การติดเชื้อข้ามชนิดสัตว์และการศึกษาทางซีรัมวิทยาของเชื้อไข้หวัดใหญ่ที่กลายพันธุ์ในประเทศไทย ภายหลังการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 ในสุกร

นายจิรภัทธ อรุโณรัตน์

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INTERSPECIES TRANSMISSION AND SEROLOGICAL STUDY OF CURRENT REASSORTANT SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC H1N1 2009 IN THAILAND

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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จิรภัทธ อรุโณรัตน์ : การติดเชื้อข้ามชนิดสัตว์และการศึกษาทางซีรัมวิทยาของเชื้อไข้หวัดใหญ่ที่กลายพันธุ์ในประเทศไทยภายหลังการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 ในสุกร (INTERSPECIES TRANSMISSION AND SEROLOGICAL STUDY OF CURRENT REASSORTANT SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC H1N1 2009 IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. สพ.ญ. ดร. เยาวลักษณ์ ปัญญสิงห์, 124 หน้า.

การติดเชื้อข้ามชนิดจัดได้ว่าเป็นประเด็นที่มีความสำคัญเป็นอย่างยิ่งของเชื้อไวรัสไข้หวัดชนิดเอ เนื่องจากเชื้อไวรัสชนิดนี้สามารถก่อโรคได้ในหลายชนิดสัตว์ รวมไปถึงโฮสต์ที่สำคัญ 3 ชนิดได้แก่ มนุษย์ สัตว์ปีก และสุกร ในปี 2552 ได้มีการระบาดของเชื้อไข้หวัดสุกรสายพันธุ์ใหม่ชนิด H1N1(H1N1pdm09) ในหลายพื้นที่ทั่วโลก โดยต้นกำเนิดของไวรัสเกิดการกลายพันธุ์ผสมระหว่างไวรัสไข้หวัดสุกร 2 ชนิด ซึ่งในปี 2553 ได้มีการเพาะแยกไวรัสชนิดนี้ได้จากฝูงสุกรในหลายพื้นที่ทั่วโลก โดยการศึกษาในครั้งนี้เป็นการศึกษาพยาธิกำเนิดของเชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่ และไวรัสกลายพันธุ์ลูกผสมของเชื้อไวรัสนี้ในเป็ด และการศึกษาความสามารถในการป้องกันการติดเชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่และไวรัสกลายพันธุ์ลูกผสมของวัคซีนป้องกันไข้หวัดใหญ่ในมนุษย์ ผลการศึกษาพบว่าเชื้อไวรัสไข้หวัดสุกรสายพันธุ์ใหม่และไวรัสกลายพันธุ์ลูกผสมสามารถติดเชื้อในเป็ดทดลอง โดยไม่แสดงอาการหรือแสดงอาการทางคลีนิกเพียงเล็กน้อย และพบการปลดปล่อยไวรัสสู่สิ่งแวดล้อมได้ ดังนั้นเป็ดจึงจัดได้ว่าเป็นแหล่งกักเก็บเชื้อไวรัสที่สำคัญอย่างหนึ่ง การสำรวจการติดไข้หวัดหวัดในเป็ดจึงมีความสำคัญอย่างยิ่ง และยังพบว่าวัคซีนป้องกันโรคไข้หวัดใหญ่ในมนุษย์ในปัจจุบัน สามารถป้องกันการติดเชื้อได้เพียงแค่เชื้อไข้หวัดสุกรสายพันธุ์ใหม่ และไวรัสลูกผสมที่มี H1 มาจากไข้หวัดสุกรสายพันธุ์ใหม่เท่านั้น เชื้อไข้หวัดสุกรทั่วไป และไข้หวัดสุกรลูกผสมอื่นๆ วัคซีนไม่สามารถป้องกันได้ ซึ่งข้อมูลนี้จะเป็นประโยชน์ต่อการพัฒนาวัคซีนป้องกันไข้หวัดใหญ่ในอนาคต นอกจากนี้จากการสำรวจการติดเชื้อไข้หวัดสุกรในช่วงปี 2555-2557 พบว่าสถานการณ์ของโรคไข้หวัดสุกรในประเทศไทยได้เปลี่ยนไป ภายหลังการระบาดของเชื้อไข้หวัดสุกรสายพันธุ์ใหม่ ซึ่งการศึกษาทางซีรัมวิทยาโดยฉพาะอย่างยิ่ง HI จำเป็นอย่างยิ่งที่ต้องมีการศึกษา โดยผลการศึกษาพบว่า เชื้อไข้หวัดสุกรสายพันธุ์ใหม่ ได้กลายเป็นไวรัสท้องถิ่นในฝูงสุกรในประเทศไทย และควรเลือกเป็นหนึ่งในไวรัสอ้างอิงที่ใช้ในการตรวจทางซีรัมวิทยาในปัจจุบัน กล่าวโดยสรุปจากการศึกษาครั้งนี้พบว่า ภายหลังการระบาดของเชื้อไข้หวัดสุกรสายพันธุ์ใหม่ การสำรวจการติดเชื้อในโฮสต์หลายๆชนิด มีความจำเป็นอย่างยิ่ง เพื่อที่จะให้ได้ข้อมูลที่เป็นประโยชน์เกี่ยวกับสถานการณ์ไวรัสในปัจจุบัน การพัฒนาวัคซีนป้องกันโรคไข้หวัดใหญ่ และการป้องกันการติดเชื้อข้ามชนิดสัตว์ต่อไป

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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JIRAPAT ARUNORAT: INTERSPECIES TRANSMISSION AND SEROLOGICAL STUDY OF CURRENT REASSORTANT SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC H1N1 2009 IN THAILAND. ADVISOR: PROF. DR. ROONGROJE THANAWONGNUWECH, CO-ADVISOR: DR. YAOWALAK PANYASING, 124 pp.

Interspecies transmission is one of the most interesting aspects for influenza A virus (IAV) research since the virus is able to infect multi-host species, especially, for three main important hosts including human, avian and swine. In 2009, pandemic H1N1 (H1N1pdm09) emerged and caused infection in both humans and pigs worldwide. The virus origin came from the reassortant between two swine influenza viruses (SIVs) and later, found circulated in pig population since 2010 in many parts of the world. In this study, the pathogenesis of H1N1pdm09 and its reassortant viruses using domestic ducks and the use of human influenza vaccines against H1N1pdm09 and its reassortant viruses of swine origin were investigated. The results demonstrated that H1N1pdm09 and its reassortant viruses could infect experimental ducks showing asymptomatic to mild clinical signs with small amount of virus shedding. This suggested that ducks could be one of the H1N1pdm09 reservoirs. Surveillance program of IAV in ducks is of importance. Additionally, current human influenza vaccines could protect only H1N1pdm09 and H1pdm reassortant viruses. The studied vaccines, however, did not completely protect all SIVs and reassortants of H1N1pdm09 origin. The finding data benefited human vaccine development and future plan. Moreover, the SIV surveillance data during 2012-2014 showed that SIV status in Thailand had changed after the H1N1pdm09 introduction. SIV serological assay especially for HI test was also investigated. The data showed that H1N1dpm09 has become endemic in the Thai pig population and should be added into the routine reference viruses for serological study. In conclusion, after the emergence of H1N1pdm09 in Thailand, multi-species influenza active surveillance is necessary for getting up to date influenza status, vaccine strategy and prevention among interspecies transmission.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

bp	base pair(s)
BSL-2	Biosafety Level 2
°C	dregree Celsius (centrigrade)
Ct	Cycle threshold
DAB	3,3-diaminobenzidine tetrahydrochloride
cDNA	complementary deoxyribonucleic acid
DPI	day post infection
DPV	day post vaccination
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
HPAI	Highly pathogenic avian influenza virus
IAV	influenza A virus
LPAI	Low pathogenic avian influenza virus
M	Matrix gene
MDCK	Madin-Darby canine kidney cell line

MEM	Minimal essential media
ml	milliliter (s)
µg	microgram (s)
µl	microliter (s)
µm	micrometer (s)
mM	micromole (s)
NA	Neuraminidase gene
NP	Nucleoprotein gene
NS	Non-structural protein gene
OIE	World Organization for Animal Health
H1N1pdm09	pandemic H1N1 2009 virus
PA	Polymerase acidic gene
PB1	Polymerase basic 1 gene
PB2	Polymerase basic 2 gene
PCR	Polymerase chain reaction
RDE	Receptor destroying enzyme
RNA	Ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SA	sialic acid
SIV	swine influenza virus
SN	Serum neutralization test

$TCID_{50}$	50% tissue culture infectious dose
TRIG	Tripple reassortant internal gene cassette
VAERD	Vaccine-associated enhanced respiratory disease
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1. Importance and rationale

Swine influenza virus (SIV), an important zoonotic respiratory pathogen, is evolving in both veterinary and human public health concern worldwide. Three subtypes of SIV commonly circulating in pig population include H1N1, H1N2, and H3N2 (Forrest and Webster, 2010). Interestingly, the bidirectional interspecies-transmission between human and swine or swine and avian have been seen due to the interface of those species (Nelson and Vincent, 2015). Moreover, swine is the main intermediate mixing vessel of influenza A viruses (IAV) and suspected to be the pandemic source. In addition, swine has the capacity to infect by both mammalian and avian viruses resulting in generating novel reassortant viruses (Rajao and Vincent, 2015). Most of reassortant or mutated viruses caused the pandemic scheme. For instance, the first pandemic of "Spanish flu" (1918) causing high morbidity and mortality worldwide, .the origin of the virus was derived from an avian mutated virus infecting directly in the human population. Other examples of pandemic outbreaks from the reassorted viruses occurred in 1957 called "Asian flu" and in 1968 called "Hong Kong flu". The virus genomes composed of PB1 and HA/NA protein from the avian species (H2, N2) and other genes were from an original H1N1 influenza virus in human. These two viruses

caused the mortality more than 1.5 million people around the world (Watanabe et al., 2012).

In April 2009, the pandemic H1N1 2009 influenza virus (H1N1pdm09) suspected to emerge from swine of unknown origin to humans, rapidly spread and caused the outbreaks in many areas around the world including Thailand (Sreta et al., 2010). The genome of this virus is closely related to the swine influenza virus (SIV) so called Swine-origin 2009 influenza A virus (H1N1) or swine flu. The supposed origin of the 2009 H1N1 influenza virus were from two different viruses, a triple reassortant internal gene virus (TRIG) from North America swine lineage (H1N2) and Eurasian avian-like swine lineage (H1N1). Specifically, TRIG was a mix of polymerase acidic (PA) and polymerase basic 2 (PB2) genes from the North American avian lineage, nonstructural (NS), nucleoprotein (NP), hemagglutinin (HA: H1) and matrix (M) genes from the classic swine lineage and Polymerase basic 1 (PB1) gene of human lineage. The gene from Eurasian avian-like lineage were NA (N1) and M gene (Arias et al., 2009). The H1N1pdm09 has been suspected to circulate in the swine population without being detected any clinical signs and possibly transmitted to humans especially the swine workers before the outbreak. The first report of H1N1pdm09-infection in pigs was found in the Canadian pigs in April 2009 and in Thailand, in December 2009 (Smith et al., 2009a). Due to H1N1pdm09 has the internal gene as TRIG virus, the virus has ability to reassort and generate the novel viruses easily (Gauger et al., 2012). After the emergence of H1N1pdm09, a novel reassortant H1N1pdm09 (rH1N1) was detected in Hong Kong in

early 2010 (Vijaykrishna et al., 2010). A genome of this novel virus contained reassortant of NA gene from H1N1pdm09 with Eurasian swine lineage. In Thailand, the reassortant H1N1pdm09 (most internal genes from the 2009 H1N1pdm09 and N1 from an endemic Thai swine influenza virus) was recently found in a commercial farm in Central division of Thailand (Sreta et al., 2010). This data suggested that novel reassortant viruses could emerge in the swine population as well as survive and circulate in the swine population. Therefore, possibility of reverse zoonosis (transmission back to the humans) or even transmitted to other species could occur (Nelson and Vincent, 2015). Therefore, many organizations and researchers raised their concern on the interspecies transmission from pig to other species, particularly in aquatic avian species including domestic ducks. Ducks are suspected to be the main reservoir of IAV, since most HA and NA surface proteins can be found in those waterfowls. Moreover, those avian species do have the seasonal migration and are able to spread the virus from one area to other areas (Kim et al., 2009).

Domestic ducks are the major waterfowls of interest since they are one of the main populations of avian species in Thailand. Four duck raising systems (close biosecurity system, open house system, grazing system and backyard ducks) exist in most Asian countries including Thailand (Songserm et al., 2006). However, grazing and backyards ducks are suspected to be the major virus reservoir transmitting the virus to other domestic species. Infected ducks can shed the virus via oropharyngeal and cloacal routes for several days without showing the clinical signs (Kida et al., 1980).

Furthermore, duck population were also suspected to be the main source of the highly pathogenic avian influenza (HPAI) H5N1, transmitted to other species (Keawcharoen et al., 2011). In 2013-2014, H7N9 avian influenza virus emerged in the human population and caused many lethal cases in China and many areas around the world. The genetic characterization was proved that the origin of H7N9 avian influenza came from the reassortant between avian viruses in the wild birds prior to the transmission to human (Gao et al., 2013; Stein, 2013). Since ducks are placed and easily interface together with other species including pigs, the main source of H1N1pdm09 and rH1N1, questions on the pathogenesis of the swine viruses in the ducks should be considered. In addition, interspecies transmission from pigs to duck was investigated previously (Charoenvisal et al., 2013c). In order to elucidate the pathogenesis of those viruses in ducks, the study on the H1N1pdm09 and the rH1N1 isolated from pigs in the experimental ducks should be performed to understand the susceptibility, clinical signs, viral shedding and lesions in the experimental ducks. This investigation would provide the essential information for the epidemiological study, disease control and prevention of those viruses.

The surveillance data of Thai swine influenza in 2010-2014 showed that the H1N1pdm09 and its reassortant viruses circulated in conventional pigs and became the predominant strains in the Thai swine population (Charoenvisal et al., 2013a; Nonthabenjawan et al., 2015). Consequently, this brings to an important question on possibility of the interspecies-transmission and efficacy of the current vaccine using in

human. Currently, the commercial sub-unit human influenza vaccines (the recombination antigen of HA and NA) are commonly used in many countries (World Health Organization, 2009). The vaccines compose of HA and NA antigens from human seasonal H3N2 influenza virus, H1N1pdm09 and influenza B virus as the recommendation of the World Health Organization (World Health Organization, 2009) with annually changed the seed viruses every year in both Northern and Southern hemispheres. However, reassortant of H1N1pdm09 viruses such as reassortant H3N2 (rH3N2) from pigs contains the genetic data not closely related to the human H3N2 virus in the vaccines (Nonthabenjawan et al., 2015). If the swine virus could accidentally transmit to the human population and cause the disease, the questions of concern would be the protection of the human influenza vaccines to the reassortant SIVs. To answer this question, the pig model for this experiment was conducted. The results would provide the information benefitting to the public health and the influenza vaccine strategy in humans.

According to SIV surveillance data in 2010-2014, Thai SIV genetic status has changed after the introduction of H1N1pdm09. Prior to the introduction of H1N1pdm09 to the Thai swine population, only three subtypes (endemic H1N1, endemic H1N2 and endemic H3N2) of SIV were found (Sreta et al., 2013). After the introduction of H1N1pdm09 to the Thai swine population, many reassortants of H1N1pdm09 were found to be co-circulated with the endemic viruses (Charoenvisal et al., 2013a). This suggested that not only the SIV status has changed, but also the serological status of

Thai SIVs might have changed particularly, when using current serological methods especially for hemagglutination inhibition test (HI test). Based on the genetic data in 2010, two of Thai SIV isolates were selected to use as reference viruses for HI test, i.e., enH1N1-2006 for subtype endemic H1 and enH3N2-2007 for subtype H3 (Sreta et al., 2013). Similar question on the reference viruses from 2010 using for serological diagnosis (HI test) does exist. Determination of the current reference viruses used for HI test is of importance and current serological SIV status must be set up to get an up-to-date information. The obtained information would benefit the serology diagnosis and the current status of swine influenza viruses in Thailand.

1.2 Objectives of the study

- To investigate the pathogenicity of the H1N1pdm09 and its reassortant virus (rH1N1) isolated from pigs in domestic ducks
- To study current human Influenza vaccines on the protection of the reassortant SIVs infection if the interspecies transmission occurs.
- To get an up-to-date information on the reference viruses used for the serological methods (HI test) and serological status of current Thai SIVs.

1.3 Literature reviews

Influenza viruses: Pandemic H1N1 influenza virus 2009 (H1N1pdm09) and its reassortant viruses.

Influenza viruses are the important contagious respiratory pathogen evolving in both veterinary and human public health concern worldwide. Influenza viruses can cause variety of clinical signs from mild to severe illness and even death in the risk population around the world (Kenah et al., 2011). The viruses are negative sense stranded segmented RNA in the Orthomyxoviridae, classified into 4 groups: influenza A, B, C and D. However, influenza type A virus (IAV) is of importance because the viruses can cause the diseases in wide host ranges, such as humans (H1N1, H3N2), pigs (H1N1, H1N2 and H3N2), domestic poultry and wild birds (H5 and H7).

