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DEVELOPMENT OF DNA VACCINE AGAINST PORCINE EPIDEMIC DIARRHEA VIRUS

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โรคพีอีดีเป็นโรคในระบบทางเดินอาหารโรคหนึ่ง ซึ่งก่อให้เกิดความเสียหายอย่างรุนแรงในลูกสุกรดูดนม เป็นเหตุให้เกิดความสูญเสียทางเศรษฐกิจเป็นอย่างมากในหลายประเทศรวมถึงในประเทศไทยเองก็ตาม ในปัจจุบันยังไม่มีวัคซีนพีอีดีตัวใดที่มีประสิทธิภาพเป็นที่น่าพอใจในท้องตลาด สาเหตุหนึ่งอาจเกิดจากความแตกต่างทางพันธุกรรมที่สไปค์ยีนระหว่างไวรัสสองสายพันธุ์ดั้งเดิมและสายพันธุ์อุบัติใหม่ โดยวัตถุประสงค์ในการศึกษาครั้งนี้ เพื่อสำรวจความแตกต่างทางพันธุกรรมที่บริเวณสไปค์ยีนของเชื้อไวรัสพีอีดีที่ระบาดอยู่ในประเทศไทย และเพื่อประเมินประสิทธิภาพของดีเอ็นเอวัคซีนต่อโรคพีอีดีที่พัฒนาขึ้นจากเชื้อไวรัสพีอีดีสายพันธุ์ที่แยกได้ในประเทศไทย ในการศึกษาครั้งนี้ การวิเคราะห์ลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนที่บริเวณสไปค์ยีนของเชื้อไวรัสพีอีดีสายพันธุ์ที่แยกได้ในประเทศไทยร่วมกับสายพันธุ์ที่แยกได้จากประเทศอื่นๆ พบว่า เชื้อไวรัสพีอีดีที่แยกได้ในประเทศไทยจัดอยู่ในสายพันธุ์อุบัติใหม่ ซึ่งลำดับพันธุกรรมที่บริเวณนิวทรัลไลซิงอิพิโทป์มีความแตกต่างกับเชื้อไวรัสสายพันธุ์ที่ใช้ในการผลิตวัคซีน ณ ปัจจุบัน นอกจากนั้นยังพบการเพิ่มและการขาดหายไปของลำดับพันธุกรรมที่บริเวณปลายด้านกรดอะมิโนของสไปค์ยีนอีกด้วย ดีเอ็นเอวัคซีนได้พัฒนาขึ้นโดยการสร้างพลาสมิดดีเอ็นเอที่มีส่วนของสไปค์ยีนแทรกอยู่ โดยการประเมินประสิทธิภาพของวัคซีนได้ดำเนินการในสุกรหย่านมและสุกรสาวอุ้มท้อง ภูมิคุ้มกันสารน้ำและภูมิคุ้มกันแบบพึ่งเซลล์ได้ถูกประเมิน ด้วยวิธีไวรัลนิวทรัลไลเซชั่น อีไลซาและการตรวจหาการเพิ่มจำนวนของลิมโฟไซท์และเซลล์ลิมโฟไซท์ที่ผลิตอินเตอร์เฟียรอนแกมม่า ผลการศึกษาพบว่า การให้ดีเอ็นเอวัคซีนไม่กระตุ้นให้เกิดการสร้างแอนติบอดีต่อเชื้อไวรัสพีอีดี แต่การเพิ่มจำนวนของประชากรลิมโฟไซท์ชนิดซีดี 4 ที่ 7 วันหลังให้วัคซีน รวมถึงจำนวนลิมโฟไซท์ชนิดซีดี 4 และซีดี 8 ที่ผลิตอินเตอร์เฟียรอนแกมม่าที่ 28 วันหลังให้วัคซีนมีการเพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเทียบกับกลุ่มควบคุม ในขณะที่ระดับแอนติบอดีและนิวทรัลไลซิงแอนติบอดีที่จำเพาะต่อเชื้อไวรัสพีอีดีในนมน้ำเหลืองและน้ำนมของแม่สุกรที่ได้รับวัคซีน มีแนวโน้มที่จะสูงกว่าในกลุ่มควบคุม จากการศึกษาครั้งนี้ได้แสดงให้เห็นถึงความเป็นไปได้ในการใช้วัคซีนเป็นตัวเลือกหนึ่งในการวางแผนป้องกันและควบคุมโรคพีอีดีต่อไป

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Porcine epidemic diarrhea (PED) is a devastating enteric disease mainly affected to the sucking piglets which causing the severe economic losses in many countries worldwide including Thailand. To date, there have no satisfied commercial vaccine for PED. The clustering system based on the spike gene demonstrated 2 variant of PEDV, classical and pandemic variant, which the difference of these variants may be affected to the protective immunity between them. The objectives of this study were to investigate the genetic diversity of S gene of PEDV in Thailand and evaluate the efficacy of DNA vaccine which developed from Thai PEDV isolate. In this study, the nucleotide and amino acid sequence of S gene of Thai isolates PEDV were analyzed with isolates from other countries. The results demonstrated Thai PEDV isolates were belong to the pandemic variant which the neutralizing epitope of these isolates were different to the vaccine isolate. In addition, insertion and deletion at N-terminal of S gene were detected in these Thai isolates. To develop the DNA vaccine against PEDV, plasmid DNA was constructed and the vaccine efficacy was evaluated in weaned pigs and gestation gilts. Humoral and cellular immunity were investigated by viral neutralization assay, ELISA, lymphocyte proliferation and detection of IFN-g producing cells. The results demonstrated the absence of PEDV-specific antibody in all groups but the lymphocyte proliferation of $CD4^+$ cell at 7 DPV and the PEDV-specific IFN-g producing cell of $CD4^+$ and $CD8^+$ cells at 28 DPV in vaccinated pigs was higher than control pigs ($p<0.05$). Meanwhile, the PEDV-specific IgA and the neutralizing antibody titer in colostrum and milk of vaccinated sows were relatively higher than control group which might be associated with the booster effect. This study reveal the possibility of the DNA vaccine to be a vaccine candidate which might be used in the control and prevention program.

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

INTRODUCTION

Important and rationale

Porcine epidemic diarrhea (PED), a devastating enteric disease characterized by vomiting, acute watery diarrhea and dehydration in pigs at all ages, is caused by PED virus (PEDV), a RNA virus in the genus *Alphacoronavirus*, the family of *Coronaviridae*. Clinical diseases, however, are significantly more severe in piglets under 7 days of age compared to pigs at other ages in which results in high mortality approaching 100%. In addition to causing enteric problem, PEDV infection can lead to a reduction of reproductive performance especially in primi-parous sows (Olanratmanee et al., 2010). Since the first case of PED reported in the United Kingdom and Belgium in 1976-1978 (Pensaert and de Bouck, 1978; Wood, 1977), the disease has continued to cause severe economic losses in several European countries (Chasey and Cartwright, 1978) and in many Asian countries, including China, Korea and Japan (Gao et al., 2013; Li et al., 2012; Park et al., 2011b; Park et al., 2007; Sato et al., 2011). Additionally, in 2013, PED outbreak was occur in United States, which all the swine herds were naïve herds, and estimated to have killed over 7 million pigs (Cima, 2013, 2014). Until now, PEDV has been reported as the major source of substantial economic losses in most swine producer countries.

In Thailand, first PED outbreak was in a farm located in the southern region of the country in 1995 (Srinuntapunt et al., 1995). At that time, the disease did not spread to other swine-producing areas. However, the re-emergence of PED was occurred again in late 2007 in the highest pig densities area of Thailand (Puranaveja et al., 2009). At this time, the disease has rapidly spread throughout the country resulting in >90% of Thai swine farms being infected by PEDV. At present, PED develops into an endemic stage in which many herds experience recurrent outbreaks of which the frequency varies depending on the control and prevention program of farms.

To control the disease following the outbreaks, planned exposure of all sows in the herd with minced PEDV-infected intestines has been a successful implementation to stop the disease outbreak. The successful prevention of re-current outbreaks, however, is still questionable. Several implementations including oral administration of minced PEDV-infected intestines in gilts prior to introduction and in multiparous sows at pre-farrow have been implemented with varying degree of success. In addition, PEDV vaccination with commercially available intramuscular injection (IM) vaccine was the alternative of the prevention program in Thailand. However, both methods have their disadvantages. In case of planned exposure, side effects of this management protocol including increased percentage of mummified fetuses and risks of contamination by other pathogens including porcine reproductive and respiratory syndrome virus, classical swine fever virus, porcine circovirus and *Salmonella* spp. (Ha et al., 2010; Park et al., 2009). Additionally, this method can possibly spread the virus into other units resulting in the recirculation of the virus within the farm. Meanwhile, vaccinations by available IM vaccines have shown variable degree of success. Several publications have questioned to the efficacy of PEDV vaccine and demonstrated partial protection of vaccine (de Arriba et al., 2002b; Song et al., 2007; Sun et al., 2012). One reason would be the nucleotide difference in spike (S) gene, where neutralizing epitopes are located. There have two variants of PEDV that was classified by the insertion and deletion of nucleotides in S gene. First is the classical variant that was identified since 1970s, which is the variant that were used to develop the existing commercial vaccine, and the pandemic variant which was just outbreak around 2005-2006 that caused the severe watery diarrhea and vomiting even in the vaccinated herds. The differentiation of S gene of PEDV between commercial vaccine, which made from classical variant, and the field isolates, which is the pandemic variant, might be the important cause of vaccine inefficiency (Hao et al., 2014).

Due to the variable degree of success from the commercial vaccine, the planned exposure which call “oral feedback” was used to control the PED outbreak. However, the most important disadvantage of this method is the risk of unexpected pathogens transmission through the infected tissues. In this point, vaccination by the DNA vaccine,

the direct injection of plasmid DNA to produce foreign antigen by using the host cellular machinery to induce a specific immune response, is the new generation of vaccine which provide many advantages. The most important advantages is DNA vaccine can induce both humoral mediated immunity (HMI) and cellular mediated immunity (CMI) that play a role in pathogen protection and also the pathogen clearance after infection. Other advantages of DNA vaccine include simplicity of manufacture, biological stability, rapidly production, cost effectiveness and safety (Dhama et al., 2008). In case of PED, DNA vaccine can be produced using S gene sequence sharing high genetic similarity with isolates responsible for outbreaks. In addition, DNA vaccination can use for disease eradication program because it did not need the virus to circulate in the farm. In addition, simplicity of manufacture of DNA vaccine supports it to compete with the rapid mutation of virus in the field.

In this study, the investigation of genetic diversity of PEDV S gene in Thailand were explored. The results of genetic differences of PEDV S genes were the information that provides a basis of knowledge for the development of an effective vaccine. Furthermore, the PEDV DNA vaccine were constructed and the efficacy of the vaccine were evaluated. The results in this study may be helpful to design the control and prevention program against PEDV for the swine farm in Thailand.

CHAPTER II

LITERATURE REVIEW

Coronaviruses

The viruses in family *Coronaviridae* can cause diseases in variety animals as well as in human. There have two subfamily in family *Coronaviridae* comprise *Coronavirinae* and *Torovirinae.* The morphology of these viruses characterized by the envelope and spherical (*Coronavirinae*) or disc, or rod shape (*Torovirinae*) that surround by a fringe or the club-shape spike projection protrude from the surface of virion that made it similar to the “corona”. Meanwhile, many members of *Coronavirinae* causing the substantial economic losses. However, the economic importance of the viruses in subfamily *Torovirinae* are not clear yet. In subfamily *Coronavirinae,* there have 4 genera that were comprised *Alpha-, Beta-, Gamma- and Delta- Coronavirus* (Fehr and Perlman, 2015). The genus *Alphacoronavirus* contain such as human disease virus HCoV-229E and animal disease virus such as PEDV, TGEV and feline infectious peritonitis virus (FIPV). Genus *Betacoronavirus* contain the important respiratory disease virus in human such as severe acute respiratory Coronavirus (SAR-CoV), Middle East respiratory Coronavirus (MER-CoV) and other disease virus in animal such as mouse hepatitis virus (MHV) and porcine hemagglutinating encephalomyelitis virus (PHEV). The genus *Gammacoronavirus* contain the viruses of birds such as infectious bronchitis virus (IBV) and the genus *Deltacoronavirus* that were isolated from pigs (PDCoV).

The Coronavirus particle contain four main structural proteins consist of spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins. The trimeric S glycoprotein is a class I fusion protein (Bosch et al., 2003) which associated with the attachment to the host receptor (Collins et al., 1982). The M protein is the most abundant structural protein in the virion which is thought to give the virion its shape (Neuman et al., 2011). The E protein, which is found small amount in the virion, is associated with the viral assembly and the release of virus process. This protein is highly divergent in the coronaviruses but

have the common feature (Godet et al., 1992). The N protein is the only protein that present in the nucleocapsid. This protein compose of two domains, N-terminal domain and C-terminal domain, which both of them can bind to the RNA but with the different mechanisms (Chang et al., 2006; Hurst et al., 2009). In addition, in group of *Betacoronavirus,* the hemagglutinin-esterase (HE) is present. This protein, that act as hemagglutinin, by binding to sialic acid and contain acetyl-esterase activity (Klausegger et al., 1999), can enhance the S protein-mediated cell entry and virus spread through the mucosa (Cornelissen et al., 1997).

The life cycle of the coronaviruses start when the virus attach to the host cell. The attachment is initiated by the interaction between the S protein and its receptor. Even though, the receptor binding domains (RBD) of coronavirus are located in S1 region but the site of RBD are vary depending on each viruses (Cheng et al., 2004; Kubo et al., 1994). The specific interaction between the S protein and receptor is the important factor reflect the tissue tropism of each viruses. Many of coronaviruses use the peptidase as the cellular receptor. However, the virus entry can occur even in the absence of enzymatic domain of these protein. Many of *Alphacoronavirus* use the aminopeptidase-N (APN) as their receptor (Delmas et al., 1992; Li et al., 2007; Yeager et al., 1992) while the other coronaviruses such as SARS-CoV and HCoV-NL63 use angiotensin-converting enzyme (ACE) as their receptor (Hofmann et al., 2005; Li et al., 2003). After receptor binding, the viruses are entry to the host cell cytosol by membrane fusion between viruses and host cells. However, before the membrane fusion, S protein is needed to be cleavage for two proposes. First is to separate the RBD from the fusion domain (Belouzard et al., 2009) and the second propose is for exposing the fusion peptide that normally located at S2 portion (Bosch et al., 2003). Then, the exposed fusion peptide will be insert in the membrane resulting in fusion and release of the viral genomic RNA into the cytoplasm. The next step is the translation of replicase gene that encode two open reading frame (ORF), rep1a and rep1b, which express two polyproteins, pp1a and pp1ab. Polyprotein pp1a and pp1ab contain the non-structural protein (nsp) 1-11 and 1-16, respectively, that subsequently cleaved into each nsps (Ziebuhr et al., 2000). Then, many of the nsps are assembly into

the replicase-transcriptase complex (RTC) that create a suitable environment for RNA synthesis, RNA replication and transcription of sub-genomic RNAs. The complex protein serve as the RNA-dependent RNA polymerase which transcribe full-length of complementary RNA. During maturation and assembly of the virion, the S protein, which is need glycosylation for the proper folding of the protein, is glycosylated in the rough endoplasmic reticulum (RER). In this place, S protein is interact with M and E protein before migrate into the endoplasmic reticulum—Golgi intermediate compartment (ERGIC) which is the virus assembly site (Krijnse-Locker et al., 1994). Then, N protein is transported to the virus assembly site and forming into the mature virion (de Haan and Rottier, 2005). After virus assembly, virions are transport in vesicle to the cell surface and be released by the exocytosis. In some coronaviruses, the remaining S protein that does not assembly into the virion can lead to the formation of multinucleated cells by mediate the cell-cell fusion between the infected cell and the uninfected cell nearby. This method is allow the virus to escape the detectable and neutralization of specific antibody and leading to the spread of the virus throughout the infected organ.

First replication site of most of coronaviruses are the epithelial cell of respiratory and enteric tract. The reason is the virion of coronaviruses, which is enveloped, are less stable in the environment when compare to the non-enveloped viruses. However, some of coronaviruses, such as SAR-CoV, is surprisingly stable in environment. In addition, some of coronaviruses, such as TGEV and PEDV, can infect to the enteric tract which is the environment that presence of the proteolytic enzyme. It is not clear how the coronaviruses can persist in the enteric tract but the glycosylated of the glycoprotein might increase the proteolytic resistant of the virion.

To date, there have no specifically therapeutics for coronaviruses. The treatment nowadays is only the supportive treatment which support the self-recovery from the host immune system. However, *in vitro* study indicate that interferons (IFNs), which is the important cytokine that activate the anti-viral stage, are partially effective to coronaviruses (Cinatl et al., 2003). To counter the limited option of coronaviruses therapy, vaccination is the most suitable method to prevent the coronaviruses infection in theory. There have

some commercial vaccines against coronaviruses, such as IBV, TGEV, PEDV and Canine CoV, have been approved nowadays. However, theses vaccine are not always used because the doubtful of vaccine efficacy. In addition, some vaccines are live attenuated vaccine which might be induce the evolution rate of coronaviruses via the recombination of vaccine stain and the circulating strains. In this point, there have many challenges in the development of the vaccine against coronaviruses (Saif, 2004). Many times in mucosal infections by coronaviruses, the primary natural infection does not prevent the following secondary infection. This indicate the immunity induced by the original viruses might not be high enough to prevent the secondary expose leading to re-infection of corona virus. Therefore, the developed vaccine should induce higher immunity than the original virus or at least reduce the clinical outcome during the secondary re-infection. In addition, the developed vaccine should not render to increase the mutation and evolution rate of the viruses via the recombination of the existing viruses strains in the field and the newly introducing vaccine strain at some point of time (Wang et al., 1993).

Porcine epidemic diarrhea

Porcine epidemic diarrhea (PED) is a contagious enteric disease characterized by vomiting and acute severe diarrhea. Pigs at all ages are susceptible to infection and display similar clinical disease. However, the degree of clinical severity, in term of mortality rate, is related to age of infected pigs. In pigs which younger than 3 weeks of age especially lower than 7 days of age, the morbidity rate can reach to 100% and mortality rate is around 50-100% (Stevenson et al., 2013). In the other hand, clinical signs of pig older than 3-weeks-old is milder compared to younger age and clinical diseases disappear within 5-10 day following infection (Madson et al., 2014). Factor that might influence the disease severity of nursing pigs is the faster turnover rate of enterocytes of suckling pigs which is approximately 5-7 days compared to 2-3 days in that of weaned pigs (Jung and Saif, 2015). Clinical diseases of PED is similar to other enteric diseases caused by other viruses in subfamily *Coronavirinae* including TGEV and PDCoV (Jung et al., 2015). Even though, the emergence of porcine respiratory coronavirus (PRCV)

reduced the prevalence of TGEV outbreak due to cross-protective immunity (Saif et al., 2012). However, there have no cross-protection activity between TGEV and PEDV (Hofmann and Wyler, 1989)

PED is caused by PED virus (PEDV), an enveloped single-stranded positive-sense RNA virus belonging to the genus *Alphacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae* and order *Nidovirales*. A distinctive morphology of PEDV is similar to the other viruses in subfamily *Coronavirinae*. The PEDV genome is approximately 28 kb in size and composed of 8 open reading frames (ORF) (Kocherhans et al., 2001). Four ORFs encode non-structural proteins consist of ORF1a and ORF1b, cover 70% of the entire genome, encode the viral polymerase and ORF3a and ORF3b encode the non-structural protein might be associated with the viral pathogenicity but the function is unclear (Sun et al., 2014). The remaining ORFs encode spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N), the major structural proteins, respectively. Among the PEDV proteins, the S protein is a glycosylated protein involve with viral pathogenesis. The S protein attaches to the host cellular receptors resulting to virus entry by membrane fusion (Bosch et al., 2003). Additionally, important role of S protein is including the induction of neutralizing antibody and the neutralizing epitopes were reported (Chang et al., 2002; Cruz et al., 2006; Sun et al., 2008). From these reasons, this gene is important for understanding the genetic relatedness of PEDV field isolates, the epidemiological status of the virus and vaccine development. Many researchers focus in the genetic diversity of S gene (Chen et al., 2014; Li et al., 2012; Park et al., 2011b; Sun et al., 2012).

Epidemiology of PEDV start in the early 1970s, the first PEDV which is named CV-777, the prototype of PEDV which belong to the “classical variant” PEDV nowadays, was isolated in Belgium by Pensaert and de Bouck (1978). Although the disease was spread and persist to an endemic form in some regions of Europe, the prevalence of PEDV were still not high (Carvajal et al., 1995). Some country reported the PED outbreak, such as Netherland (Pijpers et al., 1993), Hungary (Nagy et al., 1996) and England (Pritchard et al., 1999). The reason why the transmission rate of PEDV outbreak was not high still

unclear. However, PEDV causing a sever outbreak in Asia, especially in Japan and South Korea, in the 1980s - 1990s (Kweon et al., 1993; Takahashi et al., 1983). Extensively used of live-attenuated vaccine for the control and prevention program in many countries in Asia reduced the economic loss even though the disease was turn into endemic stage. In Thailand, the severe outbreaks start at late 2007 and the Chinese-like PEDV was reported (Puranaveja et al., 2009). However, In China in 2010-2012, many pig herds have been vaccinated with the CV-777 or CV-777 related vaccines were occurs severe outbreaks by another “new-variant” PEDV, which is named as “pandemic variant” PEDV nowaday. The similarity of nucleotides in S gene this variant and prototype strain was 93% (Li et al., 2012). In 2013, the naïve pig herd in the United States of America was reported the severe PEDV outbreak and closely genetic related with the China/2012/AH2012, Chinese PEDV reported in 2011-2012 (Chen et al., 2014; Huang et al., 2013). However, the nucleotides of S gene of US PEDV isolates exhibited 89-92% similarity with CV-777 (Chen et al., 2014).One year after first outbreak, PEDV was rapidly spread throughout the country and also spread into another country in North America, such as Canada and Mexico (Ojkic et al., 2015). At this present, the US-like PEDVs were also reported as the causative agent of diarrheic piglets in South Korea and Taiwan (Cho et al., 2014; Lin et al., 2014). However, in 2014, a less pathogenic PEDV strain, which the genetic characterization indicate that this strain was similar to the classical variant, was reported in the US. However, the wording that use for call the variant in US is different from other, especially in Asia. In US, the US prototype that first discovered, which the genetic character is similar to the pandemic-variant, is been called the “non S-INDEL” PEDV while the less-pathogenic PEDV, that has been found later in US, is been called “S-INDEL” PEDV.

