IMMUNE RESPONSE TO SWINE FEVER VIRUS IN TISSUE CULTURED C-STRAIN VACCINATED PIGS AND LAPINISED C-STRAIN VACCINATED PIGS

สำนักหอสมุดกลาง

By
Miss Jarunee Satra

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy Program in Biology
Department of Biology
Graduate School, Silpakorn University
Academic Year 2013

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ภูมิคุ้มกันต่อไวรัสอหิวาต์สุกรในสุกรที่ได้รับวัคซีนสายพันธุ์ซี ชนิดเพาะเลี้ยงในเนื้อเยื่อและสุกรที่ได้รับวัคซีนสายพันธุ์ซีชนิดเพาะเลี้ยงในกระต่าย

สำนักหอสมุดกลาง

โดย

นางสาวจารุณี สาตรา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต

สาขาชีววิทยา

ภาควิชาชีววิทยา

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

ปีการศึกษา 2556

ลิขสิทธิ์ของบัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

The Graduate School, Silpakorn University has Approved and Accredited the Thesis title of “Immune Response to Swine Fever Virus in Tissue Cultured C-strain Vaccinated Pigs and Lapinised C-strain Vaccinated Pigs” submitted by Miss Jarunee Satra as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology.

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คำสำคัญ: ภูมิคุ้มกัน / ไวรัสอหิวาต์สุกร / วัคซีนสายพันธุ์ซี

จารุณี สาตรา: ภูมิคุ้มกันต่อไวรัสอหิวาต์สุกรในสุกรที่ได้รับวัคซีนสายพันธุ์ซีชนิดเพาะเลี้ยงในเนื้อเยื่อและสุกรที่ได้รับวัคซีนสายพันธุ์ซีชนิดเพาะเลี้ยงในกระต่าย อาจารย์ที่ปรึกษาวิทยานิพนธ์: ผศ. ดร. จันทร์ดี ระแบบเลิศ ภภ. ผศ. ดร. สรวง รุ่งประกายพรรณ และ ดร. วิทวัช วิริยะรัตน์ 141 หน้า

โรคอหิวาต์สุกร มีสาเหตุเกิดจากเชื้อไวรัสอหิวาต์สุกร (classical swine fever virus, CSFV) จีนัส *Pestivirus* วงศ์ Flaviviridae เป็นโรคติดต่อในสุกรส่งผลกระทบต่อปัญหาเศรษฐกิจทั่วโลก วัตถุประสงค์ เพื่อศึกษา ระบบภูมิคุ้มกันแบบสารน้ำและระบบภูมิคุ้มกันแบบพึ่งเซลล์ ในสุกรกลุ่มฉีดวัคซีน tissue cultured C-strain และสุกรกลุ่มฉีดวัคซีน lapinised C-strain หลังฉีดวัคซีน 3 วัน และฉีดเชื้อพิษทับด้วยเชื้อ CSFV ชนิดรุนแรงมากสายพันธุ์บางเขน ผลการทดลองพบว่า สุกรกลุ่มฉีดวัคซีน tissue cultured C-strain มีอุณหภูมิร่างกายสูงขึ้นเฉลี่ยระหว่าง 39.40±0.26°ซ - 39.95±0.70°ซ. ในขณะที่สุกรกลุ่มฉีดวัคซีน lapinised C-strain มีอุณหภูมิร่างกายสูงขึ้นเฉลี่ยระหว่าง 39.25±0.34°ซ. - 39.58±0.13°ซ. สุกรกลุ่มฉีดวัคซีน tissue cultured C-strain จำนวน 3 ใน 4 ตัว ไม่มีการลดลงของจำนวนเม็ดเลือดขาวและการแสดงออกของยีน TGF-β แต่มีการเพิ่มขึ้นของยีน IFN-γ ในวันที่ 3 หลังฉีดเชื้อพิษทับ สุกรจำนวน 3 ใน 4 ตัว ในกลุ่มนี้กระตุ้นแอนติบอดีในวันที่ 14 และ 21 และไม่แสดงอาการป่วยรุนแรงหรือตายหลังฉีดเชื้อพิษทับ อย่างไรก็ตาม สุกรจำนวน 1 ตัว ซึ่งฉีดวัคซีน tissue cultured C-strain มีจำนวนเม็ดเลือดขาวและยีน TGF-β ลดลง แต่ไม่มีการเพิ่มขึ้นของยีน IFN-γ ตรวจพบแอนติบอดีในวันที่ 14 และตรวจพบแอนติเจนในวันที่ 5, 7 และ 14 หลังฉีดเชื้อพิษทับ โดยสุกรแสดงอาการป่วยรุนแรงและตายในวันที่ 18 หลังฉีดเชื้อพิษทับ เช่นเดียวกันกับสุกรในกลุ่มควบคุมบวก สุกรทั้งสี่ตัวในกลุ่มฉีดวัคซีน lapinised C-strain ไม่มีการลดลงของจำนวนเม็ดเลือดขาวและยีน TGF-β แต่มีการเพิ่มขึ้นของยีน IFN-γ ในวันที่ 3 หลังฉีดเชื้อพิษทับ สุกรทั้งสี่ตัวในกลุ่มนี้กระตุ้นแอนติบอดีในวันที่ 14 และ 21 และไม่แสดงอาการป่วยรุนแรงหรือตายหลังฉีดเชื้อพิษทับ อาการทางคลินิก อุณหภูมิร่างกาย จำนวนเม็ดเลือดขาว การแสดงออกของยีน IFN-γ และ TGF-β รวมทั้งการตรวจพบแอนติเจนและแอนติบอดีในสุกรกลุ่มที่ได้รับวัคซีน tissue cultured C-strain จำนวน 3 ใน 4 ตัว เหมือนกันกับสุกรกลุ่มที่ได้รับวัคซีน lapinised C-strain ทั้ง 4 ตัว อย่างไรก็ตาม วัคซีน tissue cultured C-strain ให้ความคุ้มโรคต่อเชื้อ CSFV ชนิดรุนแรงมากสายพันธุ์บางเขนได้เพียงบางส่วน ในขณะที่วัคซีน lapinised C-strain ให้ความคุ้มโรคได้อย่างสมบูรณ์หลังฉีดวัคซีน 3 วัน

ภาควิชาชีววิทยา บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

ลายมือชื่อนักศึกษา... ปีการศึกษา 2556

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์1..............................2..............................3................................

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JARUNEE SATRA: IMMUNE RESPONSE TO SWINE FEVER VIRUS IN TISSUE CULTURED C-STRAIN VACCINATED PIGS AND LAPINISED C-STRAIN VACCINATED PIGS. THESIS ADVISORS: ASSIST. PROF. JUNDEE RABABLERT, Ph.D., ASSIST. PROF. SUANG RUNGPRAGAYPHAN, Ph.D. AND WITTHAWAT WIRIYARAT, D.V.M., Ph.D. 141 pp.

Classical swine fever is caused by classical swine fever virus (CSFV), genus *Pestivirus*, Family Flaviviridae. It is a highly contagious disease of swine that effects to economically worldwide. The aim is to investigate humoral and cell mediated immune responses in tissue cultured C-strain vaccinated pigs and lapinised C-strain vaccinated pigs, 3 days post vaccination, and challenged with a highly virulent CSFV Bangkhen strain. In our results, tissue cultured C-strain vaccinated group had the average rising body temperature 39.40±0.26°C - 39.95±0.70°C, whereas lapinised C-strain vaccinated group had the average rising body temperature 39.25±0.34°C - 39.58±0.13°C. Three of four pigs in tissue cultured C-strain vaccinated group did not decrease white blood cells and TGF-β gene expression but increased IFN-γ gene on day 3 post challenge. Three of four pigs in this group induced antibody on day 14 and 21 and did not show severe clinical signs or died post challenge. However, one pig in tissue cultured C-strain vaccinated group decreased white blood cells and TGF-β gene but did not increase IFN-γ gene, the antibody was detected on day 14 and the antigen was detected on day 5, 7 and 14 post challenge. This pig showed severe clinical signs and died on day 18 post challenge, as well as pigs in positive control group. All of four pigs in lapinised C-strain vaccinated group did not decrease white blood cells and TGF-β gene but increased IFN-γ gene on day 3 post-challenge. All of four pigs in this group induced antibody on day 14 and 21 and did not show severe clinical signs or died post challenge. The clinical sign, body temperature, total white blood cells, IFN-γ and TGF-β gene expression, including antibody and antigen detection in three of four tissue cultured C-strain vaccinated pigs were similar to all of four lapinised C-strain vaccinated pigs. However, tissue cultured C-strain vaccine partially protected pigs while lapinised C-strain vaccine completely protected pigs against the highly virulent CSFV Bangkhen strain at 3 days post vaccination.

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List of Abbreviations

Abbreviation	Term
μl	microliter
A450	Absorbance at wavelength 450 nm
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cells
ASEAN	Association of South East Asia Nations
BDV	Border disease virus
BMHCs	Bone marrow hematopoietic cells
BT	Body temperature
BVBP	Bureau of Veterinary Biologics Production
BVDV	Bovine viral diarrhea virus
cDNA	complementary deoxynucleic acid
CMI	Cell mediated immunity
CMIR	cell mediated immune response
CSF	classical swine fever
CSFV	classical swine fever virus
C-strain	Chinese-strain
CTL	Cytotoxic T lymphocyte

สำนักหอสมุดกลาง

Abbreviation	Term
DCs	Dendritic cells
DLD	Department of Livestock Development
DNA	Deoxy nucleic acid
DP	Double positive
dpc	day post challenge
dpi	day post inoculation
dpv	day post vaccination
ELISA	Enzyme- linked immunosorbent assay
E	envelop
EU	European Union
g	gram
GPDH	Glyceraldehyde-3-phosphate dehydrogenase
HI	Humoral immunity
HIR	Humoral immune response
HRPO	horse-reddish peroxidase
HV	High virulent
IFN-α	interferon- alpha
IFN-β	interferon- beta
IFN-γ	interferon- gamma
IL	interleukin
IRF	interferon regulatory factor

Abbreviation	Term
LC	Lapinised C-strain
LD_{50}	50% Lethal Dose
MDA	Maternal derived immunity
MHC	Major histocompatibility complex
min	minute
ml	milliliter
MLV	Modified live attenuated vaccine
MV	Moderate virulent
Nab	Neutralizing antibody
NC	Negative control
NIAH	National Institute of Animal Health
NK	Natural killer
nm	nanometer
NS	Normal saline
NS protein	Non-structural protein
NT Ab	Neutralizing antibody
NTR	Non-translated region
OD	Optical density
OIE	Office International des Epizooties
ORF	Open-reading frame

สำนักหอสมุดกลาง
มหาวิทยาลัยศิลปากร

Abbreviation	Term
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PC	Positive control
PCR	Polymerase chain reaction
PCV2	porcine circovirus 2
PD_{50}	50% Protective Dose
PID_{50}	50% Pig Infectivity Dose
PRRSV	porcine reproductive and respiratory syndrome virus
PRV	pseudorabies virus
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	Reverse transcription polymerized chain reaction
SLA	Swine leukocyte antigen
TC	Tissue cultured C strain
TGF-β	Transforming growth factor-beta
Th	T helper
TNF-α	Tumor necrosis factor-alpha
VBAD	Veterinary Biologics Assay Division
VRP	Virus replication particles
WBC	White blood cell

สำนักหอสมุดกลาง มหาวิทยาลัยศิลปากร

CHAPTER I
INTRODUCTION

Classical swine fever virus (CSFV) is the enveloped RNA virus, genus *Pestivirus*, family Flaviviridae. It is the causative agent of classical swine fever (CSF) [1]. CSF is a highly contagious disease of swine that affects to economic problem and health of livestock in the world. World Organization of Animal Health (Office International des Epizooties, OIE) notifies CSF as the surveillance disease [2]. Infected pigs may run acute, sub-acute or chronic course. In general, in the acute course pigs show high fever, hemorrhage, leucopenia, abortion and high mortality. In sub-acute course, pigs show slightly clinical signs while in chronic course, pigs do not show any clinical signs or pigs have chronic sickness and may recover [3]. The severity of disease depends on phenotype and age of host and the virulence of difference strains of virus [4]. All non- immune pigs infected with a highly virulent strain die 100%, while pigs infected with intermediate or low virulent strain have chronic signs [5]. Decision on control strategies are complex, will vary for different regions, and depend on many factors, such as breed density, production systems, the presence of susceptible wildlife, impact on export trade and current opinions on economic versus ethical factors [6] [7]. Modified live vaccine (MLV) are inexpensive and can induce complete protection against virulent CSFV, so MLV are still widely used in the CSF endemic areas, including Asia and central-south America [8] [9] [10] [11]. A number of live attenuated CSF vaccines have been developed by traditional methods such as passage the vaccine virus in tissue culture (e.g. Thiverval strain), or by serial passage the vaccine virus in rabbits (e.g. the Chinese or C-strain [12]. Although live attenuated vaccines have been used for CSF prophylaxis for many years, some questions remain about the effective they would be at controlling CSF in a suppressive emergency vaccination campaign. To prevent infection of neighboring herds, suppressive vaccination should induce a rapid protection that prevents dissemination of virus. The C-strain vaccine provides solid protection against a challenge by 7 days post-vaccination (dpv) [12] and there are many reports indicated that protection occurs even earlier [13]. It was demonstrated that vaccination with C-strain on the same day as challenge prevented transmission to vaccinated pen mates [14]. However, fewer data are available on how rapidly vaccination prevents transmission of virus to susceptible animals in these very short intervals. For CSF-free area, introductions of CSFV could originate from any region of the world and so an emergency vaccine should provide protection against all CSFV strains. C-strain and the majority of live attenuated CSF vaccines belong to genotype 1.1 and are considered to be effective against all genotypes [15]. Experimental studies have demonstrated vaccine efficacy against CSFV genotype 1 [16] and genotype 2 strains [11] [17]. However, there is evidence that genotype 2 strains have replaced genotype 1 viruses as the dominant genotype in China, and area where vaccination is mandatory, implying that antigenic differences between genotypes may impact to some extent on vaccine efficacy [18] [19]. In addition, very little information is available on vaccine efficacy against viruses of the genotype 3 subgroups. The ability of C-strain to protect against clinical signs upon challenge with CBR/94/2 two weeks after vaccination has been demonstrated [20]. However, the mechanism of protection has not been elucidated and it is unclear if C-strain vaccine protects efficiently against transmission of genetic and antigenic diverse strains at very short periods post vaccination. It is generally accepted that neutralizing antibody induced by vaccination with C-strain, which is detected from 2-3 weeks post-vaccination [21] is a major protective mechanism. However, for a rapid emergency vaccination, the mechanism of protection induced prior to this time is of greater importance. Since virus-specific T cell IFN-γ response can be detected 7 dpv, it has been suggested that they may mediate protection in the absence of antibody [17] [22]. C-strain induced T cell responses have been reported in a limited number of studies. Virus specific CD4+ and CD8+ T cell interferon (IFN-γ) responses, targeting the major viral envelop € 2 and the non-structural (N) 3 protein glycoprotein, have been observed following vaccination [23] [24] [25]. MHC class I restricted cytotoxic T lymphocyte responses, directed against epitopes on both E2 and NS3 have also been described [23]

[24] [26]. It has been reported that immunization of pigs with C-strain vaccine induced a robust, virus specific, IFN-γ response detectable from at least 9 dpv but these responses could not be detected in pigs experimentally infected with a recent, moderately virulent, field isolate [27]. While IFN-γ appears to serve as a marker for anti-CSFV cell mediated responses, the data underlying a correlation with protection are limited. According to OIE Terrestrial Manual [28], potency is expressed as the number of PD_{50} (50% protective dose) contained in one vaccine dose which is at least 100 PD_{50}. Two groups of five 6-8 week old piglets are inoculated intramuscularly with a 1/40 and a 1/160 dilution of the reconstituted vaccine, respectively. The vaccinated pigs together with two controls are challenged intramuscularly with 10^5 PID_{50} (50% pig infectivity dose) of a virulent strain of CSFV, 2 weeks later. The pigs are observed for 2 weeks thereafter, during which time the controls should die. According to ASEAN Standards for Animal Vaccine [29] at least four susceptible pigs are each inoculated with 1/100 of a field dose of the product by the recommended route. After at least 2 weeks, the vaccinated pigs together with two unvaccinated controls are challenged with at least $10^{4.0}$ LD_{50} (50% lethal dose) of a virulent strain of CSFV, and observed for at least 2 weeks. The two controls must die or manifest serious signs of the disease. All vaccinates should survive and show no clinical signs of disease. In Thailand, the first incidence of CSF occurred in Bangkhen District (Chatuchak Region), Bangkok, in 1950, then CSF widely spread throughout Thailand and affected to the economic loss of pig farming, the first year that Department of Livestock Development (DLD) produced crystal violet inactivated vaccine [30]. This inactivated vaccine induces immune slowly and shortly, so that DLD developed MLV, SFA strain. However, MLV, SFA strain caused the reaction after inoculation in pigs and hyperimmune serum must be inoculated in the same time. In 1975, DLD received lapinised swine fever virus, Chinese (C) strain from Hungary and used for MLV production. This MLV, C-strain was highly safe and did not cause reaction after inoculation in pigs, and vaccine virus was not spread to other pigs. MLV, C-strain could induce rapid immunity within 5 dpv [31] and 7 dpv [32]. Bureau of Veterinary Biologics Production (BVBP), DLD, Ministry of Agriculture and Cooperatives, has produced

MLV, C-strain by culturing virus in rabbits for three days, collecting spleen, mesenteric lymphnodes and blood, then mixing suspension of spleen and lymphnodes with serum and making vaccine in the lyophilized form, that called " lapinised C-strain (LC) vaccine " [33]. For animal welfare reasons, using a lot of rabbits for lapinised C-strain vaccine production is contrary to animal ethics. Therefore, BVBP in connection with National Institute of Animal Health (NIAH), DLD, developed MLV, C-strain vaccine by propagating virus in tissue cultures that called "tissue cultured C-strain (TC) vaccine". However, the clearly data about the efficacy of both vaccines are not enough for selection of vaccine types using to control SFV in Thailand.

In this study, the researchers study the humoral immune response (HIR) and the cell mediated immune response (CMIR) in TC vaccinated pigs and LC vaccinated pigs, 3 days prior to challenge with CSFV Bangkhen strain, by observation of clinical signs, measuring of body temperatures, measuring numbers of leukocytes and lymphocytes, quantification of antigen and antibody in serum, measuring of IFN-γ and transforming growth factor-beta (TGF-β) gene expressions from peripheral blood mononuclear cells (PBMC), in TC vaccinated pigs and LC vaccinated pigs post challenged with CSFV Bangkhen strain.

สำนักหอสมุดกลาง มหาวิทยาลัยศิลปากร

CHAPTER II

OBJECTIVES

The specific aims are:

1. To observe the clinical signs and measure body temperatures of TC vaccinated pigs and LC vaccinated pigs, 3 days prior to challenge with CSFV Bangkhen strain.

2. To measure the number of leukocytes and lymphocytes of TC vaccinated pigs and LC vaccinated pigs, 3 days prior to challenge with CSFV Bangkhen strain by using the automated hematology analyzer.

3. To quantify the amount of CSFV antigen and anti-CSFV antibody from sera of TC vaccinated pigs and LC vaccinated pigs, 3 days prior to challenge with CSFV Bangkhen strain by enzyme-linked immunosorbent assay (ELISA).

4. To measure IFN-γ and TGF-β genes from PBMC of TC vaccinated pigs and LC vaccinated pigs, 3 days prior to challenge with CSFV Bangkhen by quantitative reverse transcription real-time-polymerase chain reaction (qRT-PCR).

สำนักหอสมุดกลาง
มหาวิทยาลัยศิลปากร

CHAPTER III
LITERATURE REVIEWS

Classical swine fever virus (CSFV), the infectious agent of classical swine fever (CSF), can cause important economic losses, representing a constant and serious threat for the pig industry [34]. The disease can manifest as acute hemorrhagic fever with respiratory, gastrointestinal and neurological symptoms resulting in high mortality rates, as a chronic disease with atypical symptoms, or even remain mostly unapparent [35]. Besides host factors like the age and general health status of the animals, it is mainly the virulence of the CSFV isolate that determines disease severity [2]. Virulence of a virus can be defined as fitness advantage or as the ability of the virus to cause clinical and pathological symptoms in a susceptible host. In some reports, virulence was related to cell killing *in vitro* [36]. Serological differentiation of infections caused by different pestiviruses is based on monoclonal antibodies directed against the enveloped protein E^{rns} or E2. Fig. 1 schematically shows the genome organization and structure of CSFV. The three proteins inducing detectable antibodies: E2 (major immunogene, neutralizing activity), E^{rns} (low or no neutralizing activity) and NS3 (no neutralizing activity) are highlighted. For marker diagnostics, the detection of antibodies against immunogenic proteins such as the envelope protein E^{rns} or E2 and the non-structural protein NS3 can be used. However, since E2 is the major immunogen, and part of many CSF vaccines, recently developed CSF marker assays were designed for the detection of E^{rns} -specific antibodies [37, 38].

1. Classical swine fever virus (CSFV)

The CSFV virion is a hexagonally shaped particle, which has an electron-dense inner core structure of about 30 nm, surrounded by a spherical envelope with diameters ranging between 40 and 60 nm. The genome of CSFV possess a single positive-strand RNA genome of approximately 12,300 nucleotides flanked by 5‟ and 3‟

non-translated regions (NTR). The large open reading frame encodes typically a polyprotein of 3,898 amino acids that undergoes co- and post-translational processing by cellular and viral proteases. The polyprotein processing generates the four structural proteins C, E^{rns}, E1, and E2 and the eight non-structural proteins (NS) N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (**Figure 1**) [37].

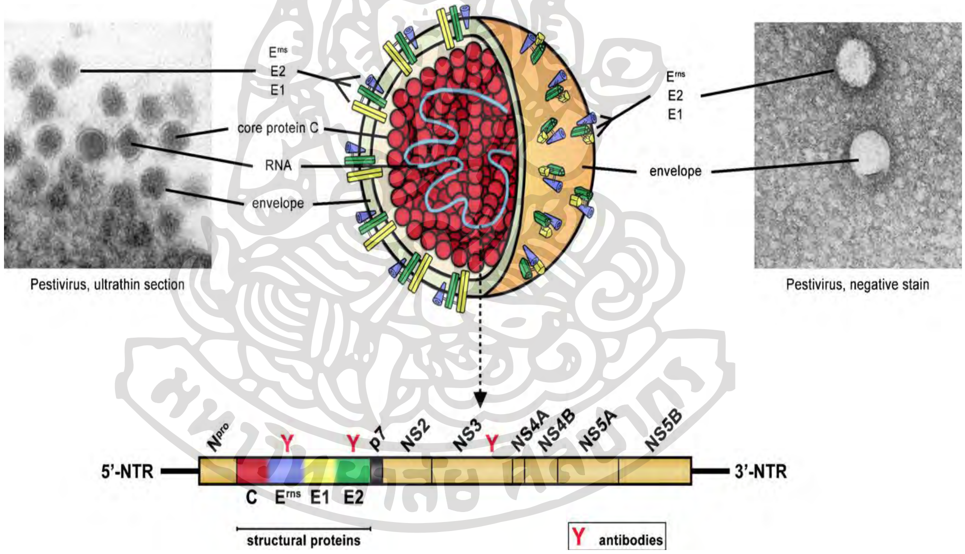

Figure 1. Schematic description of the genome organisation and virion structure of CSFV. Antibodies are directed against the envelope proteins ERNS, E2 and the non-structural protein NS3. E2 is the major immunogenic protein inducing high levels of neutralizing antibodies. [38].

สำนักหอสมุดกลาง

1.1 Structural proteins

1.1.1 C protein

The core protein (C) is a small protein which contains many basic amino acids (lysine and arginine) [39] [40]. The formation of a core protein-RNA complex inside the virion suggests a protective function of the core protein [41]. C protein can activate the promoter of heat shock protein 70 gene, and suppress the SV40 early promoter. These findings indicate that the C protein appears to function not only as a viral structural protein but also as a regulator of gene expression [42].

1.1.2 E^{rns} protein

E^{rns} (E0) glycoprotein was formally termed gp44 with an apparent size of 41-44 kDa and composed of 227 amino acids. Approximately half of the molecular mass of the mature E^{rns} glycoprotein is made up of carbohydrates [43]. Unlike E1 and E2 proteins, E^{rns} glycoprotein lacks the membrane anchor and is secreted from infected cells [44], but it is associated with mature virions and has been reported to be involved in CSFV entry into the cell. E^{rns} may take part in the initial attachment process of viral entry, rather than in the specific binding or fusion process [43]. E^{rns} may also play an important role in the post-entry stages, which may be a possible infection, while E^{rns} is considered to be the secondary glycoprotein that mediates neutralization [45]. Both E^{rns} and E2 are known to induce viral neutralizing antibodies which give protective immunity in the natural host [46]. E^{rns} glycoprotein represents a second determinant for the induction of protective immunity against CSF. It is reported that animals vaccinated with recombinant vaccinia virus expressing E^{rns} developed neutralizing antibodies against CSFV [43] [47]. Compared to other viruses in the family *Flaviviridae*, the N-terminal of the E^{rns} is unique to *Pestiviruses*, and has been implicated in the evasion of host interferon (IFN) responses [47] [48]. An important feature is that E^{rns} is heavily glycosylated with N-linked glycans and representing up to half of the apparent molecular weight [49]. It contains ribonuclease activity and its C-terminal domain controls translocation across eukaryotic cell membranes and has a critical role in the inhibition of double-stranded RNA-induced cell responses. N-glycan of CSFV E^{rns} is also essential for

Erns blocking of interferon-beta (IFN-β) induction [50] [51] [52]. CSFV E^{rns} can bind to exogenous double strand RNA (dsRNA) and inhibit dsRNA-induced IFN-β production, however it failed to inhibit TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β) but instead triggered IFN-β production. Deglycosylation of E^{rns} rendered it unable to bind to dsRNA, and thus unable to inhibit dsRNA-induced IFN-β production [53] [54].

1.1.3 E1 protein

Glycosylation is one of the most common types of protein modifications, and N-linked oligosaccharides are added to specific asparagines residues in the context of the consensus sequence Asn-X-Ser/Thr [55]. E1, with its molecular weight 33KDa and containing 195 amino acides, is the smallest envelope and type I trans-membrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor, which has been implicated in viral adsorption to host cells [45] [48]. E1 has been shown to contain three putative N-linked glycosylation sites. Glycosylation sites (at CSFV amino acid residue position N500, N513 and N594) are highly conserved among CSFV isolates and two of them (N513 and N594) also conserved among other *Pestiviruses* [48]. Removal of all three putative glycosylation sites in E1, at CSFV positions N500, N513 and N594, yielded non-viable progeny, while single or dual site mutants excluding N594 were viable. Virus glycoproteins are crucial to of the virus cycle such as attachment to host cell receptors, entry, assembly of newly produced viral progeny, and exit [56]. *In vivo*, viral glycoproteins have been shown to influence infectivity, virulence, and host immune responses. Modifications of glycosylation sites in envelope proteins E1 yielded viruses with a variable degree of attenuation [48)]. Modification of E1 glycosylation patterns could be used for developing CSFV live-attenuated vaccines [57].

1.1.4 E2 protein

Envelope protein E2 (373 amino acides, 51-55KDa) is the major envelope glycoprotein exposed on the outer surface of the virion and represents an important target for induction of the immune responses during infection [58]. The protein contains four antigenic domains (A, B, C, and D), which are located within the N-terminal half of the protein. E2 forms a heterodimer with E1 as well as homodimers, and it may play a major role in virus attachment and entry [45]. On the basis of amino acid sequence analysis and determination of the N-terminal, it was shown that processing of E2 is mediated by a host cell signalase. Typical hydrophobic signal sequences were identified upstream of the E2 N-terminus within E1-coding sequences. A transmembrane anchor of about 40 hydrophobic amino acids was demonstrated at the C-terminus of E2. The E2 glycoprotein contains sequential neutralizing epitopes, which are responsible for eliciting neutralizing antibodies which confer protective immunity and is therefore frequently used for designing DNA vaccines against CSF [59] [60].

1.2 Non-structural proteins

1.2.1 Npro protein

Npro (168 amino acids, 23KDa) is the first non-structural protein encoded in the ORF protein. Npro (N-terminal auto-proteinase) exerts two known functions: an auto-protease activity for co-translational cleavage from the nascent downstream nucleocapsid protein C and as an antagonistic effect on the IFN-α/β induction pathway [49] [51], [61]. Npro is a cysteine proteinase, that has similarities to subtilisin-like proteinases and which is not found in other viral systems [54]. Mutants lacking the Npro gene, as opposed to wild-type CSFV, induced type I interferon in cell culture, which suggests a function in the interference with the primary cellular antiviral defense [22]. Previous studies indicated that the N-terminal proteinase Npro is required for virulence of CSFV. Replacement of the Npro gene of CSFV by the murine ubiquitin gene only slightly affects the characteristics of virus replication in the porcine kidney cell line SK-6. Mutant vA187-Npro-Ubi obtained by replacement of the Npro gene by the murine ubiquitin gene in the CSFV genome, did not induce any clinical symptoms in experimentally infected pigs [52]. Npro deletion mutants are attenuated and induce protective immunity in SPF (Specific pathogen free) pigs after single oronasal inoculation [62].