Specifically, the H5 and H7 in wild birds act as the highly pathogenic avian influenza virus (HPIV) (Forrest and Webster, 2010). The virus genome composes of eight segmented RNA: polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA) form as the RNA polymerase complex and after the combine with nucleoprotein (NP) called ribonucleoprotein (RNP) which are important to the genome transcription process (Neumann and Kawaoka, 2015). Two important surface protein, hemagglutinin (HA) the major protein for binding to host SA receptor and start the viral entry phase and neuraminidase (NA) has enzymatic activity to catalyze the sialic acid (SA) receptors liberating the progeny viruses to other cells. Moreover, these two

proteins are important and used for subtyping of the influenza A viruses. The matrix (M), divided into M1 (formed as inner layer of virus and played important role in viral assembly phase) and M2 (functioned as pH-dependent ion channel and protected HA conformation). The other protein called nonstructural 1 (NS1) enhances the viral mRNA translation and nuclear exporting protein (NEP) which functioned as nuclear exporting factor. Two types of sialic acid (SA) receptor formation on glycoprotein of host cells used for HA binding are the α2,3 (avian type) and α2,6 (mammalian type) SA receptor. :The avian type receptor are found on the intestinal epithelium of avian and lower respiratory of human whereas the mammalian type receptor usually found on human and mammalian trachea. Interestingly, both SA receptors can be found in trachea of pigs, and thus, pig can be infected with both mammalian and avian viruses. (Neumann and Kawaoka, 2006; Arias et al., 2009; Bai et al., 2011; Webster and Govorkova, 2014). IAV have a highly potential in genetic variations due to the process of genetic drift and shift. The genetic drift is the point mutation occurred due to lacking of proofreading activity of RNA during the replication. This mechanism can occur in any viral genetic segment. Importantly if it occurs on the neutralizing epitopes on HA and NA, it would alter the effective immunological response. The genetic shift is the combination of the segmented genomes of two different viruses infecting into one cell and generate reassortant viruses (Arias et al., 2009; Li and Chen, 2014). A novel reassortant virus can emerge possibly leading to the pandemic outbreak worldwide such as the first recognized pandemic outbreak occurring in 1918 called the “Spanish flu” causing high

morbidity and mortality worldwide. The origin of the virus was derived from an avian mutated virus infecting directly into the human population. Later, the pandemic outbreaks occurred in 1957 called “Asian flu” and in 1968 called “Hong Kong flu”. The virus genomes composed of PB1 and HA/NA protein from the avian species (H2, N2) and other genes were from an original H1N1 influenza virus from human. These two viruses caused the mortality more than 1.5 million people around the world (Smith et al., 2009a). Recently, the H1N1pdm09 influenza virus has been recognized causing the negative impact on both public health and swine health worldwide.

The virus origin came from a triple reassortant virus from the North American triple reassortant virus (TRIG), composing of genomic segments from three different origins: North American avian lineage, classic swine lineage and human lineage. Reassortant with NA (N1) and M genes from the Eurasian avian-like swine lineage were evident (Arias et al., 2009; Bai et al., 2011). This novel virus was suspected circulating for a year in the swine population without being detected prior to the emergence in the human population (Arias et al., 2009). After the emergence of the H1N1pdm09 in humans in Mexico, there were many reports of the H1N1pdm09 in pigs. The first report was in the Canadian pigs in April 2009 (Smith et al., 2009a). The phylogenetic study of the virus found that its genome is closely related to the human H1N1pdm09 virus. Then, the virus has spread throughout the world including Thailand in December 2009 (Sreta et al., 2010). This H1N1pdm09 virus was isolated in the swine population in Saraburi and Ratchaburi province located in central Thailand. In vivo study of

H1N1pdm09 in mice showed prominent bronchitis and alveolitis with excessive cytokine production. In non-human primate study, the H1N1pdm09 virus replicated efficiently in lung and caused severe lung lesion with edematous exudates, inflammatory cells infiltration and severe thickening of alveolar wall. However, in miniature pigs, no obvious clinical signs were found but the virus could replicate in the respiratory organ of infected pigs with high titers (Itoh et al., 2009).

After the emergence of H1N1pdm09, the reassortant H1N1pdm09 (rH1N1) has been reported in the pig population in many areas around the world. The first evidence of the reassortant virus of H1N1pdm09 origin was found in Hong Kong, in January 2010. The reassortant virus contained the reassortant of NA gene from the 2009 H1N1pdm09 virus with the Eurasian swine lineage (Vijaykrishna et al., 2010). Thereafter, the virus contained most of the genes from the H1N1pdm09 and NA gene from a swine influenza virus (SIV) H1N2 were found in Italy (Moreno et al., 2010). Furthermore, the reassortant H1N1pdm09 found in England in mid-April 2010 composed of NA gene from the H1N1pdm09 virus with other 7 genes of classical England swine origin (Howard et al., 2011). Likewise, the reassortant of H1N1pdm09 isolated from pigs in central division of Thailand revealed that, the H1 gene came from the H1N1pdm09 virus and N1 gene came from an endemic swine influenza virus circulating in a commercial swine farm (Kitikoon et al., 2011a). Similarly in Argentina, the genome of the reassortant virus contained most genes from the H1N1pdm09 and only HA and NA genes from a human-like swine influenza virus (Pereda et al., 2011) and in the United States, the virus

genome came from 3 different sources: NA and HA genes from the human lineage, NP and M genes from the H1N1pdm09 and the rest of the genes from TRIG. Nowadays, the reassortant of H1N1pdm09 can be found in pigs from many subtypes worldwide such as Germany (2009), China (2010-2012), Japan (2013), Vietnam (2013), USA (2010-2013), Korea (2012), Brazil (2013) and Italy (2013) (Nelson et al., 2015).

Human Influenza Vaccines.

Currently, many types of influenza vaccine are used to control and prevent influenza infection. Inactivated influenza vaccine especially for sub-unit vaccines are commonly used worldwide. The vaccines are designed to induce the neutralizing antibodies to the HA portion especially for HA1 (head of HA protein) and inhibit the binding process to host receptor of the virus. This type of vaccine is safety by standardizing the amount of antigen and has high efficient to induce the antibody. Current commercial influenza vaccines compose of HA antigen of two influenza A (H1N1pdm09 and seasonal H3N2) and influenza B strain. Moreover, alternative inactivated vaccine are virus-like particles (VLPs). This vaccine compose of the structural protein of the virus such as HA, NA or M proteins forming virion without the internal protein. The viral vector influenza vaccine is one of candidate influenza vaccine of interest. This vaccine uses the vector viruses such as adenovirus or vaccinia Ankara virus to harbor influenza antigens to host infection. This vector vaccine is safe and has high efficient to induce the immune response. But the mutation of HA1 portion continuingly occurs from antigenic drift or

shift and the vaccine antigens used in inactivated vaccines are usually changed annually as the WHO prediction upon the circulating viruses in each area. Therefore, the influenza virus surveillance data are necessary for strain prediction. Unpredictable or inaccurate prediction may cause the mismatch between vaccine strains and circulating viruses reducing the vaccine efficacy (Schotsaert and Garcia-Sastre, 2014; Treanor, 2015).

Swine influenza virus

Pathogenesis

Most subtypes found in pigs are H1N1, H1N2 and H3N2. The infected pigs usually showed various clinical signs including asymptomatic, coughing, sneezing, ocular and nasal discharge, conjunctivitis and other systemic signs such as fever, weight loss and poor growth (Schnitzler and Schnitzler, 2009). The transmission routes occur via aerosol, droplet and direct contact from secretion and fomites. The incubation period usually takes 1-3 days and recovery period takes 4-7 days after infection. The virus causes high morbidity rate but low mortality rate and rapid recovery (Shetty, 2009). The tissue tropism of the virus is the epithelium of the upper and lower respiratory system of pigs. After infection, inflammatory cells primarily infiltrate into the lung and cause epithelial necrosis. Then, the inflammatory cells (mainly neutrophils), necrotic epithelial cells, cell debris and exudates obstruct the respiratory

airways and consequently cause the clinical signs. At 6-7 days after infection, infected pigs shed the virus via the droplets and other respiratory exudates (Arias et al., 2009).

Epidemiology

Swine influenza virus especially for endemic SIVs do not usually show clearly clinical signs but show the sign of co-infection having respiratory signs called "Porcine Respiratory Disease Complex (PRDC)". Main pathogens causing the respiratory signs or co-infection with SIV including *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumonia, Pasteurella multocida*, porcine reproductive and respiratory syndrome virus (PRRSV), Pseudorabies virus (PRV) and porcine circovirus type 2 (PCV2) (Opriessnig et al., 2011).

The first case of the SIV in North America was in 1930 which suspected from the interspecies transmission of the Spanish flu in 1918-1919 (Shope, 1931). The virus circulated in the North America known as classical swine H1N1. In 1998, there was a report of the triple reassortant internal genes cassette virus (TRIG virus) which had the HA, PB1 and NA genes derived from human, PA and PB2 from avian and NP, M and NS from classical swine H1N1. After that, the reassortant between classical swine H1N1 and TRIG virus called reassortant H1N2 (rH1N2) emerged. Recently there are classical swine H1N1 (H1 α, H1 β, H1 γ, H1 δ1, H1 δ2 and H1pdm09), H3N2 (four main clusters), H1N2 and reassortant viruses circulating in the pig population in North America (Vincent et al., 2008; Vincent et al., 2009; Forrest and Webster, 2010).

In Europe, classical swine H1N1 of avian origin circulated in pig population until 1979 and was replaced with the avian like swine lineage virus H1N1 called “avian-like swine H1N1” (Vincent et al., 2014). The human H3N2 introduction into the pig population since 1968 and this virus reassorted with the avian-like swine H1N1 (genome of the novel virus compose of the internal genes of avian like swine lineage virus H1N1 and external genes of human H3N2) and became dominant of H3N2 in European pigs (Komadina et al., 2007; Smith et al., 2009b). Additionally, in 1994 the reassortant H1N2 was introduced into the pig population in UK and rapidly spread to many countries in Western Europe. Evidently, these were the three major subtypes of SIVs circulated in Europe before the introduction of H1N1pdm09.

In Asia especially in China, there are 3 major subtypes of SIVs (H1N1, H1N2 and H3N2). H3N2 has the genetic data closely related to human H3N2. H1N1 divided into classical swine H1N1 from North America and avian like swine lineage virus H1N1 from Europe. Circulation of classical swine H1N1, H1N2 and H3N2 in swine population in Thailand was reported since 1970 (Komadina et al., 2007). The study of the virus genome in 2000-2006 found that PB2, PB1, PA and M genes closely related to the avian-like swine lineage virus H1N1 from Europe, and HA gene closely related to the classical swine H1N1 from North America. Within the subtype H1N1, the H1 gene of Thai SIVs was divided into two groups (H1α and H1δ) and the N1 gene closely related to avian-like swine lineage virus H1N1 from EU. For subtype H3N2, the H3 was divided into two groups (human H3N2 and avian-like swine from EU and Hong Kong) and the N2 was

divided into three groups which closely related to viruses form North America, Asia and Europe. The subtype H1N2 had the HA gene from classical swine H1N1 from North America and the NA gene from Europe (Komadina et al., 2007; Smith et al., 2009b). After the introduction of pdmH1N1, the SIV surveillance data during 2010-2014 revealed only classical swine H1 (H1α) co-circulated with H1N1pdm09.

Swine influenza diagnosis

IAV diagnostic methods include immunohistochemical techniques for the direct detection of IAV antigen in tissues, virus isolation in cell culture, hemagglutination test (HA test) for, and reverse transcription polymerase chain reaction (RT-PCR). Serum neutralization test (SN test), antibody ELISA and hemagglutination inhibition test (HI test). The HI test is usually used as the gold standard for serological study of the influenza virus. This method detect the antibody specific to HA antigen of the virus (Pedersen, 2014; Vemula et al., 2016).

Interspecies Transmission

Avian species and Influenza A virus

In order to understand more on influenza A virus, many researchers have raised their concerns on interspecies transmission since influenza A virus can cause disease in wide host ranges. One of the major important animals is the avian species. It should be noted that a lot of domestic poultry were killed and culled by highly pathogenic H5N1 in 2004 causing massive economic loss in the poultry industry (Songserm et al.,

2006; Watanabe et al., 2012). The control and surveillance programs for highly pathogenic avian influenza H5N1 are essential. Another group of avian species is the waterfowls. These aquatic avian species are the major reservoir for influenza viruses, harboring most of the HA and NA subtypes but showing no clinical sign. In addition, most of the waterfowls do migrate, believing to be the main reservoirs for distributing the influenza virus from one to other areas (Kim et al., 2009).

Ducks and Ducks raising systems in Thailand

Duck is an important domestic waterfowl in Thailand in term of influenza carriers. The duck population in Thailand composes of both domestic and wild ducks. The domestic ducks raised in many areas of Thailand compose of Peking or cherry valley and Muscovy ducks for meat and Khaki Campbell and native laying ducks for eggs. Four duck raising systems are found in Thailand: 1) Closed high biosecurity system, for the meat ducks raised in 50-55 days in closed and all in/all out system, 2) Open house system, raised in open air but practicing all in/all out strategy using for both meat and egg-laying ducks. In addition, most laying ducks are raised in this system, 3) Grazing system, raised in the open rice fields three weeks after hatching, especially, for egg-laying ducks. The ducks are raised for five to six months from one rice field to another rice field after the harvesting season. After that the ducks are brought back to the farm for eggs production, and 4) the last system is the backyard ducks found mostly in Asian countries providing comingling among domestic animal species in the same environment. The ducks are raised mixing with other animals such as chickens, geese

or pigs without any control or prevention of diseases among species (Kida et al., 1980; Songserm et al., 2006; Kim et al., 2009).

Ducks as a role of interspecies transmission of H1N1pdm09 and its reassortant viruses.

Ducks are known as the main reservoir of the influenza virus. The evidence was shown that ducks could carry many subtypes of the influenza virus with less or no clinical signs. Particularly, in the avian influenza H5N1, ducks were the most important reservoir and transmitted to the domestic poultry causing huge economic loss in 2004 (Songserm et al., 2006). It should be noted that ducks usually resist to most of influenza strains showing no or mild lesions or clinical signs (Kida et al., 1980). However, in the highly pathogenic strains such as H5 or H7 in avian species, clinical signs of cough, anorexia, acute respiratory syndrome, diarrhea and sudden death could be found. Upon necropsy, subcutaneous edema, multifocal hemorrhage or necrosis in multi-organs such as pericardium, proventriculus, grizzard, intestine, spleen, liver, heart, brain and kidney, thymus and bursa were also found. Histopathologically, lesions in the lung showed extensive tracheitis and bronchitis, mainly mononuclear cell infiltration in submucosal area and hemorrhage. Lesions in other organs contained diffuse hepatocellular damage or necrosis, brain degeneration with infiltration of mononuclear cells (Kida et al., 1980; Kim et al., 2009; Keawcharoen et al., 2011).

Likewise, duck raising systems especially grazing and backyard ducks are very important since ducks are main reservoirs of the influenza virus and are able to comingle with other animals in the same environment (Charoenvisal et al., 2013c;

Boonyapisitsopa et al., 2016). Interspecies transmission particularly when comingled with H1N1pdm09 or rH1N1-infected pigs is possible. Although previous studies of H1N1pdm09 in the ducks have been done, but the information about pathogenesis of the H1N1dpm09 and rH1N1 are still not clearly elucidated. In order to elucidate the pathogenesis of those viruses in ducks, the study on the H1N1pdm09 and it reassortant viruses isolated from pigs in the experimental ducks should be performed, in order to understand the susceptibility, clinical signs, viral shedding and lesions of those viruses in the experimental ducks.

CHAPTER 2

Interspecies Transmission:

PATHOGENICITY OF PANDEMIC H1N1 2009 (H1N1pdm09) AND REASSORTANT SWINE INFLUENZA VIRUSES (rH1N1) OF SWINE ORIGIN IN DOMESTIC DUCKS

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Pathogenicity of pandemic H1N1 2009 (H1N1pdm09) and reassortant swine influenza viruses (rH1N1) of swine origin in domestic ducks

Abstract

Currently, the pandemic H1N1 (pH1N1) 2009 influenza A virus and its reassortant pandemic H1N1 influenza (rH1N1) viruses have been circulating in pigs population in many countries around the world including Thailand. Many organizations and researchers have raised some concerns about interspecies transmission, particularly in aquatic avian species including domestic ducks when commingling in the back-yard farming. In order to elucidate the pathogenesis of those viruses in the domestic ducks, the study on the pH1N1and rH1N1 recently isolated from pigs was conducted to understand the susceptibility, clinical signs, viral shedding and lesions in the experimental ducks. Twenty one 6-week-old influenza A virus negative ducks were divided into 3 groups (2 challenged and 1 control group). In the challenged groups, ducks were individually inoculated with the pH1N1 or the rH1N1 in the appropriated group as mentioned previously. All experimental ducks were observed the clinical signs and collected the oropharyngeal and cloacal swabs to investigate the viral shedding using a modified real time RT-PCR. Tissues samples were collected for pathological and immunohistochemistry examination. The results demonstrated that either pH1N1 or rH1N1 did not induce significant flu-like clinical sign. However, both viruses could infect the experimental ducks which had varied gross and microscopic lesions as well

as the duration of virus shedding. Interestingly, the detectable lesions and the extended shedding period were found in the group of rH1N1-infected ducks. In conclusion, inter-species transmission should be considered when commingling different animal species.

