การติดเชื้อร่วมในสุกรอนุบาลระว่างเชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงกับเชื้อไวรัสไข้หวัดสุกร สายพันธุ์ใหม่ที่แยกได้จากสุกรในประเทศไทย

นายนัทธพงศ์ แสงประทุม



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

# DUAL INFECTION BETWEEN A THAI ISOLATE HIGHLY PATHOGENIC PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND PANDEMIC H1N1 SWINE INFLUENZA VIRUS IN WEANLING PIGS

Mr. Nattaphong Sangpratum



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2017

| | |
|---|---|
| Thesis Title | DUAL INFECTION BETWEEN A THAI ISOLATE HIGHLY PATHOGENIC PORCINEREPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND PANDEMIC H1N1SWINE INFLUENZA VIRUS IN WEANLING PIGS |
| By | Mr. Nattaphong Sangpratum |
| Field of Study | Veterinary Pathobiology |
| Thesis Advisor | Professor Doctor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP |

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science

(Professor Doctor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP)

THESIS COMMITTEE

.....Chairman

(Associate Professor Doctor Theerayuth Kaewamatawong, D.V.M., Ph.D., DTBVP)

.....Thesis Advisor

(Professor Doctor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP)

.....Examiner

(Assistant Professor Doctor Sawang Kesdangsakonwut, D.V.M., M.Sc., Ph.D.,DTBVP)

.....Examiner

(Assistant Professor Docotr Aunyaratana Thontiravong, D.V.M., Ph.D.)

.....External Examiner

(Doctor Suphattra Jittimanee, D.V.M., Ph.D., DTBVP)

นัทธพงศ์ แสงประทุม : การติดเชื้อร่วมในสุกรอนุบาลระว่างเชื้อไวรัสพีอาร์อาร์เอสสายพันธุ์รุนแรงกับเชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่ที่แยกได้จากสุกรในประเทศไทย (DUAL INFECTION BETWEEN A THAI ISOLATE HIGHLY PATHOGENIC PORCINEREPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND PANDEMIC H1N1SWINE INFLUENZA VIRUS IN WEANLING PIGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. รุ่งโรจน์ ธนาวงษ์นุเวชD.V.M., M.Sc., Ph.D., DTBVP, 40 หน้า.

ในปี ค.ศ. 2009 ได้มีเชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่ เกิดการระบาดในฟาร์มสุกรของประเทศไทย ซึ่งบทบาทของเชื้อไวรัสดังกล่าวในกลุ่มโรคทางเดินหายใจของสุกรนั้นยังไม่แน่ชัด อันเนื่องมาจากอาการแสดงทางคลินิกของสุกรที่ไม่รุนแรง ในทางตรงกันข้ามเชื้อไวรัสพีอาร์อาร์เอสชนิดสายพันธุ์รุนแรง ที่เป็นเชื้อประจำถิ่นอีกโรคหนึ่งในฟาร์มสุกร เป็นเชื้อที่มีความรุนแรงของโรคสูง รวมทั้งมีความสามารถในการกดภูมิคุ้มกัน ทำให้สุกรที่ติดเชื้อมีความไวรับต่อการติดเชื้อแทรกซ้อนเพิ่มมากขึ้น การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความรุนแรงในการก่อโรคของเชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่ เมื่อทำการติดเชื้อร่วมกับเชื้อไวรัสพีอาร์อาร์เอส ชนิดสายพันธุ์รุนแรง โดยสุกรทั้งหมด 32 ตัวที่ใช้ในการทดลองนั้นได้นำมาจากฟาร์มสุกรที่ปลอดโรคเชื้อไวรัสไข้หวัดสุกร, เชื้อพีอาร์อาร์เอส, เชื้อเซอร์โคไวรัสชนิดที่ 2 และเชื้อมัยโคพลาสมา แบ่งกลุ่มโดยใช้วิธีการสุ่มเป็น 4 กลุ่ม คือ กลุ่มควบคุม, กลุ่มที่ให้เชื้อพีอาร์อาร์เอสเพียงชนิดเดียว (PRRSV), กลุ่มที่ให้เชื้อไวรัสไข้หวัดสุกรเพียงชนิดเดียว (SIV) และกลุ่มติดเชื้อร่วม (Co-infection) โดยสุกรในกลุ่ม PRRSV และ Co-infection จะถูกให้เชื้อ HP-PRRSV ที่แยกได้จากสุกรที่ติดเชื้อในประเทศไทย ทางจมูกในวันที่ 0 ต่อมาสุกรในกลุ่ม SIV และ Co-infection จะถูกให้เชื้อ pdmH1N1 SIV ในวันที่ 6 ทางท่อลม จากนั้นทำการชันสูตรซากในวันที่ 2 และ 4 วันหลังจากให้เชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่ ซึ่งการเปรียบเทียบคะแนนรอยโรคปอดระหว่างกลุ่ม PRRSV และ Co-infection ในวันที่ 2 dsc นั้นไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ในขณะวันที่ 4 dsc นั้น คะแนนรอยโรคปอดของสุกรในกลุ่ม Co-infection มีความรุนแรงมากกว่าสุกรในกลุ่ม PRRSV อย่างมีนัยสำคัญทางสถิติ นอกจากนี้พบว่าปริมาณเชื้อ SIV ในเนื้อเยื่อปอด ในสุกรกลุ่ม Co-infection มีปริมารมากกว่าสุกรในกลุ่ม SIV อย่างเดียวอย่างมีนัยสำคัยทางสถิติ โดยการศึกษานี้สรุปว่า pdmH1N1 SIV ที่มีความสามารถในการก่อโรคด้วยตนเองต่ำ แต่สามารถก่อความรุนแรงของโรคที่ปอดเพิ่มมากขึ้นได้เมื่อทำการติดเชื้อร่วมกันกับ HP-PRRSV ซึ่งนำไปสู่ภาวะโรคระบบทางเดินหายใจแบบซับซ้อนได้ภายหลัง



ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

# # 5975303831 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: CO-INFECTION / HIGHLY PATHOGENIC PRRSV / PANDEMIC H1N1 2009 / PATHOGENESIS / SWINE INFLUENZA VIRUS

NATTAPHONG SANGPRATUM: DUAL INFECTION BETWEEN A THAI ISOLATE HIGHLY PATHOGENIC PORCINEREPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND PANDEMIC H1N1SWINE INFLUENZA VIRUS IN WEANLING PIGS. ADVISOR: PROF. DR. ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D., DTBVP, 40 pp.

After an introduction of pandemic H1N1 swine influenza virus (pdmH1N1 SIV) in the Thai swine farms, mild clinical outcome is observed and its role in porcine respiratory disease complex (PRDC) is questionable. A highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) has become endemic in the Thai swine farms. This study was conducted to determine the pathogenicity of the pdmH1N1 SIV when co-infected with a HP-PRRSV. Thirty-two weanling pigs from a free PRRSV, SIV, porcine circovirus type 2 (PCV-2) and *Mycoplasma* spp. commercial farm were randomly divided into 4 groups: Negative, PRRSV, SIV and Co-infection groups. Pigs in PRRSV and Co-infection groups were challenged intranasally with the Thai isolate HP-PRRSV at 0 days post HP-PRRSV challenge (dphc) and pigs in SIV and Co-infection groups were inoculated intratracheally with pdmH1N1 SIV at 6 dphc. The 1st and 2nd necropsies were done at 2 and 4 days post SIV challenge (dsc). A comparison of the lung lesion scores between PRRSV and Co-infection groups at 2 dsc showed no statistical difference, but at 4 dsc, lung lesions score of the Co-infection group had higher average score than that of the PRRSV only group. Also, SIV viral load in the lung of the Co-infection group showed higher viral load at 4 dsc, comparing to the SIV-infected group significantly. The results suggest that the low pathogenic pdmH1N1 SIV could enhance the lung severity when co-infected with the HP-PRRSV possibly leading to porcine respiratory disease complex (PRDC).



Department: Veterinary Pathology Student's Signature .....
Field of Study: Veterinary Pathobiology Advisor's Signature .....
Academic Year: 2017

# ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my dearest advisor Prof. Dr. Roongroje Thanawongnuwech for professional guidance, mentoring and his patience throughout my Master's degree.

