การประเมินการใช้ชุดทดสอบอีไลซาในการตรวจหาแอนติบอดีของโรคอหิวาต์สุกรจากตัวอย่างน้ำลาย

นางสาวปานจันทร์ สิทธิเจริญชัย

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EVALUATION OF A COMMERCIAL ELISA TEST KIT ON CLASSICAL SWINE FEVER ANTIBODY DETECTION USING ORAL FLUID SAMPLES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
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ปานจันทร์ สิทธิเจริญชัย : การประเมินการใช้ชุดทดสอบอีไลซาในการตรวจหาแอนติบอดีของโรคอหิวาต์สุกรจากตัวอย่างน้ำลาย. (EVALUATION OF A COMMERCIAL ELISA TEST KIT ON CLASSICAL SWINE FEVER ANTIBODY DETECTION USING ORAL FLUID SAMPLES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, 52 หน้า.

โรคอหิวาต์สุกรเป็นโรคที่ก่อให้เกิดความเสียหายต่อการผลิตสุกรเป็นอย่างมาก มาตรการในการเฝ้าระวังและตรวจสอบสถานะของโรคจัดเป็นปัจจัยหนึ่งที่มีความสำคัญต่อการควบคุมและป้องกันโรคเป็นอย่างยิ่ง การตรวจวินิจฉัยหรือการศึกษาสถานะการติดเชื้อของโรคอหิวาต์สุกรโดยมากนิยมใช้วิธีการตรวจหาแอนติบอดีที่จำเพาะต่อเชื้อไวรัสอหิวาต์สุกรจากตัวอย่างซีรัม อย่างไรก็ตามวิธีการดังกล่าวจำเป็นต้องมีการเก็บตัวอย่างเลือดจากสุกร ซึ่งอาจก่อให้เกิดความเครียดต่อตัวสุกรได้ อีกทั้งการเก็บตัวอย่างเลือดยังเป็นวิธีการที่สิ้นเปลืองเวลาและแรงงาน และจำเป็นต้องใช้อุปกรณ์ต่างๆ สำหรับเก็บตัวอย่างเลือดอีกด้วย ปัจจุบันมีการศึกษาการใช้ตัวอย่างน้ำลายในการตรวจวินิจฉัยโรคสำคัญต่างๆในสุกร เช่น การตรวจหาเชื้อ หรือ แอนติบอดีที่จำเพาะต่อไวรัสพีซีวี 2 และ ไวรัสพีอาร์อาร์เอส เป็นต้น เนื่องจากการใช้ตัวอย่างน้ำลายในการตรวจวินิจฉัยเป็นวิธีที่ก่อให้เกิดความเครียดต่อสุกรค่อนข้างน้อย และเป็นวิธีที่สามารถปฏิบัติได้ง่าย ดังนั้นการตรวจหาแอนติบอดีที่จำเพาะต่อเชื้อไวรัสอหิวาต์สุกรจากตัวอย่างน้ำลายจึงจัดเป็นวิธีที่มีความน่าสนใจเป็นอย่างมาก การศึกษาครั้งนี้ทำการศึกษาการตรวจสอบแอนติบอดีที่จำเพาะต่อไวรัสอหิวาต์สุกรในสุกร โดยใช้ตัวอย่างน้ำลายและเลือดที่ได้จากการศึกษาก่อนหน้าโดยในการศึกษาประกอบด้วยลูกสุกรจำนวน 3 กลุ่ม (อายุ 20 วัน จำนวนทั้งสิ้น 20 ตัว) ได้แก่ 1) สุกรกลุ่มที่ได้รับไวรัสอหิวาต์สุกรสายพันธุ์เอแอลดี (ALD) ซึ่งมีความรุนแรงในการก่อโรคต่ำ (กลุ่ม A) (n = 8) 2) สุกรกลุ่มที่ได้รับวัคซีนต่อโรคอหิวาต์สุกร (กลุ่ม B) (n = 8) และ 3) สุกรกลุ่มควบคุมลบ (กลุ่ม C) (n = 4) โดยที่สุกรกลุ่ม A และ B ซึ่งได้รับไวรัสหรือวัคซีน (ตามลำดับ) ในวันที่ 0 และได้รับเชื้อพิษซ้ำที่เป็นไวรัสอหิวาต์สุกรสายพันธุ์ที่มีความรุนแรงในการก่อโรคสูง (สายพันธุ์ Bangkok 1950) ในวันที่ 14 ทำการเก็บเลือดและน้ำลายจากสุกรทั้ง 3 กลุ่มในวันที่ -1 3 7 10 14 17 21 24 27 และ 30 และทำการการุณยฆาตสุกรทั้งหมดในวันที่ 30 การตรวจสอบแอนติบอดีที่จำเพาะต่อไวรัสอหิวาต์สุกรทำโดยใช้ชุดตรวจสอบอีไลซา (BioChekCSFV Antibody Test Kit, Reeuwijk, The Netherlands) โดยใช้ทั้งวิธีการตามคู่มือของชุดตรวจสอบและวิธีการที่มีการดัดแปลงในการศึกษาครั้งนี้และทำการทดสอบความไวของการทดสอบ ผลการศึกษาพบว่าชุดตรวจสอบอีไลซาสามารถตรวจพบแอนติบอดีที่จำเพาะต่อไวรัสอหิวาต์สุกรจากตัวอย่างน้ำลายได้ และระยะเวลาในการบ่มตัวอย่างน้ำลายในชุดตรวจสอบที่ยาวนานขึ้นสามารถส่งผลในการเพิ่มสัญญาณในการตรวจพบแอนติบอดีได้ นอกจากนี้วิธีการที่มีการดัดแปลงในการศึกษาครั้งนี้ยังส่งผลให้การตรวจแอนติบอดีที่จำเพาะต่อไวรัสอหิวาต์สุกรจากตัวอย่างน้ำลายด้วยชุดตรวจสอบอีไลซานั้นมีความไวสูงขึ้นอีกด้วย การศึกษาในครั้งนี้แสดงให้เห็นว่าสามารถตรวจพบแอนติบอดีที่จำเพาะไวรัสอหิวาต์สุกรจากตัวอย่างน้ำลายได้และชุดตรวจสอบอีไลซาต่อโรคอหิวาต์สุกรในปัจจุบันสามารถนำมาดัดแปลงเพื่อให้สามารถใช้ตรวจสอบแอนติบอดีดังกล่าวได้

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556

5575326931 : MAJOR VETERINARY PATHOBIOLOGY
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PANCHAN SITTHICHAROENCHAI: EVALUATION OF A COMMERCIAL ELISA TEST KIT ON CLASSICAL SWINE FEVER ANTIBODY DETECTION USING ORAL FLUID SAMPLES. ADVISOR: PROF. ROONGROJE THANAWONGNUWECH, DVM, Ph.D., 52 pp.

Classical swine fever (CSF) is a devastating disease, contributing to economic loss of swine production in endemic countries. Monitoring the swine herd status from this disease is crucial for the prevention and control program. Many diagnostic assays have been developed and used for the detection and monitoring of CSF antibody, which often include blood collection procedure causing stress for the animals, time consuming, needs labor and many equipments. Recently, the collection method for swine oral fluid samples was developed and used in the detection of pathogens and antibody such as PCV-2 and PRRSV. This particular method is not only less stressful to the animals, but it is simple and practical for both farmers and veterinarians. In addition, the use of enzyme-linked immunosorbent assays (ELISA) is a safer and less expensive way in the detection of antibody and antigen for CSF in disease free country. This experiment obtained oral fluid samples and blood samples from previous experiment which divided a total of 20 piglets (20 days old) into 3 experimental groups; challenged with ALD strain, a low virulence (A) (n=8), vaccination (B) (n=8) and negative control (C) (n=4). The animals were vaccinated with modified live vaccine (MLV) at 0 day post inoculation (dpi) and re-challenged (A&B) with Bangkok 1950 strain, a high virulence on 14 dpi and euthanized at 30 dpi. Oral fluid samples were collected daily by hanging cotton ropes in each pen and fluid was extracted from the ropes by mechanical compression and blood samples were collected on -1, 3, 7, 10, 14, 17, 21, 24, 27 and 30 dpi. A commercial classical swine fever indirect ELISA test kit (BioChekCSFV Antibody Test Kit, Reeuwijk, The Netherlands) was used in the detection of CSF antibody in oral fluid samples with normal protocol and modified oral fluid concentration. The sensitivity of the ELISA was evaluated using known negative oral fluid combined with serum of known serum neutralizing titers. The results demonstrate that CSFV antibody could be detected using indirect ELISA assay and the longer incubation time enhanced the antibody detection signal. Using the oral fluid obtained from experimental animals with the normal ELISA protocol and NPLA test, the results showed low detectable antibody titers from both tests. By using the modified ELISA protocol, the sensitivity increased in the known CSF NPLA titers of oral fluid samples. With the use of oral fluid samples from the challenged experiment, the ELISA was able to detect low amount of CSF antibody titers in the oral fluid using the modified protocol. This proved that anti-CSFV antibody could be detected from oral fluid samples with the indirect ELISA and this test could be used to further in the monitoring and surveillance program of CSFV in the future.

