ลักษณะพันธุกรรมและพยาธิวิทยาของไวรัสไข้หวัดใหญ่สุกรที่แยกได้ในประเทศไทยหลังจากพบการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009

นางสาวณทยา เจริญวิศาล

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GENETIC AND PATHOGENIC CHARACTERIZATION OF THAI SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC H1N1 2009

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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ณ ทยา เจริญวิศาล: ลักษณะพันธุกรรมและพยาธิวิทยาของไวรัสไข้หวัดใหญ่สุกรที่แยกได้ในประเทศไทยหลังจากพบการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009. (GENETIC AND PATHOGENIC CHARACTERIZATION OF THAI SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC H1N1 2009) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : อ.สพ.ญ.จุฑาทิพย์ เขียวเจริญ, 93 หน้า.

ในปี พ.ศ. 2552 ที่ผ่านมานั้น ได้มีการแพร่ระบาดของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009 หรือ pH1N1 ในมนุษย์ ซึ่งเกิดการแพร่ระบาดอย่างรวดเร็วไปทั้ง 6 ทวีปทั่วโลก และมีผู้เสียชีวิตถึง 18,449 คน หลังจากนั้นสามารถแยกเชื้อไวรัสได้จากสุกรที่ติดเชื้อตามธรรมชาติในหลายประเทศรวมถึงประเทศไทย การที่เชื้อไวรัสชนิดนี้ได้ชื่อว่าไข้หวัดสุกร หรือ ไข้หวัดหมู ในระยะแรกนั้น เนื่องจากไวรัสมีลักษณะพันธุกรรมทั้ง 8 ท่อน ใกล้เคียงกับไวรัสไข้หวัดใหญ่สุกร องค์การอนามัยโลก (WHO) และ องค์การโรคระบาดสัตว์ระหว่างประเทศ (OIE) จึงสนับสนุนให้หน่วยงานที่เกี่ยวข้องทำการสำรวจไวรัสไข้หวัดใหญ่ในสัตว์หลายชนิดรวมทั้งสุกรเพื่อเป็นแนวทางในการป้องกันและควบคุมโรค การสำรวจโรคไข้หวัดใหญ่สุกรเริ่มขึ้นในเดือนมิถุนายน 2553 จนถึงพฤษภาคม 2555 จากการสำรวจพบไวรัส pH1N1 มากที่สุดในปี 2553 ต่อจากนั้นตรวจพบไวรัสลูกผสม H1N1 H1N2 และ H3N2 เนื่องจากไวรัสลูกผสมที่พบจากการสำรวจนั้น เป็นไวรัสใหม่ที่ไม่เคยแยกเชื้อได้มาก่อน ดังนั้นจึงจำเป็นที่จะศึกษาพยาธิกำเนิดของโรค โดยการนำเชื้อไวรัส pH1N1 และไวรัสลูกผสม H1N1 (rH1N1) ใส่เข้าไปในสุกรแต่ละกลุ่ม นอกจากนี้ ยังนำเป็ดเข้าไปเลี้ยงร่วมกับสุกรแต่ละกลุ่มเพื่อดูว่าไวรัสสามารถติดจากสุกรมายังเป็ดได้หรือไม่ ผลการทดลอง พบว่าไวรัสทั้ง 2 ชนิด สามารถติดต่อและเพิ่มจำนวนได้ในสุกร โดยแสดงอาการและรอยโรคทางพยาธิวิทยาจำกัดอยู่ที่ระบบหายใจ นอกจากนี้ไวรัสยังสามารถติดต่อไปยังเป็ดได้แต่มีข้อจำกัดในการเพิ่มจำนวน เนื่องจากเป็ดจึงไม่แสดงอาการทางคลินิกและพบรอยโรคไม่ชัดเจน การศึกษาในครั้งนี้พบไวรัสลูกผสมหลายชนิด ดังนั้นการสำรวจโรคไข้หวัดใหญ่สุกรจึงมีความจำเป็นอย่างมาก ทั้งในอุตสาหกรรมการเลี้ยงสุกรและการสาธารณสุขในมนุษย์

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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NATAYA CHAROENVISAL : GENETIC AND PATHOGENIC CHARACTERIZATION OF THAI SWINE INFLUENZA VIRUSES AFTER THE INTRODUCTION OF PANDEMIC H1N1 2009. ADVISOR : PROF. ROONGROJE THANAWONGNUWECH, D.V.M., Ph.D, CO-ADVISOR : JUTHATIP KEAWCHAROEN, D.V.M., Ph.D., 93 pp.

Since pandemic H1N1 (pH1N1) 2009 influenza virus emerged in April 2009, at least 18,449 people died and was called Swine-origin 2009 A (H1N1) or “swine flu” due to its genetic character. Swine influenza virus (SIV) surveillance was conducted from June 2010 to May 2012 in Thailand. The results of the surveillance revealed that pH1N1 was the most prevalence in 2010. Later, reassortant H1N1, H3N2 and H1N2 viruses were also isolated. As a result of the discovery of the emergent SIV, pathogenesis studies of this novel virus was conducted in order to plan for future disease protection and control measures both in swine and human populations. Hence, pathogenesis studies in pigs and commingling sentinel ducks were conducted. The pH1N1 and its reassortant virus (rH1N1) isolated from pigs in Thailand were inoculated into 2 separate cohorts. A day later, sentinel ducks were commingled with pH1N1 or rH1N1 inoculated pigs in separate groups. This present studies suggested that both pH1N1 and rH1N1 viruses were able to infect and replicate in pigs and sentinel ducks. Moreover, those viruses were capable to transmit from infected pigs to sentinel ducks when animals were commingling but viral replication in ducks was limited. From the study, virological and serological surveillance of swine influenza are of importance for the prevention and control of swine influenza and for preparedness of future pandemic influenza in humans.

Department : Veterinary Pathology Student’s Signature .....
Field of Study : Veterinary Pathobiology Advisor’s Signature .....
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CONTENTS

LIST OF ABBREVIATIONS

bp	base pair (s)
°C	degree celsius (centrigrade)
Ct	Cycle threshold
DAB	3,3'-diaminobenzidine tetrahydrochloride
cDNA	complementary deoxyribonucleic acid
DPI	days post infection
enH1N1	endemic H1N1 virus
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
IHC	Immunohistochemistry staining
IgG	Immunoglobulin G
LPAI	Low pathogenic avian influenza virus
LSAB	labeled streptavidin-biotin method
M	Matrix gene
MDCK	Madin-Darby canine kidney cell line
ml	milliliter (s)
ug	microgram (s)
ul	microliter (s)
um	micrometer (s)
mM	micromole (s)
N	necropsy
NA	Neuraminidase gene

ND	not done
NP	Nucleoprotein gene
NS	Non-structural protein gene
OIE	World Organisation for Animal Health
pH1N1	pandemic H1N1 2009 virus
rH1N1	pandemic H1N1 2009 reassortant virus
PA	Polymerase acidic gene
PB1	Polymerase basic 1 gene
PB2	Polymerase basic 2 gene
PCR	polymerase chain reaction
PCV2	Porcine circovirus type 2
PRRSV	Porcine reproductive and respiratory syndrome virus
PRDC	Porcine Respiratory Disease Complex
RNA	Ribonucleic acid
rt	real-time reverse transcriptase polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
%	percentage
SA	Sialic acid
S	second
SIV	swine influenza virus
$TCID_{50}$	50% tissue culture infectious dose
TRIG	Triple reassortment internal gene cassette
VI	Viral isolation in cell line
WHO	World Health Organization
-	negative
+	positive

CHAPTER I

1.1 Research coherence

Influenza A virus is a negative-sense, single-stranded, segmented RNA virus. It is a member of family *Orthomyxoviridae* and in genus Influenza A virus. The influenza A can cause disease in wide host ranges including chicken, ducks, water fowl, humans, horses and pigs (Van Reeth, 2007). However, wild aquatic birds are known as reservoir of the virus not showing any clinical sign but able to shed the virus into the environment (Kim et al., 2009). As a result, interspecies transmission is able to occur and is a major concern about influenza virus in term of zoonosis.

Importantly, the negative sense single stranded RNA of the virus influents the antigenic drift occurring by point mutation during replication cycle. It occurs due to influenza RNA lacking of proofreading. Moreover, the segmented genes of the virus promote antigenic shift or reassortment. The reassortment occurs when at least 2 viruses infect in the same cell at the same time. Exchanging the RNA segments, then, a novel strain emerges. As soon as, the novel virus introduces itself into a new susceptible host with no immunity to the novel virus, pandemic outbreak will possibly occur. However, avian and human influenza viruses bind to different host receptors. Avian virus is not easily directly transmitting into humans unless having a mixing vessel. Thus, pig is able to serve as a mixing vessel. Antigenic drift and shift often occur in pigs, while the virus genetic is stable in human and avian species (Forrest and Webster, 2010).

The pandemic H1N1 2009 (pH1N1) emerged since 2009 in Mexico. The novel virus rapidly spread into human population and soon transmitted into pigs (Weingartl et al., 2010). The pH1N1 contains North American triple reassortant internal gene (TRIG)

cassette as an internal core (Garten et al., 2009). The TRIG cassette manipulates antigenic drift and shift, especially envelops proteins. The TRIG virus often changes its envelop and able to escape host immune system. By this way, TRIG cassette drives the genetic variation of swine influenza virus (SIV) in North American in the last 10 years. The first TRIG virus epidemic; later causing highly genetic variation of SIV is one of major concerns in North America because the viruses cause major economic loss in the swine industry (Vincent et al., 2009; Ma et al., 2010). It should be noted that TRIG virus has never been isolated in Thailand previously. However, the TRIG virus was just introduced into the Thai swine population when pH1N1 emerged in 2009. As a result, genetic and pathogenic variation of swine influenza viruses containing TRIG cassette in the Thai pig population is of interest and of public health concern.

The swine influenza virus (SIV) surveillance was conducted from June 2010 to May 2012 (Chapter II). The results showed pH1N1 was the major population in 2010 and followed by its reassortant H1N1 (rH1N1) virus in a year later. The novel swine reassortant virus was collected from nursery pigs showing mild flu-like symptoms such as sneezing and coughing. In contrast, the TRIG virus might cause severe clinical signs such as abortions and deaths among naïve sows when it was first isolated in North America (Olsen, 2002). Hence, the pathogenesis of the pH1N1 and its reassortant viruses isolated from naturally infected pigs was studied (Chapter III). The results pointed out that both viruses induced acute respiratory disease. Pigs in the rH1N1-infected cohort showed more severe clinical signs, had higher numbers of pigs shedding the virus, had increased histopathological severity of lung lesions and had increased numbers of viral antigen-containing cells in lung tissue. The clinical signs and pathological lesions in both rH1N1 and pH1N1-infected pigs were restricted only in the respiratory tract.

As mention above, aquatic birds serve as a reservoir of influenza virus and ducks are carriers of the highly pathogenic influenza H5N1 virus. The H5N1-infected ducks may not show any clinical sign but able to shed variable amounts of virus into the environments (Kim et al., 2009). Thus, commingling environment among pigs and avian species still found in swine production system in Thailand and other South-East Asian countries. In chapter IV, the experimental condition of commingling situation between pigs and sentinel ducks was conducted. Ducks were brought to comingling with pH1N1 or rH1N1 inoculated pigs in separate groups 1 day after virus inoculation in pigs. Then, clinical signs, viral shedding and pathological lesions were observed. The results showed both studied viruses were able to transmit from pigs to ducks but viral replication in ducks was limited. As a result, clinical signs were not obvious and low levels of viral shedding were detected in both sentinel duck groups.

All of three manuscripts in this dissertation partially fulfilled the requirement for the degree of Doctor of Philosophy Program in the Veterinary Pathobiology, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

1.2 Importance and rationale

Since pandemic H1N1 (pH1N1) 2009 influenza virus emerged in April 2009. The virus was responsible for 18,449 deaths (WHO, 2010a). The pH1N1 virus spreaded into more than 100 countries including Thailand in 6 continents by human to human transmission and was also called Swine-origin 2009 A (H1N1) or “swine flu” due to its genetic character. Each gene segment of the pH1N1 virus was closely related to the swine influenza virus (SIV). Interestingly, the internal gene cassette of the pH1N1 is a triple reassortant virus of human, avian and swine origin viruses. This particular character calls triple reassortant internal gene or TRIG (Garten et al., 2009). In North America, SIV containing TRIG cassette has been circulating in pigs for years with different surface proteins. The TRIG cassette includes polymerase acidic (PA) and polymerase basic 2 (PB2) genes of North American avian lineage, nonstructural (NS), Nucleoprotein (NP) and matrix (M) genes from classic swine lineage and Polymerase basic 1 (PB1) gene of human lineage (Vincent et al., 2008). However, the pH1N1 virus picked up H1 HAs from classic swine lineage, N1 NAs and M gene from Eurasian avian-like swine lineage. It is interesting if the TRIG virus has a high tendency to pick up different surface proteins and is able to continuing survive in pigs and in humans (Vincent et al., 2009; Ma et al., 2010).

In addition, the pH1N1 is unusual in its genetic characterization since it was the first report of reassorted SIV between the North American lineage and the Eurasian lineage (Garten et al., 2009; Schnitzler and Schnitzler, 2009). Phylogenetic analysis demonstrated that the reassortment probably occurred years before the pandemic outbreaks. Due to inobservance before (Smith et al., 2009), the World Health Organization (WHO) and World Organization for Animal Health (OIE) encourage routine surveillance on Influenza A virus’s natural hosts particularly in pigs.

Moreover, highly pathogenic H5N1 virus or “bird flu” emerged in Hong Kong in 1997 and re-emerged in many Asian countries including Thailand in 2003 (Suzuki, 2005; Neumann et al., 2009). From year 2003 to 2010, there were totally 510 human-infected cases and 303 deaths (WHO, 2010b). The transmission occurred by direct contact between chicken and humans and no confirmed evidence of human to human transmission. Nevertheless, H5N1 virus was isolated from pigs in Vietnam, Indonesia and China with no obvious clinical signs (Suzuki, 2005; Nidom et al., 2010). As a result, the risk of point mutation and reassortment of influenza viruses in swine may facilitate the presence of a novel high pathogenic virus with the ability to transmit among human population (Suzuki, 2005). Therefore, SIV surveillance on swine is more important since it is one of the influenza natural hosts and can also be a mixing vessel of avian and human viruses (Ma et al., 2009).

In the swine industry, swine influenza is an important respiratory disease caused by swine influenza virus. Although endemic SIV may cause low mortality rate in pigs, the virus may also have the major impact on the production performance. In addition, SIV is one of the major respiratory pathogens causing Porcine Respiratory Disease Complex or PRDC (Thacker et al., 2001). SIV itself causes acute respiratory disease in pigs showing typical clinical signs of sneezing, coughing or barking cough, respiratory distress, conjunctivitis, nasal discharge and fever. However, the severity depends on many factors such as viral strain, host immune status and other complications. Generally, the incubation period is between 1- 3 days with rapid recovery beginning at 4-7 days after the onset (Vincent et al., 2008; Sreta et al., 2009).

Influenza A virus causes a disease in wide host ranges including wild birds, poultry, pigs, horses and humans and is able to transmit among different host species. Influenza A virus composes of 8 segmented gene; 6 internal proteins and 2 surface glycoproteins: Hemagglutinin or HA gene, and Neuraminidase or NA gene. The subtype

of Influenza A virus is determined on the surface glycoproteins; HA and NA genes. Currently, there are 17 different HA and 9 different NA subtypes (Fouchier et al., 2005; Tong et al., 2012). The HA and NA genes are surface glycoproteins antigen and a target of neutralizing antibody (especially HA). As a result, virus avoids antibody recognition by amino acid substitution of HA. Moreover, HA plays an important role on virus attachment to host cell membrane, mediate virus – cell membrane fusion activity and let the virus particle enter the host cell. Influenza virus only binds to 2 specific receptors. The first is sialic acid (SA) α2,3 found in epithelial cells of gastrointestinal tract of wild aquatic birds. The second is SA α2,6 found in epithelial cells of respiratory tract of humans. NA has receptor-destroying activity (neuraminidase activity), uses for releasing the virus from host cell. Furthermore, NA is one of antiviral drugs target. Neuraminidase inhibitor such as Oseltamivir (Tamiflu®) and Zanamivir (Relenza®) block virus releasing activity. Once neuraminidase activity had been blocked, new viral particles attach to each other and form large aggregated particles. However, mutation and amino acid substitution of NA cause drug resistant (Skehel and Wiley, 2000; Neumann et al., 2009). The common influenza subtypes in pigs and humans are H1N1, H1N2 and H3N2. Although those subtypes between two hosts are similar, in fact the genetic characters are different. Generally, all of HA and NA subtypes (except H17) can be isolated from wild birds and waterfowls serving as reservoirs of the virus gene pool. Those harbored reservoirs may not show any clinical sign during the infection time. However, once they jump into a new host, the pandemic outbreaks may occur. The first Spanish influenza, occurring in 1918-1919, killed about 50 million people worldwide and the virus later identified as an avian-like H1N1 virus. The Asian influenza occurring in 1957 was a human/avian reassorted H2N2 virus and later introduced H2 and N2 to humans. Hong Kong influenza (H3N2) occurring in 1968, the virus brought in H3 and PB1genes from the avian virus to the human population. In 1976, the H1N1 swine influenza pandemic was found in soldiers'

army training base in Fort Dix, New Jersey (Ma et al., 2009; Neumann et al., 2009; Schnitzler and Schnitzler, 2009). As a result, interspecies transmission from pigs to humans was evident and this mechanism may become an important key for the incoming pandemic outbreaks.

After the emerging of pH1N1, interspecies transmission has raised the concern of pig serving as a mixing vessel. Interestingly, in late April 2009, a pig farm in Canada was suspected for influenza virus infection and phylogenetic analysis indicated SIV isolated from this farm was closely related to the human pH1N1 (Weingartl et al., 2010). After that, 2 workers in this farm developed flu-like symptoms with no contact with other symptomatic humans and pH1N1 infected pigs was suspected as a source of exposure (Howden et al., 2009). Later, in October 2009, a reassorted pH1N1 virus was isolated from pigs in a Hong Kong slaughter house (Vijaykrishna et al., 2010). The Hong Kong reassorted virus is a swine triple reassortment internal gene cassette H1N1 virus picking up the NA gene from the pH1N1 and HA gene from Eurasian swine lineage (Vijaykrishna et al., 2010). In Thailand, the pH1N1 virus was found in pig in a university farm in Saraburi province in December 2009 (Food-resources, 2009). Later, it was again found in a commercial swine farm in Ratchaburi province with no reassortment (Sreta et al., 2010). However, the reassorted virus was later found in other commercial farms in Central Thailand from the active surveillance program (Kitikoon et al., 2011a). Recent genetic characterization survey found reassorted pH1N1 virus with H1 gene from 2009 pH1N1 and NA gene from an endemic N1 Thai swine virus. The PB1 primers routinely use for differentiating the 2009 pH1N1 from the local Thai swine H1N1 in the laboratory also showed a positive result with the reassorted pH1N1. This evidence demonstrated that a reassorted virus might occur whenever a new virus has been introduced into the swine population. It should be noted that the TRIG virus has been introduced into the

Thai swine population. As a result, the genetic surveillance of Thai SIV is important and should be continuously performed (Sreta et al., 2010).

