CORRELATION OF THE CLASSICAL SWINE FEVER (CSF) ANTIBODY LEVELS DETECTED BY SERUM NEUTRALIZATON AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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ความสัมพันธ์ของระดับแอนติบอดีต่อโรคอหิวาต์สุกรที่ตรวจได้จากปฏิกิริยาลบล้างฤทธิ์และอิไลซา

น.ส.ชัยรานี ริดา มาฟิรา

จุฬาลงกรณ์มหาวิทยาลัย
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	CORRELATION OF THE CLASSICAL SWINE FEVER (CSF) ANTIBODY LEVELS DETECTED BY SERUM NEUTRALIZATON AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)
By	Miss Chairani Ridha Maghfira
Field of Study	Veterinary Science and technology
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ชัยรานี ริดา มาฟิรา : ความสัมพันธ์ของระดับแอนติบอดีต่อโรคอหิวาต์สุกรที่ตรวจได้จากปฏิกิริยาลบล้างฤทธิ์และอิไลซา. (CORRELATION OF THE CLASSICAL SWINE FEVER (CSF) ANTIBODY LEVELS DETECTED BY SERUM NEUTRALIZATON AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)) อ.ที่ปรึกษาหลัก : เยาวลักษณ์ ปัญญสิงห์

โรคอหิวาต์สุกรเป็นโรคติดเชื้อไวรัสที่สำคัญในสุกร ที่มีผลกระทบกับอุตสาหกรรมการเลี้ยงสุกรเป็นอย่างมาก การตรวจภูมิคุ้มกันต่อเชื้ออหิวาต์สุกรด้วยชุดตรวจอิไลซา (ELISA) เป็นวิธีการตรวจแอนติบอดีที่สามารถทำได้ง่ายและสะดวกในทางปฏิบัติเมื่อเทียบกับการตรวจแอนติบอดีด้วยปฏิกิริยาลบล้างฤทธิ์ (serum neutralization test) อย่างไรก็ตาม ระดับแอนติบอดีที่ตรวจได้จากชุดตรวจอิไลซาไม่สามารถแสดงระดับแอนติบอดีในการป้องกันโรคได้เหมือนการตรวจด้วยปฏิกิริยาลบล้างฤทธิ์ การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความสัมพันธ์ของระดับแอนติบอดีที่ตรวจด้วยชุดตรวจอิไลซาและปฏิกิริยาลบล้างฤทธิ์ โดยการตรวจแอนติบอดีต่อโรคอหิวาต์สุกรจากตัวอย่างซีรัมสุกรที่เป็นลบและบวก จำนวน 522 ตัวอย่าง ด้วยชุดตรวจอิไลซาและปฏิกิริยาลบล้างฤทธิ์ และประเมินความสัมพันธ์ของระดับแอนติบอดีที่ตรวจพบ ความสอดคล้องของผลตรวจที่ได้จากทั้งสองวิธีการ และวิเคราะห์ระดับแอนติบอดีที่ตรวจด้วยวิธีอิไลซาที่ระดับแอนติบอดีไตเตอร์ต่าง ๆ ผลการศึกษาพบว่า ระดับแอนติบอดีต่อโรคอหิวาต์สุกรจากชุดตรวจอิไลซามีความสัมพันธ์กับระดับแอนติบอดีไตเตอร์ที่ตรวจจากปฏิกิริยาลบล้างฤทธิ์อย่างมีนัยสำคัญทางสถิติ โดยพบความสัมพันธ์เชิงบวกระหว่างค่า sample-to-positive (S/P) จากชุดตรวจอิไลซาและระดับแอนติบอดีไตเตอร์ที่ตรวจจากปฏิกิริยาลบล้างฤทธิ์ (r_s = 0.89; p<0.0001) และพบความสอดคล้องของผลตรวจที่ได้จากทั้งสองวิธีการ (ค่าสัมประสิทธิ์แคปปา = 0.913) นอกจากนี้ พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ (p<0.0001) ของค่าเฉลี่ย S/P จากกลุ่มตัวอย่างในแต่ละระดับแอนติบอดีไตเตอร์ ได้แก่ กลุ่มตัวอย่างที่มีระดับแอนติบอดีเป็นลบ (แอนติบอดีไตเตอร์ <2) กลุ่มตัวอย่างที่มีระดับแอนติบอดีไตเตอร์ต่ำกว่าระดับป้องกันโรค (แอนติบอดีไตเตอร์ <32) และกลุ่มตัวอย่างที่มีระดับแอนติบอดีไตเตอร์ที่ระดับป้องกันโรค (แอนติบอดีไตเตอร์ ≥32) กลุ่มตัวอย่างที่มีระดับแอนติบอดีไตเตอร์ต่ำกว่าระดับที่สามารถป้องกันโรคได้ มีค่าเฉลี่ย S/P ที่ 1.132±0.587 และกลุ่มตัวอย่างที่มีระดับแอนติบอดีไตเตอร์ที่ระดับป้องกันโรคได้ มีค่าเฉลี่ย S/P ที่ 1.767±0.479 ดังนั้น ความสัมพันธ์อย่างมีนัยสำคัญทางสถิติของค่า S/P และระดับแอนติบอดีไตเตอร์ที่ได้จากการศึกษานี้ สามารถให้ข้อมูลที่เป็นประโยชน์สำหรับผลตรวจที่ได้จากวิธีอิไลซาในการตรวจหาแอนติบอดีและการประเมินสถานะของภูมิคุ้มกันในการป้องกันโรคในระดับฝูง

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Chairani Ridha Maghfira : CORRELATION OF THE CLASSICAL SWINE FEVER (CSF) ANTIBODY LEVELS DETECTED BY SERUM NEUTRALIZATON AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA). Advisor: Assoc. Prof. Dr. YAOWALAK PANYASING

Classical swine fever is an important viral disease that has a devastating impact on the swine industry. The Enzyme-linked immunosorbent assay (ELISA) provides a simpler and more practical approach to detect classical swine fever virus (CSFV) antibodies compared to the serum neutralization (SN) test. However, antibody responses detected by ELISA cannot directly exhibit a protective level. This study aimed to evaluate the correlation of classical swine fever antibody responses detected by SN assay and commercial ELISA. A total of 522 negative and positive serum samples were tested by the SN and ELISA. Correlation, an agreement between two assays, and comparisons of sample-to-positive (S/P) values among the level of SN titers were evaluated. The results revealed a strong positive relationship (r_s 0.89; p<0.0001) and excellent agreement with the Kappa value of 0.913 between the S/P values and SN titers. The comparison tests showed a statistically significant difference (p<0.0001) between the mean S/P values among three distinct levels of SN titers, i.e., negative SN titers (antibody titer <2), SN titers below protective level (antibody titer <32), and SN titers at protective levels (antibody titer ≥32). Positive samples having antibody titers below and at protective levels showed S/P values at 1.132±0.587 and 1.767±0.479, respectively. Therefore, the strong correlation between the S/P values and neutralizing antibody titers could provide useful information in terms of detecting CSFV antibodies and estimating protective status of antibody positive animals by the ELISA method.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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Chairani Ridha Maghfira

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LIST OF ABBREVIATIONS

µl	microlitre
ANOVA	analysis of variant
BAU/ml	binding antibody units per millilitre
BSA	bovine serum albumin
cELISA	competitive enzyme-linked immunosorbent assay
CI	Confidence interval
CO_2	carbon dioxide
CSFV	classical swine fever virus
DIVA	differentiating infected from vaccinated animals
dpv	days post vaccination
E2	envelope glycoprotein 2
e.g.,	exempli gratia, for example
Erns	envelope ribonuclease glycoprotein
et al.,	et alii, and others
FAS	FS-7-associated surface antigen
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
h	hour (s)
HRP	horseradish peroxidase
i.e.,	id est, that is
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IPMA	immunoperoxidase monolayer assay
log	logarithm

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M	Molar
M cells	microfold cells
mAb	monoclonal antibody
MEM	minimal essential medium
min	minute (s)
ml	millilitre
MLV	modified live vaccine
MV	mean value
nAb	neutralizing antibody
nm	nanometre
NPLA	neutralizing peroxidase linked assay
NS3	non-structural protein 3
°C	degree Celsius
OD	optical density
PBST	phosphate-buffered saline with Tween
S/P	sample-to-positive ratio
SD	standard deviation
SE	standard error
SN	serum neutralization
TCID	tissue culture infectious dose
Th	T helper cells
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
WOAH	world organization for animal health

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CHAPTER 1

INTRODUCTION

Classical swine fever (CSF) is a contagious viral disease in pigs caused by classical swine fever virus (CSFV) belonging to the family *Flaviviridae*, genus *Pestivirus* (Moennig, 2000). The CSF outbreak in domestic pigs contributes to a severe trade impact on pigs and pig products. This disease has been noted as the top ten highest causes of pig losses globally (2006-2009), up to 21,953 livestock units (WorldBank, 2011). Accordingly, CSF is also included in the World Organization for Animal Health (WOAH) important listed diseases. Implementing stamping-out policy following mass vaccination is suggested as one of eradications strategy when an outbreak occurs in previously free country (WOAH, 2022b). This approach has been proven by large parts of the European Union, which succeeded in holding CSF-free status by WOAH (Postel *et al.*, 2018). However, culling as strict eradication control is considered destructive and may affect food security issues. Thus, vaccination as an alternative eradication strategy is more likely to be considered, particularly in the endemic CSF region including Thailand (WOAH, 2022c).

