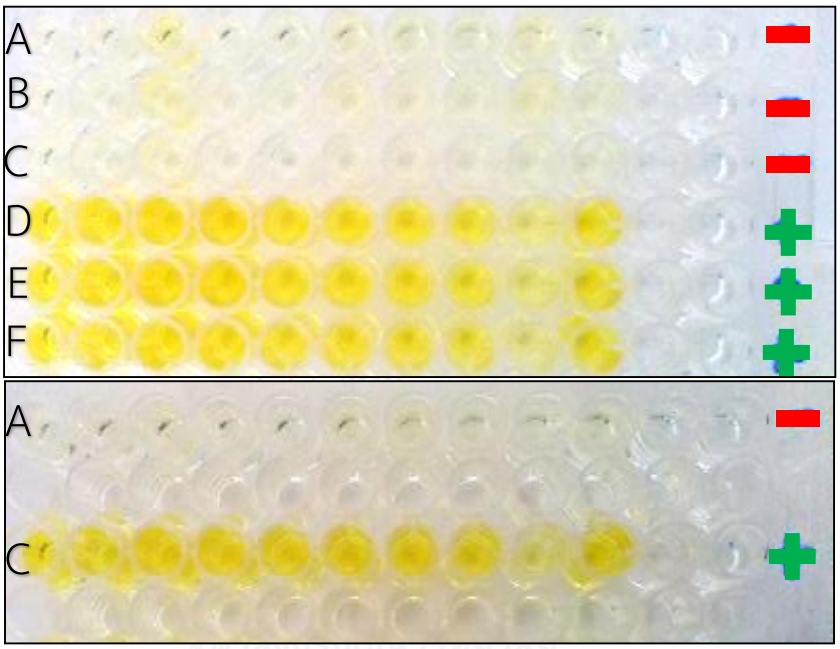


**Figure 29.** Color development of repeatability test from clear to yellow color after adding stop solution of both intra-plate and inter-plate.

**Table 17.** Repeatability test of intra-plate of negative samples showing mean OD, SD and %CV of each sample (3 replicates/sample)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Row A | 0.15 | 0.16 | 0.42 | 0.13 | 0.15 | 0.22 | 0.20 | 0.17 | 0.26 | 0.18 |
| Row B | 0.15 | 0.16 | 0.38 | 0.13 | 0.14 | 0.22 | 0.18 | 0.17 | 0.26 | 0.18 |
| Row C | 0.15 | 0.14 | 0.34 | 0.12 | 0.14 | 0.23 | 0.19 | 0.17 | 0.25 | 0.16 |
| **Mean OD** | 0.15 | 0.15 | 0.38 | 0.13 | 0.14 | 0.22 | 0.19 | 0.17 | 0.25 | 0.17 |
| **SD** | 0.00 | 0.01 | 0.04 | 0.01 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.01 |
| **%CV** | 0.89 | 6.17 | 11.27 | 5.86 | 1.80 | 2.11 | 5.05 | 0.47 | 2.19 | 5.79 |

**Table 18**. Repeatability test of intra-plate of positive samples showing mean OD, SD and %CV of each sample (3 replicates/sample)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Row D | 1.88 | 2.29 | 2.64 | 2.65 | 1.75 | 1.72 | 1.51 | 1.25 | 0.53 | 1.73 |
| Row E | 1.73 | 2.25 | 2.59 | 2.65 | 1.60 | 1.65 | 1.44 | 1.21 | 0.50 | 1.67 |
| Row F | 1.68 | 1.93 | 2.45 | 2.19 | 1.52 | 1.60 | 1.44 | 1.10 | 0.45 | 1.59 |
| **Mean OD** | 1.76 | 2.16 | 2.56 | 2.49 | 1.62 | 1.66 | 1.46 | 1.18 | 0.49 | 1.66 |
| **SD** | 0.10 | 0.20 | 0.10 | 0.26 | 0.12 | 0.06 | 0.04 | 0.08 | 0.04 | 0.07 |
| **%CV** | 5.85 | 9.20 | 3.82 | 10.58 | 7.35 | 3.74 | 2.90 | 6.57 | 8.68 | 4.25 |