I wish to express my sincere thanks to Asst. Dr. Sawang Kesdangsakonwut for his assistance on pathological analysis and useful knowledge and suggestion. My gratitude is also expressed to Assoc. Prof. Dr. Theerayuth Kaewamatawong as my chairman for his valuable suggestion about an appointment and useful advice and Asst. Prof. Dr. Aunyaratana Thontiravong and Dr. Suphattra Jittimanee for providing the useful suggestion. I am thankful to all staff of the Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL) and Department of Veterinary Pathology, Chulalongkorn University for their kindly technical assistance and support. I would like to thank Dr. Roongtham Kedkovid, Dr. Yonlayong Woonwong, Dr, Jirapat Arunorat and especially Dr. Chaitawat Sirisereewan for their endless friendship and support throughout my study. Thus, my graduate program would have been impossible without the financial support from the National Research University (NRU; NRU59-PPS023HR).

Most of all, I would like to express my special appreciation and thanks to my beloved parents (Mr. Peerasak Sangpratum and Mrs. Niramon Sangpratum), my sister (Miss Praveenuch Sangpratum) and my friends for their unending support and continuing encouragement.

**CONTENTS**

Page

THAI ABSTRACT ..... iv

ENGLISH ABSTRACT ..... v

ACKNOWLEDGEMENTS ..... vi

CONTENTS ..... vii

LIST OF TABLE ..... 1

LIST OF FIGURES ..... 2

LIST OF ABBREVIATIONS ..... 3

CHAPTER I INTRODUCTION ..... 5

1.1 Importance and rationale ..... 5

1.2 Research objective ..... 7

1.3 Research benefits ..... 7

CHAPTER II LITERATURE REVIEW ..... 8

2.2 Disease and etiology ..... 8

2.2.1 Porcine reproductive and respiratory syndrome virus (PRRSV) ..... 8

2.2.2 Swine influenza virus (SIV) ..... 9

2.2 Co-infection between PRRSV and SIV ..... 10

CHAPTER III MATERIALS AND METHODS ..... 12

3.1 Viruses and cells ..... 12

3.2 Animals ..... 12

3.3 Experimental design ..... 12

3.4 Clinical observation ..... 13

3.5 Pathological study ..... 13

Page

3.6 Quantification of viral RNA ..... 14
3.6.1 HP-PRRSV ..... 14
3.6.2 SIV ..... 14
3.7 Statistical analysis ..... 15
CHAPTER IV RESULTS ..... 16
4.1 Clinical respiratory score ..... 16
4.2 Mean rectal temperature ..... 16
4.3 Lung lesion score and histopathological findings ..... 20
4.4 SIV shedding in nasal swabs and loading in lung tissues ..... 23
CHAPTER V DISCUSSION AND CONCLUSION ..... 25
5.1 Discussion ..... 25
5.2 Conclusion ..... 26
5.3 Compliance with ethical standards ..... 27
REFERENCES ..... 28
APPENDIX ..... 36
VITA ..... 40

# LIST OF TABLE

**Table 1** Number of positive PRRSV genomic RNA in sera at -2 and 2 dphc ..... 17

# LIST OF FIGURES

**Figure 1** Clinical respiratory score of pigs.. ..... 18

**Figure 2** Mean rectal temperature of pigs ..... 19

**Figure 3** Lung lesion score after SIV challenge ..... 21

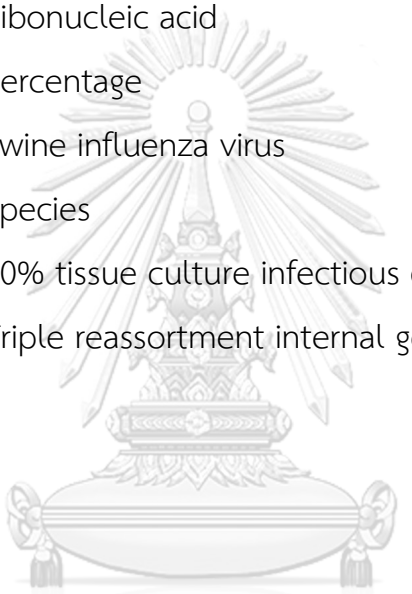
**Figure 4** Lung histopathology ..... 22

**Figure 5** SIV genomic copies per microliter in lung samples after SIV challenge..... 24

# LIST OF ABBREVIATIONS

| | |
|---|---|
| bp | Base pair (s) |
| °C | Degree Celsius |
| Ct | Cycle threshold |
| cDNA | Complementary deoxyribonucleic acid |
| dphc | Days post HP-PRRSV infection |
| dsc | Days post SIV infection |
| et al. | et alii, and others |
| ELISA | Enzyme-linked immunosorbent assay |
| g | Gram (s) |
| HA | Hemagglutinin gene |
| H&E | hematoxylin and eosin staining |
| HI | Hemagglutination inhibition test |
| HP-PRRSV | Highly pathogenic porcine reproductive and respiratory syndrome virus |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IPMA | Immunoperoxidase monolayer assay |
| M | Matrix gene |
| MDCK | Madin-Darby canine kidney cell line |
| ml | Milliliter (s) |
| µg | Microgram (s) |
| µl | Microliter (s) |
| µm | Micrometer (s) |
| pdmH1N1 | Pandemic H1N1 2009 virus |

| | |
|---|---|
| rH1N1 | Pandemic H1N1 2009 reassortant virus |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PCV-2 | Porcine circovirus type 2 |
| PAMs | Porcine alveolar macrophages |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| PRDC | Porcine respiratory disease complex |
| RNA | Ribonucleic acid |
| % | Percentage |
| SIV | Swine influenza virus |
| spp. | Species |
| $TCID_{50}$ | 50% tissue culture infectious dose |
| TRIG | Triple reassortment internal gene cassette |



# CHAPTER I
# INTRODUCTION

## 1.1 Importance and rationale

Porcine respiratory disease complex (PRDC) is one of the most common respiratory problem in the swine industry worldwide. This condition is a multiple microbial interaction causing fever, growth retardation, cough and dyspnea, 30-70% morbidity rates and 4-6% mortality rates (Opriessnig et al., 2011). Primary and secondary pathogens are mainly composed of respiratory viruses and bacteria, respectively. Primary pathogens associated with PRDC including porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine circovirus type 2 (PCV-2), *Mycoplasma hyopneumoniae* can cause damage to respiratory defense mechanism particularly in nursery pigs. PRRSV is the most common primary invader that associated with PRDC (Cheong et al., 2017). Moreover, PRRSV causes negative immunomodulatory effects by down regulation of interferon alpha (IFN-$\alpha$) and up regulation of interleukin 10 (IL-10) (Suradhat and Thanawongnuwech, 2003; Thanawongnuwech et al., 2004). Mostly, the combination of various pathogens could increase disease severity, such as lung pathological enhancement with PRRSV-SIV (Van Reeth et al., 1996), prolonged clinical signs and pneumonic lesions with PRRSV-porcine pseudorabies virus (PRV) (Shibata et al., 2003) and increased susceptibility to *Streptococcus suis* infection (Thanawongnuwech et al., 2000). Recently, a highly pathogenic PRRSV (HP-PRRSV) or atypical type 2 PRRSV (Jantafong et al., 2015) has become a dominant circulating PRRSV strain, causing more severe clinical appearance along with high morbidity and mortality rates (Tian et al., 2007; Nilubol et al., 2012).

Interestingly, pandemic H1N1 2009 (pdmH1N1) outbreak occurred in human population in Mexico and the United States and later spread worldwide (Dawood et al., 2009) including Thailand (Sreta et al., 2010). This virus genome was derived from the classical swine and Eurasian swine lineages and the North American H3N2 containing triple-reassortant internal gene (TRIG) from swine, human and avian lineages

(Pensaert et al., 1981; Brown, 2000; Smith et al., 2009b). In humans, pdmH1N1 infection showed fever, dry cough, muscle pain and dead due to respiratory failure, causing high morbidity and mortality rates (Smith et al., 2009a). However, clinical signs in infected pigs showed only fever, coughing, sneezing and nasal discharge (Pereda et al., 2010). Normally, the incubation period in pigs is between 1-3 days with rapid recovery between 4-7 days after the onset (Vincent et al., 2008). Furthermore, recent surveillance data showed pdmH1N1 and pdmH1N1 origin reassortant viruses have been circulating in the Thai swine farms after the introduction of pdmH1N1 SIV (Nonthabenjawan et al., 2015). In addition, despite zoonotic potential, pdmH1N1 SIVs have low pathogenicity and get less attention in Thailand due to it caused only mild respiratory signs and minimal gross lesions (Arunorat et al., 2017).