Comparing to other countries, Thai ecosystem is different and may facilitate any new reassorted virus. Firstly, Thailand has varieties of swine farm systems; from big industrial (over 1000 sows), medium (200-1000 sows), small scale (50-200 sows) and backyard (less than 50 sows) farms. Secondly, most of Thai swine farms have open housing system with only a few evaporation systems in the big industrial farms. Thirdly, commingling among domestic species is commonly seen in the backyard farms or even in the small to medium farms. Moreover, commingling of several animal species such as pigs, ducks and other avian species feeding and placing together in the farm are still present, particularly in the backyard farms. Hence, both pigs and ducks play their important roles in influenza virus transmission since both species can act as the intermediate host and reservoir of the virus. As a result, interfacing among animal species may facilitate sharing exotic influenza genes among species. Continuous surveillance in pigs and *in vivo* experimental infection of new reassorted virus in pigs commingling with sentinel ducks may assist the understanding of its pathogenesis, transmission factor and risks of interspecies transmission.

Since the emerging of pH1N1 2009, researchers are aware of the impact of swine influenza virus in term of interspecies transmission and pathogenesis studies. However, most of the pathogenesis studies used the pH1N1 isolated from humans inoculating in mice, turkeys, ferrets, pigs and non-human primates (Itoh et al., 2009; Munster et al., 2009; Russel et al., 2009; Belser et al., 2010; Vincent et al., 2010). Although, those experimental animals are good models for human influenza research, they rarely appear being the sources of influenza infection. In contrast, pigs are serving as influenza A virus natural host and a mixing vessel. As a result, pigs can be infected with both human and avian influenza viruses. Reassortment among human, avian and

swine influenza viruses can occur in pigs and transmits the novel virus back to the human community (Ma et al., 2009). The pathogenesis of the novel viruses will provide information of infection site, replication ability and shedding and transmission period as well as the virulence of the viruses. The information will support effective control and prevention program and also useful for further study in humans. Therefore, pathogenesis study of pH1N1 that isolated from pigs or any reassorted pH1N1 virus in pigs is necessary.

As a result, *in vivo* study in pigs and ducks, one of natural mammalian hosts and avian reservoir of influenza virus would provide the better understanding of pathogenesis and interspecies transmission among viruses. Moreover, comparing studies of the pH1N1 and reassorted pH1N1 isolated from pigs have not been done previously which would be useful information for the pig industry and also for public health concern in term of zoonosis as well as possibly preparing for the new pandemic influenza outbreak.

1.3 Research objectives

1. To understand and get an up-to-date genetic data of the Thai SIVs after the pH1N1 introduction.
2. To understand the pathogenesis of the pH1N1 (isolated from pig) and its reassorted pH1N1 virus in pigs.
3. To investigate the incident of pH1N1 and its reassorted pH1N1 virus interspecies transmission in sentinel ducks.

1.4 Research delimitation

This research composed of 2 separate parts. The first part (Chapter I) is SIV surveillance after the pH1N1 outbreak in 2009. Nasal swabs were collected from 4-8, 12, 16, 20, 24 week old pigs in North, North-eastern, Eastern, and central regions of Thailand during June 2010- May 2012. Pigs might show clinical signs of sneezing, coughing, conjunctivitis and fever. Lung tissues were collected from 5-6 week old piglets with respiratory problem. All samples were tested by Reverse transcriptase-Polymerase chain reaction (RT-PCR). Positive RT-PCR samples were conducted whole genome sequencing and genetic analysis.

The second part of the research contains pathogenesis study of pH1N1 and rH1N1 viruses in pigs and sentinel ducks. Both viruses were isolated from naturally infected pigs. The viruses were propagated in embryonated chicken eggs until the concentration reached 10^4 $TCID_{50}$/ml. Twenty one pigs and 21 ducks were divided into 3 groups. The first and second groups are pH1N1 and rH1N1 group, respectively. Each group had 9, 3 week old pigs in pigs experiment and 9, 6 week old cherry valley ducks in sentinel ducks experiment. The third group is negative control group containing 3

pigs and 3 ducks in each experiment. The viruses were intratracheally inoculated into pigs. One day after that, ducks were placed into each pigs room. Clinical signs, nasal swab from pigs, oropharyngeal and cloacal swabs from ducks were collected. Later, 3 pigs from viral inoculated groups and 1 pig from the control group were euthanized and necropsied at 2, 4, and 12 days post inoculation (DPI). On 3, 5 and 13 DPI, 3 ducks from group1 and 2, and 1 ducks from negative control group were necropsied. Then, clinical signs, viral shedding and pathological lesions were analyzed.

1.5 Research benefits

1. This research revealed SIV status and its genetic characterization in swine herds in Thailand after the introduction of pH1N1 in 2009. The information making the veterinary and farmers concern about influenza virus in the pig farms. Moreover, advantages for public health prevention and control protocols could be developed.
2. The pathogenesis study of pandemic H1N1 and reassortant pandemic H1N1 in the natural host would benefit for human health study. The study showed that the reassortant virus caused more severe disease. Although, both studied viruses were restricted only in the respiratory system, the viruses are able to shed for at least 12 days or at the end of the experiment.
3. The study demonstrated the possibility of interspecies transmission from pigs to ducks. However, the pH1N1 and rH1N1 had limited replication in ducks.

CHAPTER II

Original article: Virus Genes (Manuscript submitting)

Title: Genetic characterization of Thai swine influenza viruses after the introduction of pandemic H1N1 2009

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Genetic characterization of Thai swine influenza viruses after the introduction of pandemic H1N1 2009

2.1 Abstract

Pandemic H1N1 2009 (pH1N1) influenza virus containing triple reassortant internal genes (TRIG) from avian, human, and swine influenza viruses emerged in 2009 is highly infective and is able to transmit to pigs. During June 2010-May 2012, influenza virus surveillance was conducted in Thai pig population. Twenty three samples (1.75%) were successfully isolated from total 1,335 samples. Interestingly, pH1N1 (7 isolates, 30.34%), reassortant pH1N1 (rH1N1) (1 isolate, 4.35%), Thai endemic H1N1-like (enH1N1) (3 isolates, 13.04%), reassortant H3N2 with pH1N1 internal genes (rH3N2) (9 isolates, 39.13%) and reassortant H1N2 with pH1N1 internal genes (rH1N2) (3 isolates, 13.04%) were found. It should be noted that rH1N1, rH1N2 and rH3N2 viruses contained the internal genes of pH1N1 virus having a TRIG cassette descendant from the North American swine lineage. Although all isolates in this study obtained from mild clinically sick pigs, the viruses were still highly infective and possibly may play an important role on human-animal interfacing transmission. In addition, the TRIG cassette may influent on antigenic shift resulting in emergence of novel viruses as seen in this study. Continuing surveillance of influenza A natural hosts, particularly in pigs is necessary.

Keywords Influenza, H1N1, H1N2, H3N2, reassortant, surveillance, pigs, Thailand

2.2 Introduction

Since April 2009, pandemic H1N1 2009 (pH1N1) influenza virus emerged and spread among human population including Thailand. The molecular analysis indicated that pH1N1 is a reassortant virus between the North American triple reassortant swine influenza virus (SIV) and the European Avian-like SIV. It was the first report of the reassorted SIV between North American and Eurasian lineages containing neuraminidase (NA) and matrix (M) genes from European avian-like SIV and 6 other genes from North American triple reassortant virus (Garten et al., 2009). The North American triple reassortant virus composes of polymerase basic 2 (PB2), polymerase acidic (PA) genes from avian origin, polymerase basic 1 (PB1) gene from human origin and hemagglutinin (HA), nucleoprotein (NP), nonstructural (NS) genes from classic swine lineage. The triple reassortant internal gene (TRIG) cassette has a high tendency to pick up different surface proteins and is able to continuingly survive in pigs and in humans. As a result, the genetic characteristic of North American SIVs has had high variations after the introduction of the TRIG virus into the swine populations (Vincent et al., 2009; Ma et al., 2010).

Influenza virus circulates among different host species including wild birds, poultry, pigs, horses and humans (Forrest and Webster, 2010). The virus usually binds to 2 specific receptors, sialic acid (SA) α2,3 found in the epithelial cells of gastrointestinal tract of wild aquatic birds and SA α2,6 found in the epithelial cells of human respiratory tract. As known previously, pigs serving as “a mixing vessel” for genetic reassortant among avian and human viruses have both receptors in the tracheal epithelium (Arias et al., 2009). As a result, cross-species transmission was occasionally found (Komadina et al., 2007; Lindstrom et al., 2012). The commingling among domestic species is commonly practiced in the backyard farming system or even in the small to

medium swine farms in most Asian countries including Thailand. Hence, Thai swine ecosystem may enhance the reassortant between humans and pig viruses.

Several months after pH1N1 emergence in humans, the virus was isolated from non clinical sign pigs confirming human to pig transmission in many countries including Canada, Norway, Italy, Hong Kong and Thailand (Hofshagen et al., 2009; Moreno et al., 2009; Sreta et al., 2010; Vijaykrishna et al., 2010; Weingartl et al., 2010). Subsequently, pH1N1 reassortant (rH1N1) viruses were reported in Hong Kong, Italy, German, Thailand and US (Vijaykrishna et al., 2010; Kitikoon et al., 2011a; Moreno et al., 2011; Starick et al., 2011). It should be noted that the Thai rH1N1 virus contained the NA gene from a Thai endemic SIV and other 7 genes from pH1N1.

Retrospectively, genetic characterization of the Thai SIV during year 2000-2008 showed 3 major SIV subtypes (H1N1, H1N2, and H3N2) (Chutinimitkul et al., 2008). The endemic Thai H1N1 SIV has internal genes and NA gene closely related to the avian-like swine lineage of Eurasia but HA gene is similar to classic swine H1N1. The H3 HAs viruses were also divided into 2 clades. Clade A was similar to human H3N2 virus and clade B was similar to human-like swine SIV from European countries and Hong Kong. The N2 NAs gene of Thai H3N2 subtype was divided into 3 groups; American/Asian SIV, European SIV and Thai SIV. In contrast, H1N2 viruses pose the H1 (HA) gene from classic swine H1N1 but the N2 (NA) gene from European lineage (Komadina et al., 2007; Chutinimitkul et al., 2008; Takemae et al., 2008). It should be noted that the TRIG cassette swine influenza viruses had never been found in Thailand prior to 2009. It was not until the introduction of pH1N1 into the Thai pig population in early 2009 (Sreta et al., 2010). As a result, genetic surveillance of Thai SIV is important and necessary for disease control and prevention of the novel influenza viruses.

2.3 Materials and Methods

2.3.1 Sample collection

Total 1,314 nasal swabs and 21 lung tissues were collected from April 2010 to May 2012. The Thai influenza surveillance was performed in 28 pig farms of 14 provinces in Northern, North-eastern, Eastern and central regions. Nasal swabs were collected from 4-8, 12, 16, 20, 24 week-old pigs, gilts and sows with noticeable respiratory signs such as sneezing, coughing, conjunctivitis and fever. Lung tissues were collected from 5-6 week old necropsied pigs with lesions characterized by multifocal, dark, plum-colored consolidation at cranioventral areas.

2.3.2 Virus identification and isolation

Total RNA was extracted from nasal swabs or supernatant of lung tissue homogenate by using a commercial kit (NucleoSpin Extract Viral RNA Kit, Macherey-Nagel, Germany) as described by a manufacture and submitted for a routine reverse transcriptase polymerase chain reaction (RT-PCR) (Payungporn et al., 2006). Primers specific to Matrix (M) gene (forward primer: 5'- TGATCTTCTTGAAAATTTGCAG-3' and reverse primer: 5'- CGATGGTCATTTTGTCAACA-3') were used and RT-PCR system was performed by using AccessQuick RT-PCR system (Promega, USA). Briefly, the cycling conditions started at 48°C for 45 min, 94°C for 3 min and followed by 35 cycles of denaturation (94°C for 20 s), annealing (55°C for 20 s) and extension (72°C for 30 s). For RT-PCR positive samples, those samples were subsequently inoculated into both 9 days-old embryonated chicken eggs and MDCK cell line as described previously (Sreta et al., 2009). Isolated viruses were harvested within 2 days after inoculation in embryonated chicken eggs and MDCK cell line before genetic sequencing.

2.3.3 Virus gene sequencing and sequencing analysis

Viral RNA was extracted from inoculated samples and cDNAs were synthesized by using ImProm-II Reverse Transcriptase (Promega, USA) and universal primer (5'-AGCAAAAGCAGG-3') as described by the manufacture. Then, PCRs were performed using specific primers designed for full length of eight genes of influenza A virus (Hoffman et al., 2001). The PCR products were analyzed by using 1.2% agarose gel electrophoresis and purified by using a commercial kit (Nucleospin Gel and PCR clean-up, Macherey-Nagel, Germany). DNA sequencing was carried out by 1st BASE company (Singapore) with specific primer sets. Blast analysis was carried out on NCBI. Sequence analysis was done by using MEGA 5.1 program (Tamura et al., 2011). The nucleotide sequences of Thai SIV isolates, human pH1N1, Thai endemic swine H1N1 previously isolated in 2006-2009, North American swine, European swine and avian H1N1 viruses were included in H1 HAs and N1 NAs phylogenetic tree. In H3 HAs and N2 NAs phylogenetic trees, human H3N2, Thai endemic swine H3N2, previously isolated in 2004-2005, North American swine, European swine and avian H3N2 viruses were included. All reference isolates were obtained from Genbank. The phylogenetic tree was constructed applying neighbor-joining algorithm by using MEGA 5.1 program.

2.4 Results

2.4.1 Virus identification and Sequence analysis

Of total 1,335 samples (1,314 swabs and 21 lung tissues), only 23 samples (1.75%) were positive for influenza A virus based on Matrix gene RT-PCR results. All 23 positive samples were, then, isolated from 4-8 week old pigs. Most positive samples were obtained during cold weather from October – March in Thailand. Those positive samples were collected from the central area (Saraburi, Suphanburi and Ratchaburi),

the Eastern area (Chonburi and Chachoengsao), the North-eastern area (Nakorn Ratchasima and Burirum) and the Northern area (Chaing Mai and Lumpoon) of Thailand. Detail description and Genbank accession number of each isolate is shown in Table 1.

From total 23 RT-PCR positive samples, 7 isolates (A/swine/Thailand/CU-RP1/2010, A/swine/Thailand/CU-RP3/2010, A/swine/Thailand/CU-SPN47/2010, A/swine/Thailand/CU-SPN65/2010, A/swine/Thailand/CU-PL63/2010, A/swine/Thailand/CU-PL65/2010 and A/swine/Thailand/CU-DP83/2010) were similar to pH1N1 virus. The sequence analysis revealed these swine pH1N1 viruses had all genes including HA and NA genes closely related to the human pH1N1 virus (Table 2).

One rH1N1 (A/swine/Thailand/CU-SA433/2010) was re-isolated in the same farm as previously reported (Kitikoon et al., 2011a). This isolate contained PB2, PB1, PA, HA, NP, M and NS genes of the pH1N1 virus but the NA gene closely related to that of the endemic swine H1N1 viruses circulating in Thailand, Hong Kong and European countries (Table 2).

Three isolates were closely related to the endemic Thai H1N1 (enH1N1) virus, previously isolated in 2004-2006 (Chutinimitkul et al., 2008). These 3 isolates included A/swine/Thailand/CU-SPL2/2010, A/swine/Thailand/CU-SPL4/2010 and A/swine/Thailand/CU-PS73/2010. The PB2, PB1, HA, NP and NA genes showed 94-96% identity to the Thai enH1N1 in 2000 (A/swine/Ratchaburi/NIAH1481/2000(H1N1) (Table 2), whereas, PA, M and NS were 95-98% identity to the Thai enH1N1 in 2004 (A/swine/Chonburi/NIAH977/2004(H1N1).

Interestingly, from 2011 onward, 9 reassortant H3N2 viruses (rH3N2) and 3 reassortant H1N2 (rH1N2) viruses containing pH1N1 internal genes were found. The rH3N2 viruses included A/swine/Thailand/CU-CG43/2011, A/swine/Thailand/CU-

CG45/2011, A/swine/Thailand/CU-CG48/2011, A/swine/Thailand/CU-CG51/2011, A/swine/Thailand/CU-CG55/2011, A/swine/Thailand/CU-P43/2012, A/swine/Thailand/CU-P53/2012, A/swine/Thailand/CU-BN53/2012, A/swine/Thailand/CU-BN54/2012. These rH3N2 viruses had PB2, PB1, PA, NP, M and NS genes similar to those of pH1N1 virus but HA and NA genes of these viruses had 95% identity to the previous Thai swine H3N2 isolate (A/swine/Ratchaburi/NIAH59/2004(H3N2)) (Table 2). Three rH1N2 viruses included (A/swine/Thailand/CU-CT43/2011, A/swine/Thailand/CU-CT63/2011 and A/swine/Thailand/CU-CT83/2011) obtained from the same farm. PB2, PB1, PA, HA, NP, M and NS genes were closely related to those of pH1N1 virus but NA gene had 93% identity to human H3N2 isolate (A/Stockholm/12/1988(H3N2)) and had only 88% identity to the previous Thai swine isolate (A/swine/Ratchaburi/NIAH9426/2005(H3N2)) (Table 2).