**Table 19.** Repeatability test of inter-plate of negative samples showing mean OD, SD and %CV of each sample (3 replicates/sample)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Row A1 | 0.15 | 0.14 | 0.24 | 0.12 | 0.10 | 0.16 | 0.15 | 0.12 | 0.15 | 0.14 |
| Row A2 | 0.13 | 0.12 | 0.16 | 0.12 | 0.09 | 0.14 | 0.14 | 0.11 | 0.11 | 0.14 |
| Row A3 | 0.13 | 0.13 | 0.16 | 0.11 | 0.09 | 0.14 | 0.14 | 0.11 | 0.11 | 0.13 |
| **Mean OD** | 0.14 | 0.13 | 0.19 | 0.12 | 0.09 | 0.14 | 0.14 | 0.11 | 0.12 | 0.14 |
| **SD** | 0.01 | 0.01 | 0.04 | 0.00 | 0.01 | 0.01 | 0.00 | 0.00 | 0.02 | 0.00 |
| **%CV** | 9.98 | 7.30 | 23.00 | 2.45 | 8.61 | 7.02 | 1.01 | 3.84 | 18.99 | 2.67 |



**Table 20.** Repeatability test of inter-plate of positive samples showing mean OD, SD and %CV of each sample (3 replicates/sample)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Row C1 | 1.68 | 1.93 | 2.45 | 2.19 | 1.75 | 1.72 | 1.44 | 1.10 | 0.45 | 1.73 |
| Row C2 | 1.81 | 1.70 | 2.19 | 2.07 | 1.83 | 1.71 | 1.93 | 1.51 | 0.49 | 1.85 |
| Row C3 | 1.79 | 1.73 | 2.29 | 1.97 | 1.78 | 1.88 | 1.37 | 1.09 | 0.40 | 1.47 |
| **Mean OD** | 1.76 | 1.79 | 2.31 | 2.08 | 1.79 | 1.77 | 1.58 | 1.23 | 0.45 | 1.68 |
| **SD** | 0.07 | 0.12 | 0.13 | 0.11 | 0.04 | 0.10 | 0.31 | 0.24 | 0.04 | 0.19 |
| **%CV** | 4.01 | 6.99 | 5.68 | 5.29 | 2.27 | 5.44 | 19.36 | 19.62 | 9.75 | 11.44 |

**Table 21.** Repeatability test showed %CV with median value of each protocol which calculated from mean OD and SD

| PCV2b-ELISA | Intra-plate | | Inter-plate | |
|---|---|---|---|---|
| **Sample** | Negative | Positive | Negative | Positive |
| **%CV** | 0.47-11.27 | 2.9-10.58 | 1.01-23 | 2.27-19.62 |
| **Median** | 3.62 | 6.21 | 7.16 | 6.34 |

**4.10 Comparative Agreement of Indirect ELISA and IPMA**

The observed agreement between indirect PCV2b-based ELISA and IPMA with 657 field sera was 89.34%. Kappa value (Linear weighted kappa) was 0.757 with standard error at 0.027. Data were shown in table 22.

**Table 22.** Comparative agreement between IPMA and indirect PCV2b-based ELISA by using 657 field sera

| PCV2b-ELISA | IPMA | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 409 | 45 | 454 (69.1%) |
| Negative | 25 | 178 | 203 (30.9%) |
| **Total** | 434 (66.1%) | 223 (33.9%) | 657 |

**Figure 30.** Comparison of color development between indirect ELSIA (Left) and IPMA (Right). Color development of indirect ELISA changed from nothing to blue (Before adding stop solution)

### 4.11 Correlation Coefficient Test

Twenty positive samples were ranged from dilution of serum at 1:30 – 1:1920 to determine the relationship between end point antibody titer ($Log_{10}$) of IPMA (picture not shown) and OD values of last serum dilution giving positive result (Figure 31). Correlation coefficient test between indirect PCV2b-based ELISA and IPMA resulted in Spearman correlation of coefficient (r) at 0.74 ($p < 0.001$).