The modified live vaccine (MLV) is commonly used to control the disease, especially in the endemic area. This vaccine provides several advantages, i.e., safe for young pigs and pregnant sows, rapid onset protection with only a single dose required, and it can be applied orally (Coronado *et al.*, 2021). The drawback is this type of vaccine may interfere the serological detection due to lack of differentiating infected from vaccinated animals (DIVA) properties, in which vaccine-induced antibodies cannot be distinguished from infection-induced antibodies by serological test (Postel *et al*., 2018; Coronado *et al*., 2021). In addition, vaccination program has also made the virulency of the virus decreasing. This situation has led to the highly

variable CSF clinical signs from mild to atypical, and therefore laboratory diagnosis for CSF is indispensable.

Serum neutralization (SN) method or virus neutralization test (VNT) is serological-based detection that can be used to measure neutralizing antibodies of CSFV. Neutralizing antibodies in serum is reported to be reliably correlate of protection. This technique serves as the gold standard for classical swine fever antibody detection and commonly used for post-vaccination evaluation or surveillance of infection (WOAH, 2022a). However, serum neutralization assay is known to be laborious, time-consuming, and requires a meticulous procedure.

On the other hand, commercial serological test to distinguish between infected and vaccinated pigs, i.e., enzyme-linked immunosorbent assay (ELISA) have been developed and evaluated in previous studies (Schroeder *et al.*, 2012; Pannhorst *et al.*, 2015; Meyer *et al.*, 2017; Wang *et al.*, 2020a; WOAH, 2022a). The studies shown that commercially available ELISA in combine with marker vaccines can improve routine CSFV antibody screening. Nevertheless, ascertaining the presence of antibodies in endemic areas by serological detection such as ELISA will be challenging. In country where the pig populations have been exposed to infection and vaccination, particularly live vaccine, antibody responses detected by ELISA cannot directly exhibit the estimation of the protection level. Therefore, despite providing many advantages, ELISA has not been used for the detection of CSF antibodies in endemic areas. The commercial ELISA was designed for differentiating infected and vaccinated animals target the detection of Erns antibody which is one of the neutralizing antibodies. The estimation range of protective antibody levels that correlated with standard antibody assay could implement an application for the use of ELISA as a monitoring tool in the endemic area. Therefore, this study aimed to evaluate the correlation of the CSF antibody levels detected by the commercial Erns ELISA and SN assay.

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Objectives of study

To evaluate the correlation of the classical swine fever antibody levels detected by enzyme-linked immunosorbent assay (ELISA) and serum neutralization assay.

Keywords (Thai): แอนติบอดี อหิวาต์สุกร อิไลซา ปฏิกิริยาลบล้างฤทธิ์

Keywords (English): Antibody, classical swine fever, ELISA, serum neutralization

Hypothesis

The level of classical swine fever Erns antibody detected by enzyme-linked immunosorbent assay is correlated with the antibody titer detected by serum neutralization assay.

Advantages of the study

The relationship of antibody response detected by ELISA and serum neutralization assay was evaluated. The results from this study can be applied for evaluating the status of the herd antibody protective level against classical swine fever virus using a common method, i.e., ELISA.

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CHAPTER 2

LITERATURE REVIEW

Classical swine fever

The disease

Classical swine fever (CSF) or also known as hog cholera is a viral disease that seriously impacts the socio-economic pig industrial trade worldwide. CSF is still reported to be a significant problem and endemic in large part of the world. According to World Organization for Animal Health (WOAH), CSF-free countries are held for a long time by North America, Australia, and New Zealand then followed by some parts of South America and some west parts of Europe afterwards. Meanwhile, Africa and large parts of Asia are still variously recognized, from area without CSF official status to an endemic region (WOAH, 2022c).

Mortality in CSF acute-lethal courses can reach to 100% depend on the age and the virulence strain (Blome *et al.*, 2017; Postel *et al.*, 2018). Besides fever as its name tends to, CSF also characterized by a wide range of clinical signs from atypical i.e., general weakness, anorexia, gastrointestinal symptoms, to typical signs i.e., neurological signs and skin hemorrhages or cyanosis. Mostly the symptom is not presented exclusively to CSF. In general, the incubation period in individual pig ranges between 4 and 10 days after infection. In acute form, death usually occurs 2 to 4 weeks after CSFV infection. While progression of the infection in herds is relatively slow, hence, the infection becomes evident only after several weeks after introduction of the virus into the herd (Blome *et al.*, 2017). The chronic form may take up to a month until they are truly recognized. Atypical clinical signs can be present throughout and until death, occurring up to three months or even later after the infection. Viral shedding is observed from about four days post infection until the death of the animal (Blome *et al.*, 2017). Lastly, late onset CSF which is a

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consequence of moderately virulent strains. This form is also known as the sequelae of intrauterine or very early post-natal infection leading to persistent viremia. In the early and up to 70 days of gestation, intrauterine infections may lead to the birth of persistently viremic animals which appear clinically normal or retarded in growth (Dahle and Liess, 1992). Infected pigs in late onset courses can survive from a few weeks to several months and constantly shed large amounts of virus, thus contributing as a dangerous virus reservoir.

Etiology

Classical swine fever virus (CSFV) is an etiologic agent of CSF. This virus is a member of the genus *Pestivirus* of the *Flaviviridae* family. Recently, study has reported the expand number of pestiviruses up to 19 proposed species from *Pestivirus A* to *Pestivirus S* (Postel *et al.*, 2021). Of the 19 proposed species, *Pestivirus C* is the species that correspond to classical swine fever and has specific host-species preferences which only infected *Sus scrofa* (Smith *et al.*, 2017; Postel *et al.*, 2021). The predominant group of CSFV circulated in Thailand recently belongs to genotype 2.2, which shows similar characteristic to the CSFV of Europe origin (Suradhat *et al.*, 2007). This genogroup was first isolated in 1996, and most of this group causes milder to moderate clinical symptoms from subacute to chronic (Suradhat *et al.*, 2007). Classical swine fever virus has a small size of around 12.3 kb, enveloped, single-stranded RNA (Zhang *et al.*, 2011). The genome of CSFV encodes for several proteins, including structural and non-structural (NS) proteins. Structural protein includes the capsid protein C and envelope proteins Erns, E1, E2. Non-structural proteins are Npro, p7, NS2, NS3, NS4a, NS4b, NS5a, NS5b proteins (Beer *et al.*, 2007; Pannhorst *et al.*, 2015). Three CSFV proteins (NS3, Erns, and E2) can induce antibodies to viral infections. Nevertheless, only Erns and E2 can elicit the neutralizing antibodies and generate protective immunity (Pannhorst *et al.*, 2015). The Erns protein has an

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essential role in post-entry stages during transmission. It is also implicated in the evasion of host interferon (IFN) responses and is considered a secondary glycoprotein that mediates neutralization (Zhang *et al.*, 2011). In contrast, the E2 protein is the major neutralizing antigen. It has more significant immunogen potential for CSFV infection, hence favoured as the basis for vaccine development (Pannhorst *et al.*, 2015).

Transmission

Once CSFV enters the entry port, the virus may pass through the tonsillar crypts epithelium and M-cells for replication (Summerfield and Ruggli, 2015). The primary target sites of CSFV replication are the tonsils and oropharyngeal lymph nodes. The structural protein of envelope glycoproteins, E1 and E2, are required for virus entry into the cell through clathrin-dependent, receptor-mediated endocytosis (McCarthy *et al.*, 2019). From the primary target tissue, CSFV may spread to other lymphoid organs through the lymphatic system. Some organs that poses as the secondary target are gut-associated lymphoid tissue, lymph nodes, bone marrow, spleen, and thymus (Summerfield and Ruggli, 2015). Eventually, further replication may come off as the virus spread to all body organs through the circulatory system (McCarthy *et al.*, 2019).

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Classical swine fever virus replication is limited to the cytoplasm and it has characteristics of a non-cytopathic effect (Summerfield and Ruggli, 2015). The assembly of virion occurs on intracellular membranes of the endoplasmic reticulum (ER), and progeny virus is released from the cells at 5–6 h post-infection via exocytosis (Summerfield and Ruggli, 2015). Viremia and virus shedding may develop in CSFV-infected pigs at the early stage of clinical disease until the animal die or specific antibodies have generated (Blome *et al.*, 2017). The virus can be shed in all excretions and secretions during the clinical phase of the infection (Postel *et al.*,

2018). Mainly, it excreted through saliva, lacrimal secretions, urine, feces, and semen (Blome *et al.*, 2017). These types of body fluid can be the source of CSFV transmission to another pig through the oronasal route. In addition, CSFV can also be transmitted through the meat product and pigs that eating the CSF-infected pork meat or product may become infected. Study reported CSFV survival in several pork product and shows resistant in frozen meat at around -70 ºC (Farez and Morley, 1997).

