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SURVEILLANCE OF SWINE INFLUENZA VIRUSES IN THAILAND, 2011-2012

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เชื้อไวรัสไข้หวัดใหญ่สุกรก่อให้เกิดโรคระบบทางเดินหายใจในสุกร เชื้อไวรัสชนิดนี้สามารถพบได้ในสุกรทั่วโลก การศึกษาในครั้งนี้เป็นการศึกษาอุบัติการณ์ ชนิดสายพันธุ์ และซีรั่มวิทยาของการติดเชื้อไวรัสไข้หวัดใหญ่สุกรในประเทศไทยระหว่างปี พ.ศ. 2554-2555 โดยเก็บตัวอย่างป้ายจมูก จำนวน 745 ตัวอย่าง และเลือด จำนวน 571 ตัวอย่าง ในช่วงเดือนกันยายน 2554 ถึงเดือนมีนาคม 2555 ผลการตรวจตัวอย่างป้ายจมูกโดยวิธี real-time RT-PCR พบว่ามีตัวอย่างบวก จำนวน 186 ตัวอย่าง (24.97%) และสามารถแยกเชื้อไวรัสไข้หวัดใหญ่สุกรได้ จำนวน 13 ตัวอย่าง (1.74%) จำแนกเป็นสายพันธุ์ H1N1 จำนวน 10 ตัวอย่าง และสายพันธุ์ H1N2 จำนวน 3 ตัวอย่าง สำหรับผลการศึกษาทางซีรั่มวิทยาพบว่า สุกรมีภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่ ชนิด เอ จำนวน 262 ตัวอย่าง (45.88%) เมื่อตรวจด้วยวิธี NP-ELISA และมีภูมิคุ้มแบบจำเพาะต่อเชื้อไวรัสไข้หวัดใหญ่สุกร แบ่งเป็นสายพันธุ์ H3N2 จำนวน 88 ตัวอย่าง (15.41%) สายพันธุ์ H1N1 จำนวน 14 ตัวอย่าง (2.45%) และสายพันธุ์ pH1N1 จำนวน 28 ตัวอย่าง (4.90%) เมื่อตรวจด้วยวิธี HI test ผลการศึกษาครั้งนี้แสดงให้เห็นว่า ในช่วงปี พ.ศ. 2554-2555 มีการแพร่กระจายของเชื้อไวรัสไข้หวัดใหญ่สุกรสายพันธุ์ H1N1 และ H1N2 ในสุกรของประเทศไทย โดยพบว่าเชื้อไวรัสไข้หวัดใหญ่สุกรสายพันธุ์ H1N1 เป็นสายพันธุ์หลักที่ทำให้เกิดการติดเชื้อในช่วงเวลาดังกล่าว ข้อมูลที่ได้จากการศึกษาในครั้งนี้สามารถนำไปใช้ในการป้องกัน และควบคุมโรคไข้หวัดใหญ่ทั้งในสุกร และมนุษย์ต่อไป

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KIRANA NORADECHANON: SURVEILLANCE OF SWINE INFLUENZA VIRUSES IN THAILAND, 2011-2012. ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 56 pp.

Swine Influenza Virus (SIV) causes respiratory disease in pigs and can be found in pigs worldwide. This study provided the information on the occurrence, subtype and serological profiles of SIV infection in Thailand during 2011-2012. Seven hundred and forty-five nasal swab samples and 571 serum samples of Thai pigs were collected for SIV surveillance during September, 2011 – March, 2012. The result showed that 186 nasal swab samples (24.97%) were positive for influenza A virus by real-time RT-PCR, however only 13 samples (1.74%) were successfully isolated. For subtype identification, SIV subtype H1N1 (n=10) and H1N2 (n=3) were identified. For serological analysis, 262 serum samples (45.88%) were positive by NP-ELISA. HI test showed specific antibodies to swH3N2 (15.41%; 88/571), swH1N1 (2.45%; 14/571) and pH1N1 (4.90%; 28/571) in pig serum tested. In conclusion, our results indicated that during 2011-2012, SIV subtype H1N1 and H1N2 were circulating in Thai pig populations and the endemic SIV-H1N1 was the dominate SIV subtype in Thailand. The information gained from this study can be used for SIV prevention and control in pig and human populations.

Department: Veterinary Public Health Student's Signature .....
Field of Study: Veterinary Public Health Advisor's Signature .....
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List of Abbreviations

cDNA	Complementary deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
HA	Hemagglutinin
HI	Hemagglutination inhibition
IAV	Influenza A Virus
M	Matrix
NA	Neuraminidase
NP	Nucleoprotein
NP-ELISA	Nucleoprotein-based Enzyme-Linked Immunosorbent Assay
NS	Nonstructural protein
PA	Polymerase Acidic protein
PCR	Polymerase Chain Reaction
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
PBS	Phosphate Buffered Saline
RBC	Red blood cell
rRT-PCR	Real-time Reverse Transcription-Polymerase Chain Reaction
SIV	Swine Influenza Virus

TRIG	Triple reassortant internal genes
WHO	World Health Organization

Chapter 1 Introduction

Influenza A virus (IAV) is a virus of the family *Orthomyxoviridae.* Influenza A virus is an enveloped, negative sense, single-stranded RNA virus. Influenza A viruses are classified into subtypes by the two surface proteins; hemagglutinin (HA) and neuraminidase (NA). The viral genome contains 8 RNA segments: HA, NA, NP, M, NS, PA, PB1 and PB2 (Webster et al., 1992). Recently, there have been 18 HA subtypes and 11 NA subtypes. The IAV subtypes H17N10 and H18N11 were only found in bat (CDC, 2015). IAVs have been reported in many animal species such as birds, pigs, horses and dogs, as well as humans (Alexander and Brown, 2000). The epidemics of IAVs have been reported in human population worldwide (Cox et al., 1994).

Swine influenza viruses (SIVs) can be found in pigs worldwide. The viruses cause respiratory disease in pigs. SIVs that mainly circulated in pigs all over the world are subtypes H1N1, H1N2 and H3N2. In Thailand, early studies reported all three major subtypes of swine influenza virus isolation : H1N1, H1N2 and H3N2 (Kanai et al., 1981; Kupradinun et al., 1991; Chutinimitkul et al., 2008). Pigs can be infected with the viruses of human and/or avian origin since the respiratory tract of pig exhibits both avian and human receptors (α2,3 and α2,6, respectively) (Brown, 2001). When pigs are co-infected with influenza viruses, they can produce new influenza viruses from reassortment and may become pandemic strains (Alexander and Brown, 2000). In

addition, many evidences revealed that pigs play an important role as a mixing vessel of influenza viruses (Brown, 2001).

Some evidences showed cross species infection between human and pig. In 2011, 12 cases of human with SIV-H3N2 infection were reported. The virus contained M gene from pandemic H1N1-2009 viruses and other gene segments from triple reassortant SIV-H3N2, while the SIV surveillance program by U.S. Department of Agriculture isolated the same viruses in July 2009 (CDC, 2012[b]). Evidences of reassortant SIV in Thailand and probable zoonotic transmission have also been reported (Lekcharoensuk et al., 2010). Unlike the US, after the introduction of pandemic H1N1-2009 influenza virus, SIV-H3N2 in Thailand contained triple reassortant internal genes (TRIG) from pandemic H1N1-2009 influenza virus and envelope gene (HA and NA) from endemic SIV-H3N2 in Thai pig population (Charoenvisal et al., 2013).

As previous information, Thailand found all three major subtypes of SIV. Pigs can cause multiple cross-species transmission and the reassortant of SIV have been reported in Thailand. However, in Thailand, few information on molecular epidemiology of SIV and still intermittently reported. Swine influenza surveillance, isolation and genetic characterization combining serological study can provide information of a current status of swine influenza in Thailand. The result from our study can then be used for influenza prevention and control in both pig and human populations.

Research Questions

1. What are the occurrence and subtypes of swine influenza viruses in Thailand during 2011-2012 ?

2. Is there any evidence of genetic reassortment of swine influenza viruses in Thailand during 2011-2012 ?

Objective of study

To survey swine influenza viruses in Thailand during 2011-2012.

Chapter 2 Literature Review

Influenza A virus

Influenza virus is a member of the family *Orthomyxoviridae* and can be classified into 3 types (A, B and C). Influenza A virus can be found in human and many animal species such as birds, pigs, horses, dogs and marine mammals (Alexander and Brown, 2000). Whereas influenza B virus has been reported in human and seals (Osterhaus et al., 2000). Influenza C virus has been reported in human and pig (Yuanji et al., 1983).

Influenza A virus is 80-120 nm enveloped, negative sense, single-stranded RNA virus. The IAV has 8 RNA segments: HA (hemagglutinin), NA (neuraminidase), NP (nucleoprotein), M (matrix protein), NS (non-structural protein), PA (polymerase acidic), PB1 (polymerase basic1) and PB2 (polymerase basic2). The classification of virus is based on the viral surface proteins: hemagglutinin (HA) and neuraminidase (NA) (Webster et al., 1992). Recently, there have been 18 subtypes of HA and 11 subtypes of NA. Wild aquatic birds, well-known as natural host of influenza A viruses. They can be infected by most subtypes, while the newest subtypes (H17N10 and H18N11) were isolated from bat (CDC, 2015).

Swine influenza viruses

At present, three major subtypes of swine influenza A viruses: H1N1, H1N2 and H3N2 are found in pigs. Clinical illness by SIV in pigs are fever, depression,

coughing, nasal or ocular discharge, sneezing, eye redness or inflammation, and loss of appetite. Incubation period is 1-3 days and illness duration is about 4-7 days. Although SIV can cause high morbidity in pigs, it may result in low mortality. Moreover, SIV caused economic impact as a result of longer production period (Alexander and Brown, 2000).

SIV-H1N1 was initially isolated in 1930 in the USA (Shope, 1931). In Europe (British Isles), SIV-H1N1 can be isolated from pigs with respiratory signs during 1938 and 1940. The genetic analysis showed that the European SIV were more similar to human H1N1 influenza viruses than classical swine influenza viruses from the USA between 1931 and 1937 (Neumeier et al., 1994). In 1970s, human-like SIV-H3N2 have been reported in many countries; Italy (Ottis et al., 1982) and Hong Kong together with China (Shortridge and Webster, 1979).

During 1978-1999, SIV-H1N2 were reported in Japan, France, the United Kingdom and the United States. The viruses from Japan and France posed genes from the reassortment of swine influenza H1N1 viruses and human influenza H3N2 viruses, whereas SIV from the United Kingdom and the United States were the result of multiple reassortment from human, avian and swine influenza viruses (Karasin et al., 2000[a]).