1.2.2 P7 protein

Downstream of E2, the small hydrophobic protein p7 (70 amino acids, 7 kDa), flanked by signal peptidase cleavage sites, is essential for infectious virus production, but is not associated with virus particles [63]. In cell extracts, p7 is partially associated with E2 protein. Separation of the E2 and p7 genes with an internal ribosome entry site (IRES) resulted in a viable virus, demonstrating that the E2-p7 precursor protein is not required for virus replication [40] [64]. Generation of p7 requires microsomal membranes, but p7 is not a major structural component of the virion. p7 forms the junction between the structural and the non-structural genes in *pestiviruses*, and the same is probably true for hepatitis C virus. The nature of p7 and E2-p7 and the peculiar processing of the E2-p7-NS2 region are well conserved among *pestiviruses* [40].

1.2.3 NS2 protein

Except for the leader protein Npro, all the mature processing products and the precursor protein NS2-3 are essential for the viral life cycle, while the proteins NS3 to NS5B are sufficient for RNA replication [64] [65]. NS2 is an auto-protease associated with a cellular chaperone termed JIV (J-domain protein interacting with viral protein) and cleaves the NS2-3 protein between NS2 and NS3 [66]. NS2-mediated cleavage at the NS2-3 site is essential for the *Pestivirus* life cycle and is temporally modulated by a 1:1 stoichiometric association of NS2 with the cellular cofactor JIV [67]. Consumption of the intracellular JIV pool that remains tightly associated with NS2, and the restriction of NS2 to cis-cleavage limits the NS2 activity to shortly after infection, resulting in the virtual absence of NS2-3 cleavage at later times post-infection and, consequently, in the reduction of viral RNA replication, which is crucial for the virus to persist [37, 41]. Nevertheless, NS2 might exert other functions not required for the viral life cycle. NS2 was shown to inhibit gene expression from different cellular promoters as well as to interfere with cell proliferation [64] [68].

1.2.4 NS3 protein

Non-structural protein NS3 is multi-functional protein involved in polyprotein processing [69]. Previous reports showed that NS3 protein can induce apoptosis in host cells that present with cytopathic effects (CPE) [68]. NS3 is believed to possess three enzyme activities that are likely to be essential for virus replication: a serine protease located in the N-terminus of one-third of the protein and RNA stimulated nucleotide triphosphatase (NTPase) as well as RNA helicase activities located in the C-terminal portion [24] [70]. The presence of these activities, initially inferred from the identification of amino acid motifs and alignments with related proteins, has subsequently been demonstrated for several members of the family *Flaviviridae*. Circumstantial evidence suggested that the protease and helicase/NTPase domains of the NS3 protein were functionally interdependent [71]. Bioinformatics analysis showed that the CSFV NS3 protein contains canonical amino acid motifs present in all super-family II RNA helicases [24]. The NTPase/helicase activities of NS3, together with the NS5B (RNA-dependent RNA polymerase, RdRp), should be essential for viral replication [69]. Wen et al. reported that NS3 helicase activity was dependent on the presence of NTP and divalent cations, with a preference for ATP and Mn2+, and required the substrates possessing a 3‟-un-base-paired region on the RNA template strand [72]. NS3 protease domain enhanced the NS3 helicase activity but had no effect on its NTPase activity. For the truncated NS3 helicase domain (NS3h), both NTPase and helicase activities were up-regulated by NS5B [73]. The NS3h specifically interacted with the plus- and minus-strand 3‟-NTR. Characterization of the NS3 NTPase activity was confirmed and it was shown that both the full length and truncated NS3 enhanced IRES-mediated and cellular translation [69] [74].

1.2.5 NS4A and NS4B proteins

NS4A associates with the N-terminus of NS3 and acts as a cofactor for the serine protease activity [64]. A role in cytopathogenicity was associated with NS4B [23]. NS4A is an indispensable cofactor of uncleaved NS2-3 in the formation of infectious particles [75]. Importantly, NS4A can be supplied in trans form separately from NS2-3 without loss of efficiency [64]. NS4A is important for the correct conformation, topology and functionality of NS3 within the infected cell [69]. Another function of NS4A could be to recruit other viral or cellular proteins, as for example NS4B and NS5A for which an interaction with NS4A has been reported [76].

1.2.6 NS 5A and NS5B proteins

Cleavage between NS5A and NS5B is slow, accordingly, the precursor protein NS5A-5B can be detected in cell extracts and is processed in NS5A and NS5B with a halflife of less than 60 minutes [77]. NS5A is phosphorylated and was shown to be the only protein of the replication complex that can be complemented in trans form [64]. NS5A was also found to interact with a subunit of the translation elongation factor 1A (TEF 1A), suggesting a role of this protein in genome replication and translation [78]. NS5B is located at the extreme C-terminus of the polyprotein and binds to the 3′-NTR of the viral RNA and initiates its replication. Binding to this part of the genome will cause the degradation of the entire viral RNA, likely reducing viral replication [79]. NS5B contains motifs shared by RNA-dependent RNA polymerase (RdRp), such as the Gly-Asp-Asp (GAA) motif, which is highly conserved among RdRps and has been demonstrated to possess RdRp activity [80]. RdRp is a key enzyme which initiates RNA replication by a *de novo* mechanism without a primer and is a potential target for anti-virus therapy [81]. A three-dimensional model has been built by homology modeling based on the alignment with several virus RdRps and the results indicated that the fingers domain contains an N-terminal region that plays an important role in conformational changes [82].

1.3 Non-translated region (NTR)

The 3′-NTR of plus-strand RNA of CSFV genome is believed to be the first entry site for viral replicases for initiation of RNA genome replication. The helicase activity is needed to unwind the secondary structure present in the 3′-NTR for the replication. The 3′-NTR is most likely involved in initiation of the *pestiviral* genome replication, also involved in the coordination of the viral translation and replication. The 3′-NTR is an important site for interaction between proteins and viral RNA genome [83]. 3′-NTR mutants with deletion of 3′-terminal sequences is unable to be bound to NS5B proteins and even inhibits RNA synthesis by viral replicase [84]. The evidence suggested that 3‟-terminal sequence of 3‟-NTR is essential to replicase and helicase activities [82]. The 3‟-terminal sequence of 3‟-NTR might be also the first interactive site between helicase and the viral genome [70] [85]. The intact secondary structure of 3‟-terminal sequence of 3‟-NTR is important in helicase binding, which has been demonstrated to be necessary for replicase binding and RNA synthesis. Moreover, the 3‟-NTR may contact the 5‟-NTR by RNA-RNA interactions [69]. 3‟-NTR also is the site for the interaction between NS5B replicase and viral genome. The 5‟-NTR contains the IRES for cap-independent translation of the viral polyprotein. The 3‟-NTR may contain replication signals involved in minus-strand RNA synthesis [80]. The 5‟-NTR contains cis-elements required for replication [85] and an IRES for cap-independent translation initiation of the viral polyprotein which is co- and post- translationally processed by host cell and viral proteases, whereas the 3‟-NTR contains signals involved in the RNA replication process [86].

2. Clinical signs of CSF

CSFV infections are often fatal and characterized by fever, hemorrhages, ataxia and immunosuppression. However, diagnosis based on clinical signs and postmortem lesions are difficult, as the course of infection varies depending on host characteristics and the virus strain [4] [12]. The disease can manifest as rapidly fatal to sub-clinical. Acute forms of CSF are associated with highly virulent CSFV strains characterized by high fever, depression, hyperemia, purulent conjunctivitis, and severe neurological signs with mortality rates of up to 100% in young pigs. Persistent infections characterized as „chronic‟ or „late-onset‟ are associated with CSFV strains of moderate and low virulence [2]. Moderate forms of CSF are similar to acute infections, but characterized by a longer incubation period (6-7 days) followed by fever, chronic enteritis and muscle wasting. Transient recovery may occur followed by relapse and death [2] [87]. Low virulent infections are mild or inapparent and often very difficult to diagnose. Characteristic signs are often missed and may be absent for several months after the initial infection making early detection difficult leading to the uncontrolled spread of infection [87]. The spreading of CSF has variability of clinical signs and lesions depending on viral virulence and host factors such as age and disease resistant factor of pigs [4] [88] [89].

Pigs infected with high virulence have incubation period ~ 2-10 days, then pigs will show clinical signs with dullnes, high fever (41-42^{o}C) (**Figure 2a**), leucopenia (<9,000 cells/mm^{3}) which will find on 2-6 days post-infection, conjunctivitis, constipation followed by diarrhea, convulsion (Figure 2b), hemorrhages occur on body and various organs **(Figure 2c**), and pigs usually die within 10-20 days post-infection. In chronic case, clinical signs and lesion are not clear as acute case. Clinical signs can be separated into three periods; first period, pig has fever but not so high; second period, pig get better, recover and may produce antibody to CSFV; third period, pigs show clearly clinical signs. Because of the long time sickness, pigs usually co-infect with other microorganisms, increase severity of disease and die within 1-3 months. In-utero infection with strains of the virus of moderate or low virulence can result in „carrier sow syndrome‟ followed by prenatal or early post-natal death, the birth of diseased piglets or an apparently „healthy‟ but persistently infected litter. Infected pigs do not show clinical signs at the early period but show chronic signs at the lately period, the interval time from sick until die may long as 2-11 months that result in the long time spreading of virus from infected pigs to another pigs which is the important mechanism for virus to remain in pig herd [90].

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(a) (b) (c)

Figure 2. Clinical signs and lesions of CSFV-infected pigs; (a) dullness and high fever, (b) convulsion and (c) hemorrhages on body.

3. Pathogenesis of CSFV

The most common route of infection is the oronasal route. After exposure, CSFV first replicates in the tonsils, where it infects the crypt-epithelial cells, macrophages, lymphocytes, and endothelial cells by heparin sulfate and chondroitin sulfate B glycosaminoglycan receptors [91] [92] [93] [94]. It then spreads to regional lymph nodes and from there disseminates systemically via viremia [91] [93] [94]. The rate of CSFV spread to all systems is positively correlated with its virulence. Highly virulent CSFV can rapidly distribute throughout the body after challenge and can induce higher viral load in tissues, more shedding, and more severe pathological changes than moderately virulent strains [91] [95] [96] [97]. In its acute form, CSF can induce systemic hemorrhaging and immunosuppression by down-regulating the cytokines and immune cells, including endothelial cells, lymphocytes, monocyte/macrophage lineage cells, dendritic cells (DCs), and bone marrow hematopoietic cells (BMHCs). The systemic hemorrhaging is the result of CSFV-induced necrosis of endothelial cells with subsequent vasculitis and hemorrhaging, accompanied by severe anemia, thrombocytopenia and disturbance of fibrinogen synthesis [95] [98] [99]. The immunosuppression is the result of downregulation and necrosis of BMHCs and lymphocytes including granulocytes, B-lymphocytes, $CD3^{+}CD4^{+}CD8^{+}$ memory T-helper, $CD3^{+}CD4^{+}CD8^{-}$ naive T-helper, $CD3^{+}CD4^{-}CD8^{+}$ cytotoxic T-cells, and $CD3^{+}CD4^{-}CD8^{-}$/lowγδ T-cells [95] [100] [101] [102]. The results of lymphocyte depletion have demonstrated that CSFV directly or indirectly induced the lymphocyte apoptosis by the reduction of mitochondrial transmembrane potential and E^{rns} protein [100] [101] [103. Besides, TNF-α from CSFV-infected macrophage is able to induce the apoptosis of B-lymphocytes [104] In the T-lymphocytes, CSFV infection increases the CD49d, MHCII, and Fas expression that increase susceptibility to apoptosis mediated by Fas-FasL interaction [101]. In addition, the proliferation, cytolytic activities, and MHC-restricted lysis of T lymphocytes are markedly reduced by CSFV infection and E^{rns} after ConA stimulation [101] [105] [106]. Taken together, these CSFV-induced lymphocytic changes would lead to lymphopenia and immunosuppression in CSFV-infected pigs. Taken together, these CSFV-induced lymphocytic changes would lead to lymphopenia and immunosuppression in CSFV-infected pigs.

The amount of virus in blood is highest at 5-6 days after infection that pigs start to show clinical signs. Pigs with clinical signs show rapidly decreasing of white blood cells (WBC) that may be ≤ 9,000 cells/mm^3. Because of CSFV infection, results rapid decreasing of WBC, the researcher attempt to find out the cause of this change. The experiment of Summerfield et al., (1998) [101] found that the important cause of rapid decreasing of WBC was the apoptosis mechanism of both T-lymphocyte and B-lymphocyte. Especially in the case of T-lymphocyte, the change can be found both in CD4+ and CD8+ that can be detected since 2 days after infection. The amount of WBC is lowest on day 7 after infection [99]

Examination of pathological lesion found the severe atrophy of lymphoid organs of pigs [107]. Pigs infected with moderate/high virulent CSFV Romania (genotype 2.3) field isolate and CSFV Israel (genotype 2.1) field isolate had tonsil inflammation **(Figure 3a),** hemorrhages visible as blebs in the border of the spleen **(Figure 3b),** petechial bleedings in the lymph nodes **(Figure 3c)** and petechial bleedings in the kidney together with swellings [89]

(a) (b) (c)

Figure 3. Pathological lesions showed necrosis of (a) tonsils (b) lymphnodes and (c) spleen

It was found that the main cause of pathological change were apoptosis of lymphocytes, especially cortex of thymus was seriously destroyed. The pathological change may concern with cytokine which play role as the important mediator for apoptosis of lymphatic organs [108] [109]. It was found that various kinds of cytokine played important role for apoptosis of lymphocyte in thymus of infected pigs and could detect the synthesis of cytokines tumor necrosis factor-alpha (TNF-α) and interleukine 1-alpha (IL-1α). These two kinds of cytokine may be the important mediator to play role on apoptosis of thymocytes by working together. Working of mediator and stimulation of apoptosis may be the important mechanism which cause pathological lesion of those organs. After virus infection, the immune systems of pigs will response to virus both CMI and HI. Because of the antibody detection to CSF cannot differentiate between the maternal immunity, and immunity stimulated by infection or vaccination. In addition, the increasing of immunity level that can protect pigs takes at least two weeks. Thus, researchers are interested to study the role of CMI in CSF, including the development of CMI measurement method together with antibody level measurement method. Development of CMI measurement method by ELISPOT and find that this method can be used to detect CSFV-specific IFN-γ secreting cells in pigs vaccinated with swine fever vaccine since 6 days post-vaccination and can detect until the late period of fattening pigs. It was found that T-cells epitope was the position on antigen for specific stimulation of CSFV [65]. It was found that this epitope was between 1446-1460 amino acids of CSFV, Glenfort strain, which this T-cells epitope was in NS2-3 of virus and was the part that conserved in SFV. The experiment found that T-cells epitope could stimulate the synthesis of CSFV-specific IFN-γ and stimulate antigen response together with major histocompatibility complex (MHC) molecule to T-cells especially MHC class II. In addition, it was found that CSFV-specific cytotoxic T lymphocyte (CTL) could destroy cells at this present peptide. The study of two groups of researcher showed that CMI play important role on prevention of CSF occurrence. The results from these studies can be used for vaccination monitoring and vaccine development.

4. Immune response induced by CSFV infection in pigs

Understanding the interaction of CSFV with the cells of the porcine immune system and its role in viral pathogenesis is a key point in designing new antiviral strategies. Work carried out several decades ago described the capacity of CSFV to destroy the lymphoid follicles and these results have subsequently been confirmed [105] Among the main targets for CSFV are bone marrow cells from the monocyte-macrophage linage ($SWC3^{+}$; $SWC8^{-}$). Interestingly, while mature granulocytes ($SWC3^{+}$; $SWC8^{+}$) are not susceptible to CSFV infection, the virus does infect the less differentiated myeloid progenitor cells ($SWC3^{low}$; $SWC8^{-}$), thus explaining the presence of CSFV in peripheral blood mature $SWC8^{+}$ cells [95] [100].

Despite the high mortality and the severity of lesions in animals with virulent CSFV, one peculiarity of CSFV infection is that most infected cells in the bone marrow remain healthy, while surrounding uninfected cells succumb, mainly due to bystander apoptosis. This is most probably due to soluble factors secreted by the infected cells [99]. Infected pigs show marked immune suppression, with an altered population of T cells and depletion of lymphocytes, mainly $CD4^{+}$ and $CD8^{+}$ T cells. Depending on the virulence of the viral strain, pigs can have as much as 90% of their total T cells depleted in the final stages of the disease [105]. This effect can be observed as early as one day after infection, even before viraemia has been established [101]. Immunosuppression can be detected much earlier than seroconversion and clinical signs of the disease, which is relevant both for early diagnosis and for the study of viral pathogenesis [105] [100] [102] [110]. As with many other severe haemorrhagic diseases, CSFV infects endothelial. These cells seem to play a key role in CSFV pathogenesis, which is characterized by the development of microthrombi, disseminated intravascular coagulation and fibrinolysis, among other clinical signs [102]. While CSFV infection of endothelial cells suppresses both interferon production and apoptosis, it induces inflammatory cytokines.

CSFV exploits the migratory capacity of macrophages and dendritic cells (DCs) for dissemination throughout the body [111]. Activation of anti-apoptotic pathways in infected cells ensures virus survival, whereas induction of the expression of soluble pro-inflammatory cytokines promotes bystander apoptosis of the surrounding T cells, thus avoiding recognition of the infected cells [98].

Recent evidence suggests that CSFV might induce immune suppression not only by secreting cytokines, but also by other mechanisms, such as expressing the CSFV structural E^{rns} protein, which has been shown to be toxic for T cell lymphocytes in vitro [103][105]. CSFV also activates T cells that secrete cytokines, such as IL10, which is probably a key cytokine in the immunosuppression observed after CSF infection [25]. Considering the capacity of CSFV to escape the immune response, strategies must be designed to control the infection and to prevent CSFV-mediated immune evasion.

As mentioned above, CSF is generally a devastating disease characterized by high mortality rates. It induces a marked immune suppression in infected individuals that, depending on the virulence of the strain and the immunological status of the infected animal, might cause the death of the animals before an immune response is induced. However, some animals are able to overcome infection as a result of protective immune responses, becoming protected from re-infection for the rest of their lives [12]. In some cases, the immune responses are not sufficient to clear the virus from the body, leading to persistence of CSFV in the host. Chronic infections can be established in the presence of neutralising antibodies. Conversely, congenital infection with CSFV can result in persistently infected animals, which do not develop specific antibodies against the virus [112] [113]. This is probably due to immunotolerance developed during foetal lymphocyte differentiation. Persistently infected animals continuously shed virus and are a potential source of new CSF outbreaks, as well as creating problems in diagnosis.

Persistence of CSFV in the animal and the induction of chronic infection makes it necessary to understand not only how the virus interacts with the cells of immune system, but also the specific immune responses that are involved in protection [114]. Recent reports indicate that infected pigs are able to induce both specific $CD4^+$ T cells and cytotoxic T lymphocyte (CTL) responses ($CD8^+$ T cells) from 1 to 3 weeks after infection, even before neutralizing antibodies appear. The specific CTLs mainly recognize the structural E2 protein and the non-structural NS3 protein of CSFV [23] [24]

[110]. These results correlate with previous findings describing a CTL epitope at the interface between the NS3 and NS4 viral proteins [26]. Several swine leucocyte antigen (SLA) types I and II peptides have been characterized, most of them mapping to the NS3 polypeptide and to the structural E2 protein [65].

Neutralizing antibodies in survive CSFV-infection pigs could not be detected until 2–4 weeks post-infection and they remain relatively constant for the rest of the life [12]. Most neutralizing antibodies specifically recognize the structural E^{rns} and E2 proteins [115] [116] and their presence has been associated with protection [117]. Antibodies against the NS3 protein are also induced upon infection but, despite their ability to recognize different pestiviruses, they are non-neutralizing antibodies. The E2 glycoprotein is the most immunogenic CSFV protein and the only one capable of conferring protection against CSFV challenge by itself [117] [118] [119]. Thus, E2 has become the main candidate for inclusion in subunit and recombinant vaccines against CSFV.

5. Vaccine strategies against CSFV

5.1 CSFV classical vaccines

While the early inactivated vaccines against CSFV were not effective against virus infection [120], live attenuated vaccines were demonstrated to be very efficient at preventing disease. The first immunization experiments with live attenuated lapinised strains (attenuated after serial passages in rabbits) were performed over 60 years ago [121]. Since then, little progress has been made in this field. CSFV attenuated vaccines are safe, induce high levels of neutralizing antibodies and are capable of protecting against highly virulent strains of CSFV, even in pregnant sows as early as 5 days after vaccination. Attenuated CSFV vaccines remain the method of choice to control the disease, especially in areas in which CSF remains enzootic.

The mechanisms mediating the protection conferred by attenuated strains of CSFV are not fully understood. Vaccinated animals are protected against disease before neutralizing antibodies are detected [22]. After vaccination with the C- strain, antibodies are detectable as early as 12 days post-immunization, reaching a peak at 4–12 weeks [21] [122]. Antibodies can persist for life in animals that have received a single dose of vaccine.

Neutralizing antibodies play an important role in protection against CSFV [16] [123] [124]. However, some reports indicate a lack of complete correlation between antibody titers and protection [123] [124] and therefore other arms of the immune response might also be important in conferring protection. As a general rule, animals developing neutralizing antibodies will be protected against subsequent challenge [22] [125], but protection after vaccination in the absence of neutralizing antibodies also has been observed [22] [110] [126].

Most of the information regarding the immunological mechanisms involved in protection against CSFV infection was derived from an experiment using the modified live, Chinese (C) strain vaccine. The C strain vaccine is generally accepted to be very safe in pigs of all age groups, providing complete protection, i.e. sterile immunity in vaccinated pigs. The C strain vaccine usually induces detectable neutralizing antibodies in vaccinated pigs 2–3 weeks following primary vaccination [21] [122]. However, pigs vaccinated with the C-strain vaccine appear to be completely protected against virulent CSFV challenge as early as 1 week following vaccination [12]. It should be noted that, in some cases, vaccine induced protection was achieved in the absence of neutralizing antibodies at the time of challenge [12] [117]. These findings implicate the vital role of cell-mediated immunity (CMI) in viral protection during the early phases of immunity.

Cell-mediated immunity (CMI) is known to have both an effector and a regulatory role on the immune system and is believed to be essential for immunity against intracellular pathogens, including viruses [128]. Previous information regarding the role of CMI in vaccine induced, viral protection in pigs has been very limited, due to the lack of reagents and the impracticality of the conventional techniques for the detection of porcine CMI. Nevertheless, antigen specific lymphoproliferative activity was demonstrated in peripheral blood lymphocytes from vaccinated pigs that were protected against the CSFV challenge [129]. The existence of CSF-cytotoxic T lymphocytes

(CTL) in the peripheral blood mononuclear cells (PBMC), from immunized pigs, has been demonstrated [26] [65] [130]. An alternative technique for assessing CMI is to measure the production of cytokines that is known to influence or directly relate to cellular immunity. Among these cytokines, the role of interferon-gamma (IFN-γ) for the induction of CMI has been well characterized. IFN-γ has several immunoregulatory effects that are involved in the induction of anti-viral immunity, including the activation of CTL, natural killer (NK) cells and phagocytes [128]. The measurement of viral-specific IFN-γ production is believed to be a direct reflection of viral-specific lymphocyte activities [131] [132]. Furthermore, viral-specific IFN-γ production remains detectable for viral-specific lymphocyte activities [131] [132]. Furthermore, viral-specific IFN-γ production remains detectable for a long period of time, whereas lymphocyte proliferation tends to diminish due to the decreased ability of T cells to produce IL-2 [131]. Several reports have shown that the number of viral-specific IFN-γ producing cells is a good indicator of anti-viral immunity [131] [132] [133]. CSFV specific IFN-γ secreting cells could be detected in the PBMC from pigs vaccinated with the lapinised C strain vaccine, as early as 6 days, and lasted for up to 140 days, following vaccination [22]. Furthermore, disease protection could be demonstrated in pigs carrying high numbers of CSFV-specific IFN-γ cells at the time of challenge, while neutralizing antibodies were still undetectable [17] [22]. These findings highlight the role of vaccine-induced cellular immunity in viral protection during the early phases of immunity. In addition, the role of CMI in protection against CSFV infection was indicated even in the presence of CSFV specific neutralizing antibodies. When the pigs were challenged 140 days after a single vaccination, good correlation occurred between the level of protection and the level of IFN-γ production, more than the antibody titer [22]. Despite the protective role of the cellular immune response at the time of challenge, the role of CSFV-specific antibody following such challenge should not be excluded, since there was a significant increase in the SN titers of the vaccinated pigs, but not the control unvaccinated pigs, following the challenge [17] [22]. Generally, CSFV infection causes severe B-cell depletion [106], possibly by the induction of massive lymphocyte apoptosis

[101]. Protected pigs were probably able to control the establishment of viral infection, and therefore, B cell function was not affected. The rapid anamnestic antibody response following the CSFV challenge was certainly advantageous in reducing viral spreading in the protected animals. Recent studies revealed that PBMC from pigs vaccinated with the C-strain vaccine produced IFN-γ in response to CSFV and that the double-positive (DP), CD4+CD8+ population was the major IFN-γ producer [25]. This finding is in agreement with previous reports demonstrating that DP cells, which are the memory T helper (Th) population, can produce high levels of IFN-γ in response to a recall antigen or polyclonal T cell activator [134] [135].

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It should also be noted that the number of IFN-γ producing cells, following vaccination, was always lower than that observed following CSFV challenge [25] [136]. This finding is consistent with another report that demonstrated that immunization with a modified live vaccine induces a lower level of cell-mediated response than the actual viral infection [130]. Interestingly, the CD4^{-}CD8+ population was found to be the major IFN-γ producer, while there were a significantly less number of IFN-γ producing DP cells in the PBMC of the vaccinated pigs during the first week following CSFV challenge [25]. As it has previously been suggested, porcine memory Th population preferentially homes to the secondary lymphoid organs [133], thus, the low number of antigen-specific DP cells may simply reflect the difference in tissue homing preference among the lymphocyte subpopulations, during the effecter phase of the immune response. The increased CD8+ activity in vaccinated pigs following challenge was possibly vaccine-induced anamnestic response of the CD8+population. Alternatively, it is also possible that rapid IFN-γ production by CSFV-specific memory Th, following the challenge, might contribute to the generation of CTL [130]. Nevertheless, the data suggests that activation of memory CSFV specific Th cells and priming of a viral specific CD4^{-}CD8+ population, possibly CTL, occurred in the pigs vaccinated with the C strain vaccine. These cellular activities are likely be one of the protective immune mechanisms induced by the C strain vaccine, particularly during an early phase of immunity. When vaccinated pigs were challenged at a later stage after vaccination, there seemed to be a good correlation between the presence of neutralizing antibodies at the time of challenge and viral protection [22] [125]. It is generally accepted that vaccinated pigs with active neutralizing antibody titers of 32 were considered to be protective [125]. It should be noted that this assumption was obtained from experiments using a modified live vaccine. As the vaccine antigens are able to enter both the endogenous and exogenous antigen presentation pathway, this type of vaccine should efficiently activate both humoral (neutralizing antibody) and cell-mediated immunity (CTL). In general, active CSFV-specific antibody production following vaccination, with the C strain vaccine, correlated well with the levels of IFN-γ production [22]. Therefore, routine monitoring of SN titers, i.e. seroprofile, can still be used for assessing the effectiveness of a CSF vaccine program in the field. In fact, we strongly recommend routine monitoring of herd immunity to CSFV, in farms situated in a highly endemic area. As suggested above, neutralizing antibodies also contribute to viral protection, through the reduction of spreading and shedding of the infectious virions. The evidence that neutralizing antibodies, alone, could provide protection has also been reported. Piglets with exceptionally high levels of maternal derived neutralizing antibodies (>512) are passively protected against the CSFV challenge [137]. Furthermore, the E2 subunit vaccine, which mainly activated the CSFV-specific Th population and the production of neutralizing antibodies, confer clinical protection against the CSFV challenge [138] [139] [140]. However, it should be noted that the information [12]. As viral protection induced by the E2 subunit vaccine is likely to rely on the ability to induce CSFV-specific neutralizing antibodies [12], it may take a longer time (at least 2–3 weeks) following vaccination or more than one vaccination to obtain complete viral protection. A recent study in Thailand has shown that at least two vaccinations were required to induce clinical protection in pigs carrying a low level of maternal derived antibodies (MDA), at the time of vaccination. Nevertheless, most of the protected pigs still developed fever, leucopenia, and viraemia following the viral challenge [141]. The efficacy of the E2 subunit vaccine for prevention of horizontal and vertical viral transmission has also been previously placed in doubt [140] [142].