Department: Veterinary Pathology Student's Signature .....
Field of Study: Veterinary Pathobiology Advisor's Signature .....
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Chapter I: Introduction

Classical swine fever is a devastating disease, causing severe loss of swine production in the endemic countries. The usage of diagnostic tests is essential in the detection and monitoring of the herd health status in endemic areas and surveillance in disease free areas. In order to detect CSF antibody, the gold standard method is the neutralizing peroxidase-linked assay (NPLA) test. This technique includes time, labors, animal restraint, excessive equipment and meticulous process of viral culture. Alternatively, many tests have been developed and used in the detection of both antigen and antibody such as enzyme-linked immunosorbent assay (ELISA). The advantages of using ELISA are the rapid detection need for disease surveillance and exclusion of viral culture. Commercially available ELISAs, both indirect and competitive techniques, have been implemented with limited use in the field in order to evaluate the status of disease. Recently, oral fluid samples have been used in the detection of the presence of antibody in major swine diseases including PCV-2 and PRRSV. Oral fluid collection method is not only less stressful to the animals, but is simple and practical for both farmers and veterinarians. By combining these two methods, this experiment proved the presence of CSF antibody in oral fluid samples could be detected using a commercial ELISA and could be modified to increase the sensitivity. This evaluation will contribute not only in the facilitation of CSF antibody detection method, but nevertheless will help in the diagnosis and controlling aspects of CSFV, particularly in the CSF free farms and CSF free areas.

Chapter II: Literature Review

Classical Swine Fever Virus

CSFV is a virus in the member of the genus Pestivirus, family Flaviviridae classified to List A by the World Health Organization for Animal Health (OIE). This highly contagious virus causes devastating disease in domestic pigs and wild boars. The disease was first known as "hog cholera or swine plaque" and much described by variety of clinical symptoms and post-mortem lesions such as anorexia, lethargy, diarrhea, respiratory distress, cyanosis, diffuse petechial hemorrhages in internal organs, lung hepatization and ulceration in the large intestinal tract It was later confirmed the viral origin of the disease in 1903 (United State, 1889).

This virus is a small-enveloped positive sense single stranded RNA virus with genome of approximately 12.3 kb (Meyers et al., 1989; Moormann et al., 1996; Ruggli et al., 1996) consists of 1 open reading frame encoding 4 structural proteins (C, E^{rns}, E1 and E2) and 8 non-structural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Lindenbach et al., 2007). The structural proteins of CSFV form the nucleocapsid protein (C) and envelope glycoproteins (E^{rns}, E1 and E2) of the virus, which play an important role in the immunological response of the host. Study has shown that the formation of the neutralizing antibody is mainly via viral envelope E2 protein (Zhang et al., 2011). Additionally, the non-structural proteins of CSFV have been studied to some expense about the function and their roles on immunopathological event of the virus. The NS3, NS4A, NS4B, NS5A and NS5B are necessary for enzymatic functions used in viral replication (Meyers et al., 1996; Behrens et al., 1998). The other non-structural proteins, Npro NS2 and p7, are non-essential for the pestivirus replication in cell culture, but play other roles in the viral survival in host cells. The first translated protein, Npro, is an autoprotease that cleaves itself from the translated polyprotein of CSFV genome (Wiskerchen et al., 1991; Stark et al., 1993; Rumenapf et al., 1998). This protein is associated with immunoevasion of the virus by targeting the transcriptional factor of interferon regulatory factor 3 (IRF-3) and antagonizing the effect of type I interferon (IFN) (Seago et al., 2010), thus resulted in longer survival of the virus in infected cells. The NS2 function as an NS2-3 autoprotease, but alone had no essential function (Agapov et al., 2004; Lackner et al., 2006; Moulin et al., 2007). Recent study has suggested otherwise that the NS2 modulates the cell cycle by inducing S-phase arrest and supports the CSFV replication process (Tang et al., 2010). The small hydrophobic

protein p7 is part of the E2-p7 precursor protein and is associated with the E2 function and infectivity of the virus (Elbers et al., 1996; Harada et al., 2000). As mentioned, the envelope glycoproteins, E^{rns}, E1 and E2, have important role in the immune response of the host, viral absorption and the virulency of the virus (Tamura et al., 2012). The E^{rns} processes ribonuclease activity and is consisted of many glycosylated sites (Rumenapf et al., 1993; Branza-Nichita et al., 2004; Sainz et al., 2008). The protein is immunogenic and can induce low level of neutralizing antibody (Gavrilov et al., 2011). The E1 is smaller with 3 glycosylation sites and forms heterodimers with E2 protein (Weiland et al., 1990). Lastly, the E2 protein contains one putative O-linked and six N-linked glycosylation sites (Thiel et al., 1991; van Rijn et al., 1994; Risatti et al., 2007). This protein does not only involve in the attachment and entry into host cells (Hulst and Moormann, 1997), but it is the major immunological determination site and the major target of viral neutralizing antibody (Weiland et al., 1990).

The advanced studies in the molecular level of the virus and the information shared in the genomic databases were able to sort the virus into genotypes. Three regions of the viral genomes have been evaluated, the 3' end of the polymerase gene (NS5B) (Lowings et al., 1994; Bjorklund et al., 1999), 150 nucleotides of the 5' non-translated region (NTR) (Stadejek et al., 1996; Greiser-Wilke and Paton, 1999) and the 190 nucleotides genomic fragment encoding E2 protein (Lowings et al., 1996; Diaz de Arce et al., 1999). Due to the excessive data on the E2 encoding gene, most of the genotyping is based on the sequence of this region. This divided the CSFV into three genotypes and three to four subgenotypes each; 1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3 and 3.4 (Paton et al., 2000). More variations of the virus have been found throughout the years, for instance, in China and Taiwan, there were reports of viral isolates variation classified into 2.1a, 2.1b and 2.1c (Deng et al., 2005; Pan et al., 2005; Jiang et al., 2013). Recently in Cuba, a novel 1.4 subgenotype has been reported in 2013 (Postel et al., 2013). The classification and the phylogenetic analysis of the virus were able to characterize the virus into geographical regions and support in the epidemiological evidences of each individual outbreak (Stadejek et al., 1997; Vilcek et al., 1997; Bartak and Greiser-Wilke, 2000). The first genotype of the virus is spread throughout South America (Pereda et al., 2005) and Russia (Vlasova et al., 2003), the isolates from genotype 2 are mainly found in Europe (Blome et al., 2010) and some part of Asia (Blacksell et al., 2004; Pan et al., 2005; Kamakawa et al., 2006) and lastly, the genotype 3 is found only in Asian countries (Parchariyanon et al., 2000).

It is unclear as the time and place of the first clinical outbreak of CSFV. However, evidences suggested that it was during the 19th century when the disease spread. In the USA, the first identification of the disease was in the 1830s in Ohio (United States, Bureau of animal industry, 1889). The virus then spread throughout the continent and was finally eradicated in North America in 1978 (Terpstra, 1994). In Europe, report of early outbreaks of CSF was in United Kingdom (1879) (Beynon, 1962) and Germany (1899) (Kamphans, 1964). Multiple outbreaks records of the CSFV were reported in Germany in between 1980-1990 (Dahle and Liess, 1992). In 1992, presence of the CSFV was identified in wild boars in Germany (Moennig et al., 2003). The circulated CSFV in wild animal became an important source of viral transmission and problem of eradication in domestic pigs later on in European countries (Hofmann et al., 1999; Lowings et al., 1999). At present, this virus can be found mainly in Asia, South and Central America and the Caribbean, nevertheless, many countries had successfully eradicated the disease in domestic pigs such as United States, Canada, New Zealand, Australia and Western and Central Europe (Moennig et al., 2003). In Eastern Europe, Central America, South America and Southeast Asia, this disease is considered an endemic disease. In Thailand, the first report of CSF was in 1950 at Bangkhen district in Bangkok (Kongsamak, 1980). With the increase number of pig farms throughout the years, the disease later then spread across the country and eventually became endemic. The isolates found in Thailand in the 1980s were from 1.1, 1.3 and 1.2 subgenotype(Lowings et al., 1996; Parchariyanon et al., 2000). Subgenotype 1.3, 2.2 and 3.3 were found in the 1990s (Greiser-Wilke and Paton, 1999).

CSFV can cause severe systemic symptoms in pigs of all ages mainly due to immunopathological responses. The pathogenesis of this immunopathologic virus has been overtly studied, yet still not fully understood. The pigs that were acutely infected with CSF will commence shedding via saliva, urine, feces, ocular and nasal secretion prior to showing the clinical signs (Terpstra, 1994; Van Oirschot, 2004). The virus then can be mainly transmitted by direct or indirect oronasal contact of infected animal. Nevertheless, other possible transmission can occur by ingestion of contaminated feed (swill feeding) and insemination with infected semen (de Smit et al., 1999; Elber et al., 1999; Edwards, 2000; Floegel et al., 2000). Indirect transmission from human and farm vehicles may be possible in farm with low biosecurity level. Aerosol transmission of the virus has also been demonstrated under experimental condition with short distance of viral transmission and spread within restricted

holding area. After the animals were first exposed to the virus the primary replication site is the tonsil. The virus then spread to regional lymph nodes as the secondary replication site and disseminate to other organs such as bone marrow, visceral lymph nodes and lymphoid tissue throughout the body as the result of viremia. The virus targets many types of cell preferably the mononuclear cells and vascular endothelial cells. Recent evidence has shown the viral replication in dendritic cells, which suggested the possibility of facilitating the viral dissemination to other organs (Jamin et al., 2008). In later stage of infection, the CSFV could be found in the granulocytes (Summerfield et al., 1998a).