Immune response to CSFV infection and vaccination

Pig immune defences to CSFV infection involves both the humoral and T-cell mediated immunity (Postel *et al.*, 2018). Several immune cells are known to be the primary target of the CSFV, such as macrophages, endothelial cells, and dendritic cells (DC), either conventional (cDC) or plasmacytoid dendritic cell (pDC) (Summerfield and Ruggli, 2015). In highly virulent strains of infection, the host animal may die even before seroconversion occurs. While in the low virulent infection, controlled to chronic disease may occur indicated by transient lymphopenia. During this condition, the immune system is unable to clear the infection completely, hence the animal may shed the virus. The virulency of infection is reported to be associated with the level of interferon-alpha (IFN-**α**), where the early phase is signed with the high level of IFN-**α** (Summerfield and Ruggli, 2015). Formerly, it was known that the role of IFNs could induce the expression of antiviral proteins during viral infection. However, it cannot be proven in younger pigs since the high levels of IFN-**α** were discovered to be counterproductive. High levels of IFN-**α** in young pigs correlate with the depletion of peripheral B and T lymphocytes, leading to lymphoid depletion and lymphopenia. Clinically, this persistent deviation response presents as viral haemorrhagic fever. It indicates that IFN-**α** is unable to control the virus and turns to mediate the development of an aberrant immune response. Furthermore, it

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also discovered that CSFV-infected pigs might develop the receptors of cell death and apoptosis pathways such as TRAIL, FAS, and TNF (Summerfield and Ruggli, 2015). Besides IFNs, acute and severe CSF pathogenesis are associated with the infection and activation of macrophages. It is probably shown that macrophages are responsible for the CSFV pathogenesis by producing macrophage pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) and vasoactive mediators, including prostaglandin E2 and platelet activation. Infection and activation of dendritic cells were also observed. During CSF infection, pDC was demonstrated to produce abundant IFN-α and IL-12 by Th-1. In contrast to macrophages, cDC was discovered to contribute to CSFV response, but they may also counteract it by producing anti-inflammatory IL-10 (Summerfield and Ruggli, 2015).

Humoral-mediated immunity (HMI) occurs as an adaptation of the immune system against CSFV infection. This mechanism involved an activated B-cells to become immunoglobulin-producing plasma cells. As previously allude that not all the generating antibodies responsible for the sterilization against the pathogen. Neutralizing antibodies response is the antibody that will achieves sterilizing protection against CSFV and able to confer clinical protection against CSFV challenge (Rümenapf *et al.*, 1991). Detection of induced antibodies to reflect vaccine-induce immunity can be evaluated by serum neutralization (SN). The SN titers of equal or greater than 32 (the log2 of antibody titer $\geq$5) is recorded as acceptable protective levels for CSF, preventing viral excretion, and transmission (Terpstra and Wensvoort, 1988). Neutralizing antibodies to E2 protein, which is the major immunogenic protein, reported to produce between 10 and 20 days after infection (Terpstra and Wensvoort, 1988; Van Rijn *et al.*, 1996).

Cell-mediated immunity (CMI) is generally known to have an essential role for immunity against intracellular pathogens, including viruses. The antigen-specific lymphoproliferative activity may exist in the blood of protected pigs due to

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vaccination (Remond *et al.*, 1981; Suradhat *et al.*, 2007). Assessing CMI can be done by measuring cytokines which are known to relate to cellular immunity. Interferon gamma (IFN-**γ**) is one of the cytokines subpopulations that is known well in regard to the induction of CMI. Viral-specific IFN-**γ** producing cells is a good indicator of antiviral immunity. Furthermore, IFN-**γ** production remains detectable for a long period of time, whereas lymphocyte proliferation tends to diminish due to the decrease ability of T cells to produce IL-2 (Suradhat *et al.*, 2007). In addition, study has been reported that the early protection in vaccinated pigs has an association with the induction of the interferon stimulated genes 15 (ISG15) pathway which correspond to the elevation of several antiviral effectors of innate immune response (McCarthy *et al.*, 2019)

Regarding the immune response derived vaccination, study about the modified live Chinese strain vaccine and its immunology mechanism to induce protective immunity has been discussed. Historically, Chinese strain vaccine has been regarded as one of the most effective, safe even in pregnant sows, and provide complete protection with rapid onset. Detectable neutralizing antibodies induced by this vaccine usually shown in 2 to 3 weeks post vaccination (Suradhat *et al.*, 2007). Another study mentioned that neutralizing antibodies in C strain vaccinated pigs could not be detected until 14 dpv by NPLA, but the titer is increased at 28 dpv and reached maximum titer at 42 dpv (Freitas *et al.*, 2009; Nath *et al.*, 2016). The recent study observed that adaptive cell-mediated immunity may be shown in early phase within 8 to 10 days post vaccination (dpv), and partial protection observed in 5 dpv (McCarthy *et al.*, 2019). In addition, it was observed that piglets born from vaccinated sows during 1 month of pregnancy had a high level of passive antibody titer compared to non-immune sows (Freitas *et al.*, 2009).

Maternal derived antibodies (MDA) can be transmitted to piglets and persist until about 2 – 4 months of age (WOAH, 2022b). In wild boars, the MDA that is

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transmitted to offspring can persist for several years within the individual or wild boar population (Blome *et al.*, 2017). In regard to the antibody titer, study reported that SN titers of 1:2 to 1:4 is the proper time for CSF vaccination (Direksin *et al.*, 2016). Maternal derived antibody SN titers of greater than 1:64 may inhibit efficacy of vaccination (Suradhat and Damrongwatanapokin, 2003). Vaccination can be carried out to the young pig at the time when maternal-derived antibody (MDA) is decreasing that is around 6 – 8 weeks of age (WOAH, 2022a).

Diagnosis of CSF

Diagnosis of CSF consists of several procedures from the clinical sign, gross pathological findings, direct approach to antigen or virus isolation, and indirect approach through antibody detection. In the recent decade, the occurrence of low virulent strain has made the variety of CSF clinical signs from mild to atypical (Postel *et al.*, 2018). It may obscure the diagnosis, particularly through the antigen detection, as some of the strains also show only a short period of viremia (Postel *et al.*, 2018). This situation may lead to a suspicious condition where the infection has occurred after a long period of time, thus antibody-based detection could be an option. The worldwide guideline regarding antigen and serological detection of CSFV infections are well described in the OIE Terrestrial Manual 2022.

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Virus detection

Direct detection of the nucleic acid or antigen can be done by using RT-PCR as the most sensitive tools, virus isolation, fluorescence antibody test (FAT), immunoperoxidase staining with polyclonal or monoclonal antibody conjugates on fixed cryosections of organ material, and antigen ELISA. However, antigen-capture ELISA is not commonly use since it has lower sensitivity and specificity (WOAH, 2022a).

Detection of viral nucleic acid by RT-PCR was popularly used since early nineties (Liu *et al.*, 1991; Roehe and Woodward, 1991; Katz *et al.*, 1993). Study reported that compared to virus isolation in cell culture, detection of viral nucleic acid can be carried out at the earlier time after infection and later after the pigs recovered (Greiser-Wilke *et al.*, 2007). Apart to its advantage, RT-PCR also have the drawbacks that are vulnerable to false negative or positive results. It turns out to false negative when the nucleic acid is degraded or contributed by the inhibitors in the reaction mixture. Whereas the false positive results probably may arise because of its sensitivity to recognize any chemicals from contaminated sources (Greiser-Wilke *et al.*, 2007).

Virus isolation is the method that utilizes cell cultures. The widely used cell lines are porcine kidney cells i.e., PK-15 or SK-6. As mentioned before, CSFV does not cause a cytopathic effect, the visualization can be done by using immunofluorescence tests (IFT) or by immunoperoxidase assays (PLA). This method is considered as the gold standard for confirming CSF outbreaks even though is a time consuming and labour intensive (Greiser-Wilke *et al.*, 2007).

On the other hand, antigen detection by FAT in experimentally infected pigs showed that the detection of CSFV were observed in days 2 to 15 post infection in tonsil samples. Study reported that pigs with clear clinical signs show the high probability for the presence of virus in tonsils compared to those without clinical signs (Greiser-Wilke *et al.*, 2007). The advantages of FAT are rapid to perform and used staining method for direct CSFV visualization. This test utilizes fluorescein isothiocyanate (FITC) labelled antibodies to detect CSFV protein in the fixed tissues. However, this method may not be conclusive and specific, hence the further detection through RT-PCR or virus isolation should be done for the differentiation of CSFV from other type of pestiviruses especially BVDV or BDV in FAT positive samples. Recently, study reported that this situation can also be handled with in situ

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hybridization using specific probes of CSFV RNA (Zhang *et al.*, 2017). This method has the potential to detect CSFV in PK-15 cells. However, the applicability in swine tissues has yet to be described, and it is yet to be commercially available.

Antibody detection

Serological detection of CSFV is known as appropriate approach for surveillance and evaluating immune status either in individual animal or herd populations at post vaccination. According to OIE Terrestrial Manual 2022, antibodies detection can be evaluated appropriately at least 21 days post-infection (WOAH, 2022a). Besides ELISA, neutralizing peroxidase-linked assay (NPLA) is also the common and favoured used techniques for CSFV serological detection according to WOAH. NPLA is considered as the gold standard for monitoring and evaluation the efficacy of CSF vaccines (Wang *et al.*, 2020b).