Even though the aerosolized PEDV is still infectious but the fecal-oral route is the main route of PEDV transmission (Alonso et al., 2014). The PEDV contaminated feces, vomitus and other contaminated fomites, such as feed (Dee et al., 2014) and transport trailer (Lowe et al., 2014), can be the major source of transmission virus. In addition, the RNA of PEDV can be detected in the acute serum of infected pigs (Jung et al., 2014), which the pork plasma can be used as the feed additive. However, the infectivity of RNA

of PEDV contaminated feed is still questionable. In the farrowing unit, previous study demonstrated the RNA of PEDV can be detected in milk samples of PEDV-infected lactating sows (Li et al., 2012; Sun et al., 2012). This indicated that the vertical transmission via the PEDV contaminated milk might be the potential route of transmission. In the PEDV-positive farm, the grower and finisher pigs with a clinical or subclinical infection can be a reservoir of PEDV by shedding the virus through the subclinical diarrhea (Pijpers et al., 1993).

The incubation period of PEDV is approximately 2 days and the clinical outcome can be observed for 3-4 weeks after inoculation (Debouck and Pensaert, 1980; Puranaveja et al., 2009). The PEDV shedding in fecal sample can be detected within 48 hour after inoculation and can be detected up to 4 weeks. The gross lesions of PED are limited in only the gastrointestinal tract which characterized by the thin and transparency of intestinal walls with the accumulation of yellowish fluids in intestinal lumen (Jung et al., 2014; Lee et al., 2015; Puranaveja et al., 2009; Stevenson et al., 2013; Sueyoshi et al., 1995). In the sucking piglets from the sufficient lactation performance sows, distended stomach which filled by the undigested milk curd and the lack of lacteal duct are the common lesions that have been seen. These lesions indicated the malabsorption problem in effected piglets. In addition, the congestion of mesenteric vessels and swelling of mesenteric lymph node are also the lesions that frequently seen in PED clinical cases (Puranaveja et al., 2009). The histological lesion of PEDV infection characterized by severe acute diffuse atrophic enteritis, mild vacuolation of enterocytes with the subepithelial edema in caecum and colon (Jung et al., 2014; Sueyoshi et al., 1995). The necrosis of enterocytes causing the sloughing of epithelium and the atrophied villi are frequently fused and covered by the degenerated or regenerated flattened epithelium. These resulting to the reduced of intestinal villi length which the villous height to crypt depth rations can be lesser than 3:1 in the PEDV infected piglets.

The pathogenesis of PEDV is similar to other coronaviruses, which the viruses need the interaction between the binding domain of virus and the virus-specific receptor in the host cell for the virus entry. In PEDV, the tissue tropism of PEDV is small intestine

and the APN, which expressed a large amount in enterocytes of small intestine, was reported as the cell receptor for PEDV (Li et al., 2007). However, recently study indicates that the PEDV can infect the swine testis cell even though the APN receptor was knock out (Li et al., 2017). Therefore, the specific cell receptor for PEDV infection was still unclear. In case of TGEV, porcine respiratory coronavirus (PRCoV), which hypothesized as the mutant of TGEV, can infect to the lower respiratory tract. In contrast, the PEDV antigen cannot be detected in the lung tissue samples even in the oronasally inoculated pigs (Jung et al., 2014; Stevenson et al., 2013; Sueyoshi et al., 1995). This indicated the PEDV have no infectivity in the lower respiratory tract. After the virus entry to the enterocyte via the membrane fusion, the replication of PEDV causing acute necrosis of enterocytes and leading to the villous atrophy especially in small intestine part (Jung et al., 2014). However, previous studies demonstrated the PEDV antigen can be observed in villous enterocytes even in small intestine, from duodenum to ileum, and large intestine, but not in the rectum part (Jung et al., 2014; Madson et al., 2014). Due to the massive loss of absorptive enterocytes and the disorder function of enterocytes, leading to the malabsorption diarrhea. Previous study demonstrated PEDV infection causing the loss of electron density of the cellular cytoplasm and the degeneration of mitochondria leading to the lack of transport energy that need for absorption (Ducatelle et al., 1982). Furthermore, the ultrastructural changes and vacuolation of PEDV-infected colonic epithelial cells may interfere the water and electrolytes reabsorption (Ducatelle et al., 1982). The pathophysiology study demonstrated PEDV infection lead to the metabolic acidosis with hypernatremia, hyperkalemia, hyperchloremia and the low level of bicarbonate. In addition, the impaired gut integrity, by irregular distribution and decreased expression of tight junction protein, might lead to the leak of water into the intestinal lumen and high osmotic pressure in intestinal lumen (Jung and Saif, 2015). The severe watery diarrhea with, in some case, vomiting causing by PEDV infection resulting to severe dehydration which is the cause of death. However, the mechanism why the PEDV induce the vomiting in PEDV infected pigs is still unclear.

After the PEDV infection, mucosal and systemic immune responses were produce against the evade pathogens. At the site of infection, which is small intestine, the mucosal immune system of the host rapidly interact with the PEDV infection by the infiltration of innate immune cells into the lamina propria. The detection of these lymphocytes and mononuclear cells in small intestine were rapidly occur within 2-5 days post infection (DPI) (Jung and Saif, 2015; Sueyoshi et al., 1995). However, in sucking piglets which younger than 10 day-old, the frequency and the functionality of these cells were impaired when compare to the immunocompetent pigs (Annamalai et al., 2015). In the same time, the specific immunity against PEDV was develop. Previous study demonstrated the induction of cellular immunity was occur within 4 DPI which the proliferation of PEDV-specific lymphocytes was detected in mesenteric lymph node (de Arriba et al., 2002a) while the humoral immunity against PEDV infection require more time for development. These PED-specific lymphocyte proliferation was maintain up to 21 DPI, which was the challenge day, and the lymphocyte proliferation was correlated with the protection against the homologous challenge. Similar to mucosal CMI, the development of PEDV-specific HMI also detected within 4 DPI. The PEDV-specific IgM antibody secreting cells (ASC) were firstly detected in duodenum, ileum, mesenteric lymph node and spleen at 4 day post infection while the PEDV-specific IgA and IgG ASC in were found later at 7-12 DPI (de Arriba et al., 2002b). However, the PEDV-specific IgM was decline after 7 DPI which a small amount of PEDV-specific IgM ASC was detected in all tissues at 12 DPI. The information from the study of PEDV-specific ASC correlate with the present of mucosal antibodies which the IgA and IgG in oral fluids can be detected at 13 DPI and the titer of these antibodies can persist up to 93 days (Bjustrom-Kraft et al., 2016). In experimental infection, the production of PEDV-specific IgG in serum were detected within 10-14 DPI while the low level of neutralizing antibody can be detected at 7 DPI (Chen et al., 2016; Madson et al., 2014; Thomas et al., 2015). The field investigation in fattening pigs indicated the seroconversion can occur within 7 day after the first clinical sign was observed and the persistent of PEDV-specific IgG can be detected up to 63 days. Another study revealed the titer of PEDV-specific antibodies in PEDV exposed sows can prolong

up to six months. However, the number of ASCs in sows which exposed to the PEDV for 6 months indicated the decline of ASCs when compare to the sows which exposed for 1 month (Ouyang et al., 2015). Due to the infection via the oral route, the induction of antibody isotype was tend to be the IgA which the population of PEDV-specific IgA ASCs was higher than the PEDV-specific IgG even in ileum, mesenteric lymph node and spleen (Ouyang et al., 2015). However, the production of serum IgA was later than IgG which the first detection of serum IgA was present in 12 DPI and the titer of serum IgA was less than serum IgG (Bjustrom-Kraft et al., 2016; de Arriba et al., 2002b).

To diagnosis the PED, there have some other enteric viruses that indistinguishable by the clinically and pathologically outcome especially TGEV and PDCoV. To confirm the pathogen causing the disease, detection of the PEDV or its antigen in the clinical samples must be the best way to answer the etiology. Immunofluorescence (IF) assay, immunohistochemistry (IHC) test, *in situ* hybridization, electron microscope, virus isolation, enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase polymerase reaction (RT-PCR) technique are the list of diagnostic test that can use to identify the pathogen. However, due to the rapid response result and the high sensitivity of even the conventional or real-time RT-PCR made this methods are the most widely used method. In addition, further nucleotide sequencing analysis, which may be perform following the RT-PCR test, can give the useful information about genotype of PEDV outbreak in the farm and also the genetic diversity among the PEDV isolates. To identify the PEDV exposure in the herd, detection of PEDV-specific antibodies by immunofluorescence antibody (IFA) test, ELISA and viral neutralization (VN) test are the method have been used. Due to the passive immunity to the neonatal piglets is the most important strategy that reduced the severity of the clinical loss, the PEDV-specific neutralizing antibodies or the antibodies against S protein of PEDV in colostrum and milk are the major source of passive immunity that need to be monitor. Even though, the VN test measure the protective titer against PEDV but the time-consuming and the inability of the VN test to identify the isotype of the antibodies, which the secretory immunoglobulin A (IgA) is represent to the mucosal immunity, are the limitation of this method. In contrast,

indirect ELISA, using entire S protein or its neutralizing portion of S protein (Sun et al., 2007) as the virus antigen, could be used for antibodies detection test which the specificity is equal, less time-consuming and easier to perform.

The most important method to prevention and control the PED outbreak is strict biosecurity. The principle of biosecurity is to block any possibility of the introducing of pathogen into the farm. Many factors can introduce the virus into the herd especially by the person and fomites which can be traffic from unit to unit. The restricting human traffic between unit and limiting the contact between the external person and internal person during the loading and unloading process might be help to prevent the PEDV introduction and the spread of PEDV, which may be outbreak in the farm, to the neighbor (Lowe et al., 2014). In addition, all newly arrive or replacement animals need the suitable quarantine period to monitor the health status before introduced into herd. However, sometime the PEDV can entry to the herd by accidentally failure of the restrict biosecurity. To assure that the herd will not affected if the PEDV is outbreak in the farm, the immunization is the promising preventive and control strategy that has been used to reassure. The protection of the new-born piglets, which is the most susceptible age for PEDV, need the passive immunity against PEDV from colostrum and milk (Shibata et al., 2001). In colostrum, proportion of IgG was more than 60% of immunoglobulin content. However, the ratio between IgG and IgA was reduced day by day result in high proportion of IgA in milk. Beside, because of more resistant ability to proteolytic degradation in gastrointestinal tract and higher neutralizing ability to the virus, when compare to IgG and IgM, result more effective of IgA to neutralize oral infectious pathogens. Therefore, the target of immunization is to induce the PEDV-specific antibodies, especially the secretory IgA, titer to the protective level.

There have two immunization method which widely used in PED epidemic and endemic area. First is the vaccination which there have several vaccines that have been developed and commercialized, especially in Asian countries, including live attenuated vaccine and inactivated vaccine (Song and Park, 2012). Live attenuated vaccine was achieve by the high cell culture passages (Sato et al., 2011). The example of live

attenuated vaccines are SM98-1, which has been use as an intramuscular (IM) live attenuated or killed vaccine, and DR13, which was developed as oral live attenuated vaccine, from South Korea and 83P-5, which has been use as IM live attenuated vaccine, from Japan (Kweon et al., 1999; Sato et al., 2011; Song et al., 2007). Even though, these vaccines has been demonstrated the protection in the experimental conditions. However, the efficacy and safety of these vaccines in the field were still debated. In China 2007, although the pregnant sows were vaccinated, the protection was still not complete in nursing piglets (Song et al., 2007). In 2013, the genetically divergence of recent Korean field isolates and four vaccine strains available in the market was reported (Park et al., 2013). This reason may contribute to the reduction of vaccine efficacy. Other immunization method for control the PEDV is the planned exposure which call “feedback”. This method is the intentional exposure of pregnant sows, with the autogenous PEDV from the slurry or the minced intestine of PEDV infected piglets, which usually used in acute PED outbreak situation. The advantages of this method is the rapid inducing lactogenic immunity which, hopefully, shorten the outbreak on the farm. However, there have several complications that might be occurs when this method has been use on the farm. Due to the other pathogen, such as porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2), can be contaminated in the minced intestines or slurry (Jung et al., 2006; Park et al., 2009), this might be lead to the wide spread of unexpected pathogen throughout the herd. In addition, the infectious virus from the artificial exposed sow by this method can be shed in the feces which can be transmitted to the other unit or the other farms if the strictness of biosecurity is not good enough. Furthermore, the inconsistence of the PEDV infectious titer between the batches of feedback is questionable. This might lead to the inconsistence of herd immunity which the piglets from the insufficient infectious titer exposed sows can be the susceptible population for the PEDV repeat outbreak in the herd. However, the effectiveness of the immunization methods is depend on the passive lactogenic immunity that sows give to neonatal piglets. The most important factor that effect to the protective level in piglets is the adequate

amount of colostrum and milk that the piglets receive. Therefore, the sanitation and the health condition of lactating sows also be the important factor that need to be monitor.

In the herd which is in endemic stage, the morbidity and mortality rate of PEDV in nursing pigs is lower when compare to negative herd due to the passive immunity from PEDV-exposed sows. However, the economic loss was mainly occurs in weaned pig, which is the susceptible population for PEDV due to the reduction of passive immunity (Pijpers et al., 1993), and the PEDV can persist leading to recirculation of PEDV in the farm. In this case, the active immunity of nursing and grower pigs in the farm may be necessary to control the endemic PEDV infection, (Saif et al., 2012).

DNA vaccine

In the present day, five platforms of vaccine can be classified comprise live attenuated, inactivated or killed, subunit, toxoid and genetic based vaccines (Ada, 2005). Each platforms have pros and cons which is needed to be determined. Live attenuated vaccine can infect and multiply in host cells which providing the continuous antigenic stimulation. Due to the infection activity, this vaccine can elicit both the cellular and humoral mediated immunity by the endogenous antigen, in the infected cell, and exogenous antigen, by the phagocytosis, respectively. However, live attenuated vaccine still have a risk to return to the virulent strain. In addition, the using of live attenuated of RNA virus, which is lack the proofreading activity when replicate, might be enhance the genetic variation by increase the mutation rate and the genetic recombination. In contrast, inactivated vaccine, which a virulent pathogen was killed by chemicals or radiation, served the safety benefit because this vaccine has no risk to return to virulence and cannot affected to the mutation rate. However, this vaccine platform is elicit only the humoral immunity and require multiple booster for maintain the protection. Similar to inactivated vaccine, subunit vaccine, which is the vaccine develop from the purified antigenic protein of interested pathogen, have the same pros and cons with inactivated vaccine but this platform is easier to produce in large scale than the inactivated vaccine. Meanwhile, the toxoid vaccine is the vaccine which develop from the bacterial toxins and

induce the humoral immunity against these virulent factor. However, this vaccine also need the multiple boosters and the adverse effect may be seen in this vaccine platform. In contrast, the genetic base vaccine such as DNA vaccines have the same advantages as live attenuated vaccine which the DNA vaccine can elicit both of cellular and humoral mediated immunity (Ingolotti et al., 2010). In addition, DNA vaccine served the safety benefit and the ease to produce as same as inactivated and subunit vaccine, respectively. Furthermore, due to the stability of DNA in room temperature, this vaccine platform have more stability benefit when compare to other vaccine platforms which need the cold-chain storage.

The DNA vaccine is the administered of plasmid DNA, which contain gene of interest under the control of eukaryotic promoter, to activate protein expression that leading to the induction of immune response. The structure of DNA vaccine consist of two main part, the plasmid backbone and the transcriptional unit (Ingolotti et al., 2010). The plasmid backbone contain the genes that necessary for plasmid amplification, by the origin of replication gene, and selection by antibiotic resistance gene. Due to the plasmid amplification is usually perform in bacterial system, the pUC or pBR322, which is the *E.coli* origin of replication, is the widely use in commercially plasmid vector. Meanwhile, the antibiotic resistance gene against kanamycin and ampicillin are the mainly genes have been used in plasmid vector (Ingolotti et al., 2010). The transcriptional unit compose of promoter, gene of interest and the polyadenylation sequence (poly A tail). The viral promoters, such as cytomegalovirus (CMV), Rous sarcoma virus (RSV) and Simian virus (SV) 40, are the promoter that have been used for drive the expression of interested gene in mammalian cells. The most commonly used is the CMV promoter which is known as the strongest promoter. For the gene of interest, one of the advantages of DNA vaccine is the possibility to encode the multiple interested protein into the same construct. In addition, not only the multiple interested protein can be added, but the adjuvant sequence can be added in the same construct to enhance the vaccine potency. However, the most crucial factors for the vaccine success are the selection of immunogenic antigen for incorporate the construct and the optimization of codon usage for the best suitable

expression of interest protein. Last part of the DNA vaccine structure is the poly A tail which this sequence play an important role in protein expression by enhance the mRNA stability leading to the enhance of mRNA translation (Lutz, 2008).

From the slow rise of immunity after DNA vaccination suggests that its need a complex pathway to inducing immunity. After DNA vaccine is administered to the skin, subcutaneous or muscle, plasmid DNA is taken up by the host cell and translocate into nucleus to transcript the interested protein antigen, from the gene of interest, by using the host cellular machinery. Stromal cells, such as myocytes and epithelial cells, and antigen-presenting cells (APCs), such as dendritic cell (DC), at the administered site are the major cell types of DNA vaccine up taken (Ingolotti et al., 2010). In the general understanding, there have two route of expression that can explain the mechanism of DNA vaccine (Desmet and Ishii, 2012). First is the direct route, which the plasmid DNA is directly transfected into the stromal cell or APCs. In these cells, the endogenous expressed protein can be processed and presented by binding to MHC class I to the immune cell, especially the $CD8^+$ T cells. The other mechanism is the indirect route, which the plasmid DNA is transfected into stromal cells and the expressed proteins in the host cells have been secreted around the tissue by active secretion or the releasing after cell apoptosis. These secreted proteins can be processed to activate B cells for antibody production by themselves or can be engulfed to be presented via MHC class II by APCs. The antigen-loaded APCs can migrate to the draining lymph node to activate naïve T cell and activate B cell by the working of T cell's secreted cytokine with the shed antigen (Kutzler and Weiner, 2008). Additional, when the activated T and B cells migrate back to DNA vaccine injection site, cytotoxic T cell or CTL can lyse the myocytes presenting antigen on their MHC class I resulting to the repeat stimulation of immunity (Reyes-Sandoval and Ertl, 2001). Furthermore, the plasmid DNA itself can induce the type I interferon (IFN) production and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation via the activation of TRAF-family-member-associated NF-kB activator (TANK) binding kinase 1 (TBK1) and IkB kinase-**ε** (IKK**ε**) through stimulator of interferon gene (STING) resulting activates interferon regulatory factor 3 (IRF) and IRF 7 to stimulate type

I IFN production and NF-kB activation (Barber, 2011; Ishii et al., 2008; Ishikawa et al., 2009). The type I IFN play an important role for promoting the cross-presentation activity of DCs, differentiation of T helper 1 (T_H1) and promotion of T_H1-type isotype switching in B cells (Desmet and Ishii, 2012). However, the cytosolic DNA receptor which interact to the plasmid DNA is still unidentified. Even though the unmethylated cytosine-phosphate-guanine (CpG) motifs in foreign DNA can bind to Toll-like receptor 9 (TLR9) in the endosome and stimulate the production of type I IFN in APCs but the study in TLR9-deficient mice demonstrated this signaling molecule had minimal involvement to the immunogenicity in case of DNA vaccine (Ishii et al., 2006; Ishii et al., 2008; Rottembourg et al., 2010).

There have many potential DNA censor (Coban et al., 2013) that have been reported including DNA-dependent activator of IFN-regulatory factors (DAI), absence in melanoma 2 (AIM2), tripartite-motif 56 (TRIM56), RIG-I like receptor (RLRs), DDX41 and cyclic GMP-AMP synthase (cGAS). However, only some DNA sensors that might be affected to the production of type I IFN in case of DNA vaccine. Due to the most of DNA sensors which associated with the DNA vaccine mechanism are related to STING pathway (Barber, 2011), some of DNA sensors are reviewed in this thesis. TRIM56 is an interferon-inducible E3 ubiquitin ligase which was identified as a modulator for STING pathway (Tsuchida et al., 2010). However, the effect of TRIM56 to the induction of immunity by the DNA vaccine is not clearly identified. RLRs are known as the double stranded RNA (dsRNA) receptor that can induce type I IFN production via the modulation of STING pathway. However, with the activity of RNA polymerase III, which responsible for DNA transcription and synthesizes ribosomal and small RNAs in eukaryotes, it has been demonstrated that the cytosolic poly (dA-dT) DNA are converted into RNA and induce RIG-I-mediated type I IFN production. Moreover, the study in RIG-I gene knockdown demonstrated the reduction of dsDNA-induced type I IFN production (Chiu et al., 2009). DDX41, a member of DEXDc family of helicase, can sense the cytosolic DNA and DNA viruses leading to type I IFN and cytokines production (Parvatiyar et al., 2012). However, the interaction between this DNA sensor and STING is need further elucidate. cGAS is a

cyclic guanosine monophosphate-adenosine monophosphate (cGMP) synthase which directly bind to transfected dsDNA resulting to the synthesized cGMP which directly bind to STING. Subsequently, the protein kinase TBK1 and transcription factor IRF3 are phosphorylated and translocated into nucleus, where it activate the transcription of type I IFN and NF-kB (Shu et al., 2014; Sun et al., 2013). However, the exactly DNA sensor which responsible for cytosolic DNA sensing, in case of DNA vaccine, is need further elucidate.