The pathology of the virus is mainly by the ability of the virus to cause dysfunction and imbalance to host immune system. The virus can cause immunosuppression by direct and indirect effect. The direct effect of immunosuppression is caused by the viral replication in the reticuloendothelial cells. The viral replication in these cells may interfere with the normal cell immunological function and acquire resistant to apoptosis by reducing the effect of type I interferon (Seago et al., 2010). Leukopenia may appear early after viral infection prior to viremia as the result of indirect immunopathological effect of the virus to leukocyte subpopulations. Studies have shown that the decrease number of both lymphocyte and neutrophil was attributed to the indirect activation of apoptosis from the virus than the direct viral infection to these cells (Summerfield et al., 1998b; Sato et al., 2000; Summerfield et al., 2001). Other than the immunopathological effect of the virus, the virus itself can infect the vascular endothelial cells increase the permeability of the vessel wall. This causes generalized hemorrhages throughout the body of the susceptible hosts such as the skin, lymph nodes, kidneys, urinary bladders, spleens, intestinal tracts and lungs. In vitro, like most typical Pestiviruses, CSFV persist in infected cells and does not cause cytopathic effect (CPE). Only few reports have been made on strains of CSFV that cause CPE. The ability of these viruses to cause CPE is associated with NS3 protein of the virus. The viruses that expressed higher level of NS3 protein induced have higher chances to induce CPE in vitro and apoptosis in host cells (Meyers et al., 1996; Kummerer and Meyers, 2000; Xu et al., 2007).

The clinical manifestation of the classical swine fever showed wide range of systemic syndrome which varies between host factors such as age, immune status, environment and other concurrent infections (Depner et al., 1997; Moennig et al., 2003; Floegel-Niesmann et al., 2009). The acute form of the disease exhibits high fever, anorexia, lethargy, conjunctivitis, respiratory signs and constipation followed by diarrhea (Floegel-Niesmann et al., 2009). Upon necropsy, the acute lesions often showed diffuse systemic hemorrhages in organs such as skin, lymph nodes, bladder and kidney. In chronic form, similar clinical signs are observed with longer survival time (2-3 months) and the pathological lesions often include button ulcers in cecum and large intestine with lymphoid depletion, but hemorrhage and inflammation are less common.

Methods of diagnosis of CSFV include previous case history, clinical observation and necropsy with laboratory confirmation. The clinical signs and necropsy alone cannot differentiate CSF from other similar swine systemic diseases such as African swine fever, highly pathogenic porcine reproductive and respiratory syndrome, porcine dermatitis and nephropathy syndrome and bacterial septicemia (salmonellosis, erysipelas etc.). Other problems can cause the same clinical signs, such as coumarin poisoning and hemolytic disease in newborn piglets (Moennig et al., 2003). Laboratory tests are then utilized to distinguish between these diseases providing both the detection of the antigen and antibody of the virus. The antigen of the virus can be detected by using reverse transcriptase polymerase chain reaction (RT-PCR), viral isolation on cell culture, fluorescent antibody test or an antigen capture enzyme linked immunosorbent assay (ELISA). To detect the antibody, the gold standard method is the detection of neutralizing antibody using neutralizing peroxidase-linked assay (NPLA). Antibody detection of CSFV can routinely be used not only in disease diagnosis, but evaluation of vaccine effectiveness. In addition, the serum neutralization (SN) titer is commonly used for monitoring herd serological status in swine farm. Other methods use for antibody detection such as fluorescent antibody virus neutralization (FAVN), which also detect the CSFV neutralizing antibody, and ELISA and E^{rns}-ELISA (van Rijn et al., 1999). Several types of ELISA, direct ELISA, indirect ELISA, blocking ELISA (Have, 1984) and complex-trapping blocking ELISA (Wensvoort et al., 1988; Colijn et al., 1997) are in-house and commercially available (Colijn et al., 1997). The ELISA detection tests that differentiate infected animals from the vaccinated individuals are also available, but with limited use. CSFV

and other viruses in the family Pestivirus (bovine viral diarrhea virus (BVDV) and border disease virus (BDV)) are known to exhibit cross-reaction in the detection of antibody using serological methods. Pigs are known to be susceptible to both BVDV and BDV. Thus, in immunological based tests, it should be considered that the antibody detected maybe from other Pestiviruses. In endemic counties with other Pestiviruses such as Thailand, the use of ELISA in antibody detection is contradictory. However, it is useful in disease free herds or areas.

The prevention and control of CSF is a big issue mainly in swine production and international trade. Vaccination is the main controlling strategy in endemic areas and the evaluation of neutralizing titer is crucial. Different types of vaccine have been proven to give good protection on the animal. Commercial modified live vaccines (MLVs) are widely used in endemic area. It has proven to markedly reduce the clinical symptoms and viral shedding. CSF subunit marker vaccines have been produced, which enable to differentiate between infected and non-infected pigs. Though the use of marker vaccines is beneficial, its protective efficiency is still inferior to that of MLVs (Huang et al., 2014). The combine strategies of vaccination, early detection of CSFV and the elimination of infected animals are crucial in controlling the disease. Reports of wild boars as carrier of CSFV and many other swine diseases have been an issue in disease free countries. In the aspect of ELISA detection of antibody titers in oral fluid will facilitate on the surveillance and monitoring in the eradicated area both in farm and wild animals.

Detection of antibody from oral fluid samples using ELISA

In human medicine, saliva or oral fluid has been used routinely for diagnostic and detection of both antigen and antibody of many diseases, hormones, toxins and drug residues (Archibald et al., 1986; Connolly et al., 2004; Carr et al., 2009). It should be noted that the swine oral fluid composed of both saliva that excreted from the salivary glands and transudate that diffused from the capillaries in oral cavities. The transudates composed of substances comparable to that of serum. Depending on the species and the type of salivary gland, the saliva contains more than 99 percent water, which dilute other substances, e.g., electrolytes, immunoglobulins, proteins, enzymes, mucins, urea and ammonia (Humphrey and Williamson, 2001; Prickett and Zimmerman, 2010). These components serve 5 major functions: (1) controlling the pH in the oral cavity, (2) lubrication, (3) anti-microbial activity, (4) maintaining teeth mineralization and (5) digestion. Thus, overall IgM, IgG and IgA can be detected from the oral fluid.

In the last 5 years, the use of this technique has been applied in the diagnosis of swine diseases. This method can easily be performed in swine due to the animal behavior and also with the non-evasiveness of the oral fluid collection. This technique has rapidly applied into routine disease diagnosis in the United States. Studies have shown that oral fluid collection showed high sample coverage from all the animals in the nursery or fattening pen compared with the blood collection sampling (Prickett et al., 2008a). It has also been applied for use with individual swine (Kittawornrat et al., 2010). It is a sufficient way for routine disease surveillance and monitoring (Ramirez et al., 2012).

The method of collection is proposed by hanging cotton rope (diameter size of 1.3-1.6 cm for large pigs and 1.0) at shoulder length of the pigs for 20-30 minutes. The extraction of oral fluid can be done by manually squeezing or wrenching the ropes. The oral fluid is then collected in container and stored in refrigerator or frozen until used. Reports of detection of antibody and antigen detection using oral fluid are found in PRRSV (Prickett et al., 2008b; Kittawornrat et al., 2010), PCV-2 (Prickett et al., 2011), influenza type A virus (Irwin et al., 2010; Romagosa et al., 2012), vesicular stomatitis virus (Stallknecht et al., 1999) and APP (Loftager et al., 1993; Loftager et al., 1995). These studies had been successfully proven to be an effective way in disease diagnosis, monitoring and surveillance.

Antibody secretion in oral fluid have been detected in many diseases in swine such as *E. coli*(De Buysscher and Dubois, 1978), transmissible gastroenteritis virus (TGEV) (DeBuysscher and Berman, 1980), *Actinobacillusplueropneumoniae* (APP) (Loftager et al., 1993), porcine reproductive and respiratory syndrome (PRRS) (Wills et al., 1997), porcine circovirus type 2 (PCV2) (Allen and Ellis, 2000) and classical swine fever (CSF) (Corthier, 1976). The use of enzyme-linked immunosorbent assay in the detection of antibody is widely used in serum samples in many swine diseases. Development of commercial ELISA test kit makes this method more convenient for routine usage. In livestock production, the diagnostic laboratory results have been used as an important tool in disease management, surveillance and monitoring. The samples are submitted in large amount and routinely to serve those purposes, so other than the sensitivity and the specificity of each laboratory tests, the duration of testing has become an important aspect to consider when choosing and designing laboratory tests. In comparison with NPLA, ELISAs have the advantages on less time

consumption, no requirement of viral isolation and the ability to work with large number of samples. Originally, the ELISA diagnostic tests in swine have been designed mostly for serum samples. The ELISA method has been applied to detect the oral fluid antibody in many swine diseases such as PRRSV and PCV2 (Prickett et al., 2007; Prickett et al., 2008a; Kittawornrat et al., 2010; Kittawornrat et al., 2012). Modification of the methods are made in the serum dilutions, incubation time, concentration of the conjugate and serum heat treatment to enhance the specificity and sensitivity of the test kit followed by the development of the ELISA oral fluid test kit in the future.