Neutralizing peroxidase linked assay (NPLA) is a serum neutralizing-based detection that will help to determine the capability of a neutralizing antibody to stand against specific infection of certain antigen. As illustrated in Figure 1, the general principle of the NPLA—hereinafter SN—involves the neutralization of antigen by a specific series-dilute antibody which eventually remains the cell culture or host intact within the mixture in the well. This assay can show the concentration of antibodies that sufficient to neutralize the virus (Santana-Rodríguez *et al.*, 2022). According to WOAH, there are two point of internal control that needs to be included in the test i.e., positive control serum and back-titration of test virus. Virus back-titration used as an internal control should be between 30 to 300 $TCID_{50}$/50 µl (WOAH, 2022a). In addition, the SN assay can be used as comparative neutralization test in the end-point titration for discriminating between CSFV-infected and other typical pestiviruses-infected pigs. The procedure involved the reaction of suspected serum sample against tissue culture infectious dose (TCID 50) of representative strain

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of CSFV, BVDV, and BDV, with the cell lines that is also suitable for each strain of virus (WOAH, 2022a).

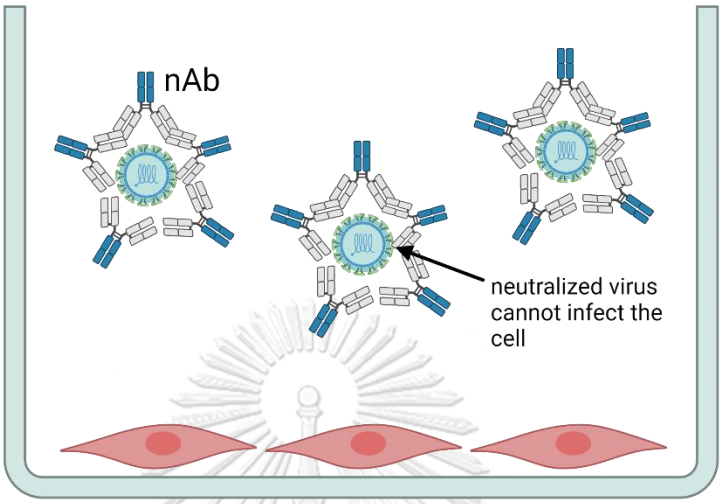

Figure 1 Illustration of serum neutralization assay principle in a well. The presence of nAb that fit to certain epitope of the virus will neutralize against the virus and prevent the infection to the cell. nAb, neutralizing antibody. (Figure created with Biorender.com)

Previous evaluation study has investigated the SN titer level according to its ability to provide the protection against the infection and reduce the clinical signs and virus excretion (Terpstra and Wensvoort, 1988). Negative SN titer (<2) indicates low to absent of neutralizing antibody. Experiment in vaccinated pigs with pre-challenge titer <12.5 showed the lowest level of protection with 3 out of 11 pigs succumbed to the challenge, indicating insufficient amount of antibody to prevent disease and death (Terpstra and Wensvoort, 1988). On the other hand, of 12 vaccinated pigs with pre-challenge titer around 25 to 49, 4 pigs were found to excrete the virus (Terpstra and Wensvoort, 1988). This SN titer range indicated the adequate protection to individual as well as herd population but insufficient protection against excretion and/or transmission of virus. Lastly, among 14 vaccinated

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pigs with pre-challenge SN titer >50, only 2 pigs experienced fever and the remaining pigs showed no significant clinical sign. Altogether, it appeared that SN titer ≥32 offer adequate protection to the disease and against excretion or transmission of the virus (Terpstra and Wensvoort, 1988).

Enzyme linked immunosorbent assay (ELISA) is a technique that involves antigen antibody complex reactions and enzyme-labeled reactant to allow quantification through the development of color after the addition of a suitable chromogen. Antibody ELISA kits are designed to capture antibody by certain match antigen that already coated within the bottom surface of the well.

Animals infected with CSFV may develop antibodies to different antigens, there are the viral envelope protein Erns or E2. Likewise, pigs vaccinated with MLV vaccine may also developed E2 antibodies. Serological methods are unable to differentiate vaccinated from infected pigs or even discriminate animals vaccinated with live attenuated or E2 subunit vaccine, thus ELISA with DIVA properties is developed correspond to the development of various marker vaccine (WOAH, 2022a).

The Pigtype® CSFV Erns Ab (Indical Bioscience, GmBH) is a double-antigen ELISA that provides DIVA properties since it will specifically detect the antibodies to the Erns protein in pig serum or plasma. This type of assay will be appropriate for evaluation of sample obtained from marker-vaccinated pigs particularly E2 subunit vaccines, which do not induce a CSFV immune response specific for the Erns protein (Pannhorst *et al.*, 2015). Therefore, Erns positive by this assay could reflect the possibility of natural infection. Diagnostic performance evaluated in prototype of CSFV Erns Ab ELISA has been discussed and the results showed that the sensitivity represent 90.2% while the specificity is 93.8% with combination of chimeric marker vaccine (Meyer *et al.*, 2017). As illustrated in Figure 2, principally, double-antigen CSFV Erns Ab ELISA is designed to capture antibodies of the CSFV Erns protein from

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the sample with the immobilized recombinant antigen Erns that coated within the plates. The unbound material will be removed by rinsing. Antibodies-antigen complex then will be detected by horseradish peroxidase (HRP) linked Erns antigen conjugate. The unbound conjugate will be removed by rinsing. A colorimetric reaction is initiated by adding chromogenic substrate e.g. TMB and stopped by stop solution (Meyer *et al.*, 2017). In this type of ELISA, if antibodies of interest are presented in the sample, blue colour product will be developed by the substrate and turned yellow after stop solution is added. The optical density (OD) is measured in a spectrophotometer 450 nm (Meyer *et al.*, 2017).

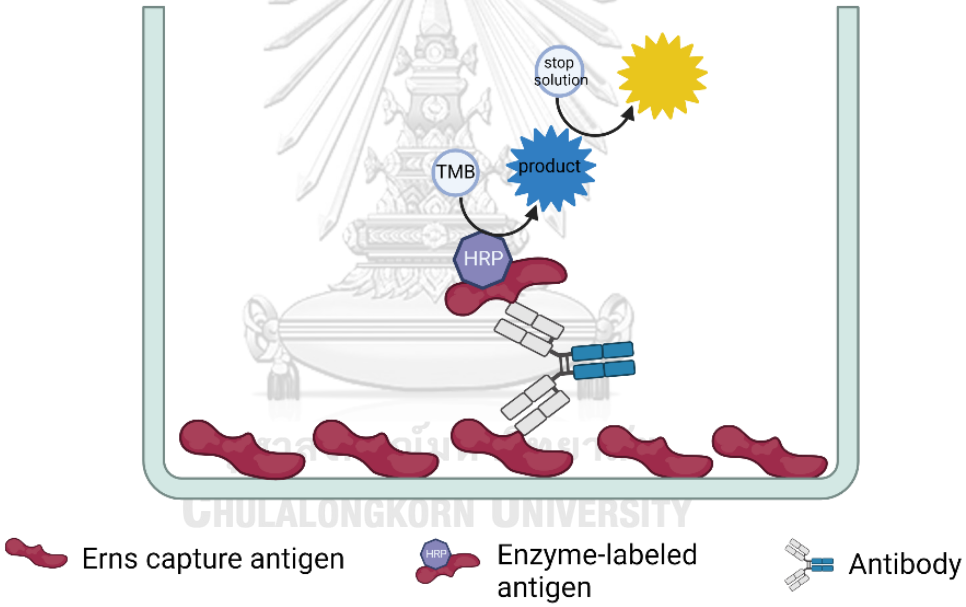

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Figure 2 Illustration of double-antigen CSFV Erns Ab ELISA principle in a well. Erns capture antigen is coated in the well plate to bind with the targeted antibody in the serum samples. TMB, 3,3,5,5-tetramethylbenzidine; HRP, horseradish peroxidase. (Figure created with Biorender.com)

Detection of CSFV Erns antibody

In recent years, while the modified live vaccines or E2-based vaccines is well-established, study has also reported the capture Erns protein or mAb against Erns in the development of DIVA assay. It is known that some certain antibodies are specific to certain Erns epitopes and aid the differentiation of vaccines strains from natural infections. The viral envelope Erns is one of the CSFV structural proteins, membrane-bound, and a second major target for neutralizing-antibody. Among the structural proteins of CSFV, Erns is the highly glycosylated with N-linked glycan (Ruggli *et al.*, 2005). N-glycan of CSFV Erns is essential for Erns blocking of IFN-ß induction (Bauhofer *et al.*, 2007; Seago *et al.*, 2007; Xia *et al.*, 2007). The Erns protein has been reported to be involved in the initial attachment process of CSFV entry into the cell and it also plays an important role in the post-entry stages (Van Gennip *et al.*, 2000; Zhang *et al.*, 2006; Zhang *et al.*, 2011).