Keywords: ducks, influenza, pandemic H1N1 2009, pathogenesis, pigs, reassortant

2.1 **Introduction**

In April 2009, the pandemic H1N1 2009 virus (H1N1pdm09) has rapidly spread causing at least 340,000 clinical cases reported and more than 4,000 deaths around the world including Thailand. The genome of the virus is closely related to the swine influenza virus (SIV), called swine-origin 2009 influenza A virus (H1N1) or swine flu. The origin of the virus came from reassorted genome segments between the triple reassortant internal gene (TRIG) virus from North America and the avian-like Eurasian swine H1N1 lineage from Europe (Dawood et al., 2009; Dubey et al., 2009; Forrest and Webster, 2010). This virus has been suspected to circulate in the swine population without undetectable clinical signs and to possibly transmit to humans, especially swine workers, with unclarified evidences. In pigs, the first report of H1N1pdm09-infection was found in Canadian pigs in April 2009 (Smith et al., 2009a). Later, several reports were found in Hong Kong in October 2009 (Vijaykrishna et al., 2010) and in Thailand in December 2009 (Sreta et al., 2010). After the emergence of H1N1pdm09, the first report of reassortant of H1N1pdm09 was found in Hong Kong in 2010. The genome of virus contain NA gene from H1N1pdm09 and the rests came from Eurasian avian-like swine lineage. Another novel reassortant H1N2 adopting the HA and NA genes from classical swine virus H1N2 and the remaining genes from H1N1pdm09 was reported in the United Kingdom. In Thailand, the first reassortant H1N1pdm09 (H1 gene from the H1N1pdm09 and N1 from an endemic Thai SIV) was found in a commercial farm in

Central division of Thailand (Kitikoon et al., 2011a). Recently, several reports of reassortant viruses between H1N1pdm09 and endemic swine influenza virus continuously occur in many areas around the world such as Germany (2009), China(2010-2012), Japan (2013), Vietnam (2013), USA (2010-2013) and in Thailand with three strains of reassortant viruses co-circulated with endemic virus in Thai swine population. These suggested, with the ability of the reassortant virus to survive and circulate in the swine population, transmission to humans or other commingled species (interspecies transmission) in the future is possible. Since the emergence of H1N1pdm09, interspecies transmission has been of concern and study in multispecies such as human, birds, ferret, mice, dogs, cats, wildlife animals, and particularly in aquatic avian species including domestic ducks (Kim et al., 2009).

Previous studies reported that H1N1pdm09 contained genes from avian-like Eurasian swine H1N1 lineage which were the avian-like swine virus and genes from TRIG which were closely related to the virus from duck origin (Arias et al., 2009; Webster and Govorkova, 2014). Moreover, most of HA and NA surface antigens can be found in ducks with no clinical signs (Kim et al., 2009) and many species of ducks have seasonal migratory behaviors leading to the spreading of the virus (Keawcharoen et al., 2008). In Thailand, domestic ducks are the most important influenza reservoirs that play an important role in the spreading of the disease as in case of highly pathogenic avian influenza H5N1 in 2004. Four duck raising systems exist in most Asian countries, including Thailand; close biosecurity system, open house system, grazing system and

backyard ducks (Songserm et al., 2006). Interestingly, the grazing and backyards ducks are suspected to be the major virus reservoir for other domestic species. Since ducks roam freely in the environment in these two types of raising systems, the chance of exposure among ducks and other susceptible species, especially pigs which are the main source of H1N1pdm09 and its reassortant viruses. Therefore, the pathogenesis of H1N1pdm09 and reassortant virus (rH1N1) in experimental ducks should be considered for the information of possible virus transmission events.

In order to elucidate the pathogenesis of those emerging viruses in ducks, the study of H1N1pdm09 and rH1N1 isolated from pigs was of interest to understand the susceptibility, clinical signs, viral shedding and lesions in the experimental ducks. This investigation will provide the information beneficial to the epidemiology, disease control and prevention of those emerging influenza viruses in the future.

2.2 **Materials and Methods**

2.2.1 Virus

Virus stocks of the pandemic H1N1 (H1N1pdm09) influenza virus, A/swine/Thailand/CU-RA29/2009 (H1N1) (Sreta et al., 2010) and its reassortant H1N1pdm09 (rH1N1) influenza virus, A/swine/Thailand/CU-SA43/2010 (H1N1) (Kitikoon et al., 2011a) were prepared by passaging in 9-day-old embryonated chicken eggs. Allantoic fluid was harvested after 72 hours post infection. Infectivity of stock viruses was determined in Mardin-Darby canine kidney (MDCK) cells according to a standard procedure routinely performed at

the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) (Charoenvisal et al., 2013b). The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by Reed and Muench method (Reed, 1938). Concentration of both viruses, which were used in this study, was 10^4 $TCID_{50}$/ml. All experiments involving live viruses were conducted under biosafety level 2 containment (BSL-2) and the viruses were aliquoted and kept in -80°C until used.

2.2.2 Animals

Six-week-old cross-bred ducks were obtained from a commercial farm considered as IAV negative farm. Serum samples, oropharyngeal and cloacal swabs were collected from all experimental ducks 1 week before starting the experiment. The sera were analyzed by using a commercial ELISA H1N1 kit (HerdChek H1N1ELISA; IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's instructions. The swabs were tested for the presence of influenza A viruses using matrix (M) gene real time reverse transcription polymerase chain reaction (rt RT-PCR) (Spackman et al., 2002). Absence of pre-existing influenza A virus antibodies and M gene were expected in the sera and swabs of all animals.

All ducks were divided into 3 groups separately in the animal facility biosafety level 2, with 3 animals in the control group and 9 animals each in two challenged groups. In the challenged groups, the ducks were individually inoculated intratracheally and intraesophageally either with 3 ml containing 10^4 $TCID_{50}$/ml of the H1N1pdm09 or the rH1N1 in the assigned group as mentioned previously. In the control group, the animals

were mock inoculated with 3 ml of minimal essential medium (MEM) media. Animal care and the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University (Protocol No. 11310052).

2.2.3 Clinical observation and sampling

All experimental ducks were observed for clinical signs for 12 day post infection (DPI). The oropharyngeal and cloacal swabs were collected at 0 to 12 DPI in the viral transport media. At each time point (3, 5 and 13 DPI), 3 ducks from each challenged group and 1 duck from the control group were randomly euthanized using intravenous administration of pentobarbital administration overdose. Tissues including air sac, trachea, lung, brain, pancreas, liver, jejunum, colon, spleen and kidney were collected. All swabs and collected tissues were performed using a modified real time reverse transcriptase-polymerase chain reaction (rtRT-PCR) to detect the presence of both influenza viruses and the collected tissues were fixed in 10% buffer formalin for pathologic examination.

2.2.4 Pathological study and Immunohistochemistry

The tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax. The 4 μm thick tissue sections were prepared for histological analysis by hematoxylin and eosin (H&E) staining and for detecting Influenza A virus antigen by immunohistochemical technique using an anti-influenza A nucleoprotein monoclonal mouse antibodies (HB654404 B.V.EUROPEAN VETERINARY LABORATORY, the

Netherlands) as a primary antibody. The biotinylated rabbit anti-mouse IgG antibody and envision polymer (Envision Polymer DAKO®, Denmark.) were used as a secondary antibody. The reactions were developed in 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as the substrates and counterstained with Mayer's hematoxylin. The SIV infected pig lung tissue section from the previous experimental study was used as a positive control. The Influenza A virus positive antigen was evaluated using the following protocol (average finding of 10 high power fields/slide): (-) no viral positive antigen could be detected, (+) 1-2 viral positive cells detected in the whole tissue, (++) 1-2 viral positive cells detected per 1 high power field, (+++) 3-10 viral positive cells detected per 1 high power field and (++++) more than 10 viral positive cells detected per 1 high power field (Haines et al., 1993; Sreta et al., 2009).

2.2.5 Modified real time RT-PCR (rtRT-PCR) for influenza A virus detection

Viral RNA was extracted from the oropharyngeal and cloacal swabs and from all tissues using the Nucleospin® RNA virus (Machery-nagel, Duren, Germany). The rtRT-PCR assays were performed on Corbett Rotor-GeneTM 6000 (Qiagen) using SuperScriptTM III Platinum® One-Step Quatitative RT-PCR System (Invitrogen, Carlabad, California, USA) according to the manufacturer's instructions. Primers and probes were taken from a recent publication (Spackman et al., 2002) specifically to amplify a portion of the M gene of influenza A virus with some modifications (forward primer (MF3; 5'TGATCTTCTTGAAAATTTGCAG 3'), reverse primer (MR1+; 5' CCGTAGMAGGCCCTCTTTTCA 3') and M-64probe (FAM-TTGTGGATTCTTGATCG-TAMRA)

(Payungporn et al., 2006). The positive CT value was 1-40 and more than 40 was determined as a negative result (Charoenvisal et al., 2013c).

The positive control of rtRT-PCR was obtained from the positive sample based on viral isolation and titration in the MDCK cell line. The viral antigen was identified using the Influenza A nucleoprotein monoclonal antibodies (HB654404), a rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, USA) and developed with chromogen aminoethyl carbazole (AEC) substrate. Virus titration ($TCID_{50}$/ml) was performed using the routine procedure of the CU-VDL (Sreta et al., 2009) and calculated using Reed and Muench method (Reed, 1938).

2.2.6 Serological study

Serum samples were collected from all experimental ducks 1 week before starting the experiment and from all animals before euthanized at day 2, 4 and 12. The serum sample were analyzed using a commercial ELISA H1N1 kit (Avian Influenza virus antibody test kit H1N1 ELISA; IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's instructions and hemagglutination inhibition test (HI test) with as described previously (Sreta et al., 2013). The serum samples obtained from 1 week before starting the experiment must have no pre-existing influenza A virus antibodies and the swabs must show no influenza positive sample from all animals. The serum samples from all ducks before euthanasia were used for the antibody measurement to the influenza virus during the study.

2.3 Results

2.3.1 Clinical observation and gross pathology

The ducks in both experimental challenged groups remained healthy with no clinical signs observed. However, the H1N1pdm09–infected ducks showed the sign of conjunctivitis at 6 (3/3), 7 (3/3) and 10 (2/3) day post infection (DPI) (Fig 2.1). Macroscopic findings of H1N1pdm09–infected ducks at 2 (3/3), 4 (2/3) and 12 DPI (1/3) predominantly showed mild airsacculitis. In addition, hemorrhage was also present in the tracheal epithelium (1/3), lung (1/3), and liver (1/3) at 2 DPI. Serosanguineous fluid in the pericardial sac (1/3) was also found at 2 DPI. Similarly, in the rH1N1-infected ducks, consistent gross lesion was found and was mild airsacculitis. Other gross lesions including multifocal white foci in caudal lobe of liver at 2 DPI (1/3) and 4 DPI (1/3) were observed. Serosanguineous fluid in the pericardial sac (1/3), lung congestion (2/3), petechial hemorrhages in the pancreas, and multifocal white foci on the surface of the spleen (1/3) and on the air sac membrane (1/3) were seen at 4 DPI. One duck from the rH1N1-infected group was removed after inoculation because of concurrent bacterial infection. None of the sham-inoculated group exhibited significant clinical signs, macroscopic findings or mortality during the study period.

2.3.2 Histopathology and immunohistochemistry

In the H1N1pdm09-infected ducks, frequent microscopic lesions included mild to moderate multifocal interstitial pneumonia at 2, 4, 12 DPI (9/9), mild to moderate

diffuse degenerative changes of the liver at 2, 4, 12 DPI (6/9), none to severe multifocal lymphocytic airsacculitis at 2, 4, 12 DPI (7/9) and mild interstitial nephritis at 2, 4, 12 DPI (8/9). Other detected lesions included none to mild pancreatic (4/6) and splenic (3/6) degeneration at 2 and 12 DPI, moderate enteritis at 4 DPI (1/3) and mild hepatitis at 2 DPI (1/3). There were no remarkable microscopic lesions observed in the trachea, jejunum or brain. No influenza antigen staining was found in the tissues of the ducks from this group.

The ducks inoculated with rH1N1 virus displayed mild to moderate multifocal interstitial pneumonia at 2, 4, 12 DPI (8/8), mild to moderate diffuse enteritis at 2, 4, 12 DPI (6/8), mild to severe diffuse degenerative changes of the liver at 2, 4, 12 DPI (5/8), and none to mild multifocal lymphocytic airsacculitis at 2, 4, 12 DPI (5/8). Moderate focal pancreatitis at 4 DPI (1/3), moderate multifocal interstitial nephritis at 2 DPI (1/3) and mild hemorrhages of the spleen at 12 DPI (1/2) were also observed. Neither specific histological lesions nor specific influenza antigen staining were presented in all collected tissues of the ducks from this group.

No significant histopathological lesions or antigen staining were presented in all collected tissue of the control ducks.

2.3.3 Viral shedding

Each duck from all groups were swabbed every day from the oropharyngeal and the cloacal routes. The real time RT-PCR was performed on both collected routes. In the H1N1pdm09-infected ducks, small amounts of virus were individually detected

sporadically of oropharyngeal swabs at 1 DPI, 2 DPI, 5 DPI and 6 DPI (detection levels ranged between 0 and $2.37x10^8$ viral RNA copies/ml) and cloacal swabs at 5DPI ($1.02x10^7$ viral RNA copies/ml) (Table 2.1). Among the rH1N1-inoculated ducks, viral shedding varied substantially in the individual ducks, which was observed from 1DPI to 6 DPI in oropharyngeal swabs (detection levels ranged between 0 and $5.56x10^8$ viral RNA copies/ml) and from 1 DPI to 7 DPI in cloacal swabs (detection levels between 0 and $2.40x\ 10^8$ viral RNA copies/ml) (Table 2.2). No viral RNA or viral infectivity was detected from the collected tissues in both infected groups and in the control group.

2.3.4 Serological examination

All ducks sera collected at the beginning of the experiment and from the necropsied ducks at 3, 5 and 10 DPI were tested negative for influenza A antibody by the commercial ELISA test kit and all ducks sera were test negative for H1N1pdm09 antibody by HI test.

2.4 Discussion

Based on the results, the studied ducks seemed resistant to either the H1N1pdm09 or rH1N1 infection. Both challenged groups exhibited asymptomatic to mild clinical signs, with low viral RNA level detected by the real time RT-PCR. However, major lesions were located only in the respiratory tract. The results found in this study were similar to previous studies using low pathogenic avian influenza virus (LPAI) inoculation in

experimental ducks (Itoh et al., 2009; Brown et al., 2012; Wibawa et al., 2013). Most distinguished lesions of LPAI were observed at 2-5 DPI and mainly located in the respiratory tract such as air sacs, bronchi and trachea (Franca et al., 2012). Interestingly, the rH1N1–infected ducks in this study had more lesions than those of the H1N1pdm09-infected ducks including the lesions in pancreas and spleen, but the lesions were limited and only found at 2-4 DPI, similar to other LPAI viruses (Brown et al., 2012; Franca et al., 2012; Brojer et al., 2013).

Aquatic poultry, especially domestic ducks, are suspected to be the main reservoir of the influenza A virus (Shoham, 2006) and the best representative model for influenza endemic transmission among the flock due to the high density population in the commercial farm. Most of the infected ducks with avian IAV showed none to mild clinical signs (Kida et al., 1980; Bao et al., 2010) similar to the H1N1pdm09 or rH1N1-challenged ducks in this study. There are some explanations why the ducks were not susceptible to the studied viruses. Firstly, ducks have higher body temperature than pigs. The higher body temperature could affect the normal replication cycles and the virus ability to survive in the host cells (Hatta et al., 2007; Beato et al., 2012). Secondly, there are variations in the expression of the sialic acid (SA) receptors in the host. The α 2, 6 sialogalactoside (SA) receptors are usually found in the respiratory epithelium of human and mammalian including pigs. This receptor is compatible to HA from the mammalian virus, including both studied viruses, which received HA gene from the classical swine lineage from North America. The avian species, including ducks, had

more expression α 2, 3 SA receptor in the epithelium of gastrointestinal tract. Possibly, the receptors of the experimental ducks were compatible to HA from the avian virus than those of the mammalian viruses (Neumann and Kawaoka, 2006; Imai and Kawaoka, 2012). Thirdly, a previous study found a rapid cell death mechanism response to the influenza virus infection, demonstrating that duck cells had faster apoptotic mechanism after influenza virus infection than chicken cells (Kuchipudi et al., 2012). This suggested the limitation of the viral replication and the resistance to the virus in duck cells, leading to undetectable virus antigen in the cells.

This study showed that both challenged groups had variable shedding patterns between 1-7 DPI in the oropharyngeal (OP) and cloacal (CL) routes based on the rtRT-PCR. It was consistent with a previous study indicating that the LPAI infected ducks could shed the virus between1-7 DPI (Franca et al., 2012). The highest number of shedding pattern of both infected group were at 3-5 DPI and the shedding ceased by 7 DPI. Interestingly, the rH1N1-challenged ducks could shed the virus in large quantity comparing to the H1N1pdm09-challenged ducks from both routes. In addition, the rH1N1 showed better replication and shedding via both respiratory and gastrointestinal tracts than the H1N1pdm09. In contrast to a previous investigation of both studied viruses in commingled experiment of sentinel ducks with infected pigs, the H1N1pdm09-infected ducks had more pathogenicity and viral shedding levels than the rH1N1-infected ducks (Charoenvisal et al., 2013c).

The result from the previous study indicated that the H1N1pdm09 had higher potential for transmitting to other hosts via the environment from the H1N1pdm09-infected pigs. The differences in the pathogenicity and transmission potential of both studied viruses could be due to the differences between the NA gene of rH1N1 and H1N1pdm09. The rH1N1 obtained the NA gene from the endemic Thai swine lineage, whereas the H1N1pdm09 obtaining it from the Eurasian swine lineage. Even though the role of NA gene in the pathogenesis of SIV is not clearly elucidated at present, a study about the NA gene related to the virulence of the reassortant virus has been reported. The viruses in this study generated from H1N1pdm09 and some of the genes including NA genes were obtained from the seasonal IAV (H3N2). The results showed that the NA gene of the reassortant virus tended to induce greater pathogenic than the original H1N1pdm09 in mice (Schrauwen et al., 2011).

As known previously, ducks play an important role as the main reservoir of the IAV (Keawcharoen et al., 2011). Most of the HA and NA subtypes can be found in ducks especially for avian influenza H5N1 and H7N9 (Kim et al., 2009; Gao et al., 2013). Ducks can reverse the highly pathogenic to low pathogenic avian influenza H5N1 virus after the infection but the viruses still continue, circulate and transmit to other avian species consequently (Hulse-Post et al., 2005; Keawcharoen et al., 2008). In 2012-2013, the novel avian influenza virus H7N9 emerged and caused severe respiratory signs and mortality in humans mostly in China. Many studies suggested that the origin of the virus originated from the reassortant among avian influenza viruses from ducks, chicken

and wild aquatic birds in China such as H9N2, H10N8 which was the H5N1 origin and suspected to have pandemic potential more than H5N1 (Gao et al., 2013; Stein, 2013). Interestingly, there were no clinical signs in those avian species. These demonstrate that aquatic birds, especially domestic ducks, are the important host generating the novel influenza A virus or maintain and transmit the virus to both avian and mammalian hosts.

