ซีรัมวิทยาและลักษณะทางพันธุกรรมของไวรัสไข้หวัดใหญ่สุกรใน 4 จังหวัดที่เลี้ยงสุกรหนาแน่นในประเทศไทยระหว่างปี พ.ศ. 2549 ถึง 2552

นางสาวดลฤทัย ศรีทะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
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ปีการศึกษา 2553

SEROLOGICAL STUDY AND GENETIC CHARACTERIZATION OF SWINE INFLUENZA VIRUS IN FOUR HIGH PIG DENSITY PROVINCES IN THAILAND BETWEEN 2006 - 2009

Miss Donruethai Sreta

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	SEROLOGICAL STUDY AND GENETIC CHARACTERIZATION OF SWINE INFLUENZA VIRUS IN FOUR HIGH PIG DENSITY PROVINCES IN THAILAND BETWEEN 2006 - 2009
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ดลฤทัย ศรีทะ : ซีรัมวิทยาและลักษณะทางพันธุกรรมของไวรัสไข้หวัดใหญ่สุกรใน 4 จังหวัดที่เลี้ยงสุกรหนาแน่นในประเทศไทยระหว่างปี พ.ศ. 2549 ถึง 2552 (SEROLOGICAL STUDY AND GENETIC CHARACTERIZATION OF SWINE INFLUENZA VIRUS IN FOUR HIGH PIG DENSITY PROVINCES IN THAILAND BETWEEN 2006 - 2009) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.น.สพ.ดร. รุ่งโรจน์ ธนาวงษ์นุเวช,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.น.สพ.ดร.อลงกร อมรศิลป์, 121 หน้า.

โรคไข้หวัดใหญ่สุกรคือโรคระบบหายใจแบบเฉียบพลันในสุกรที่มีสาเหตุจากเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ การติดเชื้อไข้หวัดใหญ่สุกรจะส่งผลให้เกิดความสูญเสียด้านเศรษกิจในการเลี้ยงสุกร และสุกรยังเป็นตัวกลางที่ทำให้เกิดการแลกเปลี่ยนพันธุกรรมของไวรัส ซึ่งอาจทำให้เกิดไวรัสสายพันธุ์ใหม่ที่ระบาดสู่คนและสัตว์ชนิดอื่นได้ ดังเช่นการระบาดของไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ เมื่อต้นปี พ.ศ. 2552 ดังนั้นการศึกษาทางไวรัสวิทยาและซีรัมวิทยาของไวรัสไข้หวัดใหญ่จึงมีความสำคัญอย่างยิ่ง จะช่วยให้เข้าใจระบาดวิทยา การพยากรณ์การระบาดของโรค และการพัฒนาวัคซีน เพื่อการป้องกันและควบคุมโรคทั้งในสุกรและมนุษย์

ลักษณะทางพันธุกรรมของไวรัสไข้หวัดใหญ่สุกรสายพันธุ์ H1N1 และ H1N2 ตั้งแต่ปี พ.ศ. 2548 ถึง 2553 พบมีความหลากหลายและสามารถแบ่งเป็นกลุ่มย่อยได้อย่างน้อยสายพันธุ์ละ 2 กลุ่มย่อย จากนั้นศึกษาคุณสมบัติความเป็นแอนติเจนของไวรัสในแต่ละกลุ่มโดยใช้ซีรัมกระต่าย โดยนำแอนติซีรัมกระต่ายมาเป็นตัวควบคุมบวกในการตรวจด้วยวิธี HI ต่อมาสำรวจทางซีรัมวิทยาทั้งสายพันธุ์ H1 และ H3 จากซีรัมสุกรใน 4 จังหวัดที่เลี้ยงสุกรหนาแน่นในประเทศไทยปี พ.ศ. 2551 ถึง 2552 พบมีซีรัมให้ผลบวกต่อโรคไข้หวัดใหญ่สุกรมากกว่าร้อยละ 50 ทั้งสายพันธุ์ H1 และ H3 ส่วนการศึกษาการติดเชื้อข้ามชนิดโฮส์ต พบมีการติดเชื้อข้ามจากสุกรสู่มนุษย์ โดยพบว่าผู้ที่สัมผัสสุกรมีโอกาสติดเชื้อไข้หวัดใหญ่จากสุกรมากกว่า 40 เท่าของผู้ที่ไม่เคยสัมผัสสุกรเลย และยังพบการระบาดร่วมกันของไวรัสสายพันธุ์ใหม่ H1N1 2009 และไวรัสไข้หวัดใหญ่สุกรสายพันธุ์ H1N1 ที่พบการระบาดอยู่แล้วในประเทศไทย จากผลการศึกษาในครั้งนี้ บ่งชี้ว่าในประเทศไทยมีการกระจายของไวรัสไข้หวัดใหญ่สุกรสูงและไวรัสมีความหลากหลายทางพันธุกรรม โดยพบร่วมกันทั้งสายพันธุ์อเมริกาและยุโรป

ภาควิชา พยาธิวิทยา ลายมือชื่อ ดลฤทัย ศรีทะ

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก R.T.L

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

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DONRUETHAI SRETA : SEROLOGICAL STUDY AND GENETIC CHARACTERIZATION OF SWINE INFLUENZA VIRUS IN FOUR HIGH PIG DENSITY PROVINCES IN THAILAND BETWEEN 2006 - 2009 . THESIS ADVISOR : PROF. ROONGROJE THANAWONGNUWECH, Ph.D, THESIS CO-ADVISOR : ASSOC. PROF. ALOGKORN AMONSIN, Ph.D., 121 pp.

Swine influenza is an acute respiratory disease in pigs caused by type A influenza viruses. Swine influenza virus (SIV) infection causes acute respiratory syndrome leading to economic loss. Moreover, swine may act as a 'Mixing vessel' and transmit a reassorted virus back to humans or vice versa similar to the pandemic influenza 2009. Importantly, both virological and serological investigation of influenza A virus in pigs will provide a better understanding of the epidemiology, epidemic prediction and vaccine development leading to effective control and prevention of the disease in both swine and humans.

Genetic characterization of Thai SIV subtype H1N1 and H1N2 during 2005-2010 were evaluated and found that at least 2 clusters were identified in each subtype. Then, the antigenic property was evaluated using hyper-immune rabbit sera as positive controls tested by HI. Later, serology study of H1 and H3 Thai SIV using swine serum in the top 4 pig density provinces in 2008-2009 in Thailand demonstrating that over 50% had seropositive to H1 and H3. Moreover, pig-to-human transmission was demonstrated on Thai swine farms and swine exposed population had over 40 times likely being infected by SIV. Finally, co-circulation of pandemic (H1N1) 2009 and seasonal swine H1N1 viruses were found in a commercial pig farm. The result showed increase SIV circulation containing genetic variation with a mix combination of the North American and European lineages.

Department : Pathology	Student's Signature Donruethai Sreta
Field of Study : Pathobiology	Advisor's Signature RTh
Academic Year : 2010	Co-Advisor's Signature AT

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LIST OF ABBREVIATIONS

CDC	=	Center for Disease Control
ELISA	=	Enzyme-linked immunosorbent assay
HA	=	Hemagglutinin
HI	=	Hemagglutination-inhibition
IHC	=	Immunohistochemistry
M	=	Matrix
MDCK	=	Madin-Darby canine kidney
NA	=	Neuraminidase
NP	=	Nucleocapsid
OIE	=	Office International des Epizooties
PIR	=	Phylogenetically important regions
PRDC	=	Porcine respiratory disease complex
RBC	=	Red blood cells
RDE	=	Receptor-destroying enzyme
RT-PCR	=	Reverse transcription-PCR
RNP	=	Ribonucleoprotein
SIV	=	Swine influenza virus
TRIG	=	Triple reassorted internal gene
WHO	=	World Health Organization

CHAPTER 1

1.1 INTRODUCTION

Swine influenza (SI) is an acute respiratory disease in pigs caused by type A influenza viruses. The influenza A viruses can be classified by viral surface proteins: hemagglutinin (HA) and neuraminidase (NA). Currently, 16 HA and 9 NA subtypes are identified (Fouchier, 2005). The H1N1, H3N2 and H1N2 are the major subtypes circulating in the swine population worldwide (Slemons, 2002; Dee, 2005). However, swine can be infected with other subtypes of influenza A such as H2N3, H1N7, H3N3, H4N6, H3N1 and H9N2 (Lekcharoensuk et al., 2006; Shin et al., 2006; Ma et al., 2007; Cong et al., 2008). Swine influenza virus (SIV) infection causes acute respiratory syndrome showing clinical signs such as coughing, dyspnea, fever, prostration and rapid recovery. Moreover, SIV can cause abortion in pregnant sow (Dee, 2005). In addition, SIV is one of major primary pathogen of porcine respiratory disease complex (PRDC) leading to economic loss in many countries all over the world (Van Reeth et al., 2001; Drolet et al., 2003; Yazawa et al., 2004). For example, SIV alone costs the British meat industry about £60 million yearly and SIV infection with PRDC loss about £7 per pig (Kay et al., 1994). The morbidity rate of SIV is high, may increase up to 100 %, whereas, the mortality rate is as low as 1%. The infected pigs will lose their weight, resulting in increasing growing period to normalize weight before going to slaughter house which is the important problem in the swine industry worldwide, including Thailand (Kay, 1994; Dee, 2005). Therefore, prevention and control of SIV in swine herds is necessary. Hence, SIV status will provide a significant data for planning of prevention and control programs (Webster et al., 2002; Cannell et al., 2008). Direct detection of the viruses and/or viral genetic materials can be problematic since SIV shedding time in the infected pigs is too short, thus the prevalence will be underestimated. Hence, the serodiagnotic assay will be helpful to detect the indirect antibodies against SIV in infected pigs.

Importantly, SIV is a public health concern as it has a zoonotic potential, due to influenza A virus can be infected in many animals including birds, horses and humans.

Many researchers occasionally reported SIV isolates in humans (Myers et al., 2006; Myers et al., 2007). Myers et al. reviewed cases of SIV in humans from the PubMed database in April 2006 and found 50 cases of apparent zoonotic SIV infection (Myers et al., 2007). Additionally, in Thailand, a 4 year old boy infected by H1N1 SIV had been reported (Komadina et al., 2007). Moreover, swine may act as a 'Mixing vessel' since their tracheal epithelium contains the viral receptors both 2,3- and 2,6-*N*-acetylneuraminic acid-galactose linkages that can be infected with either human or avian influenza A virus (Suzuki et al., 2001). Therefore, a new pandemic virus can be generated from swine through reassortment or adaptation to other hosts, especially humans. In the mid of year 2009, a novel H1N1 2009 virus widely transmits among humans, containing triple-reassortment genes with swine, avian and human viruses (Dawood et al., 2009; Garten et al., 2009). The pandemic H1N1 2009 emerged in the United Stage in April and spread worldwide, including Thailand. The pandemic H1N1 infected pigs with flu-like symptoms had been demonstrated in the swine farms in Canada, Argentina and Australia showing the transmission from humans to pigs (Cutler et al., 2009). From these reasons, the World Health Organization (WHO) encourages the surveillance of influenza viruses not only in human but in all species, especially in the pigs (Galwankar and Clem, 2009). Virological and serological investigation of influenza A virus in animals is an important key in the early recognition of outbreak threats. The influenza A virus investigation in pigs will provide the information for understanding the epidemiology, epidemic prediction and vaccine development which lead to effective control and prevention of the disease in both swine and humans.