CHAPTER III

HYPOTHESES, OBJECTIVES AND CONCEPTUAL FRAMEWORK

Research problem

1. Does the genetic difference between vaccine and field isolates play an important role in vaccine efficacy?
2. What are the genetic characteristics of PEDV outbreak in Thailand?
3. Can we develop the effective PEDV vaccine from PEDV local isolate?

Objectives of Study

1. To characterize the genetic variation of PEDV outbreak in Thailand.
2. To develop the DNA vaccine from the field isolate in Thailand to induce pig immunity against PEDV.

Keywords (Thai):

ดีเอ็นเอวัคซีน นิวทรัลไลซิ่งอิพิโทป์ ไวรัสพีอีดี ยีนสไปค์

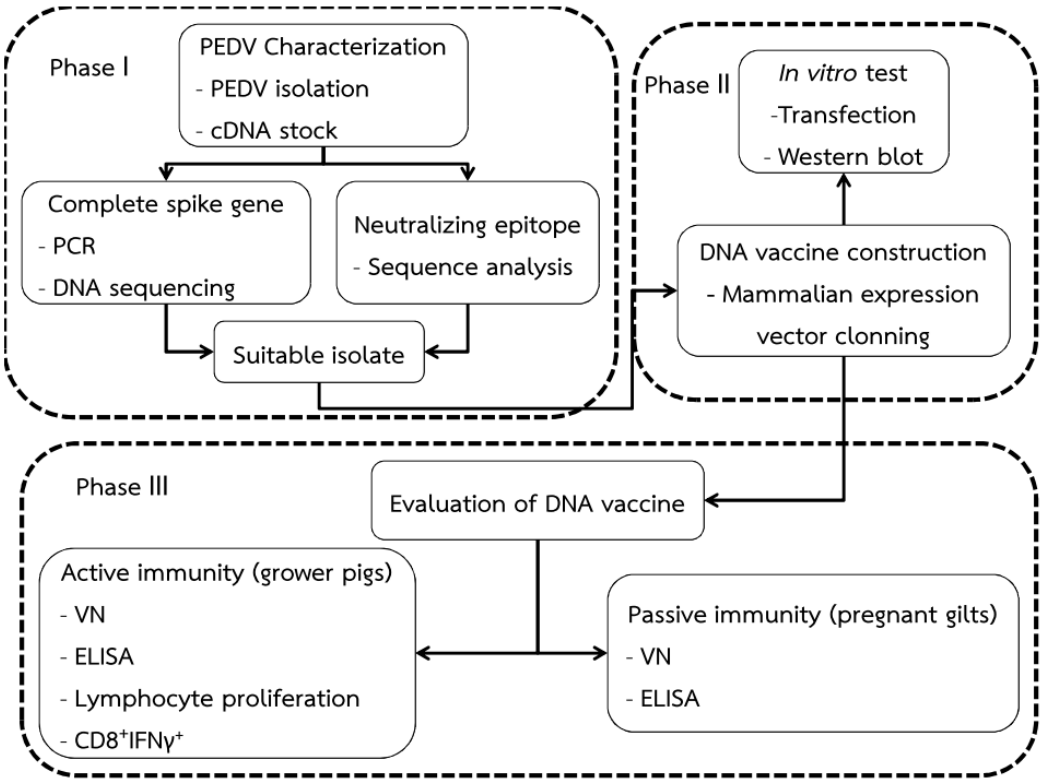
Keywords (English):

DNA vaccine, neutralizing epitopes, porcine epidemic diarrhea virus, spike gene

Hypothesis

1. There have the variation of PEDV between the field isolates and vaccine isolates
2. DNA vaccine encoding neutralizing epitope of PEDV from the field isolate can induce immunity against PEDV in pigs.

Conceptual framework



Advantages of this study

1. The genetic characteristic information of PEDV outbreak in Thailand.
2. PEDV DNA vaccine for control and prevention program

CHAPTER IV

MATERIALS AND METHODS

Phase I: Genetic characterization of PEDV in Thailand

Source of specimens and PEDV isolation

The intestinal samples were collected from the pig farms experiencing PED outbreaks. Small intestinal portions were taken from 3-4 day-old piglets displaying clinical signs associated with PED including vomiting, watery diarrhea dehydration and high mortality.

The intestinal samples were assayed for PEDV isolation using continuous Vero cell line (ATCC, CCL-81) according to the method previously described (Hofmann and Wyler, 1988). In brief, one gram of intestinal samples of piglets were minced into small pieces and suspended up to 10 ml in phosphate buffer saline (PBS; pH 7.2). Suspended samples were vortexed for homogenization and samples were clarified by centrifugation at 4,500 rpm for 10 min. Supernatant were collected, then, filtered through a 0.45 µm filter and stored at -80 °C until use for viral isolation. For viral isolation, 200 µl of supernatant samples were diluted with MEM medium in a ratio of 1:5 before inoculated into a monolayer of Vero cell, which approximate 80% of cell confluent, in 25 cm^2 flask. Then, 25 cm^2 flask was incubated at 37 ^{o}C in 5% CO_2 for 1 hour. After incubation, the inoculums were removed, then replaced with MEM (Gibco^{TM}, MD, USA) medium supplement with 5% fetal bovine serum (FBS), 2 mM L-glutamine and 100U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, referred as MEM complete media. Cytopathic effect (CPE) was observed daily for 5 days by a microscope. The CPE-positive 25 cm^2 flask was collected in -80 ^{o}C and the freeze-thaw method for 2 times were performed for break the cell. Centrifugation at 2500 rpm for 10 min were used for clarified the cell lysate. Supernatants of cell lysate were collected and stored at -80 ^{o}C until use.

Reverse transcription-polymerase chain reaction

Viral RNA from PEDV-infected Vero cells were extracted by using a Nucleospin RNA virus extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instruction. The extracted viral RNA was convert into complementary DNA (cDNA) by using M-MuLV reverse transcriptase (New England Biolabs Inc., Ma, USA) with the random hexamers. The PCR condition for converting the RNA to cDNA was 42 °C for 1 hour and 95 °C for 10 min. Each cDNA samples were confirmed for PEDV positive by using the partial S gene detection PCR (Park et al., 2007). Sequence and phylogenetic analysis of partial S gene was performed for genotype screening. The detection of complete spike gene of PEDV was performed by using 2 pairs of primer consist of S1-F (5'-ACG TAA ACA AAT GAG GTC TTT-3') and S1-R (5'-ATA CAC CAA CAC AGG CTC TGT-3') to amplified S1 and S2-F (5'-GGT TTC TAC CAT TCT AAT GAC G-3') and S2-R (5'-GTA TTG AAA AAG TCC AAG AAA CA-3') to amplified S2 (Lee et al., 2010). PCR amplification was performed by using Platinum® Tag DNA polymerase High Fidelity (Invitrogen™, CA, USA). The condition of PCR was initial denature at 94 °C for 5 min following by 35 cycles of 94 °C for 30 sec, 55 °C (58 °C for S2) for 30 sec, and 68 °C for 2 min, respectively. The final extension step was 68 °C for 10 min and PCR products were visualized by agarose gel electrophoresis. The positive products were 2,253 and 1,987 nucleotides for S1 and S2, respectively.

DNA cloning and sequencing

The PCR products were ligated into the plasmid pGEM®-T Easy Vector (Promega, WI, USA) according to the manufacturer's instruction. The heat shock method was used for plasmid transformation into the bacteria *E.coli* strain JM 109. Transformed *E.coli* were cultured in Luria-Bertani (LB) Broth (BD Difco™, MD, USA) for 1hr at 37 °C. Then, cultured *E.coli* were inoculated into 100 μg/L of ampicillin supplemented LB agar (BD Difco™, MD, USA) adding 20 μl of 50 mg/ml X-gal (Promega, WI, USA) and 50 μl of 200 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Vivantis, Malaysia) per one LB agar plate. Inoculated plate was incubated at 37 °C for 12-16 hours. Then, at least 5 white colonies were selected

to multiply by cultured in 100 µg/L of ampicillin supplemented LB Broth. Plasmid DNA (pDNA) were extracted by Nucleospin® plasmid extraction kits (Macherey-Nagel, Germany) according to the manufacturer's instruction. Nucleotides sequencing was performed by Biobasic Inc. (Ontario, Canada) by using an ABI Prism 3730XL DNA sequencer.

Sequence analyses

Nucleotide and deduced amino acid sequences were aligned using the CLUSTALW program (Thompson et al., 1994). A phylogenetic analysis was constructed, by including the partial S genes and complete S genes from this study and previously reported (Table 1), using a Bayesian Markov chain Monte Carlo (BMCMC) method in the program BEAST v1.7.4 (Drummond et al., 2012). For each analysis, three independent BEAST runs were performed; each run consist of 50 million generation with sampling of every1000 generations and the first 10% discarded as burn-in. LogCombinerv1.7.4 was used to combine logand tree files from independent BEAST runs. A 50% majority-rule consensus tree was generated from the tree files using SumTrees v3.3.1 (Sukumaran and Holder, 2010).

The percentage of homology between the isolates at the nucleotide and amino acid levels were calculated. Two measures, which include the average, pair-wise genetic distance and the maximum, pair-wise genetic distance, were also computed, as described previously (Forsberg et al., 2002). The number of synonymous and non-synonymous substitutions per site were calculated (Nei and Gojobori, 1986).

Table 1 Isolate names and accession numbers of all isolates in this study

Isolate name	Accession no.			Country
	Complete S gene	Partial S gene	ORF3 gene	
CV777	AF353511	-	AF353511	Belgium
CV777 truncated	-	-	GU372744	China
Br1/87	Z25483	-	-	UK
DR13	DQ862099	-	EU054929	Korea
Attenuated DR13	JQ023162	-	EU054930	Korea
Chinju99	AY167585	-	EU792474	Korea
Spk1	AF500215	-	-	Korea
KNU-0801	GU180142	-	-	Korea
KNU-0802	GU180143	-	-	Korea
KNU-0901	GU180144	-	-	Korea
KNU-0902	GU180145	-	-	Korea
KNU-0903	GU180146	-	-	Korea
KNU-0904	GU180147	-	-	Korea
KNU-0905	GU180148	-	-	Korea
CNU-091222-01	JN184634	-	-	Korea
CNU-091222-02	JN184635	-	-	Korea
SM98	GU937797	-	-	China
JS-2004-2	AY653204	-	-	China
LJB/03	DQ985739	-	-	China
LZC	EF185992	-	-	China
DX	EU031893	-	-	China
CH/FJND-1/2011	JN543367	-	-	China
CH/FJND-2/2011	JN315706	-	-	China
CH/FJND-3/2011	JN381492	-	-	China
CH/JL/09	-	-	GU372741	China
SJZ/2011	-	-	JQ710436	China

Isolate name	Accession no.			Country
	Complete S gene	Partial S gene	ORF3 gene	
SH5_ORF3	-	-	JQ305138	China
PF2	-	-	JQ430681	China
CH/BJSY/2011	-	-	JQ735952	China
CH/JL/08	-	-	GU372734	China
CH/HNHJ/08	-	-	GU372736	China
CH/SHH/06	-	-	GU372740	China
CH/SH/2011	-	-	JQ664299	China
CH/HLJM/07	-	-	GU372735	China
CH/IMT/06	-	-	GU372739	China
CH/HLJH/06	-	-	GU372732	China
CH/GSJI/07	-	-	GU372737	China
CH/GSJII/07	-	-	GU372742	China
CH/GSJIII/07 truncated	-	-	GU372743	China
CH/HNCH/06	-	-	GU372738	China
CH/S	-	-	GU372733	China
MC/2011	-	-	JQ710434	China
MC/2011	-	-	JQ710434	China
07NP01	-	FJ196196	-	Thailand
08RB02	-	FJ196214	-	Thailand
08RB03	-	FJ196184	-	Thailand
08NP03	-	FJ196205	-	Thailand
08NP04	-	FJ196206	-	Thailand
08RB04	-	FJ196216	-	Thailand
08CB04	-	FJ196200	-	Thailand
08RB06	-	FJ196218	-	Thailand
08UB01	-	FJ196220	-	Thailand
08CC01	-	FJ196203	-	Thailand

Isolate name	Accession no.			Country
	Complete S gene	Partial S gene	ORF3 gene	
08RB07	-	FJ196219	-	Thailand
08NP08	-	FJ196210	-	Thailand
08PC01	-	FJ196212	-	Thailand
KU03CB08	-	FJ196223	-	Thailand
KU04RB08	-	FJ196224	-	Thailand
KU06RB08	-	FJ196226	-	Thailand
6PED0108_1	-	JQ966312	-	Thailand
6PED0108_2	-	JQ966313	-	Thailand
NPPED0108_1	-	JQ966314	-	Thailand
NPPED0108_2	-	JQ966315	-	Thailand
NPPED0108_3	-	JQ966316	-	Thailand
NPPED0108_4	-	JQ966317	-	Thailand
INPED1008_1	-	JQ966318	-	Thailand
INPED1008_2	-	JQ966319	-	Thailand
V1PED0109_1	-	JQ966320	-	Thailand
V1PED0109_2	-	JQ966321	-	Thailand
NPKPED0109_1	-	JQ966322	-	Thailand
NPKPED0109_2	-	JQ966323	-	Thailand
AGPED0609_1	-	JQ966324	-	Thailand
AGPED0609_2	-	JQ966325	-	Thailand
PED0210_1	-	JQ966326	-	Thailand
PED0210_2	-	JQ966327	-	Thailand
STPED0310_1	-	JQ966328	-	Thailand
VTPED0410_1	-	JQ966329	-	Thailand
VTPED0410_2	-	JQ966330	-	Thailand
STPED0810_1	-	JQ966331	-	Thailand
STPED0810_2	-	JQ966332	-	Thailand

Isolate name	Accession no.			Country
	Complete S gene	Partial S gene	ORF3 gene	
MKPED1010_1	-	JQ966333	-	Thailand
MKPED1010_2	-	JQ966334	-	Thailand
STPED1210_1	-	JQ966335	-	Thailand
STPED1210_2	-	JQ966336	-	Thailand
SBPED0211_1	-	JQ966337	-	Thailand
SBPED0211_2	-	JQ966338	-	Thailand
SBPED0211_3	-	JQ966339	-	Thailand
SBPED0211_4	-	JQ966340	-	Thailand
SBPED0211_5	-	JQ966341	-	Thailand
SBPED0211_6	-	JQ966342	-	Thailand
SBPED0211_7	-	JQ966343	-	Thailand
SPPED0111_1	-	JQ966344	-	Thailand
SPPED0111_2	-	JQ966345	-	Thailand
SPPED0111_3	-	JQ966346	-	Thailand
SPPED0111_4	-	JQ966347	-	Thailand
SPPED0111_5	-	JQ966348	-	Thailand
SPPED0111_6	-	JQ966349	-	Thailand
SPPED0111_7	-	JQ966350	-	Thailand
SPPED0111_8	-	JQ966351	-	Thailand
SPPED0111_9	-	JQ966352	-	Thailand
SPPED0111_10	-	JQ966353	-	Thailand
SPPED0111_11	-	JQ966354	-	Thailand
SPPED0111_12	-	JQ966355	-	Thailand
SPPED0111_13	-	JQ966356	-	Thailand
SPPED0111_14	-	JQ966357	-	Thailand
SPPED0111_15	-	JQ966358	-	Thailand
SPPED0111_16	-	JQ966359	-	Thailand

Isolate name	Accession no.			Country
	Complete S gene	Partial S gene	ORF3 gene	
SPPED0111_17	-	JQ966360	-	Thailand
SPPED0111_18	-	JQ966361	-	Thailand
DTSPED0411_1	-	JQ966362	-	Thailand
DTSPED0411_2	-	JQ966363	-	Thailand
NVPED0411_1	-	JQ966364	-	Thailand
NVPED0411_2	-	JQ966365	-	Thailand
NPPED2008_2	KC764952	-	-	Thailand
NPPED2008_1	KC764953	-	-	Thailand
PED0212_1	KC764954	-	-	Thailand
SBPED0211_2	KC764957	-	-	Thailand
SPPED0212_1	KC764958	-	-	Thailand
SBPED0211_3	KC764959	-	-	Thailand
SPPED0212_2	KC764960	-	-	Thailand
6PED0108	-	-	KC344843	Thailand
SPPED1211	-	-	KC344844	Thailand
STPED0810	-	-	KC344845	Thailand
V1PED0108	-	-	KC344846	Thailand
SBPED0211	-	-	KC344847	Thailand
NPKPED0108	-	-	KC344848	Thailand
DTS2PED0612	-	-	KC344849	Thailand
PED0210_2	KC764955	-	-	Thailand
SBPED0211_1	KC764956	-	-	Thailand

Phase II: DNA vaccine construction

PEDV neutralizing epitope inserted pDNA construction

The PCR of cDNA from the represent PEDV isolate (SBPED0211_2/Thailand/ accession no. KC764957) was performed to amplify neutralizing epitope of PEDV with Platinum® Tag DNA polymerase High Fidelity (Invitrogen™, CA, USA). The PCR condition was 94 °C for 5 min following by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 68 °C for 30 sec and final extension at 68 °C for 10 min. The positive of PCR product was 450 nucleotides. The PCR product was ligated into mammalian expression pDNA vector and then transformed into One Shot® Top10 competent cells (Invitrogen™, CA, USA) by using the heat-shock method. Transformed *E.coli* were cultured in SOC medium (Invitrogen™, CA, USA) for 1 hr at 37 °C. Then, cultured *E.coli* were spread into 100 µg/L of ampicillin supplemented LB agar and incubated for 18-24 hrs at 37°C. To confirm the positive clone of transformed *E.coli*, the PCR was performed by using the same condition described above. The pDNA from the PCR-positive colony was extracted by using EndoFree® Plasmid Giga kits (Qiagen®, Germany) according to the manufacturer's instruction. The purification of extracted pDNA was measured and the A260/ A280 ratio of the extracted pDNA must be higher than 1.8.

The *In vitro* expression of PEDV neutralizing epitope inserted pDNA

Monolayer of Baby hamster kidney (BHK-21; ATCC, CCL-10) cell was prepared in 6-well plate before transfection. The percentage of cell confluent was approximate 80-90%. The pDNA transfection was processed by using Lipofectamine™2000 (Invitrogen™, CA, USA) according to the manufacturer's instruction. Briefly, 4 µg of pDNA were diluted with MEM up to 125 µl and 10 µl of Lipofectamine™2000 were diluted with 115 µl of MEM following by 5 min incubation at room temperature. Then, the diluted pDNA and diluted Lipofectamine™2000 were mixed together and incubated for 20 minutes at room temperature. After incubation, the mixture were added into BHK cell and incubated at 37 °C, 5% CO_2 for 4 hrs. After that, the mixture were removed and 5% FBS supplemented

MEM were added into the transfected cell following by incubation for 48 hours and 72 hours at 37 ^{o}C, 5% CO_2

Protein confirmation by Western blot analysis

To detect the expressed protein, pDNA transfected cells were washed 2 times by PBS and cells were collected by using cell scraper. Centrifugation at 2500 rpm for 5 min was performed to collect the cell pellets and collected cells were broken by freeze-thaw method following by centrifugation at 12000 rpm for 10 min. The supernatant from the cell lysate was mixed with SDS dye buffer at ratio 1:1. Before the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the expressed proteins by molecular weight, the samples, which mixed with SDS dye buffer, were boiled at 100 ^{o}C following by centrifugation at 12000 rpm for 5 min. SDS-PAGE was perform by using the 12% stacking gel. The expressed proteins, which separated by SDS-PAGE, were transferred to nitrocellulose membrane and Western Blotting was performed. Nitrocellulose membrane were washed by washing buffer for 5 min before the blocking step with blocking buffer, which was 5% skim milk in 0.5% PBST, for 3 hrs at room temperature. Due to the design of transcription unit of pDNA which the target gene was recombined with 6-histidine tag, the western blot was performed by using the mouse anti-Histidine Tag monoclonal antibody (R&D system®, MN, USA) in blocking buffer at 1:500 dilution as a primary antibody and the primary binding was incubated overnight in 4^{o}C. Between each step, membrane was washed 5 times by washing buffer which 5 min each. Goat anti-mouse IgG HRP polyclonal antibody (AbD serotec, NC, USA) in blocking buffer at 1:2000 dilution was used as secondary antibodies and incubated for 1 hr in room temperature. The membranes were developed by chemiluminescence using ECL (enhanced chemiluminescence) plus detection reagent for 15 min in dark place. The size of expected protein was 16.5 kDa.

Phase III: DNA vaccine evaluation

Dosage determination of DNA vaccine

Experimental design

Fifteen 3-week old pigs were randomly allocated into 3 groups of 5 pigs each. All 3 treatment groups consist of as following;

Group A) DNA-500 (pDNA0.5); pigs in this group were intramuscularly administered with SBS1-2His-3 plasmid at the dosage of 500 µg/ 2 ml.

Group B) DNA-1000 (pDNA1); pigs in this group were intramuscularly administered with SBS1-2His-3 plasmid at the dosage of 1,000 µg/ 2 ml.

Group C) Plasmid control (CTRL); pigs in this group were intramuscularly administered with control plasmid at the dosage of 1,000 µg/ 2 ml.

All pigs were vaccination twice at 0 and 14 day post vaccination (DPV) according to treatment referred in each group. Sera and anti-coagulant added blood samples were collected at 0, 7, 14, 21 and 28 DPV. Peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulant added blood samples and were assayed for lymphocyte proliferative response and detection of IFN-γ producing cells. Sera were separated and assayed for the presence of antibody using viral neutralization assay (VN) and isotype-specific enzyme-linked immunosorbent assay (ELISA) for IgG.

Passive immunity from DNA vaccinated sows

Experimental design

Ten 11-week of gestation gilts, which negative from the PED-specific ELISA test and no history of PED infection, were randomly allocated into 2 groups compose

Group 1) Treatment group (S-pDNA0.5), gestation gilts were intramuscularly administered with SBS1-2His-3 plasmid at the dosage of 500 µg/ 2 ml (n=5).

Group 2) Control group (S-CTRL), gestation gilts were intramuscularly administered with control plasmid at the dosage of 500 µg/ 2 ml (n=5).