Due to the immunosuppressive ability of PRRSV, several studies showed potentiation of co-infection between PRRSV and SIV by demonstrating an increase in post-weaning mortality (Alvarez et al., 2015), decreased SIV vaccine efficacy and also increased clinical appearance and viral shedding during an acute phase of SIV infection (Kitikoon et al., 2009). Furthermore, co-infection between HP-PRRSV and pdmH1N1 SIV has not been conducted. This study would provide useful information about the pathogenesis of co-infection of the Thai isolate HP-PRRSV and pdmH1N1 SIV in weanling pigs.

### 1.2 Research objective

To evaluate clinical, pathological and virological findings of the co-infection between the Thai isolate HP-PRRSV and pdmH1N1 SIV.

### 1.3 Research benefits

1. This study would provide the information about the role of the Thai isolate pdmH1N1 SIV associated with PRDC.

2. This study would provide more useful information about pathogenicity of pdmH1N1 SIV when co-infected with HP-PRRSV in weanling pigs.

3. This data would be used as a model of the co-infections in other countries having pdmH1N1 and HP-PRRSV circulation in the swine farms.

# CHAPTER II
# LITERATURE REVIEW

## 2.2 Disease and etiology

### 2.2.1 Porcine reproductive and respiratory syndrome virus (PRRSV)

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded positive sense RNA virus with envelope which belongs to family *Arteriviridae* in order *Nidovirales*. PRRSV genome is approximately 15 kb in size, which contains 11 open reading frames (ORFs) encoding for 14 non-structural proteins (NSP) and 8 structural proteins (Lunney et al., 2016). The virus is classified into 2 types; type I (European strain) and type II (North American strain). PRRSV infection is resulting in reproductive failure in breeding herds and respiratory problem in nursery and finishing pigs (Lunney et al., 2016).

In 2006, Chinese researchers found a deletion of 30 amino acids discontinuously in NSP2 gene (Tian et al., 2007) in a type II PRRSV genomes which is known as highly pathogenic PRRSV (HP-PRRSV) or atypical type II PRRSV. The virus has continued spreading to the Southeast Asian region including Thailand, consequently, in 2010 (Nilubol et al., 2012) and currently, become a dominant strain (Jantafong et al., 2015). Clinical signs of HP-PRRSV infection were characterized by high fever (40-42 °C) and diffuse petechial hemorrhage in various organs leading to death in adult pigs and pregnant sows. The infected pigs could spread the disease throughout the population within 3-5 days. Other clinical signs such as skin erythema and respiratory signs including coughing and dyspnea could be seen in some infected pigs (Zhou and Yang, 2010). According to the expansion of tropism ability of the HP-PRRSV via pulmonary alveolar macrophages (PAMs) and lymphoid system (Li et al., 2012), the extensive lesions were observed, showing multifocal hemorrhages in multiple organs including skin, lungs, lymph nodes and kidney. The edema and congestion could be seen in the brain. Microscopically, lymphoid depletion occurred along with lymphocytic infiltration surrounding the vessels indicate perivascular cuffing especially

in the lung, brain and kidney (Hu et al., 2013). Also, acute pulmonary congestion, hemorrhagic pneumonia, hemorrhagic nephritis can be seen.

Importantly, PRRSV has its immunosuppressive ability by interleukin-10 (IL-10) up regulation via infected PAMs (Genini et al., 2008). IL-10 has been recognized as a potent immunosuppressive cytokine, which inhibits pro-inflammatory cytokine and induce regulatory T cell (Treg) (Wongyanin et al., 2012). Treg could maintain host immune homeostasis by inflammatory responses control and immunopathology limiting (Sakaguchi et al., 2009). It is also possible that PRRSV infection could induce PRRSV-specific Treg at the infected sites, leading to ineffective lung clearance capacity, prolong infection and increase susceptibility to secondary infection.

**2.2.2 Swine influenza virus (SIV)**

Swine influenza is an influenza A virus from the family *Orthomyxoviridae* composing of eight segmented single-stranded RNA genomes including PB2, PB1, PA, HA, NP, NA, M and NS proteins. Two major proteins are haemagglutinin (HA) and neuraminidase (NA) which have been identified for 18 HA and 11 NA. In 2009, pandemic H1N1 (pdmH1N1) virus emerged in humans in Mexico and had spread globally including Thailand (Sreta et al., 2010) demonstrating the importance of zoonosis and reverse zoonosis (Dawood et al., 2009). This viral genome contained internal genes called the triple reassortant internal genes (TRIG) found in North America (PA, PB1 and PB2 segments) (Zhou et al., 1999), the Avian-like Eurasian swine H1N1 virus in Europe and Asia (NA and M segments) (Arias et al., 2009) and the classical swine lineage (HA, NP and NS segments). In humans, pdmH1N1 infection showed fever, dry cough, muscle pain and dead due to the respiratory failure which caused high morbidity and mortality rates (Smith et al., 2009a). However, pdmH1N1 infected pigs developed only asymptomatic to mild respiratory signs (Sreta et al., 2010; Arunorat et al., 2017).



Although influenza viruses have different genetic components, but clinical disease and lesions are observed similarly (Janke, 2014). Firstly, viruses enter the respiratory airways, replicate in respiratory airway epithelial cells from nasal mucosa to

alveoli and microscopically, cause cranioventral bronchointerstitialpneumonia. In mild infection, gross lesions showed small clusters of dark red lobules which mainly located in the cranial and middle lung lobes and slightly found in caudal and accessory lung lobes (Janke, 2014). For more severe infection, most lung lobes were affected with diffuse congested lung and interlobular edema with extensive foamy fluid in the bronchi and trachea. Microscopically, the hallmark lesions were necrotizing bronchitis and bronchiolitis. The airway epithelial cells were necrotic and sloughed. Lymphocytic infiltration became more prominent around the airways and vessels. Later, the fibroblastic proliferation might result in bronchial polyps or incomplete splitting of repaired airways (bronchiolitis obliterans) (Janke, 2014).

**2.2 Co-infection between PRRSV and SIV**

In swine farms, the respiratory diseases in nursery pigs mostly occurred by interaction among respiratory pathogens called PRDC. Maternal antibodies against PRRSV and SIV, did not exist at 8-12 weeks of age. Moreover, in nursery pigs, having high risk to be infected with the pathogens horizontally especially SIV is commonly found after pig grouping (Van Reeth and Pensaert, 1994). Since PRRSV and SIV are the two main respiratory pathogens, several studies showed the outcome of the co-infection (Van Reeth et al., 1996; Kitikoon et al., 2009; Alvarez et al., 2015).



Dual infection between PRRSV and SIV induced more clinical appearance than a single infection (Kitikoon et al., 2009; Dobrescu et al., 2014). The co-infected pigs developed moderate to severe respiratory signs, growth retardation and pathological changes and also viral shedding. All of these causes, led to post-weaning mortality which was one of the major causes of economic losses along with increased length of the growing phase (Alvarez et al., 2015). An increased in pathological changes observed in lungs might occur due to pathogenesis of SIV. An infection in airway epithelial cells induced cellular necrosis and inflammatory cell infiltration including phagocytic cells such as pulmonary alveolar macrophages (PAM) which were susceptible to PRRSV for viral replication (Chang et al., 2005). This phenomenon could prolong and increase pneumonia and viral shedding. Furthermore, the presence of PRRSV either from

infection or modified live vaccine (MLV) significantly reduced SIV vaccine efficacy (Kitikoon et al., 2009). In addition, co-infected pigs developed more respiratory signs and lung lesions, which indicated SIV vaccination failure.