This study indicated that the experimental ducks were not susceptible, but could be infected by both H1N1pdm09 and rH1N1 viruses. However, the rH1N1 could possibly induce more obvious pathogenicity than the H1N1pdm09. Both studied viruses caused mild clinical signs, but the relation to the influenza-induced lesions could not be confirmed due to undetectable influenza antigen. It should be noted that both viruses could be shed to the environment in low level via oropharyngeal and cloacal routes until 7 days after inoculation. It is of our concern that the study about the interspecies transmission and the influenza surveillance program of swine influenza virus should be carried out routinely in both ducks and pigs not only to prevent the spread of any novel reassortant virus to other areas, but also to prevent generation of a novel future pandemic influenza virus.

Tables & Figure

Table 2.1: Viral detection from oropharyngeal and cloacal swabs of ducks in H1N1pdm09 group using a modified real time RT-PCR

Duck ID.	Viral Detection (DPI)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Oropharyngeal swab													
1	+	-	-	-	+	+	-	-	-	-	-	-	-
2	-	-	-	-	N								
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	N							
5	-	+	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	N								
7	-	-	-	-	-	N							
8	-	-	-	-	N								
9	-	-	-	-	-	N							
Cloacal swab													
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	N								
3	-	-	-	-	+	-	-	-	-	-	-	-	-
4	-	-	-	-	-	N							
5	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	N								
7	-	-	-	-	-	N							
8	-	-	-	-	N								
9	-	-	-	-	-	N							

DPI: Day post inoculation, N: Necropsy, real time RT-PCR result (+: Ct value<40, -:Ct value≥40)

Table 2.2: Viral detection from oropharyngeal and cloacal swabs of ducks in rH1N1 group using a modified real time RT-PCR

Duck ID.	Viral Detection (DPI)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Oropharyngeal swab													
1	+	+	N										
2	-	-	+	+	+	-	-	-	-	-	-	-	-
3	+	-	N										
4	+	-	-	+	N								
5	+	+	+	+	N								
6	+	+	+	-	N								
7	-	-	N										
8	+	-	+	+	+	-	-	-	-	-	-	-	-
9	+	-	+	+	+	-	-	-	-	-	-	-	-
Cloacal swab													
1	+	+	N										
2	+	+	+	+	+	+	-	-	-	-	-	-	-
3	+	-	N										
4	+	+	+	+	N								
5	+	-	-	+	N								
6	+	+	+	+	N								
7	-	-	N										
8	-	-	+	-	+	-	-	-	-	-	-	-	-
9	+	+	+	+	-	-	-	-	-	-	-	-	-

DPI: Day post inoculation, N: Necropsy, real time RT-PCR result (+: Ct value<40, -:Ct value≥40)

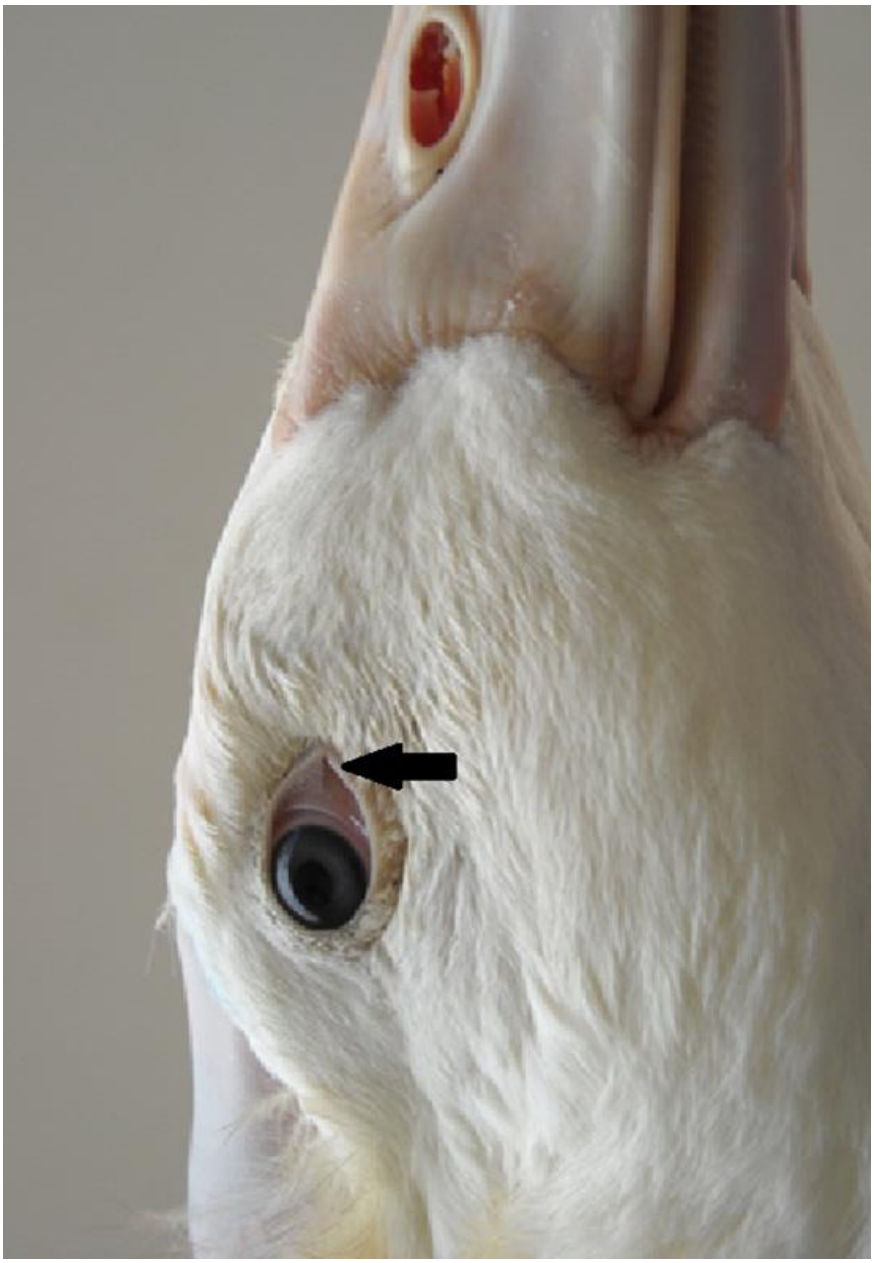

Figure 2.1: Mild conjunctivitis found in the H1N1pdm09-challenged duck

CHAPTER 3

Interspecies transmission:

PROTECTION OF HUMAN INFLUENZA VACCINES AGAINST A REASSORTANT SWINE INFLUENZA VIRUS OF PANDEMIC H1N1 ORIGIN USING A PIG MODEL.

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Protection of human influenza vaccines against a reassortant swine influenza virus of pandemic H1N1 origin using a pig model.

Abstract

Since the pandemic H1N1 emergence in 2009 (pdmH1N1), many reassortant pdmH1N1 viruses emerged and found circulating in the pig population worldwide. Currently, commercial human subunit vaccines are used commonly to prevent the influenza symptom based on the WHO recommendation. In case of current reassortant swine influenza viruses transmitting from pigs to humans, the efficacy of current human influenza vaccines is of interest. In this study, influenza A negative pigs were vaccinated with selected commercial human subunit vaccines and challenged with rH3N2. All sera were tested with both HI and SN assays using four representative viruses from the surveillance data in 2012 (enH1N1, pdmH1N1, rH1N2 and rH3N2). The results showed no significant differences in clinical signs and macroscopic and microscopic findings among groups. However, all pig sera from vaccinated groups had protective HI titers to the enH1N1, pdmH1N1 and rH1N2 at 21 DPV onward and had protective SN titers only to pdmH1N1and rH1N2 at 21DPV onward. SN test results appeared more specific than those of HI tests. All tested sera had no cross-reactivity against the rH3N2. Both studied human subunit vaccines failed to protect and to stop viral shedding with no evidence

of serological reaction against rH3N2. SIV surveillance is essential for monitoring a novel SIV emergence potentially for zoonosis.

Keywords: Influenza, Pandemic, Pigs, Reassortant, Serology, Vaccine

3.1 Introduction

In April 2009, pandemic H1N1 influenza virus (H1N1pdm09) emerged, rapidly spread and caused the pandemic scheme worldwide (Bai et al., 2011; Nelson et al., 2012). The virus was suspected circulating in the pig population prior to transmit back to humans (Peiris et al., 2009; Forrest and Webster, 2010). The H1N1pdm09 virus genome contained genes from the triple reassortant internal gene (TRIG) viruses found circulating in North America (Zhou et al., 1999) and the Avian-like Eurasian swine H1N1 virus, circulating in Asia and Europe (Arias et al., 2009; Forrest and Webster, 2010). Later, the first report of H1N1pdm09 found in the pig population in Canada was documented (Smith et al., 2009a). The H1N1pdm09-infected pigs showed asymptomatic to mild respiratory signs (Sreta et al., 2010). In early 2010, the first reassortant of H1N1pdm09 in pig was reported in Hong Kong and the genome of this novel virus contained the reassortant of NA gene from H1N1pdm09 with Eurasian swine lineage (Vijaykrishna et al., 2010) and later in many areas around the world until nowadays. In Thailand, the first reassortant virus had its internal gene called the TRIG cassette from H1N1pdm09 and obtained N1 gene form an endemic Thai swine lineage (Kitikoon et al., 2011a). Recently, the surveillance data of Thai swine influenza viruses found three types of reassortant viruses derived from H1N1pdm09 (rH1N1, rH1N2 and rH3N2) viruses (Charoenvisal et al., 2013a). These data suggested that, after the introduction of H1N1pdm09 to the Thai swine population, Thai SIV status has changed due to the

emergence of several novel reassortant viruses. Since the virus is able to survive and circulate in the swine population, the transmission to the human population or other species in the future is of concern (Nelson and Vincent, 2015).

Pig is one of appropriate animal models used for human influenza A study based on the similarity of the sialic acid (SA) receptors expression in human respiratory tract and the immune response to the influenza A infection (Rajao and Vincent, 2015). SIVs are of public health concern since the emergence of H1N1pdm09 (Arias et al., 2009). Swine H3N2 virus was found in humans in 2012 in the US (Bowman et al., 2014). Additionally, serological evidence supported that SIV-infection was found in humans (H1N1 and H1N2), particularly, in swine workers in Thailand (Kitikoon et al., 2011b), despite most SIVs circulating in human and swine population were genetically distant. These findings indicated that interspecies transmission of SIVs between pigs and humans might occur.

Recently, many types of vaccines are used for influenza protection in both human and swine. (Webster and Govorkova, 2014; Houser and Subbarao, 2015). The live attenuate virus vaccine has the high efficiency to protect the homologous virus infection. However, awareness of the virus reverse virulence or the vaccine-associated enhanced respiratory disease (VAERD) is of concern when having heterologous infection (Gauger et al., 2014; Rajao et al., 2014b; Houser and Subbarao, 2015). Killed virus vaccines are safe but having low efficiency to induce the immunity to the virus

especially for the mucosal immunity or cell mediated immunity (Rajao et al., 2014a; Houser and Subbarao, 2015). The commercial subunit vaccine, composing of viral surface antigen HA and NA, is commonly used for worldwide (Hannoun, 2013) and this particular vaccine is much safer and has its efficacy to induce the immunity to the homologous viruses and some cross protection to other viruses (Reisinger et al., 2009). The strains of the vaccine viruses are annually changed in Northern and Southern hemisphere based on the World Health Organization (WHO) recommendation. Immunity induces by vaccination or previous exposure to influenza infection usually could not protect the heterologous infection. However, previous study demonstrated that prior infection could partially induce cross protection such as HA specific antibody, viral neutralization antibody or HA antibody-dependent cell-mediated cytotoxicity (ADCC) to heterologous strain.

Interestingly, the 2014 surveillance data of Thai SIV found many reassortant viruses of H1N1pdm09 origin in both H1 (Figure 3.1) and H3 (Figure 3.2) subtypes which had the genetically distance between HA of the reassortant SIVs and the virus vaccines. Especially for the predominant reassortant H3N2 (rH3N2), the reassortant between human origin virus (H3, N2) and the rest of the genes from the H1N1pdm09 (TRIG). Moreover, this virus was prevailingly found during 2012-2014 in central Thailand (Nonthabenjawan et al., 2015) (Figure2). Protection of human influenza vaccines against the major reassortant SIV was conducted using a pig model to elucidate the preliminary

data and would benefit to human influenza vaccine development against the potentially zoonotic SIVs.

3.2 Materials and Methods

3.2.1 Viruses

Four of Thai swine influenza isolates were selected from the surveillance data in 2012 (Charoenvisal et al., 2013a). A/swine/Thailand/CU-PS73/2010 (endemic Thai swine influenza virus: enH1N1 from 2010), A/swine/Thailand/CU-PL63/2010 (pandemic H1N1 influenza virus: pdmH1N1 from 2010), A/swine/Thailand/CU-CT43/2011 (reassortant H1N2 influenza virus: rH1N2 from 2011, having H1 closely related to the pdmH1N1 and N2 obtained from the human origin virus) and A/swine/Thailand/CU-CG45/2011 (reassortant H3N2 influenza virus: rH3N2 from 2011-14) were shown in Figure 1 (square). All of those viruses were propagated in 9-day-old embryonic chicken eggs and harvested after 72 hours post infection. The infectivity of stock viruses was determined in Madin-Darby canine kidney (MDCK) cells according to standard procedures routinely performed at the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) (Sreta et al., 2009) and stored at -80°C until used. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by Reed and Muench method (Reed, 1938). All experiments involving live viruses were conducted under biosafety containment level 2 (BSL-2).

3.2.2 Vaccines

Two commercially available human subunit influenza vaccines 2014/2015 were used in this study (vaccine A and B) (Figure 1). Vaccines A (Influvac®, Abbott Biologicals B.V., Olst, Netherland: Batch No. E08) and Vaccine B (Agripal™S1, Novartis Vaccine and Diagnostics S.r.l., Italy: Lot 131501C). Both study vaccines are composed of A/California/7/2009 (H1N1) as the H1N1pdm09-derived strain, A/Victoria/361/2011 (H3N2) as the seasonal human influenza H3N2 and B/Massachusetts/2/2012 as the influenza B virus. All vaccines were stored at 4°C until used.

3.2.3 Animals and experimental design

Twenty 3-week-old cross-bred pigs were obtained from a negative influenza A virus herd. One week prior to the experiment, nasal swabs were tested for the absence of influenza A virus. Similarly, sera were tested with a commercial ELISA and a routine diagnostic HI test at CU-VDL (Sreta et al., 2013).

All pigs were firstly divided into 3 groups at the beginning (group1: N=8 and group2 and group3: N=6). Those pigs in group2 and group3 were intramuscularly vaccinated (injection site: left Hamstring with ½" sterile needles) with a single dose of vaccine A and B, respectively. Pigs in group1 were injected with the 0.5 ml of normal saline solution and served as a negative control group. All experimental pigs were observed daily on clinical signs and nasal swabs and serum samples were collected at 7, 14, 21 and 28 days post vaccination (DPV). All nasal swabs were tested using a real

time RT-PCR and all sera were tested for hemagglutinaton inhibition test (HI test) and serum neutralization (SN) test with four representative viruses.

At 29 DPV, pigs from group1 were subdivided into group1 (4 pigs) and group4 (4 pigs). Pigs in group2, group3 and group4 were intranasally inoculated with 10^6 $TCID_{50}$/ml of rH3N2 virus and the remaining pigs in group1 were inoculated using mocking media (MEM) as the negative control group. All pigs were observed and nasal swabs were collected until the end of the experiment. At 5 days post infection (DPI), all pigs were euthanized, necropsied and tissue samples were collected for histopathology and virology study. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, Kasetsart University (Protocol No. OACKU00456).

3.2.4 Serological study

Serum samples were collected from all experimental pigs 1 week before starting the experiment and from all animals before euthanized at 0,7, 14,21, 28 DPV and were analyzed using a commercial ELISA Influenza A multi-species kit (ID Screen® Influenza A antibody competition multi-species; IDvet, Louis Pasteur-Grabel, France) according to the manufacturer's instructions and hemagglutination inhibition test (HI test) with three selected local Thai SIV reference viruses, A/swine/Thailand/CU-CB1/2006(H1N1), A/swine/Thailand/CU-RA29/2009 (H1N1pdm09) and

A/swine/Thailand/CU-CB8.4/2007 (H3N2) according to the CU-VDL protocol as previously described (Charoenvisal et al., 2013b).

3.2.4.1 Hemagglutination inhibition (HI) test

All pig sera obtained at day 0, 7, 14, 21 and 28 DPV were treated with 20% kaolin for subtype H1 or with receptor destroying enzyme (RDE) for subtype H3 and then absorbed with 50% chicken red blood cells (RBCs) as described previously for elimination of the non-specific inhibition substances (Sreta et al., 2013). All Sera were diluted in concentration 1:5 and tested for HI test with four representative swine influenza viruses according to the OIE Terrestial Manual (Wood et al., 2012). The viruses used for HI test had 8 HA unit/50µl and 0.5% chicken RBCs was used for titration. Protective HI titer was determined when over or equal to 1:40 and the percentage of seroconversion was identified with a minimum four-fold of HI titers between 0 and 28 DPV (Katz et al., 2011; Sreta et al., 2013; Hsu et al., 2014).