In Thailand, in 1978, SIV-H3N2 was confirmed by virus isolation from nasal swab of pigs and serological study found that about 50% of pigs were infected with H3N2 swine influenza (Kanai et al., 1981). In 1988, SIV-H1N1 was isolated from

nasal swabs of pigs in Chonburi. Besides, in 1976, the serological surveillance from swine herds located near Bangkok indicated that SIV-H1N1 circulated in swine populations (Kupradinun et al., 1991). In 2003-2006, 12 SIV isolations were detected from the central and eastern part of Thailand. Three subtypes have been found H1N1 (n=4), H3N2 (n=7) and H1N2 (n=1) from Chonburi, Chachoengsoa, Saraburi, Ratchaburi and Nakhon Pathom. Furthermore, the SIV-H1N2 was firstly reported in Thailand (Chutinimitkul et al., 2008). During 2004-2005, serum samples of pigs from 35 provinces in Thailand were seropositive with swine influenza subtype H1N1 or H3N2 among 30% in fattening pigs and 52% in breeding sows. In 2004, 5 SIV isolations were consisted of subtype H1N1 (n=3) and H3N2 (n=2). In 2005, 14 isolates of subtypes H1N1 (n=9), H3N2 (n= 4) and H1N2 (n=1) have been reported in Thailand (Damrongwatanapokin et al., 2006). In 2004 and 2006, SIV-H3N2 7 isolates were obtained from Chonburi (n=2), Suphanburi (n=1), Nakhon Pathom (n=1) and Ratchaburi (n=3). In addition, these viruses were the first whole genome characterization of SIV-H3N2 in Thailand (Lekcharoensuk et al., 2010). In 2008, Takemae and co-worker (2011) reported two SIV-H1N1 from Ratchaburi and SIV-H3N2 from Saraburi. Then in 2009, ten SIV were isolated from Saraburi composed of H1N1 and H3N2. Serological study between 2008-2009 found seropositive with subtype H1N1 (22.15%) and H3N2 (17.47%) by ELISA test.

Reassortment of swine influenza viruses

Pigs can be infected by both avian influenza and human influenza viruses due to the presence of both avian (sialic acid-alpha-2,3-terminal saccharides (SA-alpha-2,3)) and human (SA-alpha-2,6) receptors in the respiratory tract of pigs. Thus, pigs are potential mixing vessels for influenza viruses (Brown, 2001). When influenza viruses from different species infect pigs, the viruses can reassort and new viruses with gene segments of avian, human and swine can emerge (Schultz et al., 1991). Double reassortant and triple reassortant influenza viruses from pigs have been reported in many countries such as China (Qi and Lu, 2006), the United States (Webby et al., 2000) and Canada (Karasin et al., 2000[b]). In Thailand, evidences of the reassortment of SIV were also reported (Karasin et al., 2000[b;] Chutinimitkul et al., 2008; Lekcharoensuk et al., 2010; Sreta et al., 2010; Charoenvisal et al., 2013).

Some evidences showed cross-species transmission between human and pig. Centers for Disease Control and Prevention (CDC) reported 35 cases of human infections with swine-origin influenza viruses in the United States from 2005 to 2011 (CDC, 2012[a]). Only in 2011, 12 cases with swine-origin influenza H3N2 virus infection were reported. This virus was the reassortment of 2 different influenza viruses. It contains M gene segment from pandemic H1N1-2009 viruses and the other gene segments from 1998-2011 triple reassortant SIV-H3N2 and the same viruses were isolated from SIV surveillance program in July, 2009 (CDC, 2012[b]). In contrast to the United States, the reassortment of SIV-H3N2 in Thailand were composed of TRIG

from pandemic H1N1-2009 virus and surface genes (HA and NA genes) from Thai endemic SIV-H3N2 in 2005. Moreover, after the introduction of pandemic H1N1-2009 viruses in Thai pigs, the reassortant influenza viruses in Thai pig population frequently occurred because the TRIG cassette can induce rapid antigenic drift and shift (Charoenvisal et al., 2013). Therefore, SIV surveillance program in Thailand should be regularly conducted. This surveillance program will provide important information for monitoring novel or reassorted influenza viruses with pandemic potential.

ID Screen® Influenza A Antibody Competitive ELISA Kit

The SIV have an influence on economic of farm as well as veterinary and public health concern. Swine influenza surveillance, isolation and genetic characterization combining serological study can provide occurrence of swine influenza in Thailand, which is necessary for monitoring, prevention and control the diseases in both pig and human population. The serological studies are important tools to detect the status of influenza virus infection in herds. There are many types of serum antibody assay, including hemagglutination inhibition (HI), serum-virus neutralization (SVN) and enzyme-linked immunosorbent assay (ELISA) (Ciacci-Zanella et al., 2010). The HI and SVN test will be used to identify specific subtypes of the viruses and the specific ELISAs (H1N1 and H3N2) showed poor sensitivity. Screening of the swine influenza viruses in pig population required higher sensitive tools than HI test, SVN test and specific ELISAs. Tse and co-worker (2012) evaluated performance of three commercial competitive ELISAs and revealed the sensitivity and specificity of

the ID Screen influenza A antibody competition ELISA (ID Vet-Innovative Diagnostics, France) were 95% and 79%, respectively. The ID Screen ELISA has the highest sensitivity percentage when compared with two other commercial ELISAs (Tse et al., 2012).

Chapter 3 Materials and Methods

The study consisted of 3 phases: **phase 1** Sample collection from high pig density provinces in Thailand; **phase 2** Virus isolation and serum testing and **phase 3** Genetic characterization of swine influenza viruses

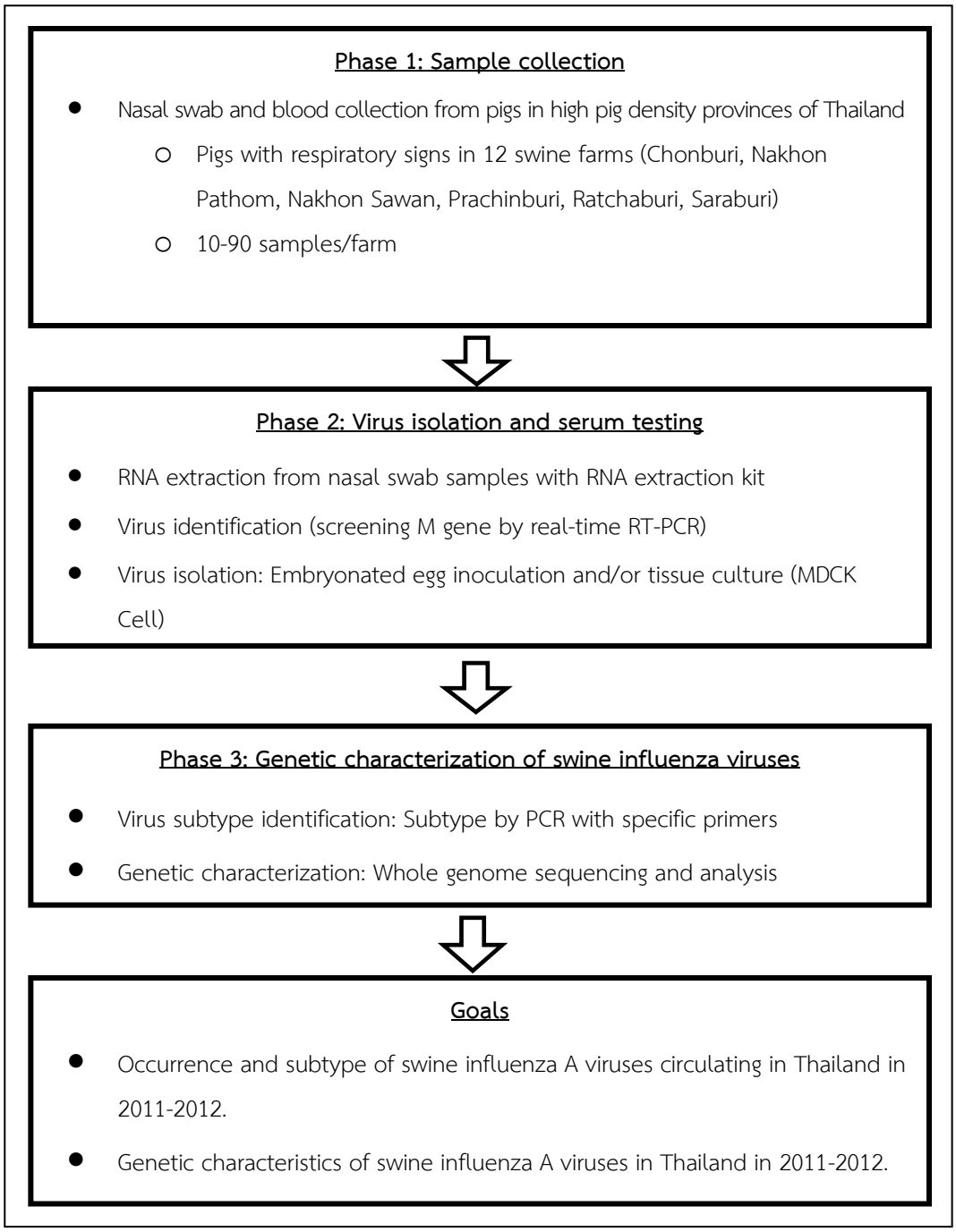

Figure 1 Flow chart of this study

Phase 1: Sample collection

Sample collection

Nasal swab and blood were collected from 12 pig farms in high pig density provinces during 2011-2012. The selection criteria of pig farms are history of respiratory problem in pig farms, farm location in high density of pig farm provinces of Thailand (Chonburi, Nakhon Pathom, Nakhon Sawan, Prachinburi, Ratchaburi, Saraburi) and farm owner participation. Seven hundred and forty-five nasal swab samples and five hundred and seventy-one blood samples were collected from pigs. In this study, pigs were classified into 5 groups ; weaning piglets (3-4 weeks), nursery (5-8 weeks), starter (9-12 weeks), mid-fattening (13-16 weeks) and finisher (more than 17 weeks).

The nasal swab samples were stored in a sterile plastic tube with 2 ml-viral transport media (VTM) at 4 $^{\circ}$C. Blood samples were stored in 10 ml syringe after collection. Sera were separated from blood at the laboratory. The samples were transferred to the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University.

Sample processing

All of the 745 nasal swab samples were centrifuged with 3,000 g x 30 seconds and divided to 2 parts for 1) RNA extraction and 2) egg inoculation and/or tissue culture. All parts were stored at -80°C until use.

All of the 571 blood samples were centrifuged with 5,000 g x 5 minutes to separate serum and divided to 2 parts for 1) NP-ELISA Test and 2) HI test. All parts were stored at -20°C until use.