At 12- and 14-week of gestation, gestation gilts were vaccinated with 2 ml of 500 µg of SBS1-2His-3 plasmid and control plasmid in PBS. Colostrum samples were collected within 3 hours post farrowing and milk samples were collected at 3, 5 and 7 days post farrowing (DPF). All samples were keep on ice while transferred to the -20 °C. Samples were assayed for the presence of antibody using VN and isotype-specific ELISA.

Samples preparation

To separate the serum, blood samples were transferred into 15 ml tube following by centrifugation at 2500 rpm for 15 min. Supernatants were collected and all serum samples were heat inactivated at 56°C for 30 min. Sera were store at -20 °C until the serological testing was performed.

Colostrum and milk samples were processed according to the method previously described (Srijangwad et al., 2015). Briefly, colostrum and milk samples were centrifuged at 4500 rpm for 30 min. Lipid layer in upper were removed and the supernatants were collected following centrifuged at 12000 rpm for 10 min. Collected supernatants were subjected for heat inactivation at 56°C for 30 min, then centrifuged again at 2500 rpm for 5 min. Supernatants were stored at -20 °C until use.

Viral neutralization assay

Each samples were 2-fold serially diluted with MEM and incubated for 1 hour with the equal volume of 10^2 $TCID_{50}$/ 50 µl PEDV suspension at 37°C, 5% CO_2. The prepared monolayer of Vero cells, which approximate 80% confluent, were washed twice by PBS following by transferred the virus suspensions into Vero cells. The virus adsorption was performed by incubated for 1 hour at 37°C, 5% CO_2. Then, the virus suspensions were removed and replaced by MEM complete medium. CPE was observed daily for 7 consecutive days. Back-titration was performed to confirm the titer of virus suspension. The lowest dilution without CPE was calculated for neutralizing titer (Reed and Muench, 1938).

Isotype-specific ELISA

Isotype-specific ELISA was performed according to Srijangwad et al. (2015). Briefly, the spike recombinant proteins of PEDV were coated into Maxisorp 96-well immuno plate (Thermo sciencetific, Denmark) by using 0.1 M carbonate buffer and were incubated for overnight at 4°C. Five times washing step with 0.05% Tween20 in PBS (PBST) were performed between every step. Blocking buffer, 5% skim milk in PBST, were added into the well and incubated for 1 hr at 37°C. Then, samples were added following by incubation for 1 hr at 37°C. Goat anti-Pig IgG (Fc): HRP (AbD Serotec, NC, USA) or Goat anti-Pig IgA: HRP (AbD Serotec, NC, USA) were used for detect IgG or IgA isotype in the samples, respectively. 3,3',5,5'-tetramethylbezidine (TMB) was used as substrate and plate was incubated for 15 min at room temperature in dark place. 1N sulfuric acid (H_2SO_4) was added to stop reaction and the OD values were measured at 450 nm with M965+ microplate reader (Metertech, Taipei, Taiwan)

Peripheral blood mononuclear cell (PBMCs) isolation

PBMCs were isolated from whole blood samples, collect in Ethylene diamine tetraacetic acid (EDTA) coated blood collecting tube, by density gradient in Lymphosep® (Biowest, France) according to the manufacturer's instructions. Briefly, blood samples were diluted with PBS (1:1) and overlaying samples on Lymphosep® following by centrifuged at 1000 x g for 30 min. The isolated PBMCs were washed twice with PBS and the PBMCs were suspended in advance RPMI-1640 medium (Gibco™, MD, USA) supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 50 µM 2-mercaptoethanol and 100U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. This solution is referred as RPMI complete medium. Viable cells were confirmed by Trypan blue solution (Gibco™, MD, USA) exclusion and count by inverted microscope. Cells concentration were calculated before use for surface/intracellular staining – flow cytometry analysis consist of lymphocyte proliferation assay and number of IFN-γ producing cells.

Lymphocyte Proliferation Assay

The lymphocyte proliferation assay is based on the coupling cell surface marker expression and the cell membrane with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; CFSE (Biolegend®, CA, USA) according to the manufacturer's instructions. Briefly, 1×10^7 PBMCs were suspended in 1 ml of PBS and an equal volume of 2.5 μM CFSE were added following by incubation for 15 min at 37°C in dark place. Reaction was stopped by adding 0.5 ml of FBS and cells were washed by sterile PBS. CFSE stained PBMCs were suspended in complete medium and cells concentration were adjusted to 1×10^7 cells/ ml. One hundred μl of cells were added to 96-well flat-bottom microtiter plates containing mock-suspension as a negative control and PEDV at a multiplicity of infection (MOI) of 0.1 as the treatment recall antigen. Cells were incubated for 5 days at 37°C in a 5% CO_2. After incubation, cells were transferred into 96-well round-bottom microtiter plate. Activated cells were washed twice by centrifugation at 1800 rpm for 5 min and suspended in PBS supplemented with 1% FBS and 0.1% sodium azide, which this reagent was referred as FACS buffer, before staining the surface marker. Surface staining was performed with primary monoclonal antibodies to swine lymphocyte surface antigens including 1:50 of PE-conjugated mouse anti-porcine CD4 (Southern Biotech, AL, USA) and 1:50 SPRD-conjugated mouse anti-porcine CD8α (Southern Biotech, AL, USA), in FACS buffer, for 30 min at 4°C in dark place. After incubation, stained cells were washed twice with FACS buffer and suspended by 200 μl of 2% formaldehyde solution. The stained cell samples were storage in 4°C in dark place, no longer than 7 days, until running flow cytometry analysis. Proliferation of lymphocyte populations were determined by flow cytometry analysis detecting 30,000 events per sample. The relative proliferative indices were calculated by using the percentage of proliferating cells in the PEDV stimulated well divided by the percentage of proliferating cells in the mock suspension well.

Detection of IFN-γ producing cells

The IFN-γ producing cells were measured by intracellular IFN-γ flow cytometry assay. In the assay, 1×10^7 PBMCs were suspended in 1 ml of complete medium. One hundred µl of cell suspension were added to 96-well flat-bottom microtiter plate containing 100 µl of mock suspension as negative control, and PEDV (MOI of 0.1) as the treatment recall antigen. Plate was incubated for 3 days at 37 °C in a 5% CO_2 and the protein transport inhibitor BD Golgistop™ (BD Biosciences, CA, USA) was added 12 hours before intracellular staining. After incubation, cells were washed twice with FACS buffer and performed the surface staining including 1:50 FITC-conjugated mouse anti-porcine and 1:50 SPRD-conjugated mouse anti-porcine CD8α as describe above. Subsequently, the stained cells were washed twice following by permeabilized cell by reagent A of Leucoperm™ (AbD Serotech®, UK) for 15 min at room temperature in the dark. After that, cells were washed and intracellular staining was performed by 1:50 of PE-conjugated mouse anti-porcine IFN-γ (BD Pharmingen™, CA, USA) in reagent B for 45 min at room temperature in dark place. Then, cells were washed twice following by cells reconstitution with 200 µl of 2% formaldehyde solution. The cells stained with the isotype control antibody (PE-conjugated IgG1 antibody) were performed and used as the background cut-off. The fluorescence minus one (FMO) staining control were performed during the establishment and validation of assay. The IFN-γ producing cells were analyzed by flow cytometry at 30,000 events per sample. The PEDV-specific IFN-γ producing cells were calculated by using the percentage of positive cells in the PEDV stimulated well minus by the percentage of positive cells in the mock suspension well.

Data analysis

Antibody titers (from VN and ELISA), lymphocyte proliferation indices and number of IFN-γ producing cells were expressed as means ± standard error of means (SEM). Dose determination experiment was analyzed and compared by the one-way ANOVA following by Bonferroni's multiple comparison test and passive immunity from sows

experiment was analyzed by unpaired *t*-test using GraphPad Prism® (Graphpad software Inc, CA, USA)

CHAPTER V

RESULTS

Genetic characterization of PEDV in Thailand

From 120 of porcine intestinal samples which were collected in 2008-2012, sixty-nine PEDV were isolated. The partial spike gene were amplified to investigate the heterogeneity of these 69 Thai PEDV isolates. The result from nucleotide sequencing revealed that the partial S gene of the 69 field isolates consisted 657 nucleotides which encoding 219 amino acids located at nucleotide positions 1462-2118, which was amino acid positions 488-706, of the full length of the S gene. Identical partial S nucleotide sequences were identified and excluded, resulting in further genetic analysis of 30 genes. The phylogenetic tree was constructed, by using the 30 partial S genes, together with another 16 previously reported Thai PEDV isolates and some partial S genes which previously reported in other countries (Park et al., 2007; Puranaveja et al., 2009). Based on the previous study, the PEDV isolates were divided into 3 groups, designated G1, G2 and G3 (Park et al., 2007). Group G1 was further divided into 3 subgroups, which were designated G1-1, G1-2 and G1-3. From this clustering system, the phylogenetic tree demonstrated that Thai PEDV isolates were further divided into 2 groups (Fig 1) which both of them were classified in G1 but in difference subgroup. Almost PEDV isolates, in this study, were clustered in subgroup G1-1, which consist all 16 previously reported Thai PEDV isolates along with 27 of 30 currently Thai isolates. The remaining isolates, 3 of 30 isolates, were designated in subgroup G1-4, including AGPED0609_1, AGPED0609_2 and SPPED0111_1. This subgroup did not fit into the previously reported clustering system which this subgroup was located between subgroups 1-2 and 1-3. When comparing the nucleotide and amino acid homology of all Thai field PEDV isolates, the nucleotide and amino acid homology demonstrated that the homology of all Thai field PEDV isolates were ranged between 94.4-100.0% and 85.8-100.0%, respectively. Meanwhile, Thai isolates in G1-1 demonstrated the nucleotide and amino acid homology

ranged from 96.4-99.7% and 92.5-100.0%, respectively. However, the nucleotide and amino acid homology of isolates in G1-1 was more diverse when compared to that of G1-4, which all 3 isolates had 100.0% similarity at the nucleotide and amino acid level. Simultaneously, the homology of nucleotide and amino acid level between subgroups G1-1 and G1-4 had 94.4-95.5% and 85.8-88.5%, respectively.

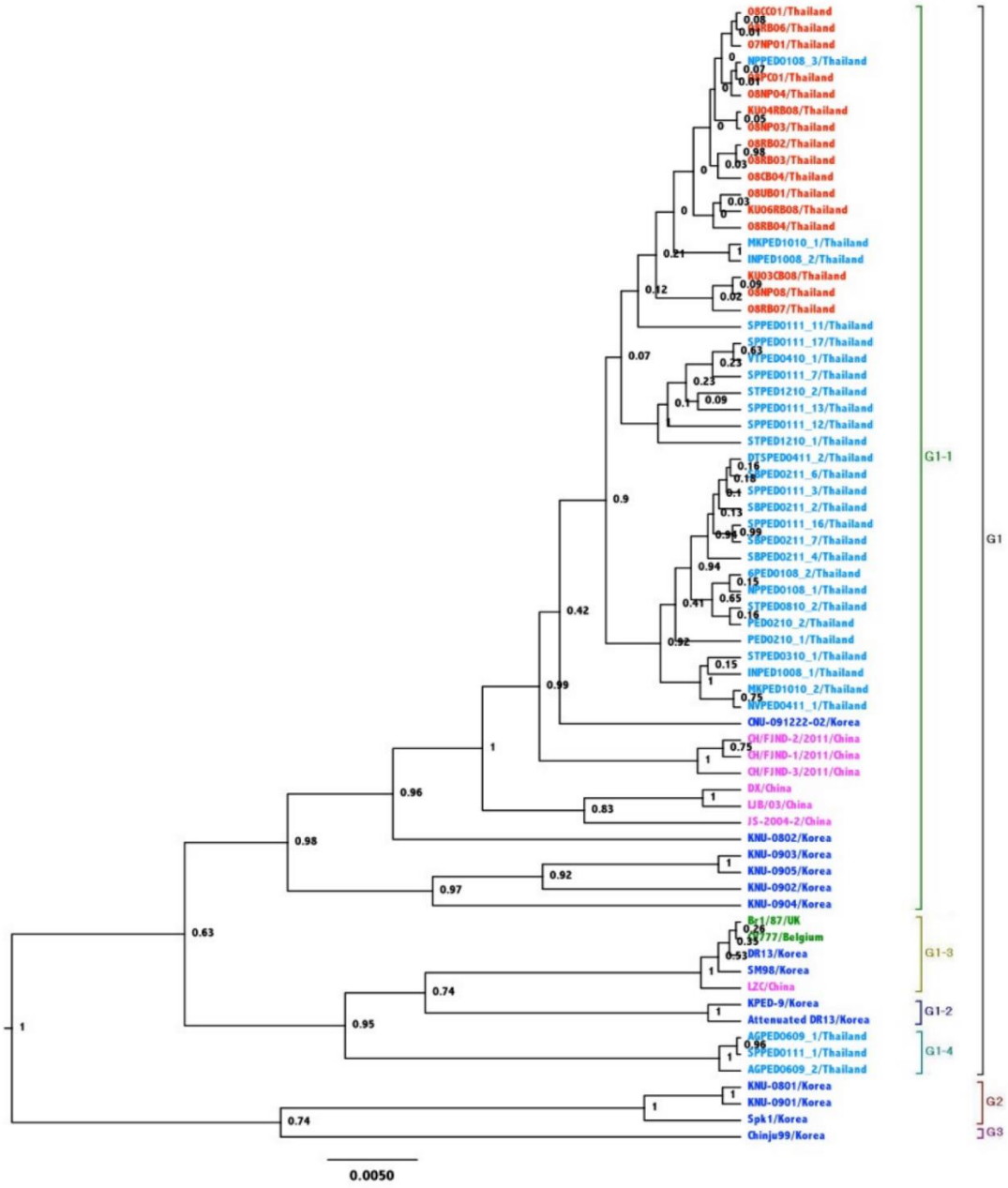


Figure 1 Phylogenetic analysis of the PEDV isolates based on the nucleotide sequences of the partial S glycoprotein genes. The trees include nucleotide sequences of Thai

isolates in this study (light blue font) and a previous study (red font) as well as sequences from China (pink font), Korea (dark blue font) and European countries (green font). The 50% majority-rule consensus tree was constructed using Bayesian MCMC method. Each internal node with the posterior probability of the corresponding clade > 0.5 is labeled.

Thirteen different Thai field PEDV isolates, including 10 isolates from subgroup G1-1 and 3 isolates from subgroup G1-4, were randomly selected to investigate the genetic diversity between this 2 groups of Thai field PEDV isolates and their genetic relationship with PEDV isolates from other countries by using the full-length nucleotide sequences of their S genes. The phylogenetic tree, based on complete S genes, was constructed together with those of other PEDV isolates from other countries (Index 1). The phylogenetic analysis, based on the complete S genes, revealed that global PEDV were divided into 3 clusters, including clusters 1, 2 and 3 (Fig 2). Cluster 1 comprised the isolates from China (CH/FJND-3, CH1, CH8, CHGD-01, HB-2011-1, HB-2011-2, HB-2011-3, HB-2011-4, HB-2012-1, HB-2012-2, HB-2012-3, HB-2012-4, BJ-2011-2, BJ-2011-3, BJ-2012-1, BJ-2012-2, ZJ-2011-1 and ZJ-2011-2) and Korea (KNU-0902, CNU-091222-01 and CNU-091222-02). Even though, all 13 PEDV Thai isolates were classified in subgroups G1-1 and G1-4, based on the partial S gene comparisons, but when analyzed based on complete S gene demonstrated that all Thai isolates in this study were classified in cluster 1. Cluster 2 comprised 8 isolates from Korea (KNU-0801, KNU-0802, KNU-0901, KNU-0903, KNU-0904, KNU-0905, Spk1 and Chinju99). Cluster 3 comprised 16 reference strains, including isolates from Korea (DR13 and SM98), China (CH2, CH3, CH4, CH5, CH6, CH7, CH/FJND-1, CH/FJND-2, JS-2004-2, LZC, LJB03 and DX) and Europe (CV777 and Br1/87).

The sequence homology between the S glycoprotein genes was measured. The nucleotide homology and amino acid homology of the isolates in cluster 1 were ranged from 94.9-99.9% and 92.9-99.9%, respectively (Table 2). Meanwhile, the homology of cluster 1 had 92.5-97.7% (90.5-96.9%) and 91.8-97.7% (90.3-97.3%) of nucleotide (deduced amino acid) sequence identity with the members of clusters 2 and 3,

respectively. The nucleotide homology and amino acid homology of all 13 Thai field PEDV isolates ranged from 94.9-99.8% and 94.0-99.7%, respectively. In addition, all 13 Thai PEDV isolates revealed 94.9-99.9% and 94.0-99.9% of nucleotide and amino acid homology, respectively, to Chinese isolates in the same cluster. Meanwhile, when compare to the Korean isolates in the same cluster (KNU0902, CNU-091222-01 and CNU-091222-02).

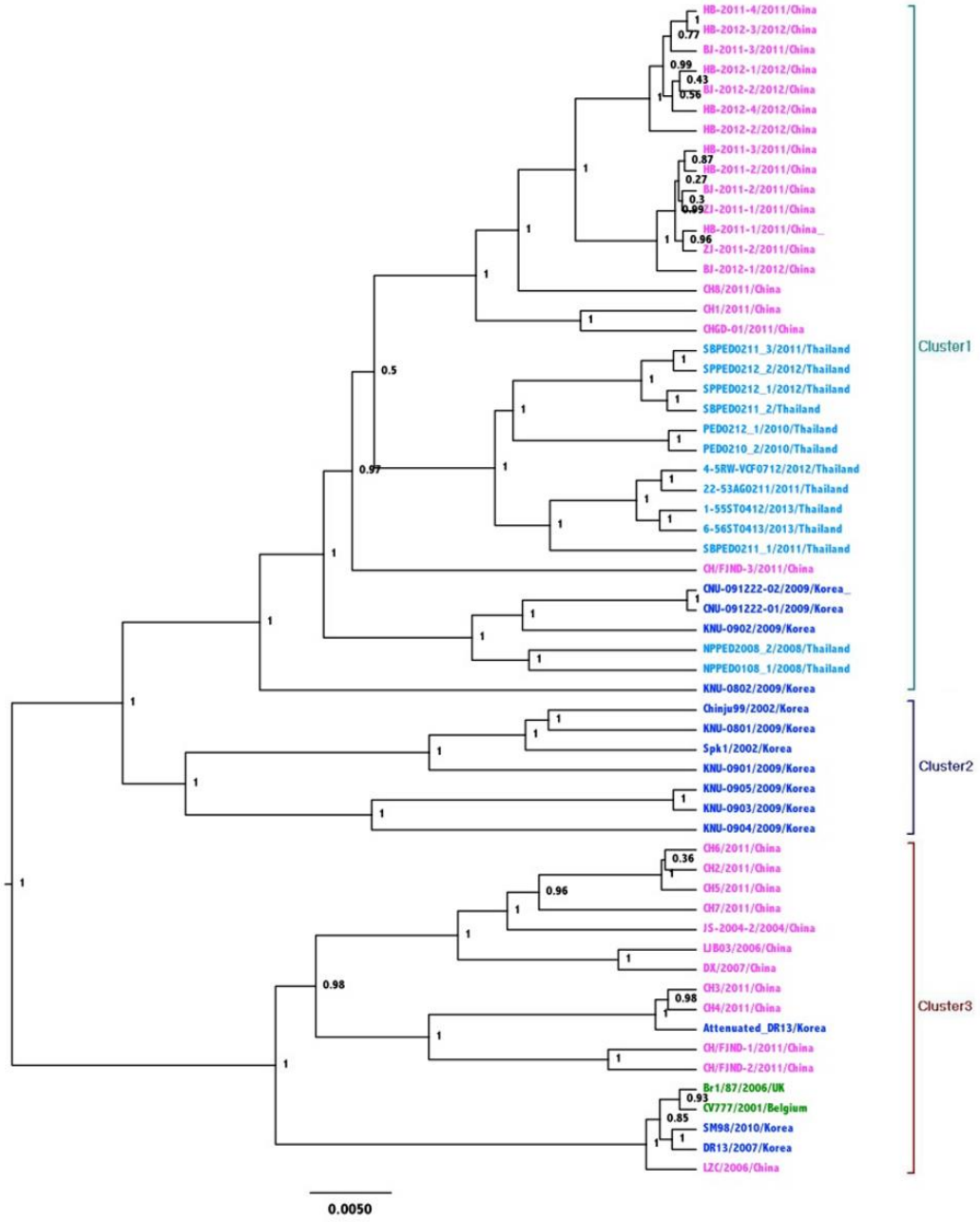


Figure 2 Phylogenetic analysis of the PEDV isolates based on the nucleotide sequences of the complete S glycoprotein genes. The trees include nucleotide sequences from Thailand (light blue font), China (pink font), Korea (dark blue font) and European countries (green font). The 50% majority-rule consensus tree was constructed using Bayesian MCMC method. Each internal node with the posterior probability of the corresponding clade > 0.5 is labeled.

Table 2 Nucleotide and amino acid identities of the complete S gene. The regular font was the nucleotide identities and the bold font was the amino acid identities.

Nucleotide/**Amino acid**	**Cluster 1**	**Cluster 2**	**Cluster 3**
Cluster 1	94.9-99.9/ **94.0-99.9**	**90.5-96.9**	**90.3-97.3**
Cluster 2	92.5-97.7	93.6-99.7/ **92.0-99.6**	**90.3-94.5**
Cluster 3	91.8-97.7	92.1-94.9	94.6-99.9/ **93.7-99.7**

It was demonstrated that the full length S glycoprotein genes of the Thai PEDV isolates are 4,158 nucleotides which encode 1,386 amino acid residues. To investigate the amino acid differences between the clusters, the nucleotide and deduced amino acid sequences of the complete S genes were aligned and analyzed, especially in the hypervariable region and four regions of neutralizing epitope. The highest difference of amino acid was located in the N-terminal of the S1 domain which was the lowest similarity portion with the CV777, the reference strain of PEDV. When focusing in the complete S gene, the PEDV isolates in cluster 1 and 2, except isolate Chinju99, demonstrated two insertions point, of four and one amino acids, at amino acid positions 55-60 (^{56}*GENQ*^{59}) and 140 (^{140}*N*). These insertions located in the hypervariable domain in the N-terminus of the S1 region. In addition, one deletion point, of two amino acids, was observed at amino acid positions 160 and 161 which all of thirteen Thai isolates, which were in cluster 1, in this study have the similar insertion and deletion patterns of amino acid.