3.2.4.2 Serum neutralization (SN) test

All sera were inactivated at 56°C for 30 minutes and diluted in concentration 1:5 with MEM before tested. Serial two-fold dilution was performed in 96 well-plates with minimal essential medium (MEM) containing 3µg/ml of TCPK trypsin (Sigma-Aldrich, St Louis, MO) and 100 $TCID_{50}$ each of four selected viruses was added to the reaction before transferring the reaction into the MDCK cells plates and incubated for 1 hour at 37°C before adding MEM with TCPK trypsin and then incubated for 72 hours

at 37°C with 5%CO_2. The neutralizing antibody titers were evaluated when the highest serum dilution was completely neutralized for 50% (Hsu et al., 2014). Later, the cells were fixed with 4% formaldehyde and the viral antigen was identified using the Influenza A nucleoprotein (NP) monoclonal antibodies (HB654404 B.V.EUROPEAN VETERINARY LABORATORY, the Netherlands), a rabbit anti-mouse IgG conjugated horseradish peroxidase (DakoCytomation, USA) and chromogenaminoethylcarbazole (AEC) substrate. Positive SN titers were determined when over or equal to 1:40 (Sreta et al., 2009; Katz et al., 2011; Wood et al., 2012; Charoenvisal et al., 2013b).

3.2.5 Pathological examination

At 5 DPI, all pigs were euthanized and necropsied. Percentages of gross lung lesion scores characterized by multifocal mottled-tan and consolidation were recorded and scored as previously described (Sreta et al., 2009; Charoenvisal et al., 2013b). Tissue mainly lung were collected from all pigs and other organs (tonsil, lymph node, trachea, liver, kidney and spleen) showing remarkable lesions were collected and fixed in 10% buffered formalin and embedded in the paraffin wax. Section cut at 4 μm thick were prepared for histological analysis by hematoxylin and eosin (H&E) staining. Intrapulmonary airways necrosis scores composed of; 0 = no change in epithelial of airways, 1= (25%) mild bronchi or bronchiolar epithelial damage, 2= (50%) moderate bronchi or bronchiolar epithelial damage, 3= (75%) moderate bronchi or bronchiolar epithelial damage and 4= (100%) bronchi or bronchiolar epithelial damage. The

alveolar septal wall thickening due to the mononuclear cell infiltration (interstitial pneumonia) was also scored; 0=no interstitial pneumonia, 1= mild interstitial pneumonia, 2= moderate multifocal interstitial pneumonia, 3= moderate diffuse interstitial pneumonia and 4= severe interstitial pneumonia (Halbur et al., 1995; Gauger et al., 2014). The average scores were used for statistical analysis.

3.2.6 Viral detection

All nasal swabs and fresh lung tissue samples were tested using a modified real time RT-PCR and running the reaction on Corbett Rotor-GeneTM 6000 (Qiagen). The primers and probe targeted specifically to amplify a portion of the M gene of influenza A virus (forward primer (MF3; 5'TGATCTTCTTGAAAATTTGCAG 3'), reverse primer (MR1+; 5' CCGTAGMAGGCCCTCTTTTCA 3') and M-64probe (FAM-TTGTGGATTCTTGATCG-TAMRA) (Spackman et al., 2002; Charoenvisal et al., 2013a). The positive ct1-40 was identified as a positive result and over or equal to 40 were identified as negative results (Charoenvisal et al., 2013b; Arunorat et al., 2014).

3.2.7 Statistical analysis

Clinical signs were analyzed descriptively. The geometric means of the HI titers and SN titers, percentages of seroconversion and macroscopic/microscopic interstitial pneumonia average were evaluated using ANOVA with 5% level of significant (p-value < 0.05) and using the Tukey-Kramer test (GraphPad Prism Version 5.00, San Diego, CA) for pair-wise mean comparison among groups.

3.3 Results

3.3.1 Serology, HI test and SN test

All pigs had negative HI and SN titers with 4 studied viruses prior to the experiment (Figure 2a, 3a). Geometric mean HI titers showed all sera in group1 were negative to the four representative viruses. Sera from group2 had cross reactivity of HI titers to the enH1N1 and rH1N2 at 14 DPV and to pdmH1N1 at 21 DPV. Sera from group3 had cross reactivity of HI titers at 21 DPV to enH1N1, pdmH1N1 and rH1N2. All tested sera had no cross-reactivity against the rH3N2 (Figure 2b) and the percentages of HI seroconversion to enH1N1, pdmH1N1 and rH1N2 were demonstrated in Figure 2c.

All pigs in group1 had no SN titers. Group2 and group3 pigs had no SN titers at 0, 7, 14 and 28 DPV to either enH1N1 or rH3N2. SN titers to pdmH1N1 from pigs in group2 and group3 were found at 21 and 28 DPV. SN titers to rH1N2 were also found at 14, 21 and 28 DPV (Figure 3b) and SN seroconversion was found only to pdmH1N1 and rH1N2 (Figure 3c).

3.3.2 Clinical signs, Histopathology and viral antigen detection

Pigs in the negative control group remained healthy with no clinical signs. Clinical observation showed only mild respiratory signs including coughing, sneezing and nasal discharge at 2-4 DPI in challenged pigs from group2 and group4. Necropsy results found mild multifocal, dark consolidation of the lung at cranio-ventral portion

regions in the pigs from group2 (3/6), 3 (4/6) and 4 (4/4) at 5 DPI. No remarkable lung lesions of pigs and other organs from all studied pigs from group1 were observed. Lung histopathological findings showed mild to moderate multifocal interstitial pneumonia in pigs from group2 (6/6), group3 (5/6) and group4 (4/4), mild necrotizing bronchitis in group2 (3/6), group3 (3/6) and group4 (3/4), moderate bronchopneumonia in pigs from group4 (3/4) and mild bronchiolar epithelial damage in pigs in group2 (3/6), group3 (3/6) and group4 (3/4). No significant gross or microscopic findings were found among the infected groups (data not shown). Averaged lung scores and histopathological scores are shown in Table 1.

Viral shedding of the challenge virus was detected by the modified real time RT-PCR in the nasal swabs and lung tissues demonstrating that virus shedding was found in all infected pigs (group2, group3 and group4) as early as 1 DPI and sporadically found later in some pigs until 5 DPI with no differences among infected groups (CT ranges 28-34). No virus detection was found in the negative control pigs.

3.4 Discussions

As expected, the studied human influenza vaccines did not completely prevent the Thai reassortant H3N2 SIV infection measured by either clinical findings or viral shedding. HI results showed seroconversion to pdmH1N1 and its reassortant virus (rH1N2) in both vaccinated groups at 21 DPV. Interestingly, HI titers did serologically cross-reactivity to the enH1N1 virus (Figure 2b). This suggested that the vaccines might

have serological cross reactivity to the enH1N1. However, serum neutralization test results suggested that the studied human influenza vaccines could only induce the neutralization antibody against the pdmH1N1 and its H1 reassortant viruses similar to the HI titers at 21 DPV but had no cross reaction to the enH1N1 virus. These data suggested that the studied human influenza vaccines did have serological response to the pdmH1N1 and its H1 reassortant viruses. In addition, H1 antigen from the pdmH1N1 in the vaccines was genetically related to the H1 of the current pdmH1N1 viruses circulating in the Thai pig population. However, the H1 antibody titers induced by the studied vaccines had no ability to neutralize the enH1N1 virus (Figure 3b).

There were no HI and SN titers or seroconversion of the studied vaccines against the rH3N2 virus measured by either serological tests or viral shedding and clinical signs. These data were supported by the rH3N2 challenge study of the vaccinated pigs. There were no significant differences in term of clinical signs, gross lesions, histopathological findings and viral shedding results among pigs from both vaccinated groups (group2 and group3) and non-vaccinated challenged pigs (group4). The challenged pigs in both vaccinated groups showed flu-like symptom characterized by mild respiratory signs, minimal gross lesions, mild to moderate histopathological lesions in the lung and low level of viral shedding similar to the non- vaccinated infected pigs (group4). In addition, phylogenetic data of H3 antigen showed that the H3 genes were from different clusters (Figure 1b). In case of the rH3N2 SIV transmitting from pigs to human population, the

studied human influenza vaccines would not completely protect the virus of the swine origin.

Commercial human subunit influenza vaccines are commonly used around the world (Houser and Subbarao, 2015). It should be noted that the variable region of the HA plays an important role in the viral tropism and entry phase of the virus. The novel vaccine composed of NA, M or NP did not protect the infection of virus but only reduce the severity and the virus shedding instead. However, the HA matching between the vaccine strains and the viruses circulating in the area is of importance (Webster and Govorkova, 2014; Houser and Subbarao, 2015; Krammer and Palese, 2015). Inducing antibody targeting to the other parts of virus such as the stem part of HA or M2 protein (HA based universal vaccine and DNA vaccine) are of interest and still under development (Gottlieb and Ben-Yedidia, 2014; Khanna et al., 2014).

At present, many organizations and researchers raise their concerns regarding the reassortant influenza viruses such as avian influenza H7N9 (Gao et al., 2013; Li and Chen, 2014), H5N1 (Watanabe et al., 2012) or the mutation occurred in the influenza B viruses (Houser and Subbarao, 2015) and particularly for the reassortant SIV. Since the H1N1pdm09 emergence, the introduction of the TRIG cassette virus into the pig population occurred worldwide. Interestingly, the TRIG virus has its ability on human to human transmission or transmitting back to the pig population and continuing reassortant with other swine influenza viruses in many areas (Li and Chen, 2014). In

Thailand, the surveillance data during 2012-2014 suggested that the rH3N2 is the predominant subtype. The genome of this virus composed of the reassortant between TRIG virus with the H3 and N2 from the endemic H3N2 virus of human origin circulating in the Thai pig population since 1997 (Charoenvisal et al., 2013a; Nonthabenjawan et al., 2015). It should be noted that most swine influenza viruses infect pigs with asymptomatic to mild respiratory signs and may transmit back to humans based on animal-human interface theory. According to the serological evidence of pig to human influenza transmission from the previous report, elevated HI titers of the enH1N1 SIV subtype in the swine workers was evident (Kitikoon et al., 2011b). Moreover, in 2012, the case of the human infected with the reassorted between enH3N2 swine influenza virus and TRIG virus was reported in the US (Kitikoon et al., 2012). It is of the public health concern regarding the efficacy of the human influenza vaccine against the emerging swine virus in the human population. Based on the results from the present study, the studied human influenza vaccines could not completely protect serologically or pathologically against the Thai reassortant H3N2 SIV infection using the pig model. Therefore, the efficacy of the commercially available human influenza subunit vaccines is of concern. The best match between the vaccine strains and the circulating strains genetically must be evaluated and updated annually, not only in humans, but also in other animals including pigs and avian species. Practically, vaccination in pigs and avian species to prevent influenza infection has been implemented in many countries. The scenario of Vaccine-Associated Enhanced

Respiratory Disease (VAERD) in the swine population and vaccination failure in avian species have been reported (Nelson and Vincent, 2015). Moreover, routine monitoring program of swine influenza viruses should carry on regularly, particularly, in the area of high density of pig raising areas (Houser and Subbarao, 2015; Nelson and Vincent, 2015). It is of great impact not only to control or prevent the emergence of novel swine viruses but also to provide benefit data for the future study on the potential spreading to the human population and on influenza vaccine improvement.

3.5 Conclusion

This present study demonstrated that the studied subunit human influenza vaccines did not completely prevent the potential zoonotic rH3N2 SIV infection. The rH3N2 SIV has become the predominant strain in the Thai swine population since 2012. It should be noted that the swine influenza viruses are continuously reassorting and circulating in the swine population after the pdmH1N1 introduction worldwide. The efficacy of current human influenza vaccines should be examined and updated. SIV surveillance program should be carried on routinely for the best prevention and control of novel emerging viruses, particularly, in the areas having high prevalence of H1N1pdm09 in the pig population.

3.6 Acknowledgements

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Table

Table 1. Comparison of the mean percentage lung lesion scores and mean microscopic lesion scores at 28 DPV

Experimental group	Average score	
	Lung score*	Histopathological score*
Group1 (NV/NC)	0[a]	0[a]
Group2 (VacA/C)	4.16±3.76[b]	2.16±1.16[b]
Group3 (VacB/C)	5.42±4[b]	1.50±0.54[b]
Group4 (NV/C)	7±3.55[b]	2.50±1.29[b]

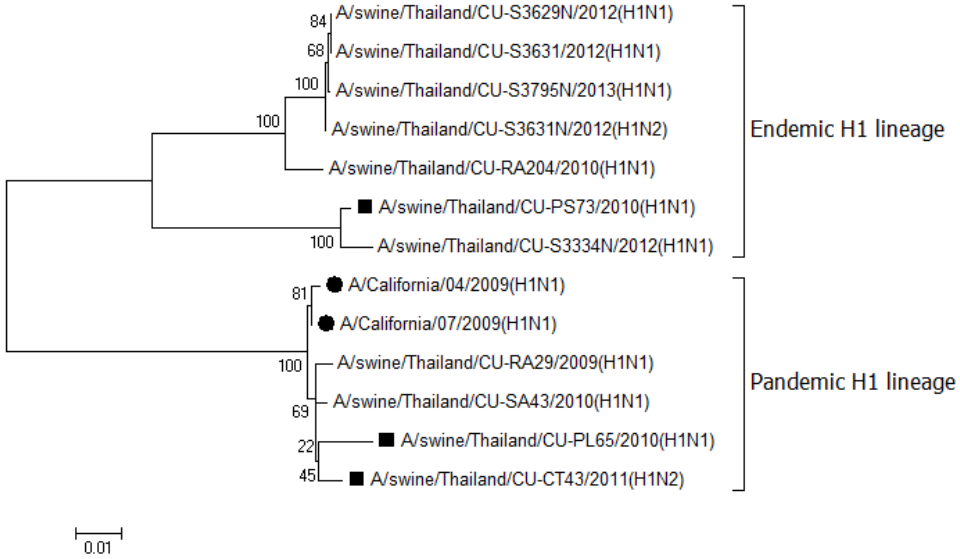
NV/NC = non-vaccinated/non-challenged, VacA/C = vaccinated vaccine A/challenged, VacB/C = vaccinated vaccine B/challenged, and NV/C = non-vaccinated/challenged;

*mean scores ± standard error

[a, b] indicating significantly different ($P \leq 0.05$) between groups.

Figures

a)



b)

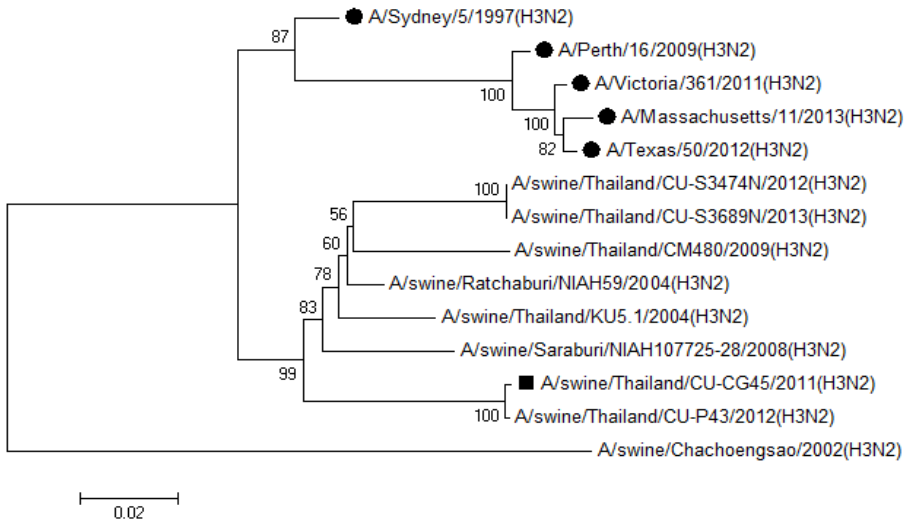


Figure 1. Phylogenetic analysis of H1 subtype (a). Phylogenetic analysis of H3 subtype (b). The phylogenetic tree was constructed with the neighbor-joining algorithm and the Kimura-2 parameter model applied to 1,000 replications of bootstrap percentage. Node label shows the bootstrap percentage, the circle and square symbols shows the human vaccine strains and the representative viruses in this experiment, respectively.