Phase 2: Virus isolation and serum testing

Virus isolation

1. Nasal swab samples (150 µl) were extracted for RNA with a commercial RNA extraction kit, Silica-membrane technology, Nucleospin® RNA Virus (MACHEREY-NAGEL,Germany). (APPENDIX)
2. Real-time reverse transcription-polymerase chain reaction (rRT-PCR) was performed to detect Matrix (M) gene of influenza A virus. The rRT-PCR reaction was produced by SuperScript III Platinum® One-step RT-PCR system (Invitrogen, Carlsbad, CA, USA) using specific primers and probe (Spackman et al., 2002). (APPENDIX)
3. The rRT-PCR positive samples were proceeded to virus isolation by egg inoculation (WHO, 2002) and/or by the cell culture, Madin-Darby canine kidney (MDCK) cells (Kitikoon et al., 2006).

Egg inoculation

The supernatant of nasal swab sample was inoculated into eggs of 9-11 days old specific antibody negative embryonated chicken eggs and then incubated at 37ºC for 72 hours. After 72 hours of incubation, the

allantoic fluid was collected from each egg and performed hemagglutination test (HA test) by using 1% chicken RBC suspension. The HA positive samples were stored at -80°C until used.

Cell culture

The supernatant of nasal swab sample were prepared to 10-fold serial dilutions with medium, inoculated onto Madin-Darby canine kidney (MDCK) cells and incubated at 37 $^{\circ}$C with 5% CO_2 for 72 hours. The cell culture was observed for cytopathic effect (CPE). The CPE positive samples were stored at -80°C until used.

Serum testing

1. All 571 serum samples were tested with Competitive ELISA for the detection of anti-nucleoprotein (NP) antibodies of the Influenza A virus (ID Screen® Influenza A Antibody Competition MS Multi Species). For the preparation process, the serum samples were diluted with dilution buffer. Then the samples were incubated in 96-well microplates coated with Antigen A (Ag A) at 37°C (± 2°C) for 1 hour ± 5 min, followed by washing each well with 300 µl of the wash solution/wash for 5 times. After that, the 50 µl of the conjugate 1X was filled to each well and incubated for 30 min ± 2 min at 21°C (± 5°C). After an additional 3 times washing with the wash solution, filled 50 µl of the substrate solution to each well, followed by incubation

for 10 min ± 1 min at 21°C (± 5°C) in the dark room. Then the stop solution 50 µl was added before reading and record the O.D. at 450 nm. Both positive and negative controls were included. The ELISA results were interpreted by the competition percentage (competition %). The competition percentage for each sample was calculated from the formula:

Competition % = (O.D. specimen / O.D. negative control) × 100

Serum samples with the competition percentage less than or equal to 45% were determined positive, those of between 45% and 50% were determined suspect and those of greater than or equal to 50% were determined negative.

2. The ELISA positive samples were tested for specific antibody subtypes by Hemagglutination-inhibition (HI) test (Kitikoon et al., 2006). In this study, three subtypes of SIVs consist of H1N1 (A/swine/Thailand/CU-CB1/2006), pH1N1 (A/swine/Thailand/CU-RA29/2009) and H3N2 (A/swine/Thailand/CU-CB8.4/2007) were used as the antigens for HI test. Serum samples were separated into two parts. The first part of serum samples were treated with RDE for 20 hour at 37°C, followed by heat inactivation at 56°C for 1 hour. RDE-treated serum were then absorbed with 100 µl of 50% chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour for H3N2 HI test. The second part of serum samples were treated with 20%

Kaolin for 30 min at room temperature, followed by centrifuge at 2000 rpm for 10 min. 20% kaolin-treated sera were then absorbed with 100 µl of 50% chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour for H1N1 and pH1N1 HI test.

Phase 3: Genetic characterization of swine influenza viruses

cDNA synthesis

After virus isolation and propagation, RNA from the HA and/or CPE positive samples were reverse transcribed into cDNA (Viseshakul et al., 2004) (APPENDIX). Then the mixture tube was heated at 70°C for 15 minutes and chilled at 4°C for 5 minutes after that the mixture of reverse transcriptase enzyme was filled. Then the mixture was set in thermocycler under the condition of 25ºC for 5 minutes, 42ºC for 60 minutes and 72ºC for 15 minutes. The cDNA was kept at -20ºC until further use.

Virus subtype and characterization

The polymerase chain reaction (PCR) were performed to identify subtype of the viruses with specific primers for HA and NA genes from our inventory (Table1). Total 30 µl of PCR mixture, contained cDNA 1.5 µl, forward primer (10µM) 1.5 µl, reverse primer (10µM) 1.5 µl, 2X KAPA Taq Master Mix 15 µl and Distilled water 10.5 µl. The mixture was set in thermocycler under the condition of 94ºC for 3 minutes (pre-denaturation), 94ºC for 30 seconds (denaturation), 48ºC for HA primers or 45ºC or NA primers for 30 seconds (annealing), 72ºC for 30-60 seconds (extension) and 72ºC for 7 minutes (final extension).

Table 1 List of primers for SIVs subtyping in this study

Gene	Primer name	Size (bp)	Primer sequence (5'-3')
H1	SwH1_F736	20	agr atg rac tat tac tgg ac
	SwH1_R1014	24	cgg gay att cct yaa tcc tgt rgc
H3	SwH3_A70_F1	22	atg aag act atc att gct ttg a
	SwH3_A70_R453	18	ttg cag gaa ttg ctt gtt
N1	NA1_A89_F83	23	tcc aaa yca raa gat aat aac ca
	NA1_A89_R541	19	ctg acc aag cra ctg act c
N2	N2_C_F736	21	agt aat gac tga tgg aag tgc
	N2_D_R1449	20	ttt tct aaa att gcg aaa gc

For whole genome sequencing, 8 gene segments were amplified by PCR with specific primers (Hoffmann et al., 2001). The PCR products were verified by gel electrophoresis using 1.2% of agarose gel in 0.5x Tris Borate EDTA (TBE). The PCR products were submitted for nucleotide sequencing at 1st Base Laboratories, Malaysia. The nucleotide sequences of each gene were then validated and assembled by using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI, USA).

For phylogenetic analysis, HA and NA gene segments were compared with the reference SIVs available at the GenBank database. The reference nucleotide sequences were consisted of the viruses from different geographical origins (North America and Eurasia) and three host origins (swine, human and avian). The nucleotide sequences were aligned by using Muscle v.3.6. (Edgar, 2004). The phylogenetic analysis was performed by the MEGA v.6.0 with neighbor-joining algorithm with the

Kimura-2 parameter model applied to 1,000 replications of bootstrap (Tamura et al., 2007). The nucleotide sequences and deduced amino acids of each gene of the viruses were aligned and compared by using MegAlign software v.6.06 (DNASTAR Inc., Madison, WI, USA).

Chapter 4 Results

In this study, swine influenza surveillance was performed from September 2011 to March 2012. In total 745 nasal swab samples and 571 blood samples were collected from 756 pigs from 12 pig farms in 6 provinces. The nasal swab samples were processed for virus isolation by egg inoculation and/or the cell culture and subtype identification by PCR. Genetic characterization of the virus was performed by using whole genome sequencing and phylogenetic analysis. The serological study, blood samples were subjected to Competitive ELISA and HI tests for specific antibody subtype identification in Thai pig populations.

Samples for Swine Influenza Surveillance

During September 2011 to March 2012, nasal swab and blood samples were collected from 12 pig farms in 6 provinces with high density of pig farms in Thailand including Chon Buri, Nakhon Pathom, Nakhon Sawan, Prachinburi, Ratchaburi and Saraburi. (Table 2)

Swine Influenza Virus identification and isolation

All Nasal swab samples were subjected for rRT-PCR to detect Matrix (M) gene of influenza A virus. In total, rRT-PCR positive samples were 24.97% (186/745) (Figure 2 and table 3). In this study, only thirteen samples (1.74%) were successfully isolated including nine SIV isolates from Ratchaburi and four SIV isolates from Chon Buri. The highest percentage of rRT-PCR positived/suspected samples was observed in farm B

(96.15%: 25/26) in Chon Buri Province (Table3). It is noted that, all of thirteen SIVs were isolated from 3-4 weeks and 5-8 weeks age groups as shown in table 4. The pigs of 3-4 weeks age group had highest percentage of rRT-PCR positived/suspected samples (46.56% : 61/131). It is noteworthy that the samples from Farm G and Farm H were collected in separated time and found rRT-PCR positive in both first and second time of sample collection.

Table 2 Description of pig farms, provinces and sample collection period in this study

Farm	**Province**	**Sample collection period**
A	Nakhon Pathom	Sep 2011
B	Chon Buri	Sep 2011
C	Ratchaburi	Oct 2011
D	Chon Buri	Nov 2011
E	Ratchaburi	Jan 2012
F	Prachin Buri	Jan 2012
G	Chon Buri	Jan 2012, Mar 2012
H	Ratchaburi	Jan 2012, Feb 2012
I	Ratchaburi	Feb 2012
J	Nakhon Sawan	Feb 2012
K	Saraburi	Feb 2012
L	Ratchaburi	Mar 2012
Total 12 Farms	**6 Provinces**	**Sep 2011-Mar 2012**

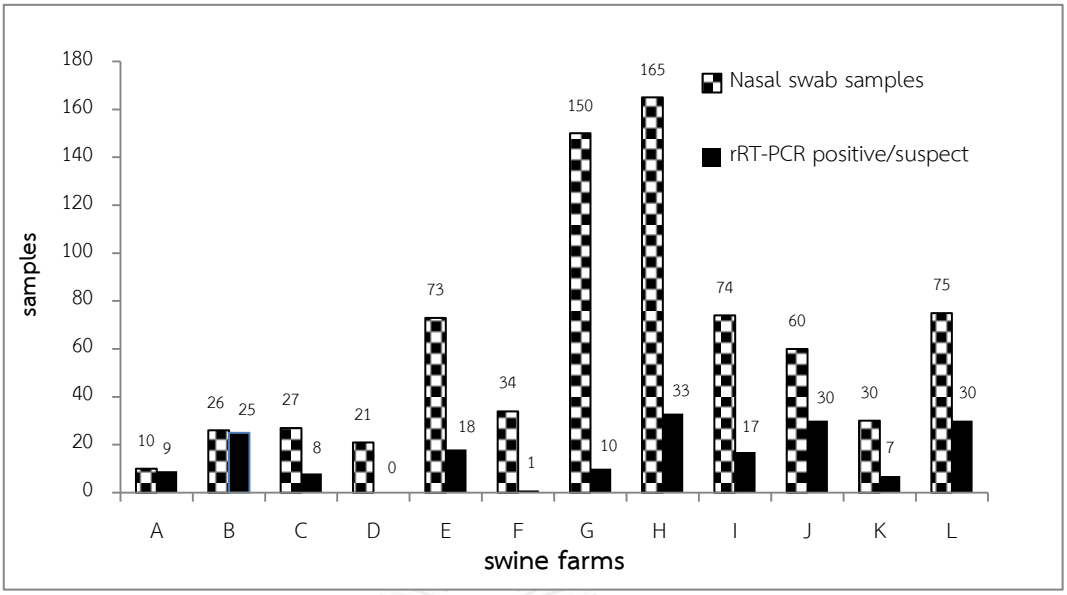

Figure 2 Number of nasal swab samples tested and rRT-PCR positived/suspected in each swine farm