Chapter III: Methodology

In vitro study on the sensitivity of the ELISA

The negative oral fluid samples from a CSFV free herd and porcine serum of known CSF SN titer obtained from Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) were used for testing the effect of oral fluid on the indirect ELISA test kit (BioChekCSFV Antibody Test Kit, Reeuwijk, The Netherlands) (see Topic 4). Prior to testing, all of the negative oral fluid was confirmed to be free of CSF antibody using NPLA (see topic 4). The serum was retested using NPLA to confirm the antibody titer level. A total of 2 batches of negative oral fluid samples (30 ml) and 6 high SN titer sera (128-256) were selected. The negative oral fluid sample and the serum were mixed together at the ratio of 1:1. Then 2-fold serial dilution was performed on the samples and each diluted samples (64, 32, 16 and 8) was tested with the indirect ELISA and used as control in the authentic oral fluid sample experiment. The lowest level of detection was recorded for the different testing procedure (see Topic 5).

In vivo oral fluid samples obtained from experimental model

The oral fluid samples were obtained from previous study using twenty PRRSV free piglets (20 days old). The piglets were randomly divided into 3 experimental groups; challenged with ALD strain, a low virulence (A) (n=8), vaccination (B) (n=8) and negative control (C) (n=4). Ear tags were placed on the animal for the purpose of identification and randomization process on the first day of animal arrival (-3 dpv/dpi (day post vaccination/day post inoculation)). The animals were housed separately in animal biosafety level 2 (ABSL-2) rooms. At 0 dpv/dpi, group A was challenged with CSFV ALD strain (10^5 $TCID_{50}$/ml) and the vaccination (B) group was vaccinated with a modified live vaccine (MLV) LOM strain (HC-VAC) and later euthanized at 30 dpi. This animal experiment received a letter of notification from the Chulalongkorn University Animal Institutional Animal Care and Use Committee (CU-IACUC).

Sample collection

Pen-based oral fluid samples were collected daily starting at -1 dpv/dpi to 30 dpv/dpi by hanging two cotton ropes overnight. The pigs were trained to chew the ropes on -1 and -2 dpv/dpi by dipping the ropes in syrup prior to hanging the ropes. The purpose was to attract the animal to chew the ropes with the sweet taste of the syrup. The damp portion of the ropes were then cut and placed in separate plastic zip-lock bags. The samples were kept in an icebox (0 - 10°C) and transferred to laboratory for further processing within 6 hours. Manual mechanical compression was used to extract the oral fluid samples. In some cases, the ropes were rather dried, 3-5 ml of PBS were added prior to extraction. After mechanical compression, the fluid will accumulate at the bottom corner of the plastic bag. The corner of the bags was then cut and the oral fluid was drained into a 15 ml centrifuge tubes. The tubes were centrifuged at 10,000 x g (4,000 rpm) for 10 minutes at 4°C to remove dirt and debris before storing at -20°C until used.

All of the animals were bled on -1, 3, 7, 10, 14, 17, 21, 24, 27 and 30 dpv/dpi to obtain the serum samples. Ten milliliters syringe and needle size 20 were used for drawing 5-10 ml of blood from the jugular or cranial vena cava of individual animal. The blood samples were kept in the syringe and stored at room temperature for 2-4 hours then placed in an icebox and transfer to laboratory within 6 hours. In the laboratory, the blood samples were transferred from the syringe into 2 ml microcentrifuge tubes and centrifuged at 6,000 rpm for 10 minutes to separate the serum. The samples were kept at -20°C until used.

Enzyme-linked immunosorbent assay (ELISA) and neutralizing peroxidase-linked assay (NPLA)

A commercial classical swine fever indirect ELISA test kit was used in the detection of CSF antibody in serum and oral fluid (BioChekCSFV Antibody Test Kit, Reeuwijk, The Netherlands). Prior to testing, washing buffer was prepared by dissolving 1 washing buffer package with 1,000 ml of distilled water and all the reagents (serum diluent, negative/positive controls, conjugate, conjugate diluent, TMB substrate and stop solution) and the ELISA plates were brought to room temperature (23-27°C). The different types of samples (serum, negative oral fluid/serum and oral fluid) were diluted at 1/30 ratio (5 μl of serum with 135 of serum diluent) in 96-well

transfer plate. The diluted samples were pipetted (100 μl/well) into 96-well CSF-E2 ELISA coated plate. The negative and positive controls provided by the test kit were added (100 μl/well) in duplication in every test run. Using the standard protocol of the test kit, the diluted samples were incubated in the ELISA plates for 60 minutes at room temperature (23-27°C) then washed 3 times with the washing buffer. Secondary antibody conjugate (anti-swine IgG antibody conjugated with horseradish peroxidase enzyme) was added in each testing well (100 μl/well) and incubated for 30 minutes at room temperature then washed 3 times with the washing buffer. The TMB color substrate was added (100 μl/well) to create the indicative yellow color. The substrate was incubated in the dark for 15 minutes at room temperature then stop solution ($MgSO_4$) (100 μl/well) was immediately added to stop the enzymatic reaction. The side of the ELISA plate was tapped to mix the substrate with the stop solution then placed in the ELISA reader to read the optical density (OD) wavelength of 405 nm. The results were calculated into S/P ratio by dividing the subtracted background OD value of the samples with the subtracted background OD value of the positive control (OD sample-OD negative control/OD positive-OD negative control).

The NPLA test was performed using a modified OIE protocol, with the virus concentration of 300 $TCID_{50}$/ml. In the preparation process, the stock CSFV (ALD strain) was grown on SK-6 cell line and kept at the concentration of 10^5 $TCID_{50}$/ml in -80°C for testing purposes. Eagle's minimum essential medium (MEM) (Gibco®, Life Technologies, USA) was prepared by diluting the MEM powder with 1,000 ml of distilled water to make the stock 10X MEM and aliquot 100 ml in bottles and kept at -20°C until further used. For the purpose of NPLA tests and cell line maintenance, 1 liter of 1X MEM was prepared by adding the 10X MEM (100 ml) with 10 ml of 200 mM L-glutamine (PAA The Cell Culture Company, Austria), penicillin-streptomycin (10,000 U/ml, 10,000 mg/ml) (Biochrom, Merck Millipore, Gernamy), pyruvate (0.11 g/L), $NaHCO_3$ (2 g/L) mixed together in 890 ml of distilled water. The growth medium was filtered and used within 2 weeks after each preparation. SK-6 cells were maintained in 25 ml or 100 ml culture flasks with 5-10% fetal bovine serum (FBS) (Sigma-Aldrich®, USA) MEM. The NPLA was performed by adding 50 μl of MEM in each 96-well cell culture plate. The serum or oral fluid samples were then added (50 μl) in the first row of the plate. Multichannel pipette were used to perform 2-fold

serial dilution of the sample down each row (one row, one sample). The virus suspension was prepared from stock and then added to each well at the concentration of 300 $TCID_{50}$/well (50 μl). After adding the virus, the plates were tapped to mix the solution and incubated for 60 minutes at 37°C in 5% CO_2 incubator. While waiting on the sample and virus incubation, SK-6 cells (2×10^5 cell/ml) were prepared in a clean laminar flow hood. The MEM of SK-6 confluent cells was discarded and the cells were washed 3 times with PBS to rinse off the dead cell debris and FBS. Trypsin-versene was added into the flasks to separate adhesive cells into individual cells and incubate at 37°C in 5% CO_2 incubator for 2-3 minutes. After incubation, the enzyme was discarded and the cells were suspended in 5% BVD and anti-BVD -free FBS MEM. One hundred microliters of the 2×10^5 cell/ml SK-6 cell suspension was pipetted into each tested well and incubated at 37°C in 5% CO_2 incubator for 48 hours. Back titration of the viral suspension was performed to confirm the concentration of CSFV added in each test run.

To evaluate the SN titer, the plates were then fixed and stained with immunoperoxidase monolayer assay (IPMA) to detect the viral infected cell. IPMA process began with taking out the plates after 48 hours of incubation and fixing the monolayer cells with 4% formaldehyde in PBS containing 0.5% Tween 20 (Amresco®, USA) (50 μl /well) for 25 minutes at room temperature. The plates were washed 3 times with PBS containing 0.5% Tween 20. Dilute the primary monoclonal CSFV antibody with distilled water containing 1% bovine serum albumin (BSA) at the ratio of 1:20 (CSFV antibody: 1% BSA). The diluted primary antibody was then added into each well (30μl /well) and incubated for 1 hour at room temperature. The plates were washed 3 times with PBS containing 0.5% Tween 20. The secondary polyclonal rabbit anti-murine IgG - horseradish peroxidase conjugate (DAKO, Denmark) was prepared by diluting the antibody with 1% BSA at the ratio of 1:300 (antibody conjugate: 1% BSA) then added into each well (30 μl /well). The plates were washed again 3 times with PBS containing 0.5% Tween 20. For the coloration, chromogen-substrate solution was prepared containing 3-amino-9-ethylcarbozole (AEC) substrate, acetate buffer and H_2O_2. The chromogenic solution was added into each well (50μl /well) and incubated for 30-60 minutes depending on the intensity of presence of coloration. Lastly, the plates were placed under running tap water for 1-2 minutes and dried prior to titer evaluation.