Several neutralizing epitopes in the S glycoprotein were reported (Cruz et al., 2008; Kang et al., 2006; Sun et al., 2008). Four regions of neutralizing epitope include amino acid positions 504-643 (Kang et al., 2006); ^{753}YSNIGVCK^{760} and ^{769}LQDGQVKI^{776} (Sun et al., 2008); and ^{1373}GPRLQPY^{1379} (Cruz et al., 2008). However, the two of neutralizing epitopes, ^{753}YSNIGVCK^{760} and ^{1373}GPRLQPY^{1379}, demonstrated the similarity between the Thai PEDV isolates and the isolates from other countries. In contrast, the amino acid positions 504-643 and ^{769}LQDGQVKI^{776} were difference. The highest differences of amino acid was located in amino acid positions 504-643. Meanwhile, in the ^{769}LQDGQVKI^{776}

region, amino acid differences, which the substitutions of S ⟶ *L* and D⟶*G* at amino acid positions 769 and 771, respectively, were observed. In addition, the genetic characterization of the deduce amino acid between the Thai isolates PEDV and the vaccine isolates in the commercial vaccine indicated the vaccine isolates had the genetic different with the Thai PEDV isolates at the amino acid positions 504-643 and 769-776. However, when comparing between the Thai isolates, only the neutralizing epitope, which located at amino acid position 504-643, was the only one region that have difference.

PEDV neutralizing epitope inserted pDNA construction

To prevent the neonatal piglets from PEDV, passive immunity from the sow colostrum and milk was the goal to the protection. Therefore, the important neutralizing epitope for the vaccine against PEDV should be the epitope that can induce the B cell activation. Previous study demonstrated that the amino acid position 753-760 and 769-776 were the B cell neutralizing epitopes (Sun et al., 2008). From the complete S gene analysis, no variation was observed between Thai isolates, which revealed as ^{753}YSNIGVCK^{760} and ^{769}LQDGQVKI^{776}, in these epitopes. Due to the information from genetic characterization, Thai PEDV isolates belong to the pandemic variant which genetically different with the classical variant (SM98 and LZC) which are the vaccine isolates. This information indicated that a represent isolate for vaccine development in Thailand should be the pandemic variant and the deduce amino acid in the neutralizing epitope should be similar to the isolate which outbreak in the field. Therefore, the isolate SBPED0211_2, a field isolate which belong to pandemic variant, was used as a represent isolate for pDNA construction for DNA vaccine against PEDV. The 450 base pair of PCR product, which covering the neutralizing epitope from S gene of PEDV recombined with 6 histidine tag, was amplified by the high fidelity DNA polymerase and cloned into the mammalian expression vector pcDNA3.1 CT-GFP TOPO (Invitrogen, CA, USA) (Fig 3). The SBS1-2His-3 plasmid was verified by DNA sequencing. The nucleotide sequences demonstrated the SBS1-2His-3 plasmid was 100% identity to the B cell neutralizing epitope of the NPPED0108_2 PEDV isolate.

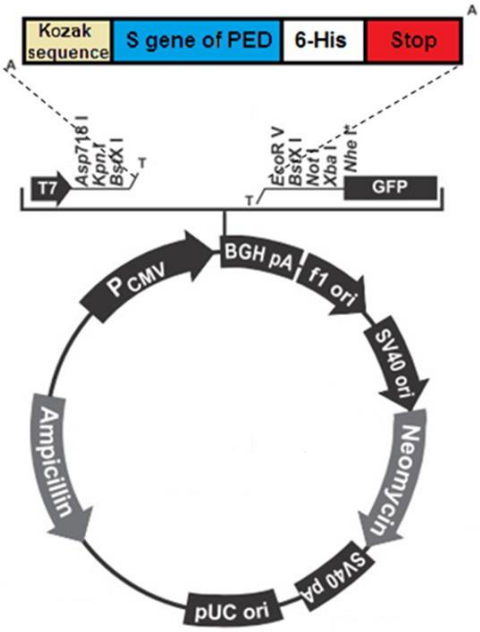


Figure 3 Map of the SBS1-2His-3 plasmid. The plasmid backbone contain CMV promoter, pUC origin of replication and ampicillin resistance gene, and transcription unit including gene of interest (S gene of PEDV), 6x-histidine tag and ribosome binding site (Kozak sequence).

PEDV S protein confirmation by Western blot analysis

To confirm the ability of SBS1-2His-3 plasmid, which containing neutralizing epitope from S gene of PEDV, to express the recombinant protein in mammalian system, the BHK-21 cell was used to be the host for protein expression. The pDNA transfection process was performed by using Lipofectamine 2000 with the incubation time at 48 and 72 hours. The predicted molecular weight of the target recombinant S protein from SBS1-2His-3 expression was 16.5 kDa. The result from western blot demonstrated the recombinant protein, which was detected by monoclonal antibody against histidine, was expressed in the expected molecular weight (16.5 kDa) (Fig 4). The intensity of the protein band from sample that incubated 48 hours was more prominent when compare to the sample that incubated 72 hours. None of the protein band, in the expected molecular weight, was detected in the sample that transfected by expression control vector

(pcDNA3.1CT-GFP expression control). This result indicated that the SBS1-2His-3 plasmid can expressed the expected recombinant protein, which include the neutralizing epitope of PEDV and the 6x histidine tag, in the mammalian cell. In addition, this SBS1-2His-3 plasmid could be use as the DNA vaccine.

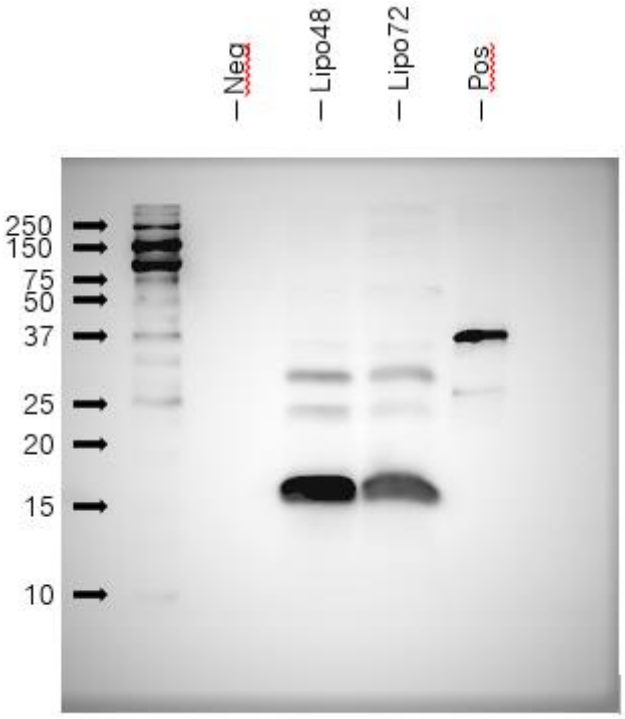


Figure 4 Expression and characteristic of recombinant S protein. Histidine tagged truncated S protein, which the expected molecular weight was 16.5 kDa, was expressed in BHK-21 cell. Plasmid expression control (Neg), 48 hrs of incubation (Lipo48), 72 hours of incubation (Lipo72) and recombinant S protein with his tag (Pos), which was 37 kDa, Western blot performed using mouse anti histidine tag monoclonal antibody as primary antibody and goat anti mouse IgG HRP polyclonal antibody as secondary antibody. Membrane was developed by chemiluminescence using ECL plus detection reagent.

PEDV-DNA vaccine induced cell-mediated immunity but not the humoral-mediated immunity

To evaluate the immunogenicity of SBS1-2His-3 plasmid, which was used as DNA vaccine against PEDV, the humoral and cell mediated immunity after vaccination with this

DNA vaccine were detected. The PEDV-specific antibodies were detected through the viral neutralization assay and PEDV-specific ELISA. Meanwhile, the PEDV-specific IFN-γ producing cells were detected by using the flow cytometry.

From the viral neutralization assay, the result demonstrated an absence of neutralizing antibodies titer against PEDV in group CTRL at any time point of study. Similar to group CTRL, serum of pigs from the group pDNA0.5 and pDNA1 also demonstrated the absence of PEDV-neutralizing antibodies titer at 0 DPV until the end of study at 28 DPV.

Similar to VN assay, the result from PEDV-specific ELISA demonstrated the absence of seroconversion in any experimental groups at any time point. However, the mean of PEDV-specific IgG from the serum of group pDNA1 seem to be slightly increased at 21 and 28 DPV while the results from CTRL and pDNA0.5 group showed a stable of PEDV-specific antibodies (Fig 5). However, there have no significantly difference of the antibody titer between any time points of all experimental groups. In addition, no significantly difference between the experimental groups at any time points was observed.

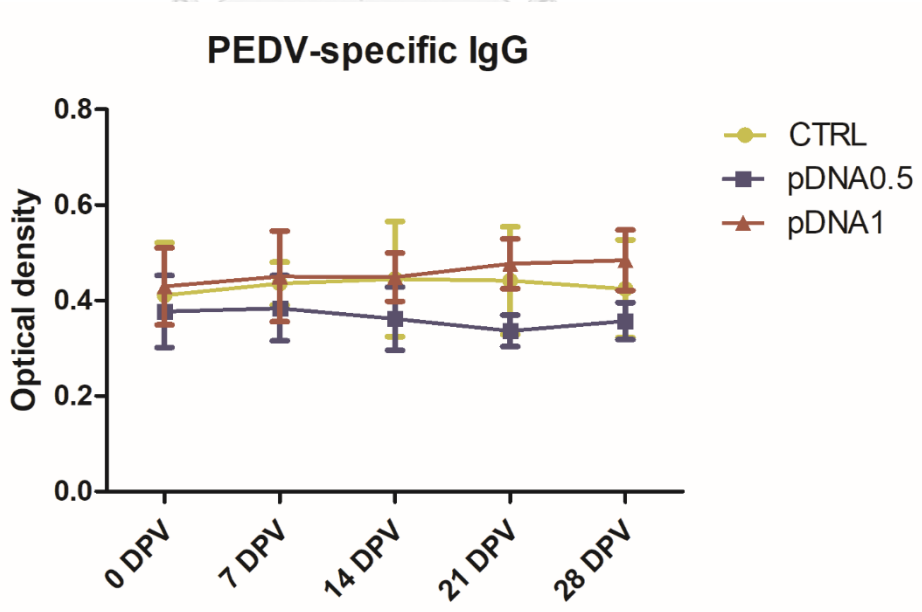


Figure 5 The optical density (OD) of PEDV-specific IgG ELISA in weaned pigs. The mean OD with SEM of control group (CTRL; yellow circle), 500 μg of SBS1-2His-3 plasmid group

(pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. No significantly difference among the experimental groups at any time point was observed.

In contrast to the antibody response, the results from proliferation assay and PEDV-specific IFN-γ producing cells detection demonstrated the immune response after the DNA vaccine administration. The lymphocyte proliferation of $CD4^+$ cell, which represent for the T helper cell (T_h), from the PBMC of pDNA0.5 and pDNA1 groups demonstrated the significantly increased of proliferative index in these groups at 7 DPV ($p<0.05$). Then, the proliferative index of these groups were slowly decreased at 14 DPV ($p>0.05$) and return to normal level, which was 1, at 21 DPV until the end of study. Meanwhile, the $CD4^+$ cells of group CTRL demonstrated a stable of proliferative index at the normal level from 0 DPV until the end of study. When compare the proliferative indices between the experimental groups, the PEDV-DNA vaccinated groups, pDNA0.5 and pDNA1, exhibited the significantly higher than the control group at 7 DPV ($p<0.05$). However, there have no significantly difference between the pDNA0.5 and pDNA1 group at any time points (Fig 6).

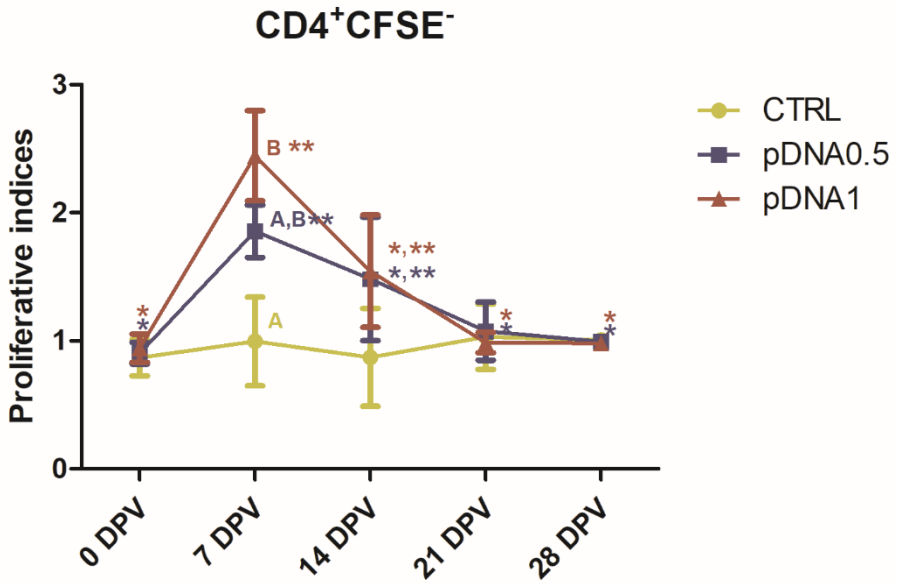


Figure 6 The proliferation index of $CD4^+$ cells in weaned pigs. The mean proliferation indices with SEM of control group (CTRL; yellow circle), 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. * and ** indicated statistical difference between the proliferation index of the time point of each group (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$). The alphabet A and B indicated statistical difference between the proliferation index of the experimental groups (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$).

Even though, the result of lymphocyte proliferation assay of $CD8^+$ cell, which represent for T cytotoxic cell (T_c), was not exhibited the significantly increasing in any experimental groups. However, the proliferative indices of the PED-DNA vaccinated groups demonstrated the relatively increasing of proliferative index at 14 and 21 DPV. At 14 DPV, the proliferative index of group pDNA1 was relatively increased, then, return to the normal level. Meanwhile, the proliferative index of group pDNA0.5 showed the highest value at 21 DPV and return to the normal level at 28 DPV. The results in control group exhibited the comparable value from the starting day until the end of study. However, there have no significantly difference between the experimental groups (Fig 7).

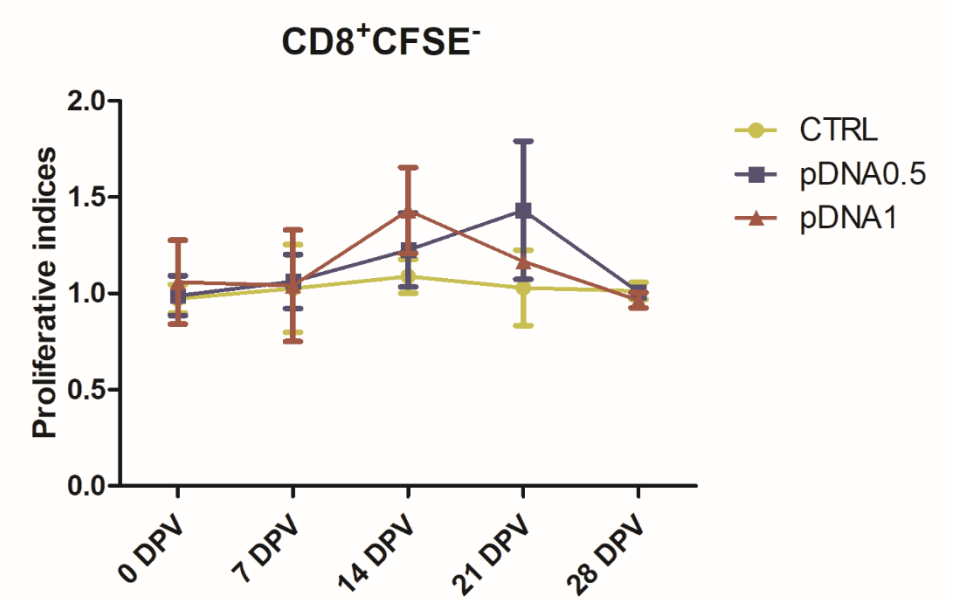


Figure 7 The proliferation index of $CD8^+$ cells in weaned pigs. The mean proliferation indices with SEM of control group (CTRL; yellow circle), 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. No significantly difference among the experimental group at any time point was observed

For the $CD4^+8^+$ cell, which represent for the T memory cell (T_m), the proliferation indices of each groups demonstrated the comparable value which indicated no significantly increasing of proliferative index at any time points in every groups. However, the result from group pDNA1 showed the fluctuate pattern of proliferative index, which was slightly increased at 7 and 21 DPV. Statistical analysis demonstrated no significantly difference between the experimental groups (Fig 8).

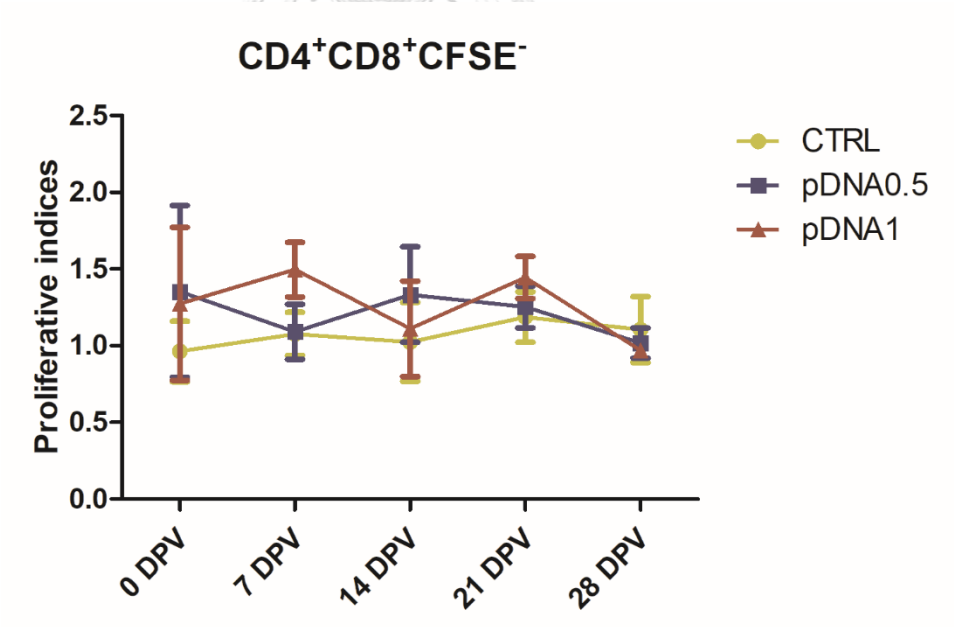


Figure 8 The proliferation index of $CD4^+CD8^+$ cells in weaned pigs. The mean proliferation indices with SEM of control group (CTRL; yellow circle), 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red

triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. No significantly difference among the experimental group at any time point was observed

The detection of PEDV-specific IFN-γ producing cell demonstrated the immunogenicity of the DNA vaccine in this study by increasing the population of IFN-γ producing cells. For the $CD4^+$ cell population, the PBMC of group pDNA0.5 showed the significantly increased ($p<0.05$) of PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells at 7 and 28 DPV when compare to 0 DPV. Meanwhile, the results of 14 and 21 DPV showed no significantly increased of PEDV-specific $CD4^+IFN\text{-}\gamma^+$ but the value of these days exhibited relatively higher than the start of experiment at 0 DPV. For group pDNA1, even though the PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells of this group was not significantly increased when compare to 0 DPV, the results of this group indicated the relatively increased from 7 DPV until the end of this study at 28 DPV. In addition, the average percentage of PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells in this group seem to be higher than pDNA0.5 group. The result from control group demonstrated the comparable of PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells at every time points which no significantly difference was observed. When compare the results among the experimental groups, only the PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells of group pDNA0.5, at 28 DPV, showed significantly higher than the control group ($p<0.05$) while there have no significantly difference between pDNA0.5 to pDNA1 and CTRL to pDNA1 group. There have no significantly difference among the experimental groups in other time points (Fig 9).

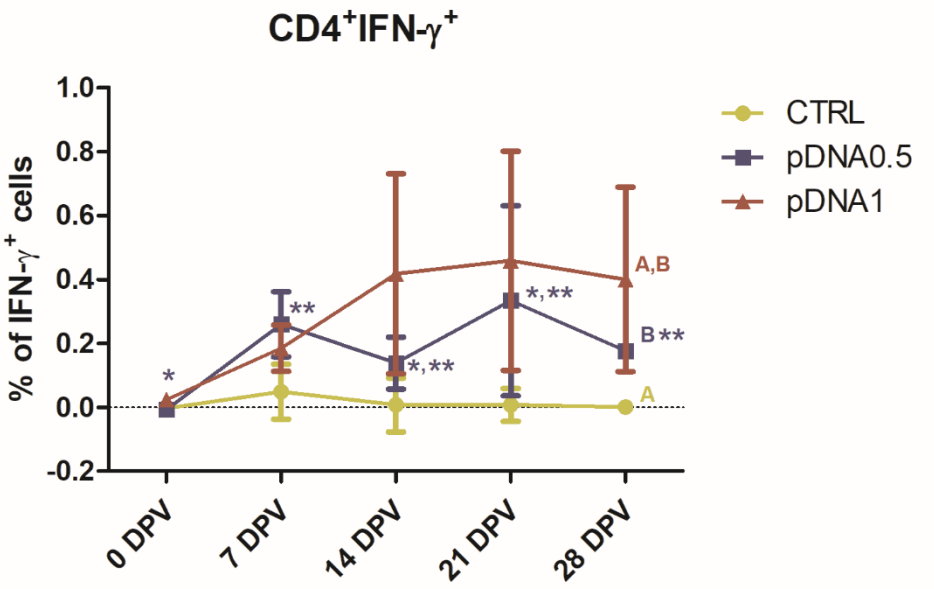


Figure 9 The percentage of PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells in weaned pigs. The mean percentage of PEDV-specific $CD4^+IFN\text{-}\gamma^+$ cells with SEM of control group (CTRL; yellow circle), 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. * and ** indicated statistical difference between the proliferation index of the time point of each group (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$). The alphabet A and B indicated statistical difference between the proliferation index of the experimental groups (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$).