Table 1 Name, collection date, origin, specimen type, subtype and Genbank accession number of the 23 swine influenza isolates in this study

Influenza virus strain	Collection date	Province	Specimen type	Subtype result	Genes sequence	Genbank accession No.
A/swine/Thailand/CU-SA433/2010	28/06/10	Saraburi	NS	rH1N1	Full PB1, PA, HA, NP, NA, M, partial PB2, NS	CY089822 - CY089829
A/swine/Thailand/CU-SPL2/2010	10/08/10	Chonburi	NS	enH1N1	Full PA, partial PB2, PB1, HA, NP, NA, M, NS	KC610038 - KC610045
A/swine/Thailand/CU-SPL4/2010	10/08/10	Chonburi	NS	enH1N1	Full PB1, PA, HA, partial PB2, NP, NA, M, NS	KC610046 - KC610053
A/swine/Thailand/CU-RP1/2010	24/09/10	Ratchaburi	Lung	pH1N1	Full PB2, PB1, PA, HA, NA, M, NS, partial NP	CY089807 - CY089814
A/swine/Thailand/CU-RP3/2010	24/09/10	Ratchaburi	Lung	pH1N1	Full PB1, PA, NP, NA, NP, partial PB2, HA, M	CY089815 - CY089821, JX293381
A/swine/Thailand/CU-PS73/2010	28/09/10	Chonburi	NS	enH1N1	Full PB1, PA, NA, M, NS, partial PB2, HA, NP	CY089800 – CY089806, JX293367
A/swine/Thailand/CU-SPN47/2010	17/11/10	Suphanburi	NS	pH1N1	Full PA, HA, NA, NS, partial PB2, HA, NP, M	KC610054 - KC610060
A/swine/Thailand/CU-SPN65/2010	17/11/10	Suphanburi	NS	pH1N1	Full PB2, PB1, PA, HA, NP, partial NA, M, NS	KC610061 - KC610068
A/swine/Thailand/CU-PL63/2010	30/11/10	Chachoengsao	NS	pH1N1	Full PB2, PB1, PA, NP, M, NS partial HA, NA	JX293382 - JX293385, CY089793 - CY089796
A/swine/Thailand/CU-PL65/2010	30/11/10	Chachoengsao	NS	pH1N1	Full PB1, HA, M, partial PB2, PA, NP, NA	JX293376 - JX293380, CY089797 - CY089799

Table 1 Name, collection date, origin, specimen type, subtype and Genbank accession number of the 23 swine influenza isolates in this study (continue)

Influenza virus strain	Collection date	Province	Specimen type	Subtype result	Genes sequence	Genbank accession No.
A/swine/Thailand/CU-DP83/2010	06/12/10	NakoRatchasima	NS	pH1N1	Full PB2, PB1, PA, M, NS, partial HA, NP, NA	CY089789 - CY089792, JX293394 - JX293397
A/swine/Thailand/CU-CG43/2011	25/02/11	Lumpoon	NS	rH3N2	Full PB2, PA, NP, NS, partial PB1, HA, NA, M	JX293386 - JX293393
A/swine/Thailand/CU-CG45/2011	25/02/11	Lumpoon	NS	rH3N2	Full PB2, PB1, PA, M, NS, partial HA, NP, NA	JX293368 - JX293375
A/swine/Thailand/CU-CG48/2011	25/02/11	Lumpoon	NS	rH3N2	Full PB2, PB1, PA, NP, M, NS, partial HA, NA	JX293398 - JX293405
A/swine/Thailand/CU-CG51/2011	14/11/11	Lumpoon	NS	rH3N2	Full NA, NS, partial PA, M	KC609993 - KC609996
A/swine/Thailand/CU-CG55/2011	14/11/11	Lumpoon	NS	rH3N2	Full PB1, PA, NS, partial HA, NP, NA, M	KC609997 - KC610002
A/swine/Thailand/CU-CT43/2011	18/12/11	Chaing Mai	NS	rH1N2	Full PB1, NS, partial HA, NP, NA, M	KC610003 - KC610008
A/swine/Thailand/CU-CT63/2011	18/12/11	Chaing Mai	NS	rH1N2	Full NS, partial PB2, PB1, HA, NP, NA, M	KC610009 - KC610015
A/swine/Thailand/CU-CT83/2011	18/12/11	Chaing Mai	NS	rH1N2	Full PB1, NA, partial PB2, HA, NP, M, NS	KC610016 - KC610022
A/swine/Thailand/CU-P43/2012	09/01/12	Chaing Mai	NS	rH3N2	Full HA, NA, NS, partial PB2, PB1, PA, NP, M	KC610023 - KC610030
A/swine/Thailand/CU-P53/2012	09/01/12	Chaing Mai	NS	rH3N2	Full HA, NS, partial PB2, PB1, NP, NA, M	KC610031 - KC610037
A/swine/Thailand/CU-BN53/2012	15/03/12	Burirum	NS	rH3N2	Full PB2, PB1, HA, NP, NA, partial M, NS	KC609978 - KC609984
A/swine/Thailand/CU-BN54/2012	15/03/12	Burirum	NS	rH3N2	Full PB1, HA, NA, partial PB2, PA, NP, M, NS	KC609985 - KC609992

Table 2 The highest degree of HA and NA homology of the studied viruses

Virus	Gene	Virus with the highest degree of homology	Identity (%)	Virus origin
A/swine/Thailand/CU-SA433/2010	HA	A/Mexico City/WR1312N/2009(H1N1)	99	Human pH1N1
	NA	A/swine/Chonburi/NIAH977/2004(H1N1)	98	Thai swine endemic H1N1
A/swine/Thailand/CU-SPL2/2010	HA	A/swine/Thailand/CU-CHK4/2009(H1N2)	98	Thai swine endemic H1N1
	NA	A/swine/Ratchaburi/NIAH550/2003(H1N1)	97	Thai swine endemic H1N1
A/swine/Thailand/CU-SPL4/2010	HA	A/swine/Thailand/CU-CHK4/2009(H1N2)	98	Thai swine endemic H1N1
	NA	A/swine/Ratchaburi/NIAH550/2003(H1N1)	97	Thai swine endemic H1N1
A/swine/Thailand/CU-RP1/2010	HA	A/Singapore/GP4436/2009(H1N1)	98	Human pH1N1
	NA	A/Singapore/ON288/2009(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-RP3/2010	HA	A/Texas/JMS377/2009(H1N1)	99	Human pH1N1
	NA	A/Singapore/ON288/2009(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-PS73/2010	HA	A/swine/Thailand/CU-CHK4/2009(H1N2)	98	Thai swine endemic H1N1
	NA	A/swine/Ratchaburi/NIAH550/2003(H1N1)	97	Thai swine endemic H1N1
A/swine/Thailand/CU-SPN47/2010	HA	A/Bangkok/INS580/2010(H1N1)	99	Human pH1N1
	NA	A/Singapore/TT117/2011(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-SPN65/2010	HA	A/Bangkok/INS580/2010(H1N1)	99	Human pH1N1
	NA	A/Singapore/TT117/2011(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-PL63/2010	HA	A/Thailand/0445-00-N0/2009(H1N1)	99	Human pH1N1
	NA	A/Finland/576/2009(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-PL65/2010	HA	A/California/VRDL91/2009(H1N1)	99	Human pH1N1
	NA	A/Finland/576/2009(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-DP83/2010	HA	A/Bangkok/INS580/2010(H1N1)	99	Human pH1N1
	NA	A/Bangkok/INS587/2010(H1N1)	99	Human pH1N1
A/swine/Thailand/CU-CG43/2011	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-CG45/2011	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	94	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-CG48/2011	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	94	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-CG51/2011	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-CG55/2011	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	94	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-CT43/2011	HA	A/Singapore/TT144/2010(H1N1)	99	Human pH1N1
	NA	A/Stockholm/12/1988(H3N2)	93	Human H3N2 (1988)

Table 2 The highest degree of HA and NA homology of the studied viruses (continue)

Virus	Gene	Virus with the highest degree of homology	Identity (%)	Virus origin
A/swine/Thailand/CU-CT63/2011	HA	A/Singapore/TT144/2010(H1N1)	99	Human pH1N1
	NA	A/Stockholm/12/1988(H3N2)	93	Human H3N2 (1988)
A/swine/Thailand/CU-CT83/2011	HA	A/Singapore/TT144/2010(H1N1)	99	Human pH1N1
	NA	A/Stockholm/12/1988(H3N2)	93	Human H3N2 (1988)
A/swine/Thailand/CU-P43/2012	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	94	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-P53/2012	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	94	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
A/swine/Thailand/CU-BN53/2012	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	95	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	96	Thai swine endemic H3N2
A/swine/Thailand/CU-BN54/2012	HA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	96	Thai swine endemic H3N2
	NA	A/swine/Ratchaburi/NIAH59/2004(H3N2)	97	Thai swine endemic H3N2

2.4.2 Phylogenetic analysis

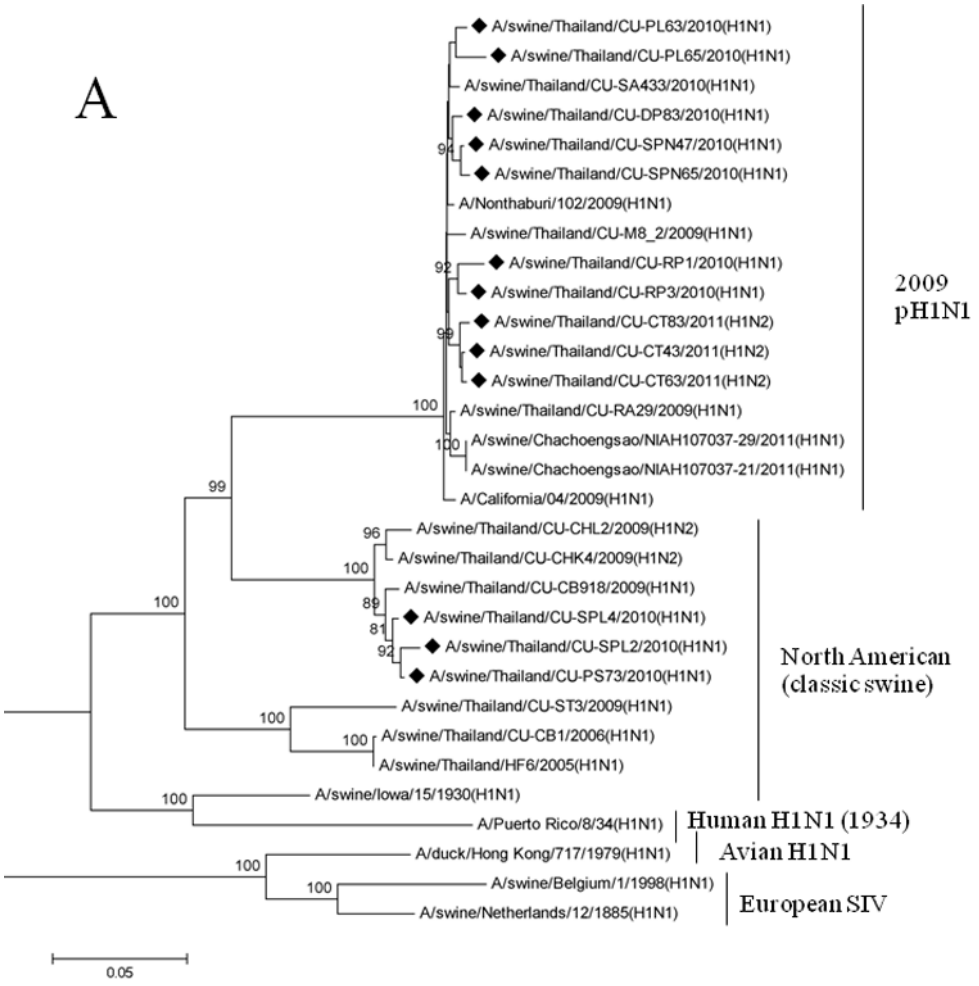
Phylogenetic tree of H1 HAs showed that HA gene of pH1N1, rH1N1 and rH1N2 viruses were grouped together with human pH1N1 isolates (A/California/04/2009 and A/Nonthaburi/102/2009), whereas, HA gene of enH1N1s belonged to North American classic swine lineage similar to the 2000-2009 endemic H1N1 SIV in Thailand (A/swine/Thailand/CU-CB1/2006, A/swine/Thailand/CU-ST3/2009) (Figure 1a).

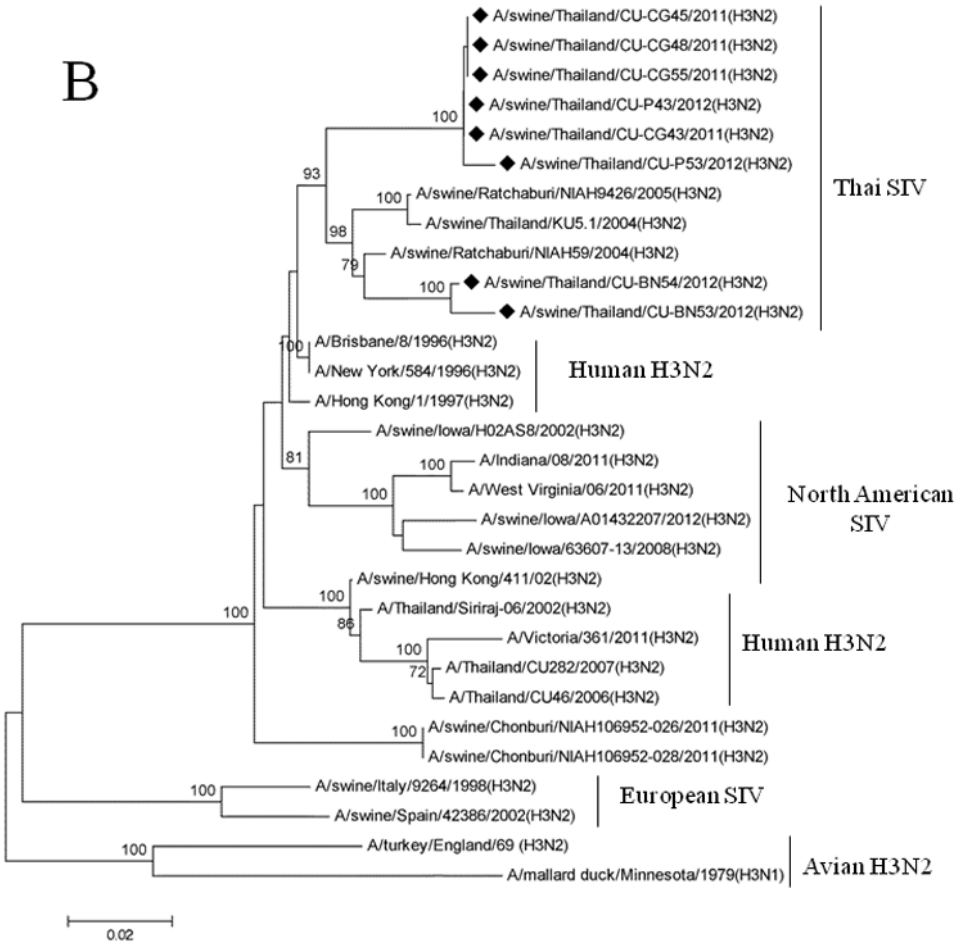
The phylogenetic tree of H3 HAs is shown in Figure 1b. All isolates in this study were clustered with endemic H3N2 viruses in Thailand during 2004-2005. These viruses were distant from avian, human and European swine lineages. In addition, the Thai rH3N2 isolates were distinguished from the 2011 reassortant H3N2 virus isolated from humans in North America (A/Indiana/08/2011 and A/West Virginia/06/2011).

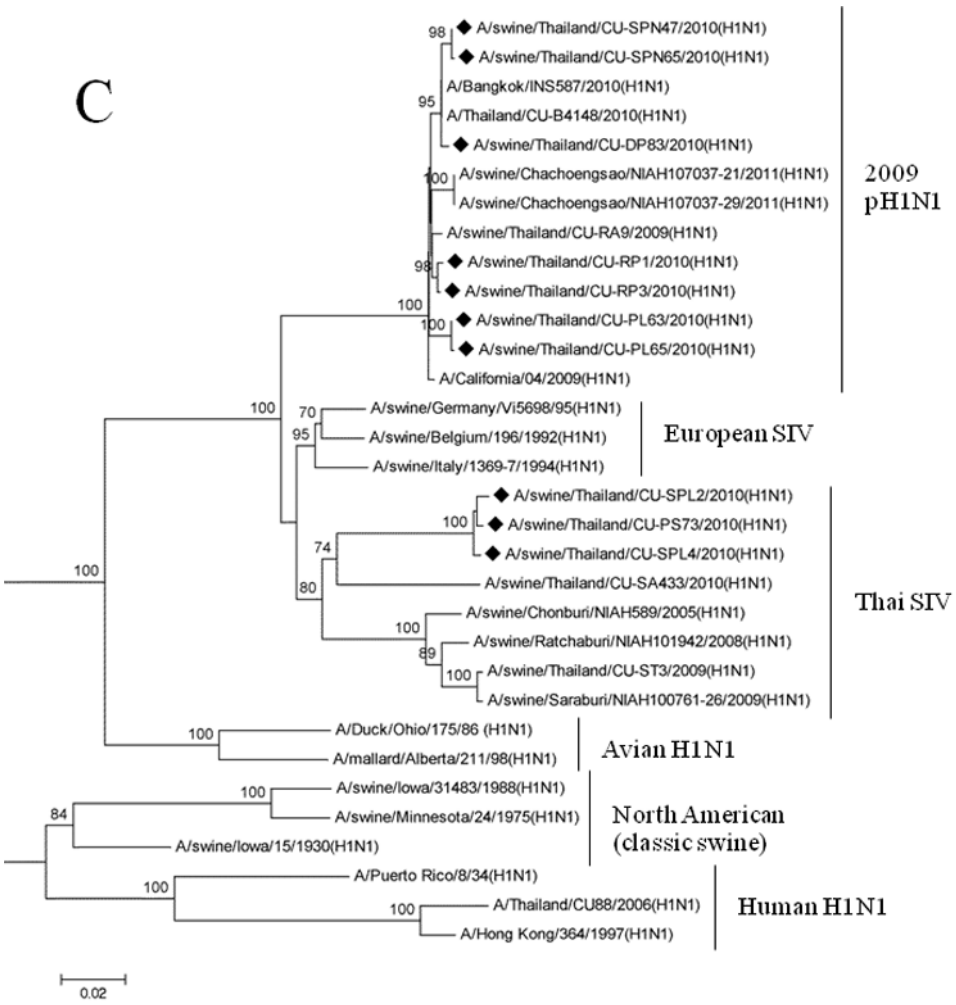
The phylogenetic tree of N1 NAs is shown in Figure 1c. NA gene of swine pH1N1 isolates was grouped with human pH1N1 virus. The NA genes of enH1N1 and

rH1N1 viruses were grouped with endemic SIV from 2005-2009. Therefore, NA gene of pH1N1, enH1N1 and rH1N1 viruses were grouped together in European avian-like swine lineage and distant from avian, human and North American classic swine lineages.