In Thailand, H1N1, H3N2 and H1N2 SIV subtypes have been recognized. The first H3N2 SIV case was reported in 1981. Subsequently, 10 years later the H1N1 SIV was isolated (Kupradinun et al., 1991). Eventually, a new subtype, H1N2 virus had been reported in 2005 containing the viral genome of both H1N1 and H3N2 SIV (Chutinimitkul et al., 2008; Takemae et al., 2008). However, SIV data in Thailand was limited. Only one pathologic study of SIV in Thailand was report (Sreta et al., 2009) showing both H1N1 (A/sw/Thailand/HF6/05) and H3N2 (A/sw/Thailand/S1/05) SIV, isolated in Thailand in

2005, were able to induce flu-like symptoms and lung lesions in infected pigs. Additionally, the HA and NA genes were also genetically evaluated. The HA gene of the Thai H1N1 belongs to classic swine lineage, whereas, the NA gene belongs to the avian like swine lineage. Therefore, both HA and NA genes of H3N2 belong to human lineage (Sreta et al., 2009). For serologic study of SIV in Thailand, a previous study reported that seroprevalence of H1N1 and H3N2 SIV in 2003 was 7.9% and 20.6%, respectively. In contrast to the study in 2005 percent seropositive of H1N1 was higher than that of H3N2 in both grower pigs and sows (Damrongwatanapokin et al., 2003; 2006). However, no evidence of serological data from 2005 to present (2010) was reported. Based on the virological surveillance in 2008, only 2.8% SIV positive had been evaluated from pigs with respiratory symptom (Nakharuthai et al., 2008). The low number of SIV cases reported in that study could be underestimated because of false negative of SIV detection due to short shedding time of SIV-infected pigs. As a result, serological surveillance is needed, since it will provide the exact SIV prevalence and it will help estimating the true SIV status. So far, there are limited serologic studies on SIV in Thailand and each study using different serological tests might yield different results. Several serodiagnostic tools have been used for antibody detection against SIV including ELISA, HI, SN and IFA tests (Webster et al., 2002). Among these tools, HI test has been used commonly in most veterinary diagnostic laboratories to detect anti-influenza virus antibody with specific subtypes in each regions and considered to be the standard test for international trade of animals by Office International des Epizooties (OIE) (Webster et al., 2002). Nevertheless, the specificity of HI test needs standard virus strains circulating in the local area or having cross-reactivity (Smith, 2003; Andreasen and Sasaki, 2006). In Thailand, 2 clusters in each H1N1 and H3N2 SIV had been found (Takemae et al., 2008). However, the SIV strain that can be used as the representative circulating viruses for the HI test had not been evaluated in Thailand. ELISA test provides rapid screening for the presence of SIV antibodies since the method can test 92 serum samples per plate in less than 2 hours (Lee et al., 1993; Webster et al., 2002). The commercial IDEXX ELISA H1N1 and H3N2 kits are used separately and are

available worldwide, but their capacity to detect antibodies to the Thai SIV has not been investigated. Moreover, there were not enough data regarding serologic surveillance and genetic characterization of SIV in Thailand. Importantly, the data since 2005 have not been studied. This information shows that current serodiagnotic tests must be evaluated continuously and genetic data of current Thai SIV is of importance for virological and epidemiological studies.

Therefore, the objectives of this study were to genetically characterize the current Thai SIV isolates and to establish an appropriate HI test then use the test for indirect survey SIV infection. Also, HI test was used for interspecies transmission study. In addition, virologic and serologic study, and genetic characterization were used for SIV monitoring on an outbreak farm. The whole dissertation is divided into six chapters. The first chapter provides a general introduction of SIV, evidence of SIV in Thailand, various laboratory techniques using to detect SIV infection and swine influenza disease control. The second chapter deals with the classical-Eurasian swine reassortment pattern of Thai swine H1 influenza viruses in 2005-2009. The results showed the genetic variation and mixed viruses from both North American and Eurasian swine lineages have been co-circulating in Thailand. Moreover, at least 2 clusters of each H1N1, H1N2 and H3N2 viruses were found in Thailand. The third chapter deals with swine influenza serological surveillance in four highest pig density provinces in Thailand during 2008-2009 using various antibody detection assays. The antigenic property of difference six Thai SIV isolates was evaluated by HI test. Evidently, the results will be useful for selection of the appropriate SIV isolate for use as antigens in the HI assay locally. The fourth chapter deals with serological evidence of pig-to-human influenza virus transmission on Thai swine farms. Serological study of swine-exposed and non-exposed participants, and swine sera were evaluated by HI test. The result demonstrated of pig-to-human influenza transmission on Thai swine farms which swine-exposure is a risk factor. This report manuscript was accepted on Veterinary Microbiology. The fifth chapter deals with evidence of pandemic (H1N1) 2009 virus in a commercial swine farm in Thailand. In this chapter, we reported the whole genome of six pandemic H1N1 2009

viruses and two endemic H1N1 viruses co-circulated in the same farm since early November 2009 to late March 2010. This report manuscript was published on the Emerging Infectious Disease, volume 16 and page 1587-1590.The last chapter is the conclusion and suggestion including prevention and control of the disease in Thailand.

1.2 OBJECTIVES

The objectives of this study are:

1. To establish an appropriate HI test for SIV infection in Thailand
2. To study the seroprevalence of SIV in pigs from June 2008 to May 2009 from swine farms in the highest pig density provinces in Thailand
3. To genetically characterize the current Thai SIV isolates from 2005 to 2010

1.3 LITERATURE REVIEW

1. Swine influenza (SI)

Swine influenza (SI) is an acute respiratory disease of swine caused by type A influenza viruses. Three subtypes that establish in pigs population worldwide are classical SIV and reassortant viruses of H1N1, H3N2 and H1N2. However, pigs are able to infect with other subtypes of influenza A virus, such as H5N2, H2N3, H1N7, H3N3, H4N6, H3N1 and H9N2 (Lekcharoensuk et al., 2006; Shin et al., 2006; Ma et al., 2007; Cong et al., 2008; Lee et al., 2009). Swine influenza causes significant loss in the swine industry. The infected pigs will lose their weight resulting in increasing growing period for slaughter which is the important problem in the swine industry worldwide, including Thailand (Kay, 1994; Dee, 2005). Clinical signs of SI are manifested as an acute respiratory disease characterized by fever, inactivity, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge. SIV infection is transmitted by direct contact using nose-to-nose contacting and airborne transmission. The virus will be excreted directly with secretion from infected pigs. Incubation period of

SI is between 1- 3 days and rapidly recover within 4-7 days. In pregnant sows, SIV can cause abortion in 3-7 days of infection (Webster et al., 2002; Dee, 2005). However, the infected pigs can easily be susceptible to the secondary infection of other respiratory pathogens resulting in porcine respiratory disease complex (PRDC). When SIV is introduced to the naive pig herds, high morbidity rate may increase up to 100% but the mortality rate is as low as 1%. SIV-associated macroscopic lung lesions observed in pigs are characterized by multifocal well-demarcated purplish-red lesions in the cranioventral areas of lung lobes known as a checker-board lung. SIV-induced microscopic lung lesions consist of epithelial disruption and attenuation in the bronchioles with later found hyperplastic proliferation of the bronchial epithelium and typical bronchiolitis obliterans. Mild to moderate peribronchiolar and perivascular lymphocytic infiltration occurs at nearly all levels of the airways (Easterday and Van Reeth, 1999).

2. Influenza A virus

Influenza viruses are members of the Orthomyxoviridae family composed of 5 genera: A, B and C viruses, *Thogotovirus* and *Isa virus* (Mateo et al., 2007). The former 3 genera are viruses causing influenza. The *Thogotoviruse* is transmitted to vertebrates via ticks but do not cause influenza, and the single *Isa virus*, infectious salmon anemia virus, is transmitted to fish through contaminated water. Influenza A, B and C are characterized by their different nucleocapsid (NP) or matrix (M) proteins. Only influenza A viruses are true zoonotic viruses (Slemons, 2002; Heinen, 2003) and are characterized their subtypes based on the antigenicity of two surface glycoproteins; HA and NA. Currently, sixteen serotypes of HA (H1-H16) and nine of NA (N1-N9) have been identified (Slemons, 2002; Dee, 2005; Fouchier et al., 2005). All 16 HA and 9 NA subtypes have been isolated from particularly aquatic birds that are believed to be a natural host and reservoir for influenza viruses (Ma et al., 2009).

Influenza A viruses are 80-120 nm enveloped viruses with negative single stranded, segmented and RNA genomes. Morphology of the virus particles is roughly spherical with glycoprotein HA and NA spikes on the surface. The HA spike appears in rod-shaped by electron microscopy, whereas, the NA spike has mushroom-shaped (Laver and Webster, 1966; Lipatov et al., 2004). M1 and M2 proteins are associated with structural viral capsule that encode viral ribonucleoprotein (RNP) complex composing of viral RNA and 4 proteins; the NP and 3 polymerase subunits (PB1, PB2 and PA) (figure 1.1). There are eight RNA segments within the viral genome composing of about 13,588 nucleotides encoded 10 proteins (table 1.1 and 1.2) (Heinen, 2003; Vincent et al., 2008).

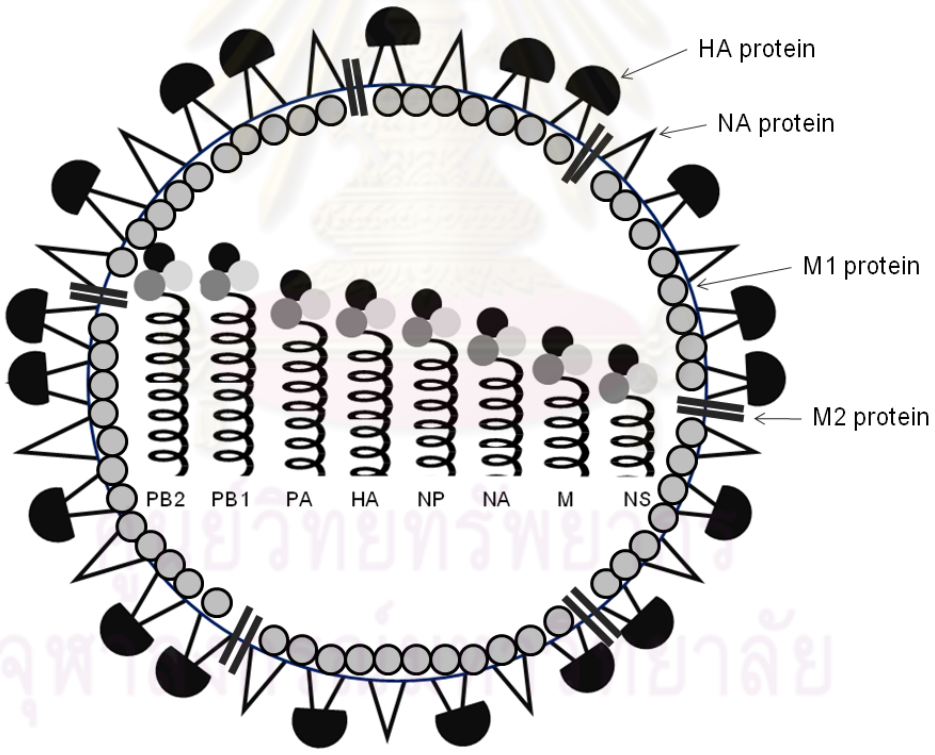

Figure 1.1 Morphology of the influenza A virus particles (adapted from Mart et al, 2010)

Table 1.1 RNA segment of influenza A virus (Heinen, 2003)

RNA segment	Nucleotides	Protein	Amino acid	Molecules/virion
1	2341	Polymerase PB2	759	30-60
2	2341	Polymerase PB1	757	30-60
3	2233	Polymerase PA	716	30-60
4	1778	Hemagglutinin HA	566	500
5	1565	Nucleoprotein NP	498	1000
6	1413	Neuraminidase NA	454	100
7	1027	Matrix protein M1	252	3000
		Matrix protein M2	97	20-60
8	890	Non-structural protein NS1	230	
		Nuclear export protein NEP	121	130-200

Table 1.2 Function of encoded proteins of the influenza A genes (Mitteiholzer, 2006).

Segment	Encoded Polypeptide	Function
1	PB2	Initiation of viral mRNA transcription through recognition and binding of the 5'cap-1 structures of host pre-mRNAs used to generate primers for viral transcription.
2	PB1	Responsible for elongation of the primed nascent viral mRNA and elongation in template RNA and vRNA synthesis. It contains the conserved motifs characteristic of RNA-dependent RNA polymerase. It also contains site for sequence-specific binding to conserved 5'- and 3'-terminal sequences of vRNA and cRNA molecules.
	PB1-F2	May have a role in modulating the host response to influenza A virus by hastening the death of immune cells.
3	PA	Thought to be involved in viral RNA replication. Strong suggestion has been made that PA is involved in the assembly of functional viral RNA polymerase complexes from their inactive intermediates.
4	HA	Binding of virion to host cell receptor and fusion between the virion envelope and the membrane of the endosome.
5	NP	Binds to and encapsidates viral RNA to from coiled ribonucleoprotein complex to which the three polymerase proteins associate.
6	NA	Cleaves terminal sialic acid from glycoproteins or glycolipids to free progeny virions from host cell receptors.
7	M1	Froms a shell surrounding the virion nucleocapsids underneath the virion envelope. Play an important role in intiating progeny virus assembly.
	M2	The membrane-spanning domain serves as a signal for transport to the cell surface. Act as a portion channel to control the pH of the cell Golgi during HA synthesis and to allow acidification of the interior of the virion during virus uncoating.
8	NS1	Regulates nuclear export of mRNA and inhibits pre-mRNA splicing. Probably inhibits IFN-mediated antiviral responses of the host.
	NEP	Provides M1 with a nuclear export signal that mediates the nuclear export of vRNA from the nucleus to the cytoplasm.