However, not to all co-infection between PRRSV and SIV cases developed more severe clinical outcomes. The disease severity also depended on the virulence of the viruses and the time interval between the infection (Van Reeth et al., 2001).

# CHAPTER III
# MATERIALS AND METHODS

## 3.1 Viruses and cells

A Thai isolate pdmH1N1 SIV (A/swine/Thailand/CU-PL65/2010(H1N1)) and a Thai isolate HP-PRRSV (HP-PRRSV/10PL01) were provided by Chulalongkorn University Veterinary Diagnostic Laboratory (CU-VDL, Bangkok, Thailand). SIV was propagated in Madin Darby Canine Kidney (MDCK) cells (ATCC, USA) and titrated to $10^{5.5}$ tissue culture infectious dose ($TCID_{50}$/ml). HP-PRRSV was propagated in MARC-145 cells and titrated to $10^2$ $TCID_{50}$/ml. All inoculums were stored at -80° C until used.

## 3.2 Animals

Thirty-two, three-week-old, crossbred pigs from a commercial farm in the central part of Thailand which had negative results against PCV-2 and *Mycolpasma* spp. antigens and antibodies were obtained. All pigs were tested negative for influenza A virus and HP-PRRSV by real time PCR and antibodies tested by a commercial ELISA kit (IDEXX PRRS X3 Ab test, USA) for PRRSV screening and a Multispecies Kit (ID Screen® Influenza A antibody competition multi-species; IDvet Louis Pasteur-Grabel, France) for SIV screening.



## 3.3 Experimental design

All pigs were divided randomly into 4 groups. Co-infection (n=9) and HP-PRRSV-inoculated groups (n=9) were inoculated intranasally with 2 ml of $10^2$ $TCID_{50}$/ml HP-PRRSV (10PL01) on day 0. The pigs in SIV positive control group (n=9) and negative control group (n=5) were mocked by using minimum essential media (MEM). In both SIV and Co-infection groups, pigs were anesthetized using 0.08 ml/kg of zolazepam and tiletamine (Zoletil®, Virbac, Thailand) intramuscularly and inoculated intratracheally with 20 ml of $10^{5.5}$ $TCID_{50}$/ml (A/Swine/Thailand/CU-PL65/2010(H1N1)) at 6 days post HP-PRRSV challenge (dphc), whereas, the negative group were inoculated with MEM with the same procedure. All pigs were housed at the BSL-2 animal facility provided

by Chulalongkorn University Laboratory Animal Center (CULAC) and freely access to feed and water. Nasal swab samples were collected at -2 dphc, 0, 1, 2, 3 and 4 dsc and kept in the transporting medium containing 5% BSA, 300 U/ml penicillin, 300 μg/ml streptomycin and 1 μg/ml trypsin at -80°C. The animal use protocols were conducted under the approval of Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (Animal Use Protocol No. 1673032).

**3.4 Clinical observation**

Clinical signs were monitored daily including appetite, rectal temperature (Fever were set at rectal temperature ≥ 40°C.) and clinical respiratory scores (0-3; 0: no clinical respiratory distress, 1: mild respiratory distress with predominant abdominal breathing, 2: Moderate exaggerated respiratory distress with entirely abdominal labored breathing, 3: Severe, very labored respiratory distress with mouth breathing along with cyanotic nose/ears)

**3.5 Pathological study**

Seventeen pigs (2 pigs from the negative group and 5 pigs from other 3 groups) were randomly selected for euthanasia using an overdosed pentobarbital sodium (Nembutal®, Ceva, Thailand) at 2 days post SIV challenge (dsc) and fifteen pigs (3 pigs from the negative and 4 pigs from other 3 groups) at 4 dsc. Lung lesion score was examined as previous described (Halbur et al., 2000). All tissue samples were fixed in 10% formalin, processed and embedded in paraffin for histopathological evaluation. Microscopic lung lesions were characterized by necrotic bronchitis and bronchiolitis, along with interstitial pneumonia and lymphocytic peribronchiolar cuffing (Landolt et al., 2003; Kitikoon et al., 2006)

### 3.6 Quantification of viral RNA

#### 3.6.1 HP-PRRSV

Viral RNA from sera were extracted using the Nucleospin® RNA virus (machery-negal, Duren, Germany). The Taqman® probe-based real-time RT-PCR was used for the quantification of PRRSV RNA (Egli et al., 2001). Reverse primer USalighnEU-R (5' AAAT|GGCTTCTC|GG|TTTT 3') and forward primer USalighnEU-F (5' TCA|CTGTGCCAG|TGCTGG 3') and US-PRRSV-specific probe FAM_US_rev (5' FAM-TCCCGGTCCCTTGCCTCTGGA-TAMRA 3') were used.

Amplification was done in a 25 µl reaction containing SuperScript™ III One-Step RT-PCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 1x Reaction Mix, 0.4 mM of each primer and probe, 0.5 µl of SuperScript® III RT/Platinum Taq Mix and 0.5 µl of viral RNA.

#### 3.6.2 SIV

Viral RNA from nasal swab samples and lung tissues were extracted using the same protocol as PRRSV test. The modified real time RT-PCR assays were done on Corbett Rotor-Gene™ 6000 (Qiagen) using SuperScript™ III Platinum® One-Step Qualitative RT-PCR System (Invitrogen, Carlabad, California, USA) according to the manufacturer's instructions. Primers and probes were used based on the previous study (Spackman et al., 2002). Amplification of a portion of the M gene (forward primer (MF3; 5' TGATCTTCTTGAAAATTTGCAG 3') and M-64 probe (FAM-TTGTGGATTCTTGATCGTAMRA)) (Payungporn et al., 2006) were done. The positive CT value >40 was considered as a negative results.

The positive control was used from the positive sample based on viral isolation and titration in the MDCK cell line from the previous study (Sreta et al., 2009). The viral antigen was detected using immunoperoxidase assay. Anti-influenza A nucleoprotein monoclonal antibodies (HB654404), a rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, USA) and aminoethyle carbazole (AEC) substrate were used.

### 3.7 Statistical analysis

All data were analysed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All statistical analyses were performed in GraphPad Prism for Windows (GraphPad Software Incorporated, San Diego, CA, USA).

# CHAPTER IV
# RESULTS

## 4.1 Clinical respiratory score

The clinical respiratory score of the experimented pigs were shown in Figure 1. After HP-PRRSV challenge, 5 pigs from the PRRSV and 4 pigs from the Co-infection groups showed mild depression and respiratory distress at 2 dphc. Most pigs from both PRRSV and co-infection groups showed clinical signs at 5 dphc. During SIV challenge period, all pigs from the PRRSV and Co-infection groups showed moderate to severe respiratory distress with no statistically significant difference ($P>0.05$) and notably, the SIV-only inoculated pigs showed no remarkable clinical respiratory distress throughout the experiment.

## 4.2 Mean rectal temperature

After HP-PRRSV challenge, 2 pigs from the PRRSV and 1 pig from the Co-infection group showed fever (40-40.3 °C) and at 6 dphc, 8/9 pigs from both groups had fever. After the SIV challenge, all pigs from the SIV-infected group showed no fever throughout the experiment. The mean rectal temperature of the PRRSV-infected groups tended to decrease. Only 2/9 pigs had fever at 6 dphc and had normal rectal temperature until the necropsy date, whereas, the remaining pigs from the Co-infection group still showed fever until the end of the experiment (Fig. 2).