Table 3 Number of nasal swab samples tested and rRT-PCR positived/suspected and SIV isolated in each swine farm

Farm	Province	Samples	rRT-PCR positive/suspect (%)	SIV isolates (%)
A	Nakhon Pathom	10	9 (90.00)	0
B	Chon Buri	26	25 (96.15)	0
C	Ratchaburi	27	8 (29.63)	1 (0.13)
D	Chon Buri	21	0 (0.00)	0
E	Ratchaburi	73	18 (24.66)	0
F	Prachin Buri	34	1 (2.94)	0
G	Chon Buri	150	10 (6.67)	4 (0.54)
H	Ratchaburi	165	33 (20.00)	8 (1.07)
I	Ratchaburi	74	17 (22.97)	0
J	Nakhon Sawan	60	30 (50.00)	0
K	Saraburi	30	7 (23.33)	0
L	Ratchaburi	75	28 (37.33)	0
Total 12 Farms	**6 Provinces**	**745**	**186 (24.97)**	**13 (1.74)**

Note : number in parentheses are percentage of positive samples in each farm

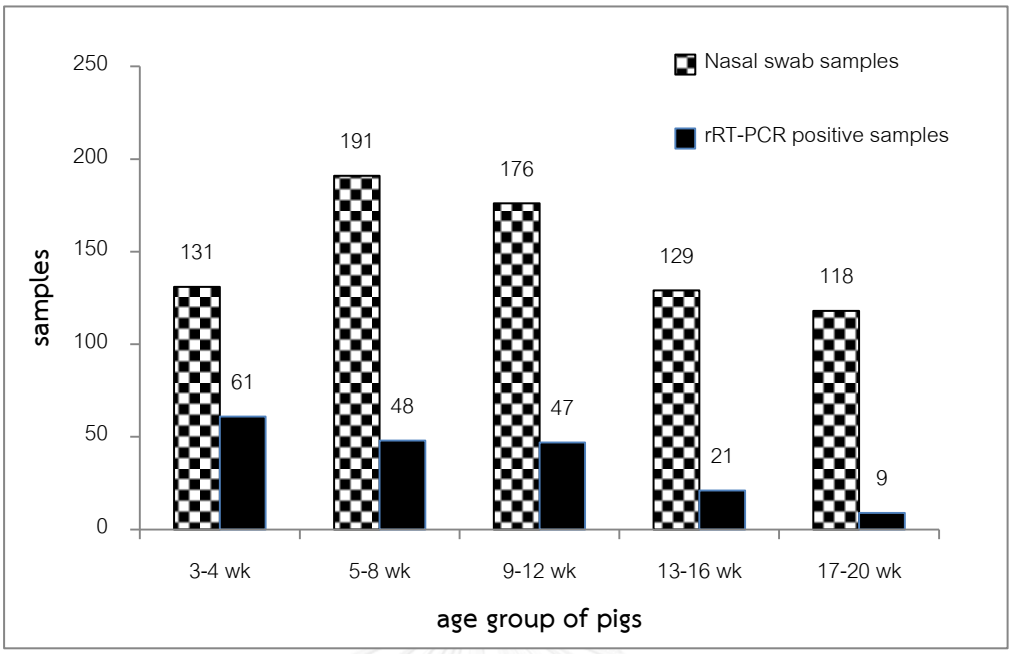

Figure 3 Number of nasal swab samples tested and rRT-PCR positived/suspected by age group of pigs

Table 4 Number of nasal swab samples tested, rRT-PCR positived/suspected and SIV isolated by age group of pigs

Group	Samples	rRT-PCR positive/suspect (%)	SIV isolates (%)
3-4 wk	131	61 (46.56)	6 (0.81%)
5-8 wk	191	48 (25.13)	7 (0.93%)
9-12 wk	176	47 (26.70)	0
13-16 wk	129	21 (16.28)	0
17-20 wk	118	9 (7.63)	0
Total 5 groups	**745**	**186 (24.97)**	**13 (1.74)**

Note : number in parentheses are percentage of positive samples in each age group

Swine Influenza antibodies

Total 571 pig sera from 10 farms were tested with competitive ELISA for the detection of anti-nucleoprotein (NP) antibodies of the Influenza A virus. In total, ELISA positive samples were 45.88% (262/571). It is noted that ELISA positive samples were 80% at farm level (8 out of 10 farms). The highest percentage of ELISA positive samples was observed in farm G (68.00%: 51/75) located in Chon Buri Province (Table5). The pigs of 17-20 weeks age group had highest percentage of ELISA positive (74.74%: 71/95) as shown in Table 6.

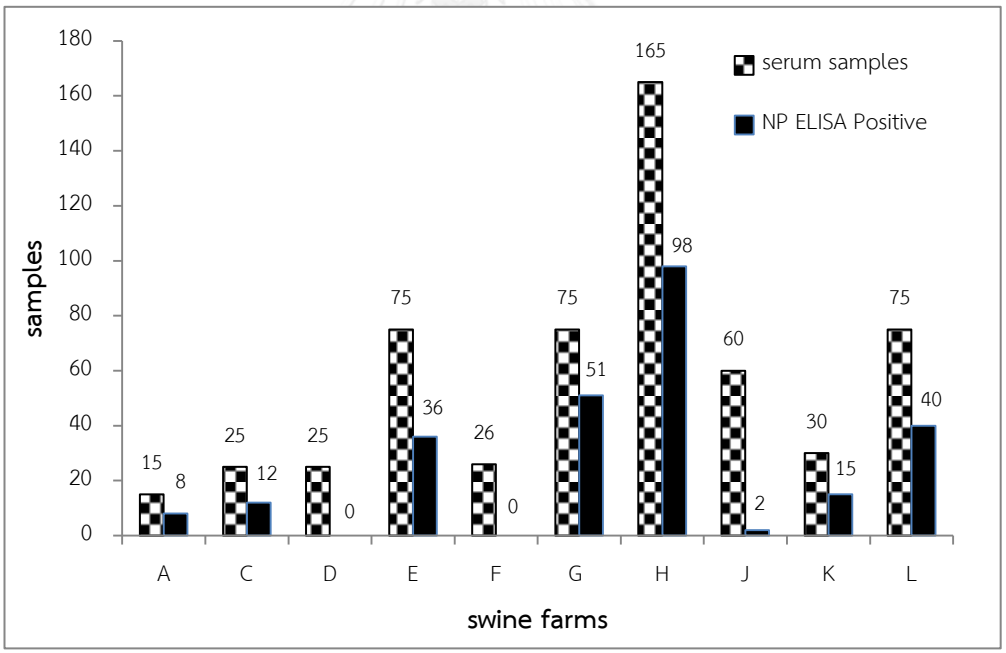

Figure 4 Number of serum samples tested and NP ELISA positived samples in each swine farm

Table 5 Number of serum samples tested, NP ELISA positived in each swine farm

Farm	Province	Samples	NP ELISA Positive (%)
A	Nakhon Pathom	15	8 (53.33)
C	Ratchaburi	25	12 (48.00)
D	Chon Buri	25	0 (0.00)
E	Ratchaburi	75	36 (48.00)
F	Prachin Buri	26	0 (0.00)
G	Chon Buri	75	51 (68.00)
H	Ratchaburi	165	98 (59.39)
J	Nakhon Sawan	60	2 (3.33)
K	Saraburi	30	15 (50.00)
L	Ratchaburi	75	40 (53.33)
Total 10 Farms	**6 Provinces**	**571**	**262 (45.88)**

Note : number in parentheses are percentage of positive samples in each farm

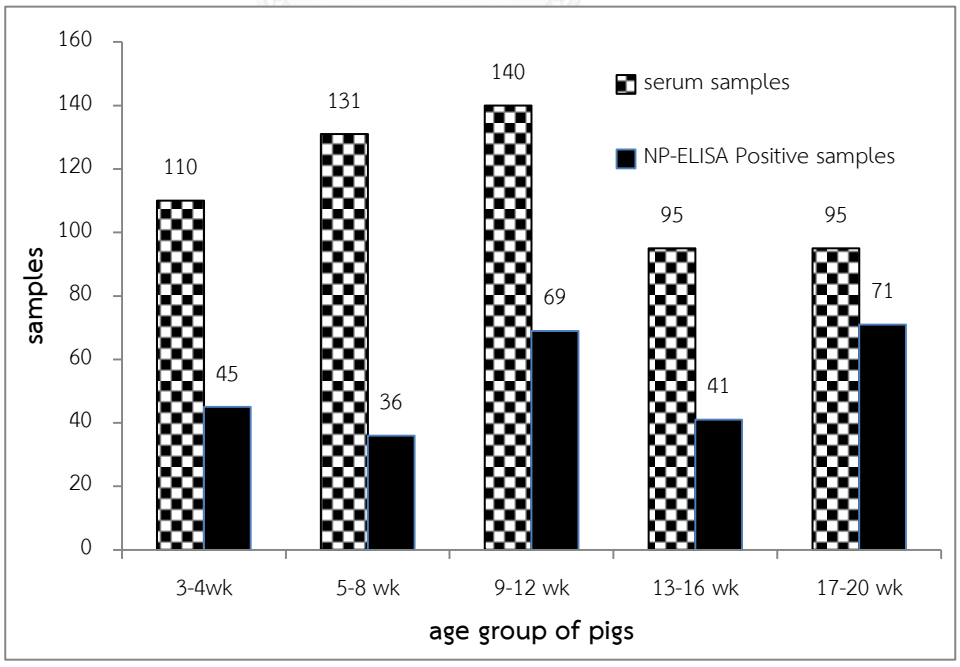

Figure 5 Number of serum samples tested and NP ELISA positived by age group of pigs

Table 6 Number of serum samples tested and NP ELISA positived by age group of pigs

Group	Samples	NP-ELISA Positive (%)
3-4 wk	110	45 (40.91)
5-8 wk	131	36 (27.48)
9-12 wk	140	69 (49.29)
13-16 wk	95	41 (43.16)
17-20 wk	95	71 (74.74)
Total 5 groups	**571**	**262 (45.88)**

Note : number in parentheses are percentage of NP-ELISA positive samples in each age group

In this study, 262 ELISA positived sera were subjected to HI test for the detection of specific antibodies SIV-H1N1 (swH1N1), pandemic H1N1-2009 (pH1N1) and SIV-H3N2 (swH3N2) subtypes. HI test result showed that Thai pigs had antibodies to swH3N2 15.41% (88/571), pH1N1 4.90% (28/571) and swH1N1 2.45% (14/571). It is noted that 12.78% (73/571) of pig sera were positive with more than one SIV subtypes (Table 7).