The phylogenetic tree of N2 NAs showed that NA gene of Thai rH3N2 viruses clustered in the same branch with endemic Thai swine H3N2 isolated during 2004-2005. However, the rH3N2 viruses were distinguished from human, North American swine lineage and the 2011 reassortant human viruses. Interestingly, NA gene of the rH1N2 isolates was grouped in the same branch with a human virus isolated in 1988 (A/Stockholm/12/1988) different from the other N2 isolates in this study (Figure 1d).




B
A/swine/Thailand/CU-CG45/2011(H3N2)
A/swine/Thailand/CU-CG48/2011(H3N2)
A/swine/Thailand/CU-CG55/2011(H3N2)
A/swine/Thailand/CU-P43/2012(H3N2)
A/swine/Thailand/CU-CG43/2011(H3N2)
A/swine/Thailand/CU-P53/2012(H3N2)
A/swine/Ratchaburi/NIAH9426/2005(H3N2)
A/swine/Thailand/KU5.1/2004(H3N2)
A/swine/Ratchaburi/NIAH59/2004(H3N2)
A/swine/Thailand/CU-BN54/2012(H3N2)
A/swine/Thailand/CU-BN53/2012(H3N2)
Thai SIV
A/Brisbane/8/1996(H3N2)
A/New York/584/1996(H3N2)
A/Hong Kong/1/1997(H3N2)
Human H3N2
A/swine/Iowa/H02AS8/2002(H3N2)
A/Indiana/08/2011(H3N2)
A/West Virginia/06/2011(H3N2)
A/swine/Iowa/A01432207/2012(H3N2)
A/swine/Iowa/63607-13/2008(H3N2)
North American SIV
A/swine/Hong Kong/411/02(H3N2)
A/Thailand/Siriraj-06/2002(H3N2)
A/Victoria/361/2011(H3N2)
A/Thailand/CU282/2007(H3N2)
A/Thailand/CU46/2006(H3N2)
Human H3N2
A/swine/Chonburi/NIAH106952-026/2011(H3N2)
A/swine/Chonburi/NIAH106952-028/2011(H3N2)
A/swine/Italy/9264/1998(H3N2)
A/swine/Spain/42386/2002(H3N2)
European SIV
A/turkey/England/69 (H3N2)
A/mallard duck/Minnesota/1979(H3N1)
Avian H3N2
100
93
100
98
79
100
100
100
81
100
100
100
86
100
72
100
100
100
100
0.02


C
A/swine/Thailand/CU-SPN47/2010(H1N1)
A/swine/Thailand/CU-SPN65/2010(H1N1)
A/Bangkok/INS587/2010(H1N1)
A/Thailand/CU-B4148/2010(H1N1)
A/swine/Thailand/CU-DP83/2010(H1N1)
A/swine/Chachoengsao/NIAH107037-21/2011(H1N1)
A/swine/Chachoengsao/NIAH107037-29/2011(H1N1)
A/swine/Thailand/CU-RA9/2009(H1N1)
A/swine/Thailand/CU-RP1/2010(H1N1)
A/swine/Thailand/CU-RP3/2010(H1N1)
A/swine/Thailand/CU-PL63/2010(H1N1)
A/swine/Thailand/CU-PL65/2010(H1N1)
A/California/04/2009(H1N1)
2009 pH1N1
A/swine/Germany/Vi5698/95(H1N1)
A/swine/Belgium/196/1992(H1N1)
A/swine/Italy/1369-7/1994(H1N1)
European SIV
A/swine/Thailand/CU-SPL2/2010(H1N1)
A/swine/Thailand/CU-PS73/2010(H1N1)
A/swine/Thailand/CU-SPL4/2010(H1N1)
A/swine/Thailand/CU-SA433/2010(H1N1)
A/swine/Chonburi/NIAH589/2005(H1N1)
A/swine/Ratchaburi/NIAH101942/2008(H1N1)
A/swine/Thailand/CU-ST3/2009(H1N1)
A/swine/Saraburi/NIAH100761-26/2009(H1N1)
Thai SIV
A/Duck/Ohio/175/86 (H1N1)
A/mallard/Alberta/211/98(H1N1)
Avian H1N1
A/swine/Iowa/31483/1988(H1N1)
A/swine/Minnesota/24/1975(H1N1)
A/swine/Iowa/15/1930(H1N1)
North American (classic swine)
A/Puerto Rico/8/34(H1N1)
A/Thailand/CU88/2006(H1N1)
A/Hong Kong/364/1997(H1N1)
Human H1N1
0.02

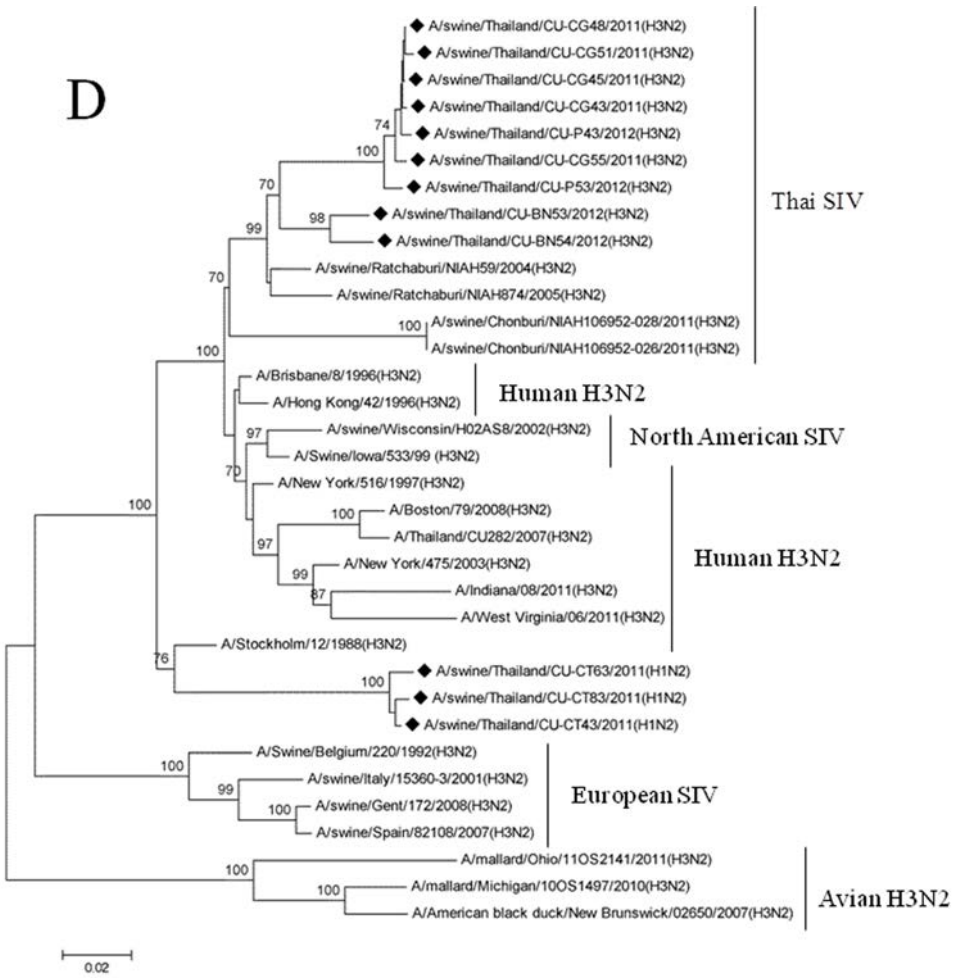


Figure 1 Phylogenetic tree of H1 HAs (a), H3 HAs (b), N1 NAs (c) and N2 NAs (d). Viruses isolated in this study were marked by filled diamonds. The trees were generated by using neighbor-joining method and bootstrapped with 1,000 replicates.

2.4.3 Molecular analysis of HA protein receptor-binding and antigenic site

The receptor binding site of H1 HAs was determined by amino acid position 204 and 239. The Aspartic acid (D) at both positions conferred binding to human influenza virus receptor (SA α 2,6), whereas, Glutamic acid (E) and Glycine (G) conferred binding to avian receptor (SA α 2,3). All swine isolates of pH1N1, rH1N1, 2 isolates of enH1N1 (A/swine/Thailand/CU-SPL2/2010 and A/swine/Thailand/CU-PS73/2010) and 2 isolates of rH1N2 (A/swine/Thailand/CU-CT43/2011 and A/swine/Thailand/CU-CT83/2011) posed Aspartic acid (D) in both positions (Table 3). One enH1N1 (A/swine/Thailand/CU-SPL4/2010) and one rH1N2 (A/swine/Thailand/CU-CT63/2011) had D at amino acid position 204 but had glycine (G) at position 239 (data not shown).

The H3 HAs receptor binding site was determined by amino acid position 242 and 244. The Glutamine (Q) and Glycine (G) at position 242 and 244 conferred binding to Sialic acid (SA) α 2,3 receptor, whereas, Leucine (L) and Serline (S) were compatible with SA α 2,6 receptor. All H3 isolates in this study had L at position 242 and S at position 244 (Table 4).

The H1 HAs posed 4 antigenic sites; Sa, Sb, Ca and Cb. The antigenic sites of swine pH1N1, rH1N1 and rH1N2 viruses were similar to the human pH1N1 virus (A/California/04/2009) but different from enH1N1 (A/swine/Thailand/CU-CB1/2006), European swine (A/swine/Belgium/1/1998) and human H1N1 lineage (A/Puerto Rico/8/1934) (Table 3). The cleavage site at amino acid position 339-350 was a typical character of H1 subtype and was found in all H1 isolates (Table 3). All H1 HAs isolates in this study had 8 potential glycosylation sites similar to North American classic swine lineage. On the other hand, the European avian-like lineage had 7 potential glycosylation sites (position 293-295 was not a glycosylation site) (data not shown).

The H3 HAs had 5 antigenic sites; A-E. The antigenic sites of rH3N2 isolates were similar to the Thai swine virus (A/swine/Ratchaburi/NIAH59/2004) and human H3N2 virus (A/Brisbane/8/1996) (Table 4). Human H3 HAs viruses had 10 potential glycosylation sites. However, Thai rH3N2 isolates had 8 potential glycosylation sites (amino acid position 149-151 and 160-162 were not glycosylation sites) (data not shown).

Table 3 Genetic analysis of antigenic sites, cleavage site and receptor binding sites of H1 HAs

	Antigenic sites												
Virus	Sa							Sb					
	142	170	172	177	179	180	201	206	207	208	210	211	212
A/California/04/2009	N	K	G	K	S	K	T	Q	S	I	Q	N	A
A/swine/Thailand/CU-DP83/2010	N	K	G	K	S	K	T	Q	S	L	Q	N	A
A/swine/Thailand/CU-PS73/2010	D	K	G	K	S	K	T	Q	C	L	Q	N	A
A/swine/Thailand/CU-CT83/2011	N	K	G	K	S	K	T	Q	S	L	Q	N	A
A/swine/Thailand/CU-CB1/2006	N	K	E	K	R	K	T	Q	S	L	Q	N	A
A/swine/Belgium/1/1998	N	K	G	K	S	K	T	Q	T	L	Q	N	A
A/duck/Hong Kong/717/1979	N	K	G	K	S	K	T	Q	T	L	Q	N	A
A/Puerto Rico/8/1934	N	E	E	K	K	N	N	Q	N	I	Q	N	E

	Antigenic sites							HA cleavage site	Recptor binding site	
Virus	Ca				Cb					
	156	158	187	239	89	90	91	339-350	204	239
A/California/04/2009	A	A	G	D	T	A	S	PSIQSRGLFGAI	D	D
A/swine/Thailand/CU-DP83/2010	A	A	G	D	T	A	S	PSIQSRGLFGAI	D	D
A/swine/Thailand/CU-PS73/2010	A	A	G	D	T	A	S	PSIQSRGLFGAI	D	D
A/swine/Thailand/CU-CT83/2011	A	A	K	D	K	A	N	PSIQSRGLFGAI	D	D
A/swine/Thailand/CU-CB1/2006	A	T	K	G	A	V	N	PSIQSRGLFGAI	D	G
A/swine/Belgium/1/1998	S	A	G	G	T	A	N	PSIQSRGLFGAI	D	G
A/duck/Hong Kong/717/1979	S	A	G	G	T	A	N	PSIQSRGLFGAI	E	G
A/Puerto Rico/8/1934	A	K	G	D	P	V	R	PSIQSRGLFGAI	D	D

Table 4 Genetic analysis of antigenic sites and receptor binding sites of H3 HAs

	Antigenic sites				
Virus	A		B	C	D
	156-162	172-177	205-215	293-298	221-237
A/swine/Ratchaburi/NIAH59/2004	KRGSVKS	KLDYKY	NDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGSRP
A/swine/Thailand/CU-CG48/2011	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP
A/swine/Thailand/CU-BN54/2012	KRGSVKS	KLDYKY	NDQTNLYVQAS	CNSGCI	STKRSQQTVIPNIGYRP
A/Victoria/361/2011	IRRSNNS	QLNFKY	KDQIFLYAQSS	CNSECI	STKRSQQAVIPNIGYRP
A/Brisbane/8/1996	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGSRP
A/swine/Iowa/HOA2S8/2002	KRGSVNS	KLDYKY	SDQTNLYVHAS	CNSECI	STKRSQQTVIPNIGSRP
A/swine/Spain/42386/2002	KRGPNNS	KSGNTY	REQTNLYIQAS	CNSECI	STKRSQQTIIPNIGSRP
A/Turkey/England/1969	KRGPDNG	KSGSSY	QEQTNLYVQAS	CISECI	STKRSQQTAIPNIGSRP

	Antigenic sites		Recptor binding site	
Virus	E			
	187-191	259-265	242	244
A/swine/Ratchaburi/NIAH59/2004	NDKFD	IRSGKSSI	L	S
A/swine/Thailand/CU-CG48/2011	NDKFD	IRSGKSSI	L	S
A/swine/Thailand/CU-BN54/2012	NDKFD	IQSGKSSI	L	S
A/Victoria/361/2011	NEQFD	IRSGKSSI	L	S
A/Brisbane/8/1996	NDKFD	IRSGKSSI	L	S
A/swine/Iowa/HOA2S8/2002	NDKFD	IRSGKSSI	L	S
A/swine/Spain/42386/2002	SDDFD	VQTGKSSV	L	S
A/Turkey/England/1969	NDNFD	MRTGKSSI	Q	G

2.4.4 Molecular analysis of drug resistance on NA and M proteins

Oseltamivir-resistance is correlated with NA gene amino acid mutation E120V, H275Y, R293K and N295S of N1 NAs and E119V, H274Y, R292K and N294S of N2 NAs. Unfortunately, all N1 NAs and N2 NAs isolates in this study contained amino acid substitution at these 4 positions considered as Oseltamivir-resistance.

Amantadine-resistance is involved with amino acid mutation L26F, V27A, A30T, S31N and G34E of M gene. All isolates in this study contained amino acid substitution S31N but no amino acid mutation at 26F, 27A, 30T and 34E.

2.4.5 Molecular analysis of virulence factor on PB2, PB1, and NS proteins

The virulence of influenza A virus may associate with mutations of amino acid position K627E and N701D of PB2, S66N of PB1-F2, and E92D of NS1 proteins. PB2 protein of swine pH1N1, rH1N1, rH1N2 and rH3N2 isolates in this study contained Glutamic acid (E) and Aspartic acid (D) at amino acid position 627 and 701, respectively. In contrast, enH1N1 isolates had E and Aspargine (N) at 627 and 701 position of PB2 protein. All pH1N1 and the reassortant isolates had stop codon inside the PB1-F2 protein. The enH1N1 isolates had no amino acid substitution at amino acid position 66 of PB1-F2 protein. All isolates in this study showed no amino acid mutation at position 92 of NS1 protein.

2.5 Discussion

The surveillance data indicated that pH1N1 viruses were mainly found among Thai pigs during year 2010 after the introduction in 2009. Most viruses were recovered from medium to large swine farms with no animal imported from outsides indicating that pH1N1 was successfully transmited from humans to pigs and becoming dominant and later becoming endemic in late 2010 and early 2011 in Thai swine population. These findings correlated well with the human influenza virus surveillance results in Thailand (The global influenza surveillance and response system, 2012) showing that pH1N1 was the most prevalent virus found in 2010 and H3N2 virus replaced and became dominated in late 2011.

Interestingly, all isolates in this study were obtained from the nursery pigs indicating that those isolated viruses became endemic in the swine population and nursery pigs are susceptible to SIV-infection due to the declining of maternal-derived antibody. It should be noted that Thai endemic SIVs (endemic H1N1, H1N2 and H3N2) were occasionally found in mid 2010 (the first 6 month of the surveillance) and never been isolated again and later pH1N1virus appeared to be the most dominated virus replacing Thai enSIVs. However, in 2011-2012, the rH3N2 viruses became the major virus population in Thai pigs, whereas, the rH1N2 viruses were found only in one swine farm at the end of 2011. Interestingly, the rH1N2 NA gene was grouped in the same branch with a human virus isolated in 1988 (A/Stockholm/12/1988) different from the other endemic H3N2 or rH3N2 in Thailand implying that the rH3N2 virus might obtain the N2 gene from human virus. Again, swine workers and veterinarians when sick with flu should not contact with pigs in order to reduce the chance of introducing new virus genes into the pig population.

Therefore, the novel reassortment H3N2 virus was isolated from humans in the United State since June 2011 (Lindstrom et al., 2012). The virus PB2, PB1, PA, HA, NP, NA and NS genes were closely related to the TRIG SIV H3N2 virus but M gene was closely related to pH1N1 virus. Thus, the novel H3N2 virus found in the US did not related to the rH3N2 isolates in this study.

Since 2009, variation of reassortant viruses were frequently found in Thai pig population after the pH1N1emergence in Thai pig population. The pH1N1 virus contains TRIG cassette and influents the virus genetic shift or reassortant as previously demonstrated in North America (Vincent at al., 2008; Vincent at al., 2009). The TRIG cassette virus was introduced into the North American swine population in 1988 inducing variation of genetic reassortant of SIVs (Vincent at al., 2008). From 1998-2008,

at least 7-8 types of influenza A viruses were isolated from the North American swine population. It should be noted that during 1930-1997 only one classic SIV was isolated and was genetically stable for almost 60 years (Vincent at al., 2008). Similarly, Thai SIV genetic charateristics had been stable before the introduction of the pH1N1 or TRIG cassette virus in 2009. The rH3N2 isolates found in this study demonstrated similar situation mentioned above also happening in Thailand. The rH3N2 viruses were isolated from 2 swine farms located 822 kilometers apart and those farms have not imported pigs from outsources. This information suggests that the TRIG cassette viruses always acquire new envelop proteins for escaping host immune response (Vincent at al., 2008).