Recent study has discovered five monoclonal antibodies (mAb) against Erns, there are 1104, 1204, 1504, 1904, and 2004 (Mi *et al.*, 2022). In addition, the study has reported 8 epitopes in Erns protein successfully mapped by mAbs, there are ^{31}GIWPEKIC^{38}, ^{65}NYTCCKLQ^{72}, ^{73}RHEWNKHGW^{81}, ^{88}DPWIQLMNR^{96}, ^{116}YDKNTDVNV^{124}, ^{127}QARNRPTT^{134}, ^{145}SFAGTVIE^{152}, and ^{161}VEDILY^{166}. Off 8 epitopes, the previous study has identified 2 conformational epitopes recognized by mAb 1204 and 1104, respectively. The broadly CSFV epitope ^{38}CKGVP^{42},W^{81} that recognized by mAb 1204 is completely conservative among different sub-genotypes. On the other hand, the mAb 1104 group has recognized highly c-strain-vaccine specific epitope with the critical determinant indicated by the amino acid combination of D^{100}/V^{107}, therefore, the elicits-mAb is able to differentiate vaccine strains from field isolates (Mi *et al.*, 2022).

Detection of CSFV-Erns antibodies can be conducted by antibody-based assay including ELISA coated with specific capture protein, western blot, SDS-page, or

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serum neutralization. Previous evaluation study has reported that the sample specimens for detecting Erns antibodies specific to CSFV is not limited to serum specimen but also oral fluid (Panyasing *et al.*, 2018). The study investigated that the detection of CSFV Erns antibodies-isotype specific in oral fluid has a satisfy and consistent results to the serum specimen testing. Diagnostic sensitivity and specificity of Erns IgG indirect ELISA in oral fluid revealed 97.5% and 100%, respectively. Whereas Erns IgA ELISA in oral fluid revealed 95.5% and 97.1%. Both oral fluid and serum specimens, the Erns IgG ELISAs were likely provided better diagnostic performance compared to Erns IgA ELISAs (Panyasing *et al.*, 2018).

Diagnostic performances of the CSFV serological assays

Evaluation study about the replacement of virus neutralization test to more rapid and convenient assay ELISA was discussed. This competitive-based ELISA utilize the capture E2 protein and specific monoclonal antibody (mAb) 6B211 against E2 protein that also generate neutralizing activity (Wang *et al.*, 2020b). The results showed the excellent agreement between cELISA and SN with Kappa value of 0.957 and high correlation of the inhibition rate of serum samples in the cELISA with the titers value in the SN test (r^2 =0.903, p<0.001). That study demonstrated that cELISA is could potentially be used as alternative assay that provide a reliable, more rapid, simpler, and affordable tool for sero-monitoring of C-strain vaccination at a herd basis.

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The commercial ELISA to evaluate CSFV antibodies against the E2 protein, NS3, or Erns protein has been developed and constructed with various type of format. The E2-ELISAs are developed for conventional screening tests of CSFV infections on a herd pig. Whereas the NS3 and Erns ELISA are designed as accompanying marker assays for CSFV E2-subunit vaccines. All these types of ELISA can be used as marker assays in accompany with the use of DIVA vaccines (Schroeder *et al.*, 2012).

The E2-ELISA assay targets the detection of antibodies against E2 glycoprotein, hence the capture protein of E2 is coated within the well. Mostly, the format of these assays comes with indirect, blocking, or competitive based principle. The E2-ELISA kits are available from various kind of supplier including Chekit* CSF-Sero (hereinafter Chekit E2), HerdChek E2 (IDEXX Laboratories), PrioCHECK E2 (Thermo Fisher Scientific Inc.), BioCheck CSFV E2 (BioCheck), ID Screen© Classical Swine Fever E2 Competition (ID VET), SVANOVIR® CSFV-Ab (Boehringer Ingelheim), VDPro®CSFV Ab C-ELISA (Median Diagnostics Inc.). Previous study reported that PrioCHECK E2 ELISA has 100% specificity and the best reproducibility up to 98.4%, which indicated the highest similarities of the samples results in all given laboratories (Schroeder *et al.*, 2012). However, PrioCHECK E2 ELISA can only show the valid results in three out of five laboratories which is the lowest number among others (Schroeder *et al.*, 2012). On the other hand, Chekit E2 has shown to be slightly more sensitive and HerdChek E2 reported to be more specific. Additionally, these two assays showed the good practicability and reproducibility test, which generally make them the most promising accompanying DIVA assays in recent years, particularly for marker vaccines contain BVDV E2 protein.

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The NS3 ELISA targets the detection of non-structural CSFV protein, NS3. This protein may elicit antibodies against viral infection but cannot specifically generate neutralizing antibodies which induce protective immunity to CSF. One of the NS3 ELISAs kits is Serelisa® HCV Ab Mono Blocking (Synbiotics Europe). The study reported that Serelisa NS3 has a low value of diagnostic performance with sensitivity and specificity were 51.8% and 44%, respectively (Schroeder *et al.*, 2012). It is reported that NS3 protein has a conserved antigenic epitope among pestiviruses (Edwards *et al.*, 1988; Edwards *et al.*, 1991). Therefore, low specificity may demonstrate that this assay is less selective in recognizing the antibodies of CSFV and other types of pestiviruses i.e., BVDV.

There were several CSFV Erns ELISA commercially available including Chekit* CSF-Marker Erns (hereinafter Chekit Erns), PrioCHECK CSFV Erns, and Pigtype® Erns. As its name suggests, this assay detects the specific CSFV antibodies against envelope ribonuclease or Erns protein. Previous evaluation study with samples from E2 subunit-vaccinated pigs reported that Chekit Erns ELISA was more sensitive than PrioCHECK CSFV Erns and Serelisa NS3 (Schroeder *et al.*, 2012). However, since this assay is not specifically designed for CSF, it has low specificity to CSFV, and hence it unable to differentiate antibodies against Erns of CSFV or ruminant pestiviruses. On the other hand, PrioCHECK CSFV Erns has been reported that this assay is suitable for DIVA ELISA detecting chimeric pestivirus vaccines CP7_E2alf and FLc11 (Schroeder *et al.*, 2012). Nevertheless, evaluation study from vaccinated pig sera showed that this test has low sensitivity and specificity with the percentage of 78% and 89%, respectively (Pannhorst *et al.*, 2015). Recent study in accordance with the DIVA assay showed improved results. Prototype of CSFV Erns with double antigen format, similar concept as applied in Pigtype® Erns, represent the sensitivity of 90.2% while the specificity is 93.8% with combination of chimeric marker vaccine (Meyer *et al.*, 2017).

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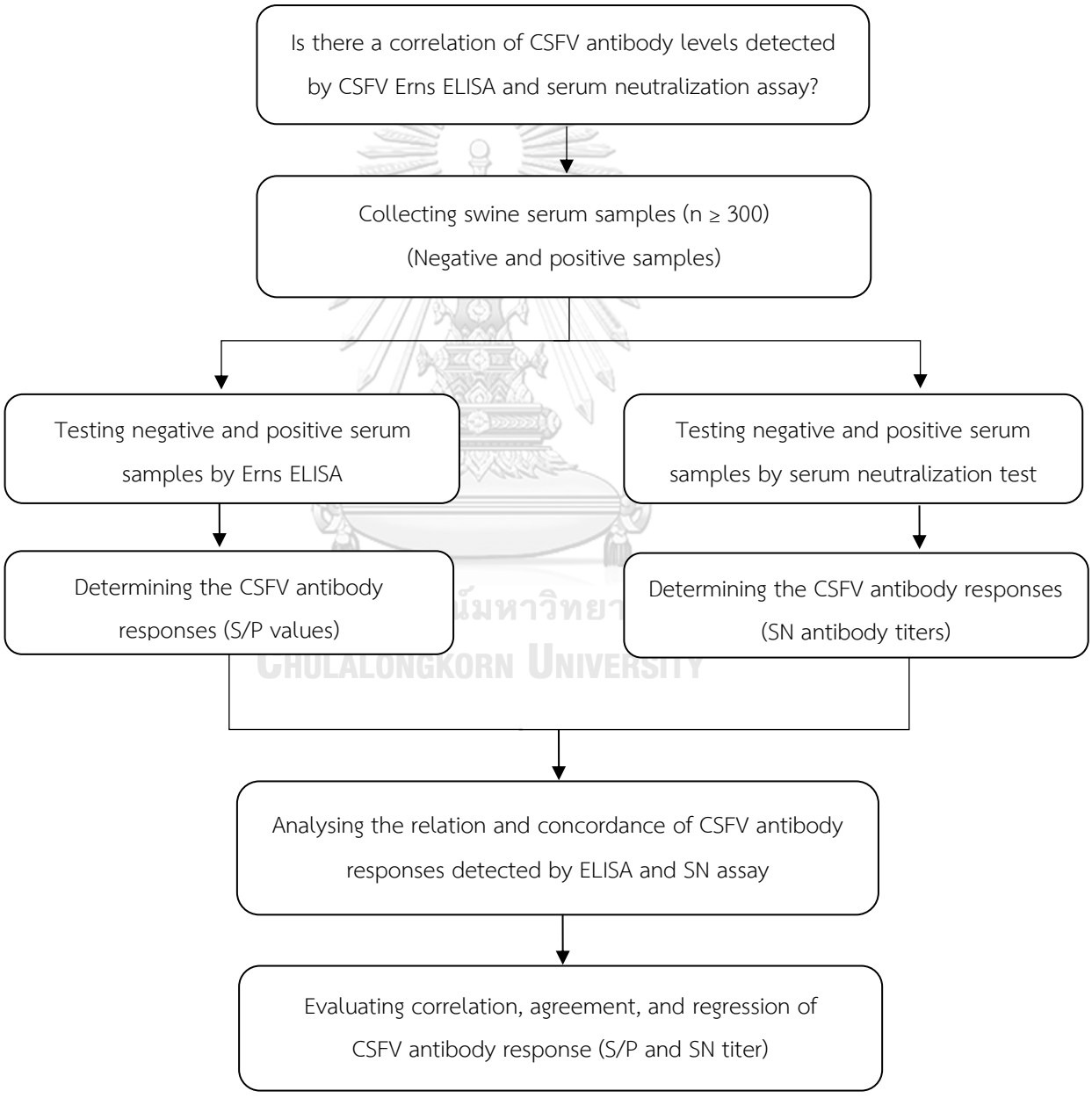
CHAPTER 3