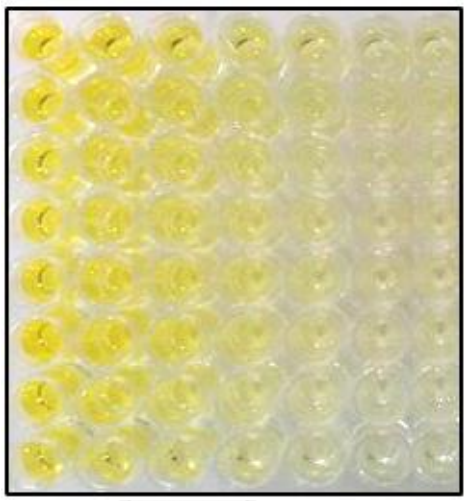

**Figure 31.** Color development of ELISA from correlation coefficient test

**Table 23.** Correlation coefficient test showing end point antibody titer ($Log_{10}$) of IPMA and end point OD value of ELISA of each sample

| Sample | Serum dilution | | | | | | | Endpoint Antibody titer ($log_{10}$) | ODValue |
|---|---|---|---|---|---|---|---|---|---|
| | 30 | 60 | 120 | 240 | 480 | 960 | 1920 | | |
| | 1.48 | 1.78 | 2.08 | 2.38 | 2.68 | 2.98 | 3.28 | | |
| 1 | 3.73 | 3.51 | 2.96 | 2.44 | 1.68 | 1.10 | 0.62 | 3.28 | 0.62 |
| 2 | 3.67 | 3.30 | 2.61 | 1.96 | 1.31 | 0.84 | 0.34 | 2.98 | 0.84 |
| 3 | 3.84 | 3.66 | 3.24 | 2.63 | 1.84 | 1.30 | 0.87 | 3.28 | 0.87 |
| 4 | 3.80 | 3.51 | 3.25 | 2.72 | 2.21 | 1.68 | 1.11 | 3.28 | 1.11 |
| 5 | 3.74 | 3.52 | 3.09 | 2.46 | 1.94 | 1.45 | 0.94 | 3.28 | 0.94 |
| 6 | 3.47 | 3.06 | 2.48 | 1.78 | 1.18 | 0.73 | 0.12 | 2.98 | 0.73 |
| 7 | 3.15 | 2.43 | 1.78 | 1.18 | 0.76 | 0.60 | 0.33 | 2.98 | 0.60 |
| 8 | 3.55 | 3.10 | 2.54 | 2.00 | 1.57 | 0.91 | 0.31 | 2.98 | 0.91 |
| 9 | 3.49 | 3.03 | 2.43 | 1.88 | 1.20 | 0.85 | 0.26 | 2.98 | 0.85 |
| 10 | 3.32 | 2.67 | 2.01 | 1.41 | 0.94 | 0.60 | 0.31 | 2.98 | 0.60 |

**Table 23.** Correlation coefficient test showing end point antibody titer ($Log_{10}$) of IPMA and end point OD value of ELISA of each sample (Cont.)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 11 | 0.73 | 0.47 | 0.30 | 0.20 | 0.16 | 0.14 | 0.12 | 1.78 | 0.47 |
| 12 | 2.58 | 1.99 | 1.44 | 1.02 | 0.68 | 0.55 | 0.24 | 2.98 | 0.55 |
| 13 | 1.20 | 0.68 | 0.44 | 0.29 | 0.20 | 0.18 | 0.10 | 2.08 | 0.44 |
| 14 | 1.35 | 1.00 | 0.71 | 0.49 | 0.29 | 0.20 | 0.11 | 2.38 | 0.49 |
| 15 | 1.51 | 0.99 | 0.76 | 0.49 | 0.34 | 0.21 | 0.16 | 2.38 | 0.49 |
| 16 | 1.20 | 0.77 | 0.49 | 0.33 | 0.23 | 0.17 | 0.12 | 2.08 | 0.49 |
| 17 | 2.80 | 2.32 | 1.55 | 1.08 | 0.70 | 0.60 | 0.32 | 2.98 | 0.60 |
| 18 | 0.82 | 0.51 | 0.33 | 0.22 | 0.16 | 0.14 | 0.11 | 1.78 | 0.51 |
| 19 | 2.47 | 1.78 | 1.27 | 0.77 | 0.49 | 0.31 | 0.16 | 2.68 | 0.49 |
| 20 | 1.16 | 0.70 | 0.44 | 0.30 | 0.20 | 0.15 | 0.10 | 2.08 | 0.44 |