In this study, all Thai reassortant viruses containing the TRIG cassette were the results of rapid antigenic drift and shift. These HA and NA genes were derived from genetic compositions of endemic SIVs from Thai pigs (excepted for rH1N2 isolates) (Komadina et al., 2007; Chutinimitkul et al., 2008; Takemae et al., 2008). This present information pointed to the fact that pH1N1, enH1N1 and Thai endemic H3N2 viruses were co-circulating within the Thai pig population. Since pH1N1 virus was the most dominated viruses during late 2010 - early 2011, numbers of pH1N1 reassortant viruses were later isolated in 2011-2012.

Most of the swine viruses isolated in this study posed receptor binding site compatible with human receptor (SA2,6) (Skehel and Wiley, 2000; Suzuki, 2005, Neumann et al., 2009; Imai and Kawaoka, 2012). In addition, pH1N1virus contains TRIG cassette making the virus become highly infective among those species. Cross-species transmission between pigs and humans could not be avoided particularly in the swine-exposed population. Veterinarians and swine workers have high exposure risk to the swine influenza virus (Kitikoon et al., 2011b). A good example of interspecies transmission was the novel reassortant influenza H3N2 virus in the United State in 2011.

The virus was isolated from 12 people but only 6 persons had history of contacting with swine suggesting probably person to person transmission (Lindstrom et al., 2012). This possibility could occur in Thailand since people do not pay much attention to flu or flu-like symptoms when working closely to swine. This ignorant may facilitate the novel reassortant virus to spread into human communities or vice versa.

Amino acid substitution of NA of all Thai swine isolates in this study showed Oseltamivir resistance. However, amino acid substitution (S31N) of M genes was also able to cause Amantadine resistance (Das et al., 2010). However, pH1N1 and its reassortant viruses were possibly of low virulence according to no amino acid substitution at position 627, 701of PB2, 92 of NS1 protein and non-translation of PB1-F2 protein (Arias et al., 2009; Neumann et al., 2009). Additionally, pathogenesis of pH1N1 and its reassortant virus found in Thai pigs was conducted demonstrating only mild clinical signs in experimentally-infected nursery pigs. The enH1N1s might cause more severe clinical signs because of having amino acid substitution N701D of PB2 protein as shown in the previous pathogenesis study of local Thai SIV (Sreta et al., 2009).

In summary, pH1N1 virus and its TRIG cassette reassotant viruses have recently established in the Thai swine population (Hiromoto et al., 2012). The TRIG cassette viruses rapidly drive antigenic drift and shift causing various reassortant viruses of pH1N1 origin. Two major concerns include having increased numbers of novel genetic reassortant viruses and public health consequences when having highly pathogenic reassortant viruses. Definitely, genetic characteristic of future Thai SIV will be more variable. Genetic variations of the future Thai SIVs may affect the swine herd health immunity differently from the endemic viruses and may cause economic loss in the Thai swine industry more or less. Public health awareness should be in focus since evidence of interspecies transmission among human and pig populations are occasionally

reported. The TRIG cassette viruses were likely to acquire new HA and NA genes, resulting in novel reassortment viruses and probably successfully replicate in humans. In addition, during 2003-2004, avian influenza H5N1 virus emerged in Thailand and caused 12 human deaths with limited human to human transmission (Keawcharoen et al., 2005; WHO, 2010b). Although no reassortant evidence between the TRIG cassette virus and the avian H5N1 virus was previously found, this particular novel virus could be reassorted from commingling environment among domestic species commonly seen in the backyard farming system. The combination of the high infectivity TRIG cassette and high virulent avian H5N1 viruses might possibly cause dangerous pandemic threat among human population in the future. Thus, continuing influenza surveillance is necessary, particularly in swine.

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CHAPTER III

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Title: Experimental infection with a Thai reassortant swine influenza virus of pandemic H1N1 origin induced disease

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Experimental infection with a Thai reassortant swine influenza virus of pandemic H1N1 origin induced disease

3.1 Abstract

Following the emergence of the pandemic H1N1 influenza A virus in 2009 in humans, this novel virus spread into the swine population. Reassortant viruses eventually emerged from the 2009 pandemic and were reported in swine populations worldwide including Thailand. As a result of the discovery of this emergent disease, pathogenesis studies of this novel virus were conducted in order that future disease protection and control measures in swine and human populations could be enacted. The pandemic H1N1 2009 virus (pH1N1) and its reassortant virus (rH1N1) isolated from pigs in Thailand were inoculated into 2 separate cohorts of 9, 3-week-old pigs. Clinical signs, viral shedding and pathological lesions were investigated and compared. Later, pigs were necropsied at 2, 4, and 12 days post inoculation (DPI). The present study demonstrated that both the pH1N1 and rH1N1 influenza viruses induced acute respiratory disease in experimentally inoculated nursery pigs. Although animals in the rH1N1-infected cohort demonstrated more severe clinical signs, had higher numbers of pigs shedding the virus, were noted to have increased histopathological severity of lung lesions and increased viral antigen in lung tissue. Interestingly, viral genetic material of both viruses could be detected from the nasal swabs until the end of the experiment. Similar to other swine influenza viruses, the clinical signs and pathological lesions in both rH1N1 and pH1N1 were limited to the respiratory tract.

Keywords: Influenza, Pandemic H1N1 2009, Pathogenesis, Reassortant, Swine, Thailand

3.2 Introduction

Influenza A viruses are highly contagious respiratory pathogens capable of transmission between various avian and mammalian species including swine and humans. Two specific receptors: sialic acid (SA) α2,3 commonly found in the epithelial cells of gastrointestinal tract of wild aquatic birds and SA α2,6 found in the epithelial cells of the respiratory tract of humans, are recognized. Pigs are known as a "mixing vessel" as they express receptors which can bind both avian and human influenza viruses within the respiratory tract. As a result, interspecies transmission from pigs to humans or vice versa is possible. A study of pig-to-human influenza virus transmission on Thai swine farms proved that swine-exposed workers had antibodies against the circulating swine influenza viruses (SIV) (Kitikoon et al., 2011b). Cross-species transmission becomes an important factor in monitoring for future human influenza outbreaks. Pandemic H1N1 (pH1N1) virus emerged in April 2009 and rapidly spread among human populations globally. The pH1N1 virus was also called Swine-origin 2009 A (H1N1) due to all of its gene segments closely related to SIV. The pH1N1 virus is a reassortant virus of the European avian-like swine virus (M and NA genes), the classic swine H1N1 virus (HA gene) and the North American triple reassortant H3N2 virus (PB2, PB1, PA, NP and NS genes (Garten et al., 2009). Following the epidemic outbreaks within the human population, the virus was also isolated from pigs in Canada, Norway, Italy, Hong Kong, South Korea and Thailand (Hofshagen et al., 2009; Howden et al., 2009; Song et al., 2009; Moreno et al., 2010; Sreta et al., 2010; Vijaykrishna et al., 2010). It should be noted that the North American triple reassortant internal gene (TRIG) virus might influence antigenic drift and shift in mammalian species (Vincent et al., 2009). As a result, the reassortant variants of pH1N1 containing TRIG cassette were occasionally found in swine and other animals including turkeys (Vijaykrishna et al., 2010; Berhane et

al., 2012; Ducatez et al., 2011; Kitikoon et al., 2011a; Moreno et al., 2011; Starick et al., 2011; Zhu et al., 2011). The recent Thai reassortant pH1N1 (rH1N1) virus has 7 genes derived from the pH1N1 virus and has only the Neuraminidase (NA) gene from an endemic Thai swine H1N1 virus (Ducatez et al., 2011). Thus, amino acid sequences of Hemagglutinin (HA) gene of the pH1N1 and rH1N1 are 98.4% identical and most antigenic sites are quite similar.

Previous pathological studies comparing the pH1N1human isolate and a seasonal human H1N1 influenza virus in pigs found that those pigs showed none of the clinical signs associated with SIV (Itoh et al., 2009). Microscopic lesions revealed only mild bronchitis and bronchiolitis with peribronchiolar lymphocytic cuffing and a mild interstitial pneumonia (Vincent et al., 2010). The pathology of the virus having undergone reassortant in pigs demonstrated in the present experiment may reflect severity of disease not only in pigs but also in humans. In addition, individuals working closely with infected swine may facilitate a human-animal interface, thereby promoting viral transmission between humans and pigs (Kitikoon et al., 2011b). Interestingly, the genetics of SIV circulating in North America in 1997-1998 were not considered to be stable when the triple reassortant H3N2 virus was introduced resulting insignificant febrile disease, severe influenza-like illness, mortality in piglets and abortion in sows. As a result, surveillance and pathogenesis studies are considered to be essential due to this highly evolved genetic variation of SIV in North America (Zhou et al., 2000; Olsen et al., 2002).

In the present experiment, a pathogenesis study of pH1N1 and its reassortant pH1N1 (rH1N1) following experimental infection of three week old piglets has demonstrated that acute respiratory disease in nursery pigs is induced by both viruses. Pigs in the rH1N1-infected group showed prominent clinical signs, with higher numbers

of animals shedding the virus, increased severity of pulmonary lesions and evidence of viral antigen in lung tissue. The information gained from the present study confirmed the increased virulence of the reassortant influenza virus in comparison with the pandemic virus.

3.3 Materials and methods

3.3.1 Viruses

A/swine/Thailand/CU-RA29/2009(H1N1) (Sreta et al., 2010), a pandemic H1N1 of pig origin (pH1N1) and A/swine/Thailand/CU-SA43/2010 (H1N1) (Kitikoon et al., 2011a), a novel reassortant virus of pig origin (rH1N1) were individually propagated 3 times in 9-day-old embryonated chicken eggs. Allantoic fluids were collected after 72 hours incubation. The virus concentrations were calculated using 50% tissue culture infectious dose (TCID50) in Madin-Darby canine kidney (MDCK) cell using Reed and Muench method. Concentrations of both viruses were adjusted to 104 TCID50/ml and kept in the -80ºC until used.

3.3.2 Experimental pigs

Twenty one, 3-week-old pigs from a local SIV, porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV)-free herd (kindly provided by the Charoen Pokphand Food public company limited, Thailand) were divided into 3 groups. Group 1 and 2 containing 9 pigs each were intratracheally inoculated with 5 ml containing 104 TCID50/ml of pH1N1 and rH1N1, respectively. A negative control group containing 3 pigs received mock cell culture media intratracheally. Clinical signs such as fever, coughing, sneezing, nasal discharge and conjunctivitis were blindly recorded daily by the same veterinarian for a week and at 10 and 12 days post infection (DPI). All pigs tested serologically negative for PRRSV and

PCV2 using commercial ELISA kits (IDEXX laboratories, USA and Synbiotics, USA, respectively). All animals were housed in the animal facility biosafety level 2 with appropriated food and clean water providing adequately throughout the experiment. The animal usage and procedures were approved by Chulalongkorn University-Faculty of Veterinary Science animal care and use committee (protocol No. 11310052).

3.3.3 Viral detection

Nasal swab were collected at 1-7, 10 and 12 DPI. Total RNA was extracted from nasal swabs, sera, fresh bronchial lymph node and lung tissue collected at necropsy by using a commercial kit (NucleoSpin Extract Viral RNA Kit, Macherey-Nagel, Germany). A modified real time reverse transcriptase polymerase chain reaction (real time RT-PCR) was performed using Superscript III platinum one-step quantitative RT-PCR system (Invitrogen, USA). Primers specific to Matrix (M) gene containingforward primer (MF3; 5' TGATCTTCTTGAAAATTTGCAG 3'), reward primer (MR1+; 5' CCGTAGMAGGCCCTCTTTTCA 3') and M-probe (FAM-TTGTGGATTCTTGATCG-MGB) were used in this study. The cycling conditions started at 48˚C for 45 min, 95˚C for 10 min and followed by 40 cycles of denaturation (94˚C for 15 s), annealing (55˚C for 30 s) and extension (72˚C for 40 s) (Payungporn et al., 2006).

Nasal swabs, lung and bronchial lymph node homogenate samples were filtrated and inoculated onto MDCK cells using ten-fold serial dilutions. The inoculated cell cultures were incubated for 72 hours. Virus was identified using anti-influenza A nucleoprotein monoclonal antibody as a primary antibody and rabbit anti-mouse IgG conjugated horseradish peroxidase as a secondary antibody (DakoCytomation, Carpinteria, California). Then, color was developed using a chromogen aminoethylcarbazole substrate (Sigma, St. Louis, Missouri) (Sreta et al., 2009).

3.3.4 Pathological examination

Three pigs from each viral inoculated group and 1 pig from the negative control group were randomly selected for euthanasia and necropsied at 2, 4 and 12 DPI. At necropsy, percentages of gross lung lesion scores characterized by multifocal mottled tan and consolidation in consistency were recorded and scored as previously described [20]. Lung, bronchial lymph nodes, ileum, tonsil, liver, kidney and spleen were collected from each animal at necropsy, immersed and fixed in 10% buffered formalin for subsequent histopathological analysis.

Formalin-fixed tissues were embedded in paraffin and processed routinely. Sections were cut approximately 4-6 μm thick for histopathological and immunohistochemistry (IHC) staining for Influenza A virus antigen detection. The IHC staining was performed using a Polymer-Based method (Envision system). Primary antibody using anti-influenza A (H5N1) nucleoprotein monoclonal mouse antibody (EVS238, B.V.EUROPEAN VETERINARY LABORATORY, the Netherlands) and secondary antibody using Biotinylated rabbit anti-mouse IgG antibody and envision polymer (Envision Polymer DAKO®, Denmark) were concurrently performed with a negative control slide. The sections were developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin. A positive control slide was also included using the SIV-infected lung section from our previous experiment (Sreta et al., 2010).

3.3.5 Hemagglutinination inhibition (HI) assay

Sera were collected from all pigs before starting the experiment and at each necropsy. All sera were pretreated with 20% kaolin and receptor destroying enzyme (Denka Seiken Co. Ltd., Japan). The antibody detection was performed used standard

HI assay [1]. Virus antigens used in this experiment were representatives of Thai endemic swine viruses; A/swine/Thailand/CU-CB1/2006(H1N1) and A/swine/Thailand/CU-CB8.4/2007 (H3N2) and pH1N1 virus (A/swine/Thailand/CU-RA29/2009(H1N1)). Samples with HI titers ≥ 40 were considered as previously exposed to the specific tested antigen.

3.4 Results

3.4.1 Clinical examination

Clinical signs were noted daily at 1-7, 10 and 12 days post infection (DPI) in both cohorts. One pig from the pH1N1-infected group (group 1) was found dead due to stress following restraint and findings associated with this animal were excluded from our evaluation. The pH1N1-infected pigs developed sneezing (3 of 8) and had ocular discharge (1 of 8) beginning at 1-2 DPI, and subsequently showed mild (2 of 8) to moderate (3 of 8) serous nasal discharge and conjunctivitis (5 of 8) at 2 DPI. In contrast, the rH1N1-infected pigs showed increased severity of clinical signs, with moderate to severe serous nasal discharge (8 of 9), sneezing (5 of 9) and conjunctivitis (9 of 9) at 1-2 DPI with resolution of the former two clinical signs and amelioration of the discharge at 3-4 DPI in 5 of 6 animals. Only mild serous nasal discharge was observed in two pigs in both cohorts at the end of the experiment (12 DPI). Pigs in the control group had no signs of disease throughout the course of the experiment.

3.4.2 Viral shedding

Viral shedding was measured from nasal swabs using a modified real time RT-PCR and viral isolation in MDCK cells (Table 1). One pH1N1-infected pig (1 of 8) demonstrated evidence of viral shedding as early as 1 DPI. One of the six remaining pigs in the same group was tested positive at 2 DPI with very low levels of viral copies

(data not shown). Subsequently, one of the two remaining pigs was tested positive at 5 DPI by both real time RT-PCR and viral isolation. By day 7 post infection, all of the pH1N1-infected pigs shed the virus with high levels of viral copies at 7, 10 and 12 DPI from collected nasal swabs (data not shown). In the rH1N1-infected cohort, one pig was tested positive as early as 1 DPI followed by five of nine animals being positive at 2 DPI, while all six remaining pigs were tested positive at 3 DPI by both real time RT-PCR and viral isolation tests. Similar to the pH1N1 group, viral shedding in nasal swabs was detected again at 10 DPI (2 of 3 pigs) and was detected in all the remaining pigs (3 of 3) at 12 DPI by the real time RT-PCR. None of the nasal swabs from the control group yielded positive results from both tests.

Subsequent to staggered endpoints within the study, euthanasia and necropsy, viral detection in bronchial lymph node and lung tissues was performed (Table 2). In the pH1N1-infected group, virus was detected in 1 lung sample and 2 bronchial lymph nodes when necropsied at 2 DPI by a real time RT-PCR but virus isolation yielded negative results. In contrast, the virus was detected in all rH1N1-infected lungs at 2 DPI and one lung sample (1 of 3) at 4 and 12 DPI by both real time RT-PCR and virus isolation tests. The virus genetic material was also detected in bronchial lymph node of two rH1N1-infected pigs (2 of 3) at 2 and 4 DPI by the real time RT-PCR. The viral genetic material could not be detected in the sera of all pigs analyzed. All control pigs were negative for influenza virus by both tests throughout the experiment.

Table 1 Viral shedding measured from nasal swabs detected by a real time RT-PCR and viral isolation

Animal ID	Virus detection																			
	0 DPI		1 DPI		2 DPI		3 DPI		4 DPI		5 DPI		6 DPI		7 DPI		10 DPI		12 DPI	
pH1N1-infected group	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI	rt	VI
1	-	-	-	-	-	-	N													
2	-	-	-	-	-	-	N													
3	-	-	-	-	-	-	-	-	-	-	N									
4	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	-	+	-
5	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-
6	-	-	-	-	-	-	-	-	-	-	N									
7	-	-	-	-	+	-	-	-	-	-	N									
8	-	-	-	-	-	-	N													
rH1N1-infected group																				
1	-	-	-	-	+	-	+	+	-	-	N									
2	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
3	-	-	-	-	+	-	+	+	-	-	-	+	-	-	-	-	+	-	+	-
4	-	-	-	-	-	-	+	-	-	-	N									
5	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+	-
6	-	-	-	-	-	-	N													
7	-	-	-	-	+	-	N													
8	-	-	+	-	+	-	N													
9	-	-	-	-	+	-	+	+	-	-	N									
Negative control group																				
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	N									
3	-	-	-	-	-	-	N													

DPI = Day post infection.

rt = A real-time RT-PCR (+ = Ct values < 40; - = Ct values ≥ 40).