Modification of ELISA for oral fluid samples

After evaluation of the normal ELISA protocol suggested by the manufacturer, the different steps in the protocol were adjusted to enhance the ELISA detection ability with *in vitro* samples. The samples were diluted in a 96-well transfer plates and 100 μl of the diluted samples were placed in each well of CSF-E2 ELISA coated plates. The negative and positive controls provided by the test kit were added (100 μl/well) in duplication in every test run. The plates were then incubated at different time (1 (room temperature), 6 (4°C), 12 (4°C) hour(s)) and temperature (4, 25 and 37°C). Incubation at 6 and 12 hours were held at 4°C due to high bacterial contamination in oral fluid samples, which may destroy the coated protein on the ELISA plates and antibody in the oral fluid samples. After incubation, the plates were washed 3 times with the washing buffer and secondary antibody conjugate (anti-swine IgG antibody conjugated with horseradish peroxidase enzyme) were added at different volume (100, 150 and 200 μl). The antibody conjugate has been diluted by the manufacturer and the concentration of the conjugate was not indicated. Only each of the considerable factors was varied, while other conditions will remain the same as normal protocol (see topic 4). The plates were then tested with the same protocol by incubating the conjugate for 30 minutes at 37°C then washed 3 times with the washing buffer. The TMB color substrate was added (100 μl/well) to create the indicative yellow color. The substrate was incubated in the dark for 15 minutes at room temperature (23-27°C) then stop solution ($MgSO_4$) (100 μl/well) was immediately added to stop the enzymatic reaction. The side of the ELISA plate was tapped to mix the substrate with the stop solution and placed in the ELISA reader to read the optical density (OD) at the wavelength of 405 nm. The results were calculated into S/P ratio by dividing the subtracted background OD value of the samples with the subtracted background OD value of the positive control (OD sample-OD negative control/OD positive-OD negative control).

Next step was to use the oral fluid obtained *in vivo* oral fluid samples from the animal model as the experimental samples. Prior to testing with the test kit, the samples (negative oral fluid/serum and oral fluid) were process with heat treatment at 56°C for 15 minutes and diluted at 1/3 fractions with the sample diluent provided in the kit based on preliminary tests and previous PRRS work (Kittawornrat et al.,

2012). The samples were diluted in a 96-well transfer plates and 100 μl of the diluted samples were placed in each well of CSF-E2 ELISA coated plates. The negative and positive controls provided by the test kit were added (100 μl/well) in duplication in every test run. The controls provided by the test kit were diluted at 1/30 ratio. The plates were then incubated at different time and temperature (1 hour at 4, 25 and 37°C; 6 hours at 4 and 25°C and 12 hours at 4°C). After incubation, the plates were washed 3 times with the washing buffer and secondary antibody conjugate (anti-swine IgG antibody conjugated with horseradish peroxidase enzyme) was added at different volume (100, 150 and 200 μl). ELISA protocol was similarly performed as normal protocol after adding the different conjugate volume. The results were calculated into S/P ratio by dividing the subtracted background OD value of the samples with the subtracted background OD value of the positive control (OD sample-OD negative control/OD positive-OD negative control).

Statistical analysis and data analysis

Descriptive data analysis was performed with S/P ratio and NPLA titer value results. Analysis of variance (ANOVA) was used to assess the differences between each variable factors based on the S/P ratio results in the *in vitro* oral fluid samples. Post hoc multiple comparison tests were used to evaluate the significant differences between different ELISA protocols. Significant differences were found when $p < 0.05$ and very significant differences were found when $p < 0.001$.

Chapter IV: Results

In vitro study on the sensitivity of the ELISA

The results from the *in vitro* study using normal protocol showed that the lowest detectable SN titer level for positive S/P ratio using the cut off level of the serum (S/P ratio ≥ 0.4) in negative oral fluid/serum sample was at the SN titer of 8. A total of 18 samples were evaluated using the normal protocol of the ELISA. The percent positive samples and the average S/P ratio in each titer groups (64, 32, 16, 8 SN titer) are shown on Table 1. Most of the samples that contained equal to or more than 16 SN titer showed S/P ratio the exceeded the cut off level (77.8%). Only one samples containing 8 SN titer has positive S/P ratio. The average S/P ratio were positive in groups containing antibody level 16, 32 and 64 SN titer, the group containing antibody 8 SN titer level was negative.

Using a total of 6 negative oral fluid/serum samples, variation in the volume of conjugate added did not show any significant differences in the S/P ratio value (Table 2). Interestingly, modification in the incubation time and temperature showed significant differences in S/P ratio value ($p<0.05$). At antibody titer level more than 16, incubation using the normal protocol at room temperature exhibited significantly higher S/P ratio level than 4°C ($p<0.001$), but no significant difference at 37°C (Table 3). The increase in the sample incubation time to 12 hours at 4°C significantly increased the S/P ratio value ($p<0.001$) (Table 4).

Table 1 :ELISA results of the *in vitro* samples. The table showed the percentage of positive samples and the average S/P ratio of each SN antibody level group. Samples test results showing S/P ratio more than or equal to 0.4 were considered positive for CSF antibody.

	***In vitro* samples: SN antibody Level**			
n=18	**64**	**32**	**16**	**8**
Positive percentage (%)	100 (18/18)	100 (18/18)	77.8 (14/18)	5.6 (1/18)
AverageS/P ratio	2.648	1.438	0.696	0.196

Table 2 : Comparison of average S/P ratio of *in vitro* oral fluid samples at different secondary antibody conjugate volumes (100, 150 and 200 μl).

	Average S/P ratio (conjugate volume)		
SN level	Normal 100μl (n=18)	150μl (n=6)	200μl (n=6)
64	2.648	2.605	2.688
32	1.438	1.452	1.517
16	0.696	0.661	0.670
8	0.196	0.281	0.235

Table 3 : Comparison of average S/P ratio of *in vitro* oral fluid samples at different sample incubation temperatures (room temperature,4°C and 37°C).

	Average S/P ratio (incubation temperature)		
SN level	Room temperature (n=18)	4°C (n=6)	37°C (n=6)
64	2.648[a]	1.290[b]	2.477[a]
32	1.438[a]	0.646[b]	1.208[ab]
16	0.696[a]	0.242[b]	0.630[ab]
8	0.196	0.037	0.165

[a,b]Indicating significant differences ($p< 0.05$)

Table 4 : Comparison of average S/P ratio of *in vitro* oral fluid samples at different sample incubation time (1, 6 and 12 hr(s)).

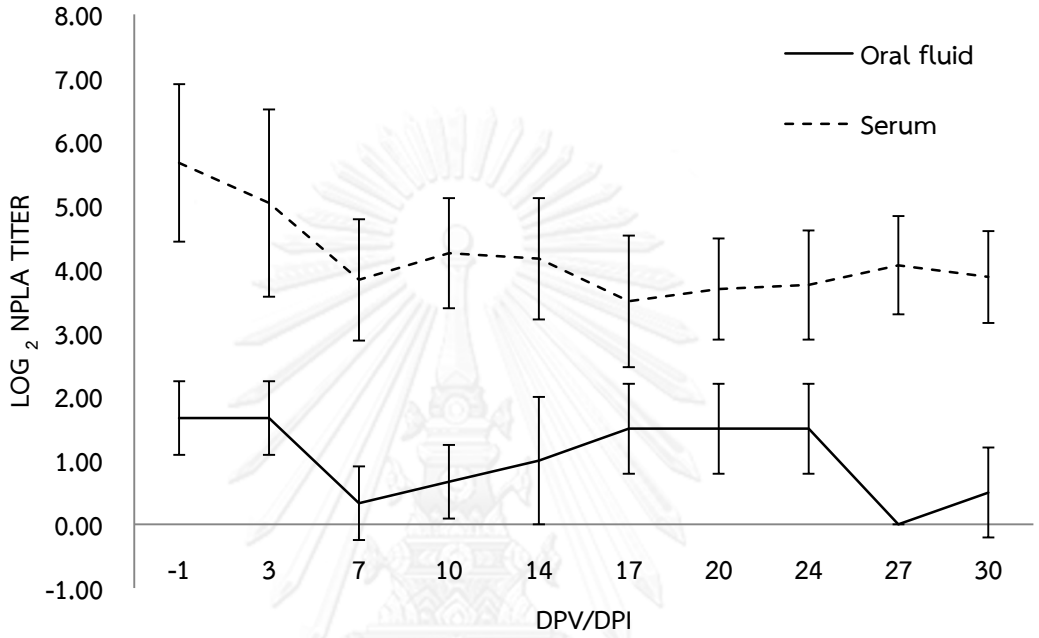
	Average S/P ratio (incubation time)		
SN level	1 hour (n=18)	6 hours (n=6)	12 hours (n=6)
64	2.648[a]	1.124[b]	4.289[c]
32	1.438[a]	0.299[b]	2.931[c]
16	0.696[a]	0.000[b]	1.599[c]
8	0.196[a]	0.000[b]	0.566[c]

[a,b,c]Indicating significant differences ($p< 0.001$)

Detection of neutralizing antibody titer of serum and *in vivo* oral fluid samples obtained from experimental animal

The SN titers of the experimental animals were high with the average titers ranging from 5.13 - 6.25 log_2 prior to vaccination and inoculation. The average titers in group A, B and C at -1 dpv/dpi were 5.63, 5.13 and 6.25, respectively. The average serum titers then decreased twice exponentially within 7 dpv/dpi (A =3.88, B= 3.63 and C= 4.00) and remained stable throughout the experimental period. Significantly lower neutralizing antibody titers were detected with *in vivo* oral fluid samples with the titer ranging from <2 to 2 log_2throughout the period of the experiment (Figure 1). One sample in group C on 0 dpv/dpi was unable to test for neutralizing antibody titer due to high bacterial contamination.