3. Epidemiology

Swine influenza is an important respiratory disease and is endemic in the swine population leading to economic loss in swine industry worldwide. Mostly, the prevalence was high in late fall and early winter months in the cold countries. However, the epidemics or pandemics of SI may occur with many reasons; an immunologically naïve population, poor husbandry, secondary bacterial or viral infections and cold weather (Poljak et al., 2008). Moreover, SIV has its zoonotic potential and has been occasionally reported in humans, including Thailand (A/Thailand/271/2005, H1N1) (Komadina et al., 2007).

SI was first recognized in 1918 coinciding with an influenza pandemic in humans known as 'Spanish flu' killing at least 20 million worldwide (Vincent et al., 2008). Although, the etiology in pigs was identified as H1N1 SIV in 1930 (Olsen, 2002). Currently, SIV circulating worldwide are of various strains and subtypes including classical swine H1N1, 'avian-like' H1N1, 'human'- and 'avian-like' H3N2 and H1N2 viruses (Webby et al., 2004; Vincent et al., 2008; Liu et al., 2009). The epidemiology of SIV occurs in two forms; epidemic or endemic. In the epidemic form, the virus quickly moves through all swine units of the naïve farm with a rapid recovery, if there are no complicating factors with secondary bacterial infections. In the endemic form, clinical signs may be less noticeable and not all pigs demonstrate typical clinical signs (Brown, 2000).

In the United States, SI was first clinically recognized in pigs in the Midwestern U.S. in summer/fall of 1918 and first SIV isolated belonged to the classical H1N1 lineage, reported in 1930 (Brown, 2000). Since then, SI has been of importance to the U.S. swine industry. It was not until 1998, subtype H3N2 was emerged and subsequently, reassortment of H1N1 and H3N2 SIV occurred producing a new subtype of H1N2 SIV in 1999 (Olsen, 2002). Genetic diversity in each subtype always is the characters of influenza. For example, in North American, subtype H3N2 isolates had been identified by phylogeny analysis of the HA1 region of HA genes into three different clusters; I, II, and III (Vincent et al., 2008). In addition, using HI and serum neutralization

tests, Gramer et al. found that the viruses in cluster II had limited cross-reactivity with viruses of other clusters (Gramer et al., 2007; Vincent et al., 2008). In addition, Vincent et al. compared the pathologic and serologic reaction of 10 H1N1 SIV isolates collected over the last 75 years and found significant variability in pathogenicity (Vincent et al., 2006). The serological results of the study showed that the classic historical H1N1 viruses tended to have better cross-reaction between historical sera and antigens, with moderate to good cross-reactivity with the modern viral antigens. However, the modern swine sera were less reactive to the historical viruses (Vincent et al., 2006). Similarly, there were only low to moderate degrees of cross-reactivity among recent H1N1 and H1N2 SIV isolates from the United States (Vincent et al., 2008). Olsen et al. reported that the sero-prevalence of SIV by HI test in 2,375 pigs showed seropositive of swine H1, avian H1 and human H3 viruses of about 27.7%, 7.6% and 8.0%, respectively. The results demonstrated that swine H1 viruses still circulating and avian H1 and human H3 were substantially found higher than those in the previous studies (Olsen et al., 2000).

In Europe, SI also causes problems in the swine industry since 1970s (Kay et al., 1994) and many researchers reported the SI prevalence. For example, Loeffen et al. reported the SI sero-prevalence in Dutch breeding herds about 16-17% seropositive of each H1N1 and H3N2 SIV in 2003 (Loeffen et al., 2003). In addition, Fraile et al reported that seropositive herds of each H1N1, H3N2 and H1N2 SIV were higher than 90% of 107 swine farms in Spain in 2007 (Fraile et al., 2009). Moreover, SIV isolated in Europe had genetic variation including classical swine H1N1, avian-like H1N1, human- and avian-like H3N2 (Brown, 2000). Furthermore, European H1N2 appears to be a double reassortant with human H1 1980 and 1986, swine N2 and avian internal genes (Van Reeth et al., 2003).

In Asia, a number of influenza viruses have been isolated from pigs in China. These mainly included classical and avian-like swine H1N1 viruses, H3N2 viruses similar to human viruses, and double-reassortant H1N2 virus containing the similar genes to those of human and swine viruses (Hai Yu et al., 2007). In Korea, Jung et al. reported the sero-prevalence of SI from total 742 pig sera in 2006. They found that seropositive of

subtypes H1, H3, and both H1 and H3 viruses were 51.2%, 43.7%, and 25.3%, respectively (Jung et al., 2007). Moreover, Pascua et al. studied prevalence of SIV from 2002 to 2007 in Korea, they found that both virological and serological prevalence in each year of SIV subtype H1N1, H3N2 and H1N2 was highest in 2007 (Pascua et al., 2008).

4. Swine influenza virus in Thailand

The SIV subtype in Thailand can actually be divided into three subtypes; H1N1, H1N2 and H3N2 (figure 1.2). Since 1978, the serologic study revealed that H3N2 SIV relating to contemporary human strains (Kanai et al., 1981; Nerome et al., 1981). In addition, in 1988, the H1N1 SIV was the first subtype isolated from pigs with an influenza-like symptom from eastern Thailand; Chachoengsao and Chonburi (Kupradinun et al., 1991). Subsequently, in 2005 a new subtype H1N2 was isolated from pigs in Saraburi province (Damrongwatanapokin et al., 2006). However, a few of genetic characterized information had been reported. For instance, our previous study reported the HA and NA genes characterization of the pathogenic SIV in Thailand (Sreta et al., 2009). We found that HA gene of H1N1 belongs to classical swine lineage, similar to the North American strain, whereas, NA gene belongs to avian-like swine lineage, similar to Eurasian strain. In addition, both HA and NA genes of H3N2 belong to human lineage, similar to Eurasian strain according to other studies (Chutinimitkul et al., 2008; Takemae et al., 2008). However, the H1N2 SIV was a reassortant from a classical swine H1N1 and a human like swine H3N2 (Takemae et al., 2008). Based on phylogenetic tree patterns, the SIV H1N2 HA gene is similar to the North American and Asian SIV H1N2 (classical swine H1), whereas the NA gene is closely related to the European SIV (human N2 lineage) (Takemae et al., 2008). However, major SIV subtype circulating in Thailand are H1N1 and H3N2, based on virological and serological surveillances. A previous study reported that only 2.8% positive SIV had been evaluated from the pig with respiratory symptom and only subtype H3N2 was found by RT-PCR (Nakharuthai et al., 2008). However, the 2003 study reported about 7.9% seropositive to H1N1 and 20.6% to H3N2

viruses, conflicting with the data in 2005 showing higher seropositive of H1N1 than H3N2 in both grower pigs and sows (Damrongwatanapokin et al., 2003; 2006). From those previous virological and serological studies of SIV in Thailand, we might conclude that in two or three years the SIV circulating subtype was changed. Since, in 2003 H3N2 subtype was dominant circulation but in 2005 was H1N1 subtype and in 2008 H3N2 subtype was dominant again. However, it needs more epidemic information, validated tools, surveillance and specific factors of SIV outbreak in Thailand for clearly understanding of epidemiology, prevention and control of the disease, and pandemic influenza preparedness specific in our country.

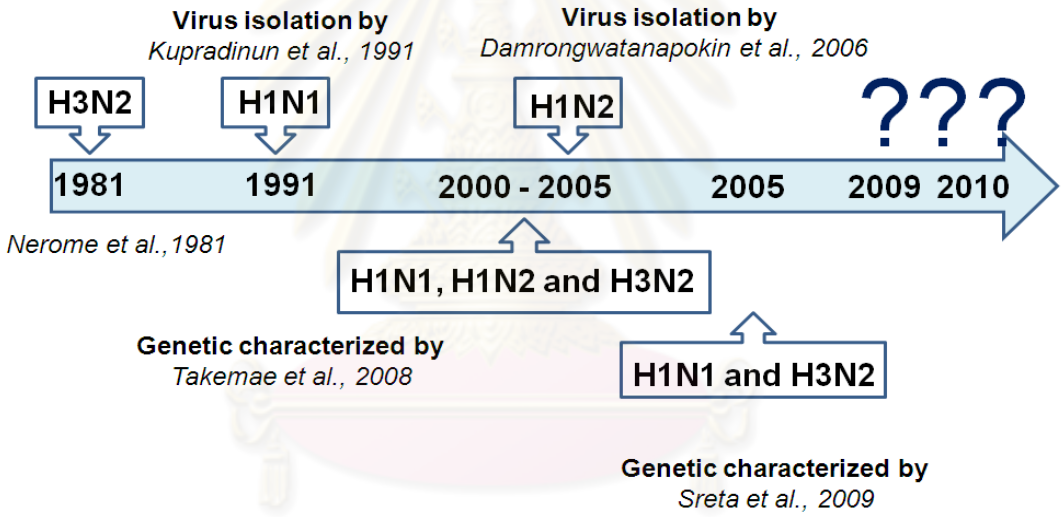

Figure1.2 SIV evidence in Thailand from 1981 to 2005

5. Antigenic variation (Antigenic drift and antigenic shift)

Antigenic change of Influenza A viruses contains two characteristics; antigenic drift and antigenic shift.

1. Antigenic shift occurs through genetic reassortment of the multiple gene segments when different influenza A viruses infect the same host cell and exchange of RNA segments. This type of mutation in the past has been the causes of major SIV outbreaks in the immunologically naïve pig populations (Zhou, 1999; Karasin et al., 2000). The major pandemic of the human H1N1 in 2009 also occurred as a result of

reassortment between human, avian and swine influenza A viruses (Dawood et al., 2009; Garten et al., 2009) (figure 1.3).

2. Antigenic drift refers to an accumulation of point mutations that is inevitable during virus replication due to the lack of proof reading activity of the viral RNA polymerase complex (Hampson, 2002). These mutations at the antigenic sites of the HA and NA proteins are the cause of immunological evasion giving rise to diverse SIV lineages in the pig population. A replication cycle can be a mistake approximately 1/10 nucleotide and consists of multi different virus progenies. However, the virus particle can infect other cells and transmit to other hosts (Hampson, 2002).

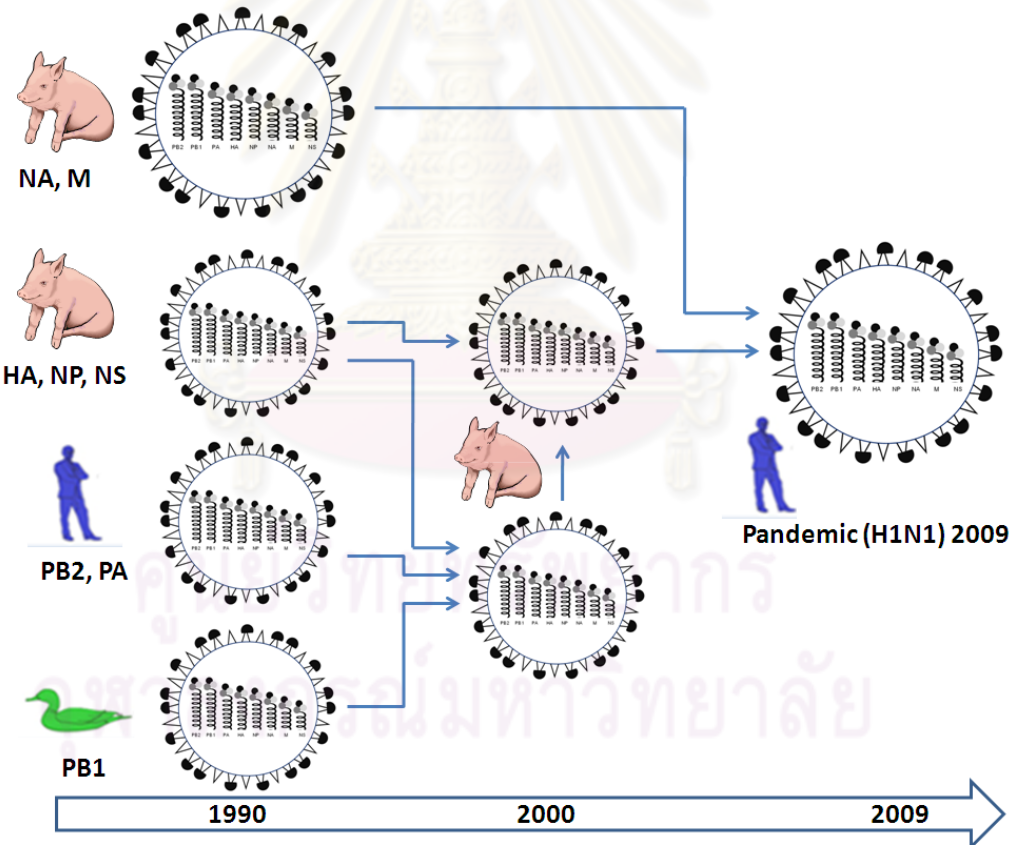

Figure 1.3 The human pandemic H1N1 in 2009 occurred as a result of reassortment between human, avian and swine influenza A viruses (adapted from Dawood et al., 2009; Garten et al., 2009)