VI = Viral isolation using MDCK cell line

N = Necropsy

3.4.3 Pathological examination

Typical SIV macroscopic lung lesions are characterized by multifocal, dark, plum-colored lungs suggestive of consolidation. In our experimental animals, pH1N1-infected pigs at 2 DPI (1 of 2), 4 DPI (2 of 3) and in all rH1N1-infected pigs at 2 (3 of 3) and 4 (3 of 3) DPI had similar lesions concentrated within the cranioventral regions of multiple lobes (Figure 1A). The lung lesions mentioned above were not related to Mycoplasma hyopneumoniae (M. hyo)-induced lesion since the PCR tested for M. hyo detection yielded negative results (data not shown). The percentages of gross lung lesions at 2 DPI demonstrated that the rH1N1-infected pigs had greater lung scores than those of the pH1N1-infected pig (Table 2). Other non-specific gross lesions in both infected groups included mild bronchial lymph node enlargement with multifocal subcapsular hemorrhage.

Microscopic pulmonary lesions were noted as a mild to moderate broncho-interstitial pneumonia in all infected pigs of both groups particularly at 2 and 4 DPI. However, immunohistochemistry (IHC) staining only demonstrated the SIV nucleoprotein in the nuclei of bronchial epithelial cells of all rH1N1-infected pigs at 2 and 4 DPI (Figure 1B and 1C) while none of the pH1N1-infected pigs were found to be immunoreactive. It should be noted that the lesions were limited only in the lungs of the infected pigs and not to any of the other examined tissues. No significant histopathological findings or IHC immunoreactivity were found in any of the control animals.

3.4.4 Hemagglutinination inhibition (HI) assay

All sera from pre-experiment and terminal blood draws showed negative results against local Thai SIV viruses, A/swine/Thailand/CU-CB1/2006(H1N1) and A/swine/Thailand/CU-CB8.4/2007 (H3N2) viruses. The HI titers of pre-experiment and terminal sera of all experimental pigs against A/swine/Thailand/CU-RA29/2009 (H1N1) or pH1N1were not significantly elevated from the base line.

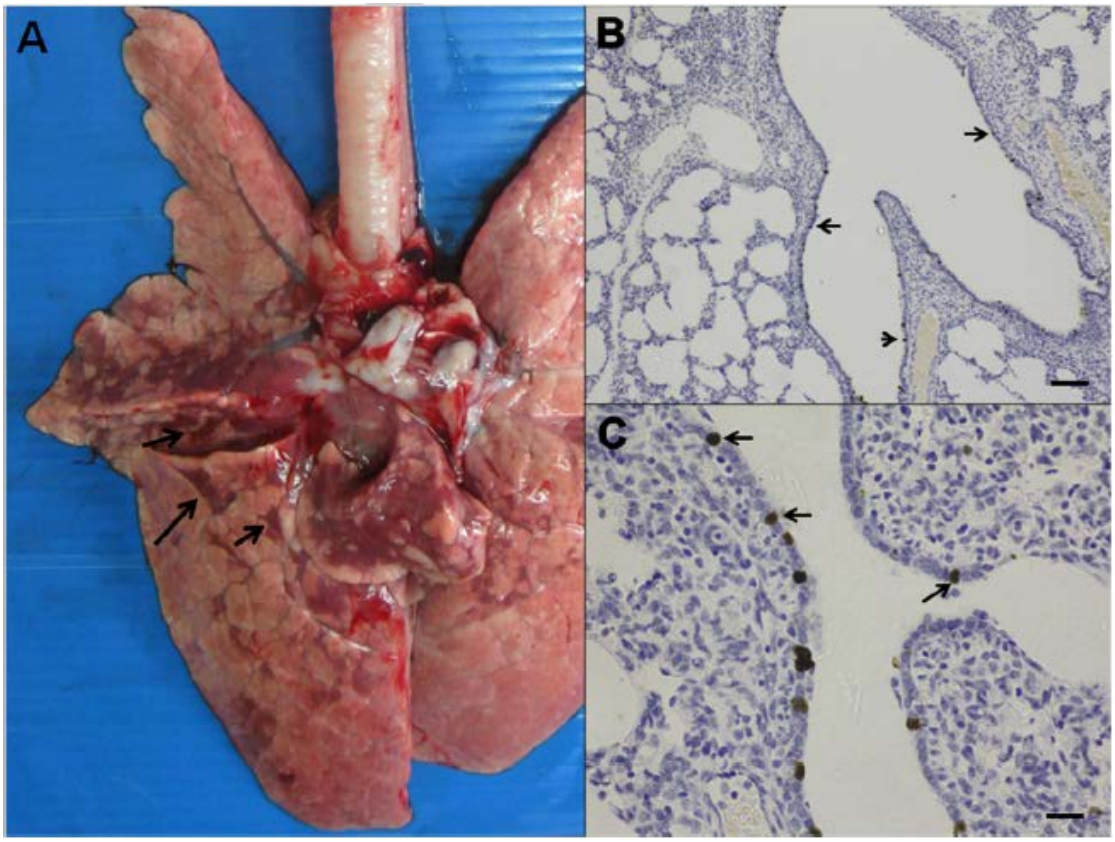

Figure 1 Gross lung lesion scoring (18%) demonstrated dark plum-color, multifocal to coalescing consolidation or “checker board lung pattern” (arrows) of the rH1N1-infected pig at 2 DPI (A). Histologically, dark brown staining of the influenza nucleoprotein demonstrating by IHC were observed in the nuclei of the infected bronchial epithelial cells (arrow) (B; bar = 200 μm, C; bar = 20 μm) from the same rH1N1-infected pig at 2 DPI.

Table 2 Percentages of gross lung lesions and virus detection in lungs and bronchial lymph nodes

Necropsy day	Animal ID	Lung lesion (%)	Viral detection in lung		Viral detection in bronchial lymph node	
	pH1N1-infected group		**rt**	**VI**	**rt**	**VI**
2 DPI	1	0	-	-	-	-
	2	8	-	-	+	-
	9	10	-	-	+	-
4 DPI	3	5	-	-	-	-
	7	0	-	-	-	-
	8	5	-	-	-	-
12 DPI	4	0	-	-	-	-
	5	0	-	-	-	-
	rH1N1-infected group					
2 DPI	6	18	+	+	-	-
	7	13	+	+	+	-
	8	13	+	+	+	+
4 DPI	1	9	+	+	+	-
	4	0	-	-	-	-
	9	0	-	-	+	-
12 DPI	2	0	+	-	-	-
	3	0	-	-	-	-
	5	0	-	-	-	-
	Negative control group					
2 DPI	3	0	-	-	-	-
4 DPI	2	0	-	-	-	-
12 DPI	1	0	-	-	-	-

DPI = Day post inoculation.

rt = a real time RT-PCR (+ = Ct values < 40; - = Ct values ≥ 40)

VI = Viral isolation using MDCK cell line

3.5 Discussion

In this study, pigs in both pH1N1 and rH1N1infected cohorts showed typical SIV clinical signs such as sneezing and coughing from 1-4 DPI (Vincent et al., 2010; Sreta et al., 2009). As expected, clinical signs of pigs inoculated with the pH1N1 virus and rH1N1 virus were unable to be distinguished from one another. It should be noted that viral isolation of nasal swabs from both infected groups demonstrated influenza A virus positivity until 6 DPI and only the real time RT-PCR showed positive results on 7-12 DPI suggesting that the duration of infectivity extended to 6 DPI. The modified RT-PCR used in this study appeared more sensitive than viral isolation. However, the infectivity from 7-12 DPI was inconclusive. The viral RNA could be detected as early as 1 DPI in both infected groups. But viral isolation results were only tested positive on 3-6 DPI in the rH1N1-infected group and 5-6 DPI in the pH1N1-infected group. Interestingly, the viral RNA was detected at 7-12 DPI in the pH1N1-infected pigs with mild concurrent clinical signs and histopathological lung lesions. Similar to the pH1N1-infected pigs, the rH1N1-infected pigs also showed prolong period of viral detection from the nasal swabs but in this cohort, the animals also had higher macroscopic lung lesions and the presence of virus antigen was noted in all sampled lung tissue.

Previous study on the pathogenesis of a Thai endemic SIV (H1N1) showed viral shedding between 2-4 DPI and a Thai endemic H3N2 had the shedding period only at 2 DPI (Sreta et al., 2009). Similarly, a study of human isolate pH1N1 in pigs demonstrated viral shedding as early as 1 DPI which persisted until the end of the experiment at 5 DPI (Vincent et al., 2010). In the present study, pigs in both infected groups showed detectable live viral shedding from 3-6 DPI based on viral isolation but the viral RNA was only sporadically detected through 12 DPI. The long shedding period may allow viral transmission among pigs as well as interspecies transmission particularly to the humans

working in close proximity with infected pigs. In contrast to the rH1N1-infected pigs, there was no SIV antigen detected in the lung of the pH1N1-infected pigs. The sporadic viral detection in the lungs of the pH1N1-infected pigs possibly resulted from limited viral replication and fast viral antigen disappearing. Similar to the previous Thai endemic SIV-infected pigs, the studied viral RNA was detected in the respiratory tract of both infected groups and was not found in any other organ system (Sreta et al., 2009). In contrast to the study of pH1N1 (human origin) in pigs, viral RNA was also detected in tonsil, and serum (Vincent et al., 2010).

Interestingly, the rH1N1-infected pigs demonstrated greater severity in term of clinical signs, pathological lesions and the overall number of pigs shedding the virus. As such, the reassortant virus theoretically could better infect pigs in comparison to the pH1N1. The only difference between the two studied viruses is the NA gene responsible for releasing the progeny viral particles from the infected cells (Cheung and poon et al., 2007). Since rH1N1 obtained the NA gene from the local Thai SIV (97.2% amino acid sequence identity), the virus might be more compatible in Thai pigs when compared with the pH1N1 (99.6% amino acid sequence identity to human pH1N1 but 95% identity to other SIV isolates) (Chutinimitkul et al., 2008). However, the role of NA gene in SIV pathogenesis has not been fully elucidated and would require further investigation.

Importantly, the pH1N1 contains the triple reassortant internal gene (TRIG) cassette composed of swine, avian and human origin genes. It has been speculated that the TRIG cassette may be able to accommodate multiple HA and NA genes providing advantages to the viral infectivity, replication and possibly mutation. As a result, the TRIG cassette might be the cause of the reported increasing genetic variation rate of SIV in the US occurring since 1998 (Vincent et al., 2008; Vincent et al., 2009; Ma et al., 2010). Since the TRIG cassette was recently introduced into the Thai pig

population by the pH1N1 virus, the emergence of the Thai reassortant virus (rH1N1) in pigs has been described (Kitikoon et al., 2011a). In addition, evidence of interspecies transmission among human and pig populations are occasionally reported (Kitikoon et al., 2011b; Howden et al., 2009; Komadina et al., 2007). Any novel rH1N1 influenza virus may be able to transmit back to the human population without being noticed and possibly causing another pandemic outbreak. As such, surveillance of influenza virus infections in both pigs and humans is critical for early recognition and prevention of a potential epidemic or pandemic outbreak.

In summary, clinical manifestations and pathological lesions of both pH1N1 and rH1N1-infected pigs in this study were most evident during the early stages of infection (1-4 DPI), consistent with studies of the pathogenesis of other SIV infections. The rH1N1-infected pigs demonstrated prominent clinical signs and pathological lesions typical of SIV infection and nasal swab tests noted that the reassortant virus had higher numbers of pigs shedding the infective virus based on the viral isolation. While result is not statistically significant, the trend observed suggests both cohorts demonstrated some animals shedding virus through the end of the study at12 DPI. Similar to other SIV studies, the studied viruses replicated well in the lung tissues and the viral antigen was only detected within the respiratory tract.

3.6 Acknowledgements

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CHAPTER IV

Original article: The Thai Journal of Veterinary Medicine (Accepted)

Title: Probable pig to duck transmission of the pandemic H1N1 2009 (pH1N1) and its reassortant in commingling experimental condition

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Probable pig to duck transmission of the pandemic H1N1 2009 (pH1N1) and its reassortant in commingling experimental condition

4.1 Abstract

The pandemic H1N1 2009 (pH1N1) virus considered as a low pathogenic influenza virus, however, rapidly spread among humans and finally found in the swine population of 6 continents. Interspecies transmissions among different animal species are of interest. In this study, sentinel ducks were commingled with pH1N1 or pandemic H1N1 reassortment virus (rH1N1) inoculated pigs in separate groups. According to the results, both studied viruses were able to cross-species transmit to a few sentinel ducks with mild or no clinical signs. Viral shedding measured by a modified real time RT-PCR detection from the oropharyngeal and cloacal swabs were also observed in both studied viruses. Interestingly, ducks commingled with pH1N1-infected pigs showed higher number of infected ducks detected by viral shedding in cloacal swabs. This present study suggested that pH1N1 and rH1N1 were able to transmit from pigs to ducks but viral replication in ducks were limited. As a result clinical signs were not obvious and low levels of viral shedding were detected in both sentinel duck groups.

Keywords: ducks, interspecies transmission, pandemic H1N1 2009, pigs, reassortant

4.2 Introduction

In April 2009, a pandemic H1N1 influenza A virus (pH1N1) emerged and spread worldwide. The pH1N1 is a reassortant virus of the North American triple reassortant (TRIG) swine virus and the avian-like Eurasian swine lineage (Garten et al., 2009). The pH1N1 virus efficiently transmitted back to pigs observed in many countries (Ducatez et al., 2011), including Thailand (Sreta et al., 2010) and the pH1N1 reassortant virus (rH1N1) was evidently found in pigs in 2010 in Thailand. The rH1N1 contains Neuraminidase (NA) gene of a Thai endemic swine influenza virus (SIV) and the other 7 genes were closely related to the pH1N1 virus (Kitikoon et al., 2011a).

Since, Influenza A virus is a highly contagious pathogen and able to cause disease in mammalian and avian species. Influenza viruses normally require 2 specific receptors; Sialic acid (SA) α2, 3receptor commonly found in the intestinal tract of avian species and SA α2, 6 receptor commonly found in the respiratory tract of humans (Forrest and Webster, 2010). Wild aquatic ducks are known as the natural reservoir of the influenza A virus. Influenza A viruses in avian species are commonly divided into highly pathogenic (HPAI) and low pathogenic (LPAI) avian influenza viruses (Kim et al., 2009) and the pH1N1 and rH1N1 viruses were classified as LPAI viruses (Babiuk et al., 2010). LPAI viruses normally cause limited lesions within 2 days in the epithelial cells of respiratory and digestive tracts showing mild pneumonia and infiltration of lymphocytes and macrophages. In addition, the replication site is mainly in the large intestine since high concentration of viral shedding is found in feces up to 7 days (Van Reeth, 2007; Kim et al., 2009). Interestingly, mallard ducks are able to act as the viral reservoir transmitting the virus to domestic poultry (Keawcharoen at al., 2008). Hence, the avian-like swine virus such as Eurasian H1N1 lineage containing PB2 and PA genes of the first isolated TRIG virus was closely related to the virus isolated from duck (Brown, 2000)

indicating that duck's viruses can effectively transmit either the whole virus or a few gene reassortment to pigs. It should be noted that Bao et al. (2010) challenged pH1N1 (human isolate) in Peking ducks and found no viral RNA detection in the lung tissue nor in the epithelial cells of the digestive tract, the main replication site of LPAI. Moreover, avian viruses such as the 1918 H1N1 and pH1N1 can cross species transmission resulting in fatal diseases in humans but no or mild clinical signs in poultry, particularly in ducks (Babiuk et al., 2010, Kalthoff et al., 2010).

Commingling among domestic species is commonly seen in pig ecosystems particularly in the backyard farming in most Asian countries. Interspecies transmission of influenza viruses is possible when pH1N1 is widespread in the domestic animals, particularly in pigs. The viruses used in this study were isolated from naturally infected pigs. The infectivity and pathogenesis of these viruses in sentinel ducks were elucidated.

4.3 Materials and methods

4.3.1 Viruses

A/swine/Thailand/CU-RA29/2009(H1N1) (Sreta et al., 2010), a pandemic H1N1 of pig origin (pH1N1) and A/swine/Thailand/CU-SA43/2010 (H1N1) (Kitikoon et al., 2011), a novel reassortant virus of pig origin (rH1N1) were individually propagated 3 times in 9 day old embryonated chicken eggs. Allantoic fluids were collected 72 hours later and virus concentrations were calculated using 50% tissue culture infectious dose (TCID50) in Madin-Darby canine kidney (MDCK) cells by Reed and Muench method. The virus concentration of both isolates was adjusted to10^4 $TCID_{50}$/ml and kept in the -80 ºC until used.

4.3.2 Animals

Twenty one, 6-week-old ducks (Anas platyrhynchos domesticus) were placed in the animal facility biosafety level 2 with adequate appropriated nutritional food and clean water. Before starting the experiment, oropharyngeal and cloacal swabs and sera were collected from each duck. All samples were tested negative using a routine RT-PCR detecting for influenza A virus (M gene).

At the same time, 21 nursery pigs were divided into 3 groups. Group 1 containing 9 pigs received intratracheally inoculation with pH1N1 virus (A/swine/Thailand/CU-RA29/2009(H1N1)). Group 2 also had 9 pigs receiving intratracheally inoculation with rH1N1 ((A/swine/Thailand/CU-SA43/2010 (H1N1)). Group 3 was a negative control group containing 3 pigs receiving mock inoculation with cell culture media.

One day post inoculation (1 DPI) in pigs, sentinel ducks were divided into 3 groups similar to the pig experiment and placed into each pig room. As a result, pigs per ducks ratio are 1:1 and those animals were commingling in each separate group though out the experiment. At 3, 5 and 13 DPI, 3 ducks from group 1 and group 2 and one duck from the control group were randomly selected for necropsy. Adequate appropriated foods and water for pigs and ducks were provided in each room. The animal usage and procedures were approved by Chulalongkorn University-Faculty of Veterinary Science Animal Care and Use committee (protocol No. 11310052).