**Table 1** Number of positive PRRSV genomic RNA in sera at -2 and 2 dphc

| | No. of positive PRRSV genomic RNA in sera | |
|---|---|---|
| Group | **-2 dphc** | **2 dphc** |
| **Negative** | 0/5 | 0/5 |
| **SIV** | 0/9 | 0/9 |
| **PRRSV** | 9/9 | 9/9 |
| **Co-infection** | 9/9 | 9/9 |

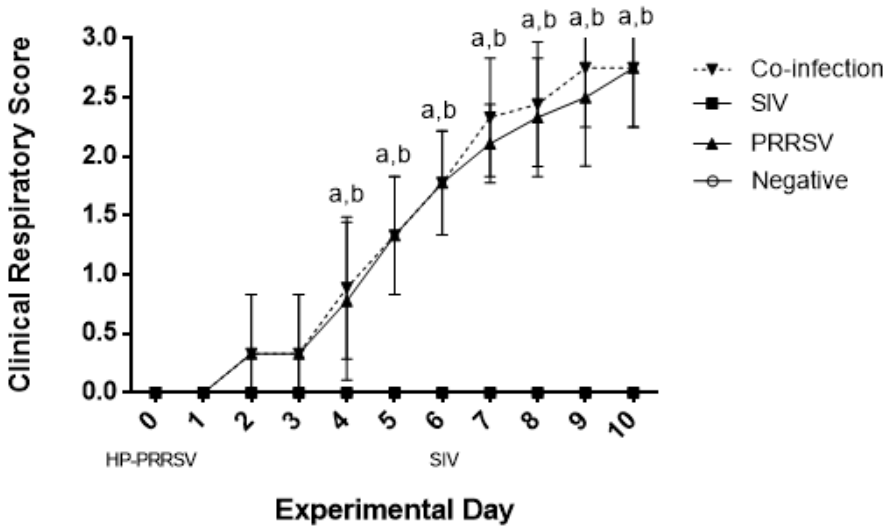



**Figure 1** Clinical respiratory score of pigs. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. ([a] indicates significant difference between the Co-infection and SIV groups. [b] indicates significant difference between the PRRSV and SIV group ($P<0.05$)).

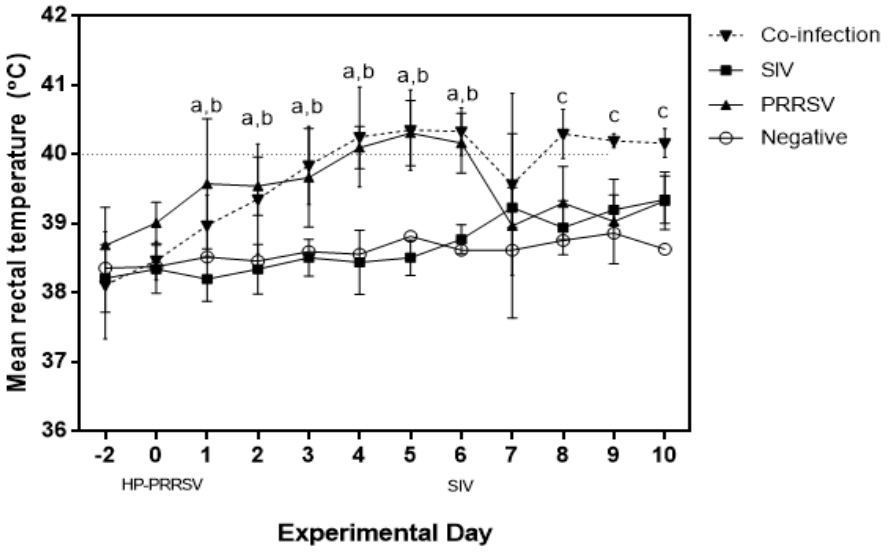



**Figure 2** Mean rectal temperature of pigs. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. ([a] indicates significant difference between the co-infection and SIV groups. [b] indicates significant difference between the PRRSV and SIV groups. [c] indicates significant difference between the co-infection and PRRSV groups ($p<0.05$)

**4.3 Lung lesion score and histopathological findings**

Gross examination at 2 and 4 dsc demonstrated that all gross lung lesions from the Negative and the single SIV-infected groups showed grossly look normal lung lesion, whereas, lungs from the PRRSV and Co-infection groups showed firm and fail to collapse with no significant differences ($p>0.05$) at 2 dsc. However, lung lesion score was more severe in the co-infection pigs than those of the single PRRSV-infected groups ($p<0.05$) at 4 dsc (Fig. 2).

Microscopically, all lungs from the Negative group show no significantly findings (Fig. 3a). Lungs from the single SIV-infected pigs, showed mild to moderate bronchointerstitial pneumonia (Fig. 3b). The single PRRSV-infected lung sections showed severe interstitial pneumonia (Fig. 3c). However, co-infected lung lesions showed severe bronchointerstitial pneumonia (Fig. 3d) and bronchiolar epithelium showed epithelium necrosis with lymphocytic peribronchiolar cuffing, along with mononuclear cells infiltration in the alveolar septum.

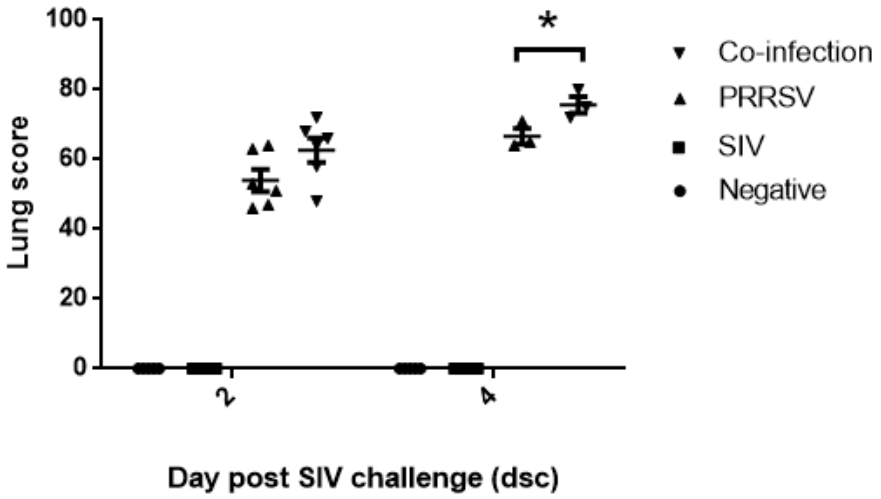



**Figure 3** Lung lesion score after SIV challenge (2 and 4 dsc) Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. (Asterisk indicates significant differences between group ($p<0.05$).)

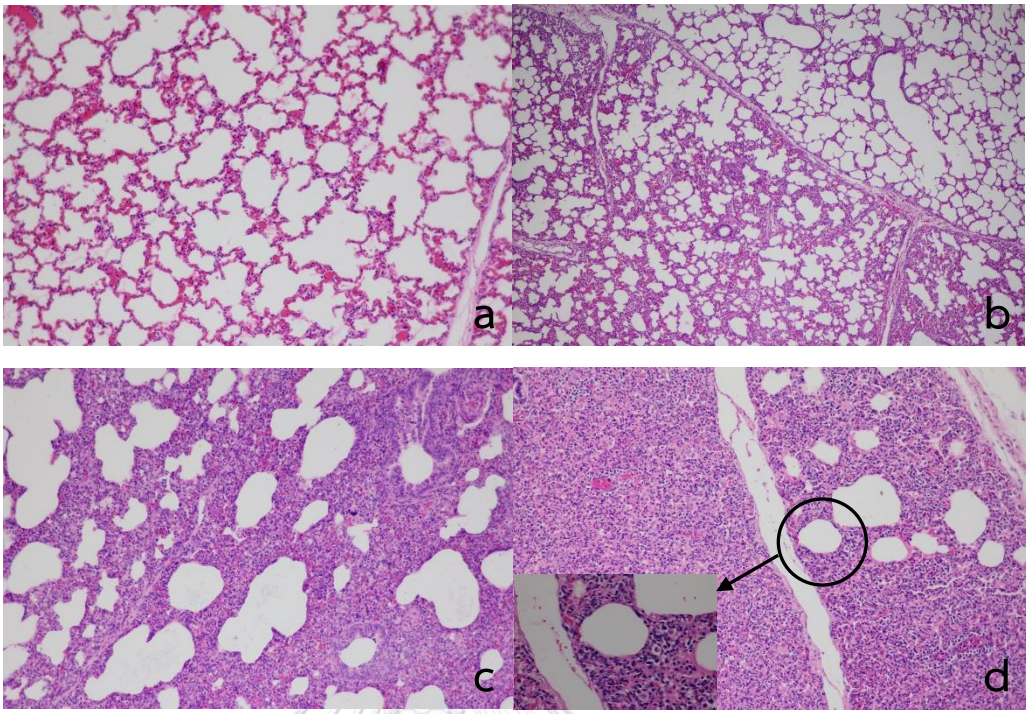



**Figure 4** Lung histopathology. (a) Negative group. No remarkable lesions (4x). (b) PRRSV group. Moderate interstitial pneumonia accompanied with lymphocytic infiltration in alveolar septa (10x). (c) SIV group. Moderate necrosis of bronchiolar epithelium, showing necrotizing bronchointerstitial pneumonia (4x). (d). Co-infection group. The left alveoli are filled with inflammatory cells with exudate and cell debris. The bronchiolar epithelium shows necrosis, indicating severe bronchointerstitial pneumonia (10x). (left corner). Necrotizing bronchiolitis (arrowhead) with lymphocytic peribronchiolar cuffing.