6. Pathogenesis of SIV

SIV is transmitted to pig by nasopharyngeal route such as nose to nose contract and airborne droplet. The respiratory lining cells attach the virus by their cilia then the virus can binds to the receptor site (sialic linkage). The virus could get inside the cell by endocytosis and multiplies in the respiratory epithelial lining. Cell destroying, mucociliary clearance and host immune response insufficiency cause focal necrosis, atelectasis and hyperemia in respiratory lining and the virus will spread to the lower respiratory track. Later, accumulation of bronchial exudates and widespread atelectasis are evident (Easterday and Van Reeth, 1999). SIV infects the epithelial lining of the respiratory tract producing clinical signs consisting of cough, fever, lethargy and anorexia. SIV-associated gross lung lesions observed in pigs are characterized by multifocal well-demarcated purplish-red lesions in the cranioventral areas of lung lobes known as a checker-board lung. SIV-induced microscopic lesions consist of epithelial disruption and attenuation in the bronchioles with later found hyperplastic proliferation and bronchiolitis obliterans. Mild to moderate peribronchiolar and perivascular lymphocytic infiltration occurs at nearly all levels of the airways (Easterday and Van Reeth, 1999; Sreta et al., 2009). Viral antigen can be detected in the epithelial cells of airways by immunohistochemistry (IHC) staining (Thacker et al., 2001). In Thailand, only one SIV pathogenesis study was done by Sreta et al (2009). The study reported that both H1N1 and H3N2 SIV subtypes (Thai isolates) were able to induce the flu-like symptoms and lesions compatible with viral pneumonia in the cranioventral areas and were able to cause broncho-interstitial pneumonia. The course of infection was limited to less than a week in both SIV-infected groups as SIV antigen detection was found positive only at 2-4 dpi. The SIV antigen was found in the nuclei of the bronchial and bronchiolar epithelial cells, pneumocytes and pulmonary macrophages with similar levels in both SIV-infected groups indicating no differences between the two subtypes in the viral protein production or replication. It should be noted that both studied Thai isolates of both subtypes replicated only in the respiratory tract of pigs and shed the virus in the nasal secretions similar to other SIV (Sreta et al., 2009).

7. Diagnosis

SIV infection needs differential diagnosis from other respiratory pathogen, since the clinical signs of SI in pigs show acute respiratory symptoms, similarly to other respiratory pathogens such as *Mycoplasma spp*. and PRRSV (Dee, 2005). Therefore, laboratory diagnosis of SIV is an important tools; both direct and indirect methods (Webster et al., 2002). The direct methods include viral isolation, RT-PCR, real time PCR, FA, IHC and EM. However, the direct methods can be problematic due to the short shedding time of SIV and the viruses can only be survived for a short period in the environment. Nevertheless, the detected antibodies producing from infected pigs are useful in the surveillances. However, the specificity of antibody detection depends on the viral antigens utilized (Leuwerke et al., 2008). Several serodiagnostic tools; ELISA, HI test, SN and IFA have been used for antibody detection against SIV. Among these assays, HI test has been used commonly in veterinary diagnostic laboratories to detect anti-influenza virus antibody and considered to be the standard test for international trade of animals by OIE. The commercial IDEXX ELISA H1N1 and H3N2 kit are used worldwide (Lee et al., 1993). Although, ELISA allows rapid screening for the presence of antibodies to SIV and the method can be testing 92 serum samples per plate in less than 2 hours, the specificity and sensitivity are still in doubt.

8. Swine influenza disease control

Since there are no specific treatments for SIV infections, prevention and control are very important (Dee, 2005). Treatment of clinically infected pigs including anti-inflammatory medicine to reduce the severity of affected pigs, antimicrobials to prevent secondary bacterial infections and importantly, all good management strategies. Prevention is largely depending on biosecurity and avoiding the introduction of carrier animals (Poljak et al., 2008). To introduce new animals into the herd, vaccination, isolation and acclimation should be done. In the United States, SI vaccine is commonly used to prevent the clinical diseases but not in Thailand due to limited information. Control program depends on biosecurity and avoiding the introduction of carrier animals.

Persons who work with swine may play an important role as a mixing host, leading to reassortment and development of novel progeny strains with pandemic potential (Ma et al., 2009). People exposing to swine may be the first person becoming infected in the event of a novel virus and serving as a bridge for transmission of the virus to their communities. A policy of vaccinating swine workers annually with human influenza vaccine would decrease the risk of reassortment events. Persons who work with swine should be considered for sentinel influenza surveillance and may be an important group to include in pandemic planning (Myers et al., 2006; 2007).

CHAPTER 2

The Classical-Eurasian swine reassortment patterns of Thai swine H1 influenza viruses in 2005-2009

2.1 Introduction

Swine influenza virus (SIV) is a type A virus in the *Orthomyxoviridae* family. The virus particle contains a genome of eight negative sense single-stranded RNA segments encoding surface envelope glycoproteins, structural proteins and an inner core of ribonucleoprotein (RNP) complexes (Olsen et al., 2006). Influenza A viruses are further subdivided by the antigenic characterization of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. A total of 16 HA and 9 NA subtypes have been identified, with minimal serological cross-reaction between the subtypes (Fouchier et al., 2005). The virus undergoes two major forms of evolution, antigenic drift and shift. Antigenic shift occurs through genetic reassortment of the multiple gene segments when two parental viruses infect the same host cell. This type of mutation in the past has caused of several major SIV outbreaks in the immunologically naïve pig populations (Zhou et al., 1999; Karasin et al., 2000). Antigenic drift refers to an accumulation of point mutations inevitably during virus replication due to lacking of proof reading activity of the viral RNA polymerase complex (Hampson, 2002). These mutations at the antigenic sites of the HA and NA proteins is the cause of immunological virus escaping mutants giving rise to diverse SIV lineages in the pig population around the world.

It is known that SIV infection remains one of the major problems in pig production throughout the world. Similar to influenza A viruses found in other species, SIV can induce flu-like symptoms such as coughing, sneezing, labored abdominal breathing and feed withdrawing (Easterday and Van Reeth, 1999; Loeffen et al., 2003; Kitikoon et al., 2006). The economic lost is primarily due to an increased time to market because of the reduced weight gain and medical cost due to secondary infections and/or synergistic infection with other swine respiratory pathogens (Kay et al., 1994; Olsen et al., 2000). Currently, three dominant subtypes, H1N1, H3N2 and H1N2, are

established among pig population throughout the world. However, H1N1 was the first subtype found in the swine species (Olsen et al, 2006). The genetic makeup of H1N1 SIV in North America prior to 1997 was designated as a 'classical-swine' since all eight gene segments were from the swine origin (Vincent et al., 2008). In contrast, the H1N1 virus introduced to the European pigs in 1979 contained wholly avian genes antigenically distinct from the classical swine H1N1 viruses. Currently, European H1N1 isolates contains H1 segment from both human and avian lineages (Zell, 2008). Contemporary H1N1 viruses in the North American swine population have changed dramatically since the emergence of the H3N2 subtype in late 1997. The introduction of H3N2 lead to the appearance of H1N2 and changed the H1N1 from a wholly-swine lineage to a triple-reassorted swine, avian and human virus with antigenically distinct characters (Webby et al., 2004; Karasin et al., 2006).

Influenza virus infection in pigs also poses a public health concern as swine influenza viruses are known zoonotic pathogens. The Center for Disease Control (CDC) claims that in the United States of America at least one case of human infection with SIV is reported every 1-2 years (Ramirez et al., 2006). Since pigs are known to be susceptible to both avian and mammalian influenza viruses, hypothetically, pig may act as a mixing host for the production of novel reassorted influenza viruses (Castrucci et al., 1993; Shu et al., 1994). The widespread human-to-human transmission of the swine-origin influenza A H1N1virus or so-called the "pandemic H1N1 (pH1N1)" in the beginning of March 2009 put the speculation on pigs as the influenza virus intermediate host into perspective. Genetic data indicates that the pandemic H1N1 virus was a product from the reassortment of several genetically diverse SIV circulating in the swine population. One unique feature that separates this virus from all other known swine influenza viruses is that it contains surface H1 from a North American (Classical swine) lineage and N1 from an Eurasian (avian-like swine)- swine lineage (Kingsford et al., 2009). Moreover, the internal gene segments comprises of a mixed gene pools derived from human, avian and swine lineages in which all had been embedded in the swine population for over a decade prior to this pandemic outbreak (Smith, 2009).

Unfortunately, there is not enough genetic evidence to conclude when and where this virus was produced due to the lack of systemic and inconsistent SIV surveillance in swine. However, it was demonstrated that certain Thai H1N1 SIV isolates obtained over 4 years ago shared the classical-Eurasian swine reassortment pattern of the HA-NA genes with the pH1N1 strain. This unique pattern is found nowhere else but in those Thai swine H1N1 isolates (Kingsford et al., 2009). There are limited numbers of published data on the genetic information of SIV in Thailand. So far there is only one study that reported the whole genomic character of six H1N1, one H1N2 and five H3N2 viruses isolated between 2000-2005 in Thailand (Takemae et al., 2008). In this study, we carried out phylogenetic analysis of eight gene segments of five more recent H1N1 isolates and the surface genes of two H1N2 viruses isolated in Thailand in 2009.

2.2 Materials and Methods

2.2.1 Viruses

Five H1N1 isolates were isolated from pigs with respiratory signs from the eastern region (Chonburi) in 2005, 2006 and 2009, central region (Saraburi) in 2009 and north-eastern region (Udon Thani province) in 2009, respectively (table 2.1). Two H1N2 isolates, K4/09 and L2/09 were isolated from pigs in Chachoengsao in 2009 (table 2.1). All viruses were propagated once in embryonated chicken eggs (Pyhala et al., 1987) and followed by two passages in Madin-Darby canine kidney (MDCK) cells as described previously (Kitikoon et al., 2006) and stored at -80°C until used.

2.2.2 Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 150 μl of each sample using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) and cDNAs were synthesized at 37°C for 3 hr using Omniscript RT kit (Qiagen, USA) consisting of 4 U of Omniscript reverse transcriptase, 2 μl of 10x reverse transcriptase buffer, 2 μl of 10mM dNTP, 20 μM/μl of universal primer (Uni12 primer 5'-AGCAAAAGCAGG-3') as described previously (Hoffmann et al., 2001).

2.2.3 DNA sequencing and phylogenetic analysis

All gene segments of SIV were amplified by conventional PCR using 1 μl of cDNA, 20 μM/μl of forward and reverse primers published (Hoffmann et al., 2001). The amplification reaction was performed in a thermocycler under the following conditions: initial denaturation at 94 ˚C for 3 min, followed by 30 amplification cycles consisting of 94 ˚C for 30 s (denaturation), 58 ˚C for 30 s (primer annealing), and 72 ˚C for 7 min (extension), and concluded with a final extension step at 72 ˚C for 7 min. The PCR products from each gene were diluted 5 times with nuclease-free water and re-amplified by specific primer pairs of each gene segment (Zhou et al., 1999). The PCR products were analyzed by 1.5% agarose gel electrophoresis and purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). DNA sequencing was carried out by 1st BASE Company, Singapore with specific primer sets. Sequences were edited by Chromas Lite version 2.01 (Technelysium Pty Ltd., Australia) and Bioedit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999). Gene sequences were submitted to GenBank database. Finally, sequences were aligned with SIV reference strains using the SeqMan program (DNASTAR, Madison, WI). The phylogenetic trees were conducted in MEGA4 (Tamura et al., 2007) using neighbor-joining method with 1000 times bootstrapping replicates (Saitou and Nei, 1987).