MATERIALS AND METHODS

Conceptual Framework

CORRELATION OF THE CLASSICAL SWINE FEVER (CSF) ANTIBODY LEVELS DETECTED BY SERUM NEUTRALIZATON AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)



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Study design

Negative and positive serum samples (n = 522) were tested with serum neutralization (SN) and enzyme-linked immunosorbent assay (ELISA). Antibody status (negative, positive) of the samples was classified by the SN assay. Subsequently, the SN results presented as antibody titer (log2 x) were classified into three categories. Negative group is categorized for samples with SN titer less than 1 (log2), below protective level group is for samples with SN titer at the range of 1 to less than 5 (log2), and at protective level group is for sample with SN titer equal or greater than 5 (log2). The same set of the samples were then tested with commercial antibody CSFV ELISA. ELISA antibody responses were presented as sample-to-positive (S/P) ratios. Antibody responses detected by ELISA and SN assays were analysed for correlation, diagnostic performances, and predicted antibody levels.

Biological samples

Diagnostic serum samples collected from either non-exposed (unvaccinated or uninfected animals) or previous exposed (vaccinated or infected animals) to CSFV were included in this study. All serum samples were stored in -20 ºC until assayed.

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Serum neutralization assay

The neutralization peroxidase linked assay (NPLA) was performed in 96-wells flat-bottomed microtiter plates. Firstly, 50 µl serum sample was serially 2-fold diluted in 50 µl of growth medium (Eagle's MEM, 5 – 10% FBS and antibiotics) in well plate. Then, 50 µl prediluted virus suspension in 100 $TCID_{50}$ growth medium was added to the wells and homogenized for 20 seconds. The plates were incubated in a CO_2 incubator in a moist chamber for 1 h at 37 ºC. After that, 100 µl of growth medium suspension containing 3 x 10^5 SK6 cells/ml were added to all wells and incubation was carried out at 37 ºC in 5% CO_2 incubator for 72 h. After discarded the

medium, the cell monolayers were fixed with 100 µl of 0.4% formaldehyde in 0.5% PBST for 30 min, and then washed 3 times with 200 µl of 0.5% PBST. Detection of viral-infected cells was continued with immunoperoxidase monolayer assay (IPMA) as a part method of serum neutralization.

Virus was visualized by adding 50 µl prediluted 1:1000 WH303 CSF-specific monoclonal antibody (RAE0826, APHA Scientific, Surrey, UK) in 0.5% PBST with 1% BSA, followed with incubation at 37 ºC for 90 min. The plates were washed 3 to 5 times with 200 µl of 0.5% PBST. Then, 50 µl of prediluted 1:300 polyclonal rabbit anti-mouse IgG/HRP (P0161, Dako Denmark A/S, Denmark) in 0.5% PBST with 1% BSA was added to the wells, followed with incubation for 1 h at room temperature. Subsequently, the plate was washed and 50 µl of chromogen–substrate solution containing 3-Amino-9-Ethyl Carbazole (1 ml; Sigma-Aldrich, USA), acetate buffer (19 ml; 0.1 M acetic acid + 0.1 M sodium acetate) and 30% H_2O_2 (20 µl; Sigma-Aldrich) was added to each well and stain for 1 h at room temperature. After that, the supernatant was discarded and washed once with deionized water. The test plate was read visually with a light microscope. Positive control of the CSFV ALD strain (10^3 $TCID_{50}$/20 µl) and negative control with MEM solution were included. The presence of virus was indicated by the reddish-brown coloration in the cytoplasm of the cells. In addition, at the low-power microscopy, the monolayer was examined to determine the endpoint of the titration. The neutralizing antibody titers (nAbs) were expressed as the reciprocal of the last or highest dilution of serum that neutralizes the virus and shows a lack of staining in the cell. Samples with neutralization titer <2 were considered negative and ≥2 were positive or logarithmically transformed (base 2) to <1 for negative and ≥1 for positive titer.

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Enzyme-linked immunosorbent assay

Serum samples were tested using commercial antibody ELISA (Pigtype® CSFV Erns Ab, Indical Bioscience, GMBH Leipzig, Germany) (Figure 3). The procedure was performed in 96-well microtiter plates and following the protocol suggested by the manufacturer. Positive and negative controls were included on each test plate. Firstly, 100 µl positive and negative control were applied in duplicates. Serum samples were diluted in the remaining 92 well in each test plate by mixing 10 µl undiluted serum sample with 90 µl sample diluents. Procedure continued with 60 min incubation at 37 ºC. Then, plates were washed 3 times using 400 µl of diluted 1:10 wash solution and conjugate with horseradish peroxidase (HRP) was added at amount of 100 µl. The plates were incubated again for 60 min at 37 ºC. After that, the amount of 400 µl diluted (1/10) wash solution was added to wash the plate three times. Subsequently, 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate was added 100 µl, followed with incubation for 10 min at room temperature in the dark. Finally, the well mixture ended with 100 µl stop solution, and plates were read using microplate reader (Envision® Multimode Microplate Reader, PerkinElmer, Waltham, MA, USA). The reactions were measured as optical density (OD) at a wavelength of 450 nm within 20 min after stopping the reaction. Data interpretations were applied following manufacture protocol. Validation criteria was determined with the mean value (MV) of the measured optical density (OD) for positive control (PC) $\geq$ 0.7 and $\leq$ 0.3 for the negative control (NC). The ratio (S/P) of sample was calculated according to the following formula:

$$S/P = OD_{sample} - MV\ OD_{NC} / MV\ OD_{PC} - MV\ OD_{NC}$$

Interpretation of S/P values is samples with S/P ratio <0.3 are considered as negative, S/P-ratio $\geq$ are 0.5 positive, and S/P-ratio $\geq$0.3 and/or <0.5 are suspect.

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Statistical analysis

Central tendency followed with Saphiro-Wilk test was done to analyze the data distribution from each assay. The titer of SN results was plotted against S/P value of ELISA and the correlation between S/P value and SN titer was examined using Spearman's-rank correlation coefficient. The concordance between two assays were analyzed using Cohen's kappa method. Comparisons of S/P values among the level of SN titers were analyzed using Welch's ANOVA and continued with Tukey's post hoc test. Diagnostic sensitivity and specificity were also evaluated. In addition, linear regression model was analyzed according to Bland (2004) approach (Bland, 2004) to estimate the 95% prediction intervals. The standard error (SE) of the predicted SN titer for an individual was estimated by the following formula,

$$\text{SE} = SD.\sqrt{\left(1+\frac{1}{n}+\frac{(x-\bar{x})^2}{\sum(x_i-\bar{x})^2}\right)}$$

x is the observed value of ELISA (S/P) for a new subject, n, x bar, xi, and SD are the number of observations, mean of S/P, i^{th} or the order of S/P, and standard deviation of the observed samples (S/P). The upper and lower 95% prediction limits were calculated by the regression line +/- 1.96 times standard errors. Statistical analyses were conducted using SAS® 9.4 version (SAS® Institute Inc., Cary, NC, USA) and *p* values of less than 0.05 were considered statistically significant.

Figure 3 The commercial double-antigen ELISA kit; Pigtype® CSFV Erns Ab, Indical Bioscience, GMBH Leipzig, Germany. Composed of (1) coated 96-well microtiter plate, (2) sample diluent, (3) negative control, (4) positive control, (5) wash buffer, (6) conjugate, (7) TMB substrate, (8) stop solution.

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CHAPTER 4

RESULTS

Serum neutralization test

Distribution of antibody titers (log2) detected by SN are shown in Figure 4. Of 522 serum samples, 208 samples (39.8%) were negative (SN titer <1 in log2), and the remaining 314 samples (60.2%) were positive. The neutralizing antibody titers in positive samples ranged between 1 to 12 (log2) with a mean ± SD of 3.367 ± 3.380 (log2).