Figure 2a

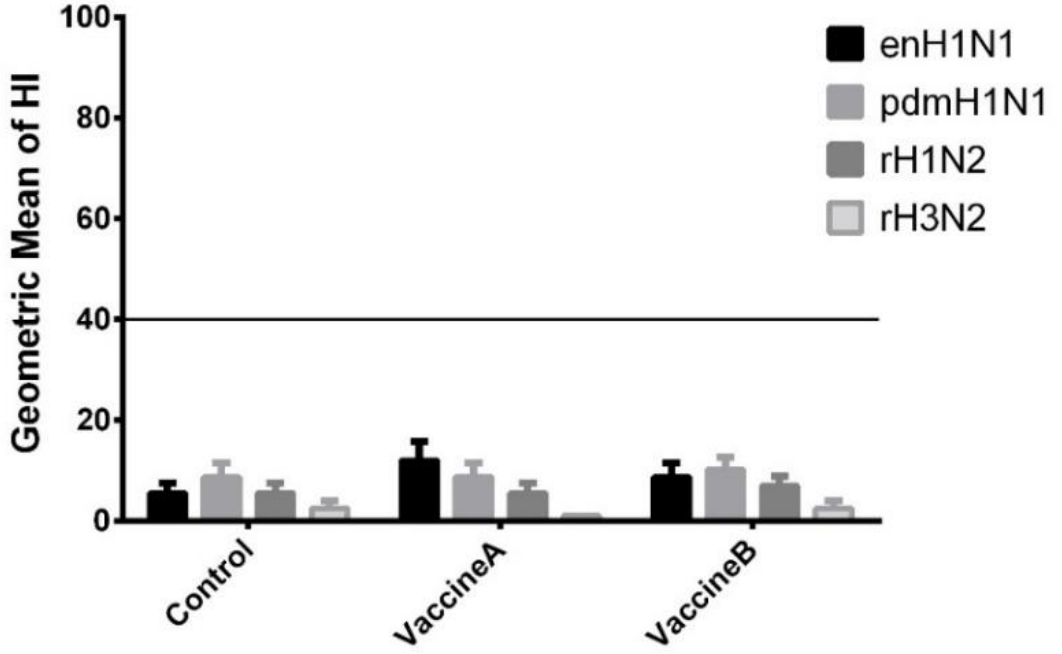
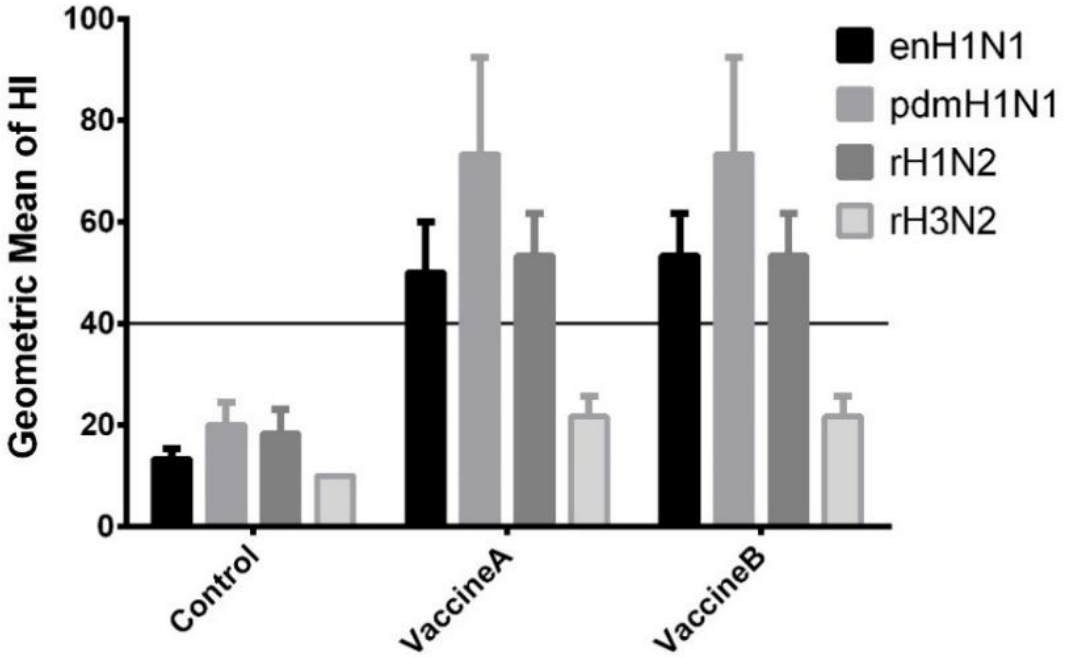


Figure 2b



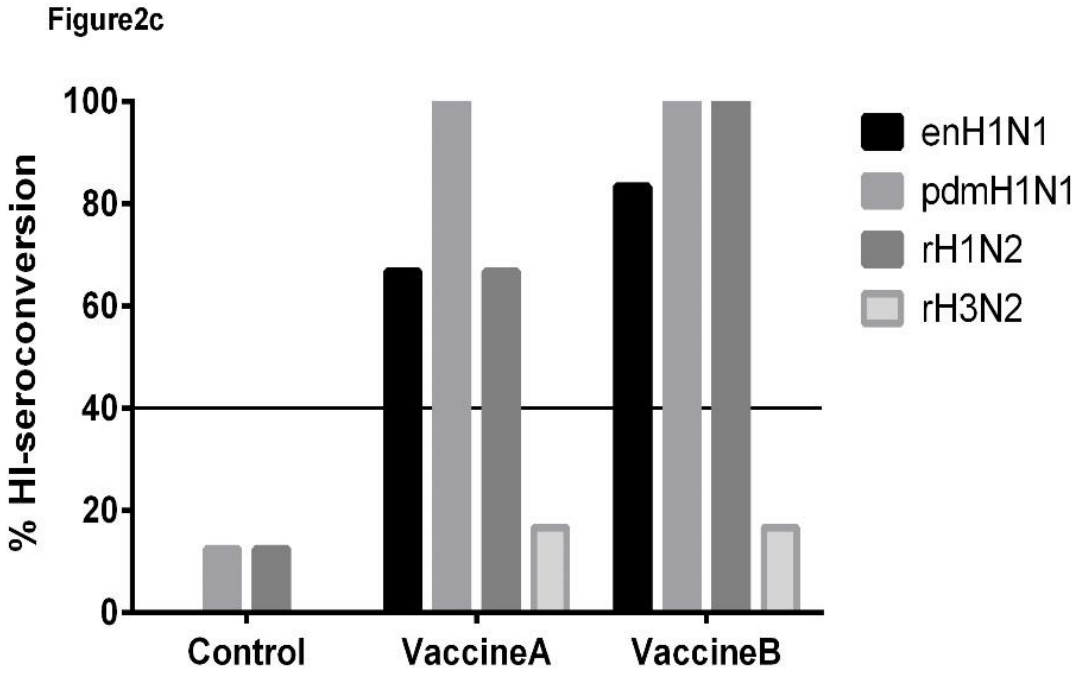


Figure 2. HI geometric mean titers of control, vaccine A, and vaccine B groups with the four representative viruses (enH1N1, pdmH1N1, rH1N2 and rH3N2) at 0 DPV (a) and at 28 DPV (b). The seroprotection was determined when ≥ 40. The HI seroconversion of control, vaccine A and vaccine B groups with the four representative viruses (enH1N1, pdmH1N1, rH1N2 and rH3N2) at 28 DPV (c). The seroconversion was calculated by the percentage of subjects with either a pre-vaccination (0DPV) HI titer ≤ 10 and at 28 DPV HI titer ≥ 40 or a pre-vaccination (0DPV) ≤ 10 and post-vaccination at 28 DPV HI titer ≥ 4 fold increase. The positive seroconversion was determined when ≥ 40.

Figure3a

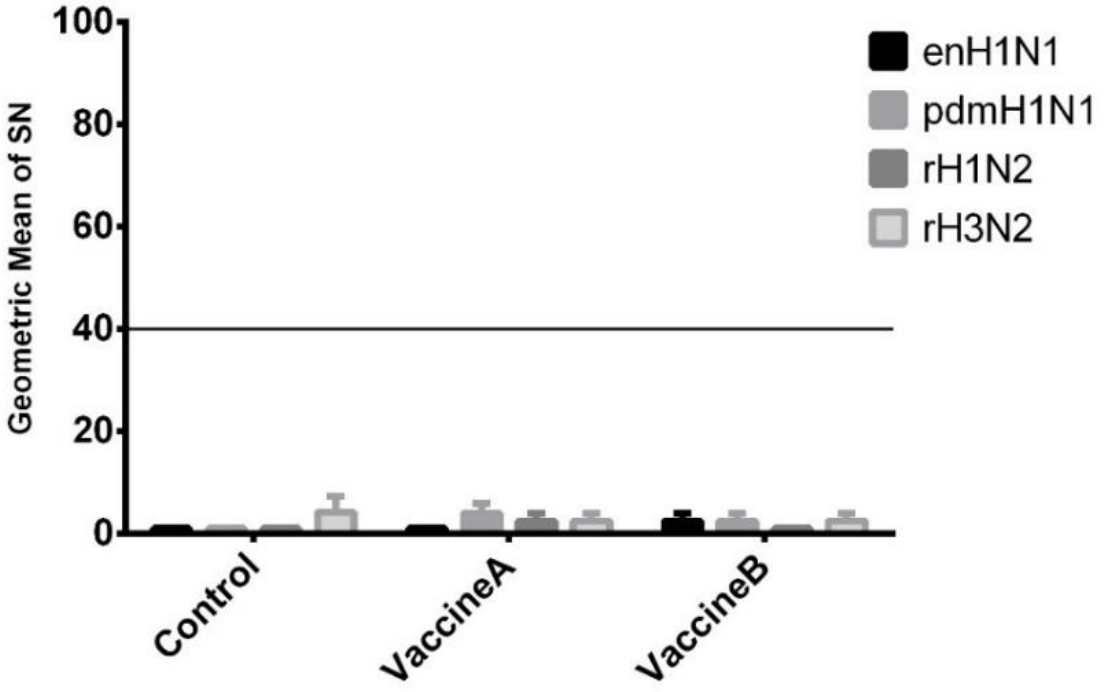
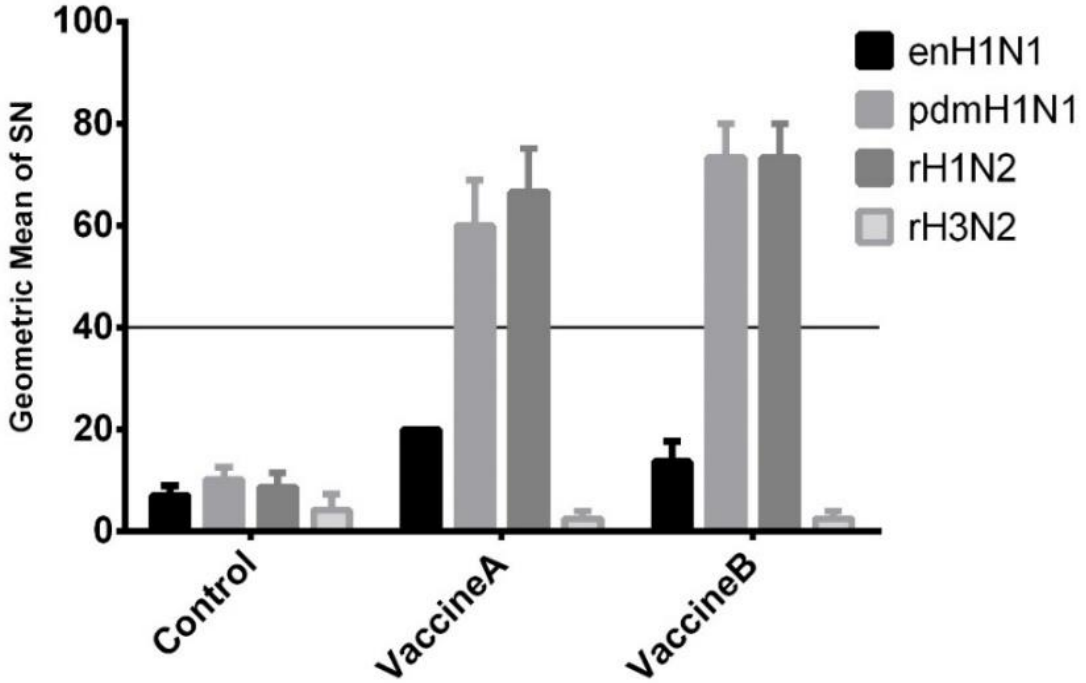


Figure3b



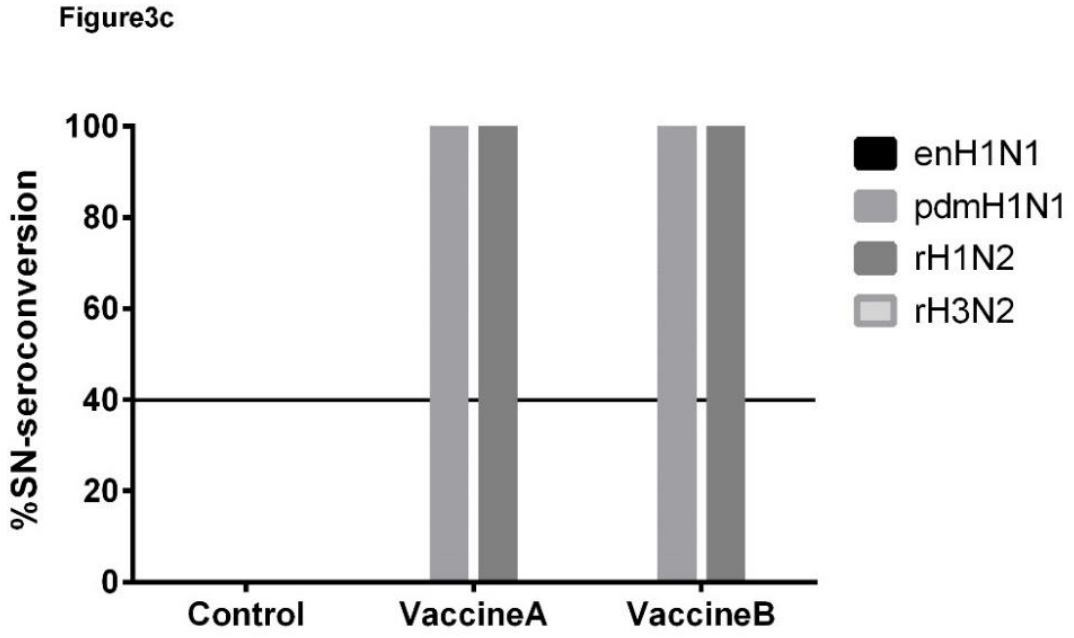


Figure 3. SN geometric mean titers of control, vaccine A, and vaccine B groups with the four representative viruses (enH1N1, pdmH1N1, rH1N2 and rH3N2) at 0 DPV (a) and at 28 DPV (b). The seroprotection was determined when ≥ 40. The SN seroconversion of control, vaccine A, and vaccine B groups with the four representative viruses (enH1N1, pdmH1N1, rH1N2 and rH3N2) at 28 DPV (c). The seroconversion was calculated by the percentage of subjects with either a pre-vaccination (0DPV) SN titer ≤ 10 and at 28 DPV SN titer ≥ 40 or a pre-vaccination (0DPV) ≤ 10 and post-vaccination at 28 DPV SN titer ≥ 4 fold increase. The positive seroconversion was determined when ≥ 40

CHAPTER 4

DETERMINATION OF CURRENT REFERENCE VIRUSES FOR SEROLOGICAL STUDY OF SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC 2009 H1N1 (H1N1pdm09) IN THAILAND

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Determination of current reference viruses for serological study of swine influenza viruses after the introduction of pandemic 2009 H1N1 (H1N1pdm09) in Thailand

Abstract

Since the introduction of pandemic H1N1 2009 virus (pdmH1N1) in pigs, status of Thai swine influenza virus has changed. The pdmH1N1 and its reassortant viruses have become predominantly circulating in the Thai swine population based on the surveillance data in 2012-2014. From this reason, the reference viruses for serology study especially for hemagglutination inhibition (HI) test and current SIV serological status in Thailand should be updated. Six anti-sera of the reference viruses from 2006-2009 (enH1N1-06, enH1N1-09, enH1N2-09, pdmH1N1-09, enH3N2-07 and enH3N2-09) were used for HI test with four available current viruses (enH1N1-10, pdmH1N1-10, rH1N2 and rH3N2) and the selected reference viruses were tested with sera collected from the field to determine the current SIV status. The results showed that anti-sera of swH1N1-06 had the highest titers against enH1N1-10. Anti-sera of pdmH1N1-09 had the highest titers against pdmH1N1-10 and rH1N2, whereas, anti-sera of enH3N2-09 had the highest titers against rH3N2. The results demonstrated that 2006-2009 SIVs (enH1N1-06, pdmH1N1-09 and enH3N2-09) should be selected as reference viruses for current serological study (HI test). The seroprevalence results from 410 samples

revealed enH1N1 (37.79%), pdmH1N1 (37.32%) and H3N2 (35.86%), respectively. The present study indicated that pdmH1N1 was widespread and commonly found in the Thai pig population increasing the risk of novel reassortant viruses and should be added as a reference virus for HI test. It should be noted that SIV surveillance program and serological study should be conducted continuously for the benefits of SIV control and prevention as well as its zoonotic potential.

Keywords: Hemagglutination inhibition; pandemic H1N1 2009; influenza; swine; Thailand

4.1 Introduction

Swine influenza virus (SIV) is an important respiratory pathogen frequently associated with the porcine respiratory disease complex (PRDC) in pigs and causing economic losses to the swine industry worldwide (Forrest and Webster, 2010; Kenah et al., 2011). SIVs cause high morbidity rate in affected herds but mortality rates are typically low. The infected pigs usually show minimal to mild respiratory signs such as fever, coughing, anorexia, sneezing, having nasal and ocular discharge and conjunctivitis (Sreta et al., 2009). Three SIV subtypes can be commonly identified in pigs including H1N1, H1N2 and H3N2. In April 2009, H1N1pdm09 influenza virus emerged and caused high morbidity and mortality in humans worldwide (Smith et al., 2009a). The virus was a reassortment between the triple reassortant virus from North America and the Eurasian avian-like virus from Europe. The virus was suspected to circulate in pig populations before transmitting to humans with unknown mechanisms and consequently causing a pandemic (Arias et al., 2009; Bai et al., 2011). After that, several reports of H1N1pdm09 circulation in the pig population with mild respiratory signs were documented (Forrest and Webster, 2010; Sreta et al., 2010; Sreta et al., 2013). During 2010, several reassortant viruses from pdmH1N1 and enH1N1 circulated in the pig population in Hong Kong (Vijaykrishna et al., 2010) and many other countries (Watson et al., 2015) including Thailand (Kitikoon et al., 2011a). This has raised concerns to

address and improve surveillance programs for influenza virus not only in humans but also in pigs.

SIV diagnosis can be done by either viral antigen or antibody detection. Viral antigen detection is dependent on duration of viral shedding time found only short periods of time, whereas, serological tests, depending on specific antibody against influenza A virus, may be helpful especially in areas where vaccines are not used. The gold standard for the detection of influenza A virus specific antibody is the hemagglutination inhibition test (HI test) (Pedersen, 2014). The viruses used in the HI assays have a major impact on the test due to the antigenic variation of viruses in many areas due to genetic diversity of hemagglutinin (HA), the most variable region and important for viral entry phase of influenza virus (Wozniak-Kosek et al., 2014). The usage of suitable reference viruses, therefore, is necessary to assess the real serological status of SIV in each specific area (Sreta et al., 2013).