For the $CD8^+$ cell population, the percentage of PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells in PBMC of group pDNA0.5 demonstrated the significantly increased, when compare to the start of experiment, at 7, 21 and 28 DPV ($p<0.05$). Meanwhile, the PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells from group pDNA0.5 at 14 DPV seem to be relatively higher than at 0 DPV. Similar to $CD4^+IFN\text{-}\gamma^+$ cells, the PBMC of group pDNA1 showed relatively increased of the PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells, when compare to 0 DPV, at 7, 14 and 28 DPV. In addition, the percentage of PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells at 21 DPV of group pDNA1

was significantly higher when compare to 0 DPV (p<0.05). There have no significantly increased of PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells in the control group was observed. When compare the results among the experimental groups, only the percentage of PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells of group pDNA0.5, at 28 DPV, was significantly higher than the control group (p<0.05) but it was not significantly difference with group pDNA1. In addition, there have no significantly difference between the experimental groups in the other time points was observed (Fig 10).

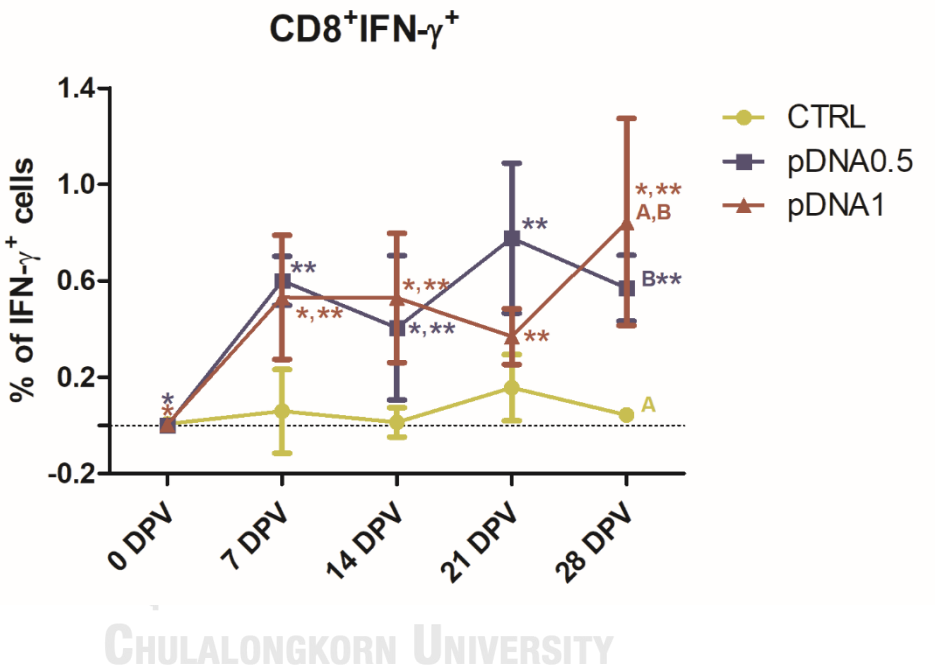


Figure 10 The percentage of PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells in weaned pigs. The mean percentage of PEDV-specific $CD8^+IFN\text{-}\gamma^+$ cells with SEM of control group (CTRL; yellow circle), 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. * and ** indicated statistical difference between the proliferation index of the time point of each group (ANOVA followed by Bonferroni's multiple comparison test, p<0.05). The alphabet A and B indicated statistical difference between the proliferation index of the experimental groups (ANOVA followed by Bonferroni's multiple comparison test, p<0.05).

For the $CD4^+CD8^+$ cell population, the percentage of PEDV-specific $CD4^+CD8^+IFN\text{-}\gamma^+$ cells in PBMC of the PED-DNA vaccinated groups (pDNA0.5 and pDNA1) demonstrated only the relatively increased, which were peaked at 21 and 14 DPV for pDNA0.5 and pDNA1 group, respectively. The results in group CTRL showed a slightly increased at 7 DPV which no significantly difference was detected. When compare the results among the experimental group, the percentage of PEDV-specific $CD4^+CD8^+IFN\text{-}\gamma^+$ cells from group pDNA0.5 demonstrated a significantly higher than the control group ($p<0.05$) at 28 DPV. However, no significantly difference between the pDNA1 and CTRL group at 28 DPV was observed. In addition, there have no significantly difference among the experimental groups at any time points was observed (Fig 11).

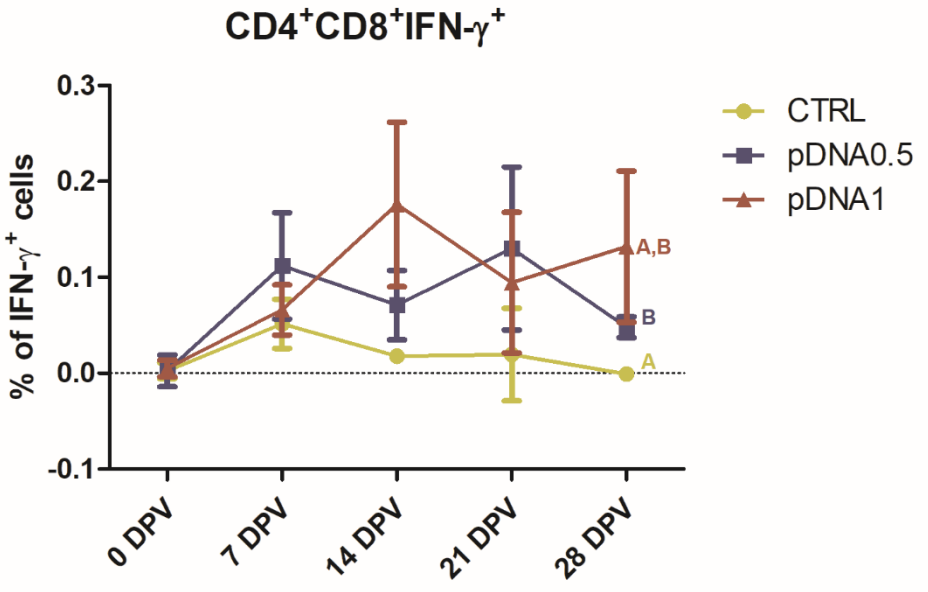


Figure 11 The percentage of PEDV-specific $CD4^+CD8^+IFN\text{-}\gamma^+$ cells in weaned pigs. The mean percentage of PEDV-specific $CD4^+CD8^+IFN\text{-}\gamma^+$ cells with SEM of control group (CTRL; yellow circle), 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; blue square) and 1000 µg of SBS1-2His-3 plasmid group (pDNA1; red triangle) at 0, 7, 14, 21 and 28 day-post-vaccination (DPV) were present. The alphabet A and B indicated statistical

difference between the proliferation index of the experimental groups (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$).

PEDV-DNA vaccine slightly enhanced the PEDV-specific antibodies in colostrum and milk

In contrast with the dosage determination study, vaccination by SBS1-2His-3 in gilts demonstrated an enhancement of PEDV-specific IgA and PEDV-specific neutralizing antibody. From the PEDV-specific ELISA test, even though the PEDV-specific IgA titer from colostrum and milk of PED-DNA vaccinated sows (S-pDNA0.5) were not significantly higher than the control group (S-CTRL), the result from the S-pDNA0.5 group showed the relatively higher PEDV-specific IgA titer than the S-CTRL group in every time points. The peak of the PEDV-specific IgA titer from pDNA0.5 was present in colostrum at 0 DPF which this titer was significantly higher than the titer from milk at 3 DPF ($p<0.05$) and relatively higher than the titer from milk at 5 and 7 DPF. Similar to pDNA0.5 group, the PEDV-specific IgA titer of CTRL group was peak at 0 DPF which the titer of this colostrum was significantly higher than the titer from milk at 3 and 5 DPF and relatively higher than titer of milk at 7 DPF (Fig 12).

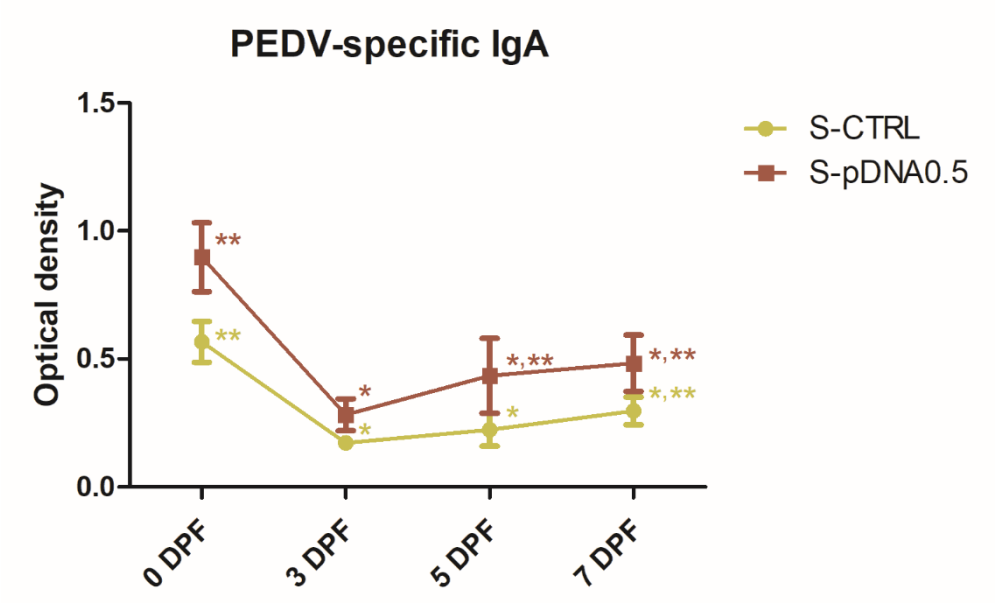


Figure 12 PEDV-specific IgA ELISA from colostrum and milk samples in gestation gilts. The mean OD with SEM of control group (S-CTRL; yellow circle) and 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; red square) were present. * and ** indicated statistical difference between the proliferation index of the time point of each group (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$).

From the viral neutralization assay, the PEDV-specific neutralizing antibody titer from the S-CTRL group demonstrated the limited neutralizing titer at 0 DPF while the milk samples from the other time points were absence. In contrast, the PEDV-neutralizing titer from the S-pDNA0.5 group were detected from colostrum and milk at 0, 3 and 7 DPF while the titer from milk at 5 DPF was absence. The neutralizing titer of colostrum from group S-pDNA0.5 was significantly higher when compare to the titer from 5 DPF ($p<0.05$). However, when compare the PEDV-specific neutralizing titer between S-pDNA0.5 and S-CTRL group, there have no significantly difference between them (Fig 13).

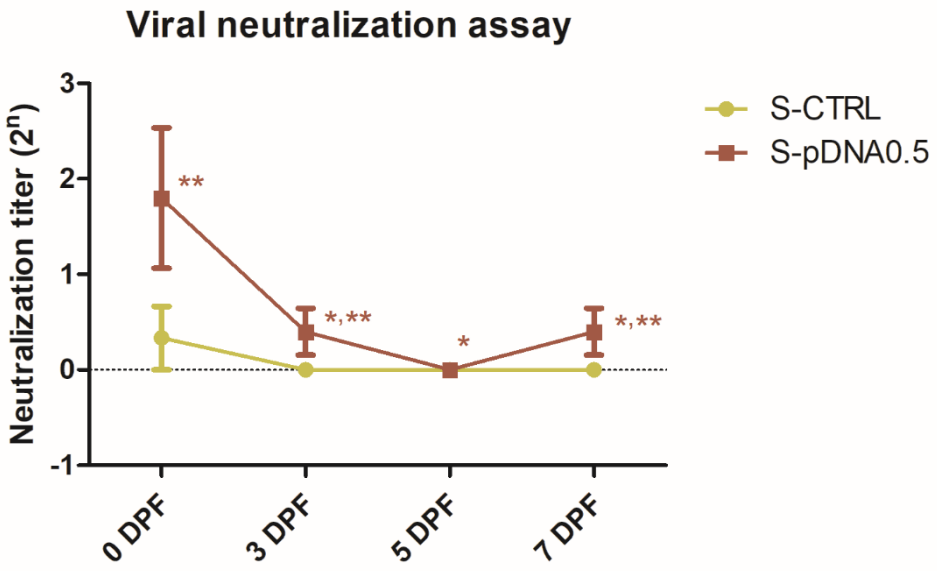


Figure 13 Viral neutralization titer against PEDV in gestation gilts. The mean neutralizing titer with SEM of control group (S-CTRL; yellow circle) and 500 µg of SBS1-2His-3 plasmid group (pDNA0.5; red square) were present. * and ** indicated statistical difference

between the proliferation index of the time point of each group (ANOVA followed by Bonferroni's multiple comparison test, $p<0.05$).

CHAPTER VI

DISCUSSION

Genetic characterization of PEDV in Thailand

Since the emergence of porcine epidemic diarrhea in 2007, PEDV has continued to cause economic losses in the Thai swine industry. The disease situation was developed to the endemic stage which several herds were experienced to the repeat outbreaks. However, the severity of the disease, as demonstrated by the mortality rate, was lessened. Even though the control and prevention program including immunization and strict biosecurity have been implemented, the degrees of success were vary. The recurrence of PED outbreaks has led to the necessity of genetic diversity investigation of PEDV. A better understanding of the genetic diversity may facilitate the development of a successful control and prevention program.

To investigate the heterogeneity of PEDV in Thailand, the partial and complete S genes were characterized. At the beginning, the partial S gene was characterized first because this portion can be used to characterize in a large number of samples. In contrast to partial S gene, the characterization of the complete S gene which is more complicated due to the large size of this gene. In addition, previous study reported the investigation of the genetic diversity of PEDV by using the partial S gene, which including the neutralization epitope of PEDV, and use this portion for clustering PEDV. This study also demonstrated that this portion exhibited high similarity when using complete S genes (Park et al., 2007). Therefore, the initially study of the partial S genes was to understand the heterogeneity of PEDV in Thailand. Based on the characterization of the partial S gene, the results in this study demonstrated that Thai PEDV isolates were divided into 2 subgroups. However, when clustering based on the complete S genes, those 2 subgroups were clustering in the same cluster which was cluster 1. Most of the Thai PEDV isolates were classified in cluster 1 which included the isolates in KNU-serial isolates, field isolates of Korean PEDV, and the isolates which outbreaks in China in recent years.

A previous report suggested that Thai PEDV isolates were China-like isolates which similar to the JS2004 that was isolated in China. However, the results from the present study are contradicted with that previous report. Based on the characterization of the complete S genes of PEDV field isolates in this current study, the field isolates that cause economic damage to the Thai swine industry, were belong to cluster 1 which possess the unique characteristic feature defined by the insertion of 4 amino acids (GENQ) between positions 55 and 56, a 1-aa (N) insertion between positions 135 and 136, and the deletion of 2 amino acids between positions 155 and 156. These unique characteristic feature of Thai isolates were genetically related to the Korean KNU-serial isolates (Lee et al., 2010) and the isolates which previously reported for the severe outbreaks in China (Chen et al., 2013; Li et al., 2012). In addition, this unique characteristics that used for identified a new variant of PEDV, which called pandemic variant, were reported in China and caused more severe outbreaks (Li et al., 2012). In this point, the isolate JS2004 was not possess these characters that mentioned above. The contradiction of the results between the previous study and present study could be explained by the coverage level of the genetic investigation which the current study use both of partial and complete S genes, whereas the previous study used only the partial S gene. The phylogenetic analysis of the partial S gene was based on only 657 nucleotides and involved in amino acid positions 488-706 of the complete S gene. This coverage level was not sufficient to represent the whole gene of the virus and, especially, the 2 insertion and the 1 deletions mentioned above are located in the N-terminus of S1 domain which is upstream to the partial S gene portion. The deduced amino acid, therefore, indicated the disadvantage of the phylogenetic analysis based on only partial S genes for investigate the molecular epidemiology or heterogeneity of PEDV isolates.

The source of pandemic PEDV variant introduction into Thailand is not known. However, it has been speculated that the pork meat and bone materials which imported from the PEDV endemic countries was a possible route of introduction. The first PED outbreak was reported in Nakorn Pathom province which located in the central region of Thailand and very distant from the border. In addition, no genetic materials, such as gilts

or boar, were imported to this farm. The only observed changes were the changed of raw material for manufacture feed from fish meal to pork meat and bone meal due to the high fish meal prices.

In Thailand, PEDV has become endemic stage and continuously damage to the Thai swine industry. Even though, the severity of the disease has been declined and only the piglets farrowed from the primi-parous sows are seriously affected following repeated outbreaks, the disease still cause the economic losses. The duration between the repeated outbreaks in herds was longer from every 3-4 months to every 6 months. To investigate whether the heterogeneity of PEDV plays an important role in disease recurrence, the genetic characterization, based on complete S genes, of 10 isolates from 3 repeated outbreaks herds were analyzed. The result demonstrated that all isolates were in cluster I and there had only 3% of amino acid difference. These suggested that the heterogeneity of the S gene might not contribute to the recurrence of the disease in herds. Other factors, including the immune status of replacement gilts prior to the herd introduction, might play an important role in disease recurrence.

To control PEDV in swine herds, several management strategies including vaccination have been implemented. In Thailand, the available commercial PED vaccines in market were imported from China and Korea. However, the efficacy of these vaccines are still questioned. Even in the heavy vaccination programs herds, which implied whole herd vaccination and pre-farrow vaccination, still experience repeated outbreaks. Due to the genetic information from this study demonstrated that Thai PEDV isolates are a pandemic variant PEDV while the commercial vaccines, including LZC and SM98, are classical variant PEDV. Even though, the genetic characterization of the Thai isolates displayed the similarity of spike gene among them, the genetic characterization between the Thai PEDV isolates and vaccine isolates were different, especially in the neutralizing epitope. This finding may explain why the current commercial PEDV vaccines, which was develop from the classical variant PEDV, were not success for control PED in Thailand. In addition, recently study demonstrated the differential gene modulation between classical and pandemic variant PEDV which the pandemic variant PEDV can suppress the immune

response by down-regulated the expression of the toll-like receptors and the signal molecules downstream resulting to the decrease of type-I IFN and pro-inflammatory cytokines production but the classical variant was not (Temeeyasen et al., 2018). These finding might be another reason why the PEDV vaccine from the classical variant PEDV could not protect the vaccinated pigs from the pandemic variant PEDV infection. Therefore, due to the genetic information from this study indicated that the PED vaccine for control and prevention program in Thailand should be develop base on the pandemic variant PEDV which outbreak in the field.

The DNA vaccine construction

Due to the variation of PEDV isolates from the field and the vaccine, the introduction of the commercial live-attenuated vaccine which genetically diverse to the existing isolated in the herd might lead to the increase of mutation rate of PEDV. Together with the variable degree of success, its indicated that the PED vaccine for control and prevention program in Thailand must be develop from the field isolate. In this point, the advantage of the DNA vaccine which simply to manufacture and rapidly production may be suitable choice for PED vaccine development. The commercial available effective plasmid platforms, which ready to use, serve the rapid response for the novel emerge of PEDV isolate or even other pathogens. These advantages of the DNA vaccine are match with the criteria for the PED vaccine development which the vaccine should be immunogenic to elicit the either HMI or CMI with the safety manner of the vaccine which should not drive the mutation rate of the virus.

Due to the genetic information of PEDV in Thailand, there have no genetically difference in the amino acid sequence among the Thai PEDV isolates. The represent isolate for vaccine development in this study was chosen based on the genetic information of neutralizing epitope and the geographical location of the farm which the PEDV were isolated. In this study, the PEDV isolate from Ratchaburi, which is the most density area of pig farms, was used and the neutralizing epitope of this isolate was similar to the other Thai isolates.

In this study, the expression of PEDV antigen was detected *in vitro* by the SDS-PAGE and Western blot analysis. The result from the Western blotting demonstrated the capability of the pDNA to express the protein in the mammalian expression system which showed in the positive band of target protein with the functional of histidine tag downstream from the transcription unit. However, due to the principal of the SDS-PAGE, the expressed protein, which tested by this method, were in a linear form which cannot represent to the conformation of the expressed protein. This might not reflected to the native expressed protein which will be used for induce the immunity *in vivo*. The native conformation of the expressed protein was needed for the further elucidated.

The immunogenicity of PEDV DNA vaccine

Several studies reported the potency of DNA vaccine to enhance the humoral mediated immunity (Karlsson et al., 2018; Park et al., 2011a). However, the results from several studies demonstrated the contradicted outcome. In pigs, vaccination by the DNA vaccine in the naïve pigs tend to induce the cell-mediated immunity than the humoral-mediated immunity (Bragstad et al., 2013). Several studies demonstrated the insignificant of antibody enhancement by the DNA vaccine (Kodihalli et al., 1999; Meunier et al., 2018; Suradhat et al., 2015). However, the DNA vaccinated animals still have the protective immunity when inoculated with the wild-type pathogens (Kodihalli et al., 1999). These indicated the potency of DNA vaccine which tend to be the induction of cell-mediated immunity which might be help for the disease protection by reduced the spread of pathogens and the pathogen clearance. Similar to the results from this study, even though the vaccination by DNA vaccine in weaned pigs showed no significant difference of PEDV-specific antibody when compared to the control pigs, the lymphocyte proliferation and the PEDV-specific IFN-γ producing cell of the pigs that vaccinated by the DNA vaccine were increased. However, the protective efficacy was not evaluated in this study due to the age limitation of the challenge experiment.