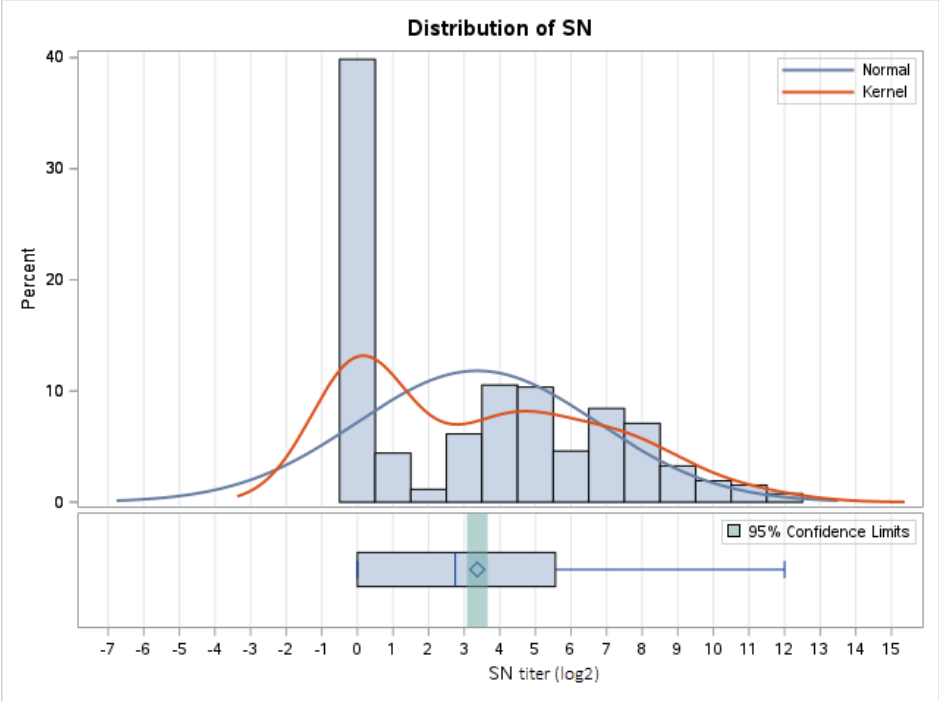

Figure 4 Distribution of antibody titers detected by serum neutralization test. The bar chart displays the percentage number of samples on each distribution value. The horizontal box plot displays the data distribution of titer (log2) measured by serum neutralization test. Blue curves represent the assumption of the normal data distribution. Red curves represent kernel distribution which is the non-parametric of the probability density function of a random variable.

Enzyme-linked immunosorbent assay

Distribution of antibody responses (S/P values) detected by ELISA are shown in Figure 5. Of 522 serum samples, 218 samples (41.8%) were negative, and 304 samples (58.2%) were positive. The S/P values of the negative samples were ranged between -0.143 to 0.258, whereas the positive samples were ranged between 0.520 to 2.859. Mean ± SD of negative and positive samples was -0.053 ± 0.064 and 1.598 ± 0.514, respectively.

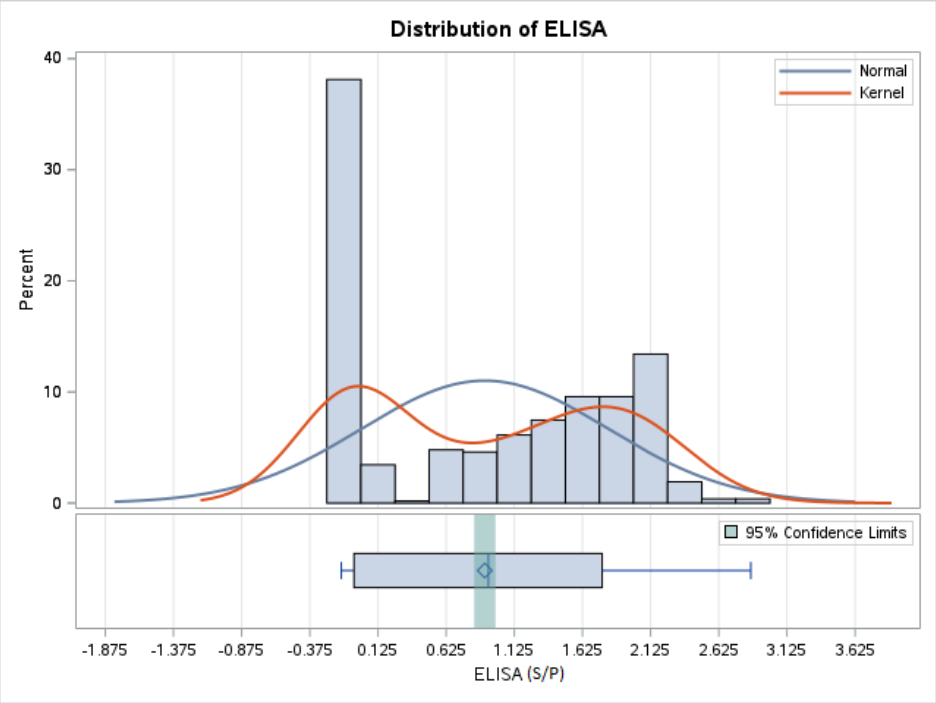

Figure 5 Distribution of sample-to-positive (S/P) values responses detected by ELISA. The bar chart displays the percentage number of samples on each distribution value. The horizontal box plot displays the data distribution of S/P values. Blue curves represent the assumption of the normal data distribution. Red curves represent kernel distribution which is the non-parametric of the probability density function of a random variable.

Correlation of antibody responses

The scatter plot and correlation of CSFV antibodies detected by ELISA and SN assay are presented in Figure 6. Correlation analysis between S/P values and SN titers using Spearman's-rank correlation coefficient test revealed a strong positive relationship. The value of r_s was 0.89 (p<0.0001), indicated a statistically significant association between S/P level of CSFV Erns ELISA and the SN titer of neutralizing antibody. The agreement between the two assays showed near perfect agreement with the value of 0.913 (95% CI 0.8772 – 0.9484).

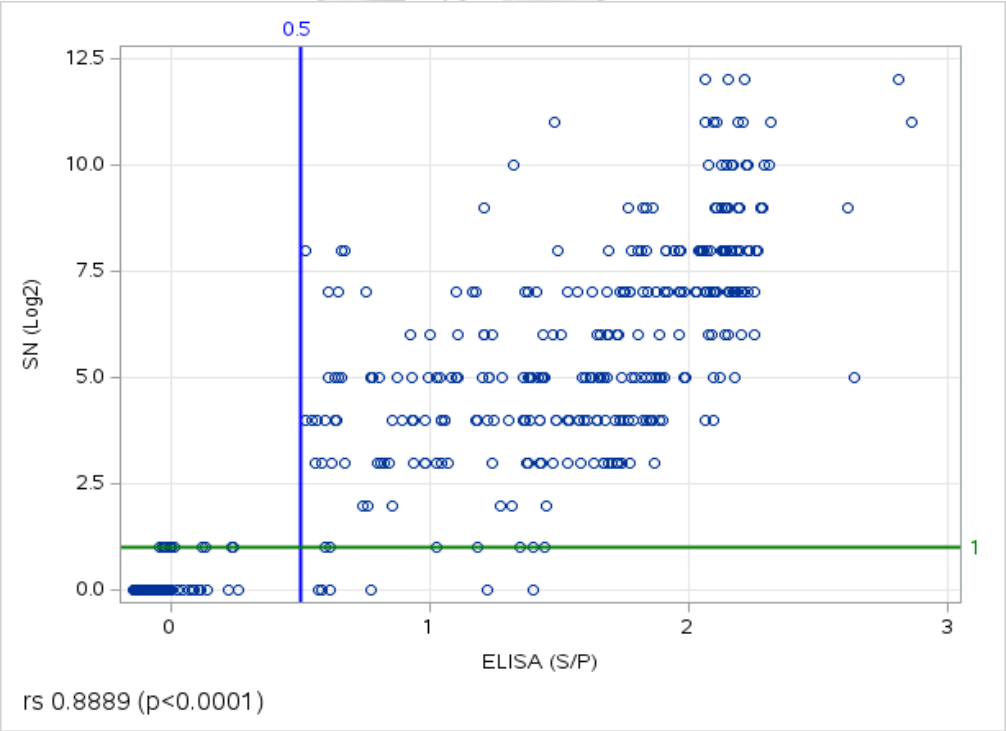

Figure 6 Correlation of antibodies response detected by ELISA towards serum neutralization. Green horizontal and blue vertical line represents positivity cut-offs of SN assay and ELISA respectively. r_s, Spearman-rank order correlation coefficient

Antibody responses detected by ELISA and serum neutralization

Serum samples were classified into 3 groups based on the SN antibody titers, i.e., negative (n = 208), below protective levels (n = 116), and at protective levels (n = 198). The S/P values (mean±SD) are shown in Table 1 by the level of the SN titers in a log base 2 unit.

Table 1 CSFV antibody responses detected by serum neutralization (SN titers) and ELISA (S/P values)

SN titer (log2)	n	Mean S/P	SD
< 1	208	-0.034[a]	0.173
1 to <5	116	1.132[b]	0.587
≥5	198	1.767[c]	0.479

[a,b,c] Within column, different superscripts indicate the differed significantly of mean S/P among the SN levels (p<0.0001); n, amount of serum samples; SD, standard deviation.