4.3.3 Clinical measurements and sampling

Clinical signs of ducks were observed at 2-8, 11 and 13 DPI. Oropharyngeal and cloacal swabs from all remaining ducks were collected at 2-8, 11 and 13 DPI. Nasal swab were also collected from all remaining pigs on 1-7, 10 and 12 DPI. All collected

swabs were tested for the presence of the influenza virus by a modified real time Reverse Transcriptase-Polymerase Chain Reaction (real time RT-PCR) and virus isolation (described below). Serum from ducks and pigs were collected at necropsy tested for influenza A virus antibody detection. Lung, pancreas and caecum of ducks and lung and bronchial lymph node of pigs were collected during necropsy for quantitative detection of viral RNA by the real time RT-PCR and viral isolation. Selected organs including brain, pancreas, liver, jejunum, caecum, spleen and kidney were fixed in 10% buffered formalin for histopathologied study.

4.3.4 Viral detection by a modified real time Reverse Transcriptase-Polymerase Chain Reaction (real time RT-PCR)

Total RNA was extracted from collected swabs, lungs, pancreas and caecum using a commercial kit (NucleoSpin Extract Viral RNA Kit, Macherey-Nagel, Germany). A modified real time reverse transcriptase-polymerase chain reaction (real time RT-PCR) was performed using Superscript III platinum one-step quantitative RT-PCR system (Invitrogen, USA). Primers were specific to the Matrix (M) gene. Forward primer (MF3; 5' TGATCTTCTTGAAAATTTGCAG 3'), reward primer (MR1+; 5' CCGTAGMAGGCCCTCTTTTCA 3') and M-probe (FAM-TTGTGGATTCTTGATCG-MGB) were used in this study as previous described (Payungporn et al., 2006). Briefly, the cycling conditions were started at 48˚C for 45 min. Then, 95˚C for 10 min and followed by 40 cycles including denaturation (94˚C for 15 s), annealing (55˚C for 30 s) and extension (72˚C for 40 s). Positive samples showed CT value over 40.

Meanwhile, homogenated tissue samples were filtrated and inoculated onto the monolayer of MDCK cells using a ten-fold serial dilution manner. The inoculated cell cultures were incubated for 72 hours. The virus was identified using anti-influenza A

nucleoprotein monoclonal antibody (EVS238, B.V.EUROPEAN VETERINARY LABORATORY, the Netherlands) as a primary antibody and rabbit anti-mouse IgG conjugated horseradish peroxidase as a secondary antibody (Dako Cytomation, Carpinteria, California). Then, the color was developed using a chromogen aminoethyl carbazole substrate (Sigma, St. Louis, Missouri) to identify the virus antigen in the nucleus of the infected cells (Sreta et al., 2009).

4.3.5 Pathological examination

Formalin-fixed tissues were embedded in paraffin and processed routinely. Briefly, sections were cut approximately 4-6 µm thick for histopathological and immunohistochemistry (IHC) staining for the influenza A virus antigen detection. The IHC staining was performed using a Polymer-Based method (Envision system). Primary antibody using anti-influenza A (H5N1) nucleoprotein monoclonal mouse antibody (EVS238, B.V.EUROPEAN VETERINARY LABORATORY, the Netherlands) and secondary antibody using Biotinylated rabbit anti-mouse IgG antibody and envision polymer (Envision Polymer DAKO®, Denmark) were concurrently performed with a negative control slide. A positive control slide was also included using the SIV-infected lung section from the previous experiment (Sreta et al., 2009). The sections was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin.

4.3.6 Serological test

All sera were tested for influenza A virus antibody by a commercial ELISA (Avian Influenza virus antibody test kit, IDEXX laboratories, USA). Concurrently, sera were randomly selected for hemagglutination inhibition (HI) test (1 sample/group/necropsy day). Since the hemagglutinin (HA) gene of pH1N1 and rH1N1 are closely related,

pH1N1 virus (A/swine/Thailand/CU-RA29/2009(H1N1) was used as the representative antigen in the assay. Samples with HI titers $\geq$ 40 were considered as previously exposed to the specific tested antigen.

4.4 Results

4.4.1 Clinical examination

Sentinel ducks in the pH1N1 group did not show any clinical sign during 2-4 DPI but only ocular and nasal discharges were observed at 5 DPI. All 3 remaining ducks in the pH1N1 group showed conjunctivitis between 6-13 DPI. Ducks in the control and the rH1N1 groups did not show any obvious clinical sign throughout the experiment. It should be noted that the inoculated pigs in both groups showed obvious clinical signs of SIV infection including nasal discharge, coughing and sneezing.

4.4.2 Virus detection

Both groups of inoculated pig showed viral detection in the nasal swabs as early as 1 DPI and could be detected in low levels until 12 DPI (data not shown). In addition, the rH1N1-inoculated group showed higher number of pigs shedding the virus based on the nasal swab results (data not shown). In the experimental ducks, viral detection in the pH1N1 group was detected by the real time RT-PCR from the oropharyngeal swabs at 3 DPI (1 of 9) and cloacal swabs at 2 (1 of 9), 3 (2 of 9), 11 (1 of 3) and 13 (1of 3) DPI. Based on the viral isolation results in the pH1N1 group, only one oropharyngeal swab and one cloacal swab showed positive results with low levels of virus concentration at 4 DPI (Table 1). In the rH1N1 group, 1 oropharyngeal and 1 cloacal swab at 2 DPI were tested positive and the virus isolation of the rH1N1 group yielded negative results (Table 2). No viral detection was found in the negative control ducks throughout the

experiment. Low levels of viral genetic material was detected only in the lung of one necropsied duck in the pH1N1 group by the real time RT-PCR at 3 DPI (data not shown).

Table 1: Viral detection measured from oropharyngeal and cloacal swabs of sentinel ducks in the pH1N1 group using a modified real-time RT-PCR and viral isolation

Duck No.	Virus detection																			
	1 DPI		2 DPI		3 DPI		4 DPI		5 DPI		6 DPI		7 DPI		8 DPI		11 DPI		13 DPI	
Oropharyngeal swab	r	V	r	V	r	V	r	V	r	V	r	V	r	V	r	V	r	V	r	V
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

r = a real time RT-PCR (+ = Ct values < 40; - = Ct values ≥ 40)

V = viral isolation using MDCK

N = necropsy.

Table 2: Viral detection measured from oropharyngeal and cloacal swabs of sentinel ducks in rH1N1 group using a modified real time RT-PCR and viral isolation

Duck No.	Virus detection																			
	1 DPI		2 DPI		3 DPI		4 DPI		5 DPI		6 DPI		7 DPI		8 DPI		11 DPI		13 DPI	
Oropharyngeal swab	r	V	r	V	r	V	r	V	r	V	r	V	r	V	r	V	r	V	r	V
1	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	N									
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	N													
5	-	-	-	-	-	-	N													
6	-	-	-	-	-	-	N													
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	N									
9	-	-	-	-	-	-	-	-	-	-										
											N									
Cloacal swab																				
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	N									
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	N													
5	-	-	-	-	-	-	N													
6	-	-	+	-	-	-	N													
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	N									
9	-	-	-	-	-	-	-	-	-	-	N									

DPI = day post inoculation

rt = a real time RT-PCR (+ = Ct values < 40; - = Ct values ≥ 40)

VI = viral isolation using MDCK

N = necropsy.

4.4.3 Pathological examination

In both infected groups of pigs, typical lesions of SIV infection characterized by multifocal consolidated lung lesions with a checker-board lung pattern at the cranioventral area were observed. The viral antigens were detected by IHC only in the lung and bronchial lymph node of the rH1N1 infected pigs (data not shown). However, sentinel ducks in both inoculated pig groups as well as in the control group showed only mild interstitial pneumonia and mild air saculitis. None duck tissues were tested positive by IHC technique.

4.4.4 Serological examination

At the beginning of the experiment, all duck sera were tested negative for influenza A virus antibody using a commercial ELISA. Sera collected from necropsied ducks at 3, 5 and 13 DPI were also tested negative for influenza A antibody using a commercial ELISA. Similarly, all duck sera were tested negative for the pH1N1 virus using HI test.

4.5 Discussion

In this experiment, pigs and ducks were commingling in close contact and might share the same water basins. Although, appropriate foods for ducks and pigs were provided in separate bowls, the animals randomly consumed foods in all bowls. Mimicking the field situation in backyard farming, ducks, chickens and birds freely share the same environment and might have the direct contact among those animals. The poultry not only consume the leftover feed stuff but might also contaminate the pigs from their excretions or vice versa. As a result, cross-transmission of influenza virus from pigs to ducks or vice versa could happen in the mimicking pigs to ducks interfacing environment.

As expected, the sentinel ducks did not showed obvious clinical signs or significant lesions. However, viral RNA was detected in the oropharyngeal and cloacal swabs of ducks in both inoculated pig groups. In addition, pH1N1 virus could be isolated from sentinel ducks in MDCK cells with low titers implying that both studied viruses could infect, replicate, and probable transmit from pigs to ducks when commingling. Previously, ducks were demonstrated not susceptible to the human pH1N1 infection and no viral RNA was detected from swabs or lungs (Swayne et al., 2009; Bao et al., 2010). It should be noted that the 1918 H1N1 influenza virus could replicate in ducks with low levels of virus titers (Babiuk et al., 2010).

Based on the present study, the pH1N1 virus obtained from pigs was able to infect and replicate in the duck intestine better than the respiratory tract. This result is correlated well with the evidence that the LPAI viruses shed via fecal-oral route and persist in duck population. As a result, all subtypes of the viruses, especially LPAI, can be isolated from duck feces (Kim et al., 2009).

It should be noted that the viral RNA was also found in the duck lung tissue of the pH1N1 group in this study. However, viral antigen could not be detected in all collected tissues of both inoculated groups. The results suggested that both pH1N1 and rH1N1 viruses had limited replication in sentinel ducks possibly due to lacking of SA α2, 6 receptors in ducks (Matrosovich et al., 2008). The HA gene of both pH1N1 and rH1N1 is similar compatible with the SA α2, 6 receptor and in theory able to infect pigs. In addition, the differences of body temperature in avian and mammals may affect the replication process (Forrest and Webster, 2010). As a result, both studied viruses had possibly limited replication in the sentinel ducks.

In the sentinel ducks, viral detection in the pH1N1 group yielded higher numbers of positive ducks than those of the rH1N1 group. In contrast, viral detection in the parallel infected pigs was observed more in the rH1N1-infected pigs. Since the NA gene of pH1N1 and rH1N1 were 89% homology and the 7 other genes had at least 99% homology, the difference of the NA gene might cause the variation in infectivity and virus shedding in the sentinel ducks. It should be noted that NA gene is responsible for releasing progeny viral particles from the infected cells (Suzuki, 2005). The NA gene of the studied rH1N1 obtained from the local swine influenza virus must be well adapted in the Thai pig population but might have the limitation on releasing of new progeny virus in ducks.

Unfortunately, all collected sera showed negative results on influenza A antibody suggestive of limited infection and replication of the studied viruses. In contrast, antibody against HPAI (H5N1) could be detected as early as 4 DPI and gradually increased by 14 DPI in experimental ducks (Jeong et al., 2009).

In conclusion, the results suggested that pH1N1 and rH1N1 isolated from pigs could probably be transmitted to the sentinel ducks, when commingling in closely contact with the infected pigs. However, the studied viruses had limited replication in ducks. The pH1N1 isolated from pigs appeared to have the likely potential in pig to duck transmission. Interspecies transmission may influence the antigenic drift and shift resulting in the emerging of a novel influenza virus. Each novel virus may adapt in the specific hosts before emerging and causing severe diseases and may transmit to another host or vice versa and so on. Since backyard farming commonly seen in most Asian countries creates a perfect interspecies transmission together with closely human-animal interface scenario. Influenza A virus surveillance, monitoring, and pathogenesis study are necessary.

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CHAPTER V

5.1 Research synopsis

The pH1N1 influenza virus emerged in 2009 and reached pandemic level 6 within 3 months (Schnitzler and Schnitzler, 2009). The virus spreads into more than 214 countries and responsible for 18,449 deaths (WHO, 2010a). It successfully replicates in human as well as in varies species such as mice, ferrets, pigs and turkeys (Itoh et al., 2009; Munster et al., 2009; Russel et al., 2009). The pH1N1 composed of PB1 gene from human, PB2 and PA from avian, HA, NP, NS from classic swine and M, NA from Eurasian swine virus (Garten et al., 2009). The combination of human, avian and swine influenza virus genes was previously found in the North American pig population since 1998 and was called triple reassotant internal gene (TRIG) cassette. The TRIG cassette is likely to pick up different HA and NA gene, in order to escape from the host immune system (Vincent et al., 2009; Ma et al., 2010). In 2009, the TRIG cassette was introduced into Thai swine population by cross transmission of pH1N1 from humans to pigs.

As a result, the purpose of this study is to investigate the consequences of the pH1N1 in the Thai swine population. The study was divided into 2 major parts; the genetic characterization (Chapter II) and the pathogenesis of the pH1N1 virus and its reassortant virus (Chapter III and Chapter IV). The SIV surveillance and genetic characterization were conducted during June 2010 – May 2012. The results showed that pH1N1 was the most isolated virus in 2010 and early 2011. However, endemic H1N1 (enH1N1) was occasionally isolated in the first 6 months of the study. The obtained information indicated that pH1N1 was established in Thai swine population and co-circulated with Thai endemic influenza viruses. In 2011 and 2012, the genetic

variation of the reassortant viruses of pH1N1 viruses; rH1N1, rH1N2, rH3N2 were isolated. The rH1N1 viruses had been isolated since early 2010 from one farm in central Thailand. Later, rH3N2 viruses were isolated since February 2011 and became major viruses obtained between 2011 and 2012. Interestingly, the reassortant viruses picked up the HA and NA genes from Thai endemic H3N2, previously isolated in 2004-2005. The rH1N2 was also isolated from 1 farm and it had the NA gene of human H3N2 virus previously isolated in 1988. The other 7 genes of the reassortant virus were similar to pH1N1. The rH1N1, rH1N2 and rH3N2 viruses confirmed that TRIG cassette influenced influenza virus genetic variation (antigenic drift and shift). Once the TRIG cassette virus has established itself in the Thai pig population, the genetic characterization of Thai SIV would have highly genetic variation similar to the SIV in North America.

Moreover, the SIV surveillance results of viruses obtained in this study had similar pattern to human influenza surveillance in Thailand. The chart in figure 1 shows that pH1N1 was the most prevalence in 2010 after being introduced into the Thai swine population. The H3N2 virus replaced and became dominant since late 2011 onward (WHO, 2012).

Molecular analysis of H1 and H3 HA gene indicated that most isolates in this study were compatible with human receptor (SA α2,6). The antigenic sites of pH1N1, rH1N1 and rH1N2 isolates were similar to the antigenic sites of the 2013 H1N1 human vaccine strain (A/California/7/2009 (H1N1)). However, the H1 HA antigenic sites of enH1N1 were not similar to the H1N1 human vaccine strain. The Thai rH3N2 had 3 antigenic sites similar to the 2013 H3N2 human vaccine strain (A/Victoria/361/2011H3N2)) but the other 2 viruses were different. All isolates described in chapter II had NA gene amino acid substitution E119V, H274Y, R292K, N294S and M gene amino acid substitution S31N. These amino acid mutation leads to Oseltamivir and

Amantadine resistance. Fortunately, the pH1N1 and the reassortant viruses showed low virulent pathogenesis based on the genetic analysis.

It should be noted that pH1N1 and its reassortant viruses are highly infective. However, both parts of this study indicated that those were low virulent. The *in vivo* experiment of pH1N1 and rH1N1 viruses showed mild clinical signs such as sneezing, coughing, conjunctivitis and having nasal discharge. However, pH1N1 and rH1N1-infected experimental pigs did not show any different clinical sign. Additionally, the commingling sentinel ducks did not demonstrate any obvious clinical sign.

The modified real-time RT-PCR detected viral RNA of both viruses in pig nasal swabs from 1-12 days post inoculation (DPI). Moreover, the viruses had successfully been isolated in MDCK cells only between 3-6 DPI. The obtained information suggested that the virus infectivity of both studied virus was evident until 6 DPI. However, the viral RNA was detected until 12 DPI or at the end of the experiment. Again, the rH1N1 virus showed higher number of pigs shedding the virus via nasal swabs. In commingling sentinel ducks, viral RNA of both pH1N1 and rH1N1 viruses were detected from oropharyngeal and cloacal swabs. Unlike the results in pigs, the viral RNA was detected from pH1N1 group more than that of from rH1N1 group. In addition, viral RNA was detected in the cloacal swabs more than that of in the oropharyngeal swabs. It should be noted that the pH1N1 virus was able to infect and replicate in ducks intestine more than the respiratory system. This finding is correlated well with the previous study showing that the low pathogenic influenza (LPAI) virus was able to shed though fecal-oral route (Kim et al., 2009). The oropharyngeal and cloacal swab samples from pH1N1-infected group showed low levels of virus titers isolated in MDCK cells. In summary, both pH1N1 and rH1N1 viruses were able to infect and replicate in pigs and sentinel

ducks. Moreover, those viruses were capable to transmit from infected pigs to sentinel ducks when animals were commingling.

The results in experimental pigs demonstrated that pathologic lesions of both studied viruses were restricted only in the lung. Macroscopic lesions showed dark plum-color, multifocal to coalescing consolidation or “checker board lung pattern” in cranioventral lobes. Microscopic lesions were mild to moderate broncho-interstitial pneumonia. The immunohistochemistry staining showed SIV antigens in the nuclei of infected-bronchial epithelial cells. The lung lesions were evidently observed in pigs when necropsied at 2 and 4 DPI in the rH1N1-infected group. In contrast, lesions and influenza virus antigen were not detected in the sentinel ducks. However, viral RNA was detected in duck lung tissue by a modified real-time RT-PCR. The results suggested that the studied viruses were well replicated in pigs but having limited replication in the sentinel ducks. These results correlated with the amino acid analysis of HA genes in the SIV surveillance study. The receptor binding sites of most of the isolates in this study were compatible with the human receptor (SA α2,6) more than those of the avian receptor (SA α2,3).