Due to the expression mechanism of DNA vaccine, the enhancing of the CMI with the absent of a significant increase of the HMI from this study suggested that the

intramuscularly vaccination by DNA vaccine seem to be expressed through the direct expression route which the expressed protein were processed and presented through the MHC class-I as the endogenous antigen. However, even though the $CD4^+$ cells, which represent for the helper T cells, were increased in this study, the antibody production which was the product of the cross-presentation between the helper T cells and B cells were absent. These might be explanation by the production of type-I IFN, which induced by the reaction between the cytosolic DNA receptor and plasmid DNA, and the function of the B cell to recognize the antigen. Due to the function of type-I IFN, which important for the T_{H1} differentiation, this cytokine can drive the host immune response to the CMI resulting to the increased of $CD4^+$ and $CD8^+$ IFN-γ producing cell. However, the function of the B cell to recognize the antigen is depend on the conformation of the antigen. Meanwhile, the gene of interest for the vaccine development in this study was the partial S gen which the conformation of the expressed protein from the plasmid DNA might have not enough immunogenicity for B cell. However, the conformation of the *in vivo* expressed protein from this pDNA was needed further elucidated.

To evaluate the efficacy of vaccine, the best parameter is the protective efficacy against the challenge model. Generally, weaned piglet, which usually 3- or 4-week-old, are the widespread age of pig that used for vaccine evaluation due to the immunocompetent of this age. However, in case of PEDV, the difficulty of the challenge experiment of PEDV is the susceptible age of this virus which an obvious clinical outcome is occur in sucking piglets, especially less than 2-week-old. Meanwhile, pigs that older than 3-week-old showed just transient diarrhea with the quickly recovery even in the inoculation of naïve pigs. In this point, the evaluation of protective efficacy can be interfered by the age-resistant of the weaned pig. In addition, during the period of inducing the complete immune response after vaccination, which usually at least 2 week or may be longer for DNA vaccine, pigs were more resist to the PEDV-infection resulting to the mild clinical signs that were difficult to observe.

In contrast to the study in weaned pigs, vaccination in gestation gilts by DNA vaccine against PEDV demonstrated an enhancement of PEDV-specific antibody. The

reason why the induction of PEDV-specific antibody between the studies in weaned pigs and the study in gestation pigs was contradicted is still doubtful. One explanation of this contradicted results might be associated with the age of experimental animals. Previous study demonstrated the different in humoral immune response of pigs in different ages after infected by PEDV (Stadler et al., 2018). In addition, the innate immune response after PEDV infection in pigs with different ages were also different (Annamalai et al., 2015).

Even though the intramuscular DNA vaccine against PEDV might not significantly induced the PED-specific antibody when compared to the control pigs. However, in endemic area, such as in Thailand, which almost pigs in the positive herds were exposed with the PEDV, the PEDV-exposed pigs, especially in grower and finisher pigs, can be the carrier of the PEDV without any sign of the disease. To eliminate the PEDV that may be carry in these pigs, cell-mediated immunity, especially the cytotoxic T cell, was needed for the virus clearance. This propose raise the possibility of the usage of this DNA vaccine for eradicate the PEDV instead of the using the oral feedback which continuously circulate the PEDV in the farm. In addition to the level of passive immunity, the induction of the active immunity against PEDV, to reduce the PEDV load in the farm, in grower and finisher pigs with the strictly biosecurity might be the important factors that fulfill the effective control and prevention program for PED. Vaccination by the DNA vaccine in wean pigs, which might be carry the virus from the farrowing unit, and grower pigs, which might be selected for the replacement gilts, should be implemented in the one-site system farm which all units are located in the same place that also have a high risk of disease transmission between units. However, strictly biosecurity level to prevent the newly introduction of PEDV is still necessary.

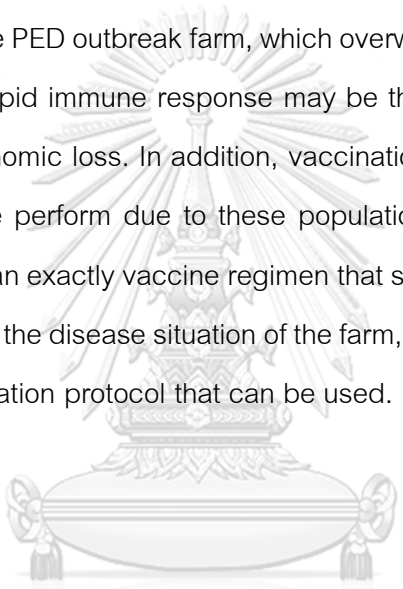
To further evaluate the efficacy of the vaccine that developed in this study, the dose of plasmid DNA used for vaccination might be affected to the host immune response. The result in this study demonstrated the trend of higher immune response in high dose of vaccination group (pDNA1) although the statistical analysis showed no significant difference with low dose of vaccination (pDNA0.5). These indicated the efficacy of the

DNA vaccine might related to the dose-dependent and the adjustment of DNA vaccination should be further determined.

To improve the efficacy of the vaccine that developed in this study, the transfection efficacy which can be improved by using the delivery system should be determined. Several studies reported the development of mucosal vaccine which used the bacterial-vector (Guimaraes et al., 2006; Xu et al., 2012b) or viral-vector (Lin et al., 2012; Meseda et al., 2018; Xu et al., 2012a) as the delivery system of the gene of interest. However, the limitation of these delivery system is the decrease of vaccine potency after repeat vaccination which host can produce the immunity against the vectors resulting to the interference of the expression of the gene of interest. Another widely use delivery system is the cationic polymer which served several advantages such as low immunogenicity, safety and the capacity to deliver the large genes. Several studies reported the successful of cationic polymer which used as the transfection agent (Boletta et al., 1997; Demeneix et al., 1998; Goula et al., 1998). An up taken of the polyplex into the cell was processed through the adsorption endocytosis which resulting from the positively charge of the polyplex (Boussif et al., 1995). However, the positive charge at the surface of polyplex rising some problem in *in vivo* condition such as the non-specific interaction with the non-target tissue or blood components. To reduce the problem, the modification of the surface polyplex by using the non-ionic and hydrophilic polymer coating demonstrated a better transfection efficacy when compare with the native cationic polymer (Patnaik et al., 2006). This nanocomposite might be a good choice for improve the vaccine efficacy of the DNA vaccine in this study.

Another factor need to be consider is the vaccine regimen. Previous study indicated the priming effect due to enhancing the cellular-mediated immunity would be improve the immune response after the booster by vaccination or natural exposure, even in the cell-mediated or humoral-mediated immunity (Meunier et al., 2018; Suradhat et al., 2016). In addition, the booster effect of mucosal immunity via the systemic vaccination by intramuscular administered were reported in the sows previously exposed to the natural infection by oral route (Chattha et al., 2015; Saif, 1999; Yuan et al., 2005). The vaccine

regimen can be design for the specific propose. For example, in the stable herd which farm was not affected by the PED outbreak, prime exposure by oral feedback or oral vaccine should be perform during the gilt acclimatization process which pigs have enough time for the induction of specific immunity and virus clearance. The second and third dose of vaccination can be perform, at 5 and 3 week before the farrowing, by the non-live virus vaccine, such as inactivated, subunit or DNA vaccine, to enhance the antibody titer before farrowing. The objective of the non-live virus vaccine using is to reduce the viral load in farrowing unit that might be transmitted from the sows to their piglets. In contrast, in the PED outbreak farm, which overwhelm with the viral load, the oral feedback that induce rapid immune response may be the best choice for the farrowing unit to diminish the economic loss. In addition, vaccination in grower and finisher by the DNA vaccine should be perform due to these population can be the carrier of PEDV. However, there has not an exactly vaccine regimen that suitable to all farm. The design of vaccine regimen is up to the disease situation of the farm, the strictness of biosecurity and the available of immunization protocol that can be used.



CHAPTER V

GENERAL CONCLUSION AND FUTURE RECOMMENDATIONS

Since the pandemic variant of PEDV was outbreak in many countries worldwide, the research about PEDV was become the hot issue which many researchers try to find the information in every aspect to solve this problem. The genetic characterization, pathogenesis, an interaction between PEDV-host and the development of PEDV vaccine were the example of research topics. In this study, the genetic characterization of Thai PEDV isolates indicated the PEDV isolates, which outbreaks in Thailand, were belong to the pandemic variant. In contrast to the field isolates, the commercial available vaccine was developed from the classical variant. The sequence analysis in this study demonstrated the difference of nucleotide and amino acid sequence at the neutralizing epitope between the vaccine isolate and the field isolates in Thailand. This finding might be the reason why the degree of success of this commercial vaccine was unsatisfied. In addition, the clearest evidence used to differentiate the pandemic variant out of classical variant was the insertion and deletion at the S gene. Several studies demonstrated the role of PEDV to interfere the innate immune response at the cellular level (Cao et al., 2015; Ding et al., 2014). Recently study demonstrated the differential gene modulation between the classical and pandemic variant. The results from this study indicated the pandemic variant can suppressed the pro-inflammatory cytokine and type 1 IFN production while the classical variant was not (Temeeyasen et al., 2018). This finding might be another one reason why the vaccinated pigs, by the classical variant, was not protected from the pandemic variant. However, the different pathogenesis which might be associated with the insertion and deletion at the S gene and the cross protection between the classical and pandemic variant were need to be further elucidated.

Even though, the intramuscular DNA vaccine against PEDV in this study was not significantly induced the antibody production in weaned pigs, however, the detection of the cell mediated immune response demonstrated the immunogenicity of this vaccine.

The results from this study demonstrated the significantly increasing of the PEDV-specific effector T cells, either $CD4^+$ or $CD8^+$ T cells, at 28 DPV. In case of enteric viral disease such as rotavirus, the $CD4^+$ T cell was reported as an important lymphocyte population that protected the host from the rotavirus infection (McNeal et al., 2006; McNeal et al., 2002) while the $CD8^+$ T cell was a population which important for the virus clearance (Franco and Greenberg, 1999). These finding indicated the potential of this developed DNA vaccine that might be used in the grower and finisher pigs to reduce the pig population that can be the carrier of the PEDV in the farm. However, the further evaluation of this DNA vaccine such as the potential of the vaccine to reduce the virus shedding period and the protection efficacy in the challenge experiment should be further investigated.

In contrast to the vaccination in weaned pigs, intramuscular injection of DNA vaccine against PEDV in the gestation gilts demonstrated a relatively increased of the PEDV-specific antibody. This result was concur with the previous study that demonstrated the potential of intramuscular DNA vaccine to induced the virus specific mucosal IgA in pigs previously primed with the oral exposure (Yuan et al., 2005). Due to the dose-dependent might be affected to the efficacy of the vaccine, the dose adjustment to get the best efficacy should be further evaluated. In addition, the route of vaccination which might affect to the isotype of antibody should be determined.

REFERENCES

Ada, G., 2005. Overview of vaccines and vaccination. Mol Biotechnol 29, 255-272.

Alonso, C., Goede, D.P., Morrison, R.B., Davies, P.R., Rovira, A., Marthaler, D.G., Torremorell, M., 2014. Evidence of infectivity of airborne porcine epidemic diarrhea virus and detection of airborne viral RNA at long distances from infected herds. Vet Res 45, 73.

Annamalai, T., Saif, L.J., Lu, Z., Jung, K., 2015. Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs. Vet Immunol Immunopathol 168, 193-202.

Barber, G.N., 2011. Cytoplasmic DNA innate immune pathways. Immunol Rev 243, 99-108.

Belouzard, S., Chu, V.C., Whittaker, G.R., 2009. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. Proc Natl Acad Sci U S A 106, 5871-5876.

Bjustrom-Kraft, J., Woodard, K., Gimenez-Lirola, L., Rotolo, M., Wang, C., Sun, Y., Lasley, P., Zhang, J., Baum, D., Gauger, P., Main, R., Zimmerman, J., 2016. Porcine epidemic diarrhea virus (PEDV) detection and antibody response in commercial growing pigs. BMC Vet Res 12, 99.

Boletta, A., Benigni, A., Lutz, J., Remuzzi, G., Soria, M.R., Monaco, L., 1997. Nonviral gene delivery to the rat kidney with polyethylenimine. Hum Gene Ther 8, 1243-1251.

Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J Virol 77, 8801-8811.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 92, 7297-7301.

Bragstad, K., Vinner, L., Hansen, M.S., Nielsen, J., Fomsgaard, A., 2013. A polyvalent influenza A DNA vaccine induces heterologous immunity and protects pigs against pandemic A(H1N1)pdm09 virus infection. Vaccine 31, 2281-2288.

Cao, L., Ge, X., Gao, Y., Herrler, G., Ren, Y., Ren, X., Li, G., 2015. Porcine epidemic diarrhea virus inhibits dsRNA-induced interferon-beta production in porcine intestinal epithelial cells by blockade of the RIG-I-mediated pathway. Virol J 12, 127.

Carvajal, A., Lanza, I., Diego, R., Rubio, P., Carmenes, P., 1995. Evaluation of a blocking ELISA using monoclonal antibodies for the detection of porcine epidemic diarrhea virus and its antibodies. J Vet Diagn Invest 7, 60-64.

Chang, C.K., Sue, S.C., Yu, T.H., Hsieh, C.M., Tsai, C.K., Chiang, Y.C., Lee, S.J., Hsiao, H.H., Wu, W.J., Chang, W.L., Lin, C.H., Huang, T.H., 2006. Modular organization of SARS coronavirus nucleocapsid protein. J Biomed Sci 13, 59-72.

Chang, S.H., Bae, J.L., Kang, T.J., Kim, J., Chung, G.H., Lim, C.W., Laude, H., Yang, M.S., Jang, Y.S., 2002. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. Mol Cells 14, 295-299.

Chasey, D., Cartwright, S.F., 1978. Virus-like particles associated with porcine epidemic diarrhoea. Res Vet Sci 25, 255-256.

Chattha, K.S., Roth, J.A., Saif, L.J., 2015. Strategies for design and application of enteric viral vaccines. Annu Rev Anim Biosci 3, 375-395.

Chen, Q., Gauger, P.C., Stafne, M.R., Thomas, J.T., Madson, D.M., Huang, H., Zheng, Y., Li, G., Zhang, J., 2016. Pathogenesis comparison between the United States porcine epidemic diarrhoea virus prototype and S-INDEL-variant strains in conventional neonatal piglets. J Gen Virol 97, 1107-1121.

Chen, Q., Li, G., Stasko, J., Thomas, J.T., Stensland, W.R., Pillatzki, A.E., Gauger, P.C., Schwartz, K.J., Madson, D., Yoon, K.J., Stevenson, G.W., Burrough, E.R., Harmon, K.M., Main, R.G., Zhang, J., 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol 52, 234-243.

Chen, X., Zeng, L., Yang, J., Yu, F., Ge, J., Guo, Q., Gao, X., Song, T., 2013. Sequence heterogeneity of the ORF3 gene of porcine epidemic diarrhea viruses field samples in Fujian, China, 2010-2012. Viruses 5, 2375-2383.

Cheng, P.K., Wong, D.A., Tong, L.K., Ip, S.M., Lo, A.C., Lau, C.S., Yeung, E.Y., Lim, W.W., 2004. Viral shedding patterns of coronavirus in patients with probable severe acute respiratory syndrome. Lancet 363, 1699-1700.

Chiu, Y.H., Macmillan, J.B., Chen, Z.J., 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell 138, 576-591.

Cho, Y.Y., Lim, S.I., Kim, Y.K., Song, J.Y., Lee, J.B., An, D.J., 2014. Complete Genome Sequence of K14JB01, a Novel Variant Strain of Porcine Epidemic Diarrhea Virus in South Korea. Genome Announc 2.

Cima, G., 2013. Viral disease affects U.S. pigs: porcine epidemic diarrhea found in at least 11 states. J Am Vet Med Assoc 243, 30-31.

Cima, G., 2014. PED virus reinfecting U.S. herds. Virus estimated to have killed 7 million-plus pigs. J Am Vet Med Assoc 245, 166-167.

Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H., Doerr, H.W., 2003. Treatment of SARS with human interferons. Lancet 362, 293-294.

Coban, C., Kobiyama, K., Jounai, N., Tozuka, M., Ishii, K.J., 2013. DNA vaccines: a simple DNA sensing matter? Hum Vaccin Immunother 9, 2216-2221.

Collins, A.R., Knobler, R.L., Powell, H., Buchmeier, M.J., 1982. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell--cell fusion. Virology 119, 358-371.

Cornelissen, L.A., Wierda, C.M., van der Meer, F.J., Herrewegh, A.A., Horzinek, M.C., Egberink, H.F., de Groot, R.J., 1997. Hemagglutinin-esterase, a novel structural protein of torovirus. J Virol 71, 5277-5286.

Cruz, D.J., Kim, C.J., Shin, H.J., 2006. Phage-displayed peptides having antigenic similarities with porcine epidemic diarrhea virus (PEDV) neutralizing epitopes. Virology 354, 28-34.

Cruz, D.J., Kim, C.J., Shin, H.J., 2008. The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize Porcine epidemic diarrhea virus. Virus Res 132, 192-196.

de Arriba, M.L., Carvajal, A., Pozo, J., Rubio, P., 2002a. Lymphoproliferative responses and protection in conventional piglets inoculated orally with virulent or attenuated porcine epidemic diarrhoea virus. J Virol Methods 105, 37-47.

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

de Haan, C.A., Rottier, P.J., 2005. Molecular interactions in the assembly of coronaviruses. Adv Virus Res 64, 165-230.

Debouck, P., Pensaert, M., 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV 777. Am J Vet Res 41, 219-223.

Dee, S., Clement, T., Schelkopf, A., Nerem, J., Knudsen, D., Christopher-Hennings, J., Nelson, E., 2014. An evaluation of contaminated complete feed as a vehicle for porcine epidemic diarrhea virus infection of naive pigs following consumption via natural feeding behavior: proof of concept. BMC Vet Res 10, 176.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjostrom, H., Noren, O., Laude, H., 1992. Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. Nature 357, 417-420.

Demeneix, B., Behr, J., Boussif, O., Zanta, M.A., Abdallah, B., Remy, J., 1998. Gene transfer with lipospermines and polyethylenimines. Adv Drug Deliv Rev 30, 85-95.

Desmet, C.J., Ishii, K.J., 2012. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol 12, 479-491.

Dhama, K., Mahendran, M., Gupta, P.K., Rai, A., 2008. DNA vaccines and their applications in veterinary practice: current perspectives. Vet Res Commun 32, 341-356.

Ding, Z., Fang, L., Jing, H., Zeng, S., Wang, D., Liu, L., Zhang, H., Luo, R., Chen, H., Xiao, S., 2014. Porcine epidemic diarrhea virus nucleocapsid protein antagonizes beta interferon production by sequestering the interaction between IRF3 and TBK1. J Virol 88, 8936-8945.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29, 1969-1973.

Ducatelle, R., Coussement, W., Debouck, P., Hoorens, J., 1982. Pathology of experimental CV777 coronavirus enteritis in piglets. II. Electron microscopic study. Vet Pathol 19, 57-66.

Fehr, A.R., Perlman, S., 2015. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol Biol 1282, 1-23.

Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Botner, A., 2002. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38-47.

Franco, M.A., Greenberg, H.B., 1999. Immunity to rotavirus infection in mice. J Infect Dis 179 Suppl 3, S466-469.

Gao, Y., Kou, Q., Ge, X., Zhou, L., Guo, X., Yang, H., 2013. Phylogenetic analysis of porcine epidemic diarrhea virus field strains prevailing recently in China. Arch Virol 158, 711-715.

Godet, M., L'Haridon, R., Vautherot, J.F., Laude, H., 1992. TGEV corona virus ORF4 encodes a membrane protein that is incorporated into virions. Virology 188, 666-675.

Goula, D., Remy, J.S., Erbacher, P., Wasowicz, M., Levi, G., Abdallah, B., Demeneix, B.A., 1998. Size, diffusibility and transfection performance of linear PEI/DNA complexes in the mouse central nervous system. Gene Ther 5, 712-717.

Guimaraes, V.D., Innocentin, S., Lefevre, F., Azevedo, V., Wal, J.M., Langella, P., Chatel, J.M., 2006. Use of native lactococci as vehicles for delivery of DNA into mammalian epithelial cells. Appl Environ Microbiol 72, 7091-7097.

Ha, Y., Shin, J.H., Chae, C., 2010. Colostral transmission of porcine circovirus 2 (PCV-2): reproduction of post-weaning multisystemic wasting syndrome in pigs fed milk from PCV-2-infected sows with post-natal porcine parvovirus infection or immunostimulation. J Gen Virol 91, 1601-1608.

Hao, J., Xue, C., He, L., Wang, Y., Cao, Y., 2014. Bioinformatics insight into the spike glycoprotein gene of field porcine epidemic diarrhea strains during 2011-2013 in Guangdong, China. Virus Genes 49, 58-67.

Hofmann, H., Pyrc, K., van der Hoek, L., Geier, M., Berkhout, B., Pohlmann, S., 2005. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proc Natl Acad Sci U S A 102, 7988-7993.

Hofmann, M., Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. J Clin Microbiol 26, 2235-2239.

Hofmann, M., Wyler, R., 1989. Quantitation, biological and physicochemical properties of cell culture-adapted porcine epidemic diarrhea coronavirus (PEDV). Vet Microbiol 20, 131-142.

Huang, Y.W., Dickerman, A.W., Pineyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig, T., Meng, X.J., 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio 4, e00737-00713.

Hurst, K.R., Koetzner, C.A., Masters, P.S., 2009. Identification of in vivo-interacting domains of the murine coronavirus nucleocapsid protein. J Virol 83, 7221-7234.

Ingolotti, M., Kawalekar, O., Shedlock, D.J., Muthumani, K., Weiner, D.B., 2010. DNA vaccines for targeting bacterial infections. Expert Rev Vaccines 9, 747-763.

Ishii, K.J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., Sato, S., Yamamoto, M., Uematsu, S., Kawai, T., Takeuchi, O., Akira, S., 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol 7, 40-48.

Ishii, K.J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., Uematsu, S., Takeuchi, O., Takeshita, F., Coban, C., Akira, S., 2008. TANK-binding kinase-1

delineates innate and adaptive immune responses to DNA vaccines. Nature 451, 725-729.

Ishikawa, H., Ma, Z., Barber, G.N., 2009. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 461, 788-792.

Jung, K., Ha, Y., Ha, S.K., Kim, J., Choi, C., Park, H.K., Kim, S.H., Chae, C., 2006. Identification of porcine circovirus type 2 in retrospective cases of pigs naturally infected with porcine epidemic diarrhoea virus. Vet J 171, 166-168.