Amantidine and Ostelnamivir resistance were determined by analysis of the M2 and NA genes (Gubareva et al., 2002; Furuse et al., 2009). The molecular changes associated with high pathogenicity are polygenic traits and specific mutations have been identified in the PB2 (E627K, G590S), PB1-F2 (N66S), and NS (F92D) genes (Forrest and Webster, 2010)

2.2.4 Statistical analysis

Genetic analysis was performed by comparison of each gene within the same subtypes by descriptive analysis.

2.3 Results

2.3.1 Sequence analysis

All eight genes from all 7 Thai H1-SIV isolates were sequenced, and the sequences were deposited in GenBank database. The sequences generated were then compared to previous Thai SIVs (Takemae et al, 2008) and SIVs genes from the NCBI website (http://www.ncbi.nlm.nih.gov/genomes/FLU) from North American and European pigs. Nucleotide comparison of the five H1N1 and two H1N2 in this study was shown in table 2.2.

The results of the whole genome analysis indicated that all three H1N1 SIVs; HF6/05, CB1/06 and CB918/09 isolated from pigs in the eastern region (Chonburi) in 2005, 2006 and 2009 have the classical (HA)-Eurasian (NA) reassorted gene pattern (table 2.2). The HA and NS genes of those viruses belonged to the classical-swine lineage while the rest of the genome were clustered with the Eurasian-swine lineage. Thai SIVs isolated from pigs in 2005 and 2006 were similar to one another (over all 99% homology) and were closely related to H1N1 viruses isolated from pigs in the same province during 2004-2005. In contrast, nucleotide homology of the CB918/09 isolate was different from the rest of the previous Thai SIVs (2000-2006).

The H1 HA gene of CB918/09 in particular showed only 86.7% homology with HF6/05 and CB1/06. The ST3/09 virus isolated from central Thailand (Saraburi) had 91.7% homology of the H1 HA gene to the previously reported H1N2 virus (A/sw/Saraburi/NIAH13021/05 H1N2) isolated in 2005. The H1 HA gene of M8.2/09 isolate, however, showed 99% similar to the pandemic H1N1 virus. The K4/09 HA gene was 98.9% similar to the L2/09.

The N1 NA genes of the five H1N1 isolates in this study were clustered in Eurasian swine N1. The HF6/05, CB1/06, CB918/09 and ST3/09 N1 NA gene was similar to the Thai swine H1N1 in 2000-2008, while, M8.2/09 was similar to pH1N1 (table 2.2). Nucleotide sequence of NA gene of HF6/05 and CB1/06 was 99% identity, 92% to CB918/09 (Chonburi, differ year) and 91.2% to ST3/09. The homology between CB918/09 and ST3/09 was 90.9% (isolated in the same year in different provinces) by

using local pairwise alignment (http://www.ebi.ac.uk.). The N2 NA gene of both H1N2 isolates (K4/09 and L2/09) was grouped in human lineage circulating in human 1990s and it was different from the previous swine H1N2 in Thailand (Takemae et al., 2008). N2 NA gene of K4/09 showed 99.5% homology to L2/09, while both K4/09 and L2/09 showed 82.7-82.8% homology to other NA gene of H1N2 subtype (A/sw/Saraburi/NIAH13021/05). Furthermore, both K4/09 and L2/09 showed 84.1-91.1% homology to other SIV H3N2 2003-2005 in Thailand.

Six internal genes of HF6/05, CB1/06, CB918/09, ST3/09, K4/09 and L2/09 were similar to the Eurasian swine lineage sharing the highest identity to other Thai H1N1 SIVs (table 2.2). However, M8.2/09 shared the highest identity to pH1N1 having PB2 and PA genes from avian-North American, PB1 gene from human-like, NP and NS genes from classical swine and M gene from Eurasian swine.

2.3.2 Phylogenetic analysis

As shown in figure 2.1(a) phylogenetic analysis of the HA gene places the HA of the five H1N1 isolates and two H1N2 isolates into the classical swine lineage similar to the North American swine lineage. Base on sub-blanching and blanch length (% nucleotide divergence), three clusters of Thai swine H1 influenza virus could be divided; cluster I, II and III. HF6/05, CB1/06 and ST3/09 were placed in cluster I. CB918/09, K4/09 and L2/09 were placed in cluster II and M8.2 was placed in cluster III. In the United States, HA H1 swine virus had been divided into four clusters consisting of H1α (classic H1N1), H1β (reassortant H1N1-like), H1γ (H1N2-like) and H1δ (human-like H1). Thai swine H1 cluster I and II were similar to the H1α in the United States while Thai swine H1 cluster III was similar to the H1γ (pandemic H1N1 2009 virus). All 6 Thai H1 HA genes (except M8.2/09) were grouped in the SwH1α cluster along with the Thai H1N1 viruses previously found in Chonburi and the H1N1 viruses circulating in the US swine population prior to 1997 (before the introduction of the H3N2 subtype). Furthermore, HF6/05, CB1/06 and ST3/09 were in the same sub-branch different from

CB918/09, K4/09 and L2/09. In contrast, M8.2/09 HA gene was clustered with the SwH1γ group similar to the pandemic H1N1 viruses in humans (figure 2.1a).

Base on phylogenetic analysis, the N1 NA genes were classified into 4 groups; avian N1, human N1, Eurasian swine N1 and North American swine N1 (figure 2.1b). All five Thai H1N1 SIVs were grouped in the Eurasian swine N1. In addition, M8.2/09 NA gene was in the same sub-branch with the pandemic H1N1. While, the N2 NA genes contained 3 groups composing of avian N2, Eurasian swine N2 and human N2 1990s-2000s (figure 2.1c). Both K4/09 and L2/09 NA genes were grouped in the human N2 1990s-2000s lineage similar to the North American swine N2. Previous Thai H1N2 SIV 2005 (A/sw/Saraburi/NIAH/05) NA gene was in the different branch since it placed in the Eurasian swine N2.

The polymerase genes of all 7 SIV isolates in this study categorized in avian-like swine lineage similar to the Eurasian SIV except for PB2 gene of M8.2/09 clustered in the North American swine lineage. The PB2 gene of M8.2/09 was grouped in the swine lineage similar to the North American SIV as well as the pandemic H1N1. The PB2 of other 6 SIVs (HF6/05, CB1/06, CB918/09, ST3/09, K4/09 and L2/09) were grouped in the avian origin similar to Eurasian SIVs (figure 2.1d). The PB1 gene of M8.2/09 was from the humanH3 origin similar to the pandemic H1N1 but other 6 SIVs (HF6/05, CB1/06, CB918/09, ST3/09, K4/09 and L2/09) were similar to Eurasian swine which is from avian origin (figure 2.1e). However, PA gene of all 7 isolates was from avian origin (figure 2.1f).

Phylogenetic tree of M and NP genes contained 3 lineages; human, swine and avian origin (figure 2.1g and 2.1h). M and NP genes of all 7 SIV isolates in this study were clustered in the avian origin. At least 2 distinct evolutional pathways existed among Thai SIV isolates in this study. Both M and NP genes of M8.2 were separated in different sub-blanch from other 6 isolates.

Phylogenetic tree of NS gene have 3 lineages; human, swine and avian origin. NS gene of ST3/09 was grouped in the avian origin, similar to Eurasian swine lineage. Other 6 SIV isolates were grouped in the classic swine origin similar to the North

American swine lineage (figure 2.1i). Phylogenetic analysis showed that at least 3 distinct evolutional pathways existed among Thai SIV isolates in this study.

2.3.3 Genetic analysis

The receptor binding site on HA gene was analyzed and showed residue types at four positions in table 2.3. The 255 residue of Thai H1 HA gene had 3 residue types composing of Glycine, Aspartate and Asparagine. The other residues 190, 193 and 222 had unique residue type similar to other H1 swine viruses.

Genetic analysis on M2 gene of Amantadine resistance contained S31N in all 7 Thai H1 SIV. On residue 27, ST3/09 was V27F, while, other 6 Thai SIVs were V27.

Drug resistant residues on NA gene were analyzed at amino acid residues E119, H275, R293 and N295. No changing of those amino acid on drug resistance residues were observed in all isolates.

The molecular changes associated with high pathogenicity are polygenic traits and specific mutations have been identified in PB2 (E627K, G590S), PB1-F2 (N66S), and NS (F92D) (table 2.4). In addition, PB1-F2 protein with 90 amino acid residues was found in all 6 Thai H1 SIVs except M8.2/09 (table 2.4).

2.4 Discussion

Our result showed that Thai SIV H1 subtype had genetic variation more than the previous study since the pandemic H1N1 was introduced into the Thai pig population (Sreta et al., 2010). A previous study of Thai SIVs in 2000 to 2005 reported that there were 2 clusters of Thai H1 HA gene (Takemae et al., 2008). In this study, we found that at least 3 clusters of Thai H1 HA gene were found. However, all Thai H1 HA genes were classical swine origin similar to the North American swine lineage which was sub-grouped into three clusters; classical swine cluster I, II and III. Thai H1 cluster I and II were thought to have derived from a hypothetical Classical swine H1N1 common ancestor in 1981 and 1990, respectively (Takemae et al., 2008). Moreover, the pandemic H1N1 2009 has currently been circulating in Thai pig population.

As time goes by, accumulation of point mutation causes antigenic drift and nucleotide change under selective pressure (Shen et al., 2009). Similarly in Thailand, the nucleotide of H1 virus in 2009 has changed showing only 86.7% homology to the previous 2005-2006 SIVs. Moreover, different geographic areas showed distinct genetic evolution since the eastern part of Thailand, Chonburi province had genetic diversity of SIVs from the central part, Saraburi province isolated in the same year 2009. For example, in both areas the H1 HA gene viruses having separating evolution line, result in the antigenic differences and are able to make the immune system of naïve pigs failing to recognize the virus. Thus, moving infected pig into the naïve swine population may cause the outbreaks (Zhou et al., 1999; Karasin et al., 2000). The HA function is important for binding receptor site and it is of importance for host neutralizing antibody (Forrest et al., 2010). From our study, the HA gene sequence of different times and different geographic areas are about 10-15% divergence, but antigenic drift were not determined.

There are two evolutional lineages of H1N1 SIVs; the North American which is a classic swine origin and Eurasian swine lineage which is an avian origin mostly circulating in Europe and Asia (Forrest et al., 2010). In Thailand, SIV isolates were mixed of both H1N1 lineages and the internal genes were originated from either avian, swine or human virus. Moreover, in late 2009 the pandemic H1N1 was introduced and has still been circulating in pig population (Sreta et al., 2010). Especially, the pandemic H1N1 contains the SIV triple reassorted internal gene (TRIG) backbone which may potentially increase the risk of emerging disease (Kingsford et al., 2009). Previously, we investigated a commercial swine farm and found the pandemic H1N1 circulating in the farm for at least 4 months (Sreta et al., 2010). In this study, we found the pandemic H1N1 in different geographic area, indicating that co-circulation of both pandemic and endemic viruses already occurred. Undoubtedly, the reassortant between both viruses can happen and the novel reassorted virus may have high pandemic potential. Nevertheless, the pandemic H1N1 from humans could produce respiratory signs and shed in both respiratory and digestive system in non-immune pigs experiment (Brookes et al., 2010).

But the pathogenesis of the pandemic H1N1 isolated from swine has not yet been evaluated. The adaptation of the pandemic H1N1 in swine might produce different disease patterns. It should be noted that pathogenesis study of the pandemic H1N1 from swine should be investigated to provide a better understanding of epidemiology and outbreak treat both in swine and humans. In Thailand, H1 SIVs have shown genetic variation, therefore, study on pathology and serology are needed for prevention and control program. In addition, serologic diagnosis of the disease requires specific virus antigen that can represent the endemic prevalence in the local areas. Moreover, the receptor-binding site is correlated with the pathogenesis. The amino acid position 190 and 225 play predominant roles in determining the receptor-binding specificity (Shen et al., 2009). Our study found that position 190 of all 7 isolates was aspartate similar to both human and swine viruses. However, amino acid substitution was found at position 225 containing glycine, asparagine and/or aspartate (table 2.4). Changing at the position 225 is definitely correlated with the pathogenesis but it could occur by adaptation in eggs (Shen et al., 2009). Therefore, the pathogenesis of different Thai H1 viruses should be evaluated.