The rH1N1 infected pigs showed greater severity of clinical signs, pathologic lesions and higher numbers of pigs shedding the virus. In contrast, the sentinel ducks demonstrated higher viral detection in the pH1N1 group. In addition, only pH1N1 viral RNA was detected from the lung tissue. The difference between pH1N1 and rH1N1 was the NA gene. Both NA genes were classified in swine Eurasian SIV but having only 89% identity. The NA gene of rH1N1 was closely related (97.2% identity) to the NA gene of Thai swine endemic H1N1 previously isolated in 2005-2006. The NA gene is responsible for releasing progeny viral particles from infected cells. Therefore, the biology feature of NA gene affords host range restriction. The LPAI with low–pH stable sialidase activities

is not degraded in acidic condition of duck's stomach and gizzard. As a result, the LPAI is able to isolate from duck feces (Suzuki, 2005). The low pH stability of sialidase activities was determined by amino acid position 344 and 466 of NA gene. Amino acid substitution K344R and L466F were obviously decreased stability of sialidase activity. This character was found in human H2N2 and H3N2 viruses causing the epidemic outbreaks before 1970s. Therefore, the evidence of amino acid substitution related sialidase activities in N1 NAs was missing. However, this study showed the difference of NA genes causing differences in the infectivity, replication and virus shedding of influenza virus in different hosts.

In summary, the SIV surveillance after the introduction of pH1N1 indicated that pH1N1 virus established in Thai swine population and later became endemic. The pH1N1 virus has caused variation of Thai swine virus genetic reassortant in 2010-2012 due to its TRIG cassette. The TRIG cassette virus contains internal genes derived from human, avian and swine influenza viruses, making it successfully replicated in different host species. Moreover, the study confirmed probably cross-transmission from pigs to ducks.

The interspecies transmission ability and high infectivity of pH1N1 virus demonstrated the novel human pandemic in 2009. The novel virus could be generated without being noticed. In the swine industry, genetic variation of SIV may affect herd health immunity of naïve population causing economic loss. However, the current interest is of public health concerns. It should be noted that SIV were occasionally isolated from humans (Komanida et al., 2007; Linstrom et al., 2012). Serologic study using human sera was conducted in 2 Thai swine farms comparing to the unexposed cohort group. Swine workers directly contacted with pigs showed highest percentage of positive specimens as well as 50% of Veterinarians sera were also positive with SIV

(Kitikoon et al., 2010b). Unawareness of flu and flu-like symptoms possibly provide the virus opportunity spreading into the human population. Therefore, the most concern pandemic is reassortment between the highly infectivity pH1N1 with TRIG cassette and highly pathogenic H5N1 avian virus. As found previously, H5N1 virus has been isolated from pigs in Vietnam, Indonesia and China with no obvious clinical signs (Suzuki, 2005; Nidom et al., 2010). As a result, continuing SIV surveillance is advantageous for disease prevention and control in term of public health concerns.

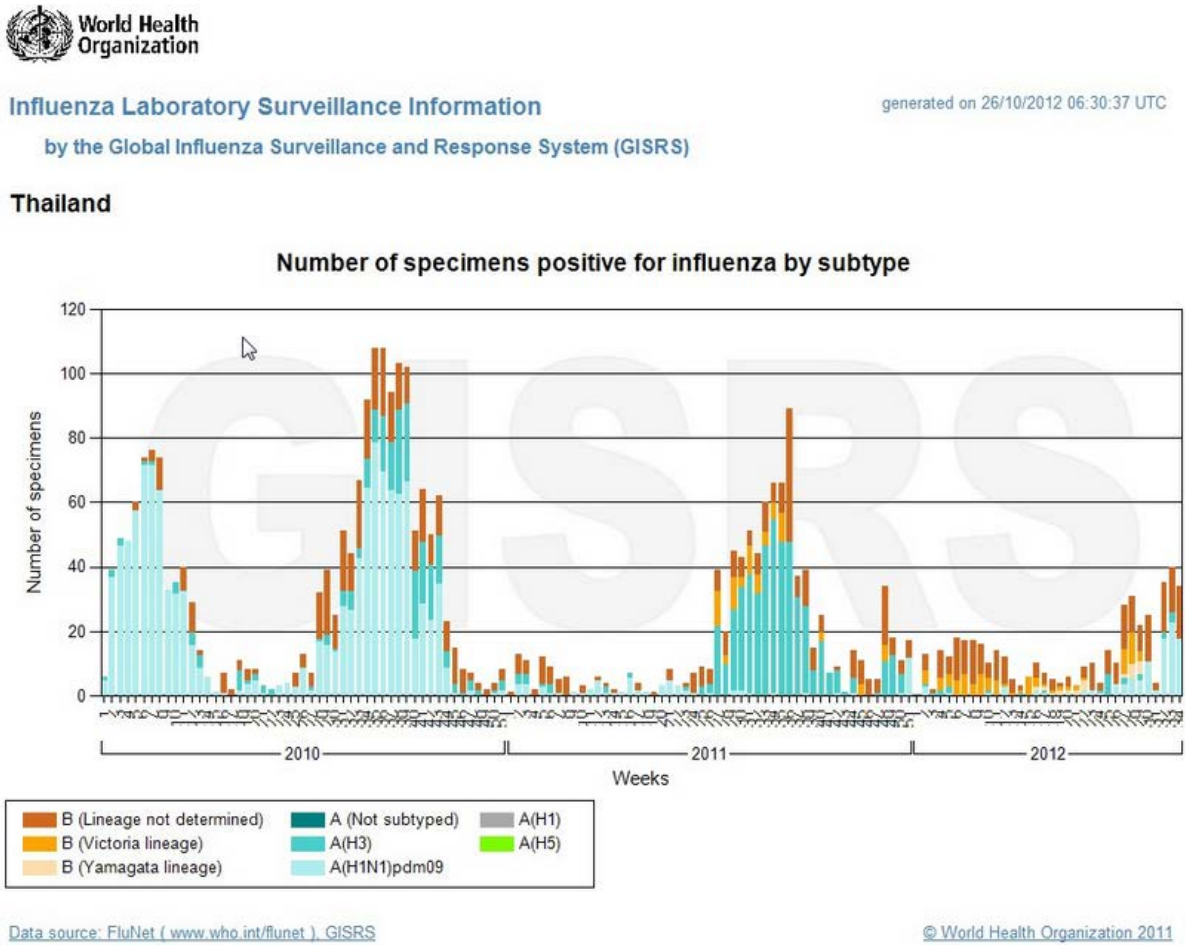

Figure 1: The chart showed number of human specimens positive for influenza by subtype in Thailand during year 2010-2012 (WHO, 2012).

5.2 Research limitation

The SIV surveillance was conducted un-continuously. The first interruption was in late 2010 due to the outbreaks of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) in Thai swine farms. Secondly, the surveillance program was paused for almost 4 months during the Thai mega-flood in 2011.

The whole genome sequences were not fully completed in some isolates. The genetic of Influenza virus is highly variable due to antigenic drift. As a result, the primers for whole genome sequence were often changed to yield the best sequences. Therefore, lots of time and money would be required to finish all sequencing data.

5.3 Suggestion for further investigation

The SIV surveillance should be continuously performed since pigs are a mixing vessel for influenza viruses. The obtained information benefits swine farm management as well as public health concerns. In addition, the H1-H13 HA viruses are able to replicate in the pig respiratory system more or less (Kida et al., 1994). As a result, influenza viruses with the HA subtypes never been found epidemic or endemic in humans might cause severe respiratory disease in humans leading to a pandemic threat in the future. Moreover, the commingling environment between pigs and avian species are still found in Thailand and other South-East Asian countries particularly in the back-yard farms. The reassortant between swine and avian viruses is possible based on previous evidences.

The results from the *in vivo* experiments indicated that NA gene might play an important role on viral shedding and altered viral stability between different hosts.

However, most researches on NA gene focus on antiviral drugs resistant. Further study using advance technique such as reverse genetic could be an alternative tool for specific gene study to elucidate the role of NA gene.

The study demonstrated that the major populations of virus circulated in Thailand between 2010-2012 were pH1N1and rH3N2. The amino acid sequence of HA protein of pH1N1 SIV were similar to the H1N1 virus using as a component for human 2013 vaccine. Therefore, the enH1N1 SIV was different from vaccine. Moreover, amino acid at antigenic sites of HA gene of rH3N2 SIV were different from human 2013 H3N2 vaccine strain. If SIV cross-transmission to human, would the antibodies produced by human 2013 inactivated vaccines cross-protect these viruses? This suspicion should be investigated.

Moreover, Hemagglutinin Inhibition (HI) test is a standard test for influenza virus antibody detection. In Thailand, the reference viruses for Thai SIV were selected in 2007 -2009. Therefore, the most isolated viruses were changed into rH3N2 in 2011-2012. The antigenic sites of the viruses were different from those references virus in 2009. As a result, the most appropriate new reference viruses for HI test should be elucidated for the best results. The HI test with suitable reference viruses will give the right picture of SIV serological profiles and will help to implement the proper management for SIV control.

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APPENDICES

APPENDICES

Appendix A: The procedure for preparation of the tissue processing

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

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

1. Deparaffinization (heat 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, Absolute alcohol II = 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% H_20_2 (Absolute methanol 150 ml and 30% H_20_2 15 ml) for 10 min at room temperature
3. Wash in Distill water for 5 min
4. Wash in PBS for 5 min, 2 times
5. Pretreated slides with 0.05% Protenase K for 10 min at 37°C
6. Wash in PBS for 5 min, 3 times
7. Block non-specific antigen with 1%BSA for 30 min at 37°C
8. Wash in PBS for 5 min, 3 times
9. Apply primary antibody: anti-influenza A (H5N1) nucleoprotein monoclonal mouse antibodies, dilution 1:300, incubate at 4°C overnight
10. Wash in PBS for 5 min, 3 times
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 times
13. Developed 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 C: Virus titration and Immunoperoxidase monolayer assay (IPMA)

1. Prepare monolayer of MDCK cells in 96-well tissue culture plate.
2. Wash confluent cell monolayers 3 times with cell culture medium containing 5% BSA and 5mg/ml TPCK-treated trypsin
3. Make 10-fold dilution of sample and apply 100 ul into each well, follow the prepared dilution (4 well per 1 dilution)
4. Incubate in 37°C humidified incubator with 5% CO_2 for 72 hours.
5. Fix the cell with 4% formalin in PBS-0.5% tween
    5.1 Discard all culture media (into disinfectant)
    5.2 Apply 100 ul of 4% formalin in PBS-0.5% tween in each well
    5.3 Incubate for 25 min at room temperature
    5.4 Wash with PBS-0.5% tween, 3 times
6. Apply antibody: anti-influenza A (H5N1) nucleoprotein monoclonal mouse antibodies, dilute with 1% BSA in PBS-0.5% tween (dilution 1:1,000), 50 ul/ well, incubate for 1 hour at room temperature
7. Wash with PBS-0.5% tween, 3 times
8. Apply conjugate: rabbit anti- mouse IgG dilute with 1% BSA in PBS-0.5% tween (dilution 1:300), 50 ul/ well, incubate for 1 hour at room temperature
9. Wash with PBS-0.5% tween, 3 times
10. Apply AEC substrate 50 ul/ well, incubate for 10 min at room temperature (dissolve AEC 8 tablets n Dimethyformamide 40 ml, then making AEC substrate by add 5% AEC and 3% H_20_2 in acetate buffer)
11. Wash with tap water, 3 times
12. Dry plate and observe under phase-contrast microscope
13. Calculate $TCID_{50}$ by Reed and Muench method

Appendix D: Primer designed for genetic characterization in this study

Primer name	Sequence	Tm	Position	basepair	Product size
PB2					
PB2-1F	ATGGAGAGAATAAAAGAACTA	54	1-21	21	863
PB2-863R	TGTGTGCTGTGGCACATT	54	846-863	18	
PB2-727F	GTGAGAAATGATGATGTTGA	54	727-749	20	931
PB2-1658R	ATCCATTGATAAGTGTTGAC	54	1639-1658	20	
PB2-1597F	TCATCAATGATGTGGGAGAT	54	1597-1616	20	682
PB2-2279R	TAATTGATGGCCATCCGAA	54	2261-2279	19	
PB1					
PB1-1F	ATGGATGTCAATCCGACT	52	1-18	18	831
PB1-831R	TTCATTGCCCCCTACTG	52	815-831	17	
PB1-679F	ATGACCAAAGATGCAGAGA	54	679-697	19	934
PB1-1613R	TCATTGTTTATCATGTTGTTC	54	1593-1613	21	
PB1-1369F	AATACAAGCAGGAGTGGA	52	1374-1391	18	901
PB1-2275R	ATTATTTTTGCCGTCTGAG	52	2257-2275	19	
PA					
PA-1F	ATGGAAGACTTTGTGCGG	54	1-18	18	858
PA-858R	GCATCCATCAGCAGGAAT	54	840-858	18	
PA-743F	AAATGTCCAAAGAAGTGAA	52	743-761	19	861
PA-1604R	TTGTGTGGCTCCAGTCT	52	1588-1604	17	
PA-1468F	AGGACCAAAGAAGGAAGG	54	1468-1485	18	663
PA-2131R	GGAAGGAGTTGAACCAAGA	54	2113-2131	19	
H1 HAs					
H1-1F	ATGAAGGCAATACTAGTAGTTCTG	50	1-24	24	883
H1-883R	CCCTTGGGTGTCTGACAAGT	51	883-902	20	
H1-778F	GAAGCAACTGGAAATCTAGT	56	778-797	20	812
H1-1590R	AATCTGGTAAATCCTTGTTGA	56	1570-1590	21	
H1-1313F	ACAATGCCGAACTGTTGGTT	56	1313-1332	20	457
H1-1652R	AAAGACCCATTGGAGCACATC	55	1631-1652	21	
H3 HAs					
H3-7F	ACTATCATTGCTTTGAGC	55	1-18	18	804

Primer name	Sequence	Tm	Position	basepair	Product size
H3 HAs (continue)					
H3-804R	GGCAATTAGATTCCCTGT	52	787-804	18	
H3-698F	TCGGGCCTAGACCCTGG	54	698-714	17	719
H3-1417R	CCCATGTCTTCAGCATTTTC	51	1417-1436	20	
H3-1286F	TTGAAGACACTAAAATAGAT	50	1286-1305	20	484
H3-1770R	TCAAATGCAAATGTTG	55	1754-1770	16	
NP					
NP_1F	ATGGCGTCTCAAGGCACCAAA	51	1-21	21	520
NP_537R	GGAAGTGTTGAACCTTGCATT	51	516-537	21	
NP-493F	ATGGATCCCAGAATGTGC	54	493-510	18	492
NP-985R	CTGGGTTTTCATTTGGTCT	54	967-985	19	
NP-850F	GCTTGTGTGTATGGGCTT	52	850-866	18	665
NP-1515R	TCAATTGTCAAACTCCTCTG	56	1496-1515	20	
N1 NAs					
N1_1F	ATGAATCCAAACCAAAAGATAA	50	1-22	22	396
N1_377R	GAAGGTTCTGCATTCCAAGG	50	377-396	20	
N1_318F	AAGAATCGGTTCCAAGGGGGA	53	318-338	21	593
N1_910R	ACCCACGGTCGATTCGAGC	50	891-911	19	
N1_830F	AGGAATGCTCCTGTTATCCTG	52	830-851	21	467
N1-1297R	CAAAAGGATATGCTGCTCCC	56	1277-1297	20	
N2 NAs					
N2-1F	ATGAATCCAAATCAAAAGATA	52	1-21	21	484
N2-484R	CATTCATCAATAGGGTTCG	54	466-484	19	
N2-352F	AGAGAACCTTATGTGTCATGCGA	53	352-374	23	499
N2-851R	CAGACACATCTGACACCAGGAT	50	851-872	22	
N2-631F	GATAGTATTGGTTCATGGT	52	631-655	19	726
N2-1357R	ATGTACCTGAGGTGCCAC	56	1340-1357	18	
M					
M-1F	ATGAGTCTTCTAACCGAGGT	58	1-20	20	681
M-681R	AGCACTGGAGCTAGGATGA	58	663-681	19	
M-621F	TCAGGCTAGGCAGATGGTG	51	621-639	19	347
M-968R	TGTTGACAAAATGACCATCG	49	949-968	20	

Primer name	Sequence	Tm	Position	basepair	Product size
NS					
NS-1F	ATGGACTCCAACACCATGTCA	50	1-21	21	638
NS-638R	CTCCCATTCTCATCACAGTT	48	619-638	20	
NS-213F	GAAAGAGGAATCCAGCGAG	51	213-231	19	630
NS-820R	TAAATAAGCTGAAACGAGAAAG	52	820-843	22	

BIOGRAPHY

Miss Na taya Charoenvisal was born on May 17, 1982 in Bangkok, Thailand. She is an alumnus of Satit Pathumwan Secondary School, Bangkok, Thailand. She graduated with a Bachelor Degree, Doctor of Veterinary Medicine, (DVM) in academic year 2006 from the Faculty of Veterinary sciences, Chulalongkorn University. During her master program, she was appointed the scholarship as an exchange student for 1 year (April 2006 – April 2007) from the Japan Student Services Organization (JASSO) to study and conduct her research work at the Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Japan. Then, she was awarded her Master's degree in Master of Science (Veterinary Pathobiology) in 2008. She was a Ph.D. candidate of Pathobiology program at the Department of Pathology, Chulalongkorn University. During her Ph.D. program, she attended the Training Course for Zoonosis Control at the Faculty of Veterinary Medicine, Hokkaido University, Japan, twice. She has published a number of international scientific articles as the following;

General publication during her study:

1. Sreta D, Jittimanee S, Charoenvisal N, Amonsin A, Kitikoon P, Thanawongnuwech R. 2013. Retrospective swine influenza serological surveillance in the four highest pig density provinces of Thailand before the introduction of the 2009 pandemic Influenza A virus subtype H1N1 using various antibody detection assays. J. Vet. Diagn. Invest. 25, 45-53.

The publication from her thesis:

1. Charoenvisal, N., Keawcharoen, J., Sreta, D., Tantawet, S., Jittimanee, S., Arunorat, J., Amonsin, A. and Thanawongnuwech, R. 2013. Experimental infection with a Thai reassortant swine influenza virus of pandemic H1N1 origin induced disease. Virol. J. 10:88

2. Charoenvisal, N., Keawcharoen, J., Sreta, D., Tantawet, S., Jittimanee, S., Arunorat, J. Poonsuk, K., Amonsin, A. and Thanawongnuwech, R. 2013. Probable pig to duck transmission of the pandemic H1N1 2009 (pH1N1) and its reassortant in commingling experimental condition. Thai. J. Vet. Med. (Accepted)

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

Poster presentation:

1. "Surveillance of a swine influenza virus (SIV) in thai commercial swine farms during year 2010-2011" The 22nd conference of IPVS 2012 on 11-13 June 2012, Jeju, Korea.