ANOVA followed with Tukey's multiple comparisons test showed statistically significant differences between the mean S/P value in negative group and every level of positive groups (p<0.0001), i.e., below and at protective levels.

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Diagnostic performances

The categorical results of CSFV Erns antibody detection by ELISA and SN are presented in Table 2. In a total of 314 positive results from SN as reference assay, an amount of 298 serum samples were detected positive by ELISA while the remaining 16 samples were classified as false negative. Additionally, 202 out of 208 negative serum samples by SN were also detected negative by ELISA and the other 6 samples

were false positive. Accordingly, the sensitivity and specificity of CSFV Erns ELISA in this study amounted to 94.9% and 97.1%, respectively.

Table 2 Contingency table of CSFV Erns antibody detection by ELISA and SN assay

ELISA	**SN**		**Total ELISA**
	Positive	Negative	
Positive	298	6	304
Negative	16	202	218
Total SN	314	208	522

Regression analysis

Linear regression analysis was performed according to Bland (2004) approach. Predictions of antibody responses between sample-to-positive ratio and antibody titers are shown in Figure 7. In this study, the standard error (SE) of the predicted SN titers by ELISA in individual data was estimated to be 0.908. The predicted SN values by S/P of CSFV Erns ELISA were given as 3.3306x + 0.3413. The calculation of 95% limits for the prediction can be measured by the regression line +/- 1.96 standard errors (SE) or written as follows,

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$$= (3.3306x + 0.3413) +/- 1.96 * 0.908$$

From the formula above, the upper and lower 95% prediction limits for serum neutralization (log2) were +/- 1.96 times standard errors (SE) or equal to +/- 1.78 of the predicted SN titers, respectively.

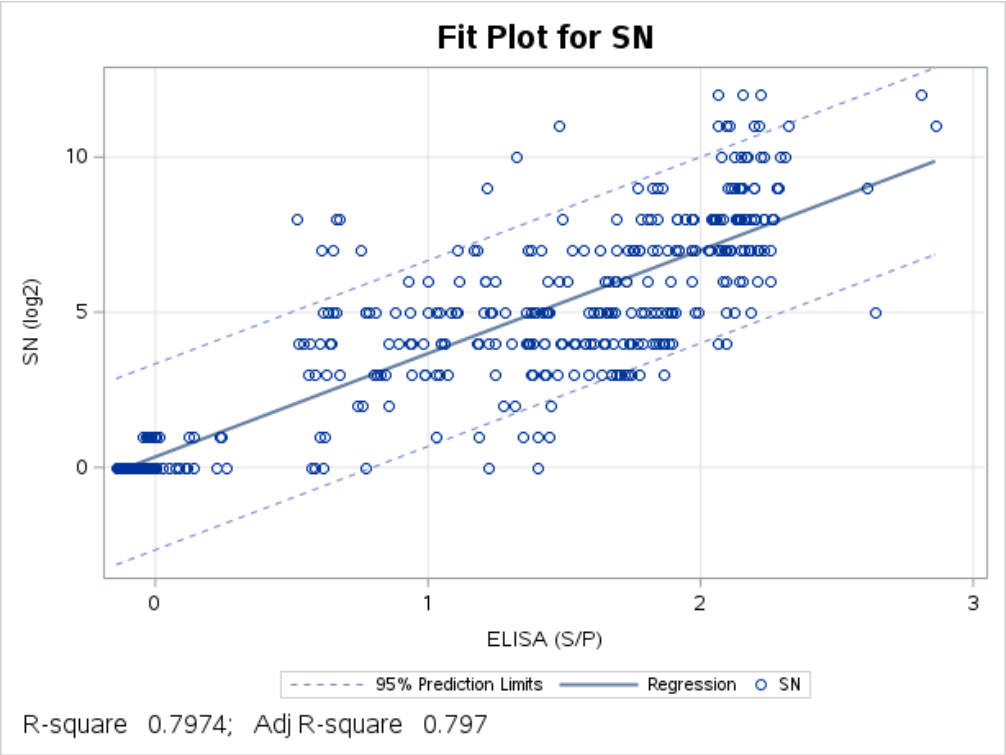

Figure 7 Scatter plots and regression analysis displaying CSF specific antibody predictions between sample-to-positive ratio of CSF Erns ELISA and base 2 log scale titers of serum neutralization (SN). Circle hollows represent individual antibody levels. Solid blue line represents the regression or predicted mean for SN measurement by ELISA. Dashed lines represent the 95% prediction limits which indicate the interval for a single SN value.

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CHAPTER 5

DISCUSSION AND CONCLUSION

Comparability assessments are usually conducted to evaluate the performance of the modified assay or the new alternative method toward the previously existing assay or standard assay for certain purposes (OIE, 2019). Serum neutralization test is considered a gold standard for classical swine fever antibody detection. Neutralizing capacity of this assay also correlates with protection level (Terpstra and Wensvoort, 1988; Santana-Rodríguez *et al.*, 2022). In this study, serum neutralization assay was performed to provide information regarding the antibody status of the samples and to categorize groups of antibody titer levels for evaluating the correlation with ELISA measurement.

Sample size of diagnostic samples may influence the accuracy in predicting levels of neutralizing antibodies between the assays (Dolscheid-Pommerich *et al.*, 2022). In this study, the sample size was determined by considering the rule-of-thumb of large sample that may increase the accuracy of the statistical analysis particularly in diagnostic test. Studies had suggested to obtain at least 300 samples to reach the likely estimated statistics to be the same as the true values within the intended population (Bujang and Adnan, 2016). Therefore, in correspond to this present study, we consider that the total sample used was at acceptable range and represent the robustness of the analysis.

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Correlation of antibody responses detected by different serological techniques were evaluated in several diseases, either in animals or humans (Graham *et al.*, 1997; Paudel *et al.*, 2014; Nyiro *et al.*, 2019; Wang *et al.*, 2020b; Bonifacio *et al.*, 2022; Dolscheid-Pommerich *et al.*, 2022; Lee *et al.*, 2022). For example, Wang *et al.* (2020) evaluated the correlation between competitive ELISA (cELISA) and neutralization assay in 139 serum samples for monitoring CSFV-titer antibody post-

vaccination. The results showed that the inhibition rate of serum samples in the cELISA is highly correlated with the titers value in the SN test (r^2 =0.903, p<0.001), which indicates a promising cELISA to replace virus neutralization test (VNT), particularly for C-strain post-vaccination monitoring. Another study in humans investigated the correlation between QuantiVac ELISA, the quantitative detection of SARS-CoV-2 IgG, and microneutralization assay with 123 plasma samples (Dolscheid-Pommerich *et al.*, 2022). The study showed the potential implementation of QuantiVac ELISA estimating the quantitative immunity of SARS-CoV-2 infections, with ELISA values ≥480 BAU/ml predicting neutralizing titer of ≥10 in 72% of cases and <480 BAU/ml predicting low neutralizing titer (<10) in 90.8% cases.

Commercial ELISA (Pigtype® CSFV Erns Ab) used in this study is one of the antibody ELISA designed as accompanying assay for marker vaccines and provide DIVA properties. The assay was designed to detect antibodies against the Erns protein which is one of the CSFV specific neutralizing antibodies (Meyer *et al.*, 2017). The strong correlation results between the commercial ELISA and SN assay suggests a high potential to estimate neutralizing antibody titers by the observed S/P value. The strong correlation between these two assays is possibly contributed from the similarity of the target antibody detection that is immunogen glycoprotein Erns.

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The results from this study revealed the significant differences in S/P responses among the level of SN titers (negative, below protective level, and at protective level). That is, the group of positive samples having S/P values at 1.132±0.587 and 1.767±0.479 were estimated the antibody titer as below and at the protective level, respectively. The results could be applied for estimating antibody status on a herd basis, but interpretation of individual result should be an attentive consideration.

Regression analysis showed that the corresponding SN titer values could be estimated with the observed S/P ELISA at 95% prediction limits within a width of 3.56

log2 SN titer. Prediction limits represent the maximum and minimum of SN predicted value. This result may indicate that the estimated SN titer of given serum sample by the observed S/P value would probably fall within 1.78-fold higher or lower than the true SN value. Furthermore, adjusted R-square (0.797) showed a moderate accuracy of the regression model between ELISA and SN, which explaining that 79% variability of SN titer is fairly influenced by the ELISA S/P value. This finding assumed to be caused by the high variability of serum sample characteristics and thus makes the direct estimation of single SN value being arbitrary. Therefore, careful consideration is needed for the direct estimation of individual SN value by the formula.

The utilization of the commercial ELISA to estimate protective antibody or SN titer against CSFV could be used to accompany the standard assay and not suggests being stand-alone in diagnosing the disease. In addition, the S/P value for predicted SN titer should not be used inclusively since the data in this study does not represent all types of ELISA. The predicted SN titer by the observed S/P value could be used as the approach to assist the functional standard assay in evaluating the status of protective immunity but not for the specific titer value.

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CONCLUSION

This study evaluated the correlation of antibody responses detected by commercial ELISA and serum neutralization assay. The strong correlation between the S/P values and neutralizing antibody titers could provide useful information in terms of detecting CSFV antibodies and estimating protective status of antibody positive animals by the ELISA method.

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