Table 7 Number of serum samples tested, NP ELISA and HI test positived in each swine farm

Farm	serum samples	NP ELISA positive samples	HI Test							
			swH1N1	pH1N1	swH3N2	swH1N1 &pH1N1	swH1N1& swH3N2	pH1N1& swH3N2	swH1N1& pH1N1&swH3N2	Negative all of 3 subtypes
A	15	8	5	-	-	1	-	-	-	2
C	25	12	-	6	-	1	-	-	2	3
D	25	0	-	-	-	-	-	-	-	-
E	75	36	-	-	17	1	1	9	4	4
F	26	0	-	-	-	-	-	-	-	-
G	75	51	1	12	9	7	-	3	2	17
H	165	98	7	2	57	5	2	5	4	16
J	60	2	-	-	-	-	-	-	1	1
K	30	15	-	4	4	1	-	4	1	1
L	75	40	1	4	1	13	1	1	4	15
Total	**571**	**262**	**14**	**28**	**88**	**29**	**4**	**22**	**18**	**59**

Table 8 Number of serum samples tested, NP ELISA and HI test positived by age group of pigs

Age group	Serum samples	NP ELISA positive samples (%)	HI Test							
			swH1N1	pH1N1	swH3N2	swH1N1& pH1N1	swH1N1& swH3N2	pH1N1& swH3N2	swH1N1&pH1N1& swH3N2	Negative all of 3 subtypes
3-4 wk	110	45 (40.91)	1	2	17	4	1	7	6	7
5-8 wk	131	36 (27.48)	0	9	17	0	0	0	0	10
9-12 wk	140	69 (49.29)	9	8	18	7	1	2	1	23
13-16 wk	95	41 (43.16)	3	5	9	11	1	2	0	10
17-20 wk	95	71 (74.74)	1	4	27	7	1	11	11	9
	571	**262**	**14**	**28**	**88**	**29**	**4**	**22**	**18**	**59**

Genetic characteristics of Thai swine influenza viruses

In this study, thirteen SIV viruses were as H1N1 (n=10) and H1N2 (n=3) and four SIV isolates (S3073N, S3248N, S3334N and S3350N) were selected for whole genome sequencing (Figures 6, 7 and 8).

Table 9 Description of swine influenza A viruses isolated from this study

Sample ID	Collection Time	Age (weeks)	Farm	Province	Subtype
S3073N	Oct-11	4	C	Ratchaburi	H1N2
S3243N	Jan-12	8	H	Ratchaburi	H1N1
S3245N	Jan-12	8	H	Ratchaburi	H1N1
S3246N	Jan-12	8	H	Ratchaburi	H1N1
S3248N	Jan-12	8	H	Ratchaburi	H1N1
S3334N	Jan-12	8	G	Chon Buri	H1N1
S3340N	Jan-12	8	G	Chon Buri	H1N1
S3343N	Feb-12	4	H	Ratchaburi	H1N1
S3345N	Feb-12	4	H	Ratchaburi	H1N1
S3347N	Feb-12	4	H	Ratchaburi	H1N1
S3350N	Feb-12	8	H	Ratchaburi	H1N1
S1027N	Mar-12	4	G	Chon Buri	H1N2
S1032N	Mar-12	4	G	Chon Buri	H1N2

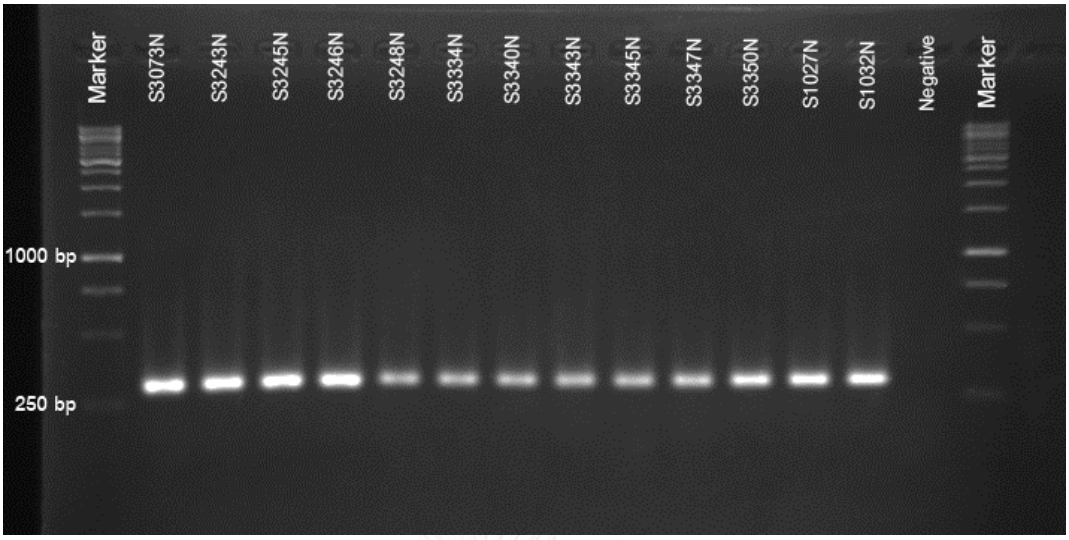

Figure 6 H1 identification by PCR assay using subtype specific primer; Marker: 1,000-bp marker, H1: expected PCR product 278 bp

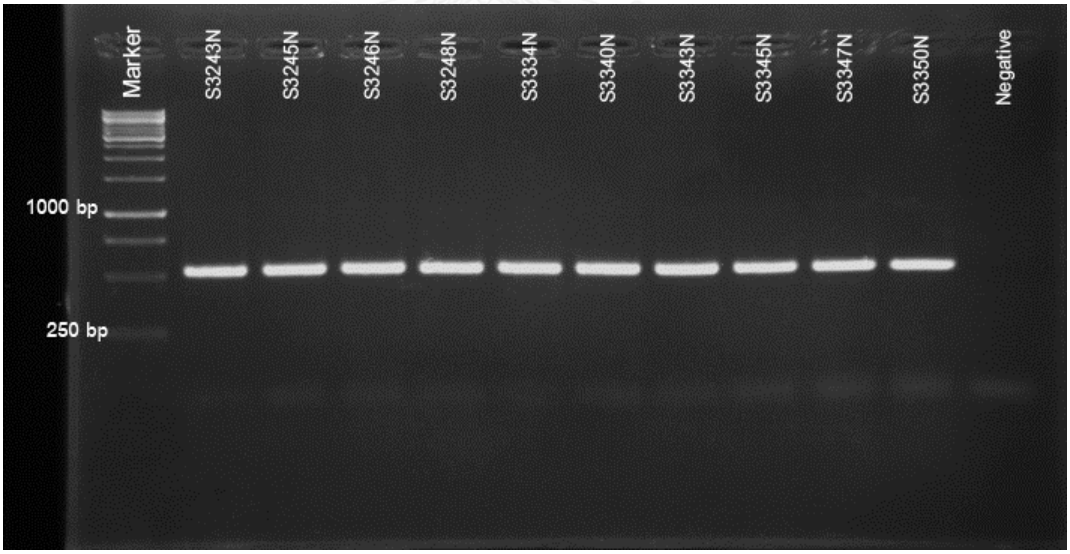

Figure 7 N1 identification by PCR assay using subtype specific primer; Marker: 1,000-bp marker, N1: expected PCR product 541 bp

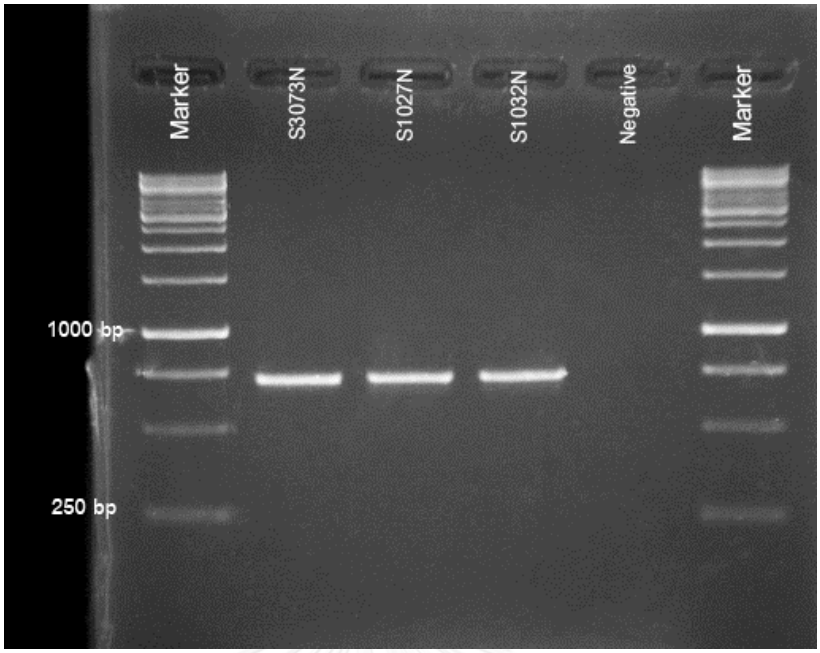

Figure 8 N2 identification by PCR assay using subtype specific primer; Marker: 1,000-bp marker, N2: expected PCR product 713 bp

Table 10 Nucleotide identities of each gene of S3073N (H1N2) comparing to reference nucleotide sequences available in GenBank

Gene	Position	Virus with the highest percentage of nucleotide identity	GenBank accession number	% Nucleotide identity
PB2	1-2280	A/Toronto/T5362/2009(H1N1)	KM355361.1	99%
PB1	1-2274	A/Hong Kong/H090-779-V10/2009(H1N1)	KM355362.1	99%
PA	1-2151	A/Hong Kong/H090-787-V10/2009(H1N1)	KM355363.1	99%
HA	1-1701	A/Singapore/GP1142/2009(H1N1)	KJ162042.1	98%
NP	1-1435	A/swine/Thailand/UD402/2009(H1N1)	KM355364.1	99%
NA	1-1410	A/swine/Ratchaburi/NIAH59/2004(H3N2)	KJ162043.1	96%
M	1-982	A/Toronto/3141/2009(H1N1)	KM355365.1	99%
NS	1-838	A/New York/3753/2009(H1N1)	KM355366.1	99%

Table 11 Nucleotide identities of each gene of S3248N (H1N1) comparing to reference nucleotide sequences available in GenBank