Findings on the efficacy of the E2 subunit vaccine suggest that nature of an antigen presented to the immune system (i.e. endogenously or exogenously produced form) can significantly affect the ability of the vaccines to induce protective immunity against CSFV. Generally, exogenous protein antigen does not efficiently activate CTL activity [128]. It should be noted that CSFV is cell-associated and a non-cytopathic virus. Replication is restricted in the cytoplasm of the cell and the virus can spread directly from the infected cell to adjacent cells [90]. Thus, inefficient priming of viral specific CTL could be one of the explanations for incomplete viral protection induced by the subunit vaccine. Interestingly, plasmid encoding E2 protein of CSFV conferred complete protection in the vaccinated- challenged pigs, even in the absence of neutralizing antibodies at the time of challenge [110]. Together, these findings emphasize the vital role of cell-mediated immunity (i.e. viral specific CTL activity) for controlling viral spreading in the challenged animals.

Although classical live attenuated vaccines against CSFV have been shown to be effective, they also have several disadvantages. Antibodies induced upon vaccination are indistinguishable from those elicited in natural infections, which impairs their differential diagnosis [114]. Also, errors in the conservation and/or manipulation of the vaccines and incomplete coverage of the overall susceptible population with the vaccine, might lead to protection failures and to the appearance of carrier animals that cannot be distinguished with the current diagnostic tools [143] [144] [145]. In addition, attenuated vaccines are able to replicate to a limited degree in lymphoid organs, mainly in the tonsils, for at least 15 days, and are indistinguishable from the virus in the field using standard diagnostic tests [146].

Vaccination against CSFV currently is not permitted in the EU, except in emergency outbreaks. The strict limitations and restrictions of the animal movement policy make further implementation of vaccination difficult, thus favouring the culling of animals. Apart from ethical reasons, the slaughter of non-infected animals in infected control zones has caused major economic losses in affected countries in the EU. Therefore, the policy of non-vaccination or limited emergency vaccination may not to be sustainable. Clearly, new strategies have to be implemented to control CSF, avoiding the sacrifice of large numbers of pigs if an outbreak occurs within EU borders [56] [147].

5.2 Subunit vaccines: immunogenicity of the CSFV E2 glycoprotein

Several strategies have been developed to generate DIVA vaccines and accompanying diagnostic tests in order to fulfill the need for new and safer "marker vaccines" to control CSF [90]. The most efficient subunit vaccines developed against CSFV to date are based on the E2 major glycoprotein from the CSFV envelope. A variety of degrees of protection have been afforded by using different forms of the recombinant E2 glycoprotein expressed in the baculovirus system [60] [119] [148]. One of the most immunogenic versions of the recombinant E2 glycoprotein lacks the transmembrane domain, thus facilitating its secretion and purification from supernatant of baculovirus infected cells.

So far, most of E2 subunit marker vaccine formulations are in the experimental phase and only two vaccines licensed by the European Agency for the Evaluation of Medicinal Products (EMEA) are commercially available (Bayovac CSF, Bayer; Porcilis Pestis, Intervet). Vaccinated animals develop specific antibodies that exclusively recognise the E2 glycoprotein, whereas pigs infected with field strains of CSFV also develop antibodies against the E^{rns} protein, which can be distinguished with a specific enzyme-linked immunosorbent assay (ELISA) [138]. Despite the advantages of using subunit vaccines, their protective efficacy is still under evaluation [56] [149]. Compared with classical attenuated vaccines, the protection conferred with subunit vaccines is much more limited, especially among pregnant sows, and there is a higher risk of establishment of persistently infected individuals [56] [126] [138] [149] [150] [151] [152].

Two final reasons, both related to the lack of efficient differential diagnostic methods, have contributed to the non-implementation of these vaccine strategies as an emergency measure in the field [152]. Firstly, specific antibodies against E^{rns} are not developed until 3–6 weeks after natural infection with field strains of CSFV (depending on the virulence of the CSFV strain), which makes the differential diagnosis at early stages of infection difficult [113]. Secondly, the available E^{rns} based ELISAs are still far from being as sensitive and specific as the E2 based ELISAs [149].

Using the baculovirus expression system, it has been possible to map protective domains within the E2 glycoprotein [148] [153] and, more recently, to obtain a detailed characterization of some protective epitopes within E2 [150] [151]. All these studies now would permit the inclusion of defined neutralising B cell epitopes in new recombinant vaccines, with the aim of inducing protective immune responses that facilitate the differential diagnosis of vaccinated versus CSFV infected individuals.

In addition to the development of new, improved CSF vaccines, parallel efforts should be made to develop new diagnostic methods capable of differentiating vaccinated from infected individuals. Unfortunately, as for many other diseases, commercial interests might have contributed to the slow development of new and more efficient diagnostic methods and vaccines against CSFV [149].

The E2 based vaccines show limitations similar to those found for other CSF vaccine strategies. Some animals do not produce specific CSFV antibodies in response to vaccination and there is not a complete correlation between protection and the induction of neutralizing antibodies. Research on new DIVA vaccines capable of conferring high levels of protection against CSFV should be complemented by research on the development of new diagnostic tools [12].

5.3 New vaccines against CSFV

As for many other viruses, the generation and use of infectious clones has provided new opportunities for understanding and characterizing mechanisms of viral replication and pathogenesis [156]. Infectious clones of attenuated strain C, Alfort/Tübingen, Eystrup and Brescia strains of CSFV have allowed the determination of E^{rns}, N^{pro} and E2 as potential virulence factors [62] [156] [157] [158]. Information generated using reverse genetics has facilitated further the identification of CSFV proteins or protein domains that determine viral virulence and host range, as well as the design and development of new rational vaccines against CSF. The observation that virulent CSFV strains in the field can evolve into more attenuated forms of the virus under certain epidemiological conditions has opened new avenues to study specific changes in the viral genome involved in virulence, particularly in the E2 gene [159].

It has been possible to generate deletion mutant viruses lacking the entire E2 or E^{rns}. These deletion mutants have been shown to be safe vaccines, due to their ability to grow only in competent cell lines that provide the deleted protein in *trans*. Upon in vivo injection, the mutant viruses induced strong immune responses without producing new viral progeny [160]. Similar strategies have been used to develop several chimaeras by exchanging the E2 or the E^{rns} proteins from the attenuated C strain of CSFV with those from antigenically related pestiviruses [161]. Finally, virus replicon particles (VRP) are emerging as alternative and safe vaccine candidates, due to their inability to be transmitted [162]. In order to be more efficient, these future suicidal vaccines should contain the E2 glycoprotein that contains most of the known cellular and humoral protective determinants of CSFV [23]. One of the main concerns regarding this type of vaccination focuses on the possibility of viral recombination between the mutated vaccine strain and CSFV strains present in the field [163].

Several other strategies have been used to develop DIVA vaccines against CSFV, with variable success regarding their capacity to reduce viral replication in animals or to avoid transplacental transmission of the virus. Most of these vaccines include the full-length CSFV E2 glycoprotein or fractions of this protein. E2 has been expressed in different viral vectors, such as those derived from vaccinia and adenovirus expression vectors [117] [164] [165], or species-specific vectors, such as porcine poxviruses or Aujeszky‟s disease virus [118] [166]. In all cases, these recombinant vectors have conferred protection against clinical disease. Bovine viral diarrhoea virus (BVDV), a pestivirus antigenically related to CSFV, has been used as a vector to express the CSFV E2 glycoprotein, inducing protection against CSFV challenge [167]. Despite the protective potential of these vaccines, much remains to be done to characterise the immune responses they induce and their protective efficacy in terms of viral dissemination and transplacental transmission of CSFV [167].

5.4 DNA vaccines against CSFV: a real alternative?

DNA immunisation is one of the most promising strategies of vaccination developed in the last decade. DNA vaccines are capable of inducing both humoral and cellular immune responses. They are easy to produce and have been demonstrated to be safe, even for immunization of neonates in the presence of maternal antibodies [168] [169]. Due to its simplicity, DNA vaccination also permits customised design of vaccines [170] and has the potential to direct encoded antigens to specific pathways [171] [172] [173]. DNA immunization can be a useful tool in understanding the intrinsic immunological mechanisms involved in protection against a given pathogen [174] [175] or to map immunogenic protective determinants [176].

CSFV has been included in this revolution within the field of vaccine development. Most of the DNA vaccines developed against CSFV are based on the expression of its major immunogenic determinant, the E2 glycoprotein, from different viral strains. Some authors report the use of DNA vaccines encoding the full-length E2 glycoprotein, while others use mutant forms lacking its transmembrane domain [177] [178][179].

Comparison of the results obtained in vaccination and challenge studies by different groups using DNA vaccines against CSFV is difficult due to different forms of E2, different doses of challenge virus and differences in the virulence of challenge strains of CSFV. Even though immunization with large amounts of a DNA vaccine encoding the entire E2 glycoprotein (including its transmembrane region) was capable of protecting pigs from clinical CSF, no direct correlation was observed with the presence of neutralising antibodies against CSFV [177]. Similar levels of protection have been observed by using prime-boost protocols of vaccination with DNA plasmids followed by injection of recombinant adenovirus expressing the entire CSFV E2 protein [165].

Protection against CSFV in the absence of neutralizing antibodies has been demonstrated with an experimental DNA vaccine encoding the full-length CSFV E2 glycoprotein [110]. Although no antibodies were detectable prior to CSFV challenge, an accelerated humoral response was observed in some of the vaccinated animals, with neutralising antibodies being detectable as early as at day 6 post-infection. Interestingly, lack of viraemia, protection from clinical disease and protection from immunosuppression correlated with the induction of both neutralising antibodies and major histocompatibility complex (MHC) class II restricted cellular responses against CSFV [110]. This suggests that other arms of the immune response, such as the specific induction of MHC class II restricted $CD4^+$ T cells, may play a role in protection against CSFV. An ideal vaccine against CSFV therefore should induce both humoral and cellular responses. Recently, it has been shown that the induction of neutralising antibodies can [180] and IL8 plus the porcine cell surface molecule CD154 [181].

6. Towards defining viral genetic determinants of CSFV virulence

6.1 Attenuation of CSFV through serial passages in non-natural hosts

Avirulent vaccine strains are derived from highly virulent strains by attenuation through multiple passages in cell culture or non-natural animal hosts. One example is the "GPE⁻" vaccine [NCBI: D49533] that was derived from the strain "ALD" [NCBI:D49532]. With the aim of identifying the mutations responsible for the loss of virulence, the genomes of the vaccine and parent viruses were completely sequenced [182]. In this study, 225 nucleotide differences spanning the whole genome were found. Six of these differences were located in the non-coding regions. As mutations in the NTR of hepatitis C virus are responsible for the loss of virulence [183], it was postulated that the exchanges in the NTR of strain "ALD" may also be relevant concerning virulence. However, no conclusive statements on the involvement of specific nucleotide and amino acid exchanges in attenuation of the "GPE⁻" strain could be drawn.

6.2 Gain of pathogenicity by passaging the "GPE⁻" vaccine strain in pigs

Under field conditions, the "GPE⁻" vaccine virus is not transmitted. Artificial serial passages of the attenuated "GPE⁻" vaccine strain in pigs by injection of infected tonsil homogenates resulted in the recovery of a pathogenic virus after 11 passages [184]. Sequence analyses revealed one exchange in the E2 protein and two exchanges in the NS4B protein. By introducing these exchanges into the "GPE⁻" genome using reverse genetics, it was demonstrated that these three exchanges were responsible for the regaining of pathogenicity. While the exchange in the E2 protein may influence viral entry and release, the exchanges in the NS4B protein enhanced the replication efficiency in porcine cells. Evidences from other studies on the potential involvement of E2 and NS4B in virulence are discussed below. The study of Tamura and colleagues is the first that identified specific amino acid sequences of CSFV related to virulence based on gain of pathogenicity. It is postulated that the recovery of virulence is due to re-adaptation of the "GPE^{-}" virus to its original host, the pig. The process of virus adaptation involves probably both genome mutation and selection of sequences from the quasispecies population. This is underlined by the findings that exchanges in the viral NS4B protein found after several "GPE^{-}" passages in pigs were already present as a minor quasispecies in the original "GPE^{-}" vaccine. From these experiments it could not be concluded whether these genome positions are universal CSFV virulence determinants.

6.3 Codon and codon pair usage are not related to virulence.

It is well established that differences in codon usage influence protein expression [185]. In addition, the codon pair usage was found to influence virulence of poliovirus and influenza virus, for which attenuation was generated by artificially modifying the codon pair bias. This was referred to as de-optimization of the codon pair usage [186] [187]. In case of CSFV, no remarkable differences in codon usage related to virulence were observed [188]. This is supported by the observation that codon usage of RNA viruses is related to the host [189]. Comparative analyses of the codon pair usage of highly, moderately, and avirulent CSFV strains did not reveal any direct correlation between codon pair usage and virulence either [190]. Instead, it was observed that differences in codon pair usage of CSFV isolates cluster the strains into genotypes, similar to the way primary nucleotide sequence analysis and codon usage do.

6.4 A possible role of quasispecies distribution in virulence

Another aspect that has never been investigated in the context of CSFV virulence so far is the role of the quasispecies distribution of the viral RNA. Deep sequencing facilities and accompanying analysis software have made in-depth quasispecies analyses possible. A potential role of the quasispecies composition of CSFV isolates in influencing their virulence should be considered, as it had already been determined to affect the virulence of other viruses like foot-and-mouth disease virus [191] [192]. Several reports show the occurrence of different quasispecies in CSFV isolates [193] [194] [195]. In order to further investigate this, the quasispecies composition of the E2 und NS5B encoding genes of highly and low to moderately virulent strains was analyzed. Here, differences in the nucleotide variability and quasispecies entropy were observed [196]. The highest genome diversity was found in the highly virulent strains “Brescia” and “Koslov” compared to the low and moderately virulent isolates “Paderborn”, “Hennef”, and “Uelzen”. From these limited preliminary data it is not possible to state whether the higher genome variability observed in highly virulent isolates does contribute to the virulence phenotype. As severe clinical signs were observed after infections with CSFV “Eystrup” and “Brescia” derived from cDNA which have a rather homogeneous genome composition[197] [198], a high quasispecies diversity is unlikely to be a virulence determinant *per se*, but its potential involvement in influencing virulence cannot be excluded yet. For conclusive argumentation, further in depth analyses are required.

6.5 The potential role of selected individual viral proteins in CSFV virulence

Different viral proteins of CSFV were associated with virulence. The N^{pro} protein possesses two different functions: an auto-proteolytic function as protease [61] [201] [202] [203] [204] [205] by mediating the degradation of the interferon regulatory factor 3 (IRF3). Animal experiments showed that CSFV mutants missing the N^{pro} protein were attenuated [62], whereas mutations in the N^{pro} protein abolishing IRF3 degradation did only slightly alter virulence [61]. This indicates that N^{pro} is not responsible for the different degrees of virulence among CSFV isolates. More likely, inhibiting the IFN response via the IRF3 pathway may help the virus to overcome the initial innate immune defense of the host. It may also influence the persistence of the virus in the host as suggested for BVDV [206]. Furthermore, an interaction of N^{pro} with

IRF7 was reported [207]. This manipulation of the IRF7 function is another way for CSFV to circumvent the host‟s innate immune defense. But again, this is probably similarly regulated in the different CSFV isolates, independent of virulence. The structural glycoproteins *ERNS*, E1, and E2 are parts of the viral envelope membrane and are essential for replication, virus attachment, and entry [208]. In addition, the *ERNS* protein possesses ribonuclease activity [209]. Mutations that abrogate the ribonuclease activity led to virus attenuation [157]. For BVDV a role of *ERNS* in the control of IFN-β activation is described [210]. The *ERNS* proteins are found as homodimers on the surface of the virion. This dimerization is not essential for viral replication and infection [211], but the loss of dimerization causes virus attenuation as well [212]. Mutations in the *ERNS* of strain "Brescia" C1.1.1 combined with adaptive mutations in the E2 protein led to reduced virulence [158].

The E2 protein has also been proposed as a virulence determinant [158] [198] [213]. E2 is the most immunogenic protein of CSFV and induces the formation of neutralizing antibodies, especially against the highly immunogenic TAV-epitope therein (amino acids TAVSPTTL) [44] [45] [214] [215] [216]. Replacing the TAV-epitope in CSFV with the corresponding epitope of BVDV strain NADL resulted in attenuated CSFV mutants [213]. The exchange of the complete E2 protein of the highly virulent strain "Brescia" with that of a CSFV vaccine strain resulted in a less virulent phenotype of the chimeric pestivirus [198]. By performing biostatistical analyses of evolutionary patterns within the E2 protein of CSFV, a correlation between adaptation and virulence was considered [217]. These findings, and the findings made by Tamura and colleagues, point towards a potential role of the E2 protein in virulence [184].

Besides the E2 protein, the NS4B protein appears to influence virulence too. As mentioned above, two amino acid exchanges in NS4B were involved in the partial recovery of virulence after artificial re-adaptation of the "GPE$^-$ "vaccine virus to pigs [184]. This suggested that the mutations found in E2 and NS4B of CSFV during this re-adaptation process may act synergistically to influence pathogenicity in pigs. Importantly also, NS4B of CSFV harbors a putative Toll/interleukin-1 receptor-like domain [218]. This domain is located closely to the NS4B mutations identified above. Mutations in this domain in the highly virulent “Brescia” backbone resulted in an attenuated virus, probably by affecting the modulation of the host immune response. NS4B carries also a nucleoside triphosphatase motif. This activity is essential for CSFV replication, but there is no evidence of involvement in virulence [219].

7. Immune responses and protective efficacy of CSFV commercial modified live and E2 subunit vaccines (Table 1)

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7.1. Modified live vaccines (MLV)

MLV have routinely been used in CSF endemic areas, including Asia and central-south America. These are often the Chinese (C) strain (C-strain), Japanese guinea-pig exaltation-negative (GPE−) strain, Thiverval strain of France, and Mexican PAV-250 [8] [12]. The C-strains have been attenuatedby serial passage for hundreds of times in rabbits, such as the lapinized Philippines Coronel (LPC) strain with an insertion of more than 12 U-rich continuous nucleotides in the 3′ NTR of the viral genome [12] [220]. The GPE− vaccine strain is derived from the virulent ALD strain by serial passage in different cell cultures at 30 ◦C and differs from its parental strain by 225 nucleotides [12] [182] [221]. The Thiverval strain is derived from the virulent Alfort strain by more than 170 serial passages at 29–30 ◦C [12] [222]. The Mexican PAV-250 is derived from the A-PAV-1 strain by 250 serial passages and has been used in Mexico [8]. The C-strain is avirulent and safe, since no clinical signs are noted in any immunized piglets. Even at gestation [8] [12]. The C-strain vaccinated pigs show a slight decrease in IgM+ lymphocytes and CD4+ lymphocytes, and a moderate increase in monocytes, which is significantly different from those of non-vaccinated pig [223]. The C-strain is able to replicate in lymphoid organs, mainly in the tonsils, between 6 and 42 days post-vaccination (dpv) [224]. Completed protection in C-strain vaccinated pigs is launched as early as 5 dpv and lasts for 6–18 month [11] [12] [15] [225]. The induced immune responses can protect pigs [15] [17] [220] [226] [227]. The mechanisms mediating this protection are the activation of both antibody and T-cell responses. The anti-CSFV

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Table 1. Comparison of the efficacy between modified live CSFV vaccines and E2 subunit vaccines [238].

Parameters	Modified live CSFV vaccines	E2 subunit vaccines
Immunogens	Replicating antigens, its amount increases with successive cycles of replication in regional lymph node or tonsil.	Non-replicating antigen, its amount decay since dpv and a second dose is likely required in most field situations.
	E2 presented membrane-anchored, in correct 3-D folding, in dimers.	Single antigen E2 in truncated form, presented not anchored, in unknown 3-D folding, in either dimer or monomer.
	A multitude of antigens include full length E2 in dimer, E^{rns}, E1 and nonstructural proteins (for inducing CTL).	
Immune responses	Antibody responses – neutralizing and non-neutralizing antibody. The antibody titer may be mounted so high to circumvent the antigenic variations problem among different genotypes.	Antibody responses only.
	T-cell responses – IFN-γ secreting cells, cytotoxic T lymphocytes.	Protect against genotypically homologous strain better. Critical to cross-neutralize with the prevalent strain in the field.
	Early onset immunity suited better for emergency vaccination.	No T-cell responses demonstrated.
		Need combinations or co-formulation to stimulate T-cell responses.
		Later onset immunity not suited for emergency vaccination.
Anti-CSFV neutralizing antibody	12 dpv (peak at 4–12 wpv)	Single vaccination: 21 dpv [138] [150] [231]
		Double vaccinations: 7 dpv or 14 after booster
CSFV-specific IFN-γ secreting cells	6–9 dpv	None
Clinical protection	3–5 dpv	Single vaccination: 14 dpv
		Double vaccinations: 14 dpv after booster
Complete protection	5 dpv	Either single or double vaccination reduced horizontal or vertical transmission, but incomplete in vertical transmission
DIVA by serology	No	Yes

neutralizing antibodies (NT Ab) are detected as early as 12 dpv and peak at 4–12 weeks post-vaccination (wpv) [11] [21]. There is a positive correlation between the levels of NT Ab and protective efficacy against virulent CSFV challenge [137]. In the early stage after vaccination, before the presence of NT Ab, the protection in C-strain vaccinated pigs is thought to be mediated by T-cell responses [11] [12] [57] [225] [226]. The number of virus-specific IFN-γ secreting cells is a marker of T cell protection mediated mainly by CD4+CD8+ lymphocytes that are detectable from 6 to 140 dpv [22] [225] [226]. There are also significantly cross-reactive T cell epitopes among various genotypes of CSFV [11] [12] [22] [226]. Specific cytotoxic T lymphocytes (CTL) activated by the C strain are mainly CD4−CD8+ lymphocytes [26] [130].

7.2. E2 subunit vaccines

The primary drawback of MLV is the lack of DIVA. The use of various candidate subunit vaccines based on glycoprotein Erns or E2 overcomes this DIVA problem [116] [119] [228]. E2 is the major envelope glycoprotein and Erns is the second [115] [116] to induce neutralizing antibodies during CSFV infection. E2 protein has proven to be a most potent immunogen, and the discriminatory ELISA detecting antibodies against E^{rns} protein allows for DIVA [229] [230] [231] [232]. However, candidate Erns vaccines have not been successfully protective.

Two baculovirus-expressed E2 recombinant protein subunit vaccines are commercialized and well-studied. One is the BAYOVAC (R) CSF Marker (now Advasure (R), Pfizer, UK, [8] with an accompanying discriminatory ELISA Ceditest Markerฎ (now PrioCHECK(R) CSFV Erns, Prionics, Lelystad B.V., the Netherlands [232]. The other is Porcilis(R) , Pesti with an accompanying is criminatory ELISA Chekit CSF Marker(R) , (now IDEXX CSF marker, IDEXX Europe B.V., the Netherlands). The BAYOVAC(R), CSF Marker is derived from strain Brescia [119] of genotype 1.2, while Porcilis(R) , Pesti is from the virulent strain Alfort Tubingen [116] of genotype 1.1.

A single vaccination is sufficient to prevent clinical signs and mortality from lethal CSFV challenge at 14 dpv to 13 months [46] [138] [139] [151] [231]. Single dose vaccination, however, is not protective at 7 dpv, and it is only partially protective at

10 dpv [46] [139]. A single vaccination induces NT Ab at 21 dpv [138] [231], which persists for 6 months [138]. A second dose booster vaccination protects pigs clinically after the second vaccination and persists for 3 months after challenge [150]. A single vaccination can prevent horizontal transmission at 10 [46] or 14 dpv [14] [46] remain effective for 6 months [138] [231]. However, another study showed that viral transmission could still occur at 14 and 21 dpv [139]. This discrepancy may be due to the challenge virus being genotypically homologous or heterologous with the vaccine strain.

The E2 subunit vaccines can reduce, but cannot totally prevent, vertical transmission. Transplacental transmission occurs after single dose vaccination [113] [151] [231] and even after two doses of vaccine [140] [150] [151]. The BAYOVAC ® CSF Marker appeared to have a lower probability of fetal infections than Porcilis® Pesti in a large-scale laboratory trial [151]. However, this impression may not be absolute because of the various experimental conditions and the variable sensitivity and specificity of the accompanying discriminatory E^{rns} ELISAs used [232]. Other studies have reported complete prevention of the double-vaccinated sows from transmitting challenge virus to the fetus using BAYOVAC ® CSF [113] [231].

Since the E2 subunit vaccines can only induce antibody responses, there is a need to enhance the T-cell immunity of the vaccine. Several nonstructural proteins of CSFV, including NS2, NS3, and NS4A [23] [26] [65], have been identified as having CTL epitopes. However, addition of NS3 to an E2 subunit vaccine does not obviously improve detectable antibody and T-cell responses [24]. Addition of cytokines to elicit T-cell responses is another option. Vaccination of E2 antigen co-formulated with IFN-α could prevent clinical signs and viremia as early as 7 dpv [234]. This information seems to suggest a potential direction for future vaccine improvement.

7.3. Comparison of protective efficacy of commercial modified live and E2 subunit vaccines

MLV can induce both antibody and T-cell responses, while E2 subunit vaccines alone can induce antibody responses but without T-cell responses demonstrated. MLV induce anti-CSFV neutralizing antibody as early as 12 dpv, which is an earlier onset than that of E2 subunit vaccines. Complete protection can be induced at 5 dpv in C-strain vaccinating pigs, without viremia and shedding after virulent CSFV challeng [11] [12] [225], whereas the E2 subunit vaccines induce clinical protection until 14 dpv, with total prevention of horizontal transmission but incomplete prevention from vertical transmission [46] [151] [231]. In double-dose vaccination with E2, the NT Ab can be detected early, at 7 days after booster, and the blocking of horizontal transmission is also noted. These above results indicate that the efficacy of the E2 subunit vaccines is lower than that of MLV. However, the E2 subunit vaccines can be combined with DIVA [229] [232] to facilitate rapid screening of infected pigs and help in the control and elimination of CSF.

8. The factors influencing the efficacy of CSFV vaccine

8.1 Modified live CSFV vaccine, MLV **(Table 2)**

In endemic areas, MLV are routinely used for prevention and control of CSFV. This vaccine is able to induce [11] [12] [15] [225]. However, several factors, including the viral loads of vaccines, route and age of vaccination, and co-present pathogens, can interfere with the vaccine"s efficacy [11] [12] [22].

8.1.1 Viral loads of vaccine

The efficacy of MLV is correlated with the amount of viral particles contained in each dose of the vaccine. Pigs vaccinated with 1/100 dose of C-strain viral particles show significantly lower titers of NT Ab than those vaccinated with 1 dose at 10 dpv and often have viremia and viral shedding after challenged with virulent CSFV [235] [236].

8.1.2 Route of vaccination

In pigs orally immunized with C-strain vaccine, the production of NT Ab is first found at 14 dpv, and complete protection is noted at 10 dpv, in both cases later than in intramuscularly vaccinated pigs [237]. In a recent study, pigs delivered with C-strain by either intranasal or intramuscular routes provided the complete protection against virulent CSFV challenge at 5 dpv. he IFN-γ secreting cells in the intranasally vaccinated pigs are higher than those of intramuscularly vaccinated pigs [225].

8.1.3 Piglet age of vaccination

Piglets vaccinated at 5 weeks old develop a greater number of CSFV-specific IFN-γ secreting cells and higher neutralizing antibody than those vaccinated at 3 weeks old. This difference may be explained by the fact that in 4 weeks old piglets, the porcine immune system is fully matured [11].

8.1.4 Maternal derived antibody

Maternal derived antibody is the most common factor that influences the efficacy of MLV in the field [11] [12] [17]. All piglets acquire CSFV-specific maternal derived antibody by colostrums intake, and the level of MDA gradually declines over time. The levels of anti-CSFV neutralizing antibody at the vaccinated stage, ≥1:64, impair the efficacy of MLV [11] [12] [17]. Since the levels of maternal derived antibody are correlated with the neutralizing antibody of sows, vaccination programs of both sows and piglets need to be considered together. In Taiwan, the authority recommends two vaccination programs for the sow and piglets in the fields. One is that when sows are vaccinated with LPC vaccine in the pre-farrowing period, their piglets are vaccinated at 6 and 9 weeks old. The second is that when sows are vaccinated with only double vaccinations at the nursery stage, their piglets are vaccinated at 3 and 6 weeks old. To reduce the MDA- and age-derived interferences of LPC vaccine, the second dose is routinely given at a3-week interval. However, the vaccination plan in the fields should be based on the knowledge of the herd immune status.