**4.4 SIV shedding in nasal swabs and loading in lung tissues**

During 1-3 dsc, no SIV shedding samples were positive in both single SIV and Co-infection groups. However, at 4 dsc, the single SIV-infected group had 75% positive pigs (3/4), whereas, none of Co-infection pigs showed positive result from the nasal swabs. At 2 dsc, SIV RNA quantification from lung homogenate from the single SIV (4/5) and Co-infection (3/5) groups showed no significant difference ($p>0.05$), whereas, at 4 dsc, all remaining lung tissues had significant SIV loading with higher detection in the Co-infection group than that of the single SIV group ($p<0.05$) (Fig. 4).

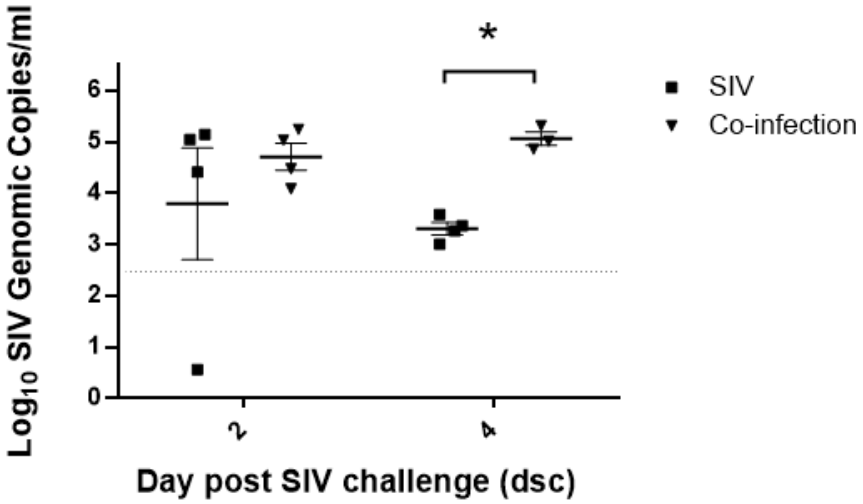



**Figure 5** SIV genomic copies per microliter (log10) in lung samples after SIV challenge (2 and 4 dsc) Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. (The asterisk indicates significant difference between groups ($p<0.05$).)

# CHAPTER V
# DISCUSSION AND CONCLUSION

## 5.1 Discussion

Based on the study objective, we confirmed the hypothesis that the Thai isolate HP-PRRSV could enhance the pathogenicity of the Thai pdmH1N1 SIV. It should be noted that this is the first co-infection report between HP-PRRSV and pdmH1N1 SIV in weanling pigs.

Our results demonstrated that the Thai HP-PRRSV isolate could induce the clinical disease on its own as previously reported (Sirisereewan et al., 2017). All HP-PRRSV inoculated pigs exhibited viremia at 2 dphc (Table 1) and had shown moderate to severe respiratory distress and depression. After the pdmH1N1 SIV inoculation, a few SIV-infected pigs only had sporadic nasal shedding and did not showed obvious clinical signs. This might due to route of infection using in this study. According to recent study (Hemmink et al., 2016), that showed the pdmH1N1 could induce more clinical signs and virus shedding via intranasal route than that of intratracheal route due to the virus bypassed the upper respiratory tract (URT), which is known to be the natural route of infection. For mean rectal temperature, co-infection pigs showed fever during 1-4 dsc whereas the only PRRSV-inoculated pigs had normal rectal temperature after SIV challenge. This might be due to increased cytokines production during the acute stage of SIV infection such as IL-6 (Barbe et al., 2010), inducing febrile response in the HP-PRRSV-induced immunocompromised pigs in this experiment.

Interestingly, a comparison of the lung lesion scores between the PRRSV and Co-infection groups at 2 dsc showed no statistical differences, but at 4 dsc, the lung lesion score of the Co-infection group had higher average scores than that of the PRRSV only group. Similarly, the SIV lung viral loading of the Co-infection group showed higher viral load at 4 dsc comparing to the single SIV-infected group. Exact mechanisms of the co-infection between these studied two viruses are not clearly clarified due to differences in time interval, virulent strain usages. However, PRRSV has its tropism via

pulmonary alveolar macrophages (PAMs) (Li et al., 2012), which could interfere the SIV clearance effectively. Additionally, IL-10 up regulation could inhibit monocyte/macrophage functions also leading to poor clearance of the virus (Thanawongnuwech et al., 2000; Moore et al., 2001). In addition, HP-PRRSV did not induce interleukin-1 receptor antagonist (IL-1Ra) comparing to type II PRRSV during an acute phase of infection (Nedumpun et al., 2017). IL-1Ra plays an essential role in anti-inflammatory cytokines induction (Arend et al., 1998), leading to excessive cytokines production. Thus, inflammatory cytokines (IL-1, IL-6 and TNF-**α**) are also induced by PAMs and increase during SIV co-infection possibly due to PAMs did not undergo apoptosis (Seo et al., 2004). The results showed the potentiation of the co-infection of HP-PRRSV and pdmH1N1 SIV.

Importantly, this study showed that the co-infected lungs had higher SIV loading, which means the SIV could replicate better than that of the only SIV-infected pigs. This suggests an increased chance to antigenic drift, which had been shown in weanling pigs with and without immunity during an early infection (Diaz et al., 2013). Furthermore, since swine is susceptible to infection with both avian and human influenza A virus (IAV) and considered as mixing vessel or intermediate host for IAV of human pandemic potential (Webster et al., 1992), reassortment is important in IAV evolution (Dugan et al., 2008) and host switching (Garten et al., 2009) especially swine workers (Kitikoon et al., 2011).



**5.2 Conclusion**

This study suggests that pdmH1N1 SIV disease severity could be enhanced by HP-PRRSV infection comparing to the single infection. Therefore, routine surveillance and diagnosis of SIVs should be done in order to reduce production losses and zoonotic prevention and control.

**5.3 Compliance with ethical standards**

This study protocol was approved by the Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (IACUC#1673032).

## REFERENCES


Alvarez J, Sarradell J, Kerkaert B, Bandyopadhyay D, Torremorell M, Morrison R and Perez A 2015. Association of the presence of influenza A virus and porcine reproductive and respiratory syndrome virus in sow farms with post-weaning mortality. Prev Vet Med. 121(3-4): 240-245.

Arend WP, Malyak M, Guthridge CJ and Gabay C 1998. Interleukin-1 receptor antagonist: role in biology. Annu Rev Immunol. 16: 27-55.

Arias CF, Escalera-Zamudio M, Soto-Del Rio Mde L, Cobian-Guemes AG, Isa P and Lopez S 2009. Molecular anatomy of 2009 influenza virus A (H1N1). Arch Med Res. 40(8): 643-654.

Arunorat J, Charoenvisal N, Woonwong Y, Kedkovid R, Jittimanee S, Sitthicharoenchai P, Kesdangsakonwut S, Poolperm P and Thanawongnuwech R 2017. Protection of human influenza vaccines against a reassortant swine influenza virus of pandemic H1N1 origin using a pig model. Res Vet Sci. 114: 6-11.

Barbe F, Saelens X, Braeckmans D, Lefevre F and Reeth KV 2010. Role of IFN-alpha during the acute stage of a swine influenza virus infection. Res Vet Sci. 88(1): 172-178.

Brown IH 2000. The epidemiology and evolution of influenza viruses in pigs. Vet Microbiol. 74(1-2): 29-46.