Gene	Position	Virus with the highest percentage of nucleotide identity	GenBank accession number	% Nucleotide identity
PB2	1-2280	A/swine/Thailand/PB485/2009(H3N2)	KJ162015.1	98%
PB1	1-2262	A/swine/Thailand/PB483/2009(H3N2)	KJ162016.1	98%
PA	1-2141	A/swine/Thailand/CM480/2009(H3N2)	KJ162017.1	98%
HA	1-1701	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	KJ162018.1	95%
NP	1-1484	A/swine/Thailand/PB486/2009(H3N2)	KJ162019.1	98%
NA	1-1412	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	KJ162020.1	95%
M	1-982	A/swine/Ratchaburi/NIAH550/2003(H1N1)	KJ162021.1	98%
NS	1-844	A/swine/Thailand/CM480/2009(H3N2)	KJ162022.1	98%

Table 12 Nucleotide identities of each gene of S3334N (H1N1) comparing to reference nucleotide sequences available in GenBank

Gene	Position	Virus with the highest percentage of nucleotide identity	GenBank accession number	% Nucleotide identity
PB2	1-2310	A/swine/Thailand/CU-SPL2/2010(H1N1)	KJ162027.1	99%
PB1	1-2281	A/swine/Thailand/CU-SPL4/2010(H1N1)	KJ162028.1	99%
PA	1-2223	A/swine/Thailand/CU-PS73/2010(H1N1)	KJ162029.1	99%
HA	1-1659	A/swine/Thailand/CU-PS73/2010(H1N1)	KJ162030.1	99%
NP	1-1497	A/swine/Thailand/CU-CBP18/2009(H1N1)	KJ162031.1	99%
NA	1-1410	A/swine/Thailand/CU-PS73/2010(H1N1)	KJ162046.1	99%
M	1-968	A/swine/Thailand/CU-PS73/2010(H1N1)	KJ162032.1	99%
NS	1-842	A/swine/Thailand/CU-PS73/2010(H1N1)	KJ162033.1	99%

Table 13 Nucleotide identities of each gene of S3350N (H1N1) comparing to reference nucleotide sequences available in GenBank

Gene	Position	Virus with the highest percentage of nucleotide identity	GenBank accession number	% Nucleotide identity
PB2	1-2280	A/swine/Thailand/PB485/2009(H3N2)	KJ162034.1	98%
PB1	1-2298	A/swine/Thailand/PB483/2009(H3N2)	KJ162035.1	98%
PA	1-2151	A/swine/Thailand/CM480/2009(H3N2)	KJ162036.1	98%
HA	1-1701	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	KJ162037.1	95%
NP	1-1497	A/swine/Thailand/PB486/2009(H3N2)	KJ162038.1	98%
NA	1-1410	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	KJ162039.1	95%
M	1-982	A/swine/Ratchaburi/NIAH550/2003(H1N1)	KJ162040.1	98%
NS	1-838	A/swine/Thailand/CM480/2009(H3N2)	KJ162041.1	98%

In detail, four SIVs (S3073N, S3248N, S3334N and S3350N) were subjected to whole genome sequencing. The remaining eight SIVs were subjected for HA gene sequencing and six SIVs were subjected for NA gene sequencing. The nucleotide sequences of four SIVs were submitted to the GenBank and analyzed by using the NCBI nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the genetic relatedness of SIVs from this study with other reference SIVs available at the GenBank database (Table 10-13). The results showed that most of SIVs from this study were related to Thailand SIVs except the sample S3073N most of genes related to human influenza viruses. It is noted that influenza viruses transmission between human and pigs were occurred.

For H1 phylogenetic analysis, the nucleotide sequences of twelve H1 gene of this study and the other H1 subtype nucleotide sequences from Eurasian and Classical Swine lineages were performed for phylogenetic analysis. The phylogenetic tree showed that all H1 of SIVs in this study were grouped in classical swine lineage (Figure 9). The H1 sequences of SIVs-H1N1 in this study closely related to endemic Thai SIVs (A/swine/Thailand/CU-RA20/2009(H1N1), A/swine/Thailand/CU-RA204/ 2010(H1N1) and A/swine/Ratchaburi/NIAH1481/2000(H1N1)). In contrast, the H1 sequences of SIVs subtype H1N2 in this study closely related to pandemic H1N1-2009 viruses (A/swine/Thailand /CU-RA15/2010(H1N1)) (Figure 9).

For N1 phylogenetic analysis, N1 of SIVs from this study were grouped in the eurasian swine lineage (Figure 10). All N1 gene sequences closely related to endemic Thai SIVs (A/swine/Ratchaburi/NIAH550/2003(H1N1) and A/swine/Thailand/CU-RA20 /2009(H1N1)) (Figure 10).

For N2 phylogenetic analysis, N2 of SIVs from this study were grouped with the N2 viruses in the triple reassortants lineage (Figure 11). N2 gene sequences were closely related to A/swine/Korea/Hongsong2/2004(H1N2).

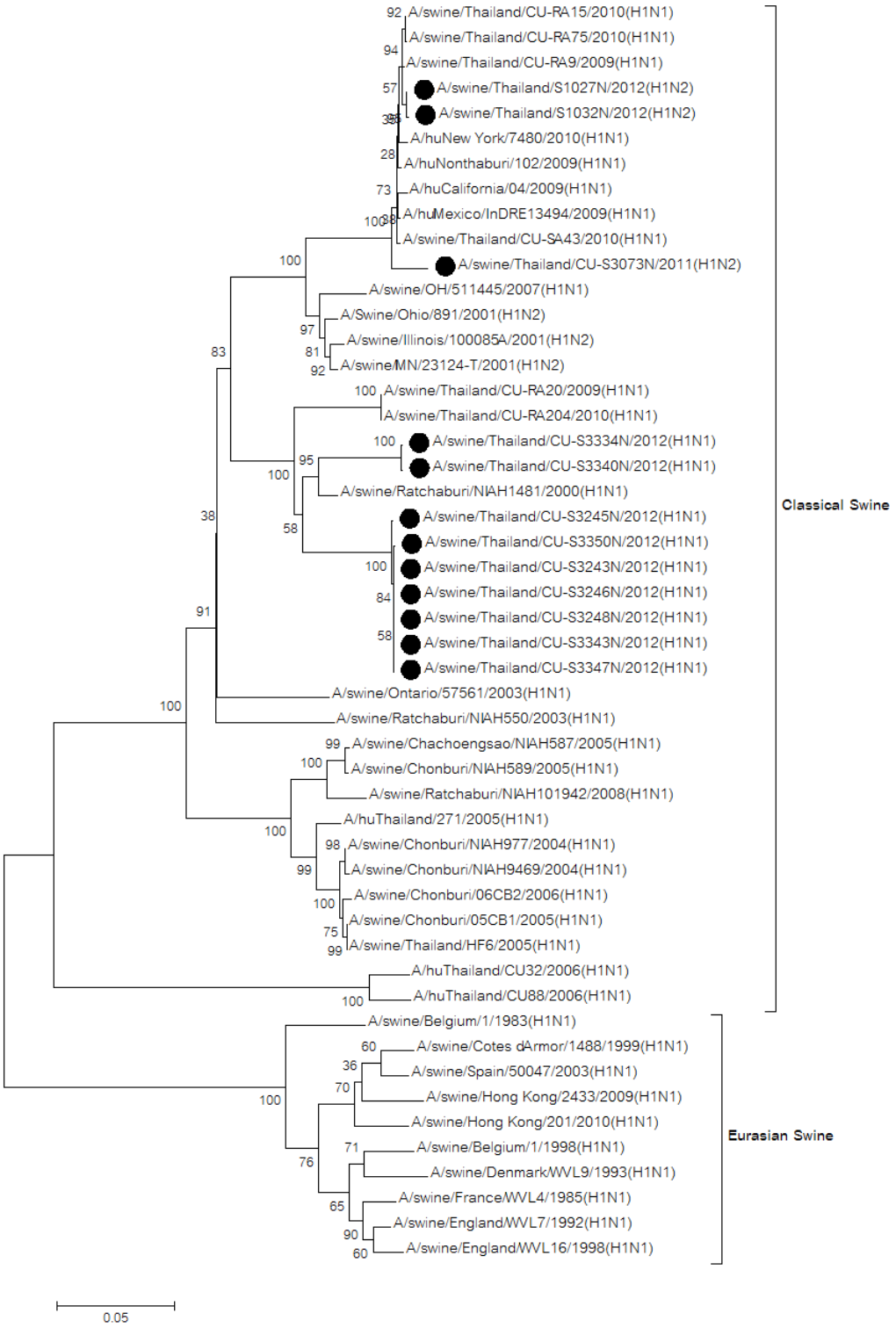

Figure 9 Phylogenetic analysis of the H1 gene of twelve SIVs from this study; Circle indicate HA gene of this study

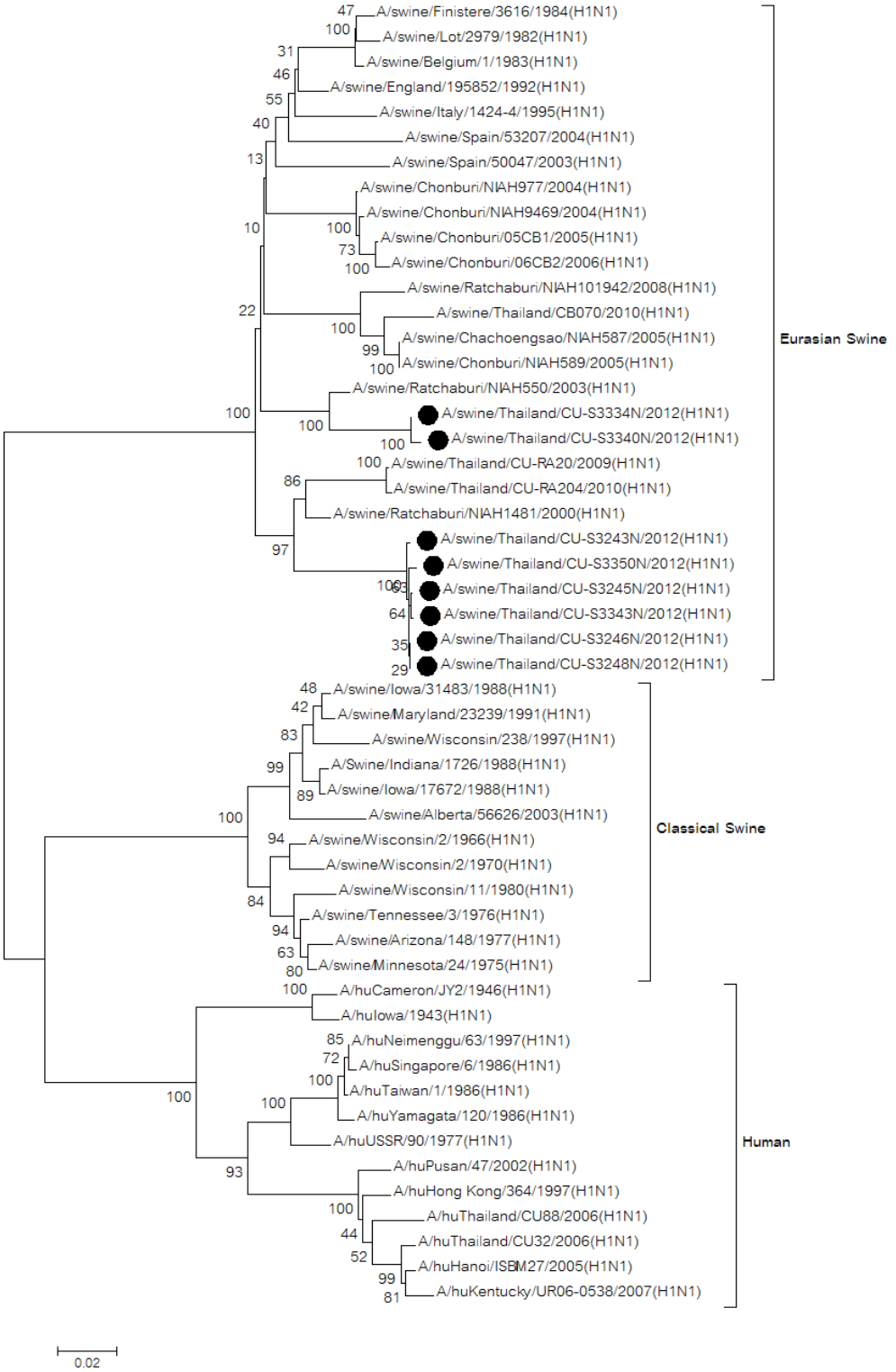

Figure 10 Phylogenetic analysis of the N1 gene of eight SIVs from this study; Circle indicate NA gene of this study