8.1.5 Other pathogens

Several swine pathogens, such as pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus 2 (PCV2), are known to induce immune suppression in pigs and therefore are the potential interference factors for MLV [248] [249] [250]. Pigs that have PRV-co-infection, when vaccinated with MLV develop fatal infection after virulent CSFV challenge [11]. When the C-strain and the live gE-deleted PRV vaccines are combined for immunization, a lower number of CSFV-specific IFN-γ secreting cells is noted, but there are no significant differences in the levels of neutralizing antibody or clinical protection against challenge. Pigs that receive this combined vaccination clearly exhibit more feverish days and pathological changes than pigs vaccinated with the CSF vaccine alone after challenge [22]. The live gE-deleted PRV vaccine most likely impairs the efficacy of MLV through the down-regulation of CSFV-specific IFN-γ secreting cells. In C-strain vaccinated pigs followed by challenge with both CSFV and PRRSV, they show the fatal CSFV infection [11]. In addition, PRRSV pre-infection significantly reduces the immunity efficiency of C-strain vaccination and leads to fatality and clinical signs after CSFV challenge. The reduction of C-strain induced immunity resides in the lower NT Ab and CSFV-specific IFN-γ secreting cells [11] [136]. The PRRSV can induce an amount of IL-10 to suppress the production of CSFV-specific IFN-γ secreting cells in vaccinated pigs [11] [136] [245] [246]. A pre-existing PCV2 infection could affect the efficacy of LPC vaccine against wild-type CSFV infection [227]. Fever/clinical signs and transient viremia/viral shedding of CSFV in saliva and feces are noted in pigs, after virulent CSFV challenge, with PCV2 pre-infection and C-strain vaccination. The PCV2 interferes with activation and proliferation of lymphocyte sub-groups, IgM+, CD4+, CD8+, and CD4+CD8+cells [227]

Table 2. Factors influencing the efficacy of modified live vaccines and E2 subunit vaccines of CSFV

Modified live vaccines (C-strain)	**E2 subunit vaccines**
Viral loads [235] [236]	Antigen variation among various genotypes of CSFV [214] [239] [240]
Route of vaccination [144] [225] [237]	Maternal derived antibody [12] [139] [241] [242] [243] [244]
Age of piglets at vaccination [11]	
Maternal derived antibody [11] [12] [17]	
Other pathogens	
PrV [11] [22]	
PRRSV [11] [136] [245] [246]	
PCV2 [227] [247]	

[247], whose numbers are significantly lower than those in pigs with LPC vaccination alone. This PCV2-derived interference results in reductions in antibody and T-cell responses, such as transient delays in the production of NT Ab against CSFV and inhibited proliferation of CSFV-specific PBMCs [227] [247] This down-regulation is partially mediated via the inhibition of CD25expression, as has been found in both in vivo and ex vivo experiments [227] [247].

2.2 E2 subunit vaccine **(Table 2)**

6.2.1 Antigenic variations among various genotypes of CSFV

Understanding the E2 antigenic structure and definition of epitopes provides scientific grounds for the improvement of the marker vaccines **(Figure 4).** Based on competitive binding studies and antigen capture assay with a panel of mAbs against E2 [251], four antigenic domains, in the order of B/C/D/A, have been identified **(Figure. 4)** [252] [253]. Domain A is subdivided into A1, A2, and A3, of which A1 and A2 are conserved among CSFV strains. The non-conserved epitopes on E2 are located in domains B/C [254]. Furthermore, the four antigenic domains of conserved and non-conserved epitopes on E2 have been mapped, and all domains are located in the N-terminal half of E2 (**Figure. 4**) [239] [240] [243] [255]. A model of the antigenic structure of E2 is proposed: The structural unit of domains B/C is anchored by a putative disulfide bond between C693and C737, and the other unit of domains A/D is formed by two disulfide bonds, one between C792 and C856 and the other between C818 and C828. Domains B/C and domains D/A each represent a globular portion with a pan-handle structure link in between [243] (**Figure. 4**).

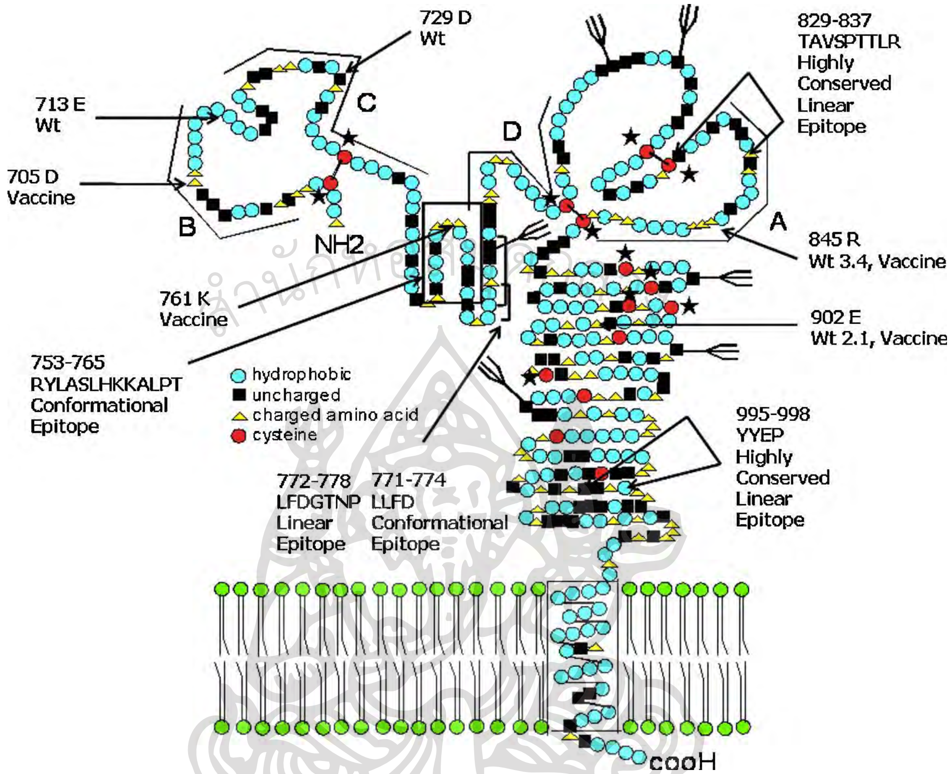

Figure 4. Proposed 2-D antigenic structure with identified conformational and linear epitopes of the CSFV E2 glycoprotein. The key residues responsible for antigenic specificity of the various strains are also indicated. The critical cysteines responsible for antibody recognition are indicated by asterisks. The model is based on the sequences of the CSFV strain LPC/AHRI. The residues 713E and 729D are specific for the field strains of genotype 2.1 and 3.4. Wt means wild type field CSFV strains [243].

There are several important conformational epitopes defined within the E2 (**Figure. 4**) [239] [240] [243] [244] [255] based on the premise that the structural integrity is maintained by the disulfide bonds. The six cysteines at domains B/C/D/A are essential for the correct folding of four antigenic domains [239] [240] [243]. In domains B/C, the neutralizing antigenic motifs 753RYLASLHKKALPT765 and 771LLFD774 are essential in maintaining the structural integrity of conformational epitopes [239] [256]. In domains B/C, the residues E713 and D729 are responsible for the antigenic specificity of field strains belonging to genotypes 2 and 3, while the residues D705 and K761are for the LPC vaccine strain of genotype 1 [257]. In domainsD/A, the neutralizing conformational epitopes are also present, and the residue R845 are responsible for the antigenic specificity of genotypes 1 and 3 [240]. Regarding the cysteines in the C-terminal half of E2, the five proximal cysteines in the C-terminal region are important in determining the to pography of E2 [240]. Neutralizing conformational epitopes with the antigenic determinant, residue E902, responsible for the antigenic specificity of genotypes 1 and 2, are also present in the C-terminal region [240].

In addition, there are several important linear epitopes [44] [45] [258] [259] [260] present within the E2 **(Figure 4).** No linear epitope has been identified within domains B/C. There is a linear epitope at motif 772LFDGTNP778 bordering domains B/C and A [259]. In domain A, a highly conserved neutralizing linear epitope 829TAVSPTTLR837 [44], identified by monoclonal antibody (mAb) WH303, has been used to develop epitope-based vaccines [215] [216] [261] [262] [263] [264] [265] and serodiagnosis [265]. This mAb WH303 has been used extensively in immunohistochemistry, but WH303 can mark the viral antigen of MLV and cannot be used for DIVA. In the C-terminal region of E2, a linear epitope9 95YYEP998 highly conserved among pestiviruses has been mapped [260]. Note that the three cysteines present at positions 945, 966 and 983 in C-terminal of the E2 function in mediating homodimerization [240].

In brief, it is clear that MLV present a multitude of viral anti-gens in addition to E2, which presumably presents anchored in the envelope of the virion, in correct 3-D folding, and as a homodimer (**Table 2**). Because of the limited viral replication within regional lymph nodes and the tonsils, the amount of presented antigen is mounted after every replication cycle and the immune system is successively stimulated, resulting in both mounted antibody and T-cell responses. It is also clear that the subunit vaccine presents a non-replicating single antigen that is not anchored in the envelope of the virion, in unknown 3-D folding, in either monomer or dimer (**Table 2**). The amount of injected E2 antigen, like any other protein, will decay over time after vaccination, so a second-dose booster vaccination is most likely required in most field situations.

8.22. Antigenic differences influence the efficacy of the E2 subunit vaccines

It is critical to clarify how antigenic differences influence cross-protection between subunit vaccines and field CSFV isolates. Two commercial CSFV E2 subunit vaccines are derived from strain Brescia of genotype 1.2 [119] and Alfort Tubingen of genotype 1.1 [116]. Recently, however, field isolated CSFVs have shifted from the historical genotype 1 or 3 to genotype 2 in most European and Asian countries [9] [26] [266] [267] [268]. Note that the genotypes of vaccine viruses are different from those of the currently prevalent field viruses [18]. The sequence variation may change the amino acid residues, thus altering the antigenicity of E2. It is clear that the recombinant E2 proteins are effective against challenge with genotypically homologous CSFV strains [60] [116] [117] [118] [119]. In a trial, however, these two commercial CSFV E2 subunit vaccines of genotype 1.1 and 1.2 did not offer complete protection against heterologous strains, such as the Paderborn strain of genotype 2.1 [139]. Imperfect protection by recombinant E2 protein vaccines against heterologous challenge has also been reported by others [12] [116]. This poor vaccine performance may be due to the antigenic variations among heterologous strains.

The antigenic variations among various genotypes of CSFV can be evaluated overall by cross-neutralization assays with genotype-specific antisera from pigs experimentally infected with genotypically heterologous strains. The neutralizing antibody was significantly higher between homologous strains than between heterologous strains ($P < 0.05$), as also demonstrated in other studies [214] [242]. All studies have indicated that antigenic variations exist among different genotypes.

Since E2 is the most potent immunogen to induce neutralizing antibody [116] [119] [228], the crossreactivity among E2 proteins of various genotypes of CSFVs can also be evaluated overall. As predicted, the CSFV genotype-specific pig antisera binds to heterologous E2 proteins less efficiently than to homologous E2 protein [214] [241], indicating that the E2 variation among CSFVs is crucial to cross-neutralization. Genotype-specific E2 antisera from pigs immunized with recombinant E2 proteins of various genotypes were cross neutralized. As predicted, the titers of NT Ab were significantly lower to heterologous strains than to homologous strains ($P < 0.05$), which may explain the incomplete E2 vaccine protection with heterologous strains [139].

The fact that antisera cross-neutralize genotypically homologous strains better than heterologous strains can be traced to amino acid levels with mAbs. The antigenic variation among the various genotypes of CSFV has been further demonstrated by various reaction patterns with CSFV-specific mAbs [19] [214] [239] [240] [241] [252] [269] [270] [271]. E2-specific mAbs had higher binding [214] [241] and neutralization efficacy [214] against the homologous strains, suggesting that alteration of antigenic epitopes on E2 of CSFV strains is responsible for the neutralization differences among various CSFVs. Single-point mutations, identified by residues H710, H724, N725, D729, K734, M738 at domains B/C and T823, P833, T834, R837 at domains D/A, could lead to loss of neutralizing mAbs binding [243]. In addition, the residue at position 710 affected binding and neutralization to mAbs or pig antisera [244]. Furthermore, the differences of antigenicity between LPC vaccine and field strains resided in single amino acids. The residues D705, E713, D729 and K761 at domains B/C [257], the residue R845 at domain A, and the residue E902 at the C-terminal region [240] are crucial for antigenic specificity of genotype 1, 2 or 3; also, their specific E2-specific mAbs has higher neutralization titer against the homologous strains. In addition, the single amino acid substitutions of residues D705N, L709P, G713E, N723S, or S779A at domains B/C from vaccine strain to field strain significantly increase binding to heterologous field strain pig antiserum [214]. Taken together, these facts indicate that variability by a single amino acid could affect the antigenicity of E2, thus influencing the cross-neutralization reactivity. The important antigen-specific residues contributing to the neutralization differences have been identified mostly at domains B/C, suggesting that the domains B/C are the main regions related to the antigenic evolution to escape from the antibody neutralization induced by vaccine strain, and are associated with a strong reduction in neutralizing titers on the heterologous virus.

8.3. Maternal derived antibody

The highest level of maternal derived antibody has been observed in 3 weeks old piglets, and higher neutralization antibody has been found in piglets vaccinated with a first dose of subunit vaccine at 5 or 7 weeks old and boostered 4 weeks later. After vaccination at 2 weeks old, when maternal derived antibody is still present, the neutralization antibody titers in maternal derived antibody -positive piglets are significantly lower than the titers of MDA-negative piglets after 3 and 6 months, possibly indicating that protection is less effective after 6 months [272].

CHAPTER IV
MATERIALS AND METHODS

This project has been approved by the Committee of Handling and Using of Animals for Science, Department of Livestock Development (DLD).No. ID#001/2010, 21 July 2010.

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1. Animals

Twelve healthy pigs used in this study were Large White/Landrace/Durox Jersey cross breed, both male and female pigs, eleven weeks old, 20-25 kgs, purchased from Nakhonratchasima Animal Research and Development Center, DLD. Pigs were held in Experimental Animal Building of VBAD, DLD, at Pakchong District, Nakhonratchasima Province, marked by ear-tag, and bled for testing of specific CSFV antigen and antibody to CSFV. All pigs were tested as free of CSFV-specific antigen and CSFV-specific antibody by ELISA. The number and treatment of animal using for this experiment were based on the minimum requirement of ASEAN Standards for Animal Vaccine [29]. Twelve healthy pigs were randomly selected and divided into four groups:(I) Negative control group (N1 and F8), (II) Positive control group (D6 and D0), (III) Lapinised C-strain vaccinated group (N2, N4, N7 and V2), and (IV)Tissue cultured C-strain vaccinated group (N5, N6, V1 and V0). To avoid contaminating each other by secretions and excretions, each group was housed in an independent and strictly hygienic facility of VBAD, DLD.

2. Viruses and Vaccines

The seed of virulent wild-type CSFV Bangkhen strain was isolated from sick pig [30]. The virus seed was prepared by culturing in sensitive pigs, subsequent collecting viraemic blood when body temperature (BT) was upper 40ºC and showed typical clinical signs of swine fever approximately 7 days post inoculation, and separated blood into small test tubes, 1 ml for each tube, kept at -70ºC. The titer of seed virus stock (lot 1/2011) was 1 X 10^6 PID_{50}/ml (50% pig infectivity dose)as quantified by endpoint titration in susceptible pigs. The stock virus was diluted 1:10 with PBS to get virus for inoculation containing 1 X 10^5 PID_{50}/ml comply OIE Terrestrial Manual [28].

Two kinds of attenuated live swine fever vaccine; lapinised C-strain vaccine (lot 1/2011, expiry date 26-1-2013) and tissue cultured C-strain vaccine (lot 1/2012, expiry date 3-4-2013) was produced by Bureau of Veterinary Biologics Production (BVBP), DLD, Thailand. The lapinised C-strain (LC) vaccine was prepared as following [33].

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LC seed was propagated in healthy rabbits, incubated 3 days, collected infected spleen (SP), mesenteric lymphnodes (MLN) and infected rabbit serum (IRS), then mixed the suspension with 2% SP & MLN + 10% IRS + 1% AB +87% stabilizer, placed in freeze dryer to get the lyophilized vaccine.

The tissue cultured C-strain (TC) vaccine was prepared as following:

LC seed is cultured in porcine kidney cell line, FS-L3 for 10 passages. The seed vaccine virus was propagated in FS-L3 (porcine kidney cell lines), incubated at 30°C for 7 days, harvested virus fluid and centrifuged at 1,000 rpm for 10 minutes, then mixed 50% virus fluid containing more than 3.0 log $TCID_{50}$ with 50% stabilizer, placed in freeze dryer to get the lyophilized vaccine.

Both lots of vaccine contained virus content equal to 100 PD_{50}/ml (50% protective dose) as quantified by titration in 14 days-vaccinated pigs challenged with virulent CSFV, Bangkhen strain, containing 1X10^5 PID_{50}/mlthat complied OIE Terrestrial Manual [28] and ASEAN Standards for Animal Vaccine [29]. Seed virus and both vaccines were courtesy by BVBP, DLD.

3. Test of vaccine and challenge

During the acclimatization period (at least seven days) of experimental pigs, rectal body temperatures (BT) and clinical signs were recorded twice daily. Each pig in group 3 (N2, N4, N7 and V2) was vaccinated with 1 ml of lapinised C-strain vaccine containing 100 PD_{50}/ml and challenged with 1 ml of virulent CSFV Bangken strain containing $1X10^5$ PID_{50}/ml at 3 days post-vaccination.Each pig in group 4 (N5, N6, V1 and V0) was vaccinated with 1 ml of tissue cultured C-strain vaccine containing 100 PD_{50}/ml and challenged with 1 ml of virulent CSFV Bangkhen strain containing $1X10^5$ PID_{50}/ml at 3 days post-vaccination. On the same day, each pig in group 2 (D6 and D0) was intramuscularly inoculated with 1 ml of virulent CSFV Bangkhen strain containing $1X10^5$ PID_{50}/ml. On the same day as well, each pig in group 1(N1 and F8) was inoculated intramuscularly with 1 ml of 0.85% normal saline (NS). Blood samples wereaseptically collected from anterior vena cava (20 ml for each time of pig blood sample collection) at 0, 1, 3, 5, 7, 14 and 21 days post-inoculation (DPI). Twenty ml of each blood sample was separated into three tubes; 5 ml kept in non-EDTA tube stood clotting and taken for serum preparation, 5 ml kept in EDTA tube was sent to a commercial lab (Patching Central Lab, Pakchong District, Nakhonratchasima Province) for measuring total white blood cell number, another 10 ml kept in EDTA tube was used for peripheral blood mononuclear cell (PBMC) preparation.

4. Study of clinical signs and fever

Throughout the duration of the study (21days post inoculation), BT and clinical signs of each pig were recorded twice daily. The upper BT of each pig in a day was used for calculation. All survive pigs are euthanized on day 22 post inoculation. All carcasses of pigs including pigs died from infection were examined for pathological change.

The clinical scoring system developed by Mittelholzer *et al*. [273] was used with some modification to record and evaluate clinical signs of infected and sham-inoculated pigs . Clinical scores consisted of nine parameters: depression, body tension (stiffness, kyphosis), body shape (body condition, thin musculature), central nervous system (CNS) signs, respiratory signs, skin condition, conjunctivitis, appetite, and diarrhea. Each parameter was given a numerical score from 0-3, where a zero indicates no clinical signs, and 1 to 3, indicates an increasing scale or severity of clinical signs.

The maximum average score (27) for any dpi was calculated as the number of parameters, multiplies by the maximum possible score of 3. Clinical scoring was based on the comparison of the sham-inoculated control pigs to the CSFV infected animals.

5. Serum and Peripheral blood sample preparation

Serum samples were collected from 5 ml of non-EDTA blood samples, centrifuged at 800 g for 20 min, collected and kept at -70 ºC until used. These serum samples were used for detection of specific CSFV antigen and antibody to CSFV by ELISA.

Peripheral blood sample (PBMC) was collected from 15 ml of EDTA stabilized blood samples. The PBMC was two- fold diluted in 0.1 mM phosphate buffer saline (PBS), pH 7.4. Diluted blood was overlaid on Lymphoprep (density 1.077±0.001 g/ml, Robbins Scientific Cooperation, Synnyvale, CA, USA) ratio 1:1, and PBMC were isolated from interface after density gradient centrifugation at 400g for 30 min. Then PBMC were washed twice with PBS and centrifuged at 400g for 5 min, and counted by colorized with Trypan-blue (Invitrogen, Grand Island, NY, USA). PBMC were separated into small vials and kept at -70^{o}C until used. These PBMC samples were used for measurement of interferon gamma (IFN-γ) cytokine gene and transforming growth factor-beta (TGF-β) cytokine gene.

6. Measurement of leukocytes

Five milliliters of EDTA-blood sample was sent to a commercial lab (Patching Central Lab) for measuring of blood values by using automated hematology analyzer. Total leukocyte number of each blood samples were analyzed.

7. Measurement of the amount of serum CSFV antigen by ELISA

The CSFV antigen in serum was measured by using commercially available HerdChek*CSFV Ag ELISA kit and followed according to manufacturer‟s instructions (IDEXX laboratories, USA). Briefly, 50 µl of test sera, positive and negative control were

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added into microtiter plates coated with CSFV antibody and incubated at 4°C overnight. The capture plate was then washed with 300 µl of wash solution five times. After washing, 100 µl of Anti-CSFV HRPO was added into each well, and the plate was incubated at room temperature for 30 min. Finally, the plate was washed with 300 µl of wash solution five times. Then, 100 µl of TMB substrate solution was added into each well, and the plate was incubated at room temperature for 10 min. Then, 100 µl of stop solution was added into each well, and the plate was incubated at room temperature for 10 min. The optical density of each well was read at 450 nm by using a microplate reader (Science Tech). Each sample was analyzed in duplicate, and the OD of duplicates was average calculated and converted into the amount of antigen. The result was analyzed and interpreted by using the formula:

Test sample = $SampleA_{450} - NCA_{450}$

Remarks:

Test sample = The average amount of antigen in test sample

$SampleA_{450}$ = The average OD of test sample measured at 450 nanometer

NCA_{450} = The average OD of negative control measured at 450 nanometer

Interpretation:

The result of antigen detection was negative when test samples$\leq$ 0.300.

The result of antigen detection was positive when test samples> 0.300.

These are cut off values for CSFV non-infected and CSFV-infected pigs, positive CSFV-infected pigs should be slaughtered.

8. Measurement of the amount of serum antibody to CSFV by ELISA

The CSFV-specific antibody in serum was measured by using commercially available HerdChek*CSFV Abs ELISA kit and followed according to manufacturer"s instructions (IDEXX laboratories, USA). Briefly, 50 µl of 1:2 diluted test sera, positive and negative control were added into microtiter plates coated with CSFV antigen and incubated at 4 °C overnight. The capture plate was then washed with 300 µl of wash solution three times. After washing, 100 µl of Anti-CSFV HRPO was added into each well, and the plate was incubated at room temperature for 30 min. Finally, the plate was washed with 300 µl of wash solution three times. Then, 100 µl of substrate solution was added into each well, and the plate was incubated at room temperature for 10 min. Then, 100 µl of stop solution was added into each well, and the plate was incubated at room temperature for 10 min. The optical density of each well was read at 450 nm by using a microplate reader (Science Tech). Each sample was analyzed in duplicate, and the OD of duplicates was averaged. The result was analyzed and interpreted by using the formula:

$$\text{Test samples} = \frac{NCA_{450} - SampleA_{450}}{NCA_{450}} \times 100$$

Percent blocking interpretation;

The result of antibody detection was negative when the test samples ≤ 30 %.

The result of antibody detection was positive when the test samples≥ 40 %

These are cut off values for CSFV non-infected and CSFV-infected pigs, positive CSFV-infected pigs should be slaughtered.

If the value upper 30% but less than 40%, tested pigs are suspected be infected with CSFV and should be confirmed by repeating test or using another method for testing.

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9. Measurement of cytokine gene expression by quantitative reverse transcription real-time-polymerase chain reaction (qRT-PCR)

IFN-γ and TGF-β cytokine genes from PBMC samples were measured by qRT-PCR. RNeasy Minikit (Qiagen, Valencia, CA, USA) and followed according to manufacturer‟s instructions. The first strand cDNA was synthesized by using Reverse Transcription Kit (TagMan ® microRNA Reverse Transcription Kits, Valencia, CA, USA) and random hexamer primers (Invitrogen, Carlsbad, CA, USA), and diluted ten times. Then 1 µl of cDNA was mixed with iQ SYBR Green supermix containing gene specific primers

[Applied Biosystems (Roche, Basal, Switzerland)], IFN-γ; 5′-CGATCCT AAAGGACTATTTTAATGCAA-3′(F); 5′TTGTCACTCTCCTCTTTCCAAT-3′(R) and TGF-β; 5′-CCTGCAAGACCATCGACATG-3′(F); 5′-CCGAAGCTTGGACAGAATC-3′(R) by using Chromo 4 Real-Time System (Bio-Rad) **(Appendix 1).** Each reaction composed of activation of polymerase at 95^{o}C for 10 min, followed by 40 cycles of denature at 95^{o}C for 15 min, annealing and extension at 60^{o}C for 1 min. The amount of cytokine gene amplification was interpreted by using melting curve analysis. A fold-change of cytokine gene expression between pre-inoculation and post-inoculation with CSFV at 0, 1 and 3days post-inoculation was used for interpretation.

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CHAPTER V
RESULTS

1. Clinical signs

To study clinical signs of pigs after CSFV infection, twelve pigs were observed for clinical signs and measured rectal body temperature (BT) twice a day (morning and afternoon) for 21 days as following: Negative control group (2 pigs), post-inoculated intramuscularly with 0.85% normal saline (NS); Positive control group (2 pigs), Lapinised C-strain vaccinated group (4 pigs), and Tissue cultured C-strain vaccinated group (4 pigs), post- inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain.

During the acclimatization period (at least seven days) of experimental pigs, rectal body temperatures (BT) and clinical signs were recorded twice daily. All experimental pigs had the normal body temperature, the BT were ranging from 38.4ºC to 38.6ºC. All pigs did not show any clinical signs or symptoms (**Figure 5**).

Figure 5. Experimental pigs were healthy and did not show any clinical signs during the acclimatization period.

For negative control group, the BT of pre-inoculated pig N1 was 38.4ºC. The BT of pig N1 was ranging from 38.4ºC to 38.6ºC during 1 to 21 day post-inoculation (dpi) with NS. Likewise, the BT of pre-inoculated pig F8 was 38.7ºC. The BT of pig F8 was ranging from 38.3ºC to 38.6ºC during 1 to 21 dpi (**Figure 6a, Appendix 2**).

For positive control group, the BT of pre-inoculated pig D6 was 38.5ºC. The BT of pig D6 began increasing from 39.6ºC to 40.8ºC during 2 to 8 dpi with CSFV Bangkhen strain. Pig D6 had the highest BT of 40.8ºC at 8 dpi and then died at 13 dpi. The BT of pre-inoculated pig D0 was 38.5ºC. The BT of pig D0 began increasing from 39.6ºC to 39.8ºC during 2 to 14 dpi. Pig D0 had the highest BT of 39.8ºC at 3, 5, 7 and 14 dpi and then died at 18 dpi. **(Figure 6b, Appendix 2**).

For lapinised C-strain vaccinated group, the BT of pig N2 was 38.6ºC at 0 dpi. The BT of pig N2 was ranging from 39.1ºC to 39.7ºC during 3 to 14 dpi with CSFV. The highest BT of 39.7ºC was observed at 3 dpi, while the lowest BT of 38.4ºC was observed at 21 dpi. The BT of pig N4 was 38.5ºC at 0 dpi. The BT of pig N4 was ranging from 39.3ºC to 39.4ºC during 3 to 8 dpi with CSFV. The highest BT of 39.4ºC was observed at 5, 7 and 8 dpi, while the lowest BT of 38.6ºC was observed at 21 dpi. The BT of pig N7 was 38.6ºC at 0 dpi. The BT of pig N7 was ranging from 39.3ºC to 39.6ºC during 3 to 14 dpi with CSFV. The highest BT of 39.6ºC was observed at 7 dpi, while the lowest BT of 38.6ºC was observed at 21 dpi. The BT of pig V2 was 38.4ºC at 0 dpi. The BT of pig V2 was ranging from 39.3ºC to 39.7ºC during 3 to 14 dpi with CSFV. The highest BT of 39.7ºC was observed at 7 and 8 dpi, while the lowest BT of 38.5ºC was observed at 21 dpi. Although all of four lapinised C-strain vaccinated pigs had fever, all pigs still survived (**Figure 6c, Appendix 2**).