Chang HW, Jeng CR, Liu JJ, Lin TL, Chang CC, Chia MY, Tsai YC and Pang VF 2005. Reduction of porcine reproductive and respiratory syndrome virus (PRRSV) infection in swine alveolar macrophages by porcine circovirus 2 (PCV2)-induced interferon-alpha. Vet Microbiol. 108(3-4): 167-177.

Cheong Y, Oh C, Lee K and Cho KH 2017. Survey of porcine respiratory disease complex-associated pathogens among commercial pig farms in Korea via oral fluid method. J Vet Sci. 18(3): 283-289.

Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB and Uyeki TM 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 360(25): 2605-2615.

Diaz A, Allerson M, Culhane M, Sreevatsan S and Torremorell M 2013. Antigenic drift of H1N1 influenza A virus in pigs with and without passive immunity. Influenza Other Respir Viruses. 7 Suppl 4: 52-60.

Dobrescu I, Levast B, Lai K, Delgado-Ortega M, Walker S, Banman S, Townsend H, Simon G, Zhou Y, Gerdts V and Meurens F 2014. In vitro and ex vivo analyses of co-infections with swine influenza and porcine reproductive and respiratory syndrome viruses. Vet Microbiol. 169(1-2): 18-32.

Dugan VG, Chen R, Spiro DJ, Sengamalay N, Zaborsky J, Ghedin E, Nolting J, Swayne DE, Runstadler JA, Happ GM, Senne DA, Wang R, Slemons RD, Holmes EC and Taubenberger JK 2008. The evolutionary genetics and emergence of avian influenza viruses in wild birds. PLoS Pathog. 4(5): e1000076.

Egli C, Thur B, Liu L and Hofmann MA 2001. Quantitative TaqMan RT-PCR for the detection and differentiation of European and North American strains of porcine reproductive and respiratory syndrome virus. J Virol Methods. 98(1): 63-75.

Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr., Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI and Cox NJ 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 325(5937): 197-201.

Genini S, Delputte PL, Malinverni R, Cecere M, Stella A, Nauwynck HJ and Giuffra E 2008. Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. J Gen Virol. 89(Pt 10): 2550-2564.

Halbur P, Thanawongnuwech R, Brown G, Kinyon J, Roth J, Thacker E and Thacker B 2000. Efficacy of antimicrobial treatments and vaccination regimens for control of porcine reproductive and respiratory syndrome virus and Streptococcus suis coinfection of nursery pigs. J Clin Microbiol. 38(3): 1156-1160.

Hemmink JD, Morgan SB, Aramouni M, Everett H, Salguero FJ, Canini L, Porter E, Chase-Topping M, Beck K, Loughlin RM, Carr BV, Brown IH, Bailey M, Woolhouse M, Brookes SM, Charleston B and Tchilian E 2016. Distinct immune responses and virus shedding in pigs following aerosol, intra-nasal and contact infection with pandemic swine influenza A virus, A(H1N1)09. Vet Res. 47(1): 103.

Henningson JN, Rajao DS, Kitikoon P, Lorusso A, Culhane MR, Lewis NS, Anderson TK and Vincent AL 2015. Comparative virulence of wild-type H1N1pdm09 influenza A isolates in swine. Vet Microbiol. 176(1-2): 40-49.

Hu SP, Zhang Z, Liu YG, Tian ZJ, Wu DL, Cai XH and He XJ 2013. Pathogenicity and distribution of highly pathogenic porcine reproductive and respiratory syndrome virus in pigs. Transbound Emerg Dis. 60(4): 351-359.

Janke BH 2014. Influenza A virus infections in swine: pathogenesis and diagnosis. Vet Pathol. 51(2): 410-426.

Jantafong T, Sangtong P, Saenglub W, Mungkundar C, Romlamduan N, Lekchareonsuk C and Lekcharoensuk P 2015. Genetic diversity of porcine reproductive and respiratory syndrome virus in Thailand and Southeast Asia from 2008 to 2013. Vet Microbiol. 176(3-4): 229-238.

Kitikoon P, Nilubol D, Erickson BJ, Janke BH, Hoover TC, Sornsen SA and Thacker EL 2006. The immune response and maternal antibody interference to a heterologous

H1N1 swine influenza virus infection following vaccination. Vet Immunol Immunopathol. 112(3-4): 117-128.

Kitikoon P, Sreta D, Tuanudom R, Amonsin A, Suradhat S, Oraveerakul K, Poovorawan Y and Thanawongnuwech R 2011. Serological evidence of pig-to-human influenza virus transmission on Thai swine farms. Vet Microbiol. 148(2-4): 413-418.

Kitikoon P, Vincent AL, Jones KR, Nilubol D, Yu S, Janke BH, Thacker BJ and Thacker EL 2009. Vaccine efficacy and immune response to swine influenza virus challenge in pigs infected with porcine reproductive and respiratory syndrome virus at the time of SIV vaccination. Vet Microbiol. 139(3-4): 235-244.

Landolt GA, Karasin AI, Phillips L and Olsen CW 2003. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J Clin Microbiol. 41(5): 1936-1941.

Li L, Zhao Q, Ge X, Teng K, Kuang Y, Chen Y, Guo X and Yang H 2012. Chinese highly pathogenic porcine reproductive and respiratory syndrome virus exhibits more extensive tissue tropism for pigs. Virol J. 9: 203.

Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B and Renukaradhya GJ 2016. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. Annu Rev Anim Biosci. 4: 129-154.

Moore KW, de Waal Malefyt R, Coffman RL and O'Garra A 2001. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 19: 683-765.

Nedumpun T, Wongyanin P, Sirisereewan C, Ritprajak P, Palaga T, Thanawongnuwech R and Suradhat S 2017. Interleukin-1 receptor antagonist: an early immunomodulatory cytokine induced by porcine reproductive and respiratory syndrome virus. J Gen Virol. 98(1): 77-88.

Nilubol D, Tripipat T, Hoonsuwan T and Kortheerakul K 2012. Porcine reproductive and respiratory syndrome virus, Thailand, 2010-2011. Emerg Infect Dis. 18(12): 2039-2043.

Nonthabenjawan N, Chanvatik S, Chaiyawong S, Jairak W, Boonyapisusopha S, Tuanudom R, Thontiravong A, Bunpapong N and Amonsin A 2015. Genetic diversity of swine influenza viruses in Thai swine farms, 2011-2014. Virus Genes. 50(2): 221-230.

Opriessnig T, Gimenez-Lirola LG and Halbur PG 2011. Polymicrobial respiratory disease in pigs. Anim Health Res Rev. 12(2): 133-148.

Payungporn S, Chutinimitkul S, Chaisingh A, Damrongwantanapokin S, Buranathai C, Amonsin A, Theamboonlers A and Poovorawan Y 2006. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J Virol Methods. 131(2): 143-147.

Pensaert M, Ottis K, Vandeputte J, Kaplan MM and Bachmann PA 1981. Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man. Bull World Health Organ. 59(1): 75-78.

Pereda A, Cappuccio J, Quiroga MA, Baumeister E, Insarralde L, Ibar M, Sanguinetti R, Cannilla ML, Franzese D, Escobar Cabrera OE, Craig MI, Rimondi A, Machuca M, Debenedetti RT, Zenobi C, Barral L, Balzano R, Capalbo S, Risso A and Perfumo CJ 2010. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis. 16(2): 304-307.

Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P and Yamaguchi T 2009. Regulatory T cells: how do they suppress immune responses? Int Immunol. 21(10): 1105-1111.

Seo SH, Webby R and Webster RG 2004. No apoptotic deaths and different levels of inductions of inflammatory cytokines in alveolar macrophages infected with influenza viruses. Virology. 329(2): 270-279.

Shibata I, Yazawa S, Ono M and Okuda Y 2003. Experimental dual infection of specific pathogen-free pigs with porcine reproductive and respiratory syndrome virus and pseudorabies virus. J Vet Med B Infect Dis Vet Public Health. 50(1): 14-19.

Sirisereewan C, Nedumpun T, Kesdangsakonwut S, Woonwong Y, Kedkovid R, Arunorat J, Thanawongnuwech R and Suradhat S 2017. Positive immunomodulatory effects of

heterologous DNA vaccine- modified live vaccine, prime-boost immunization, against the highly-pathogenic PRRSV infection. Vet Immunol Immunopathol. 183: 7-15.

Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, Webster RG, Peiris JS and Guan Y 2009a. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A. 106(28): 11709-11712.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y and Rambaut A 2009b. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 459(7250): 1122-1125.

Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT and Suarez DL 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 40(9): 3256-3260.

Sreta D, Kedkovid R, Tuamsang S, Kitikoon P and Thanawongnuwech R 2009. Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial. Virol J. 6: 34.

Sreta D, Tantawet S, Na Ayudhya SN, Thontiravong A, Wongphatcharachai M, Lapkuntod J, Bunpapong N, Tuanudom R, Suradhat S, Vimolket L, Poovorawan Y, Thanawongnuwech R, Amonsin A and Kitikoon P 2010. Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. Emerg Infect Dis. 16(10): 1587-1590.

Suradhat S and Thanawongnuwech R 2003. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. J Gen Virol. 84(Pt 10): 2755-2760.

Thanawongnuwech R, Brown GB, Halbur PG, Roth JA, Royer RL and Thacker BJ 2000. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to Streptococcus suis infection. Vet Pathol. 37(2): 143-152.

Thanawongnuwech R, Thacker B, Halbur P and Thacker EL 2004. Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. Clin Diagn Lab Immunol. 11(5): 901-908.

Tian K, Yu X, Zhao T, Feng Y, Cao Z, Wang C, Hu Y, Chen X, Hu D, Tian X, Liu D, Zhang S, Deng X, Ding Y, Yang L, Zhang Y, Xiao H, Qiao M, Wang B, Hou L, Wang X, Yang X, Kang L, Sun M, Jin P, Wang S, Kitamura Y, Yan J and Gao GF 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PLoS One. 2(6): e526.

Van Reeth K, Nauwynck H and Pensaert M 1996. Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. Vet Microbiol. 48(3-4): 325-335.

Van Reeth K, Nauwynck H and Pensaert M 2001. Clinical effects of experimental dual infections with porcine reproductive and respiratory syndrome virus followed by swine influenza virus in conventional and colostrum-deprived pigs. J Vet Med B Infect Dis Vet Public Health. 48(4): 283-292.

Van Reeth K and Pensaert M 1994. Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. Vet Rec. 135(25): 594-597.

Vincent AL, Ma W, Lager KM, Janke BH and Richt JA 2008. Swine influenza viruses a North American perspective. Adv Virus Res. 72: 127-154.

Webster RG, Bean WJ, Gorman OT, Chambers TM and Kawaoka Y 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 56(1): 152-179.

Wongyanin P, Buranapraditkul S, Yoo D, Thanawongnuwech R, Roth JA and Suradhat S 2012. Role of porcine reproductive and respiratory syndrome virus nucleocapsid protein in induction of interleukin-10 and regulatory T-lymphocytes (Treg). J Gen Virol. 93(Pt 6): 1236-1246.

Zhou L and Yang H 2010. Porcine reproductive and respiratory syndrome in China. Virus Res. 154(1-2): 31-37.

Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S and Webster RG 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol. 73(10): 8851-8856.

# APPENDIX

**Appendix A:** The procedure for preparation of the tissue processing

| Objective | Reagent | Time (minutes) |
|---|---|---|
| Dehydration | 80% ethyl alcohol | 30 |
| | 80% ethyl alcohol | 30 |
| | 95% ethyl alcohol | 30 |
| | 95% ethyl alcohol | 30 |
| | 100% ethyl alcohol | 40 |
| | % ethyl alcohol | 40 |
| Clearing | Xylene | 30 |
| | Xylene | 30 |
| Infiltration | Melted paraffin | 30 |
| | Melted paraffin | 30 |

**Appendix B:** Viral titration and Immunoperoxidase monolayer assay (IPMA) for SIV

1. Prepare monolayer of MDCK cells 96-well tissue culture plate
2. Wash confluent cell monolayer 3 times with cell culture medium containing 5% BSA and 5 mg/ml TPCK-treated trypsin
3. Make 10-fold dilution of sample and apply 100 µl in to each well, follow the prepared dilution (4 well per dilution)
4. Incubate in 37°C humidified incubator with 5% $CO_2$ for 72 hours
5. Fix the cell with 4% formalin in PBS-0.5% tween
    5.1 Discard all culture media
    5.2 Apply 100 µl of 4% formalin in PBS-0.5% tween in each well
    5.3 Incubate for 25 min at room temperature
    5.4 Wash with PBS-0.5% tween for 3 times
6. Apply antibody (Influenza A nucleoprotein monoclonal antibodies (HB654404)) dilute with 1% BSA in PBS-0.5% tween (dilution 1:1,000), 50 µl/well and incubate for 1 hour at room temperature
7. Wash with PBS-0.5% tween for 3 times
8. Apply conjugate (A polyclonal rabbit anti-mouse IgG conjugated horseradish peroxidase) dilute with 1% BSA in PBS-0.5% tween (dilution 1:300), 50 µl/well and incubate for 1 hour at room temperature
9. Wash with PBS-0.5% tween for 3 times
10. Add 5% AEC and 3% $H_2O_2$ in acetate buffer, apply AEC substrate 50 µl/well and incubate for 10 min at room temperature
11. Wash with tap water, 3 times, dry plate and observe under phase-contrast microscope
12. Calculate $TCID_{50}$ by Reed and Muench method

**Appendix C:** Viral titration and Immunoperoxidase monolayer assay (IPMA) for PRRSV

1. Prepare monolayer of MARC-145 cells 96-well tissue culture plate
2. Wash confluent cell monolayer 3 times with cell culture medium
3. Make 10-fold dilution of sample and apply 100 µl in to each well, follow the prepared dilution (4 well per dilution)
4. Incubate in 37°C humidified incubator with 5% $CO_2$ for 72 hours
5. Fix the cell with 4% formalin in PBS-0.5% tween
    5.1 Discard all culture media
    5.2 Apply 100 µl of 4% formalin in PBS-0.5% tween in each well
    5.3 Incubate for 25 min at room temperature
    5.4 Wash with PBS-0.5% tween for 3 times
6. Apply antibody (Monoclonal antibody for PRRSV detection (SDOW17 and SR30)) dilute with 1% BSA in PBS-0.5% tween (dilution 1:1,000), 50 µl/well and incubate for 1 hour at room temperature
7. Wash with PBS-0.5% tween for 3 times
8. Apply conjugate (A polyclonal rabbit anti-mouse IgG conjugated horseradish peroxidase) dilute with 1% BSA in PBS-0.5% tween (dilution 1:300), 50 µl/well and incubate for 1 hour at room temperature
9. Wash with PBS-0.5% tween for 3 times
10. Add 5% AEC and 3% $H_2O_2$ in acetate buffer, apply AEC substrate 50 µl/well and incubate for 10 min at room temperature
11. Wash with tap water, 3 times
12. Dry plate and observe under phase-contrast microscope
13. Calculate $TCID_{50}$ by Reed and Muench method

# VITA

Mr. Nattaphong Sangpratum was born on Octorber, 2th, 1991 at Nakhon Pathom province. He graduated with Doctor of Veterinary Medicine, second class honor from the Faculty of Veterinary Science, Chulalongkorn University in 2016. Soon after the graduation, he had enrolled in the Master program in Veterinary Pathobiology program since 2016.

During the Master degree study, He received a scholarship from the National Research University (NRU).

Academic publication

Kedkovid R, Woonwong Y, Arunorat J, Sirisereewan C, Sangpratum N, Kesdangsakonwut S, Tummaruk P, Teankum K, Assavacheep P, Jitimanee S, Thanawongnuwech R. 2018. Porcine circovirus type 3 (PCV3) shedding in sow colostrum. Veterinary Microbiology. 220: 12-17.

Kedkovid R, Woonwong Y, Arunorat J, Sirisereewan C, Sangpratum N, Lumyai M, Kesdangsakonwut S, Teamkum K, Jitimanee S, Thanawongnuwech R. 2018. Porcine circovirus type 3 (PCV3) infection in grower pigs from a Thai farm suffering from porcine respiratory disease complex (PRDC). Veterinary Microbiology. 215: 71-76.