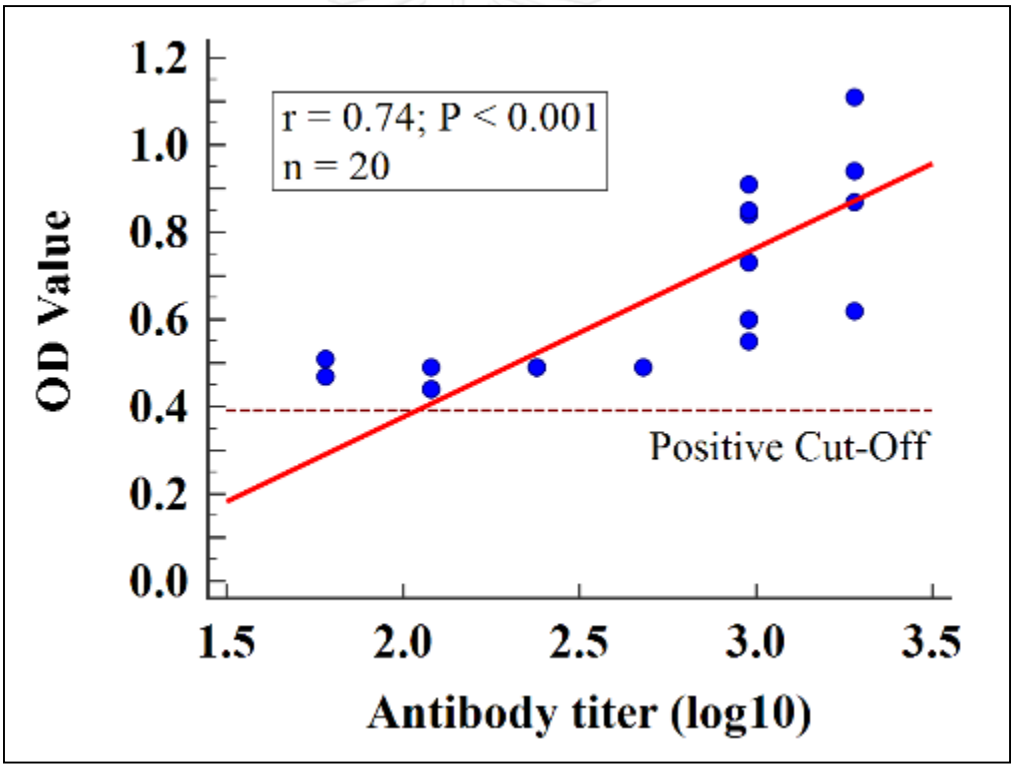


**Figure 32.** Graph showing relation between end point OD value of ELISA and end point antibody titer ($Log_{10}$) of IPMA and calculation of Spearman correlation coefficient (r) with positive cut-off at 0.39.

# CHAPTER V
# DISCUSSION

Many samples (n=10) collected from pigs with clinical signs of PMWS or PDNS were performed using PCR to detect the PCV2b viral agents and confirm positive or negative. Samples with giving significantly high PCR products at 802 bp were sent to do the sequencing and the phylogenetic analysis. Our results found that 5 of 9 laboratory strains were identified as PCV2 subtype b including L466.1, L466.2, L466.4, TF6 and KH_1 (Table 6 and Figure 19). From phylogenetic analysis, it indicated that positive samples defined as PCV2 subtype b could be distinguished from other reference strains derived from Genbank with genetic distance more than 0.035 (0.039) (Grau-Roma et al., 2008). Thus, primers and conditions (Takahagi et al., 2008) using in this study which focused on ORF2 of PCV2 could be used to confirm the result of suspicious samples and submit to do sequencing and phylogenetic analysis of PCV2. Nevertheless, 4 of 9 laboratory strains in this study that identified as PCV2 subtype d should not be abandoned. In spite of the fact that prevalence of PCV2 subtype d has increased and shifted from subtype b to d since 2012 according to previous study in Thailand (Buapaichit et al., 2013). However, PCV2 subtype d used to be in subtype b and 5 of 9 laboratory strains in this study belonged to subtype b which means PCV2 subtype b still remains the major subtype to be focused.