For tissue cultured C-strain vaccinated group, the BT of pig N5 was 38.6ºC at 0 dpi. The BT of pig N5 was ranging from 39.1ºC to 39.8ºC during 3 to 21 dpi with CSFV. The highest BT of 39.8ºC was observed at 8 dpi, while the lowest BT of 39.1ºC was observed at 21 dpi. The BT of pig N6 was 38.6ºC at 0 dpi. The BT of pig N6 was ranging from 39.0ºC to 39.7ºC during 2 to 21 dpi with CSFV. The highest BT of 39.7ºC was observed at 5 and 7 dpi, while the lowest BT of 38.5ºC was observed at 21 dpi. The BT of pig V1 was 38.4ºC at 0 dpi. The BT of pig N7 was ranging from 39.3ºC to 39.6ºC during 2 to 14 dpi with CSFV. The highest BT of 39.6ºC was observed at 14 dpi, while the lowest BT of 38.4ºC was observed at 21 dpi. The BT of pig V0 was 38.6ºC at 0 dpi. The BT of pig V0 was ranging from 39.1ºC to 41.0ºC during 2 to 14 dpi with CSFV. The highest BT of 41.0ºC was observed at 8 dpi, while the lowest BT of 39.1ºC was observed at 2 dpi. Pig Do died at 18 dpi (**Figure 6d, Appendix 2).**

Comparison of the average BT among four groups was shown in **Figure 6e and Appendix 2**. The average BT of negative control group (N1 and F8) was 38.55±0.21ºC at 0 dpi. The average BT of this group was ranging from 38.30±0.0ºC to 38.55±0.07ºC during 1 to 21 dpi with NS. Similar pattern of BT from negative control group was observed during 1 to 21 dpi, suggesting that the NS did not affect the BT of inoculated pigs. The average BT of positive control group (D6 and D0) was 38.5±0.0ºC at 0 dpi. The average BT of this group was ranging from 39.4±0.14ºC to 40.25±0.78ºC during 2 to 12 dpi with CSFV. The BT from the positive control group was 1.7 ºC higher than the negative group, suggesting that the CSFV caused high fever of positive control pigs. The average BT of lapinised C-strain vaccinated group (N2, N4, N4 and V2) was 38.53±0.10ºC at 0 dpi. The average BT of this group was ranging from 39.25±0.35ºC to 39.58±0.13ºC during 3 to 14 dpi with CSFV. The BT from the lapinised C-strain vaccinated group was 1.0 ºC higher than the negative group, suggesting that the CSFV caused slightly high fever of lapinised C-strain vaccinated pigs. The average BT of tissue cultured C-strain vaccinated group (N5, N6, V1 and V0) was 38.55±0.10ºC at 0 dpi. The average BT of this group was ranging from 39.10±0.36ºC to 39.6±0.2ºC during 2 to 18 dpi with CSFV. The BT from the tissue cultured C-strain vaccinated group was 1.1 ºC higher than the negative group, suggesting that the CSFV caused slightly high fever of tissue cultured C-strain vaccinated pigs.

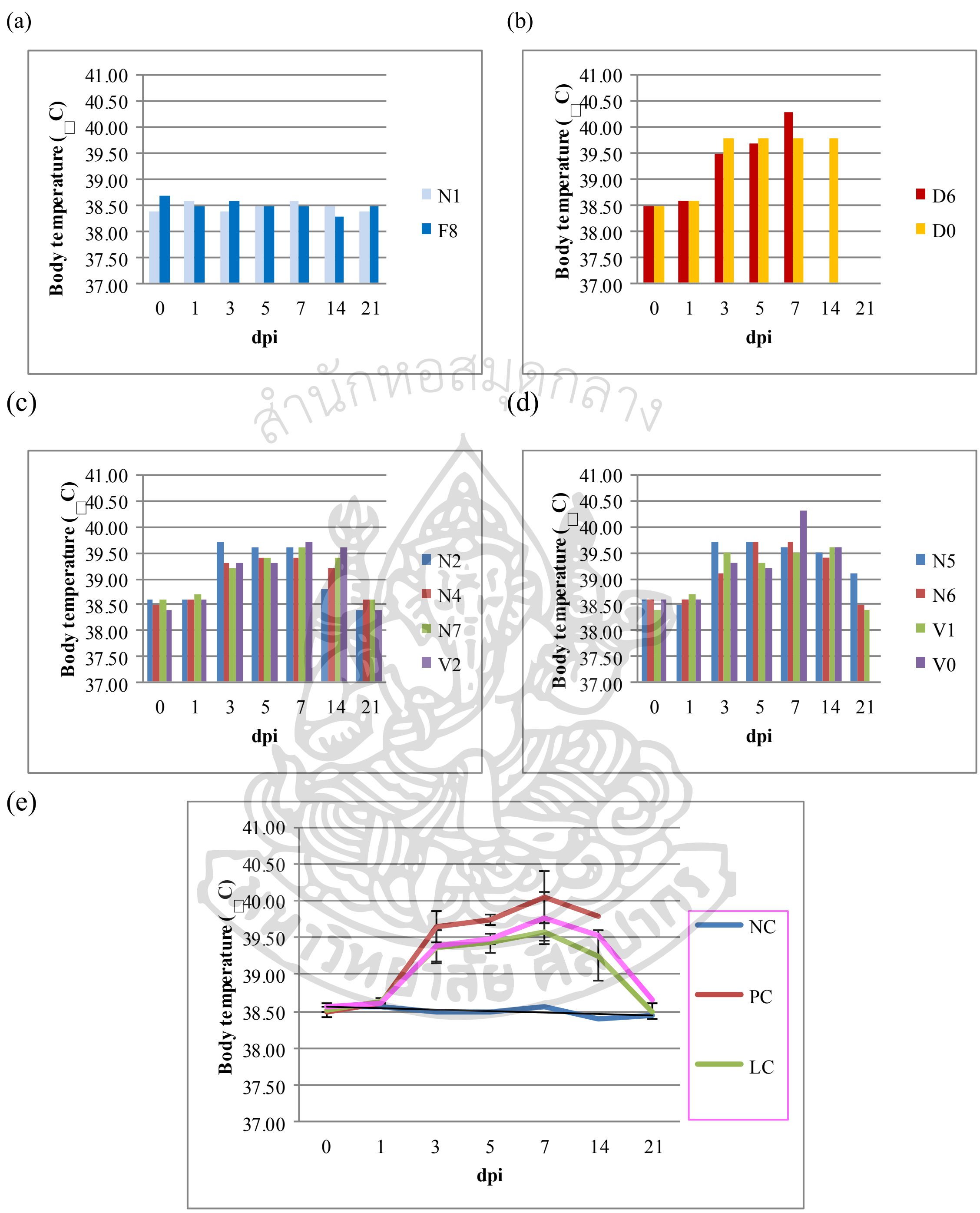

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Figure 6. Body temperature (BT) of (a) negative control (NC), (b) positive control (PC) (c) lapinised C-strain (LC) vaccinated and (d) tissue cultured C-strain (TC) vaccinated, pre- and post-inoculation with NS or CSFV Bangkhen strain (1.0 x10^5 PID_{50}) were measured twice a day at indicated times. (e) The average BT of PC , LC vaccinated or TC vaccinated groups was compared with NC group.

Both negative control pigs (N1 and F8) were clinically healthy and did not had any pathological changes post-inoculated with NS throughout the experiment (**Figure 7).**

Both positive control pigs (D6 and D0) began to show clinical signs and high fever as early as 2 dpi with CSFV Bangkhen strain. With the onset of fever, both pigs developed severe depression, loss of appetite, constipation followed by diarrhea, paresis, body stiffness, respiratory distress, ataxia, tremors, skin hyperemia (data not shown), thin musculature, purulent conjunctivitis and died at 13 dpi and 18 dpi respectively. At necropsy post mortem examination confirmed pathological change consistent of CSF including tonsil inflammation (data not shown), splenic infarction, petechial hemorrhage of kidneys and generalized strawberry lymphnodes **(Figure 8).**

All of four lapinised C-strain vaccinated pigs (N2, N4, N7 and V2) were clinically healthy and did not have any pathological changes or died after challenged with CSFV Bangkhen strain **(Figure 9).**

Three of four tissue cultured C-strain vaccinated pigs (N5, N6, V1) were clinically healthy and did not have any pathological changes or died after challenged with CSFV Bangkhen strain **(Figure 10).** However, pig V0 that vaccinated with tissue cultured C-strain 3 days prior to challenge with CSFV Bangkhen strain had typical clinical signs and pathological changes of CSF and died at 18 dpc **(Figure 11).**

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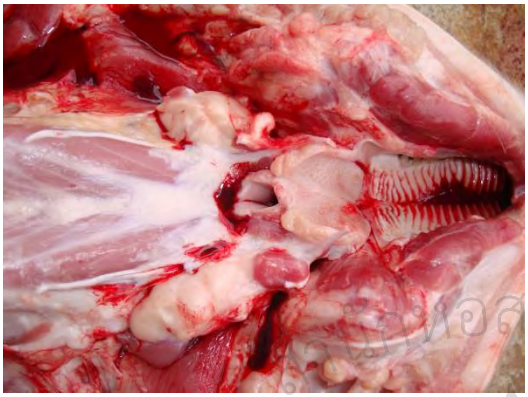
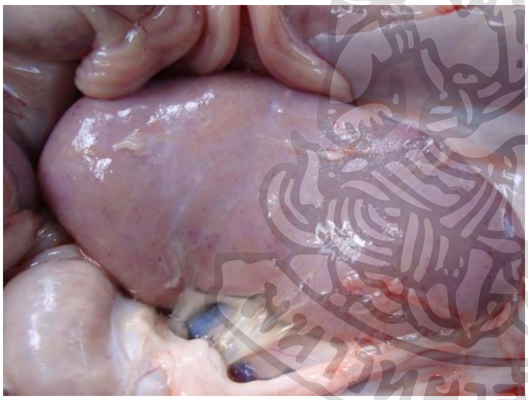
(a)



(b)

(c)



(d)

Figure 7. Negative control pigs did not have any pathological changes of (a) tonsils and (b) spleen, small intestine and colon from pig No. N1, (c) mesenteric lymphnodes, small intestine and colon as well as (d) kidney from pig No. F8.

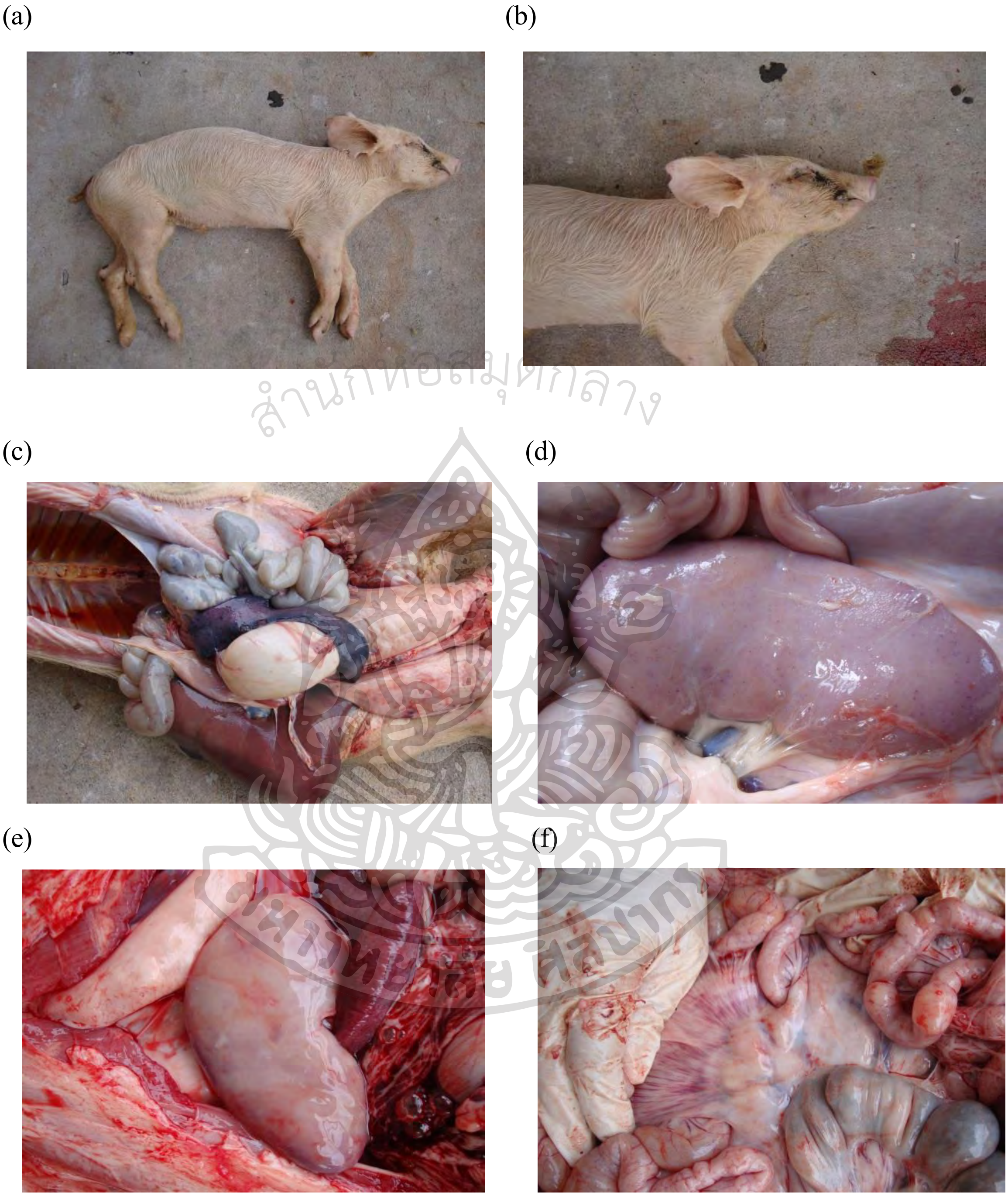


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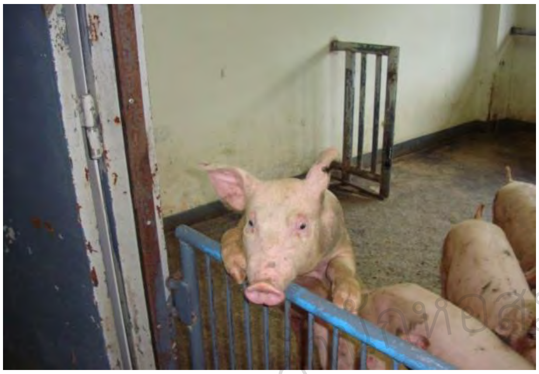
Figure 8. Pathological changes of positive control pigs; (a) thin musculature of pig D0, (b) purulent conjunctivitis of pig Do, (c) hemorrhage of spleen, liver, intestine and colon of pig D0, (e) pethechial hemorrhage of kidney of pig D0, (e) hemorrhage of kidneys of pig D6 and (f) hemorrhage of mesenteric lymphnodes, small intestine and colon of pig D6.

(a) (b)



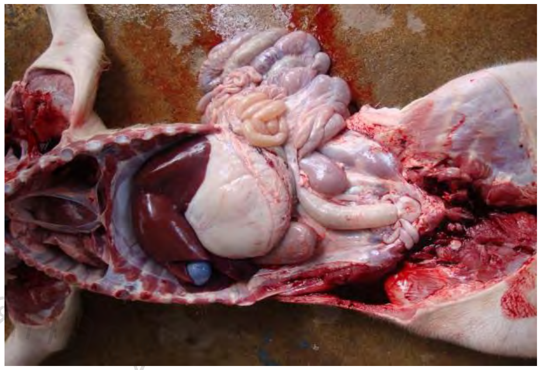


Figure 9. Clinical signs and pathological changes in lapinised C-strain vaccinated pigs (N2, N4, N7 and V2) post challenged with CSFV Bangkhen strain. All vaccinated pigs were (a) clinically healthy and (b) did not have any pathological changes of various organs.

(a) (b)

Figure 10. Clinical signs and pathological changes in 3 of 4 tissue cultured C strain vaccinated pigs (N5, N6 and V1) post challenged with CSFV Bangkhen strain. Three of four vaccinated pigs were (a) clinically healthy and (b) did not have any pathological changes of various organs.

(a)

(b)

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(c)

(d)

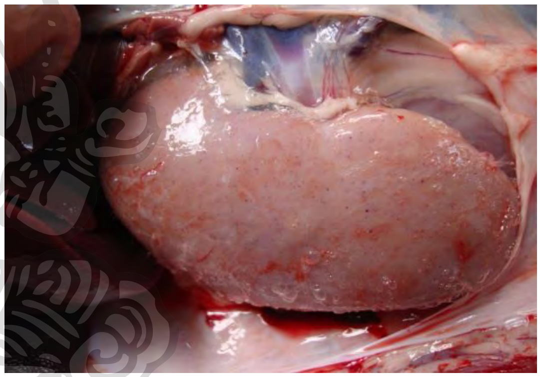

Figure 11. Clinical signs and pathological changes in 1 of 4 tissue cultured C strain vaccinated pigs that died post challenged with CSFV Bangkhen strain. Pig No. V0 had (a) thin musculature, (b) purulent conjunctivitis, (c) hemorrhage of spleen, stomach, intestine and colon, and (d) pethechial hemorrhage of kidney .

2. Leukocyte quantification

To study leukocyte number of EDTA-blood samples from pre-and post-inoculated pigs, eighty samples from NC group (14 samples), PC group (11 samples). LC group (28 samples) and TC (27 samples) were quantified by an automated hematology analyzer.

Negative control pigs (N1 and F8) had the average leukocyte number pre-inoculated with NS which equal to 22.55±3.18 x 10^3 cells/mm^3 (24.8 x 10^3cells/mm^3 and 20.3 x 10^3cells/mm^3, respectively).The average leukocyte number from day 1 through day 21 post-inoculated with NS was between 20.8±1.41 x 10^3 cells/mm^3 and 22.5±2.4 x 10^3 cells/mm^3which did not change (**Figure 12a, 12e and Appendix 3**).

Positive control pigs (D6 and D0) had the average leukocyte number pre-inoculated with CSFV which equal to 23.1±6.08 x 10^3 cells/mm^3 (18.8 x 10^3 cells/mm^3 and 27.4 x 10^3 cells/mm^3, respectively). Pig D6 had the decreasing of leukocyte number with the lowest value which equal to 9.1 x 10^3 cells/mm^3 at 7 dpi and died at 13 dpi, and pig D0 had the decreasing of leukocyte number with the lowest value which equal to 9.4 x 10^3 cells/mm^3 at 14 dpi and died at 18 dpi (**Figure 12b, 12e and Appendix 3**).

Lapinised C-strain vaccinated pigs (N2, N4, N7 and V2) had the average leukocyte number at 3 dpv which equal to 22.58±2.90X10^3 cells/mm^3 .The average leukocyte number from day 1 through day 21 post-challenge with CSFV Bangkhen strain was between16.98±2.53X10^3 cells/mm^3 and19.78±4.29X10^3 cells/mm^3 which did not change (**Figure 12c, 12e and Appendix 3**).

Tissue cultured C-strain vaccinated pigs (N5, N6, V1 and V0) had the average leukocyte number at 3 dpv which equal to 24.48±2.0 x 10^3 cells/mm^3. The average leukocyte number from day 1 through day 14 post-challenge was between14.30±3.80 x 10^3 cells/mm^3 and 24.35±2.21 x 10^3cells/mm^3which did not change. Three out of four pigs(N5, N6 and V1) had the average leukocyte number at 3 dpv which equal to 24.90±2.21x10^3 cells/mm^3, the average leukocyte number from day 1 through day 21 post-challenge was between14.30±3.80 x10^3 cells/mm^3 and 24.35±2.21 x 10^3 cells/mm^3 which did not significantly change. While one pig (V0)had the decreasing of leukocyte number with the lowest value which equal to 9.8 x 10^3cells/mm^3at 14 dpi and died at 18 dpi (**Figure 12d,**

12e, Appendix 3).

Both positive control pigs and one pig vaccinated with tissue cultured C-strain that had typical clinical signs and symptoms of CSF and died after inoculated with CSFV Bangkhen strain, had the significant decrease of leukocyte number (leucopenia). Whereas all pigs vaccinated with lapinised C-strain and three out of four pigs vaccinated with tissue cultured C-strain that did not have any notable clinical signs or symptoms or died after challenge, did not decrease of leukocyte number. The result showed that the decrease of leukocyte number affected to the typical clinical signs and symptoms of CSF and death of positive control group (D6 and D0) and tissue cultured vaccinated group (V0) after received CSFV Bangkhen strain. Both lapinised C-strain vaccine and tissue cultured C-strain vaccine could protect leucopenia in vaccinated pigs after challenge except one pig (V0) that vaccinated with tissue cultured C-strain vaccine could not protect leucopenia after received CSFV Bangkhen strain.

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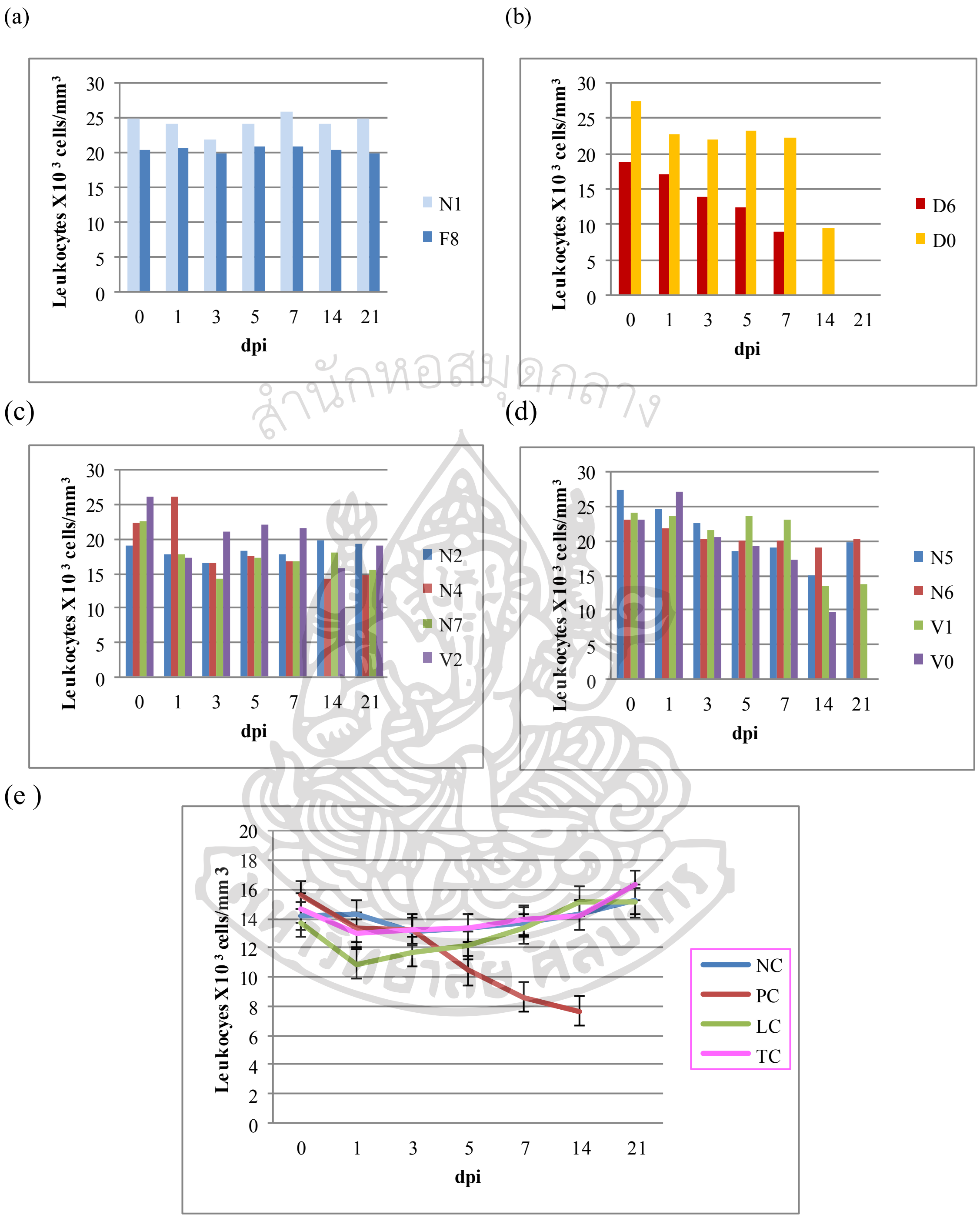

Figure 12. Leukocyte number of (a) negative control (NC), (b) positive control (PC), (c) lapinised C- strain (LC) vaccinated , and (d) tissue cultured C-strain (TC) vaccinated pigs, pre- and post-inoculation with NS or CSFV were measured by automated hematology analyzer and (e) The average leukocyte number of PC, LC vaccinated, or TC vaccinated groups were compared with NC group at indicated times.

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3. Lymphocyte quantification

To study lymphocyte number from whole blood of pigs after CSFV infection, eighty samples of whole blood collected from experimental pigs on day 0, 1, 3, 5, 7, 14 and 21 were quantified for lymphocytesby using automated hematology analyzer as following: Negative control group, pre- and post-inoculated intramuscularly with NS (14 samples). Positive control group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (11 samples). Lapinised C-strain vaccinated group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (28 samples). Tissue cultured C-strain vaccinated group pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (27 samples).

Negative control pigs (N1 and F8) had the average lymphocyte number pre-inoculated with NS which equal to 14.18±1.69 x 10^3 cells/mm^3 (15.38 cells/mm^3 and 12.99 cells/mm^3, respectively).The average lymphocyte number from day 1 through day 21 post-inoculated with NS was between 13.10±2.16 x 10^3 cells/mm^3 and 15.28±2.95 x 10^3 cells/mm^3which did not change **(Figure 13a, 13e and Appendix 4).**

Positive control pigs (D6 and D0) had the average lymphocyte number pre-inoculated with CSFV which equal to 15.62±3.48 x 10^3 cells/mm^3 (13.16 x 10^3cells/mm^3 and 18.08 x 10^3 cells/mm^3, respectively). Pig D6 had the decreasing of lymphocyte number with the low value which equal to 4.7 x 10^3 cells/$mm^3$and 1.88 x 10^3 cells/mm^3 at 5 dpi and 7 dpi, respectively, and died at 13 dpi. Pig D0 had the decreasing of lymphocyte number with the lowest value which equal to 7.67 x 10^3 cells/mm^3at 14 dpi and died at 18 dpi (**Figure 13b, 13e and Appendix 4**).

Lapinised C-strain vaccinated pigs (N2, N4, N7 and V2) had the average lymphocyte number at 3 dpv which equal to 13.70±1.20 x 10^3 cells/mm^3. The average lymphocyte number from day 1through day 21 post-challenge with CSFV Bangkhen strain was between10.87±2.00 x 10^3 cells/mm^3 and15.18±1.95 x 10^3 cells/mm^3 which change (**Figure 13c, 13e and Appendix 4**).

Tissue cultured C-strain vaccinated pigs (N5, N6, V1 and V0) had the average lymphocyte number at 3 dpv which equal to 14.69±1.42 x 10^3 cells/mm^3. The average lymphocyte number from day 1 through day 14 post-challenge was between 12.98±1.50 x 10^3 cells/mm^3 and14.23±5.35 x 10^3 cells/mm^3 which did not change. Three out of four pigs (N5, N6 and V1) had the average lymphocyte number at 3 dpv which equal to 15.18±1.26 x 10^3 cells/mm^3, the average lymphocyte number from day 1 through day 21 post-challenge was between12.98±1.50 x 10^3 cells/mm^3 and16.28±0.24 x 10^3 cells/mm^3 which did not change. While one pig (V0) had the decreasing of lymphocyte number with the lowest value which equal to 6.26 x 10^3 cells/mm^3at 14 dpi and died at 18 dpi (**Figure 13d, 13 and Appendix 4**).

Both positive control pigs and one pig vaccinated with tissue cultured C-strain that had typical clinical signs and symptoms of CSF and died after inoculated with CSFV Bangkhen strain, had the decrease of lymphocyte number (lymphopenia). Whereas all pigs vaccinated with lapinised C-strain and three out of four pigs vaccinated with tissue cultured C-strain that did not have any notable clinical signs or symptoms or died after challenge, did not decrease of lymphocyte number. The result showed that the decrease of lymphocyte number affected to the typical clinical signs and symptoms of CSF and death of positive control group (D6 and D0) and tissue cultured vaccinated group (V0) after received CSFV Bangkhen strain. Both lapinised C-strain vaccine and tissue cultured C-strain vaccine could protect lymphopenia in vaccinated pigs after challenge except one pig (V0) that vaccinated with tissue cultured C-strain vaccine could not protect lymphopenia after received CSFV Bangkhen strain.