Figure 1 : Comparison of average neutralizing antibody titers in serum and *in vivo* oral fluid on -1, 3, 7, 10, 14, 17, 20, 24, 27 and 30 dpv/dvi



Detection of antibody in oral fluid samples obtained from experimental animal using commercial indirect ELISA

All the oral fluid samples tested for SN titer were also tested for antibody using the indirect ELISA. The results showed low levels of titers were detected in all oral fluid samples and no correlation between the SN titer level and the OD level detected using the normal protocol (Figure 2). Due to low volume of oral fluid samples and the very low level of neutralizing antibody titer tested using NPLA, the samples obtained at 25-30 dpv/dpi were excluded from the ELISA modification study. When calculated the OD results to S/P ratio, all 66 in vivo oral fluid samples were negative (positive S/P ratio ≥ 0.4). The increase of conjugate volume did not increase the S/P ratio value of the antibody detected in the oral fluid. The higher incubation temperature and longer incubation time appeared to give slight increase in S/P ratio compared to normal protocol (Table 5).

Figure 2: OD level detected in each SN titer level of oral fluid samples (SN titer level (2^0) n= 23, (2^1) n= 21, (2^2) n= 15, (2^3) n=4)

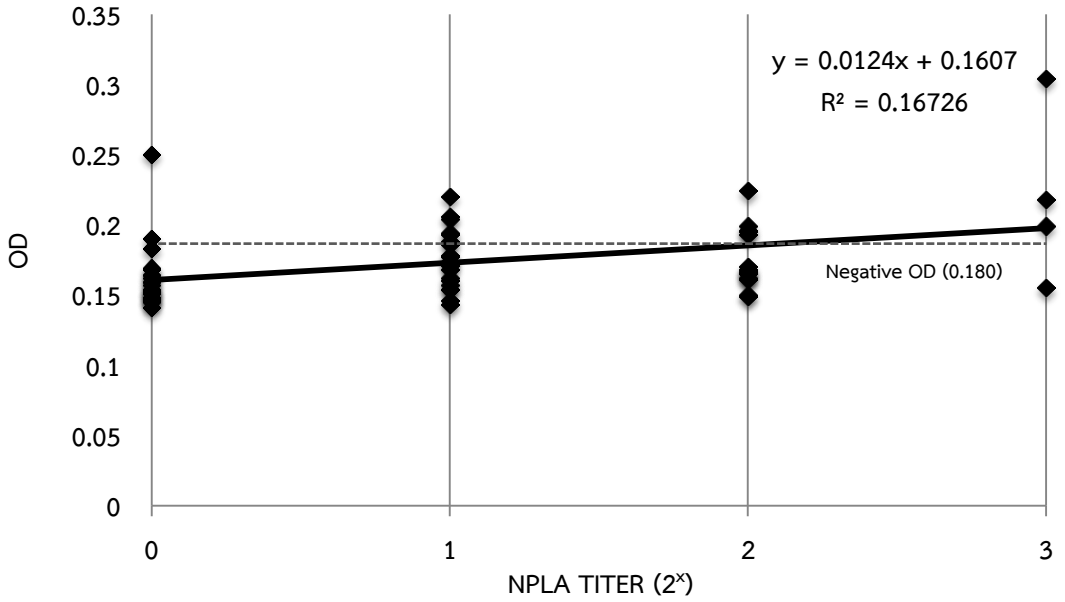


Table 5 : The S/P ratio ranges of *in vivo* oral fluid samples in different conditions of ELISA protocol

Conditions		S/P ratio range
Normal		0.002-0.059
Conjugate volume	150 μl	0.001-0.055
	200 μl	0.003-0.040
Incubation temperature	4°C	0.002-0.050
	37°C	0.002-0.070
Incubation time	6 hrs	0.001-0.074
	12 hrs	0.001-0.082

Chapter V: Discussion and Conclusion

The use of oral fluid in swine disease detection has spread widely as an alternative tool in disease diagnosis and surveillance. For CSFV, this method is convenient compared to individual blood collection. Most tests that have been developed for oral fluid disease detection were PCR based tests, which served different purposes than the serological based tests. At present, the PRRS ELISA oral fluid test kit is the only commercially available test kit for swine disease detection. More recent serological based work has been focusing on swine oral fluid samples for detecting the history of infection in influenza A virus (Panyasing et al., 2014) and surveillance of the highly lethal African swine fever virus (Mur et al., 2013). In terms of antigen detection, oral fluid has been used as a specimen of choice for PCR, sequencing and viral isolation method in swine diseases. Many veterinary diagnostic laboratories in the United States provide routine services PCR detection for SIV, PCV-2, PEDV, TGEV and PRRSV. Other than in commercial swine production, this method has proven to be a potential use for disease surveillance in wild boars (Mouchantat et al., 2014). With the limitation in serum collection from wildlife, alternative specimen such as oral fluid and fecal samples were used. Detection of CSFV antibody has been studied in fecal samples and exhibited good results in disease surveillance to define high-risk area of disease distribution (Seo et al., 2012). Recent study have modified the rope sampling technique for wild boar in the detection of CSFV (Mouchantat et al., 2014). Further studies should be performed to enhance the performance of antibody and antigen detection of CSFV from oral fluid samples to detect the disease distribution in wildlife animals.

By using the negative oral fluid adding the serum of known SN titers, we have established a model that represent the oral fluid with known levels of antibody and were able to demonstrate the detectable antibody in oral fluid. The sensitivity of the antibody detection using ELISA decrease when compared with antibody detection using serum specimen. Preliminary study using the Biochek E2-ELISA using serum samples showed positive S/P ratio (S/P ratio ≥ 0.4) in serum containing equal to or more than 3 $\log_2$ SN titer. The oral fluid contains different kinds of substances; electrolytes, immunoglobulins, proteins, enzymes, urea and ammonia (Humphrey and Williamson, 2001; Prickett and Zimmerman, 2010) and these substances may interfere with the detection of immunoglobulins in the oral fluid. The presence of protease enzymes and bacteria in the oral fluid can degrade some immunoglobulins

containing in the samples (Chiappin et al., 2007). Nurkka et al., 2003 reported that IgA contained in human saliva could be degraded when stored at room temperature. Another investigation reported that rubella IgG in oral fluid samples were stable up to 1 week at 10°C and 20°C (Morris et al., 2002). For long-term storage to avoid immunoglobulin degradation, many measures should be taken into account. The oral fluid samples should be frozen at -20°C or -80°C. Though studies have shown that multiple freeze-thawing cycles in serum samples did not significantly effect on the ability to evaluate the antibodies using ELISA methods (Pinsky et al., 2003), no tests to date have been performed in the oral fluid samples. Thus, repeat freeze-thawing cycles should be avoided because protein generally can be degraded in the process. Addition of enzyme inhibitors, leupeptin, aprotinin and 4-[2-aminoethyl] benzenesulfonyl fluoride, can facilitate in the maintenance of the immunoglobulins (Nurkka et al., 2003). It should be noted that addition of sodium azide, bacteria growth inhibitor, in oral fluid samples can interfere with horseradish peroxidase, commonly presence in enzyme immunoassay tests (Whembolua et al., 2006).

Once the establishment of antibody detection in oral fluid was made, enhancement in the detection was performed using the model *in vitro* samples. The longer incubation time (12 hours at 4°C) best enhanced the S/P ratio signal in this commercial indirect ELISA assay. The increase in the sample incubation time gave more chances for the antibody to come in contact with the coated antigen and formed chemical bond with the specific antigen coated on the ELISA plate. The increase in temperature and the conjugate volume did not display significantly changes in the S/P ratio. The higher temperature in previous studies showed increase molecular kinetic of the antibody and the formation of the chemical bond, thus the results displayed high ELISA signal. In our study, at 37°C, the S/P ratio value was not significantly different compared to the normal protocol. The increase in the incubation time with higher temperature may be performed to enhance the signal. Nevertheless, when using higher temperature with long incubation time it should be noted that false positive might occur and the error should be evaluated when using these conditions. The higher volume of the conjugate added to the ELISA in this test gave no changes to the S/P ratio due to the concentration factor. In theory, the higher concentration of the conjugate added to the ELISA would enhance the signal detected in the ELISA reader by capturing multiple Fc portion of the secondary antibody conjugate to one Fab of the antibody in the samples. The more concentrated the conjugate, the higher number of secondary antibody would be

attached to the Fab portion of the antibody in the sample and stronger color signal would be given out in indirect ELISA. In our study, the conjugate was increased in the volume not the concentration, since the secondary antibody conjugate was diluted and ready to use in the kit provided by the company. The higher volume did increase the number of secondary antibody conjugate incubated in the test, but was not enough to give high detection signal.