From the result of IFAT showing apple-green color glowing of infected cells with fluorescence microscope indicated that all positive samples provided sufficient results. SST-SW1 cell could be used for multiplication the virus from these positive samples but the level of virus titer and severity might depend on permissiveness of the cell to each individual type of PCV2. The PCV2 subtype b with highest severity would be chosen for coating antigen in further study. In this study, positive sample named "L466.2" was chosen due to highest level of infection by IFAT with the concentration of virus stock from sample named "L466.2" at $5 \times 10^{7.5}$ $TCID_{50}$/ml calculated by Reed & Muench method. However, percentage of identity of PCV2b sequences needs to be defined from the step of phylogenetic analysis because coating

antigen that was chosen in this study can be degraded in the future so that other samples of PCV2b antigen can be replaced and chosen to be alternative coating antigen.

After harvesting and purifying antigen with sucrose gradient technique. Sample named "40" which defined as coating antigen from sucrose concentration at 40% was chosen due to highest viral protein concentration as 1.87 µg/ml. In general, the in-house ELISA rely on ORF2 (Capsid protein) due to immunogenicity of ORF2 of PCV2 and yielding more amount of protein to be used as coating antigen. By the way, coating antigen of ELISA based on whole viral particle of PCV2b antigen was proposed as alternative to prepare and also provide high diagnostic performance (Nawagitgul et al., 2002). It is possible that coating antigen derived from this research would provide a good result. At any rate, other purifying techniques should be performed such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot with monoclonal antibodies in order to ensure the pure of coating antigen to increase more specificity of the test.

From the results of antigen titration in which positive and negative sera (Table 10, 11) were tested demonstrated that there was a good color development if there was sufficient for coating antigen binding with antibodies from serum. From Table 10, the color development could be seen up to column 5 and 6 (1:800 and 1:1600) throughout the column. However, the high OD value and color development could be seen in column 12 indicated that the tested serum had high background but it disappeared quickly due to insufficient of coating antigen. From Table 11, negative serum was used and found that there was lower color development due to no coating antigen binding with antibodies from serum but the color could be found at dilution of serum at 1: 30. This indicated that nonspecific background of the negative serum could alter this effect. Thus, selection of the best condition of dilution of antigen must be selected by using the last dilution of antigen in which giving good titration curve of positive serum (Figure 23). Dilution of antigen at 1:800 was selected due to high plateau, end point and height maxima with giving maximum OD about 2.0 and the color development still detected to row G.

Titration of sera and conjugate was performed (Table12, 13) and using the single dilution of antigen selected from the step of titration of antigen in which optimal dilution of antigen was set at 1:800. From table 12, titration of positive serum showed a good endpoint of antibodies from serum (OD value equal to OD of background) and conjugate also gave high background if it was too much in an optimal amount of conjugate. The endpoint of serum and conjugate could not be seen when using negative serum (Table 13) due to low background and no antibodies bound with coating antigen. In this study, maximum height plateau and titration endpoint from table 12 could be seen at dilution of conjugate at 1:1000-1:2000. To select the optimal sera and conjugate, binding ratios (BRs) between titration plates from table 12 (Positive serum) and table 13 (Negative serum) was applied by dividing the OD value of positive and negative serum at given dilution (Table 14 and Figure 24). As a result, BRs of conjugate dilution at 1:2000 provided optimal titration of conjugate due to maximum height plateau with highest P/N value (Figure 24). The optimal serum dilution was 1:120 due to the endpoint was seen at last dilution of conjugate (Table 12) and the BRs of last dilution of serum was still more than 1.0 (Table 14).

From IPMA result, SST-SW1 cells (lung cell derived from piglet) which inoculated with PCV2 pure culture were added with positive serum containing antibody providing color development. According to previous study (McNair et al., 2004), PK-15 cell line had been used instead of using SST-SW1 cells which performed in this study. This factor might affect the diagnostic performances of IPMA. But, only positive serum could affect the result in which antibodies from positive serum could bind with the PCV2 antigen derived from PCV2 pure culture so that monoclonal antibody used in this study was specific enough to give the good result. However, IPMA should be validated by comparing the effect from different cell culture.