Interestingly, a novel reassorted H1N2 swine virus was found in our study since we got H1N2 2009 containing different HA and NA gene clusters from the previous H1N2 2005 (Takemae et al., 2008). The Thai H1N2 SIV reported in 2005 contained a combination of H1N1 (H1-classical swine) and H3N2 (N2-human 1970s similar to the European swine N2). The origin of the N2 of both 2009 H1N2 viruses in this study appeared to be similar to the human-like 1990s similar to the North American swine N2 and closely related to the Thai human influenza viruses. No pathogenesis or epidemiological data was done with the 2005 H1N2 SIV (Takemae et al., 2008). Both 2009 H1N2 isolates were associated with the severe respiratory signs in pigs. This study demonstrated that a novel reassortant H1N2 swine virus had been demonstrated and showing on-going diversity of the H1N2 in the Thai swine population. The study also highlights the importance of SIV as a contributor to PRDC which remains as a major problem in the Thai pig industry.

In conclusion, Thai H1 SIVs are on-going genetic evolution and variation both H1N1 and H1N2 subtypes. It should be noted that the Thai swine population had not been vaccinated for influenza A virus. The genetic changing of Thai SIVs possibly occurs in the nature environment. We suggest preventing and controlling the diseases caused by SIVs using good management strategies and biosecurity in swine farms. For preventing interspecies transmission, vaccination the swine workers or people exposing to pigs is recommended.

Table 2.1 SIV isolates described in this report

Virus name	Designation	Date of isolation	Sample	Province	Characteristics of disease on the farm of origin*
A/Sw/Thailand/HF6/05	HF6/05	August, 2005	Nasal swab	Chonburi	Isolated from a 6-week-old piglet during an outbreak of wide spread illness among nursery pigs
A/Sw/Thailand/CU-CB1/06	CB1/06	September, 2006	Lung	Chonburi	Isolated during an outbreak of wide spread illness among feeder pigs
A/Sw/Thailand/CU-CB918/09	CB918/09	January, 2009	Lung	Chonburi	Isolated from a 6-week-old piglet during an outbreak of wide spread illness among nursery pigs
A/Sw/Thailand/CU-ST3/09	ST3/09	November, 2009	Nasal swab	Saraburi	Isolated from a 4-week-old piglet during an outbreak of wide spread illness among nursery pigs
A/Sw/Thailand/CU-M8.2/09	M8.2	November, 2009	Nasal swab	Udon Thani	Isolated from a 8-week-old piglet during an outbreak of wide spread illness among nursery pigs
A/Sw/Thailand/CU-CHK4/09	K4/09	May, 2009	Lung	Chacheongsao	Isolated from a 5-week-old piglet during an outbreak of wide spread illness among nursery pigs
A/Sw/Thailand/CU-CHL2/09	L2/09	May, 2009	Lung	Chacheongsao	Isolated from a 6-week-old piglet during an outbreak of wide spread illness among nursery pigs

* All Thai swine farms do not use swine influenza virus vaccines

Table 2.2 Homology analysis of 8 segment genes of the Thai SIV H1viruses

Gene	H1 virus	Nucleotide compared	Virus with the highest degree of homology	Identity (%)	Virus origin
PB2	HF6/05	20-2280	A/sw/Chonburi/NIAH977/2004(H1N1)	99	Eurasian swine
	CB1/06	14-2268	A/sw/Chonburi/NIAH977/2004(H1N1)	98	
	CB918/09	24-2261	A/sw/Ratchaburi/NIAH1481/2000(H1N1)	95	
	ST3/09	29-2280	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	98	
	M8.2/09	37-2304	A/Taiwan/137/2009(H1N1)	99	Avian-North American
	K4/09	22-2281	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	95	Eurasian swine
	L2/09	6-2164	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	95	
PB1	HF6/05	1-2274	A/sw/Chonburi/NIAH9469/2004(H1N1)	99	Eurasian swine
	CB1/06	25-2271	A/sw/Chonburi/NIAH9469/2004(H1N1)	99	
	CB918/09	20-2274	A/sw/Udon Thani/NIAH464/2004(H3N2)	97	
	ST3/09	1-2274	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	98	
	M8.2/09	18-2278	A/Boston/153/2009(H1N1)	99	Human-like
	K4/09	1-2274	A/swine/Udon Thani/NIAH464/2004(H3N2)	96	Eurasian swine
	L2/09	18-1688	A/swine/Udon Thani/NIAH464/2004(H3N2)	96	
PA	HF6/05	1-2159	A/sw/Chonburi/NIAH977/2004(H1N1)	99	Eurasian swine
	CB1/06	25-2151	A/sw/Chonburi/NIAH977/2004(H1N1)	99	
	CB918/09	1-2166	A/sw/Chonburi/NIAH977/2004(H1N1)	98	
	ST3/09	1-2151	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	98	
	M8.2/09	13-2199	A/Blagovechensk/01/2009(H1N1)	99	Avian-North American
	K4/09	1-2120	A/Thailand/271/2005(H1N1)	98	Eurasian swine
	L2/09	12-2120	A/Thailand/271/2005(H1N1)	98	
HA	HF6/05	1-1707	A/sw/Chonburi/05CB1/2005(H1N1)	100	Classical swine
	CB1/06	65-1700	A/sw/Chonburi/05CB1/2005(H1N1)	99	
	CB918/09	13-1694	A/sw/Ratchaburi/NIAH1481/2000(H1N1)	96	
	ST3/09	1-1319	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	97	
	M8.2/09	274-1049	A/Thailand/0419-00-N0/2009(H1N1)	99	
	K4/09	35-1735	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	96	
	L2/09	116-1699	A/swine/Ratchaburi/NIAH1481/2000(H1N1)	96	
NP	HF6/05	1-1443	A/sw/Chonburi/NIAH977/2004(H1N1)	95	Eurasian swine
	CB1/06	1-1482	A/sw/Chonburi/NIAH977/2004(H1N1)	99	
	CB918/09	2-1530	A/sw/Italy/839/1989(H1N1)	95	
	ST3/09	1-1478	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	98	
	M8.2/09	8-1523	A/New York/6943/2009(H1N1)	99	Classical swine
	K4/09	35-1531	A/swine/Ratchaburi/NIAH59/2004(H3N2)	97	Eurasian swine
	L2/09	1-1451	A/swine/Ratchaburi/NIAH59/2004(H3N2)	97	

Table 2.2 Homology analysis of 8 segment genes of the Thai SIV H1viruses (continuous)

Gene	H1 virus	Nucleotide compared	Virus with the highest degree of homology	Identity (%)	Virus origin
NA	HF6/05	12-1718	A/sw/Chonburi/05CB1/2005(H1N1)	100	Eurasian swine
	CB1/06	29-1410	A/sw/Chonburi/05CB1/2005(H1N1)	99	
	CB918/09	22-1431	A/sw/Ratchaburi/NIAH550/2003(H1N1)	97	
	ST3/09	1-1406	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	98	
	M8.2/09	16-1406	A/Boston/153/2009(H1N1)	99	
	K4/09	1-1404	A/Christchurch/1/1996(H3N2)	94	Human 1990s
	L2/09	1-1388	A/Christchurch/1/1996(H3N2)	94	
M	HF6/05	29-996	A/sw/Chonburi/NIAH9469/2004(H1N1)	99	Eurasian swine
	CB1/06	27-994	A/sw/Chonburi/NIAH9469/2004(H1N1)	99	
	CB918/09	26-993	A/Thailand/271/2005(H1N1)	97	
	ST3/09	26-994	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	99	
	M8.2/09	1-1032	A/Taiwan/126/2009(H1N1)	99	
	K4/09	1-980	A/Thailand/271/2005(H1N1)	98	
	L2/09	622-984	A/Thailand/271/2005(H1N1)	98	
NS	HF6/05	28-845	A/sw/Chonburi/NIAH9469/2004(H1N1)	99	Classical swine
	CB1/06	30-835	A/sw/Chonburi/NIAH9469/2004(H1N1)	98	
	CB918/09	1-838	A/sw/Chonburi/NIAH977/2004(H1N1)	98	
	ST3/09	1-838	A/sw/Ratchaburi/NIAH101942/2008(H1N1)	98	
	M8.2/09	1-888	A/Taiwan/137/2009(H1N1)	100	
	K4/09	1-838	A/swine/Chonburi/NIAH977/2004(H1N1)	98	
	L2/09	15-822	A/swine/Chonburi/NIAH977/2004(H1N1)	98	

Table 2.3 Amino acid changes in the receptor binding site on HA gene

Residue of HA protein	HF6/09	CB1/06	CB918/09	ST3/09	K4/09	L2/09	M8.2/09	1918-human*	1930-swine**	pandemic H1N1***	Avian***
190	D	D	D	D	D	D	D	D	D	D	E
193	S	S	S	S	S	S	S	S	S	S	S
222	K	K	K	K	K	K	K	K	K	K	K
225	G	G	N	G	D	D	D	D, G	G	D, G	G

*1918-human; A/New York/1/1918(H1N1)

**1930-swine; A/swine/Iowa/1930(H1N1)

***pandemic H1N1 and Avian according to Shen et al. (2009)

Table 2.4 Amino acid changes in associated with high pathogenicity are polygenic traits and specific mutations have been identified in PB2 (E627K, G590S), PB1-F2 (N66S), and NS (F92D)

Residue	HF6/09	CB1/06	CB918/09	ST3/09	K4/09	L2/09	M8.2/09
PB1-F2 (90)	90	90	90	90	90	90	58
PB1-F2, N66S	N	N	N	S	N	N	N
PB2, E627K	E	E	E	E	E	E	E
PB2, G590S	G	G	G	G	G	G	S
NS, F92D	D	D	D	E	D	D	D

Figure 2.1 Phylogenetic trees of two glycoprotein surface genes and three polymerase genes base on nucleotide sequences from Thai swine H1 (indicated by *filled diamond*) and other sequences available from GenBank; **a** HA (other Thai SIV indicated by *opened diamond* and human Thai indicated by *opened square*), **b** NA (N1), **c** NA (N2), **d** PB2, **e** PB1, **f** PA. The trees were constructed by the neighbor-joining method in MEGA4 and bootstrap test 1,000 replicates are show next to the branches. The trees are drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The reference viruses used in the analysis are abbreviated with host (sw; swine, hu; human), their state and year of origin, subtype and GenBank accession number.