The neutralizing antibody in oral fluid samples was able to be detected using NPLA method, but did not give high antibody level as the serum at the same period. In the study, the serum samples average SN titers range from 3.5 - 6.25 $\log_2$. In the same group of pigs and on the same day of serum collection, the SN titer detected range from 0 - 2 $\log_2$. This may be due to the low amount of antibody secretion in the oral fluid. Previous study showed lower CSFV neutralizing antibody detected in bucco-pharyngeal swab samples when compared with serum (Corthier and Aynaud, 1977). In addition, the method of oral fluid collection, specimen handling protocol and oral fluid sample processing may have impacts on the test result. The ropes should be hung for 20-30 minutes for the animals to chew on and should be processed immediately and not be left to dry. Some of the ropes collected in this study were dried when transported back to process in the laboratory and resulted in the inefficiency to extract adequate amount of oral fluid from the ropes. Another problem encountered in this study was the contamination of bacteria in the oral fluid, which affect the SK-6 cell that used in NPLA method and could affect the degradation of immunoglobulins in the sample. The oral fluid samples were resuspended by centrifugation to clean up the debris, filtration and antibiotic was added in the cell culture media to prevent the bacterial growth. However, centrifugation and filtration using 0.22 syringe filter may reduce the concentration of immunoglobulin in the oral fluid samples (Olsen et al., 2013). Clarifying agents, such as sodium alginate, are being developed to removing suspended debris in the oral fluid by inducing flocculation.

With the evaluation of the neutralizing titer using NPLA as the gold standard, the oral fluid samples were evaluated with the commercial indirect ELISA. The results showed slight tendency of higher level of OD level in higher SN titer (Figure 2) with serum samples, CSFV neutralizing antibody level and the antibody level detected using ELISA do not have correlation. By comparing the result of NPLA and ELISA in the detection of oral fluid samples, the NPLA showed higher sensitivity in

the detection of CSFV antibody. This was similar with many laboratory validating tests using serum samples in which NPLA exhibited higher sensitivity than ELISA in detection of CSFV antibody (Floegel-Niesmann and Moennig, 2004; Floegel-Niesmann et al., 2009). Since the sensitivity of the normal commercial indirect ELISA test kit protocol was unable to detect sufficient amount of CSF antibody in the oral fluid samples, optimization of the test was performed with oral fluid treatment and modification of the commercial indirect ELISA test. The oral fluid was treated at 56°C for 15 minutes prior to testing to inactivate any non-specific protein that may interfere with the testing assay.

After oral fluid treatment, the concentration of the oral fluid sample was increased by diluting the samples at 1:3 ratio and tested with different temperature, time of oral fluid incubation and secondary antibody conjugate volume. Incubation time played a significant role at increasing the ELISA signal detected in the oral fluid samples concurrent with the previous *in vitro* study. Nevertheless, the results obtained in this study were not significantly clear due to the low levels of antibody in the oral fluid samples. In future tests to establish the standard protocol for detection of CSFV in oral fluid samples, longer incubation is suggested.

In conclusion, the detection and monitoring of CSFV is crucial worldwide. The use of serological detection assay and the pen-based oral fluid sample will give a simple, cost effective, humane method in the future CSFV disease diagnosis and surveillance. This study has proven that the CSFV antibody could be detected in oral fluid using NPLA and ELISA assay and the modification of the ELISA was made to enhance the outcome of the antibody detection. Oral fluid concentration and the incubation time have significant effect in the ELISA assay detection ability of the oral fluid. The specimen process and storage is also crucial in the result outcome of antibody detection in oral fluid samples. This assay, when enhanced, can be used in the CSFV monitoring program in the future.

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APPENDIX

APPENDIX A

In vitro data results

Table 6: S/P ratio results of *in vitro* oral fluid samples tested with a normal protocol of commercial indirect ELISA

Normal protocol	S/P ratio of each SN titer level			
	64	32	16	8
SAM1	3.982	2.646	1.25	0.533
SAM2	3.15	1.786	0.81	0.392
SAM3	3.048	1.42	0.707	0.398
SAM4	2.842	1.319	0.707	0.275
SAM5	2.311	1.117	0.576	0.259
SAM6	1.503	0.846	0.337	0.081
SAM7	3.030	1.713	0.882	0.314
SAM8	2.349	1.253	0.715	0.282
SAM9	2.022	1.139	0.609	0.255
SAM10	2.302	1.198	0.698	0.259
SAM11	1.734	0.877	0.440	0.167
SAM12	1.439	0.805	0.305	0.047
SAM13	3.662	2.736	2.123	0.081
SAM14	2.736	1.567	0.648	0.109
SAM15	3.954	1.768	0.581	0.025
SAM16	3.063	1.546	0.648	0.042
SAM17	2.729	1.303	0.394	0.000
SAM18	1.810	0.852	0.092	0.000
Average S/P ratio	2.648	1.438	0.696	0.196

Table 7 *: In vitro* S/P ratio results of different variation of secondary antibody conjugate volume

Conjugate volume modification	S/P ratio of each NPLA titer level							
	150 µl				200 µl			
	64	32	16	8	64	32	16	8
SAM1	3.618	2.449	1.081	0.524	3.651	2.056	1.061	0.322
SAM2	2.931	1.571	0.726	0.345	2.951	1.606	0.835	0.329
SAM3	2.637	1.353	0.684	0.299	2.699	1.342	0.632	0.239
SAM4	2.619	1.306	0.613	0.21	2.904	1.899	0.591	0.229
SAM5	2.157	1.166	0.512	0.222	2.231	1.289	0.53	0.209
SAM6	1.665	0.866	0.352	0.086	1.692	0.908	0.369	0.082
Average	**2.605**	**1.452**	**0.661**	**0.281**	**2.688**	**1.517**	**0.670**	**0.235**

Table 8 *: In vitro* S/P ratio results of different variation of sample incubation temperature

Temperature modification	S/P ratio of each NPLA titer level							
	4°C				37°C			
	64	32	16	8	64	32	16	8
SAM1	2.364	1.163	0.437	0.027	3.743	2.063	0.993	0.211
SAM2	1.163	0.784	0.337	0.084	2.366	1.313	0.794	0.271
SAM3	1.372	0.644	0.285	0.051	2.347	1.000	0.614	0.173
SAM4	1.366	0.655	0.246	0.058	2.274	1.004	0.704	0.224
SAM5	0.783	0.340	0.076	0.000	2.744	0.890	0.253	0.036
SAM6	0.694	0.290	0.072	0.000	1.391	0.978	0.423	0.074
Average	**1.290**	**0.646**	**0.242**	**0.037**	**2.477**	**1.208**	**0.630**	**0.165**

Table 9 *: In vitro* S/P ratio results of different variation of sample incubation time

Time modification	S/P ratio of each NPLA titer level							
	6 hrs*				12 hrs*			
	64	32	16	8	64	32	16	8
SAM1	1.528	0.655	0.000	0.000	4.736	3.590	2.305	1.015
SAM2	0.655	0.549	0.000	0.000	3.590	3.260	1.723	0.729
SAM3	1.433	0.077	0.000	0.000	4.436	2.948	1.579	0.646
SAM4	1.771	0.514	0.000	0.000	5.270	3.483	1.943	0.604
SAM5	0.782	0.000	0.000	0.000	3.839	2.288	1.212	0.368
SAM6	0.577	0.000	0.000	0.000	3.863	2.020	0.833	0.032
Average	**1.124**	**0.299**	**0.000**	**0.000**	**4.289**	**2.931**	**1.599**	**0.566**

*The samples were incubated at 4°C.

Table 10 : Neutralizing antibody titer of serum and *in vivo* oral fluid from each experimental group

Average SN titer of serum samples in each experimental group										
DPV/DPI	-1	3	7	10	14	17	20	24	27	30
A	5.63	5.00	3.88	4.63	4.25	3.50	3.88	4.13	4.00	3.63
B	5.13	5.125	3.63	4.13	4.25	3.50	3.5	3.38	4.13	4.13
C	6.25	5.00	4.00	4.00	4.00	ND	ND	ND	ND	ND
Average SN titer	5.67±1.23	5.04±1.47	3.84±0.95	4.25±0.86	4.17±0.95	3.50±1.03	3.69±0.79	3.76±0.86	4.07±0.77	3.88±0.72
Neutralizing antibody titer of *in vivo* oral fluid samples in each experimental group										
A	2	2	0	1	2	1	2	2	0	1
B	2	2	1	1	1	2	1	1	0	0
C	1	1	0	0	0	ND	ND	ND	ND	ND
Average SN titer	1.67±0.58	1.67±0.58	0.33±0.58	0.67±0.58	1.00±1.00	1.50±0.71	1.50±0.71	1.50±0.71	0.00±0.00	0.50±0.71

ND: No data

Table 11 : Result S/P ratio of *in vivo* oral fluid sample tested with modification in secondary antibody conjugate volume

	S/P ratio of different conjugate volume								
	A			B			C		
DPV/DPI	100 µl	150 µl	200 µl	100 µl	150 µl	200 µl	100 µl	150 µl	200 µl
-1	0.024	0.017	0.009	0	0	0	0	0	0
0	0.006	0	0	0.005	*	*	**	**	**
1	0.013	0.009	0	0	*	*	0	0	0
2	0.01	0.001	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	*	*
4	0	0	0	0	0	0	0	0	0
5	0.013	0	0	0	0	0	0	0	0
6	0	0	0	0.001	0.001	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0.008	0.007	0	0.011	0.008	0.004	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0.059	0.055	0.04	0	0.003	0
12	0	0	0	0	0	0	0	*	*
13	0.002	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0			
15	0.003	0	0	0	0	0			
16	0	0	0	0	0	0			
17	0.01	0	0	0	0	0			
18	0	0	0	0.003	0	0			
19	0.004	0.002	0	0	0	0			
20	0	0	0	0.025	0	0			
21	0.038	0	0	0.015	0.006	0			
22	0	0	0	0.01	0	0			
23	0	0	0	0.023	0.01	0			
24	0	0.012	0	0.013	0.001	0.01			