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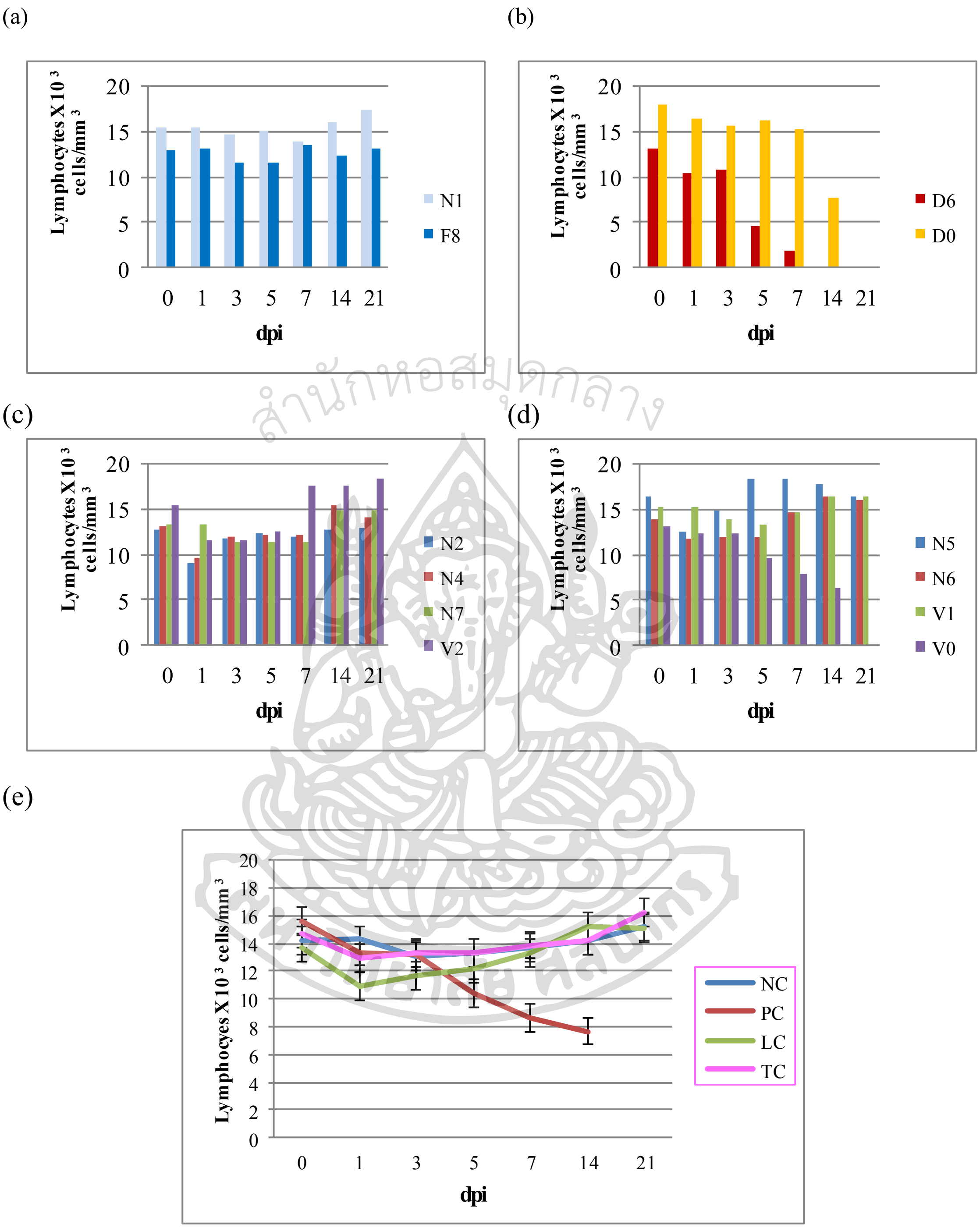

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Figure 13. Lymphocyte number of (a) negative control (NC), (b) positive control (PC), (c) lapinised C- strain (LC) vaccinated , and (d) tissue cultured C-strain (TC) vaccinated pigs, pre- and post-inoculation with NS or CSFV were measured by automated hematology analyzer and (e) The average lymphocyte number of PC, LC vaccinated, or TC vaccinated groups were compared with NC group at indicated times.

4. CSFV antigen quantification

To detect CFSV antigen in serum of pigs after CSFV infection,eighty samples of serum collected from experimental pigs on day 0, 1, 3, 5, 7, 14 and 21 were measured for CSFV antigen by using CSFV Ag ELISA Kit (IDEXX; Liebefeld-Bern-Switzerland)as following: Negative control group, pre- and post-inoculated intramuscularly with NS (14 samples). Positive control group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (11 samples). Lapinised C-strain vaccinated group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (28 samples). Tissue cultured C-strain vaccinated group pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (27 samples).

Negative control pigs (N1 and F8) were not detected CSFV antigen in serum, pre-inoculated with NS (average OD value = 0.0000±0.0000) and post-inoculated with NS which the highest OD value ≤0.0070(<0.300) throughout the experiment **(Figure 14a, 14e, Appendix 5).**

Positive control pigs (D6 and D0) were not detected CSFV antigen in serum, pre-inoculated with CSFV (average OD value = 0.0000±0.0000). Pig D6 was detected CSFV antigen in serum at 7 dpi which OD value equal to 2.6520 (>0.300) and pig D0 was detected CSFV antigen in serum at 14 dpi which OD value equal to 2.1030 **(Figure 14b, 14e, Appendix 5).**

Lapinised C-strain vaccinated pigs (N2, N4, N7 and V2)were not detected CSFV antigen in pig serum at 3 dpv(average OD value = 0.0080±0.0068) and from day 1 through day 21 post-challenge with CSFV Bangkhen strain which the highest average OD value equal to 0.0120±0.0106 **(Figure 14c, 14e, Appendix 5).** .

Tissue cultured C-strain vaccinated pigs (N5, N6, V1 and V0)were not detectedCSFV antigen in serum at 3 dpv(average OD value = 0.0264±0.0395). Three out of four pigs (N5, N6 and V1) those survived after challenge were not detected CSFV antigen from day 1 through day 21 post-challenge with CSFV Bangkhen strain which the highest average OD value equal to 0.0365±0.0437,while one pig (V0) that showed typical clinical signs and symptoms and died at 18 dpi was detected CSFV antigen at 5, 7 and 14 dpc which OD values equal to 2.6100, 2.7100 and 2.7800, respectively **(Figure 14d, 14e, Appendix 5).**

All of pigs vaccinated with lapinised C-strain were not detected CSFV antigen in serum and survived after challenge, whereas only three out of four pigs vaccinated with tissue cultured C-strain were not detected CSFV antigen in serum and survived after challenge. In contrast, one pig (V0) vaccinated with tissue cultured C-strain and two positive control pigs (D6 and D0) were detected CSFV antigen in serum and died after received CSFV, Bangkhen strain. The result showed that the detection of CSFV antigen in serum was related to the death of two positive control pigs (D6 and D0) and one tissue cultured vaccinated pig (V0) after received CSFV Bangkhen strain. Both lapinised C-strain vaccine and tissue cultured C-strain vaccine could protect the accumulation of CSFV antigen in serum of vaccinated pigs except one pig (V0) that vaccinated with tissue cultured C-strain vaccine could not protect the accumulation of CSFV antigen in serum and died after received CSFV Bangkhen strain.

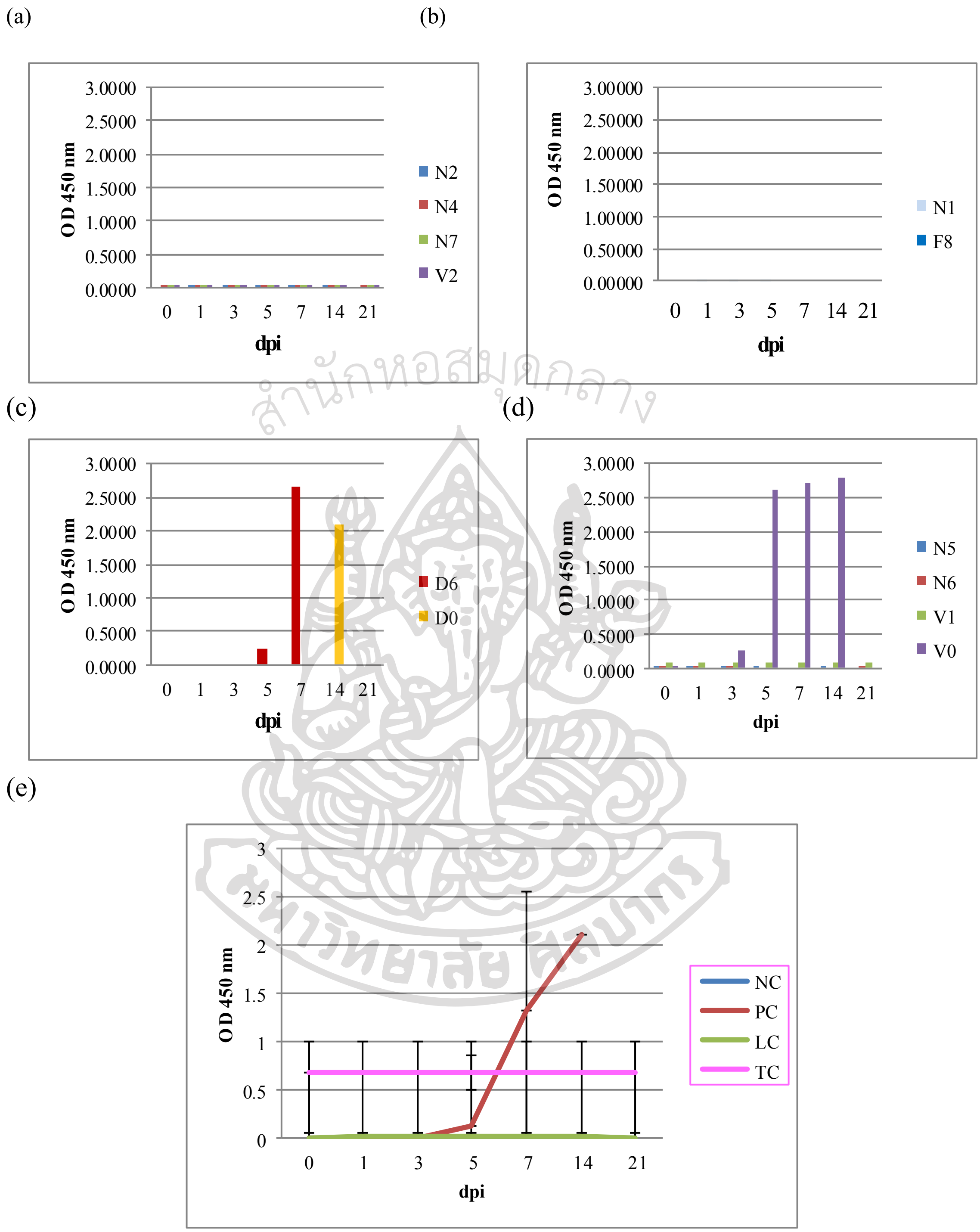

Figure 14. Antigen quantitation of (a) negative control (NC), (b) positive control (PC), (c) lapinised C- strain (LC) vaccinated , and (d) tissue cultured C-strain (TC) vaccinated pigs, pre- and post-inoculation with NS or CSFV were measured by ELISA reader and (e) The average antigen quantitation of PC, LC vaccinated, or TC vaccinated groups were compared with NC group at indicated times.

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5. Anti-CSFV antibody quantification

To detect antibody to CSFV in serum of pigs after CSFV infection, eighty samples of serum collected from experimental pigs on day 0, 1, 3, 5, 7, 14 and 21 were measured for antibody to CSFV by using CSFV Ab ELIS Kit (IDEXX; Liebefeld-Bern-Switzerland) as following: NC group, pre- and post-inoculated intramuscularly with NS (14 samples). PC group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (11 samples). LC vaccinated group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (28 samples). TC vaccinated group pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (27 samples).

Negative control pigs (N1 and F8) were not detected antibody to CSFV in serum, pre-inoculated with NS which the highest % blocking ELISA equal to 10.60±2.12% (9.10% for pig N1 and 12.01% for pig F8) and post-inoculated with NS which the highest % blocking ELISA equal to 12.18±0.91% (≤ 30%) throughout the experiment **(Figure 15a, 15e, Appendix 6).**

Positive control pigs (D6 and D0) were not detected antibody to CSFV in serum pre-inoculated with CSFV (% blocking ELISA = 12.18±0.91%). Pig D6 was not detected antibody to CSFV in serum post-inoculated with CSFV Bangkhen strain with the highest % blocking ELISA at 7 dpi which equal to 16.08% (≤ 30%) but pig D0 was detected antibody to CSFV in serum at 14 dpi which % blocking ELISA equal to 49.64% (≥ 40%). Pig D6 and pig D0 showed CSFV typical clinical signs and symptoms and died at 13 dpi and 18 dpi, respectively **(Figure 15b, 15e, Appendix 6).**

Lapinised C-strain vaccinated group (N2, N4, N7 and V2) had the average % blocking ELISA at 3 dpv which equal to 18.62±1.17%, and had the increasing of average % blocking ELISA which equal to 55.24±10.08% and 74.67±7.19% on 14 and 21 dpi respectively **(Figure 15c, 15e, Appendix 6).**. Tissue cultured C-strain vaccinated group (N5, N6, V1 and V0) had the % blocking ELISA at 3 dpv which equal to 21.01±1.28%, and had the increasing of average % blocking ELISA which equal to 56.67±10.59% and 83.97±5.78% on 14 and 21 dpi respectively. Three of four pigs (N5, N6 and V1) those survived after challenge had the increasing of average % blocking ELISA which equal to 58.95±11.70% and 83.97±5.78% at 14 and 21 dpi, while pig V0 had the slightly increasing of the % blocking ELISA at 14 dpi which equal to 49.83%, and died at 18 dpi **(Figure 15d, 15e, Appendix 6).**. The average % blocking ELISA of lapinised C-strain vaccinated group and tissue cultured C-strain vaccinated group was higher than negative control group and positive control group at 14 and 21 dpi **(Figure 15e, Appendix 6).** However, pig V0 in tissue cultured C-strain vaccinated group and pig D0 in positive control group that had slightly increasing of % blocking ELISA (~49%) at 14 dpi, died at 18 dpi. The result showed that the increasing of % blocking ELISA after challenge affected to the survival of vaccinated pigs, the average % blocking ELISA ≥74.6774.67±7.19% at 21 dpi could protect vaccinated pigs against a challenge of CSFV Bangken strain, while the % blocking ELISA ≤ 49% (V0 and D0) could not protect pigs against high virulent CSFV Bangken strain.

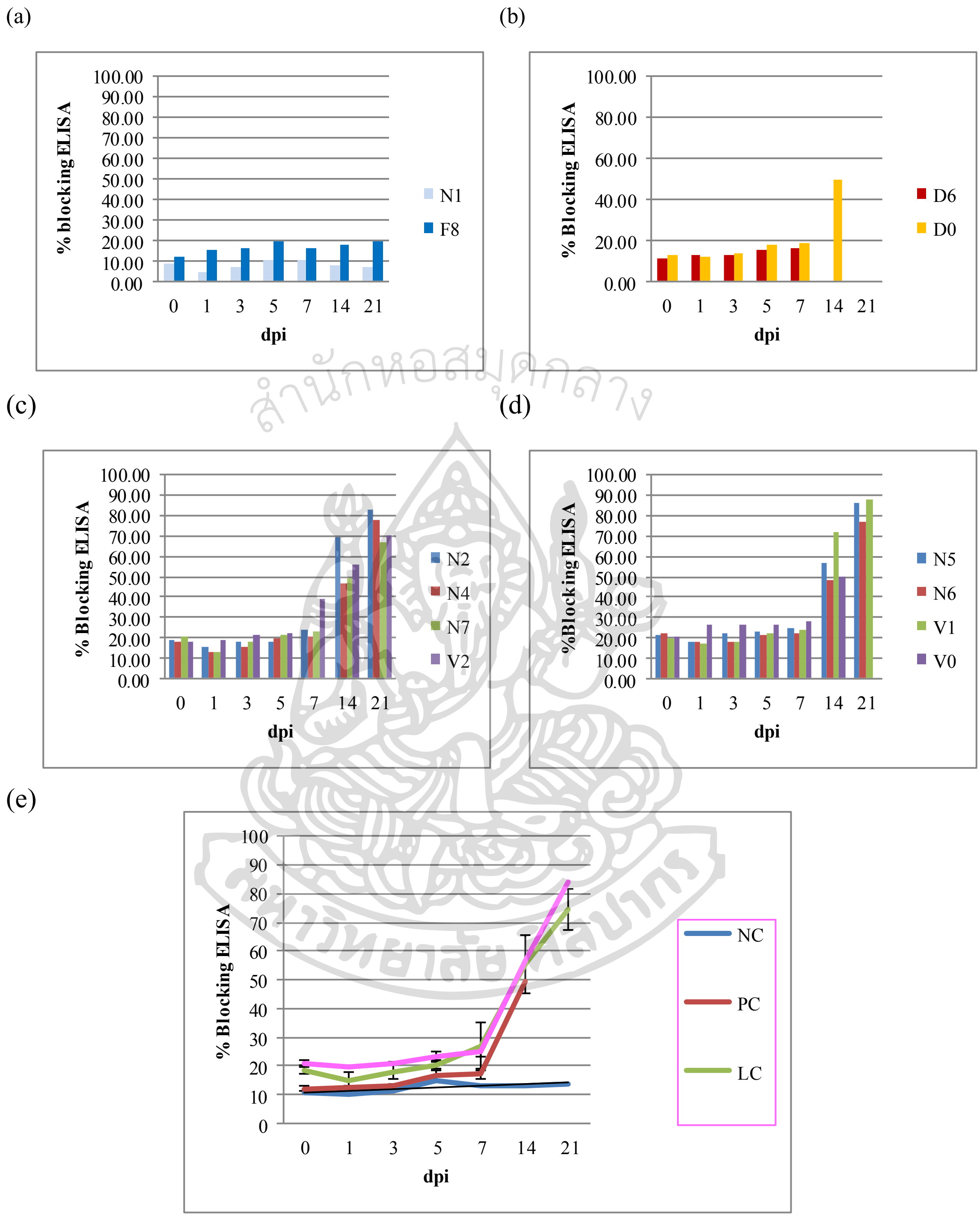

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Figure 15. Antibody quantitation (% blocking ELISA) of (a) negative control (NC), (b) positive control (PC), (c) lapinised C- strain (LC) vaccinated , and (d) tissue cultured C-strain (TC) vaccinated pigs, pre- and post-inoculation with NS or CSFV were measured by ELISA reader and (e) The average antibody quantitation (blocking ELISA) of PC, LC vaccinated, or TC vaccinated groups were compared with NC group at indicated times.

6. Interferon-gamma gene expression

To study IFN-γ cytokine gene expression from PBMC of pigs after CSFV infection, eighty samples of PBMC collected from pig blood at 0, 1, 3, 5, 7, 14 and 21 dpi were measured for IFN-γ cytokine gene expression by qRT-PCR as following: NC group, pre- and post-inoculated intramuscularly with NS (14 samples). PC group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (11 samples). LC vaccinated group, pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (28 samples). TC vaccinated group pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangkhen strain (27 samples).

NC group (N1 and F8) did not change the average IFN-γ cytokine gene expression from PBMC which equal to 0.99±0.0738 and 0.95±0.0039 folds post-inoculated with NS at 1 and 3 dpi, respectively **(Figure 16a, 16e, Appendix 7).** PC group (D6 and D0) did not increasing of IFN-γ cytokine gene expression from PBMC which equal to 1.21± 0.1042 and 1.65±0.2412 folds post-inoculated with CSFV Bangken strain at 1 and 3 dpi, respectively **(Figure 16b, 16e, Appendix 7).** LC vaccinated group (N2, N4, N7 and V2) had increasing of IFN-γ cytokine gene expression from PBMC which equal to 2.56± 0.2910 folds at 3 dpi **(Figure 16c, 16e, Appendix 7).** TC vaccinated group (N5, N6, V1 and V0) did not increase of IFN-γ cytokine gene expression from PBMC which equal to 1.04± 0.0532 and 1.93±0.6755 folds at 1 and 3 dpi, respectively (**Figure 16d, 16e, Appendix 7**). However, LC vaccinated pigs had changing folds of IFN-γ cytokine gene expression from PBMC bigger than TC vaccinated pigs because pig V0 in TC vaccinated group did not increase of IFN-γ cytokine gene expression from PBMC which equal to 1.07 and 1.18 folds at 1 and 3 dpc, although another three vaccinated pigs (N5, N6 and V1) had increasing of IFN-γ cytokine gene expression from PBMC which equal to 1.04±0.07 and 2.16±0.1214 folds at 1 and 3 dpc, respectively (figure 9d, 9e).The result showed that the increasing of IFN-γ cytokine gene expression from PBMC affected to the survival of all LC vaccinated pigs and three of four TC vaccinated pigs **(Figure 16e, Appendix 7)** .

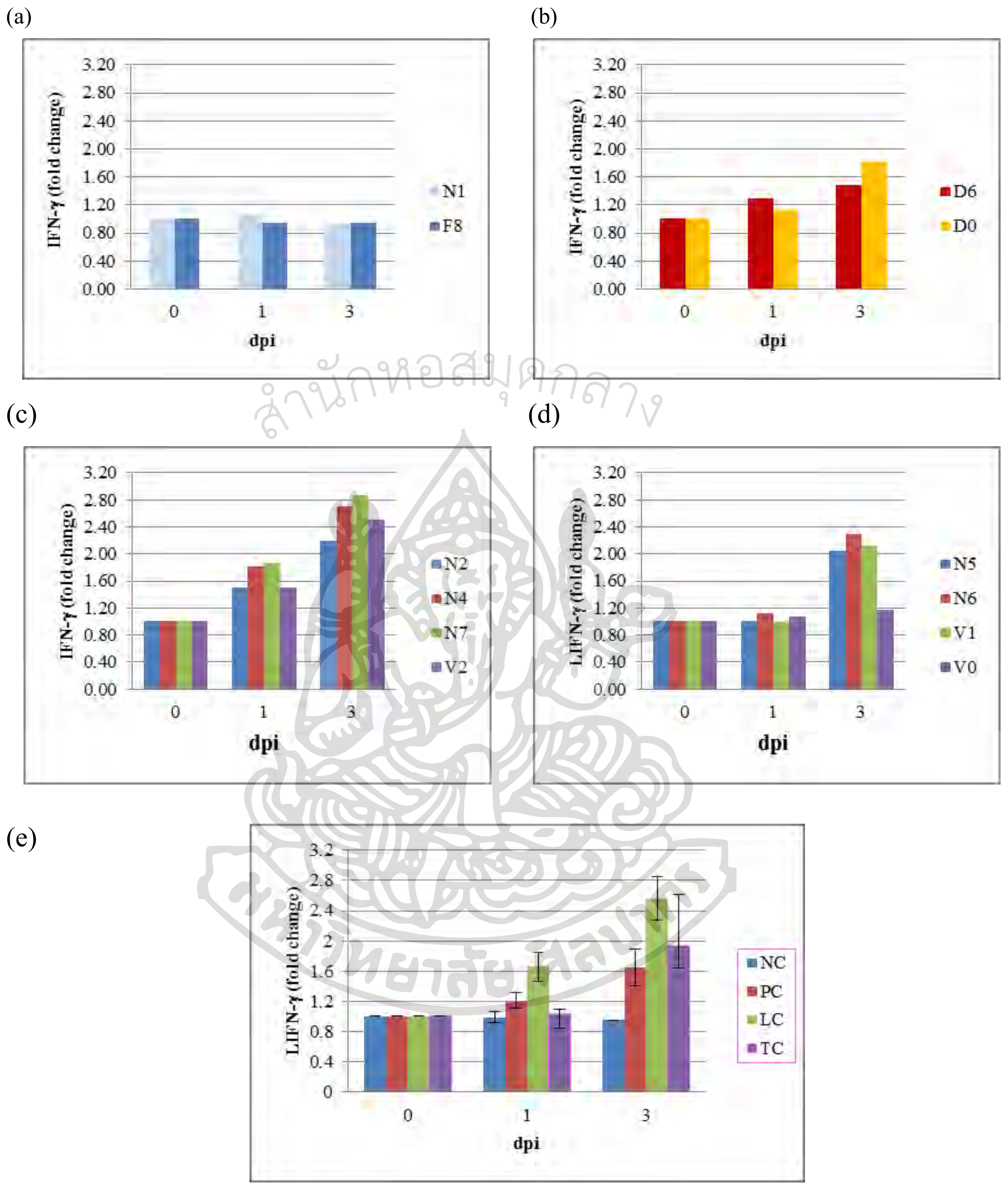

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Figure 16. The amount of IFN-γ genes from PBMC of (a) negative control (NC), (b) positive control (PC), (c) Lapinised C- strain (LC) vaccinated, and (d) Tissue cultured C- strain (TC) vaccinated pigs, pre- and post-inoculated intramuscularly with NS or CSFV (1.0X10^5 PID_{50}) were measured by qRT-PCR;. (e) Comparison of the average IFN-γ genes of PC, LC vaccinated, or TC vaccinated groups were compared with NC group at indicated times.

7. Trans-forming growth factor beta (TGF-β) gene expression

To study TGF-β cytokine gene expression from PBMC of pigs after CSFV infection: Eighty samples of PBMC collected from pig blood at 0, 1, 3, 5, 7, 14 and 21 dpi were measured for IFN-γ cytokine gene expression by qRT-PCR: Negative control group, pre- and post-inoculation with NS (14 samples), Positive control group pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangken strain (11 samples), Lapinised C-strain vaccinated group (28 samples) and Tissue cultured C-strain vaccinated group (27 samples), pre- and post-inoculated intramuscularly with 10^5 PID_{50} of virulent CSFV Bangken strain.

Negative control group (N1 and F8) did not change the average TGF-β cytokine gene expression from PBMC which equal to 1.05±0.0131 and 1.09±0.0117 folds post-inoculated with NS at 1 and 3 dpi, respectively **(Figure 17a, 17e, Appendix 8)**. Positive control group (D6 and D0), had the decreasing of TGF-β cytokine gene expression from PBMC which equal to 3.06± 1.9711 and 2.90±0.7547 folds post-inoculated with CSFV Bangken strain at 1 and 3 dpi, respectively **(Figure 17b, 17e, Appendix 8).** Lapinised C-strain vaccinated group (N2, N4, N7 and V2) did not decrease TGF-β cytokine gene expression from PBMC which equal to 1.05± 0.0408 and 1.03± 0.0407 folds at 1 and 3 dpi, respectively **(Figure 17c, 17e, Appendix 8).** Tissue cultured C-strain vaccinated group (N5, N6, V1 and V0) did not decrease TGF-β cytokine gene expression from PBMC which equal to 1.04± 0.0695 and 1.36±0.6584 folds post-inoculated with CSFV Bangken strain at 1 and 3 dpi, respectively**.** However, Tissue cultured C-strain vaccinated pigs had changing folds of TGF-β cytokine gene expression from PBMC bigger than Lapinised C-strain vaccinated pigs because Tissue cultured C-strain vaccinated pig (V0) had the decreasing of TGF-β cytokine gene expression from PBMC which equal to 2.35 folds post-inoculated with CSFV Bangken strain at 3 dpi **(Figure 17d, 17e, Appendix 8).** The result showed that the decreasing of TGF-β cytokine gene expression from PBMC affected to the dead of positive control group (D6 and D0) and Tissue cultured vaccinated group (V0) after received CSFV Bangken strain **((Figure 17b, 17e, Appendix 8).**

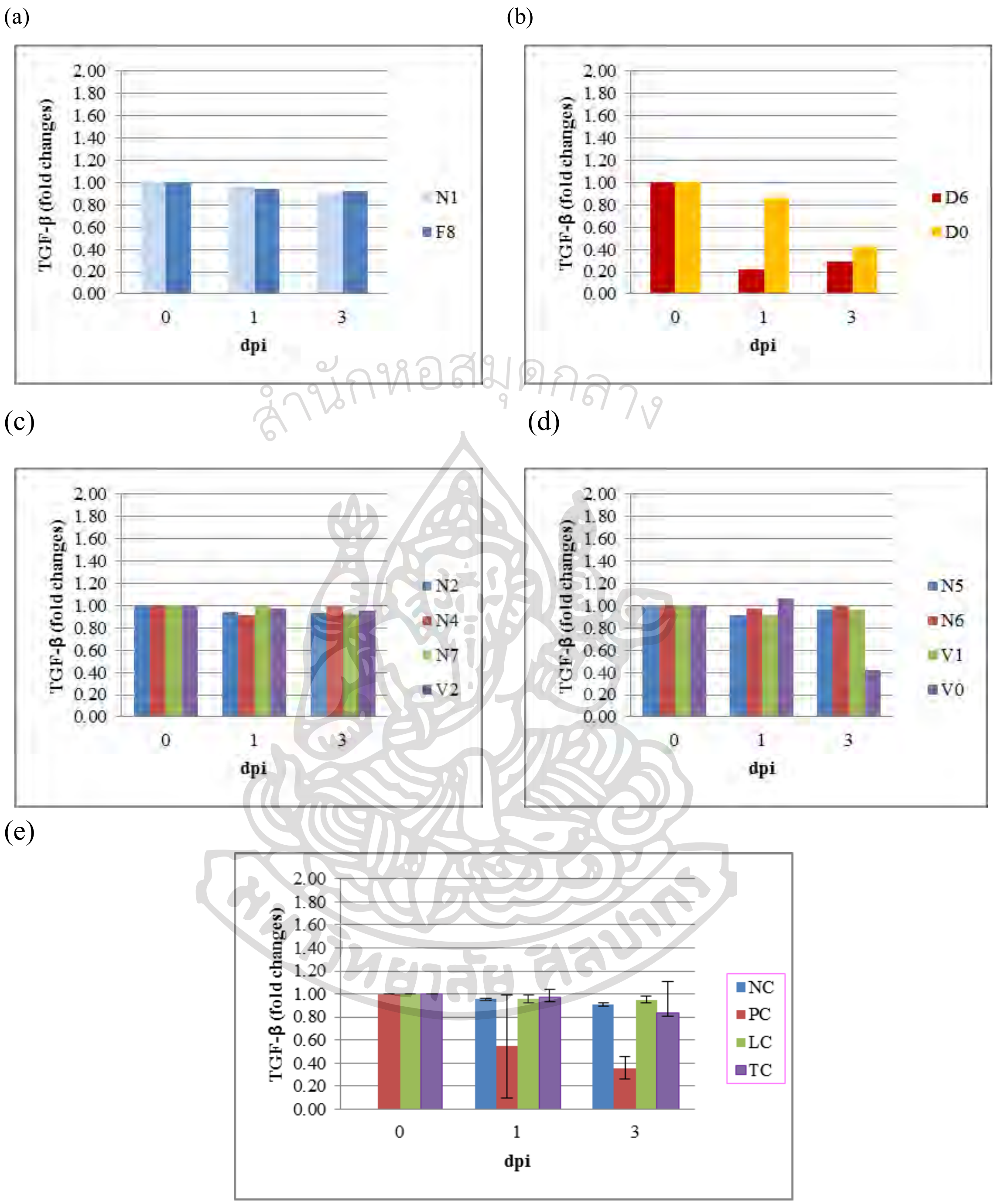

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Figure 17. The amount of TGF-β genes from PBMC of (a) negative control (NC), (b) positive control (PC), (c) Lapinised C- strain (LC) vaccinated, and (d) Tissue cultured C- strain (TC) vaccinated pigs, pre- and post-inoculated intramuscularly with NS or CSFV (1.0X10^{5} PID$_{50}$) were measured by qRT-PCR;. (e) Comparison of the average TGF-β genes of PC, LC vaccinated, or TC vaccinated groups were compared with NC group at indicated times.