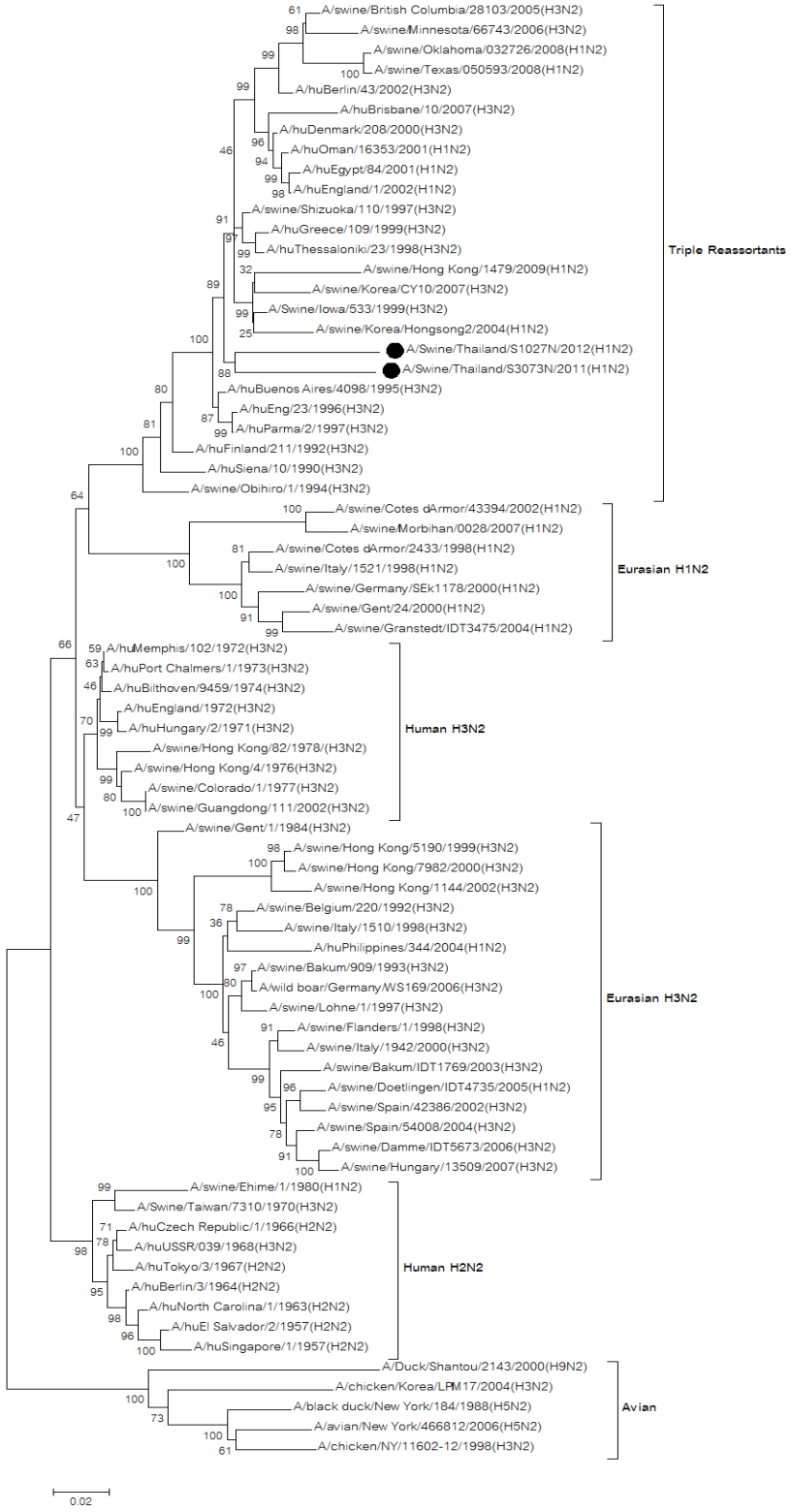

Figure 11 Phylogenetic analysis of the N2 gene of two SIVs from this study; Circle indicate NA gene of this study

Assessment of swine farms and status of swine influenza infection

In this study, 9 out of 12 swine farms were the open-housing with continuous production system in medium to large scale farms. While 3 swine farms, farm C, K and L, have All-In/All-Out production system. From swine influenza surveillance in this study, in farms with All-In/All-Out production system, we found 23.33 - 37.33 % rRT-PCR positive samples. On the other hand, in farms with continuous production system, we found wide range of rRT-PCR positive samples (2.94 - 96.15%) (Table 3). Serological result by NP-ELISA test showed that farms with All-In/All-Out production system had 48.00 - 53.33% sero-positive, while the continuous production system had 3.33-68.00% sero-positive. It is noted that herd immunity in All-In/All-Out production system are more stable than continuous production system.

Chapter 5 Discussion

1. Occurrence and subtypes of swine influenza viruses during 2011-2012

The occurrence of swine influenza viruses in Thailand during September 2011 - March 2012 was 1.74%. Our result showed that the occurrence of SIVs was similar to other studies such as SIVs in June 2008 - November 2009 as 1.6% (Takemae et al., 2011) and SIVs in June 2010 – May 2012 as 1.75% (Charoenvisal et al., 2013). At farm level, SIVs were isolated from 3 out of 12 pig farms (25%). Our finding showed lower percentage of positive farms than previous study during June 2008 - November 2009 with 50% SIVs positive farms. In this study, the virus isolation rates were 4.58% in pigs at 3-4 weeks of age, 3.66% in pigs at 5-8 weeks of age and none in pigs age more than 9 weeks. This findings differed from prior study that virus isolation rates were 4.2% of piglets aged 3 to 5 weeks, 4.2% in piglets aged 6 to 10 weeks, 0.5% of fattening pigs aged 12 to 16 weeks and none of pigs aged more than 18 weeks were positive for SIVs (Takemae et al., 2011). This information suggested that piglets at 4 – 8 weeks of age is the high-risk age group of SIV infection. Thus sample collection for SIV surveillance should be focused on pigs at these age groups. The possible explanation for high SIV infection at piglets of 4-8 weeks consist 3 risk factors; 1) decrease in maternally derived antibodies 2) stress due to change new environment and crowed condition and 3) exposed to growing pigs that may carrying the viruses. In this study, 12 out of 13 SIVs were isolated from swine farms of open-housing with

the continuous production system in the medium to large scale, while one isolate (S3073N) was collected from farm of close housing (evaporation cooling system) with All-In/All-Out system. This suggested that SIVs circulation affected by type of housing and production system.

SIVs isolated in this study included H1N1 (n=10) and H1N2 (n=3) subtypes, which were differed from previous study in 2008 - 2009 with H1N1 and H3N2 subtypes circulation in Thailand (Takemae et al., 2011). Similarly, SIV surveillance in 2010 – 2012, SIVs subtype H1N1, H3N2 and H1N2 were identified (Charoenvisal et al., 2013). In 2011, SIVs subtype H1N1 and H3N2 were isolated from healthy weaning pigs, this finding was remarkable that pig farm workers should pay attention to prevent influenza infection by using Personal Protective Equipment (PPE) (Hiromoto et al., 2012).

2. Genetic characteristics of swine influenza viruses isolated during 2011-2012

In this study, Thai SIV-H1N1 contained H1 and NS genes of classical swine lineage and other 6 genes (N1, M, NP, PA, PB1 and PB2 genes) of Eurasian swine. This observation suggested that SIV-H1N1 isolated in this study had genetic characteristics of endemic swine influenza in Thailand. On the other hand, Thai SIV-H1N2 contained H1, NS, M, NP, PA, PB1 and PB2 genes of pandemic H1N1-2009 and N2 gene of endemic H3N2 virus. This finding suggested that SIV-H1N2 is reassortant viruses from pandemic H1N1-2009 virus and endemic SIV-H3N2.

In general, Thai endemic SIV-H1N1 circulating in Thailand has two patterns of genetic composition. The first pattern is endemic SIV-H1N1 (7+1), which consist of H1 gene from the classical swine lineage and seven other genes from Eurasian swine lineage. The second pattern is endemic SIV-H1N1 (6+2), which consist of H1 and NS genes from the classical swine lineage and the six other genes from Eurasian swine lineage (Kitikoon et al., 2011). Both patterns of SIV-H1N1 were found circulating in swine populations in Thailand since 2005. Then endemic SIV-H1N1 (7+1) were disappeared from Thai pig populations, whereas endemic SIV-H1N1 (6+2) was consistently existed (Nonthabenjawan et al., 2015). In this study, three representative SIV-H1N1 isolates (S3248N, S3334N and S3350N) contained H1 and NS genes of classical swine lineage and six other genes of the Eurasian swine lineage similar to former study in 2009 – 2010 (Kitikoon et al., 2011) (Figure 12). In contrast, the study in 2009 – 2012 SIV-H1N1 isolates in Japan were originated from the pandemic H1N1-2009 virus and demonstrated high identities with human pandemic H1N1-2009 viruses (Matsuu et al., 2012).

Phylogenic analysis of the N1 gene showed that all SIVs-H1N1 in this study belonged to Eurasian swine lineage and resembled prior Thai swine viruses. Phylogenic analysis of the N2 gene showed that two SIVs-H1N2 belonged to triple reassortant lineage from triple reassortants of human, avian and swine Influenza viruses. In contrast, a study in Poland, the N2 gene was grouped with Eurasian SIV-H3N2 (Kowalczyk et al., 2012).