Before the introduction of H1N1pdm09 to the Thai pig population, a retrospective serological study conducted in 2013 showed that enH1N1-06 (H1α group) and enH3N2-07 (H3a subgroup) were the main subtypes circulating in the Thai pig population. Therefore, these viruses, enH1N1-06 for subtype H1 and enH3N2-07 for subtype H3 were recommended as the representative viruses for HI test (Sreta et al., 2013). Based on surveillance data from 2012-2014, H1N1pdm09 and its reassortant viruses were predominantly co-circulating with Thai endemic SIV indicating the dynamic

changing of current SIV status in Thailand (Charoenvisal et al., 2013a; Nonthabenjawan et al., 2015). This has raised concerns on suitable reference viruses for the HI test currently used in Thailand. The objective of this study was to identify the most appropriate reference viruses for usage in the HI test in Thailand. The up to date information from this serological study benefits the SIV diagnosis, control, and prevention in Thailand and can be used as a model in other regions.

4.2 Materials and Methods

4.2.1 Viruses

Four representative SIVs from Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL: Bangkok Thailand) in 2010 -2011 were selected based on the HA gene from each group (Charoenvisal et al., 2013a): A/swine/Thailand/CU-PL65/2010(H1N1) (a pandemic H1N1 virus from 2010: H1N1pdm-10), A/swine/Thailand/CU-PS73/2010(H1N1) (the endemic SIV that circulated prior to emergence of H1N1pdm09: enH1N1-10), A/swine/Thailand/CU-CT43/2011(H1N2) (a reassortant H1N2 first detected in 2011: rH1N2-11 and A/swine/Thailand/CU-CG45/2011(H3N2) (the reassortant H3N2 most frequently found in 2010–2013: rH3N2-11). All four viruses were propagated in nine day old chicken embryonic eggs and titered by hemagglutination assay for HA titer as described previously (Sreta et al., 2013).

4.2.2 Rabbit anti-sera preparation

Six rabbit anti-sera prepared against SIVs isolated during 2006 - 2009, enH1N1-06 (H1α-cluster I), enH1N1-09 (H1α-cluster II), enH1N2-09 (H1α-cluster II), H1N1pdm09 (pdmH1), enH3N2-05 (H3b) and enH3N2-07 (H3a), (Sreta et al., 2013; Nonthabenjawan et al., 2015) were used to characterize the contemporary viruses described above. All anti-sera were treated to remove non-specific inhibitors with 10% kaolin and absorbed with 50% chicken red blood cells for testing with subtype H1 viruses or with receptor destroying enzyme (RDE) (Denka Seiken Co. Ltd., Tokyo, Japan) and absorbed with 50% chicken red blood cells for testing with subtype H3 viruses. All the treated anti-sera were stored at −20 °C until used (Sreta et al., 2013).

4.2.3 The hemagglutination inhibition test (HI test)

All rabbit anti-sera (enH1N1-06, enH1N1-09, enH1N2-09, H1N1pdm09, enH3N2-05 and enH3N2-07) were 1:5 diluted for HI testing. Two-fold serial dilution with PBS was performed in V-shape 96-well microplates. Eight HA unit of each homologous strain and the current SIV (enH1N1-10, H1N1pdm10, rH1N2-11 and rH3N2-11) were individually added and incubated for 30 minutes at room temperature and 0.5% chicken RBCs were used in HI assays (OIE 2010 Terrestial Manual:http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.08_SWINE_INFLUENZA.pdf). All HI titers were evaluated in duplicate and geometric mean titers (GMTs) were calculated (GraphPad Prism Version 5.00, San Diego, CA). HI titers were considered positive when ≥1:40 (Sreta et al., 2013).

4.2.4 Swine serum collection

Four hundred and ten serum samples were cross-sectionally collected from pigs with various ages (weaning and growing pigs, replacement gilts and sows) from 10 farms in 5 pig dense provinces. All swine sera were treated similar to rabbit anti-sera preparation and HI performed with the chosen appropriate reference viruses. Positive HI titers were determined when ≥1:40.

4.2.5 HA and NA characterization

Two hundred and fifty nasal swabs were collected and stored in viral transport medium from nursery, growing pigs, replacement gilts and sows showing respiratory signs (sneezing, coughing or having ocular and nasal discharge). All swabs were tested using CU-VDL real time RT-PCR screening (Targeting the M gene of influenza A virus) (Charoenvisal et al., 2013a). RT-PCR positive samples were grown in 9 day old chicken embryonic eggs and after successful isolation subtyped with an appropriate set of primers to amplify the HA and NA genes (Nonthabenjawan et al., 2015). The sequences were analyzed by MEGA5 software (Tamura et al., 2007).

4.3 Results

4.3.1 Determination of reference viruses for HI test

Geometric mean titers (GMTs) of rabbit anti-sera with the homologous strain showed that enH1N2-09, H1N1pdm09 and enH3N2-07 had high HI titers against the

homologous strains (Table 4.1). In the endemic H1 group, all three anti-sera (enH1N1-06, enH1N1-09 and enH1N2-09) had cross-reactive HI titers to enH1N1-10 with the anti-sera against enH1N1-06 having the highest titer. In the pandemic H1 group, enH1N2-09 and H1N1pdm09 had cross-reactive HI titers to pdmH1N1-10 and only H1N1pdm09 had cross-reactive HI titer to rH1N2-11. In the H3 group, only enH3N2-07 had cross-reactive HI titer to rH3N2-11. There were no cross-reactive HI titers between subtype H1 and H3 (Table 4.2). The results indicated that enH1N1-06 had the highest titer against enH1N1-10 with no cross-reaction to other groups, pdmH1N1-09 had the highest titer against H1N1pdm10 and rH1N2-11 and lastly, enH3N2-07 had the highest titer against rH3N2. Therefore, enH1N1-06, H1N1pdm09, and enH3N2-07 were selected as appropriate reference viruses and could be used for current serological diagnosis in Thailand.

4.3.2 Serological study of current swine influenza virus and HA and NA genetic characterization

Of the 410 sera samples, 208 (50.73%) samples were positive for HI titer with at least one of the three reference viruses (enH1N1, H1N1pdm09 or H3N2). Consequently, seropositive samples were found when tested with enH1N1 (37.79%), H1N1pdm09 (37.32%) and H3N2 (35.86%), respectively (Table 4.3). In addition, eight of ten farms were found seropositive with all 3 subtypes in the same farm. Five viruses were isolated from nursery-growth pigs in 5 farms and genetic characterization revealed

four H1N1pdm09 and one rH3N2 (Table 4.4). Percentage of seropositive pigs included 34.69% in nursery-growth, 89.24% in sows, and 80.55% in replacement gilts.

4.4 Discussion

Before the introduction of H1N1pdm09 in Thailand, enH1N1-06, and enH3N2-07 were predominant subtypes used as reference viruses for serological diagnosis (Sreta et al., 2013). Since 2009, H1N1pdm09 was introduced into the Thai swine population and consequently became the predominant subtype. Genetic data indicated that the antigenic status of Thai SIVs has changed and the antigens used in serological methods, particularly the HI test, should be reconsidered for accurate serological study. The results of the present study showed that the previously studied viruses had none or low cross reactive HI titers to the more contemporaneous heterologous viruses (enH1N1-10, H1N1pdm09, rH1N2-11 and rH3N2-11) compared to the homologous viruses (Table 4.2). These suggested that antigenic drift has recently occurred in the HA gene. The most appropriate reference virus for enH1N1 was enH1N1-06 (H1α group) since this anti-sera had the highest HI titer against enH1N1-10 and had limited cross reactivity against either H1N1pdm09 or H3N2 viruses. To distinguish H1N1pdm09 from enH1N1 and enH1N2-09, positive HI titers were found against H1N1pdm09 and H1N1pdm10, and had cross reactivity against rH1N2, albeit with a 3.5-fold loss in cross-reactivity, since the H1 gene of those viruses belong to the

H1N1pdm09 cluster (Charoenvisal et al., 2013a). Therefore, H1N1pdm09 was the suitable reference virus for H1N1pdm and its H1 reassortant viruses. In addition, enH3N2-07 (H3a subgroup) was the suitable reference virus for Thai H3N2 compared to enH3N2-05.

The genetic diversity of the HA genes of SIVs in Thailand has recently changed. In 2005 to 2009, only enH1N1 and enH3N2 viruses circulated in the Thai pig population (Sreta et al., 2013). Thai H1 was classified into two endemic sub-lineages (H1α group-cluster I, II, III) and Thai H3 was classified into H3a and H3b subgroups. Since the introduction of H1N1pdm09, 2010-2014 surveillance data revealed that Thai H1 was divided into two sub-lineages, North America classic swine lineage similar to 2005 to 2009 SIVs (H1α group) and the pandemic (H1pdm group) and H3 was only found in Ha subgroup of human H3N2 lineage (Nonthabenjawan et al., 2015). The H1 Eurasian lineage was not found in the surveillance data from 2009 to 2014. In this study, 4 of 10 farms had evidence of H1N1pdm09 circulation. Interestingly, rH3N2 isolated from farm No.5 was a reassorted virus between H3 and N2 of the enH3N2-07 with the remaining six internal genes from H1N1pdm09 (Charoenvisal et al., 2013a). Moreover, rH3N2 has become one of the predominant subtypes of current Thai SIVs status (Nonthabenjawan et al., 2015). It should be noted that not only the H1N1pdm09 continued circulating in the Thai pig population but also its reassortant viruses. Since H1N1pdm09 contains some TRIG-lineage internal genes, the virus might have its ability

for genetic variation and interspecies transmission (Steel and Lowen, 2014). Similarly, in Vietnam, one of the highest pig producing countries in Southeast Asia (SEA), a high sero-prevalence of H1N1pdm09 was found (Baudon et al., 2015). Other countries in southeast Asia such as Malaysia and Cambodia reported serologic evidence of SIV subtype H1N1 and H3N2 circulating in the swine population prior the introduction of H1N1pdm09 (Suriya et al., 2008; Netrabukkana et al., 2015). Myanmar and Laos have no data on SIVs (Trevennec et al., 2011). It should be noted that each country must establish its own recent SIV genetic data before setting up serological diagnosis.

In this study, several age groups of pigs were collected for serum samples. Gilts and sows showed higher percentages of positive HI titer (HI-titer ≥40), whereas, nursery to growing pigs had lower percentages of positive HI titer. However, SIVs were often isolated from weaning pigs. The results suggested that SIV-infection might occur after weaning when waning of maternal immunity and the virus continuingly circulated in the sow herd after gilt acclimatization period. Interestingly, not only most replacement gilts and sows in the studied farms had high percentages of seropositive samples but also had positive HI titer with 2-3 subtypes. It should be noted that multiple infection of different SIV subtypes in the same farm commonly occurred. In addition, our cross sectional serological study showed positive HI titers of those 3 subtypes (Table 4.3) and pdmH1N1 had the highest cross-reaction after the introduction of H1N1pdm09 into Thailand.

After the introduction of H1N1pdm09 into Thailand, alterations in the genetic and antigenic profiles of Thai SIVs are of interest. The H1N1pdm09 and its reassortant viruses became widespread and circulating in the Thai pigs similar to the sero-surveillance data in other countries (Snoeck et al., 2015). Prior to the introduction of H1N1pdm09, three SIV subtypes were predominantly circulating in the Thai swine population and used as the reference viruses for serological diagnosis. SIV surveillance data in European countries during 2010-2013 showed that three endemic subtypes co-circulated with H1N1pdm09 virus such as in Greece (Kyriakis et al., 2015), and in United Kingdom (Fragaszy et al., 2015) and co-circulated with novel ressortant viruses (H1N1pdm09, rH1N2$_{hu}$ and rH3N1) with high percentage of positive HI titer to pdmH1N1 in Germany (Durrwald et al., 2010; Lange et al., 2013). Therefore, the European Surveillance Network for Influenza in Pigs (ESNIP) suggested adding pdmH1N1 as one of reference viruses for serological diagnosis (Simon et al., 2014). Interestingly, the cross-reactive antibody of Eurasian avian-like H1N1 virus or endemic H1N1 to H1N1pdm09 were investigated by HI test (Kyriakis et al., 2015) and neutralization test in Germany and Italy (Durrwald et al., 2010; De Marco et al., 2013). The findings were similar to this study, with some of endemic H1 (enH1N2-09) antiserum being cross-reactive to H1N1pdm09 and its reassortant virus. These suggested that virologic investigation and updating reference viruses for serological diagnosis should be performed concurrently with the SIV surveillance.

Two possibilities for virus transmission included importation of infected pigs from the H1N1pdm09 outbreak countries and transmission from infected humans to pigs especially from infected-workers (Arias et al., 2009). The risk of H1N1pdm09 infection between pigs and swine workers was investigated by serological evidence in many countries in Europe such as in UK. The results showed that pigs were initially infected by human transmission, then, the virus continually circulated in the pig population, and later, the virus might cause reverse zoonosis to humans (Fragaszy et al., 2015). In Italy, serological evidence in swine workers of SIV transmission from pigs to humans was also reported (De Marco et al., 2013). Additionally, SIV serological study of German farmers found significantly higher titers in both HI and neutralizing assays (Krumbholz et al., 2010; Krumbholz et al., 2014). Similarly, zoonotic transmission of SIV was confirmed in people working closely to swine in Ohio, USA (Bowman et al., 2014) and SIV serological evidence in Thai swine workers as well as in swine veterinarians was reported (Kitikoon et al., 2012). This indicates that SIV transmission between pigs and humans or vice versa has been found in many areas. Control and prevention strategies should be implemented to prevent the future pandemic threat of influenza (Nelson and Vincent, 2015).

In this study, H1N1pdm09 was found co-circulating with endemic viruses in pig population in Thailand. The introduction of pdmH1N1 into the pig population has altered the epidemiology, virology and serology of SIVs. Current serological study (HI

test) must include pdmH1N1. In addition, herds positive for pdmH1N1, endemic viruses, and their reassortant viruses have been reported in many regions. Potential reverse zoonosis of the reassortant viruses derived from the pdmH1N1 2009 may occur. To control the swine influenza viruses in pigs and prevent the interspecies transmission, regular influenza surveillance programs are of importance.

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Tables

Table 4.1: Geometric mean of rabbit anti-serum of reference viruses in 2006-2009. HI titers were evaluated against the homologous viruses.

	Anti-sera	enH1N1-06	enH1N1-09	enH1N2-09	pdmH1N1-09	enH3N2-05	enH3N2-07
H1	enH1N1-06	640	-	-	-	-	-
	enH1N1-09	-	183.79	-	-	-	-
	enH1N2-09	-	-	735.17	-	-	-
	pdmH1N1-09	-	-	-	844.48	-	-
H3	enH3N2-05	-	-	-	-	139.29	-
	enH3N2-07	-	-	-	-	-	735.17

Table 4.2: Geometric mean titers (GMTs) of rabbit anti-serum reference viruses in 2006-2009. HI titers were evaluated against current SIV subtypes (H1 and H3).

Anti-sera		Virus			
		enH1N1-10	pdmH1N1-10	rH1N2-10	rH3N2-10
H1	enH1N1-06	**320**	30.31	8.33	0
	enH1N1-09	211.12	16.65	19.13	0
	enH1N2-09	183.80	211.12	91.90	0
	pdmH1N1-09	183.79	**735.17**	**242.51**	0
H3	enH3N2-05	0	0	0	2.89
	enH3N2-07	0	0	0	**735.17**

Table 4.3: Titer distribution of enH1N1, pdmH1N1 and H3N2 viruses from pig farms in Thailand using hemagglutination inhibition (HI) assay.

GMTs	Number of sera (%) n=410		
	enH1N1	H1N1pdm09	enH3N2
<40 (negative)	251 (62.21%)	257 (62.68%)	263 (64.14%)
40	25 (6.09%)	37 (9.02%)	46 (11.21%)
80	33 (8.04%)	46 (11.21%)	57 (13.90%)
160	38 (9.26%)	33 (8.04%)	26 (6.34%)
320	18 (4.39%)	22 (5.36%)	7 (1.70%)
640	45 (10.01%)	15 (3.96%)	11 (2.71%)

Table 4.4: Percentage of enH1N1, pdmH1N1 and H3N2 viruses found in 8 studied farms using a hemagglutination inhibition (HI) assay and HA characterization of isolated viruses

Farm	enH1N1 (%)	H1N1pdm09 (%)	enH3N2 (%)	HA, NA characterization
1	36.53	42.3	42.3	pdmH1N1
2	20.5	20.5	14.7	pdmH1N1
3	32	42	32	pdmH1N1
4	97.77	97.77	42.22	pdmH1N1
5	19.5	13.04	54.34	rH3N2
6	8.54	9.54	13.54	-
7	46.66	40	33.33	-
8	41.07	44.67	41.07	-

CHAPTER 5

CONCLUSION

5.1 Research summary

According to the influenza A virus (IAV) causing the infection in wild host ranges, the possibility that the novel virus can infect many host species may occur. Two main mechanisms of IAV genetic variation are antigenic drift and antigenic shift (reassortment). These may generate the novel viruses which can cross the species barrier or infect human with pandemic scheme consequently. Currently, many organizations and researchers in both human and veterinary public health areas have raised their concern about IAV interspecies transmission. Three main host species are human, avian and swine have raised the concern for IAV control and prevention. Pigs are known as mixing vessels and become the host-linked between avian and mammalian viruses because pigs express both avian and mammalian influenza receptor types in their respiratory tract. Swine influenza virus (SIV) is one of the most important IAV raising more consideration after the emergence of pandemic H1N1 2009 (H1N1pdm09) and bidirectional transmission between human and swine population. Moreover, H1N1pdm09 was commonly found co-circulating and continuing reassortment with endemic viruses in Thai swine population. Therefore, the main

objective of this study was to focus on interspecies transmission of H1N1pdm09 and on serological assay for current SIV diagnosis in Thailand.