(A) challenged with CSFV ALD strain, (B) vaccination and (C) negative control

*Not enough amount of sample for testing , **Sample highly contaminated and was discarded

Table 12 : Result S/P ratio of *in vivo* oral fluid sample tested with modification sample in incubation temperature

	S/P ratio of temperature variation								
	A			B			C		
DPI	RT	4°C	37°C	RT	4°C	37°C	RT	4°C	37°C
-1	0.024	0.016	0.019	0	0	0	0	0	0
0	0.006	0.002	0	0.005	*	*	**	**	**
1	0.013	0.023	0.007	0	*	*	0	0	0
2	0.01	0.008	0.005	0	0	0	0	0	0
3	0	0	0	0	0.002	0	0	*	*
4	0	0	0	0	0.013	0	0	0	0
5	0.013	0.005	0.008	0	0	0	0	0	0
6	0	0	0	0.001	0.005	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0.008	0.011	0	0.011	0.01	0.005	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0.002	0	0.059	0.05	0.07	0	0	0
12	0	0	0	0	0	0	0	*	*
13	0.002	0	0	0	0	0	0	0	0
14	0	0.009	0	0	0	0			
15	0.003	0	0	0	0.005	0			
16	0	0	0	0	0.018	0			
17	0.01	0	0	0	0	0			
18	0	0.005	0	0.003	0	0			
19	0.004	0.017	0	0	0.013	0			
20	0	0	0	0.025	0.014	0			
21	0.038	0.007	0	0.015	0.017	0.002			
22	0	0.022	0	0.01	0.011	0			
23	0	0.001	0	0.023	0.018	0			
24	0	0.019	0	0.013	0.02	0			

(A) challenged with CSFV ALD strain, (B) vaccination, (C) negative control, (RT) room temperature

*Not enough amount of sample for testing, **Sample highly contaminated and was discarded

Table 13 : Result S/P ratio of *in vivo* oral fluid sample tested with modification in sample incubation time

	S/P ratio of different incubation time								
	A			B			C		
DPI	1 hr	6 hrs	12 hrs	1 hr	6 hrs	12 hrs	1 hr	6 hrs	12 hrs
-1	0.024	0.024	0.049	0	0	0	0	0	0
0	0.006	0	0.002	0.005	*	*	**	**	**
1	0.013	0.008	0.029	0	*	*	0	0	0
2	0.01	0.001	0.017	0	0	0	0	0	0
3	0	0	0.009	0	0	0.011	0	*	*
4	0	0	0	0	0	0	0	0	0
5	0.013	0.012	0.025	0	0	0	0	0	0.001
6	0	0	0	0.001	0	0.004	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0.001	0	0	0
9	0.008	0.005	0.008	0.011	0.018	0.027	0	0	0
10	0	0	0	0	0	0	0	0	0.014
11	0	0	0	0.059	0.074	0.082	0	0	0.003
12	0	0	0	0	0	0	0	0	0
13	0.002	0	0.001	0	0	0	0	0	0
14	0	0	0.004	0	0	0			
15	0.003	0	0	0	0	0			
16	0	0	0	0	0	0.001			
17	0.01	0	0	0	0	0			
18	0	0	0	0.003	0	0			
19	0.004	0.003	0.004	0	0	0			
20	0	0	0	0.025	0	0.005			
21	0.038	0	0.006	0.015	0.003	0.013			
22	0	0.006	0.006	0.01	0	0.012			
23	0	0	0	0.023	0	0.008			
24	0	0	0	0.013	0.002	0.006			

(A) challenged with CSFV ALD strain, (B) vaccination and (C) negative control

*Not enough amount of sample for testing, **Sample highly contaminated and was discarded

Table 14 : Individual OD from normal ELISA protocol and NPLA results of oral samples from experimental animals

		A		B		C	
AGE	DPI	OD (NP)	NPLA	OD (NP)	NPLA	OD (NP)	NPLA
20	-1	0.224	2	0.161	2	0.143	1
21	0	0.183	0	0.181	0	*	*
22	1	0.199	3	0.16	1	0.148	0
23	2	0.193	2	0.15	2	0.154	0
24	3	0.17	2	0.165	2	0.152	1
25	4	0.157	0	0.154	1	0.157	1
26	5	0.199	2	0.146	1	0.16	1
27	6	0.149	2	0.172	1	0.151	0
28	7	0.145	0	0.171	1	0.159	0
29	8	0.155	3	0.165	2	0.164	0
30	9	0.188	1	0.195	2	0.141	0
31	10	0.168	1	0.146	0	0.148	0
32	11	0.153	0	0.304	3	0.152	0
33	12	0.154	0	0.162	1	0.147	0
34	13	0.174	1	0.154	1	0.168	0
35	14	0.162	2	0.162	0		
36	15	0.177	1	0.166	2		
37	16	0.151	0	0.169	0		
38	17	0.193	1	0.168	2		
39	18	0.164	0	0.177	1		
40	19	0.178	1	0.159	0		
41	20	0.25	0	0.206	1		
42	21	0.22	1	0.196	2		
43	22	0.186	1	0.19	0		
44	23	0.218	3	0.204	1		
45	24	0.167	2	0.194	1		

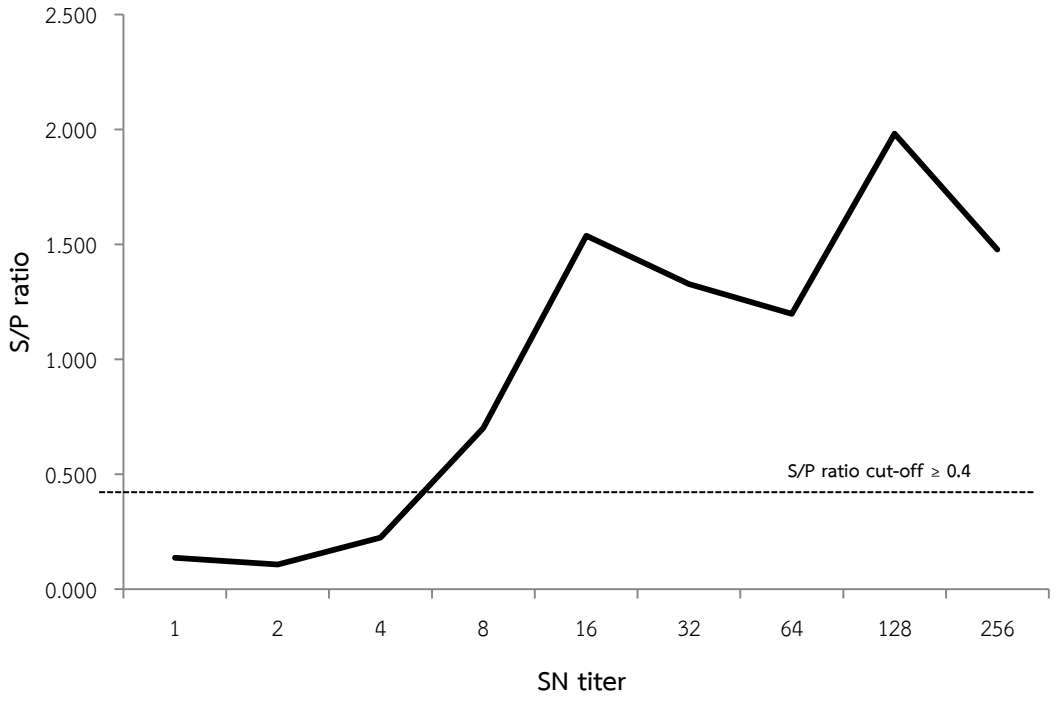
(A) challenged with CSFV ALD strain, (B) vaccination, (C) negative control

* Not enough amount of sample for testing, **Sample highly contaminated and was discarded

APPENDIX B

Preliminary study of detection of serum antibody in ELISA and SN titer

Figure 3 : The average S/P ratio of serum samples with different SN titer tested with Biochek® indirect ELISA. Positive cut of S/P ratio at more than 0.4.



Preliminary study of oral fluid ELISA condition

Using in vitro sample with serum of know SN titer in negative oral fluid samples, ELISA tests were ran to determine the if heating inactivation would enhanced the antibody detection in oral fluid samples using ELISA. Using one sample showed higher S/P ratio level at 15 minutes heat treatment (56°C) prior to testing.

Table 15 : S/P ratio level of *in vitro* samples process in heat treatment at 0, 15 and 30 minutes. The serum of known CSFV SN level was also diluted in PBS and in negative serum for comparison of S/P ratio with the serum diluted in negative oral fluid.

	S/P ratio				
SN titer	0 min	15 min	30 min	PBS	Serum
4	0.000	0.000	0.000	0.000	0.151
8	0.000	0.000	0.000	0.000	0.243
16	0.271	0.281	0.148	0.246	0.506
32	0.651	1.003	0.565	0.824	1.178

APPENDIX C

Figure 4 : Pictures of oral fluid collection

VITA

Miss Panchan Sitthicharoenchai has received her degree of doctor of veterinary medicine at Chulalongkorn University in 2012. She focused her work on veterinary pathology and large animal infectious disease and continue her graduate degree program in veterinary pathology at Chulalongkorn University. Her previous researches such as studying the characteristics of feline mammary gland tumour, optimising diagnostic techniques for classical swine fever virus and testing vaccination proficiency of swine diseases.