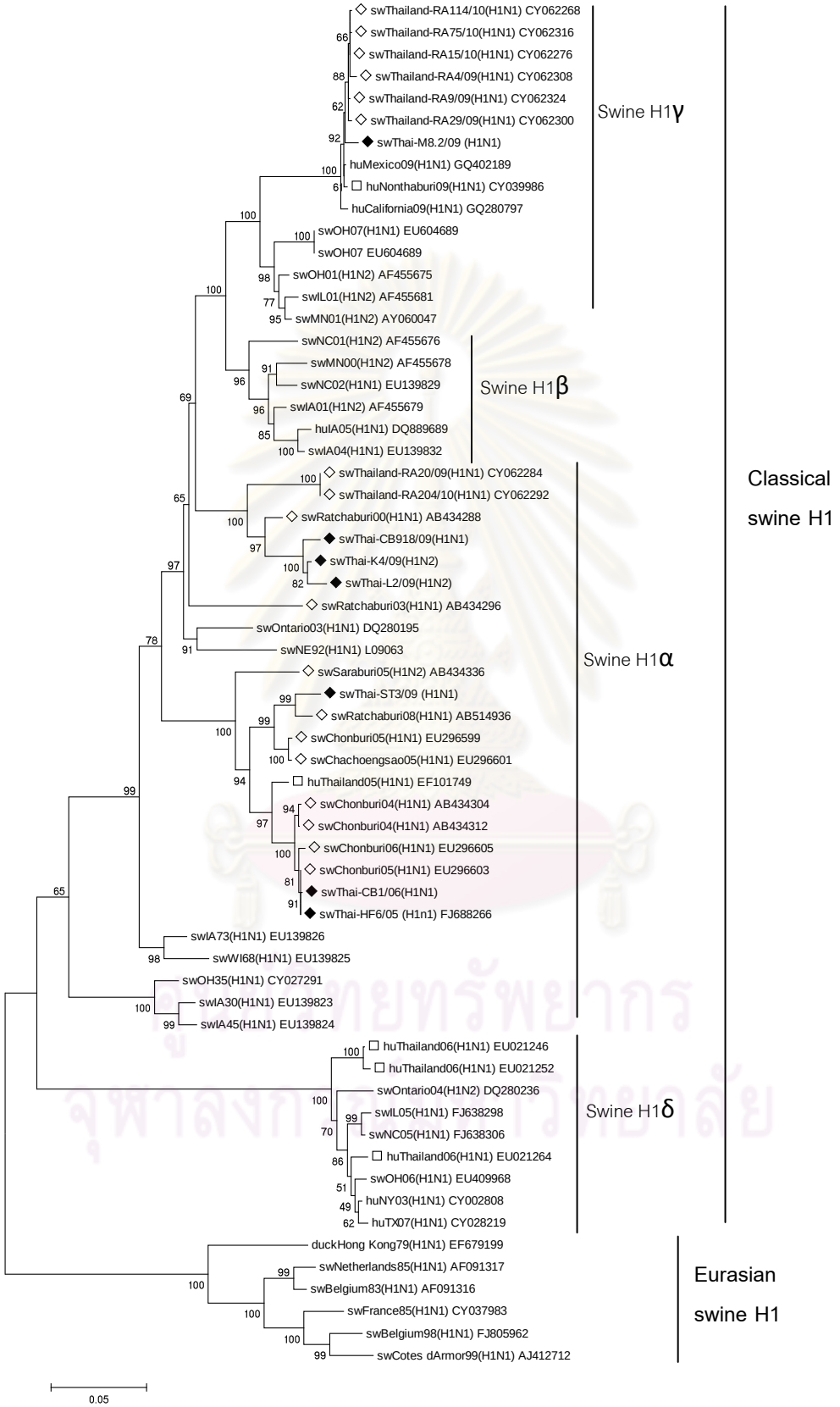

Figure 2.1(a) Phylogenetic tree of HA H1 gene

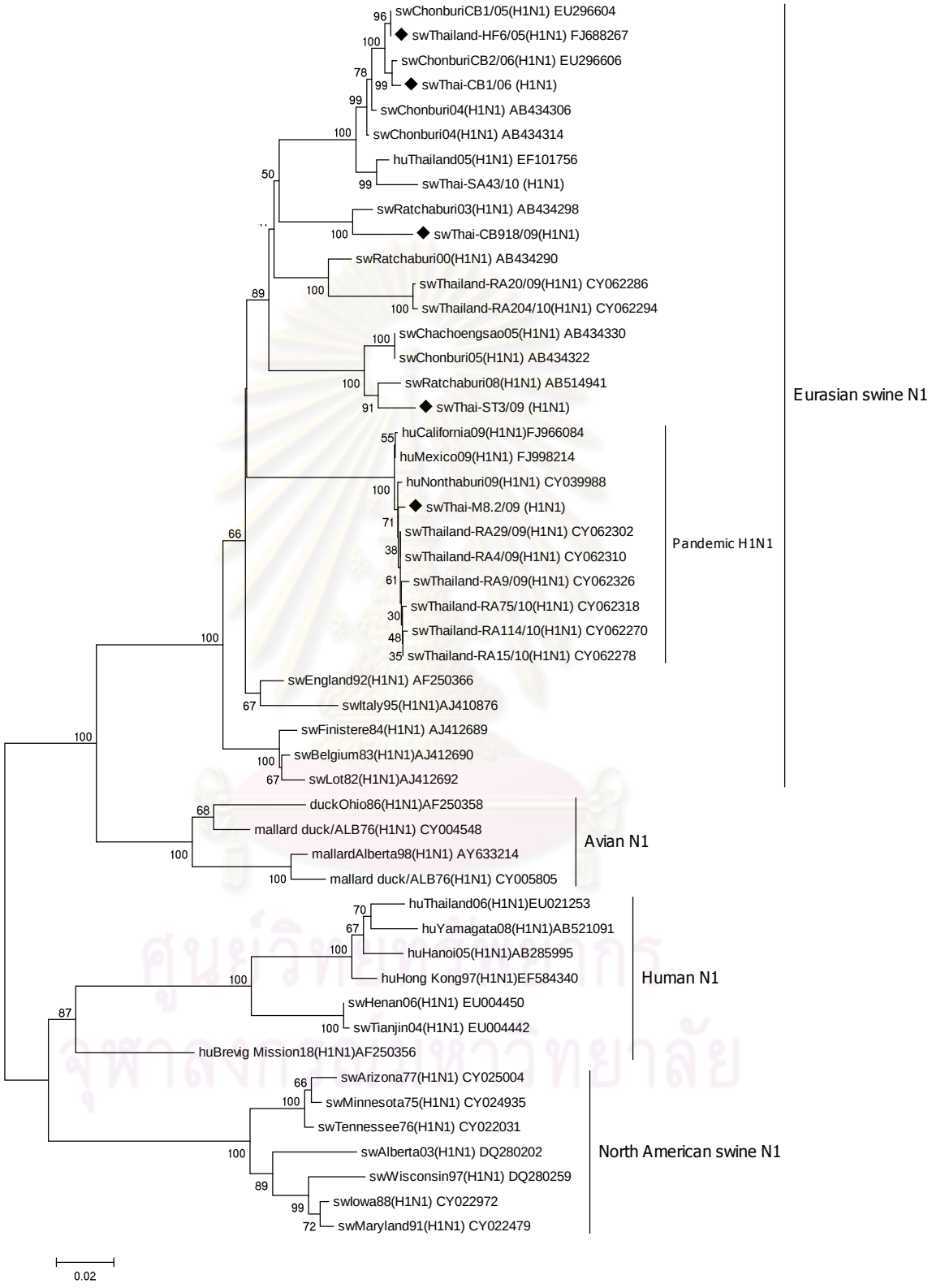

Figure 2.1(b) Phylogenetic tree of NA N1 gene

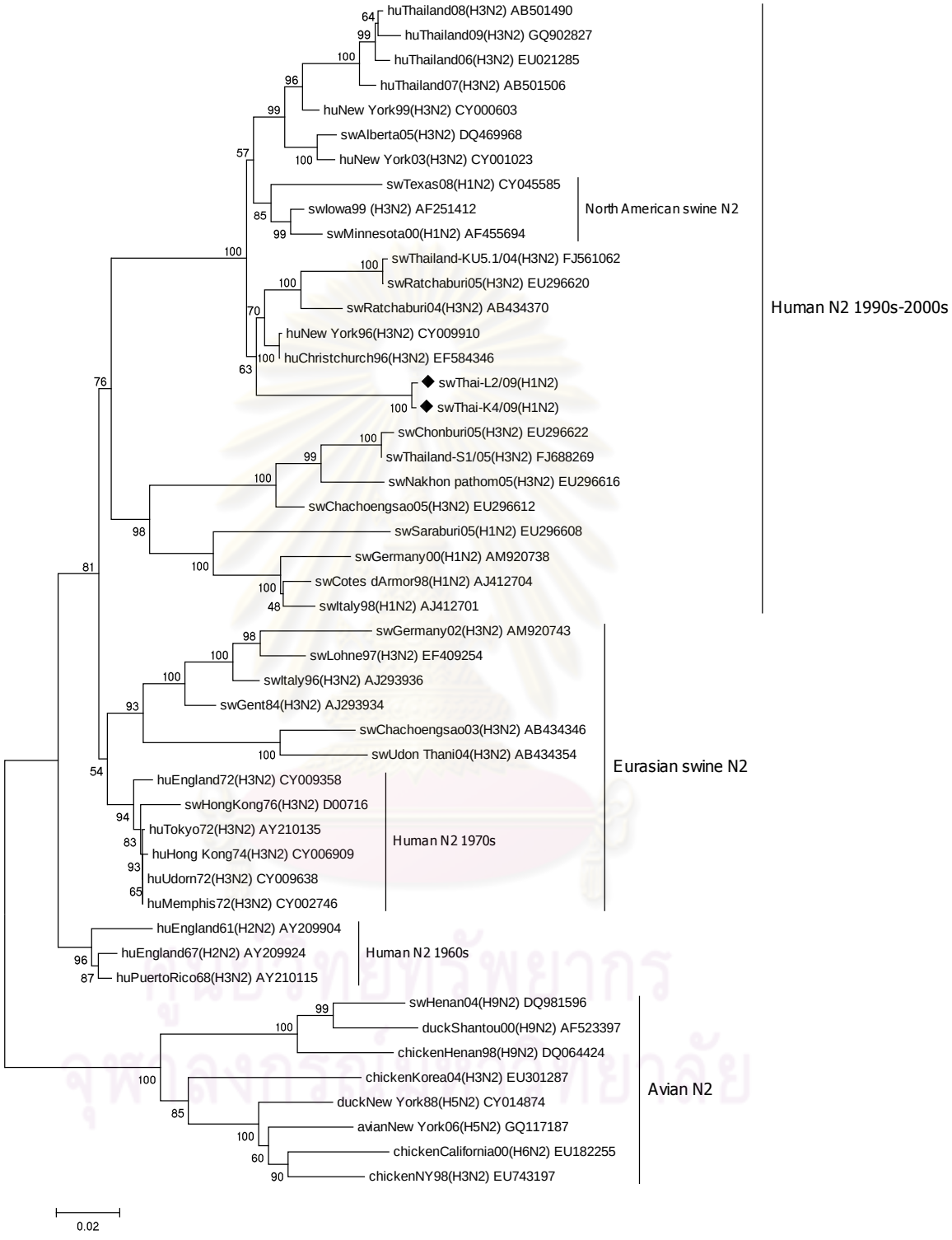

Figure 2.1(c) Phylogenetic tree of NA N2 gene

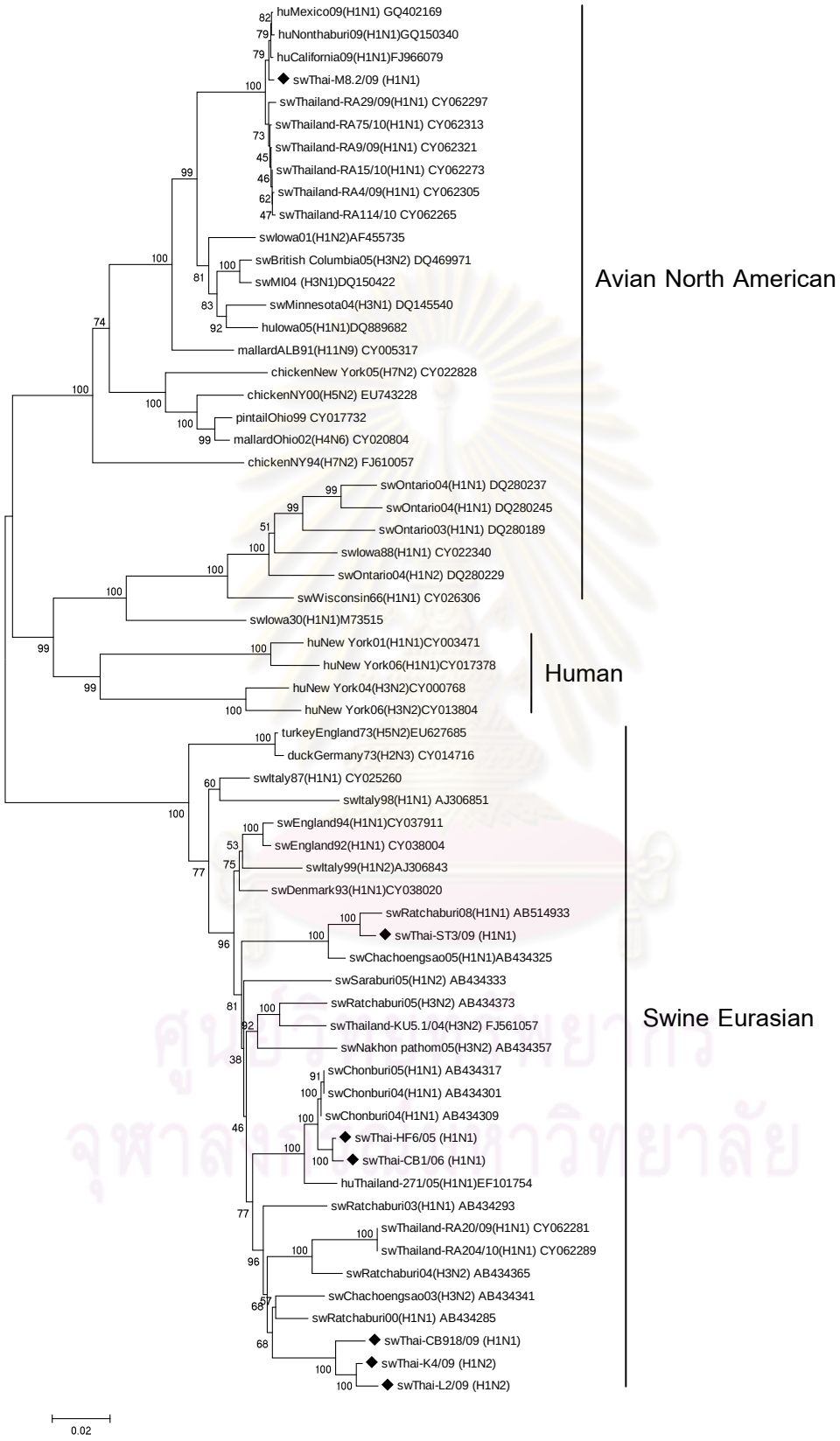