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

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

Jung, K., Wang, Q., Scheuer, K.A., Lu, Z., Zhang, Y., Saif, L.J., 2014. Pathology of US porcine epidemic diarrhea virus strain PC21A in gnotobiotic pigs. Emerg Infect Dis 20, 662-665.

Kang, T.J., Han, S.C., Yang, M.S., Jang, Y.S., 2006. Expression of synthetic neutralizing epitope of porcine epidemic diarrhea virus fused with synthetic B subunit of Escherichia coli heat-labile enterotoxin in tobacco plants. Protein Expr Purif 46, 16-22.

Karlsson, I., Borggren, M., Rosenstierne, M.W., Trebbien, R., Williams, J.A., Vidal, E., Vergara-Alert, J., Foz, D.S., Darji, A., Sistere-Oro, M., Segales, J., Nielsen, J., Fomsgaard, A., 2018. Protective effect of a polyvalent influenza DNA vaccine in pigs. Vet Immunol Immunopathol 195, 25-32.

Klausegger, A., Strobl, B., Regl, G., Kaser, A., Luytjes, W., Vlasak, R., 1999. Identification of a coronavirus hemagglutinin-esterase with a substrate specificity different from those of influenza C virus and bovine coronavirus. J Virol 73, 3737-3743.

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

Kodihalli, S., Goto, H., Kobasa, D.L., Krauss, S., Kawaoka, Y., Webster, R.G., 1999. DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. J Virol 73, 2094-2098.

Krijnse-Locker, J., Ericsson, M., Rottier, P.J., Griffiths, G., 1994. Characterization of the budding compartment of mouse hepatitis virus: evidence that transport from the RER to the Golgi complex requires only one vesicular transport step. J Cell Biol 124, 55-70.

Kubo, H., Yamada, Y.K., Taguchi, F., 1994. Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus spike protein. J Virol 68, 5403-5410.

Kutzler, M.A., Weiner, D.B., 2008. DNA vaccines: ready for prime time? Nat Rev Genet 9, 776-788.

Kweon, C.H., Kwon, B.J., Jung, T.S., Kee, Y.J., Hur, D.H., Hwang, E.K., Rhee, J.C., An, S.H., 1993. Isolation of porcine epidemic diarrhea virus (PEDV) infection in Korea. Korea J Vet Res 33, 249–254.

Kweon, C.H., Kwon, B.J., Lee, J.G., Kwon, G.O., Kang, Y.B., 1999. Derivation of attenuated porcine epidemic diarrhea virus (PEDV) as vaccine candidate. Vaccine 17, 2546-2553.

Lee, D.K., Park, C.K., Kim, S.H., Lee, C., 2010. Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea. Virus Res 149, 175-182.

Lee, S., Kim, Y., Lee, C., 2015. Isolation and characterization of a Korean porcine epidemic diarrhea virus strain KNU-141112. Virus Res 208, 215-224.

Li, B.X., Ge, J.W., Li, Y.J., 2007. Porcine aminopeptidase N is a functional receptor for the PEDV coronavirus. Virology 365, 166-172.

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

Li, W., Luo, R., He, Q., van Kuppeveld, F.J., Rottier, P.J., Bosch, B.J., 2017. Aminopeptidase N is not required for porcine epidemic diarrhea virus cell entry. Virus Res.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426, 450-454.

Lin, C.N., Chung, W.B., Chang, S.W., Wen, C.C., Liu, H., Chien, C.H., Chiou, M.T., 2014. US-like strain of porcine epidemic diarrhea virus outbreaks in Taiwan, 2013-2014. J Vet Med Sci 76, 1297-1299.

Lin, H.X., Ma, Z., Fan, H.J., Lu, C.P., 2012. Construction and immunogenicity of recombinant swinepox virus expressing capsid protein of PCV2. Vaccine 30, 6307-6313.

Lowe, J., Gauger, P., Harmon, K., Zhang, J., Connor, J., Yeske, P., Loula, T., Levis, I., Dufresne, L., Main, R., 2014. Role of transportation in spread of porcine epidemic diarrhea virus infection, United States. Emerg Infect Dis 20, 872-874.

Lutz, C.S., 2008. Alternative polyadenylation: a twist on mRNA 3' end formation. ACS Chem Biol 3, 609-617.

Madson, D.M., Magstadt, D.R., Arruda, P.H., Hoang, H., Sun, D., Bower, L.P., Bhandari, M., Burrough, E.R., Gauger, P.C., Pillatzki, A.E., Stevenson, G.W., Wilberts, B.L., Brodie, J., Harmon, K.M., Wang, C., Main, R.G., Zhang, J., Yoon, K.J., 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol 174, 60-68.

McNeal, M.M., Stone, S.C., Basu, M., Bean, J.A., Clements, J.D., Hendrickson, B.A., Choi, A.H., Ward, R.L., 2006. Protection against rotavirus shedding after intranasal immunization of mice with a chimeric VP6 protein does not require intestinal IgA. Virology 346, 338-347.

McNeal, M.M., VanCott, J.L., Choi, A.H., Basu, M., Flint, J.A., Stone, S.C., Clements, J.D., Ward, R.L., 2002. CD4 T cells are the only lymphocytes needed to protect mice against rotavirus shedding after intranasal immunization with a chimeric VP6 protein and the adjuvant LT(R192G). J Virol 76, 560-568.

Meseda, C.A., Atukorale, V., Soto, J., Eichelberger, M.C., Gao, J., Wang, W., Weiss, C.D., Weir, J.P., 2018. Immunogenicity and Protection Against Influenza H7N3 in Mice by Modified Vaccinia Virus Ankara Vectors Expressing Influenza Virus Hemagglutinin or Neuraminidase. Sci Rep 8, 5364.

Meunier, M., Guyard-Nicodeme, M., Vigouroux, E., Poezevara, T., Beven, V., Quesne, S., Amelot, M., Parra, A., Chemaly, M., Dory, D., 2018. A DNA prime/protein boost vaccine protocol developed against Campylobacter jejuni for poultry. Vaccine 36, 2119-2125.

Nagy, B., Nagy, G., Meder, M., Mocsari, E., 1996. Enterotoxigenic Escherichia coli, rotavirus, porcine epidemic diarrhoea virus, adenovirus and calici-like virus in porcine postweaning diarrhoea in Hungary. Acta Vet Hung 44, 9-19.

Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 3, 418-426.

Neuman, B.W., Kiss, G., Kunding, A.H., Bhella, D., Baksh, M.F., Connelly, S., Droese, B., Klaus, J.P., Makino, S., Sawicki, S.G., Siddell, S.G., Stamou, D.G., Wilson, I.A., Kuhn, P., Buchmeier, M.J., 2011. A structural analysis of M protein in coronavirus assembly and morphology. J Struct Biol 174, 11-22.

Ojkic, D., Hazlett, M., Fairles, J., Marom, A., Slavic, D., Maxie, G., Alexandersen, S., Pasick, J., Alsop, J., Burlatschenko, S., 2015. The first case of porcine epidemic diarrhea in Canada. Can Vet J 56, 149-152.

Olanratmanee, E.O., Kunavongkrit, A., Tummaruk, P., 2010. Impact of porcine epidemic diarrhea virus infection at different periods of pregnancy on subsequent reproductive performance in gilts and sows. Anim Reprod Sci 122, 42-51.

Ouyang, K., Shyu, D.L., Dhakal, S., Hiremath, J., Binjawadagi, B., Lakshmanappa, Y.S., Guo, R., Ransburgh, R., Bondra, K.M., Gauger, P., Zhang, J., Specht, T., Gilbertie, A., Minton, W., Fang, Y., Renukaradhya, G.J., 2015. Evaluation of humoral immune status in porcine epidemic diarrhea virus (PEDV) infected sows under field conditions. Vet Res 46, 140.

Park, J.S., Ha, Y., Kwon, B., Cho, K.D., Lee, B.H., Chae, C., 2009. Detection of porcine circovirus 2 in mammary and other tissues from experimentally infected sows. J Comp Pathol 140, 208-211.

Park, K.S., Seo, Y.B., Lee, J.Y., Im, S.J., Seo, S.H., Song, M.S., Choi, Y.K., Sung, Y.C., 2011a. Complete protection against a H5N2 avian influenza virus by a DNA vaccine expressing a fusion protein of H1N1 HA and M2e. Vaccine 29, 5481-5487.

Park, S.J., Kim, H.K., Song, D.S., Moon, H.J., Park, B.K., 2011b. Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) field isolates in Korea. Arch Virol 156, 577-585.

Park, S.J., Moon, H.J., Yang, J.S., Lee, C.S., Song, D.S., Kang, B.K., Park, B.K., 2007. Sequence analysis of the partial spike glycoprotein gene of porcine epidemic diarrhea viruses isolated in Korea. Virus Genes 35, 321-332.

Park, S.J., Song, D.S., Park, B.K., 2013. Molecular epidemiology and phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) field isolates in Korea. Arch Virol 158, 1533-1541.

Parvatiyar, K., Zhang, Z., Teles, R.M., Ouyang, S., Jiang, Y., Iyer, S.S., Zaver, S.A., Schenk, M., Zeng, S., Zhong, W., Liu, Z.J., Modlin, R.L., Liu, Y.J., Cheng, G., 2012. The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. Nat Immunol 13, 1155-1161.

Patnaik, S., Aggarwal, A., Nimesh, S., Goel, A., Ganguli, M., Saini, N., Singh, Y., Gupta, K.C., 2006. PEI-alginate nanocomposites as efficient in vitro gene transfection agents. J Control Release 114, 398-409.

Pensaert, M.B., de Bouck, P., 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch Virol 58, 243-247.

Pijpers, A., van Nieuwstadt, A.P., Terpstra, C., Verheijden, J.H., 1993. Porcine epidemic diarrhoea virus as a cause of persistent diarrhoea in a herd of breeding and finishing pigs. Vet Rec 132, 129-131.

Pritchard, G.C., Paton, D.J., Wibberley, G., Ibata, G., 1999. Transmissible gastroenteritis and porcine epidemic diarrhoea in Britain. Vet Rec 144, 616-618.

Puranaveja, S., Poolperm, P., Lertwatcharasarakul, P., Kesdaengsakonwut, S., Boonsoongnern, A., Urairong, K., Kitikoon, P., Choojai, P., Kedkovid, R., Teankum, K., Thanawongnuwech, R., 2009. Chinese-like strain of porcine epidemic diarrhea virus, Thailand. Emerg Infect Dis 15, 1112-1115.

Reed, L.J., Muench, H., 1938. A Simple Method of Estimating Fifty Percent Endpoints. Am. J. Epidemiol 27, 493-497.

Reyes-Sandoval, A., Ertl, H.C., 2001. DNA vaccines. Curr Mol Med 1, 217-243.

Rottembourg, D., Filippi, C.M., Bresson, D., Ehrhardt, K., Estes, E.A., Oldham, J.E., von Herrath, M.G., 2010. Essential role for TLR9 in prime but not prime-boost plasmid DNA vaccination to activate dendritic cells and protect from lethal viral infection. J Immunol 184, 7100-7107.

Saif, L.J., 1999. Enteric viral infections of pigs and strategies for induction of mucosal immunity. Adv Vet Med 41, 429-446.

Saif, L.J., 2004. Animal coronavirus vaccines: lessons for SARS. Dev Biol (Basel) 119, 129-140.

Saif, L.J., Pensaert, M.P., Sestak, K., Yeo, S.G., Jung, K., 2012. Coronaviruses. In: Diseases of Swine. Wiley-Blackwell, Iowa university. Ames. IA. USA.

Sato, T., Takeyama, N., Katsumata, A., Tuchiya, K., Kodama, T., Kusanagi, K., 2011. Mutations in the spike gene of porcine epidemic diarrhea virus associated with growth adaptation in vitro and attenuation of virulence in vivo. Virus Genes 43, 72-78.

Shibata, I., Ono, M., Mori, M., 2001. Passive protection against porcine epidemic diarrhea (PED) virus in piglets by colostrum from immunized cows. J Vet Med Sci 63, 655-658.

Shu, C., Li, X., Li, P., 2014. The mechanism of double-stranded DNA sensing through the cGAS-STING pathway. Cytokine Growth Factor Rev 25, 641-648.

Song, D., Park, B., 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes 44, 167-175.

Song, D.S., Oh, J.S., Kang, B.K., Yang, J.S., Moon, H.J., Yoo, H.S., Jang, Y.S., Park, B.K., 2007. Oral efficacy of Vero cell attenuated porcine epidemic diarrhea virus DR13 strain. Res Vet Sci 82, 134-140.

Srijangwad, A., Nilubol, D., Chongcharoen, W., Phoolcharoen, W., Chuanasa, T., Tantituvanon, A., 2015. Production of spike and nucleocapsid recombinant proteins of porcine epidemic diarrhea virus for antibody detection by ELISA. Asia J Pharm Sci [Article in press].

Srinuntapunt, S., Trongwongsa, L., Antarasena, C., Sangsuwan, W., Prommuang, P., 1995. Porcine epidemic diarrhea in Trang province. J. Thai. Vet. Med. Assoc 46, 11-19.

Stadler, J., Moser, L., Numberger, J., Rieger, A., Strutzberg-Minder, K., Stellberger, T., Ladinig, A., Ritzmann, M., Fux, R., 2018. Investigation of three outbreaks of Porcine Epidemic Diarrhea in Germany in 2016 demonstrates age dependent differences in the development of humoral immune response. Prev Vet Med 150, 93-100.

Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest 25, 649-654.

Sueyoshi, M., Tsuda, T., Yamazaki, K., Yoshida, K., Nakazawa, M., Sato, K., Minami, T., Iwashita, K., Watanabe, M., Suzuki, Y., et al., 1995. An immunohistochemical investigation of porcine epidemic diarrhoea. J Comp Pathol 113, 59-67.

Sukumaran, J., Holder, M.T., 2010. DendroPy: a Python library for phylogenetic computing. Bioinformatics 26, 1569-1571.

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

Sun, D.B., Feng, L., Shi, H.Y., Chen, J.F., Liu, S.W., Chen, H.Y., Wang, Y.F., 2007. Spike protein region (aa 636789) of porcine epidemic diarrhea virus is essential for induction of neutralizing antibodies. Acta Virol 51, 149-156.

Sun, L., Wu, J., Du, F., Chen, X., Chen, Z.J., 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339, 786-791.

Sun, R., Leng, Z., Zhai, S.L., Chen, D., Song, C., 2014. Genetic variability and phylogeny of current Chinese porcine epidemic diarrhea virus strains based on spike, ORF3, and membrane genes. Sci World J 2014, 208439.

Sun, R.Q., Cai, R.J., Chen, Y.Q., Liang, P.S., Chen, D.K., Song, C.X., 2012. Outbreak of porcine epidemic diarrhea in suckling piglets, China. Emerg Infect Dis 18, 161-163.

Suradhat, S., Wongyanin, P., Kesdangsakonwut, S., Teankum, K., Lumyai, M., Triyarach, S., Thanawongnuwech, R., 2015. A novel DNA vaccine for reduction of PRRSV-induced negative immunomodulatory effects: A proof of concept. Vaccine 33, 3997-4003.

Suradhat, S., Wongyanin, P., Sirisereewan, C., Nedumpun, T., Lumyai, M., Triyarach, S., Chaturavittawong, D., Paphavasit, T., Panyatong, R., Thanawongnuwech, R., 2016. Transdermal delivery of plasmid encoding truncated nucleocapsid protein enhanced PRRSV-specific immune responses. Vaccine 34, 609-615.

Takahashi, K., Okada, K., Ohshima, K., 1983. An outbreak of swine diarrhea of a new-type associated with coronavirus-like particles in Japan. Nihon Juigaku Zasshi 45, 829-832.

Temeeyasen, G., Sinha, A., Gimenez-Lirola, L.G., Zhang, J.Q., Pineyro, P.E., 2018. Differential gene modulation of pattern-recognition receptor TLR and RIG-I-like and downstream mediators on intestinal mucosa of pigs infected with PEDV non S-INDEL and PEDV S-INDEL strains. Virology 517, 188-198.

Thomas, J.T., Chen, Q., Gauger, P.C., Gimenez-Lirola, L.G., Sinha, A., Harmon, K.M., Madson, D.M., Burrough, E.R., Magstadt, D.R., Salzbrenner, H.M., Welch, M.W., Yoon, K.J., Zimmerman, J.J., Zhang, J., 2015. Effect of Porcine Epidemic Diarrhea

Virus Infectious Doses on Infection Outcomes in Naive Conventional Neonatal and Weaned Pigs. PLoS One 10, e0139266.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-4680.

Tsuchida, T., Zou, J., Saitoh, T., Kumar, H., Abe, T., Matsuura, Y., Kawai, T., Akira, S., 2010. The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. Immunity 33, 765-776.

Wang, L., Junker, D., Collisson, E.W., 1993. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virology 192, 710-716.

Wood, E.N., 1977. An apparently new syndrome of porcine epidemic diarrhoea. Vet Rec 100, 243-244.

Xu, J., Huang, D., Liu, S., Lin, H., Zhu, H., Liu, B., Lu, C., 2012a. Immune responses and protective efficacy of a recombinant swinepox virus expressing HA1 against swine H1N1 influenza virus in mice and pigs. Vaccine 30, 3119-3125.

Xu, X.G., Zhao, H.N., Zhang, Q., Ding, L., Li, Z.C., Li, W., Wu, H.Y., Chuang, K.P., Tong, D.W., Liu, H.J., 2012b. Oral vaccination with attenuated Salmonella enterica serovar Typhimurium expressing Cap protein of PCV2 and its immunogenicity in mouse and swine models. Vet Microbiol 157, 294-303.

Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellichio, C.B., Shapiro, L.H., Look, A.T., Holmes, K.V., 1992. Human aminopeptidase N is a receptor for human coronavirus 229E. Nature 357, 420-422.

Yuan, L., Azevedo, M.S., Gonzalez, A.M., Jeong, K.I., Van Nguyen, T., Lewis, P., Iosef, C., Herrmann, J.E., Saif, L.J., 2005. Mucosal and systemic antibody responses and protection induced by a prime/boost rotavirus-DNA vaccine in a gnotobiotic pig model. Vaccine 23, 3925-3936.

Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E., 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J Gen Virol 81, 853-8

APPENDIX

REAGENT AND BUFFER PREPARATION

Phosphate buffer saline (PBS) 10X

Sodium chloride (NaCl)	80 g
Disodium orthophosphate (Na_2HPO_4)	11.5 g
Potassium chloride (KCl)	2 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	2 g
Distilled water to	1000 ml

MEM complete media

Fetal bovine serum	5 ml
100X L-glutamine	1 ml
100X antibiotics/ antimycotic	1 ml
MEM to	100 ml

Luria-Bertani (LB) broth

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Distilled water to	1000 ml
(For LB agar, added 17 g of agar)	

Bis-acrylamind 30%

Acrylamide	29 g
Bis-acrylamind	1 g
Distilled water to	100 ml

SDS dye buffer

Trisma base	0.15 g

Sodium dodecyl sulfate (SDS)	0.4 g
2-mercaptoethanol	1 ml
Glycerol	2 ml
Bromophenol blue	0.02 g
Distilled water to	10 ml

APS (10%)

Ammoniumpersulfate	1 g
Distilled water to	10 ml

SDS (10%)

SDS	10 g
Distilled water to	100 ml

Running buffer 10X

Glycine	69.75 g
Trisma base	15 g
SDS	5 g
Distilled water to	500 ml

Tris-HCl 1.125M pH 8.8

Trisma base	13.62 g
Distilled water to	100 ml

Tris-HCl 0.625M pH 6.8

Trisma base	6.05
Distilled water to	100 ml

Comassie brilliant blue (CBB)

Comassie brilliant blue (R250)	4.85 g

Methanol	900 ml
Acetic acid	180 ml
Distilled water	900 ml

Destaining buffer

Methanol	50 ml
Acetic acid	75 ml
Distilled water	875 ml

12% separating gel

Distilled water	4.9 ml
30% Bis-acrylamind	6.0 ml
Tris-HCl pH 8.8	3.8 ml
10% SDS	0.15 ml
10% APS	0.15 ml
TEMED	0.006 ml

5% stacking gel

Distilled water	3.4 ml
30% Bis-acrylamind	0.83 ml
Tris-HCl pH 8.8	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

Transfer buffer

Glycine	28.8 g
Trisma base	6 g
SDS	2 g
Methanol	400 ml
Distilled water	1600 ml

Washing buffer (0.5% PBST)

Tween 20	0.5 ml
PBS 1X	100 ml

Blocking buffer (for western blot)

Skim milk	5 g
0.5% PBST	100 ml

Washing buffer (0.05% PBST)

Tween 20	0.05 ml
PBS 1X	100 ml

Blocking buffer (for ELISA)

Skim milk	5 g
0.05% PBST	100 ml

RPMI complete media

Fetal bovine serum	10 ml
100X L-glutamine	5 ml
100X antibiotic/ antimycotic	5 ml
HEPES	12.5 ml
50 mM 2-mercaptoethanol	0.5 ml
Advance RPMI-1640 to	500 ml

1 M 2-mercaptoethanol

14.2 M 2- mercaptoethanol	0.7 ml
Distilled water	9.3 ml

50 mM 2-mercaptoethanol

1 M 2-mercaptoethanol	0.25 ml

Distilled water	4.75 ml

FACS buffer

Fetal bovine serum	1 ml
Sodium azide (NaN_3)	0.5 g
PBS 1X to	500 ml

Formaldehyde (2%)

Formaldehyde (37%)	27 ml
PBS 1X	473 ml

VITA

Mr. Gun Temeeyasen was born on March 10th, 1985 at Buriram province, Thailand. He graduated Doctor of Veterinary Medicine from Chulalongkorn University, Thailand in 2009. After graduation, he worked as a veterinarian in Veterinary Diagnostic Laboratory of Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University for 1 year. Then, he worked as a swine veterinarian in Thai Food Group for 1 year. After that, he pursue his PhD at the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. His field of interest was swine virology and vaccinology especially in PEDV and PRRSV.