The first objective was to investigate the interspecies transmission of H1N1pdm09 between swine and aquatic birds especially for domestic ducks. The domestic ducks were selected to study as an interspecies-transmission model because of the high density of the raising system in Thailand and their highly potential interfacing with pigs and human. In this study, pathogenicity and viral shedding of H1N1pdm09 and its reassortant virus (the reassortant between N1 from endemic Thai swine virus with H1N1pdm09: rH1N1) in domestic ducks were studied. The results showed that ducks were not susceptible to H1N1pdm09 or rH1N1 infection. However, some ducks could be infected showing asymptomatic to mild clinical signs. The main lesions of both studied viruses were found in the respiratory tract similar to other LPAI. Moreover, rH1N1 trended to induce more pathogenicity than H1N1pdm09. It should be noted that ducks could shed both studied viruses to the environment at low level via both oropharyngeal and cloacal routes during 7 DPI. In summary, the duck could be the important transmission hub of H1N1pdm09 and other reassortant SIVs from pig if interspecies transmission occurs. Interestingly, the free grazing duck is the main duck population of interest in South East Asia (SEA), due to the short or long distance movement of the flocks when interfacing with other flocks or species. Since ducks harbored many IAV subtypes including H1N1pdm09 and its reassortant viruses, the possibility of the virus transmission from duck population to other avian or mammalian

hosts could generate the novel reassortant virus. Nowadays, many reassortant viruses are generated from different origins found in wild ducks and aquatic birds worldwide. Some of them including avian H5N1 (since1997-present), H7N9, H9N2 (during 2013-2014) in China could infect human and cause death. Therefore, interspecies transmission of H1N1pdm09 from pig to duck or other aquatic birds is important. These should be noted that IAV surveillance in duck population should be continuously monitoring. Not only to prevent the spread of the virus to other host species, but also to prevent the generation of the novel avian-mammalian reassortant influenza virus in the future.

After the introduction of H1N1pdm09 into the pig population worldwide, the reassortant viruses of H1N1pdm09 origin and endemic viruses continuingly occur. Additionally, the IAV from swine-avian transmission and swine-human transmission are of interest. Since H1N1pdm09 was characterized and proved that the origin of the virus came from the reassortant between SIVs from two different areas and caused pandemic scheme in 2009. Interestingly, if H1N1pdm09 reassortant viruses could possibly transmit back from pig to human, the protection to these reassortant viruses using current human influenza vaccine was still questionable. Therefore, the objective of the second study focused on the protection of human commercial subunit influenza vaccines to the current H1N1pdm09 reassortant viruses of swine origin. The results demonstrated that the current influenza vaccines provided protection only to H1N1pdm09 and the reassortant virus having H1 from H1N1pdm09. Those vaccines did

not cross protect to the predominant strains (enH1N1 and rH3N2) of the current Thai pig population. Since current commercial subunit vaccines contain the HA and NA antigens of human seasonal H3N2, H1N1pdm09 and influenza B virus compose of genetic data not closely relate to current Thai reassortant SIV. After the emergence of H1N1pdm09, many organizations have raised their concern and watch over on SIV genetic variation. Since the virus has TRIG cassette as the internal gene facilitating for reassortment and interspecies transmission. The results of this study demonstrated that current commercial vaccines did not completely cross protect of the studied SIV infections. In order to prevent the vaccination failure, the best matching between influenza vaccines and the endemic influenza viruses is the best choice of interest. Therefore, the efficacy of current human influenza vaccines should be updated focusing on SIVs as well as in the countries having human-animal interface like the countries in Asia. SIVs continuously reassorting and circulating in the pig population since the emergence of H1N1pdm09. In addition, SIVs surveillance program should be routinely carried on for the best prevention of novel emerging viruses. Additionally, the data would benefit for vaccine seeding determination, particularly, in the areas having high prevalence of H1N1pdm09 in the pig population.

The successful interspecies transmission of IAV depends on several factors including 1) Virus has to cross the barrier for IAV cross-transmission dividing into barrier among species (the opportunity of host interface and contact), barrier between virus-cell binding (the host immunity and viral receptors and successful viral replication) and

animal to animal transmission (the transmission of virus among hosts), 2) The mutation of HA and polymerase complex of IAV, HA is the most important protein binding to the host receptors and initiating the viral entry phase to host cells. The mutation of HA can alter the binding ability to α2, 3 SA (avian) receptor or α2, 6 SA (mammalian) receptor of IAV. The polymerase complex (PB1, PB2 and PA) play an important role in adjusting the host-range IAV infection, especially, for the amino acid substitution of PB2 which can increase the replication of IAV in different host species (Urbaniak et al., 2014). This suggested that the IAV surveillance data and collaborative research in various species are crucial for identifying the current IAV status and preparing for IAV interspecies transmission in the future.

The third objective of this study involved in HI test verification since HI test is a gold serological standard for IAV recommended by WHO. In this method, the representative viruses from each areas are required for HA antibody detection. Based on the SIV surveillance data in 2012-2014, H1N1pdm09 and its reassortant viruses became predominantly circulating in the Thai pig population and dramatically changing the status of current SIV in Thailand. This suggested that determination of current reference viruses used for Thai SIV serological study especially for HI test was necessary. Six SIV anti-sera from the retrospective surveillance data prior to the introduction of H1N1pdm09 were performed using HI test with the representative viruses from surveillance data during 2010-2012. The results showed three subtypes of HA currently

circulating in the Thai pig population, H1α (endemic group), H1pdm (pandemic group) and H3a (H3 human origin). These viruses were chosen as the appropriate reference viruses for current Thai HI test. Comparing to the HI reference viruses before the introduction of H1N1pdm09, only two HA subtypes H1α and H3a were positive. H1N1pdm09 or H1 derived from the pandemic group should be added as the references virus since H1N1pdm09 has been co-circulating with the endemic viruses in the Thai pig population.

It should be noted that influenza virus genetic does change by time as known previously. The virus is classified into A, B, C and D and has been found more in many host species including bat (H17, H18), horse (H7N7, H3N8), dog (H3N8, H3N2) and cattle (influenza D virus). In addition, novel reassortant viruses have been continuously reported in many host species. Interestingly, the pandemic influenza viruses, including Spanish flu (1918), Asian flu (1957), Hong Kong flu (1968) and the lasted H1N1pdm09 (2009) were generated from mutation or reassortant between two different viruses crossing barrier to the new hosts. This demonstrated that the antigenic variation of influenza virus continuingly occur and threaten the pandemic risk in the future. Interspecies transmission employs various mechanisms including the alternation of receptor preference and the mutation in protein involving virulence factors of the viruses. H1N1pdm09 was the good example for host barrier crossing and the pandemic scheme.

Based on the current surveillance and serological data, H1N1pdm09 commonly found co-circulating and reassorting with the endemic swine viruses in the pig population including Thailand. The first objective showed that H1N1pdm09 could infect duck with some limitations. However, the possibility of the emergence reassortant between avian and mammalian viruses and interspecies transmission to other hosts could occur. The second objective showed that current commercial human influenza vaccines did not provided completely protection to the current studied reassortant SIV. These demonstrated that the vaccination failure would occur due to the novel pandemic scheme in the future. The last objective suggested the alternation in Thai SIV genetic status after the H1N1pdm09 introduction. The serological data could provide helpful data combining with the viral genetic characterization to get the up to date data for SIV status. However, the key point of serological tool depends on the use of reference viruses. In order to obtain the accurate serological data, the reference viruses are of importance. In conclusion, this study and the SIV surveillance data in many areas around the world indicated that H1N1pdm09 and its reassortant viruses have been currently circulating in the pig population. Interestingly, the potential interspecies transmission and reverse zoonosis of the novel reassortant viruses could possibly be found sooner or later. For this reason, the study about influenza virus especially for H1N1pdm09 and other SIVs in multi-host species should be contunuingly conducted. To control the SIV in pigs and to prevent the interspecies transmission, not only the vaccine strategy, but the precisely influenza diagnostic methods including routine SIV

surveillance and serological study also are important in order to obtain the current status of SIVs which would provide benefit to SIVs control strategy in each area and would eventually prevent the generation of novel pandemic viruses.

5.2 Research limitation and further investigation

In Thailand, current SIV surveillance does not continously carry on, especially, for the serological study and lack of good collaboration among Thai and regional SIV researchers. Moreover, some regions in Thailand does not have SIV surveillance. Therefore, the whole picture of current Thai SIVs status is insufficent. In addition, most infected pigs usually show asymptomatic to mild respiratory signs and the SIV prevalence might be underestimated. Since SIV usually found co-infected with other respiratory swine pathogens such as Porcine Reproductive and Respiratory Syndrome virus (PRRSV), Porcine Circovirus type2 (PCV2), *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumonia* (APP) and *Pasteurella multocida* and those infected pigs may not show obvious clinical SIV symptoms or might have similar respiratory signs to SIV. However, most co-infections with SIV or porcine respiratory disease complex (PRDC) might cause severe clinical signs leading to economic losses comparing to the single virus infection. Accordingly, SIV diagnosis might be mis-diagnosed if the veterinarians are inexperienced. Therefore, the pathogenesis of the co-infection between SIV and other swine respiratory pathogens should be studied. This will

provide the benefit data for differentiation SIV from other swine respiratory pathogens and will benefit the farmers for implementation the appropiate management to control and prevent the interspecies transmission of SIV in pig population.

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APPENDIX

Appendix A: Immunohistochemistry staining for Influenza A virus (NP protein)

1. Deparaffinization

Preheat slide at 60°C for 15 min »» Xylene I = 5 min »» Xylene II = 5 min »» Xylene III = 5 min »» Xylene and alcohol solution = 2 min »» Absolute alcohol I = 2 min »» 95% alcohol = 2 min »» 80% alcohol = 2 min »» 70% alcohol = 2 min »» running water = 5 min »» Distill water = 5 min »» PBS = 5 min

2. Block endogenous peroxidase with 3% H2O2 (Absolute methanol 150 ml and 30% H2O2 15 ml) for 10 min at room temperature

3. Wash in Distill water for 5 min

4. Wash in PBS for 5 min, 2 min, 2 time

5. Pretreat slide with 0.05% Protenase K for 10 min at 37°C

6. Wash in PBS for 5 min, 3 time

7. Block non-specific antigen with 1%BSA for 30 min at 37°C

8. Wash in PBS for 5 min, 3 time

9. Apply primary antibody (anti-influenza A nucleoprotein monoclonal mouse antibodies, dilution 1:300, incubate at 4°C overnight

10. Wash in PBS for 5 min, 3 time

11. Apply secondary antibody (Biotinylated rabbit anti-mouse IgG antibody and envision polymer incubate at room temperature for 45 min

12. Wash in PBS for 5 min, 3 time

13. Developped with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) substrate for 2 min
14. Stop reaction in distill water
15. Counter stain with hematoxylin 45 sec
16. Wash in running water 5 min
17. Dehydration (95% alcohol = 2 min, Absolute alcohol II = 2 min, Absolute alcohol I = 2 min, Xylene and alcohol solution = 2 min, Xylene III = 5 min, Xylene II = 5 min, Xylene I = 5 min)
18. Mount slide with mounting media

Appendix B: Virus titration and Immunoperoxidase monolayer assay (IPMA)

1. Prepare monolayer of MDCK cells in 96-well tissue culture plate.
2. Wash cell monolayer with 1X PBS 3 times with cell culture medium containing 6% BSA and 5mg/ml TPCK-treated trypsin.
3. 10-fold dilution of samples and transfer 100 µl to each well (4 well per dilution).
4. Incubate in 37°C for 72 hours.
5. Discard all culture media and fix the cell with 4% formalin in PBS-0.5% tween (100µl per well) for 25 min at room temperature, wash with PBS-0.5%tween 3 times.
6. Apply antibody (anti-influenza A nucleoprotein monoclonal mouse antibodies, dilution 1:1000 with 1%BSA in PBS-0.5%tween) 50 µl/well, incubation for 1 hour at room temperature, wash with PBS-0.5%tween 3 times.
7. Apply conjugate (rabbit anti-mouse IgG dilute with 1% BSA in PBS-0.5% tween) in 1:300, 50 µl/well, incubate 1 hour at room temperature, and wash with PBS-0.5% tween 3 times.
8. Apply AEC substrate 50µl/well, incubate for 10 min at room temperature (AEC 8 tablets: Dimethyformamide 40 ml: 3%H2O2 in acetate buffer), wash in tap water 3 times.
9. Dry plate and read plate under phase-contrast microscope.
10. Calculate TCID50 by Reed and munch method.

Appendix C: Hemaglutination test (HA) and Hemagglutination inhibition test (HI)

Reagents

20% Kaolin Suspension in PBS (20 g Kaolin in PBS 100 ml / Receptor destroying enzyme (RDE)

1 X PBS pH7.5

- NaCl 8.5 g
- Na2HPO4 1.15 g
- NaH2PO4 (H2O) 0.2 g
- Distill water 1000 ml

50% Chicken's RBC

- Obtain chicken RBC's in Alsevers (1:1), Centrifuge at 1500 rpm for 10 min at 4°C.
- Discard supernatant, add 30 ml of PBS and mix gently, Centrifuge at 1500 rpm for 10 min at 4°C

- Discard supernatant, repeat steps for a total of 3 washes.
- Make a 50% RBC solution by adding equal volumes of packed RBCs and sterile PBS
- Store packed RBCs at 4°C for up to 1 week.

Serum treatment

H1 Treatment

1. Heat inactivate sera by incubating at 56°C for 30 min.

2. Add 100ul heat inactivated sera to 400ul of a 20% Kaolin suspension. Mix and incubate at room temp for 30 min, Centrifuge at 2000 rpm for 10 min.
3. Add 100ul 50% chicken RBC. Mix and incubate at room temperature for 1 hour. (Mix at beginning of 20 min., at 10 min., and at end of 20 min.).
4. Centrifuge at 2000 rpm for 10 min.
5. Transfer supernatant as 1:5 dilution for H.I. assay or transfer and store at -20°C.

H3 Treatment

1. Heat inactivate sera by incubating at 56°C for 30 min.
2. Add 100ul heat inactivated sera to 300ul of RDE, Mix and incubate at 37°C for 18-20 hours. Centrifuge at 2000 rpm for 10 min.
3. Add 100ul 50% chicken RBC. Mix and incubate at room temperature for 1 hour. (Mix at beginning of 20 min., at 10 min., and at end of 20 min.).
4. Centrifuge at 2000 rpm for 10 min.
5. Transfer supernatant as 1:5 dilution for H.I. assay or transfer and store at -20°C.

HA unit determination

1. Add 50ul PBS to all wells needed.

2. Add 50ul of stock virus/sample to first well and perform serial 2-fold dilutions across the plate.
3. Add 50ul of 0.5% RBC solution to all wells. Cover with a plate and tap gently to mix.
4. Incubate the plates at room temperature for 30 min or when RBCs have settled in control wells.
5. The highest dilution with agglutinated RBCs is the endpoint and represents the HA units of the stock. (The working dilution of virus for the HI test is 8 HA units per 50ul).
6. For back titration of virus working dilution, repeat steps of HA unit determination with virus dilution of 8 HA units/50ul.
7. Read plates for highest dilution with agglutinated RBCs (matte) for the endpoint. Adjust diluted virus to 8 HA units before using in the HI test.

Hemagglutination inhibition (HI)

1. Add 25ul of PBS to all treatment wells except row A.
2. Add 25ul of treated sera (already at 1:5 dilution) to rows A (serum controls), B Make 2-fold serial dilutions.
3. Add 25ul of virus working dilution (8 HA unit) to all wells except row A (serum controls). Cover the plate and tap gently to mix. Incubate at room temperature for 30 min.

4. Add 50 ul of 0.5% RBCs. Cover and mix. Incubate at room temperature for 30 min.

5. Read plates and record titer as the highest dilution well with a tight RBC button. HI positive is given when inhibition of hemagglutination occurs (button) and HI negative for wells with hemagglutination (matte). A positive well button should run at the same rate as the serum control well.

6. No inhibition of hemagglutination (-) at 1:10 is NEGATIVE. Inhibition of hemagglutination (+) at 1:10 or 1:20 is SUSPECT and at 1:40 or greater is POSITIVE

VITA

Mr. Jirapat Arunorat was born on October 5, 1986 in Bangkok, Thailand. He graduated with Doctor of Veterinary Medicine (DVM), second class honor, from Faculty of Veterinary Medicine, Khon Kaen University in academic year 2010. Soon after graduated, He enrolled in the Ph.D program in Veterinary Pathobiology program since 2011. During his Ph.D program, he got a grant from Chulalongkorn University (G-RSAB) to practice some bioimformatic work at School of Biological Sciences, Faculty of Science, Hong Kong University, Hong Kong under the supervision of Prof. Dr. Frederick C.C. Leung for 3 months.

During his PhD program, He received a scholarship from the Royal Golden Jubilee PhD Program from the Thailand Research Fund (TRF) (PHD/0075/2556). He used the exchanged program from this scholarship at the Animal Research Service (ARS), National Animal Disease Center (NADC), United State Department of Agriculture (USDA), USA under the supervision of Dr. Amy L. Vincent for 6 months.

The publication from his thesis.

Arunorat J, Charoenvisal N, Keawcharoen J, Sreta D, Amonsin A, Thanawongnuwech R. A Reassortant Virus of A Thai Swine Influenza Virus (SIV) and The Pandemic H1N1 of Pig Origin Did Not Induce Severe Disease in Experimental Ducks. The Thai Journal of Veterinary Medicine. 2014;44:9

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Charoenvisal, N., Keawcharoen, J., Sreta, D., Chaiyawong, S., Nonthabenjawan, N., Tantawet, S., Jittimanee, S., Arunorat, J., Amonsin, A. and Thanawongnuwech, R. 2013. Genetic characterization of Thai swine influenza viruses after the introduction of pandemic H1N1 2009. Virus Genes.

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Meetings, Conferences and Presentation

Jirapat Arunorat, Nataya Charoenvisal, Yonlayong Woonwong, Roongtham Kedkovid, and Roongroje Thanawongnuwech. Determination of current reference viruses for HI test of swine influenza viruses in Thailand.