CHAPTER VI
DISCUSSION

This is the first report that MLV, C-strain cultured in FS-L3 (pig kidney cell lines) namely tissue cultured C-strain (TC) vaccine partially protects vaccinated pigs, while MLV, C-strain cultured in rabbits namely lapinised C-strain (LC) vaccine completely protects vaccinated pigs against a highly virulent CSFV Bangkhen strain, studying in commercial breeds as Large White/Landrace/Durox Jersey pigs aged eleven weeks, 3 days prior to challenge with a highly virulent CSFV Bangken strain.

Pigs vaccinated with TC vaccine, 3 days prior to challenge with a highly virulent CSFV Bangkhen strain, partially induced CSFV-specific antibodies in sera and expressed IFN-γ genes from PBMC, while pigs vaccinated with LC vaccine completely induced CSFV-specific antibodies in sera and expressed IFN-γ genes from PBMC. At the same time, pigs vaccinated with TC vaccine, 3 days prior to challenge, partially protect the decreasing of leukocytes, lymphocytes and TGF-β gene from PBMC, and the increasing of CSFV-specific antigens in sera, while pigs vaccinated with LC vaccine completely protect the decreasing of leukocytes, lymphocytes and TGF-β gene from PBMC, and the increasing of CSFV-specific antigens in sera, post challenged with a highly virulent CSFV Bangkhen strain. All of these parameters resulted the partially protection of severe clinical signs, pathological changes and death in TC vaccinated pigs, and the completely protection in LC vaccinated pigs.

This study demonstrated that CSFV Bangkhen strain caused typical clinical signs and high fever (39.6^{o}C) in both PC (unvaccinated) pigs as early as 2 dpi. Pig No.D6 had persistent high fever (39.6°C - 40.8°C) during 2 to 12 dpi, and pig No.D0 had persistent high fever (39.6°C - 39.8°C) during 2 to 14 dpi (**Figure 6b, Appendix 2**). With the onset of fever, both pigs developed severe depression, loss of appetite, constipation followed by diarrhea, paresis, body stiffness, respiratory distress, ataxia, tremors, skin hyperemia **(data not shown),** thin musculature **(Figure 8a),** purulent conjunctivitis **(Figure 8b)** and died at 13 dpi and 18 dpi, respectively. Typical clinical signs in unvaccinated pigs that caused by CSFV Bangkhen strain were correlated to the previous report that pigs infected with a highly virulent CSFV Brescia strain developed the same clinical signs [274]. The result of the study was also correlated to the previous report that acute forms of CSF associated with highly virulent CSFV strains [2]. The clinical scoring system developed by Mittelholzer *et al.* [273] was used with some modification to record and evaluate clinical signs of PC pigs infected with CSFV Bangkhen strain, suggesting that isolated CSFV Bangkhen strain was a highly virulent CSFV strain. In addition, at necropsy post mortem examination of both PC pigs confirmed pathological change consistent of CSF including tonsil inflammation **(data not shown)**, hemorrhage of spleen **(Figure 8c),** petechial hemorrhage of kidneys **(Figure 8d, 8e),** hemorrhage of mesenteric lymph nodes, small intestine and colon **(Figure 8c, 8f),** which were typical pathological lesions of CSFV. This finding was correlated to the previous report that pigs infected with moderate/high virulent CSFV Romania (genotype 2.3) field isolate and CSFV Israel (genotype 2.1) field isolate had hemorrhages visible as blebs in the border of the spleen, petechial bleedings in the kidney together with swellings and petechial bleedings in the lymph nodes [89].

In this study, we also found that both PC pigs had the decreasing of leukocytes which the lowest number equal to 9,100 cells/mm^3 at 7 dpi for pig No.D6 and equal to 9,400 cells/mm^3 at 14 dpi for pig No.D0 **(Figure 12b, Appendix 3)**. Both infected pigs also had the decreasing of lymphocytes which the lowest number equal to 1,880 cells/mm^3 at 7 dpi for pig No.D6, and equal to 7,670 cells/mm^3 at 14 dpi for pig No.D0. **(Figure 13b, Appendix 4)**. The result indicated that CSFV Bangkhen strain was a highly virulent strain that could destroy leukocytes including lymphocytes and caused typical clinical signs and death in infected pigs. This result was corresponded to previous report, the CSFV Margarita strain caused the apoptosis of infected cells and affected to the decreasing of lymphocyte in infected pigs [275] [276].

Previous studies also reported that CSFV infected pigs with clinical signs showed rapidly decreasing of leukocytes may be $\leq$ 9,000 cells/mm^3. The important cause of rapid decreasing of leukocytes was the apoptosis mechanism of both T-lymphocyte and B-lymphocyte, and the amount of WBC was lowest on day 7 after infection [99]. Pigs infected with highly virulent CSFV that showed clinical signs had leucopenia condition which leukocytes rapidly decreased, and remained ≤ 9,000 cells/mm^3 [12]. The severe lymphocyte depletion accompanied by an immunosuppression state may account the failure of the adaptive response in the pigs that developed severe clinical signs of disease [110] [275] [277]. In contrast, three out of four TC vaccinated group (No. N5, N6 and V1), 3 days prior to challenge with a highly virulent CSFV Bangkhen strain, did not decrease the number of leukocytes and lymphocytes **(Figure 12d and 13d, Appendix 3 and 4)** and did not develop any severe clinical signs **(Figure 10a)** or pathological changes **(Figure 10b)** , while one TC vaccinated pig (No. V0) had the decreasing of leukocyte number and lymphocyte number, reaching the lowest number which equal to 9,800 cells/mm^3 and 6,260 cells/mm^3 at 14 dpc, respectively **(Figure 12d and 13d, Appendix 3 and 4)**. This pig (No. V0) developed typical clinical signs **(Figure 11a, 11b)** and pathological changes **(Figure 11c, 11d)** and died after challenge. The result indicated that TC vaccine partially protected leucopenia and severe clinical signs in vaccinated pigs (only 3 out of 4 vaccinated pigs) after challenged with a highly virulent CSFV Bangkhen strain. This is the first report of the efficacy study of C-strain vaccine cultured in FS-L3 cell lines for partially protection of severe clinical signs, pathological changes and the decreasing of leukocytes and lymphocytes after challenged with a highly virulent CSFV Bangkhen strain.

While all of four pigs vaccinated with LC vaccine (Pig No. N2, N4, N7 and V2), 3-days prior to challenge with a highly virulent CSFV Bangkhen strain, did not decrease the number of leukocytes and lymphocytes (**Figure 12c and 13c, Appendix 3 and 4)** and did not develop any severe clinical signs **(Figure 9a)** or pathological changes **(Figure 9b)**. The result indicated that LC vaccine completely protected leucopenia and severe clinical signs in vaccinated pigs (all of four vaccinated pigs) after challenged with a highly virulent CSFV Bangkhen strain. This result was corresponded to previous report, all pigs vaccinated with MLV, Riemser® C-strain CSFV vaccine, 5 days prior to challenge with either CSFV genotype 2.1 (UK2000/7.1) or genotype 3.3 (CBR/93) prevented all typical clinical signs and remained normal of leukocyte and platelet numbers after challenge. None of pigs vaccinated with MLV, Riemser® C-strain vaccine, 3 days prior to challenge with genotype 3.3 (CBR/93) had any notable clinical changes or decreased in leukocyte number. However, some of the pigs vaccinated with MLV, Riemser® C-strain vaccine, 1 day prior to challenge with either of the challenge strain had clinical signs from 7-10 dpc [15]. It has been reported that lapinised C-strain vaccine induced complete protection against CSFV within one week post vaccination [11] [12]. However, we found that that lapinised C-strain vaccine induced complete protection against CSFV within 3 day post vaccination.

In this study, CSFV-specific antigens were detected in sera of both PC pigs. Pig No. D6 had the increasing of CSFV-specific antigen which the peak OD value equal to 2.6520 at 7 dpi, and pig No.D0 had the increasing of CSFV-specific antigen which the peak OD value equal to 2.1030 at 14 dpi (>0.300). The CSFV antigen was also detected in 1 out of 4 TC vaccinated pigs (No.V0) which the peak OD value equal to 2.6100, 2.7100 and 2.7800 at 5, 7 and 21 dpc, respectively **(Figure 14b and 14 d, Appendix 5.2**). During this time, these three pigs were not detected CSFV-specific antibodies by ELISA at 7 dpc. Only pig No.D0 and pig No.V0 had the slightly increasing of CSFV-specific antibodies which the peak value of % blocking ELISA equal to 49.64% and 49.83% (> 40%) at 14 dpc, respectively and died at 18 dpc, while pig No.D6 was not detected CSFV- specific antibody which the peak value of % blocking ELISA equal to 16.08% (≤ 30%) at 7 dpc and died at 13 dpc **(Figure 15b and 15 d, Appendix 6.2**). In our result, CSFV Bangkhen strain infected pigs had the high amount CSFV-specific antigen in blood circulation, but not induce the high level of CSFV- specific antibody. This result was consistent with previous report, viral RNA could be detected in sera and nasal swabs of pigs infected with CSFV Cat01 strain or Margarita strain since 7 days post inoculation [279]. However, Renson et al. [278] reported that during CSF infection, viraemia is generally highly dependent on serum neutralizing antibody level, viraemia decreased from 7 dpi onwards in vaccinated pigs and was already well advanced when neutralizing antibody level was detected in the serum at 12 dpi (14 dpv).

In contrast, three out of four TC vaccinated group (No. N5, N6 and V1), 3 days prior to challenge, were not detected CSFV-specific antigens post challenge with a highly virulent CSFV Bangkhen strain **(Figure 14d, Appendix 5),** but induced the increasing of CSFV-specific antibodies, reaching the peak which the average value of % blocking ELISA equal to 58.95±11.70% and 83.97±5.78% at 14 and 21 dpc, respectively **(Figure 15d, Appendix 6)** and survived after challenge. The result indicated that TC vaccine partially induced the high level of CSFV-specific antibodies that could protect pigs against a challenge (only 3 out of four vaccinated pigs). While all of four LC vaccinated pigs (No. N2, N4, N7 and V2), 3 days prior to challenge, were not detected CSFV-specific antigens post challenge with a highly virulent CSFV Bangkhen strain **(Figure 14c, Appendix 5),** but induced the increasing of CSFV-specific antibodies, reaching the peak which the average value of % blocking ELISA equal to 55.24±10.08% and 74.67±7.19% at 14 and 21 dpc, respectively **(Figure 15c, Appendix 6)** and survived after challenge. The result indicated that LC vaccine completely induced the high level of CSFV-specific antibodies that could protect pigs against a challenge (all of four vaccinated pigs). The result of this study was correlated to the previous study that viral RNA were not detected in blood of pigs vaccinated 5 days prior to challenge with genotype 2.1 (UK2000/7.1), and vaccinated pigs did not show any clinical signs while positive control pigs that showed severe clinical signs had the high level of viraemia after received CSFV [280]. The further studies also reported that pigs vaccinated with C-strain vaccine, 5 days prior to challenge, that did not show any clinical signs were not detected RNA in blood and nasal secretion. However, pigs vaccinated with C-strain vaccine, 3 days prior to challenge with genotype 2.1 (UK2000/7.1), were detected the low level of CSFV antigen in blood and nasal swabs, while in group that challenged with genotype 3.3 (CBR/93), viral RNA were not detected in nasal swabs of all pigs but was detected in blood of one pig [15]. The result was also correlated to the further report that either E2-specific antibodies as detected by ELISA or neutralizing antibodies as detected by NPLA were not detected in pigs before 13 dpi. Interestingly, E2-specific antibodies as detected by ELISA were detected from 13 to 17 dpi in almost of the pigs that were infected with the CSFV Cat01 strain. However, neutralizing antibodies as detected by NPLA were detected in two pigs that were infected with the Cat01 strain at 13 dpi and in four of these pigs at 17 dpi [279]. From the previous report, C-strain vaccine could protect vaccinated pigs, 7 days prior to challenge with CSFV, pigs had the slightly increase of body temperatures, coincided with viraemia, increasing of CSFV neutralizing antibody, and survived post challenge [12]. In this study the researchers found that C-strain could protect vaccinated pigs, 3 days prior to challenge with a highly virulent CSFV, pigs had the slightly increase of body temperatures, coincided with the increasing of CSFV-specific antibodies, and did not develop typical clinical signs and survived after challenge. However, CSFV-specific antigen in blood circulation was not detected by ELISA in the condition with high titer level of CSFV-specific antibodies. This finding was corresponded to the previous study reported that the limited replication of CSFV accompanied by a strong boost of immune response as evidenced by the antibody titers towards CSFV-E2. Neutralizing antibodies were probably able to block further the dissemination and shedding of challenged virus [281].

Previous study have shown that the protection from the disease is commonly associated with a humoral (Th2) immune response with the production of CSFV-specific antibodies or neutralizing antibodies that usually appear about 2 weeks post-vaccination [12]. However, the neutralizing antibody titers did not differ between vaccinated group and unvaccinated group, suggesting that others immune responses, in addition to the neutralizing antibody response, are involved in the early onset of partial protection [278]. The importance of humoral antibodies in the protection against CSF is well known [167] [216]. After intramuscularly inoculation of CSFV Bangkhen strain, the E2-specific humoral antibody titers in the sera of pigs increased in the four LC vaccinated pigs and three TC vaccinated pigs compared to NC and PC group. Along with the humoral antibody titer, the importance of the IgG1/IgG2 balance has also been reported in previous studies [130] [282]. High IgG1 production associated with IFN-γ is considered more important in protection against viral infection than IgG2 [130] [282]. In the present study, four LC vaccinated pigs and three TC vaccinated pigs after challenged with CSFV showed an increase in antibody titers compared to the NC and PC group. These result shows that both vaccines are able to induce the humoral immune response, although TC vaccine induced not complete. In this study, we also found that the production of CSFV specific antibodies did not detected at 3 days post vaccination. However, all pigs vaccinated with lapinised C-strain vaccine and 3 out of 4 pigs vaccinated with tissue cultured C-strain vaccine, 3 days prior to challenge, could protect against a challenge with a highly virulent CSFV Bangkhen strain. Protection from the disease is mainly associated with the humoral immune response but CSFV-neutralizing antibodies usually appear about 2 weeks post-vaccination. Thus, the early protection in C-strain vaccinated pigs indicated that other immune responses may also be involved, such as cell-mediated immunity with significant IFN-γ production in relation to protection [226]. It is noteworthy that previous studies have indicated the role of specific IFN-γ producing cells, play a role in the elicited protection against CSFV [22] [225] [226] [283].

Previous studies have indicated the role of specific IFN-γ-producing cells, mainly the CD4+CD8$^{\alpha}$ low double-positive T cell (memory), play a role in the elicited protection against CSFV [22] [225] [226] [283]. IFN-γ is a good indicator of cell-mediated immunity (Th1 immune response) and IgG2 production is enhanced in some porcine B-cells treated with recombinant porcine IFN-γ [284]. Previous studies have shown a correlation between IFN-γ production and the protection induced by vaccination with MLV C-strain vaccine during CSFV infection [15] [226]. CSFV C-strain protected pigs from CSF by producing IFN-γ from PBMC cells to protect against CSFV prior the presenting of antibody. It showed that CMIR played role in CSFV protection [22]. It has been reported that CD4+ lymphocytes produced IFN-γ to protect CSFV in pigs [283]. The rapidly increase of IFN-γ provided the early protection against CSFV [15]. The study in vivo found that IFNγ resisted to CSFV [226]. In this study, the IFN-γ gene expression from the PBMCs of PC pigs, LC vaccinated pigs and TC vaccinated pigs compared with NC pigs were investigated. We found that the IFN-γ genes from both PC pigs (No. D6 and D0) and 1out of 4 TC vaccinated pigs (No. V0) did not increased at 1 and 3 dpi, and these three pigs developed severe clinical signs, pathological changes and died after received a highly virulent CSFV Bangkhen strain. In contrast, all LC vaccinated pigs (No. N2, N4, N7 and V2) induced the increasing of IFN-γ gene which the average value equal to 2.56±0.2910 fold changes at 3 dpc **(Figure 16c, Appendix 7)**. Neither of pigs developed any severe clinical signs, pathological changes or death after challenge with a highly virulent CSFV Bangkhen strain. The result indicated that LC vaccine completely stimulated the increasing of IFN-γ gene that could earlier protect all vaccinated pigs against a challenge. In the same time, 3 out of 4 TC vaccinated pigs, 3 days prior to challenge, induced the increasing of IFN-γ gene which the average value equal to 2.16±0.1224 fold changes at 3 dpc **(Figure 16d, Appendix 7)**. These three pigs did not develop any severe clinical signs or pathological changes and survived after challenge. The result indicated that TC vaccine partially stimulated the increasing of IFN-γ gene that could earlier protect only 3 out of 4 vaccinated pigs against a challenge. The result suggested that the increasing of IFN-γ played a role in protection the vaccinated pigs from severe clinical signs, pathological changes and death after challenge with a highly virulent CSFV. In our study, we found that IFN-γ also enhanced the increasing of antibody to block virus, all of four LC vaccinated pigs and 3 of 4 TC vaccinated pigs that induced the increasing of IFN-γ gene at 3 dpc, also induced the high level of CSFV specific antibodies. This result was corresponded to the previous report that the IFN-γ levels in response to CSFV were detected in addition to the humoral immune response after infection with Cat01 strain [279]. Both T cells (CMIR) and CSFV specific antibodies (HIR) that were generated in all LC vaccinated pigs and the 3 out of 4 TC vaccinated pigs that challenged with a highly virulent CSFV Bangkhen strain could be related to the protection of the progression of clinical signs and pathological changes against a challenge **(Figure 9a, 9b, 10a,10b).** It was concluded that MLV, C-strain cultured in FS-L3 (pig kidney cell lines) namely tissue cultured C-strain (TC) vaccine partially stimulate antibody and IFN-γ that could protect only three out of four vaccinated pigs while MLV, C-strain cultured in rabbits namely lapinised C-strain (LC) vaccine completely stimulated antibody and IFN-γ that could protect all of four vaccinated pigs against a highly virulent CSFV Bangkhen strain.

TGF-β1 is produced by all types of leukocytes including macrophages, dendritic cells, and lymphocytes. TGF-β is important for growth and development, inflammation and repair, and host immunity. TGF-β action is also involved in hepatocyte apoptosis, indicating that “chronic” TGF-β action leads to progressive disease [290]. TGF-β controls immune response stimulated by CD4 + CD25 + regulatory T cell [285]. Following moderate and high virulent CSFV infections, the levels of the antiviral cytokine (IFN-α) and the cytokine coordinating with innate and adaptive immune responses (IL-12) were enhanced, while the anti-inflammatory cytokine (TGF-β1) was reduced [278][286]. In this study, the TGF-β gene expression from the PBMCs of PC pigs, LC vaccinated pigs and TC vaccinated pigs compared with NC pigs were also investigated. We found that the TGF-β genes from both PC pigs (No. D6 and D0) which the average value equal to 3.06±1.97 and 2.90±0.75 fold changes at 1 dpc and 3 dpc, respectively. One out of four TC vaccinated pigs (No. V0) also decreased TGF-β genes which the average value equal to 2.35 fold changes at 3 dpc, these three pigs developed severe clinical signs, pathological changes and died after received a highly virulent CSFV Bangkhen strain. The result showed that the decrease of TGF-β gene affected to the inflammation of various organs and death of pigs post challenged with high virulent CSFV Bangken strain and affected to the replication of viruses in pig sera, which was corresponded to the previous report, TGF-β gene from PBMC cells of pigs decreased at 1 day post inoculation with CSFV HV strain [287] and the decrease of TGF-β gene induced the replication of CSFV [47]. In contrast, all LC vaccinated pigs (No. N2, N4, N7 and V2) did not decrease TGF-β genes and did not develop any severe clinical signs, pathological changes or death after challenged with a highly virulent CSFV Bangkhen strain. The results indicated that LC vaccine could completely protect the decreasing of TGF-β genes resulted all vaccinated pigs did not develop any severe clinical signs, pathological changes or death after challenge. While only 3 out of 4 TC vaccinated pigs did not decrease TGF-β genes and did not develop any severe clinical signs, pathological changes or death after challenged with a highly virulent CSFV Bangkhen strain. The results indicated that TC vaccine could partially protect the decreasing of TGF-β genes resulted only 3 out of 4 vaccinated pigs did not develop any severe clinical signs, pathological changes or death after challenge. In addition to the humoral antibody response, cell-mediated immune responses from PBMCs are a critical component of protection by the immune system against pathogens and adaptation to invasion [130] [288] [289]. Cytokine released from PBMCs play a primary role in the induction of immune responses to infection and the regulation of these responses [290]. IFN-γ is involved mainly in Th1 responses and is key cytokine in viral protection and clearance [289]. Our study showed that LC vaccinated and TC vaccinated elicits immune response that is closer to Th1 than Th2, response to CSFV challenge. This is in agreement with the results of the IgG1 responses in mice, which are known as to be mainly induced by the Th1 immune system [130]. IFN-γ not only reduces viral replication after CB3 infection, but also reduces TGF-β inflammation after the virus has been cleared [292]. IFN-γ inhibits transcription of TGF-β1 by direct transcriptional modification via STAT1 pathway. The absence of IFN-γ and the decrease of TGF-β genes in two PC pigs and one TC-vaccinated pig in our study indicate both IFN-γ and TGF-β cytokine genes play a role in CSFV infection. It was concluded that 3 days prior to challenge with a highly virulent CSFV Bangkhen strain, TC vaccine partially protected the decreasing of leukocyte number, lymphocyte number and TGF-β1gene from PBMC, and the increasing of CSFV-specific antigens in sera of three out of four vaccinated pigs, while LC vaccine completely protected the decreasing of leukocyte number, lymphocyte number and TGF-β1gene from PBMC, and the increasing of CSFV-specific antigens in sera of all four vaccinated pigs. At the same time, TC vaccine partially induced the increasing of CSFV-specific antibodies in sera and IFN-γ from PBMC, while LC vaccine completely induced the increasing of CSFV-specific antibodies in sera and IFN-γ from PBMC. All of these parameters resulted the partially protection of severe clinical signs, pathological changes and death in TC vaccinated pigs, and the completely protection of severe clinical signs, pathological changes and death in LC vaccinated pigs.

CHAPTER VI
CONCLUSION

This is the first report that modified live classical swine fever vaccine, C-strain cultured in FS-L3 (pig kidney cell lines) namely tissue cultured C-strain vaccine partially protects vaccinated pigs, while C-strain cultured in rabbits namely lapinised C-strain vaccine completely protects vaccinated pigs against a highly virulent CSFV Bangkhen strain, studying in commercial breeds as Large White/Landrace/Durox Jersey pigs aged eleven weeks, 3 days prior to challenge with a highly virulent CSFV Bangkhen strain. Pigs vaccinated with tissue cultured C-strain vaccine, 3 days prior to challenge, partially induced CSFV-specific antibodies in sera and IFN-γ gene from PBMC, while pigs vaccinated with lapinsed C-strain vaccine completely induced CSFV-specific antibodies in sera and IFN-γ gene from PBMC. At the same time, pigs vaccinated with tissue cultured C-strain vaccine, 3 days prior to challenge, partially protect the decreasing of leukocytes, lymphocytes and TGF-β gene from PBMC, and the increasing of CSFV-specific antigens in sera, while pigs vaccinated with lapinised C-strain vaccine completely protect the decreasing of leukocytes, lymphocytes and TGF-β gene from PBMC, and the increasing of CSFV-specific antigens in sera, post challenged with a highly virulent CSFV Bangkhen strain.

All of these parameters resulted the partially protection of severe clinical signs, pathological changes and death in tissue cultured C-strain vaccinated pigs, and the completely protection of severe clinical signs, pathological changes and death in lapinised C-strain vaccinated pigs

However, the efficacy test of tissue cultured C-strain vaccine should be further studies for advances. In addition, for animal welfare reasons, tissue cultures or cell lines should be used for swine fever vaccine production instead of rabbits. Therefore, the tissue cultured C-strain vaccine should be developed for the efficiency and used for CSF control and eradication.

However, the efficacy test of both vaccines should be repeated and compared for advances. In addition, for animal welfare reasons, tissue cultures or cell lines should be used for swine fever vaccine production instead of rabbits, the tissue cultured C-strain vaccine or another kinds of vaccine such as subunit vaccine should be developed for the efficacy.

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สำนักหอสมุดกลาง

APPENDIX

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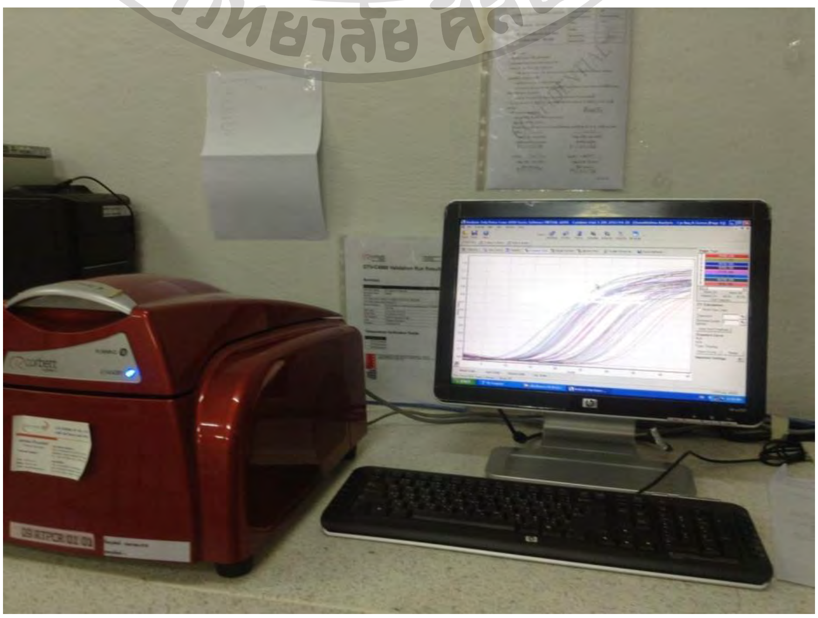
Appendix 1. Measurement of cytokine gene expression by qRT-PCR

(a) IFN-γ and TGF-β cytokine genes from PBMC samples were measured by using Chromo 4 Real-Time System (Bio-Rad)



(b) The amount of cytokine gene amplification was interpreted by using melting curve analysis.