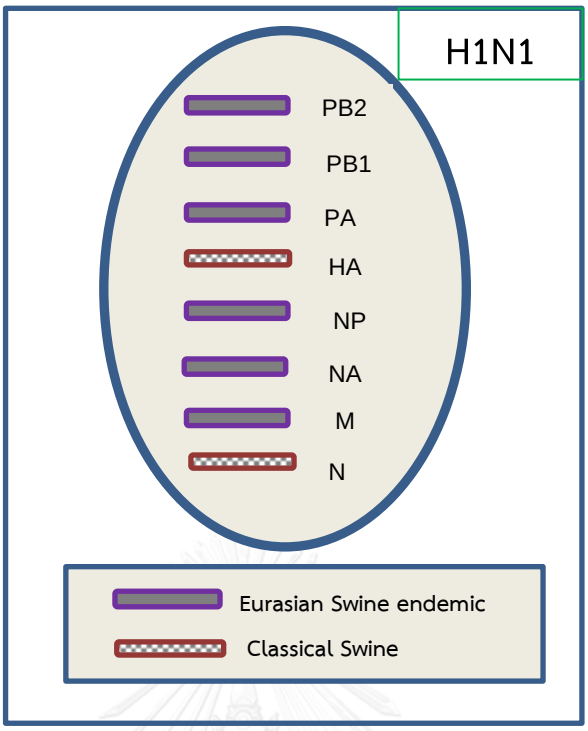

Figure 12 Representative swine influenza viruses subtype H1N1 in this study contained H1 and NS genes from the classical swine lineage and the six other genes from the Eurasian swine lineage; endemic H1N1 SIV (6+2)

3. Evidence of genetic reassortment of swine influenza viruses during 2011-2012

The genetic pattern of endemic SIV-H1N2 in Thailand in 2005 contained five genes (PB2, PB1, PA, NP and M) from the Eurasian swine lineage, two genes (HA and NS) from the classical swine lineage and NA gene of Human origin (Takemae et al., 2008). While in this study, the representative SIV-H1N2 isolate (S3073N) contained seven genes from the pandemic H1N1-2009 and N2 gene from endemic H3N2 viruses (Figure 13). In Japan, 2009-2012, the reassortant SIV-H1N2 contained HA and NA genes of Japanese SIVs and internal genes (PB2, PB1, PA, NP, M and NS) of pandemic H1N1-

2009 virus (Matsuu et al., 2012). In Poland, 2011, the first isolate of SIV-H1N2 was reassortant of Eurasian Human-like swine H1N1 and Eurasian human-like swine H3N2 origin (Kowalczyk et al., 2012).

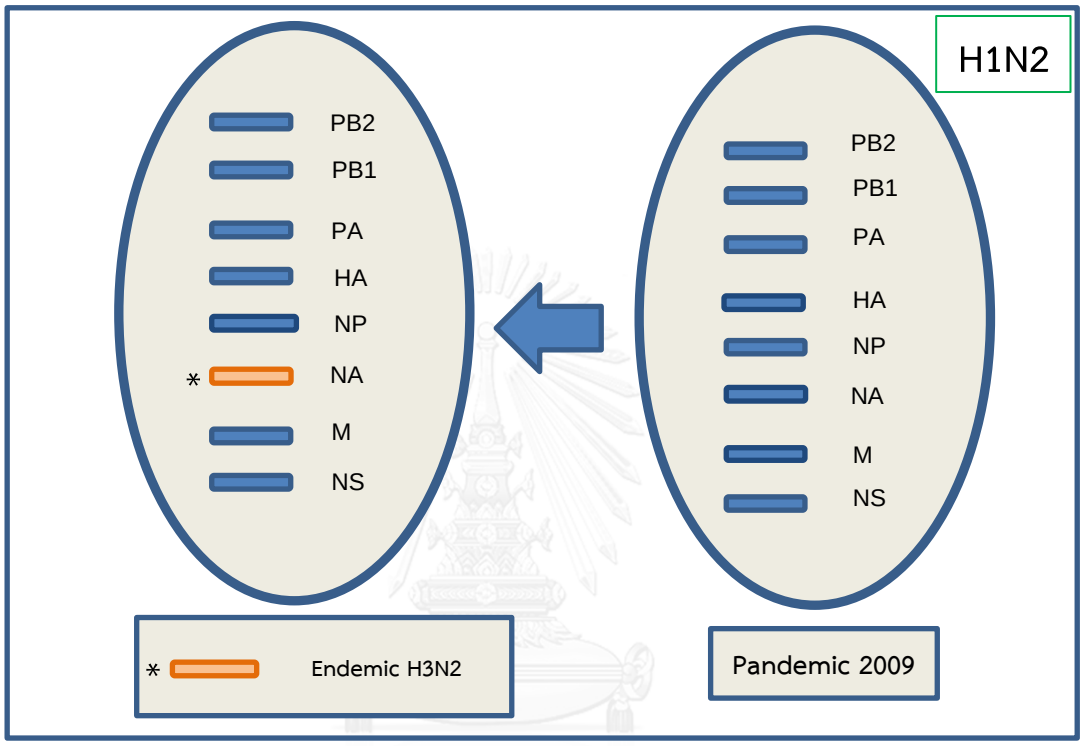

Figure 13 Representative swine influenza viruses subtype H1N2 in this study contained seven genes from the pandemic H1N1-2009 and N2 gene from endemic H3N2.

In this study, SIV-H1N2 was isolated from 4 week-old piglets in closed housing (Evaporation cooling system) farm with an All-In/All-Out system. This result suggested that the possibility of human-to-pig transmission in swine farm. Since, the reassortant SIV-H1N2 contained the internal genes from pandemic H1N1-2009 virus, which may from human pandemic viruses. It should be noted that the triple reassortant internal gene (TRIG) of pandemic H1N1-2009 virus could be an important factor for genetic

reassortment of influenza viruses. Thus, the result from our study indicated reassortment of swine influenza viruses in Thailand during 2011-2012.

4. Serological status of swine influenza infection during 2011-2012

Serological study indicated that 80% (8/10 farms) of pig farms were seropositive. At animal level, 45.88% of pigs was seropositive, which was higher than previous study in 2004 - 2005 with 55% of pig farms seropositive and 31.8% of pig seropositive (Damrongwatanapokin et al., 2006). In this study, 2 pig farms were negative for SIV, this may related to farm management system (All-In/All-Out system). Base on age of pigs, serological results showed that nursery piglets (4-8 weeks) had 0.41% HI positive titer for SIV-H1N1, 4.56% for pandemic H1N1-2009, and 14.11% for SIV-H3N2. In this study, the positive HI titer for SIV-H1N1 from nursery piglets was lower than previous study in 2010, which 38% positive for SIV-H1N1. The positive HI titer for pandemic H1N1-2009 was lower than previous study, which 9.5% positive for pandemic H1N1-2009 (Sreta et al., 2010). Our result showed that the immunity of SIV subtypes in Thai pig populations is vary and may depend on dominant SIV subtypes during study period.

It is noted that ELISA test, HI test and virus isolation could provide sufficient information for the status of SIV infection including SIV subtype and antibodies against SIV. However, microneutralization (MN) assay can help confirmation of SIV subtype and antibodies against the viruses. MN assay is an assay used to detect the level of

neutralizing antibody in serum sample. The neutralization assay can detect low level of influenza-specific antibodies and show higher sensitivity percentage than HI test (Kitikoon and Vincent, 2014).

Conclusions and suggestions

Our results indicated that in 2011-2012, SIV-H1N1 and SIV-H1N2 were circulating in Thai pig populations. The endemic SIV-H1N1 was the dominant SIV subtype. Serological study showed antibodies against SIV-H3N2, SIV-H1N1 and pandemic H1N1-2009. In addition, this study showed evidence of genetic reassortment of SIVs during 2011-2012. The proper age of pigs for virus detection is 4-8 weeks-old. Most of infected swine farms are middle to large scale with open housing and continuous production system that made a great impact on SIVs infection in pig population.

The result from this study lead to the suggestion on swine influenza prevention and control in swine farms as following

1.) Routine SIV surveillance should be conducted in Thailand. The information from SIV surveillance could help monitoring novel reassortant influenza viruses with public health concern.

2.) Appropriate personal hygiene and Personal protective equipment (PPE) are recommended for veterinarian and pig farm workers who usually contact with pigs. Since human and animal influenza viruses could be transmitted between human and pigs.

3.) Flu-like symptoms workers should avoid contact with pigs to decrease the risk of introducing new viral genes into pig population. Since our study

showed the evidence of new reassortant H1N2 viruses in pigs originated from human pandemic H1N1-2009.

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APPENDIX

1. Protocol for RNA extraction

1.1 Added 600 µl Lysis Buffer RAV1 mixed 150 µl of the sample, incubated for 5 min at 70 $^{\circ}$C.

1.2 Added 600 µl ethanol (96-100%) and mixed by vortexing for 10-15 seconds.

1.3 Added 700 µl of mixture to the NucleoSpin® RNA Virus Columns with the collection tube and centrifuged for 1 minute at 8,000 x g.

1.4 Added 500 µl Buffer RAW to NucleoSpin® RNA Virus Columns with the collection tube and centrifuged for 1 minute at 8,000 x g.

1.5 Added 600 µl Buffer RAV3 to NucleoSpin® RNA Virus Columns with the collection tube and centrifuged for 1 minute at 8,000 x g.

1.6 Added 200 µl Buffer RAV3 to NucleoSpin® RNA Virus Columns with the collection tube and centrifuged for 2-5 minutes at 11,000 x g.

1.7 Added 50 µl RNase-free H_2O (Preheated to 70 $^{\circ}$C) to NucleoSpin® RNA Virus Columns with sterile 1.5 ml microcentrifuge tube incubated for 1-2 minutes and centrifuged for 1 minute at 11,000 x g.

2. Protocol for Real-time reverse transcription-polymerase chain reaction (rRT-PCR)

	Volume
RNA	4 µl
10 µM of forward and reverse primer	0.5 µl
2.5 µM 64 probe	0.5 µl
2xMaster Mix	6.25 µl
SuperScript III	0.25 µl
Distilled water	0.42 µl
50 µM of $MgSO_4$	0.08 µl
Final volume	**12.0 µl**

3. Protocol for cDNA synthesis

First step

	Volume
RNA	5 µl
Random primer	5 µl

Second step

	Volume
Distilled water	3.7 µl
5X cDNA buffer	4 µl
2.5 mM MgCl2	2 µl
0.5 mM dNTP	1 µl
RNase inhibitor	0.3 µl
ImProm-II™ Reverse Transcriptase	1 µl
Final volume	**22 µl**

VITA

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