According to the result of ROC analysis, AUC was high because the value was close to 1. Therefore, cut-off value at 0.39 ($OD_{450}$) could be used to differentiate positive and negative samples. Not only AUC was high, but its sensitivity, specificity and accuracy also was high with 92%, 100% and 96%, respectively. Despite of the fact that higher specificity (100%) was better in confirm of true negative samples. However, the sensitivity of 92% in this study were lower than previous ELISA studies with 98.2%

and 98.33% (Blanchard, 2003; Jittimanee et al., 2012), respectively. The lower in sensitivity of the tested compared to previous studies might be due to the difference in coated antigen and difference sets of tested sera. In general, lower sensitivity of diagnostic test might influence the status of true positive samples. To improve diagnostic performances, more samples and source of pigs should be replaced by cesarean-derived colostrum-deprived (CD/CD) pigs to confirm the true disease of status of positive and negative sera. Moreover, positive and negative predictive values of this ELISA that were 100% and 92.59% resulted in reliable confirm of true disease status with prevalence of 50% from 100 tested sera. Thus, this indirect PCV2b-based ELISA might be suitable for being the confirmation test.

In the comparative agreement test, the level of observed agreement between indirect PCV2b-based ELISA and IPMA was 89.34% with 0.757 kappa statistic showing substantial inter-rater reliability (McHugh, 2012). These indicated that PCV2b-based ELISA was reliable and could be used to detect PCV2 antibodies as well as IPMA.

Repeatability test revealed that intra-plate and inter-plate analysis also provided low percentage of coefficient of covariance (%CV) from the median value of both tests (Less than 15%) and could be repeatable.

Correlation between indirect PCV2b-based ELISA and IPMA was reliable with linear regression with spearman correlation coefficient (r) = 0.74 ($p<0.001$) and endpoint of antibody titers of IPMA were ranged from 1.78-2.98 and OD value from 0.44-1.11. According to the results, OD value of indirect PCV2b-based ELISA could be used to estimate the level of antibody titer. By the way, neutralizing antibodies (NA) which showing significant protection and clearance of viral load from PCV2 infection was related to antibody titer of IPMA (Fort et al., 2007; Pileri et al., 2014). Consequently, indirect PCV2b-based ELISA might be used to determine the level of protection by analyzing PCV2 antibodies response and would be more accurate if the correlation between IPMA was excellent. However, correlation and spearman correlation coefficient (r) between both tests could be better if OD value was replaced by S/P ratio due to the variability from non-specific background of each ELISA plate. According to previous study about anti-PCV2 IgM (Fort et al., 2007), comparison of anti-PCV2 IgG which used to analyzing in this study and anti-PCV2 IgM needs to be studied due to

the apparent relationship between anti-PCV2 IgM and the level of protection by focusing on seroconversion of the pig.

# CHAPTER VI
# CONCLUSION AND SUGGESTION

In conclusion, porcine circovirus type 2 (PCV2) still causes serious economic impact on pig production globally including Thailand. Serological diagnosis has been proved to be efficient way to know disease status such as immunoperoxidase monolayer assay (IPMA) that known as gold standard assay to detect PCV2 antibodies. However, IPMA takes times and needs expert interpretation of the results. Comparing to IPMA, indirect ELISA based on whole viral particle of PCV2b antigen was developed in this study to overcome limitations of IPMA with more advantages including convenient, fast diagnosis and easier to test for large scale of samples. According to results and discussion of this study, PCV2b antigen derived from sucrose gradient technique could be use as good candidate for coating as antigen for indirect PCV2b-based ELISA. Based on our validation results, phylogenetic analysis had been used to confirm the subtype of PCV2b for use as coating antigen. High viral titer of antigen indicated by the green fluorescence of the virus in the cell. The IPMA using SST-SW1 cells originated from lung could be use as gold standard for detection of PCV2 antibodies response and comparable with an indirect PCV2b-based ELISA. Our study also used checker board titration technique to verify the optimal conditions of indirect PCV2b-based ELISA. The results showed that we obtained high sensitivity, perfect specificity, and high accuracy of the test. Therefore, an indirect PCV2b-based ELISA could be used for detection of antibody against PCV2 infection in pigs as well as IPMA.

Further investigation should be performed by setting a longitudinal study in order to find level of antibody response after infection or vaccination. Moreover, the effect of maternally derived antibodies from piglets should be conducted to see the pattern of PCV2 infection and appropriate time of vaccination and overall herd immune status. Moreover, our indirect PCV2b-based ELISA was based on whole cell of PCV2b as antigen. Therefore, cross-reactivity of ORF1 of PCV1 and PCV2 needs to be confirmed.

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