Appendix 2. Body temperature (BT) of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times.

Pig No.	**Body temperature (°C) at day post-inoculation (dpi)**																
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Negative control group																	
N1	38.4	38.6	38.3	38.4	38.4	38.5	38.5	38.6	38.5	38.6	38.4	38.6	38.6	38.5	38.5	38.4	38.
F8	38.7	38.5	38.3	38.6	38.4	38.5	38.4	38.5	38.4	38.5	38.3	38.5	38.5	38.5	38.3	38.2	38.
Mean	38. 5	38.6	38.3	38.5	38.4	38.5	38.4	38.6	38. 5	38.6	38.4	38.6	38.6	38.5	38.4	38.3	38.
SD	0.21	0.07	0.00	0.14	0.00	0.00	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00	0.14	0.14	0.0
Positive control group																	
D6	38.5	38.6	39.6	39.5	39.6	39.7	39.8	40.3	40.8	39.5	39.6	39.7	39.6	dead			
D0	38.5	38.6	39.6	39.8	39.7	39.8	39.8	39.8	39.7	39.3	39.4	39.8	39.5	39.4	39.8	39.5	39.
Mean	38.5	38.6	39.6	39.65	39.7	39.8	39.8	40.1	40.3	39.4	39.5	39.8	39.6	39.4	39.8	39.5	39.
SD	0.00	0.00	0.00	0.21	0.07	0.07	0.00	0.35	0.78	0.14	0.14	0.07	0.07				
Lapinised C-strain vaccinated group																	
N2	38.6	38.6	38.9	39.7	39.6	39.6	39.6	39.6	39.5	39.4	39.3	39.2	39.1	39.0	38.8	38.7	38.
N4	38.5	38.6	38.8	39.3	39.4	39.4	39.4	39.4	39.4	39.4	39.3	39.3	39.2	39.2	39.2	39.2	38.
N7	38.6	38.7	38.8	39.3	39.3	39.4	39.4	39.6	39.5	39.5	39.4	39.4	39.4	39.4	39.4	39.2	38.
V2	38.4	38.6	38.9	39.3	39.3	39.3	39.3	39.7	39.7	39.6	39.6	39.6	39.6	39.5	39.6	39.7	39.
Mean	38.5	38.62	38.9	39.4	39.4	39.5	39.4	39.7	39.5	39.5	39.5	39.4	39.4	39.3	39.3	39.2	38
SD	0.10	0.05	0.06	0.20	0.14	0.13	0.13	0.13	0.13	0.10	0.10	0.14	0.17	0.22	0.22	0.48	0.
Tissue cultured C-strain vaccinated group																	
N5	38.6	38.5	38.8	39.7	39.6	39.7	39.3	39.6	39.8	39.6	39.5	39.4	39.3	39.5	39.5	39.7	39.
N6	38.6	38.6	39	39.1	39.3	39.7	39.4	39.7	39.2	39.4	39.3	39.4	39.4	39.6	39.4	39.4	39.

V1	38.4	38.7	39.5	39.5	39	39.3	39.3	39.5	39.5	39.8	39.8	39.7	39.7	39.6	39.6	39.1	39.2
V0	38.6	38.6	39.1	39.3	39.1	39.2	39.1	40.3	41.0	41.1	40.6	40.0	40.2	39.4	39.6	37.6	37.5
Mean	38.5	38.60	39.1	39.4	39.3	39.5	39.3	39.8	39.9	40.0	39.9	39.6	39.7	39.6	39.5	39.0	38.9
SD	0.10	0.08	0.29	0.26	0.26	0.26	0.13	0.36	0.79	0.77	0.57	0.29	0.40	0.10	0.10	0.93	0.94

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Appendix 3. Leukocytes number of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated time was measured by automated hematology analyzer.

Pig No.	Leukocytes number (X10^3 cells/mm^3) at day post-inoculation (dpi)						
	0	1	3	5	7	14	21
Negative control group							
N1	24.8	24.2	21.8	24.2	25.8	24.2	24.8
F8	20.3	20.7	19.8	20.8	20.8	20.4	19.8
Mean	22.55	22.45	20.8	22.5	23.3	22.3	22.3
SD	3.18	2.47	1.41	2.40	3.54	2.69	3.54
Positive control group							
D6	18.8	17.2	14	12.4	9.1	dead	
D0	27.4	22.8	22	23.2	22.2	9.4	dead
Mean	23.1	20.0	18.0	17.8	15.65	9.4	
SD	6.08	3.96	5.66	7.64	9.26		
Lapinised C-strain vaccinated group							
N2	19.1	17.8	16.5	18.3	17.7	19.9	19.2
N4	22.3	26.2	16.6	17.6	16.9	14.2	14.8

N7	22.7	17.8	14.3	17.4	16.9	18.1	15.5
V2	26.2	17.3	21.2	22.2	21.7	15.7	19.1
Mean	22.58	19.78	17.15	18.88	18.30	16.98	17.15
SD	2.90	4.29	2.90	2.25	2.30	2.53	2.33
Tissue cultured C-strain vaccinated group							
N5	27.4	24.6	22.7	18.5	19.1	14.9	19.8
N6	23.2	21.9	20.4	20	20.1	19	20.2
V1	24.1	23.7	21.5	23.7	23	13.5	13.8
V0	23.2	27.2	20.6	19.2	17.2	9.8	dead
Mean	24.48	24.35	21.30	20.35	19.85	14.30	17.93
SD	2.00	2.21	1.05	2.32	2.42	3.80	3.59

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Appendix 4. Lymphocyte number of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times were measured by automated hematology analyzer.

Pig No.	Lymphocyte number ($X10^3$ cells/mm^3) at day post-inoculation (dpi)						
	0	1	3	5	7	14	21
Negative control group							
N1	15.38	15.38	14.63	15.13	13.89	16.12	17.36
F8	12.99	13.20	11.57	11.57	13.60	12.38	13.20
Mean	14.18	14.29	13.10	13.35	13.74	14.25	15.28
SD	1.69	1.54	2.16	2.52	0.20	2.64	2.95
Positive control group							
D6	13.16	10.34	10.90	4.7	1.88	dead	
D0	18.08	16.44	15.62	16.17	15.34	7.67	dead
Mean	15.62	13.39	13.26	10.43	8.61	7.67	
SD	3.48	4.31	3.33	8.11	9.52		
Lapinised C-strain vaccinated group							
N2	12.80	8.98	11.84	12.42	12.03	12.80	12.99
N4	13.16	9.59	12.04	12.27	12.27	15.39	14.05

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N7	13.39	13.39	11.35	11.35	11.35	14.98	14.98
V2	15.46	11.53	11.53	12.58	17.55	17.55	18.34
Mean	13.70	10.87	11.69	12.15	13.30	15.18	15.09
SD	1.20	2.00	0.31	0.55	2.86	1.95	2.31
Tissue cultured C-strain vaccinated group							
N5	16.44	12.60	14.80	18.36	18.36	17.81	16.44
N6	13.92	11.83	12.06	12.06	14.62	16.47	16.01
V1	15.18	15.18	13.98	13.26	14.70	16.39	16.39
V0	13.22	12.30	12.30	9.74	7.89	6.26	dead
Mean	14.69	12.98	13.28	13.36	13.89	14.23	16.28
SD	1.42	1.50	1.32	3.64	4.37	5.35	0.24

Appendix 5.1 CSFV antigen (SampleA_{450}) in serum of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times were quantified by ELISA.

Pig No.	CSFV antigen (SampleA_{450}) at day post-inoculation (dpi)						
	0	1	3	5	7	14	21
Negative control group							
N1	0.0720	0.0850	0.0815	0.0820	0.0720	0.0690	0.0655
F8	0.0640	0.0780	0.0775	0.0680	0.0635	0.0660	0.0685
Mean	0.0680	0.0815	0.0795	0.0750	0.06775	0.0675	0.0670
SD	0.0057	0.0049	0.0028	0.0099	0.0060	0.0021	0.0021
Positive control group							
D6	0.0730	0.0735	0.0665	0.3305	2.7300	dead	dead
D0	0.0760	0.0750	0.0670	0.0630	0.0680	2.1810	dead
Mean	0.0745	0.0743	0.0668	0.1968	1.3990	2.1810	
SD	0.0021	0.0011	0.0004	0.1892	1.8823		
Lapinised C-strain vaccinated group							
N2	0.0750	0.0800	0.0810	0.0810	0.0790	0.0850	0.0735
N4	0.0850	0.0895	0.0860	0.0895	0.0875	0.0875	0.0875

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N7	0.0865	0.0835	0.0865	0.0845	0.0890	0.0860	0.0910
V2	0.0945	0.1010	0.1040	0.1050	0.0915	0.0925	0.0880
Mean	0.0853	0.0885	0.0894	0.0900	0.0868	0.08775	0.0850
SD	0.0080	0.0092	0.0101	0.0106	0.0054	0.0033	0.0078
Tissue cultured C-strain vaccinated group							
N5	0.0850	0.0890	0.0905	0.0845	0.0705	0.0825	0.0745
N6	0.0895	0.0895	0.0825	0.0715	0.0765	0.0690	0.0785
V1	0.0775	0.0870	0.0790	0.0790	0.0855	0.0775	0.0835
V0	0.0875	0.0775	0.0735	0.3340	2.6880	2.7880	dead
Mean	0.0849	0.0858	0.0814	0.1423	0.7301	0.7543	0.0788
SD	0.0053	0.0056	0.0071	0.1279	1.3053	1.3558	0.0045

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Appendix 5.2 The amount of CSFV antigen (SampleA_{450} - NCA_{450}) in serum of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times were quantified by ELISA.

Pig No.	CSFV antigen (OD_{450}) at day post-inoculation (dpi)						
	0	1	3	5	7	14	21
Negative control group							
N1	0.0000	0.0070	0.0035	0.0040	0.0000	0.0000	0.0000
F8	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Mean	0.0000	0.0035	0.0018	0.0020	0.0000	0.0000	0.0000
SD	0.0000	0.0049	0.0025	0.0028	0.0000	0.0000	0.0000
Positive control group							
D6	0.0000	0.0000	0.0000	0.2525	2.6520	dead	dead
D0	0.0000	0.0000	0.0000	0.0000	0.0000	2.1030	dead
Mean	0.0000	0.0000	0.0000	0.1263	1.3260		
SD	0.0000	0.0000	0.0000	0.1785	1.8752		
Lapinised C-strain vaccinated group							
N2	0.0000	0.0020	0.0030	0.0030	0.0010	0.0070	0.0000
N4	0.0070	0.0115	0.0080	0.0115	0.0095	0.0095	0.0095
N7	0.0085	0.0055	0.0085	0.0065	0.0110	0.0080	0.0130
V2	0.0165	0.0230	0.0260	0.0270	0.0135	0.0145	0.0100

Mean	0.0080	0.0105	0.0114	0.0120	0.0088	0.0098	0.0081
SD	0.0068	0.0092	0.0101	0.0106	0.0054	0.0033	0.0056
Tissue cultured C-strain vaccinated group							
N5	0.0070	0.0110	0.0125	0.0065	0.0000	0.0045	0.0000
N6	0.0115	0.0115	0.0045	0.0000	0.0000	0.0000	0.0005
V1	0.0775	0.0870	0.0790	0.0790	0.0855	0.0775	0.0835
V0	0.0095	0.0000	0.2560	2.6100	2.7100	2.7800	dead
Mean	0.0264	0.0274	0.0880	0.6739	0.6989	0.7155	0.0280
SD	0.0395	0.0437	0.0409	0.0439	0.0494	0.0435	0.0481

Remark: Negative Control Average OD_{450} (NCA_{450}) = 0.078

The result of CSFV antigen quantification was negative when test sample ≤ 0.300

The result of CSFV antigen quantification was negative when test sample > 0.300

Appendix 6.1 Antibody to CSFV ($SampleA_{450}$) in serum of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times were quantified by ELISA.

Pig No.	CSFV antibody ($SampleA_{450}$) at day post-inoculation (dpi)						
	0	1	3	5	7	14	21
Negative control group							
N1	0.8890	0.9190	0.9015	0.8630	0.8675	0.8890	0.8960
F8	0.8500	0.8160	0.8100	0.7800	0.8130	0.7950	0.7775
Mean	0.8695	0.8675	0.8558	0.8215	0.8403	0.842	0.83675
SD	0.0276	0.0728	0.0647	0.05869	0.03854	0.0664	0.0838
Positive control group							
D6	0.8555	0.8435	0.8400	0.8185	0.8115	dead	
D0	0.8430	0.8478	0.8360	0.7935	0.7880	0.4870	dead
Mean	0.8493	0.8457	0.8380	0.8060	0.7998	0.4870	
SD	0.0088	0.0030	0.0028	0.01768	0.0166		
Lapinised C-strain vaccinated group							
N2	0.7867	0.8190	0.7943	0.7883	0.7387	0.2977	0.1637
N4	0.7945	0.8440	0.8195	0.7730	0.7675	0.5145	0.2130

N7	0.7710	0.8435	0.7915	0.7600	0.7435	0.4920	0.3170
V2	0.7955	0.7805	0.7605	0.7540	0.5910	0.4270	0.2860
Mean	0.7869	0.8218	0.7915	0.7688	0.7102	0.4328	0.2449
SD	0.0113	0.0299	0.02417	0.01521	0.0804	0.0974	0.0695
Tissue cultured C-strain vaccinated group							
N5	0.7620	0.7930	0.7535	0.7430	0.7270	0.4175	0.1330
N6	0.7485	0.7940	0.7935	0.7560	0.7500	0.4985	0.2185
V1	0.7785	0.8015	0.7925	0.7555	0.7350	0.2750	0.1135
V0	0.7665	0.7130	0.7121	0.7095	0.6955	0.4851	dead
Mean	0.7639	0.7754	0.7629	0.7410	0.7269	0.4190	0.1550
SD	0.0124	0.0418	0.0387	0.0218	0.0230	0.1024	0.0559

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Appendix 6.2 Percentage of CSFV blocking ELISA in serum of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times were quantified by ELISA.

Pig No.	Percentage of CSFV blocking ELASA at day post-inoculation (dpi)						
	0	1	3	5	7	14	21
Negative control group							
N1	9.10	4.96	6.77	10.76	10.29	8.07	7.34
F8	12.10	15.62	16.24	19.34	15.93	17.79	19.60
Mean	10.60	10.29	11.51	15.05	13.11	12.93	13.47
SD	2.12	7.53	6.69	6.07	3.99	6.88	8.67
Positive control group							
D6	11.53	12.77	14.12	15.36	16.08	dead	
D0	12.82	13.13	13.55	17.94	18.51	49.64	dead
Mean	12.18	12.95	13.83	16.65	17.30		
SD	0.91	0.26	0.40	1.83	1.72		
Lapinised C-strain vaccinated group							
N2	18.65	15.31	17.86	18.48	23.61	69.22	83.08
N4	17.84	12.72	15.25	20.06	20.63	46.80	77.97
N7	20.27	12.77	18.15	21.41	23.11	49.12	67.22
V2	17.74	19.29	21.36	22.03	38.883	55.84	70.42

Mean	18.62	15.02	18.15	20.49	26.56	55.24	74.67
SD	1.17	3.09	2.50	1.57	8.32	10.08	7.19
Tissue cultured C-strain vaccinated group							
N5	21.20	17.99	22.08	23.16	24.82	56.83	86.25
N6	22.60	17.89	17.94	21.82	22.44	48.45	77.40
V1	19.49	17.12	18.05	21.87	23.99	71.56	88.26
V0	20.73	26.27	26.36	26.63	28.08	49.83	dead
Mean	21.01	19.82	21.11	23.37	24.83	56.67	83.97
SD	1.28	4.32	4.00	2.26	2.38	10.59	5.78

Remark: Negative Control Average OD_{450} (NCA_{450}) = 0.967

$$\text{Percentage of CSFV blocking ELISA} = \frac{NCA_{450} - SampleA_{450}}{NCA_{450}} \times 100\ (\%)$$

Percent blocking interpretation;

The result of antibody quantification was negative when the test samples ≤ 30 %.

The result of antibody quantification was positive when the test samples ≥ 40 %

Appendix 7. IFN-γ gene expression from PBMC of pigs after inoculated with 0.85% Normal saline (NS) or CSFV Bangkhen strain at indicated times were measured by qRT-PCR.

Pig No.	IFN-γ gene expression from PBMC at dpi			Fold changes at dpi		
	0	1	3	0	1	3
Negative control group						
N1	27.63	28.94	26.32	1	1.05	0.95
F8	27.91	26.32	26.43	1	0.94	0.95
Mean	27.77	27.63	26.38	1	0.99	0.95
SD	0.20	1.85	0.08	0	0.07	0.00
Positive control group						
D6	27.63	35.56	40.88	1	1.29	1.48
D0	26.43	30.12	48.12	1	1.14	1.82
Mean	27.03	32.84	44.50	1	1.21	1.65
SD	0.85	3.85	5.12	0	0.10	0.24
Lapinised C-strain vaccinated group						
N2	27.43	41.45	60.25	1	1.51	2.20
N4	24.46	44.23	66.23	1	1.81	2.71

N7	24.46	45.65	70.23	1	1.87	2.87
V2	27.43	41.24	68.58	1	1.50	2.50
Mean	25.95	43.14	66.32	1	1.66	2.56
SD	1.71	2.16	4.37	0	0.19	0.29
Tissue cultured C-strain vaccinated group						
N5	27.67	27.89	56.78	1	1.01	2.05
N6	23.65	26.52	54.21	1	1.12	2.29
V1	27.67	27.47	58.96	1	0.99	2.13
V0	23.65	25.26	27.80	1	1.07	1.18
means	25.66	26.79	49.44	1	1.04	1.93
SD	2.84	1.56	22.03	0	0.05	0.68

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Appendix 8 TGF-β gene expression from PBMC of pigs after inoculated with 0.85% normal saline (NS) or CSFV Bangkhen strain at indicated times were measured by qRT-PCR.

Pig No.	TGF-β gene expression from PBMC at dpi			Fold changes at dpi		
	0	1	3	0	1	3
Negative control group						
N1	27.36	26.36	24.91	1	1.04	1.10
F8	26.17	24.77	24.19	1	1.06	1.08
Mean	26.77	25.57	24.55	1	1.05	1.09
SD	0.84	1.12	0.51	0	0.01	0.01
Positive control group						
D6	27.20	6.11	7.93	1	4.45	3.43
D0	26.51	15.93	11.22	1	1.66	2.36
Mean	26.86	11.02	9.58	1	3.06	2.90
SD	0.49	6.94	2.33	0	1.97	0.75
Lapinised C-strain vaccinated group						
N2	28.27	26.63	28.60	1	1.06	0.99
N4	27.25	24.73	27.09	1	1.10	1.01

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N7	27.12	26.91	25.14	1	1.01	1.08
V2	28.27	27.43	27.01	1	1.03	1.05
Mean	27.73	26.43	26.96	1	1.05	1.03
SD	0.63	1.18	1.42	0	0.04	0.04
Tissue cultured C-strain vaccinated group						
N5	27.89	25.52	26.79	1	1.09	1.04
N6	26.32	25.63	26.18	1	1.03	1.01
V1	27.89	25.70	26.77	1	1.09	1.04
V0	26.32	27.93	11.22	1	0.94	2.35
Means	27.11	26.20	22.74	1	1.04	1.36
SD	0.91	1.16	7.69	0	0.07	0.66

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BIOGRAPHY

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PUBLICATION:

1. Jarunee Satra (1983) Studies on properties of Pseudorabies virus and adjuvants for Pseudorabies vaccine. A Thesis for the Degree of Master of Science (Microbiology) Mahidol University.

2. จารุณี สาตรา และคณะ (2526) ระดับภูมิคุ้มกันในสุกรซึ่งฉีดวัคซีนโรคปากและเท้าเปื่อยไทป์โอชนิดน้ำมัน ประมวลเรื่องประชุมวิชาการทางการสัตวแพทย์ ครั้งที่ 10 หน้า 209-218.

3. จารุณี สาตรา และคณะ (2526) การทดลองเบื้องต้นเกี่ยวกับการนำเอาออยล์อิมัลชั่นมาใช้ในการผลิตวัคซีนโรคปากและเท้าเปื่อยสำหรับสุกรประมวลเรื่องการประชุมทางวิชาการปศุสัตว์ ครั้งที่ 2 หน้า 249-259.

4. จารุณี สาตรา และคณะ (2527) วิธีการเพื่อให้ได้ปริมาณไวรัสที่สูงสำหรับการผลิตวัคซีนโรคปากและเท้าเปื่อยไทป์เอเชียวันสุกรชนิดเชื้อตาย ประมวลเรื่องประชุมวิชาการทางสัตวแพทย์ ครั้งที่ 11 หน้า 121-127.จารุณี สาตรา และคณะ (2527) การทดลองผลิตวัคซีนโรคปากและเท้าเปื่อยไทป์เอเชียวันสุกรจากเชื้อท้องที่ ประมวลเรื่องประชุมวิชาการทางสัตวแพทย์ ครั้งที่ 11 หน้า

138-150.

5. จารุณี สาตรา และคณะ (2527) การศึกษาอายุของการเก็บวัคซีนโรคปากและเท้าเปื่อยไทป์เอเซียวันสุกร ประมวลเรื่องประชุมวิชาการทางสัตวแพทย์ ครั้งที่ 11 หน้า 151-158.

6. จารุณี สาตรา (2530) สองวิธีการของเอ็นไซม์ลิงค์อิมมูโนซอบเบ็นท์ เอสเส สำหรับการตรวจโรคออเจสกี้ในสุกร สัตวแพทยสาร ปีที่ 38 เล่มที่ 3 สัตวแพทยสมาคมแห่งประเทศไทย ในพระบรมราชูปถัมภ์ หน้า 29-35.

7. จารุณี สาตรา และคณะ (2533) การศึกษาเชิงเปรียบเทียบวัคซีนป้องกันโรคออเจสกี้ชนิดต่าง ๆ (3) ผลการตรวจสอบวัคซีนป้องกันโรคออเจสกี้เชื้อตายชนิดน้ำมัน 4 ชนิด เวชสารสัตวแพทย์ ปีที่ 20 ฉบับที่ 4 หน้า 29-35.

8. จารุณี สาตรา และสุนีจิต คงทน (2534) การตรวจหาแอนติบอดี้ต่อไกลโคโปรตีนวันของไวรัสโรคออเจสกี้โดยวิธีอีไลซ่า (การใช้ ClinEase-PRV เพื่อตรวจหาแอนติบอดี้ต่อไกลโคโปรตีนวันสำหรับการตรวจแยกทางซีรั่มวิทยาระหว่าง สุกรที่ติดเชื้อ และสุกรที่ฉีดวัคซีนหรือไม่ติดเชื้อ) รวมงานวิจัยการประชุมวิชาการทางสัตวแพทย์ ครั้งที่ 18 หน้า 131-144.

9. จารุณี สาตรา และสุนีจิต คงทน (2536) การศึกษาเชิงเปรียบเทียบวัคซีนป้องกันโรคออเจสกี้ชนิดต่าง ๆ (1) การศึกษาความปลอดภัยของวัคซีนชนิดตัดยีนส์ที่สร้างเอ็นไซม์ ไทมิดีนไคเนส และไกลโคโปรตีนเอ็กซ์กับวัคซีนเชื้อตายสองชนิด สัตวแพทยสาร ปีที่ 44 เล่มที่ 1-2 สัตวแพทยสมาคมแห่งประเทศไทย ในพระบรมราชูปถัมภ์ หน้า 43-52.

10. จารุณี สาตรา และสุนีจิต คงทน (2537) การศึกษาเชิงเปรียบเทียบวัคซีนป้องกันโรคออเจสกี้ชนิดต่าง ๆ (2) การศึกษาเชิงเปรียบเทียบต่อประสิทธิภาพของวัคซีนเชื้อเป็นชนิดตัดยีนส์ที่สร้าง

เอ็นไซม์ไทมิดีนไคเนสและไกลโคโปรตีนเอ็กซ์กับวัคซีนเชื้อตายสองชนิด สัตวแพทยสาร ปีที่ 45 เล่มที่ 1 สัตวแพทยสมาคมแห่งประเทศไทย ในพระบรมราชูปถัมภ์ หน้า 13-21.

11. จารุณี สาตรา และสุนีจิต คงทน(2538) การศึกษาเชิงเปรียบเทียบวัคซีนป้องกันโรคออเจสกี้ ชนิดต่าง ๆ (4) การศึกษาประสิทธิภาพของวัคซีนป้องกันโรคออเจสกี้เชื้อเป็นชนิดต่าง ๆ ประมวลเรื่องการประชุมวิชาการปศุสัตว์ ครั้งที่ 14 หน้า 57-74.

12. จารุณี สาตรา และสุนีจิต คงทน (2538) ความคุ้มโรคแรกเริ่มหลังฉีดวัคซีนอหิวาต์สุกรชนิดต่าง ๆ สัตวแพทยสาร ปีที่ 46 เล่มที่ 4 สัตวแพทยสมาคมแห่งประเทศไทย ในพระบรมราชูปถัมภ์ หน้า 23-29.

13. จารุณี สาตรา (2544) มาตรฐานสำหรับวัคซีนที่ใช้ในอุตสาหกรรมปศุสัตว์ สัตวแพทยสาร ปีที่ 52 เล่มที่ 3 สัตวแพทยสมาคมแห่งประเทศไทย ในพระบรมราชูปถัมภ์ หน้า 61-64.

14. จารุณี สาตรา (๒๕๔๖) โรคออเจสกี้และทิศทางการใช้วัคซีนในประเทศไทย ที่ระลึกวาระเกษียณอายุราชการรองศาสตราจารย์ นายสัตวแพทย์ บุญมี สัญญสุจจารี คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย หน้า ๘๓-๑๐๐.

15. ฐิตวัฒน์ จันทวร, สวนีย์ ตระการรังสี และจารุณี สาตรา (๒๕๔๘) การตรวจสอบวัคซีนที่สงสัยว่าเป็นวัคซีนไข้หวัดสัตว์ปีก ประชุมวิชาการทางสัตวแพทย์และการเลี้ยงสัตว์ ครั้งที่ ๓๑ สัตวแพทยสมาคมแห่งประเทศไทยในพระบรมราชูปถัมภ์ หน้า ๑-๘.

16. จารุณี สาตรา และฐิตวัฒน์ จันทวร (๒๕๔๙) ศึกษาเปรียบเทียบปริมาณไวรัสและการฉีดพิษทับในไก่ของวัคซีนนิวคาสเซิลชนิดเชื้อเป็น ประมวลเรื่องการประชุมวิชาการทางสัตวแพทย์และการเลี้ยงสัตว์ ครั้งที่ ๓๒ ฉลองสิริราชสมบัติครบ ๖๐ ปี สัตวแพทย์รวมใจเทิดไท้องค์ราชัน สัตว

สำนักหอสมุดกลาง มหาวิทยาลัยศิลปากร

แพทยสมาคมแห่งประเทศไทยในพระราชูปถัมภ์ หน้า ๑๙๙-๒๐๗.

17. จารุณี สาตรา, ฐิตวัฒน์ จันทวร, สวนีย์ ตระการรังสี และ Macelo T. Paniago (๒๕๔๙) ศึกษาเปรียบเทียบความปลอดภัย ความคุ้มโรคแรกเริ่ม และระดับความคุ้มโรคของวัคซีนนิวคาสเซิลสเตรนไม่ก่อให้เกิดในลำไส้ ๓ สเตรน ประมวลเรื่องการประชุมวิชาการทางสัตวแพทย์และการเลี้ยงสัตว์ ครั้งที่ ๓๒ ฉลองสิริราชสมบัติครบ ๖๐ ปี สัตวแพทย์รวมใจเทิดไท้องค์ราชัน สัตวแพทยสมาคมแห่งประเทศไทยในพระราชูปถัมภ์ หน้า ๑๘๗-๑๙๗.

18. จารุณี สาตรา และฐิตวัฒน์ จันทวร (๒๕๕๑) ประสิทธิภาพของวัคซีนนิวคาสเซิลชนิดเชื้อเป็น ๓ ชนิด ในลูกไก่ SPF อายุ ๑ วัน วารสารวิชาการ BQCLP e-journal ปีที่ ๑ เล่มที่ ๑ หน้า ๒๒-๓๐.

19. จารุณี สาตรา และสวนีย์ ตระการรังสี (๒๕๕๑) ประสิทธิภาพของวัคซีนรวมชนิดเชื้อตาย ๓ ชนิด ในไก่ SPF ต่อไวรัสโรคนิวคาสเซิล วารสารวิชาการ BQCLP e-Journal ปีที่ ๑ เล่มที่ ๑ หน้า ๓๑-๓๙.

20. J. Satra, T. Chanthaworn, S. Trakarnrungsee, W. Thaopech and A. Chaising. (2008) 15th FAVA Congress. สัตวแพทยสมาคมแห่งประเทศไทยในพระราชูปถัมภ์

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