Figure 2.1(d) Phylogenetic tree of PB2 gene

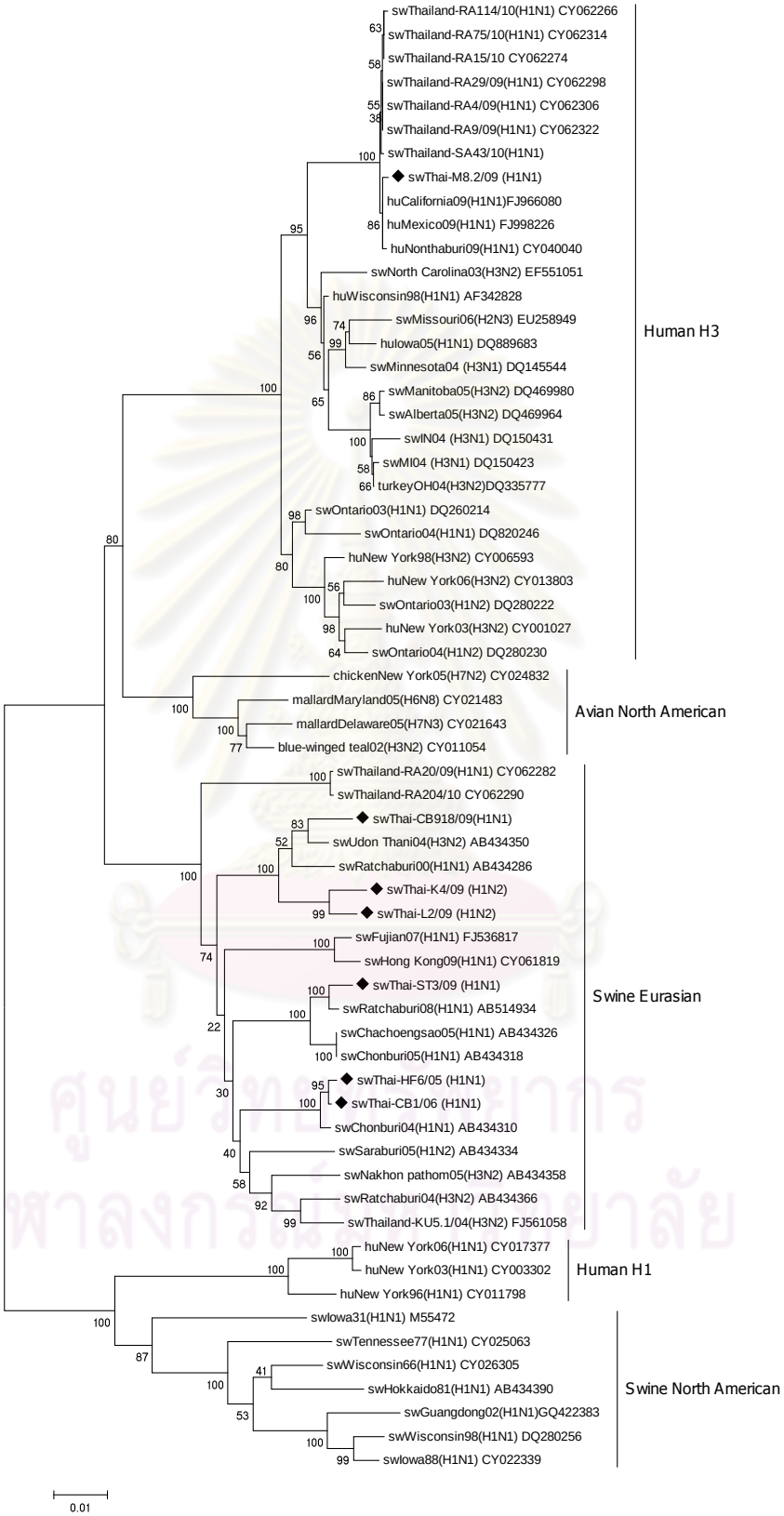

Figure 2.1(e) Phylogenetic tree of PB1 gene

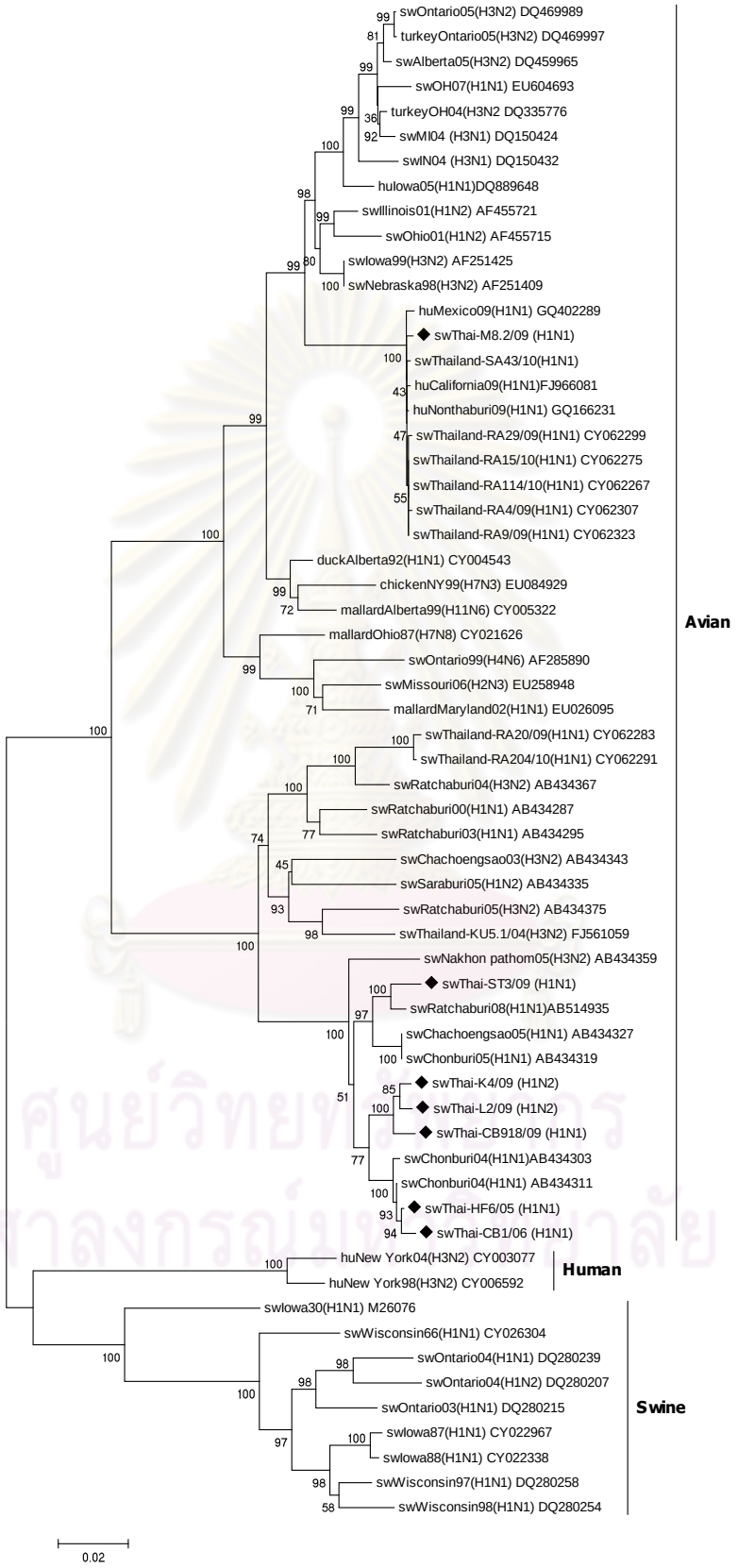

Figure 2.1(f) Phylogenetic tree of PA gene

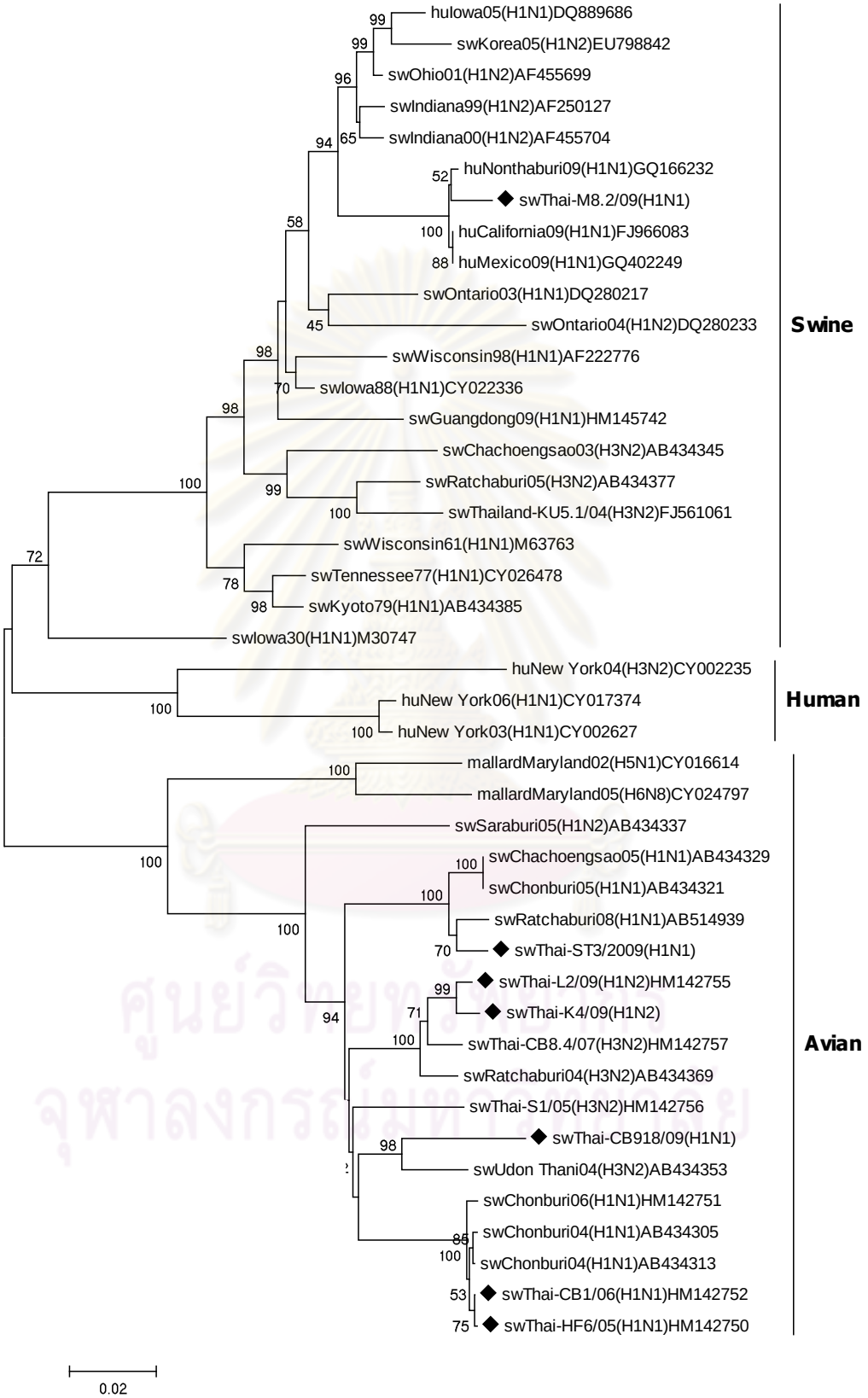

Figure 2.1(g) Phylogenetic tree of NP gene

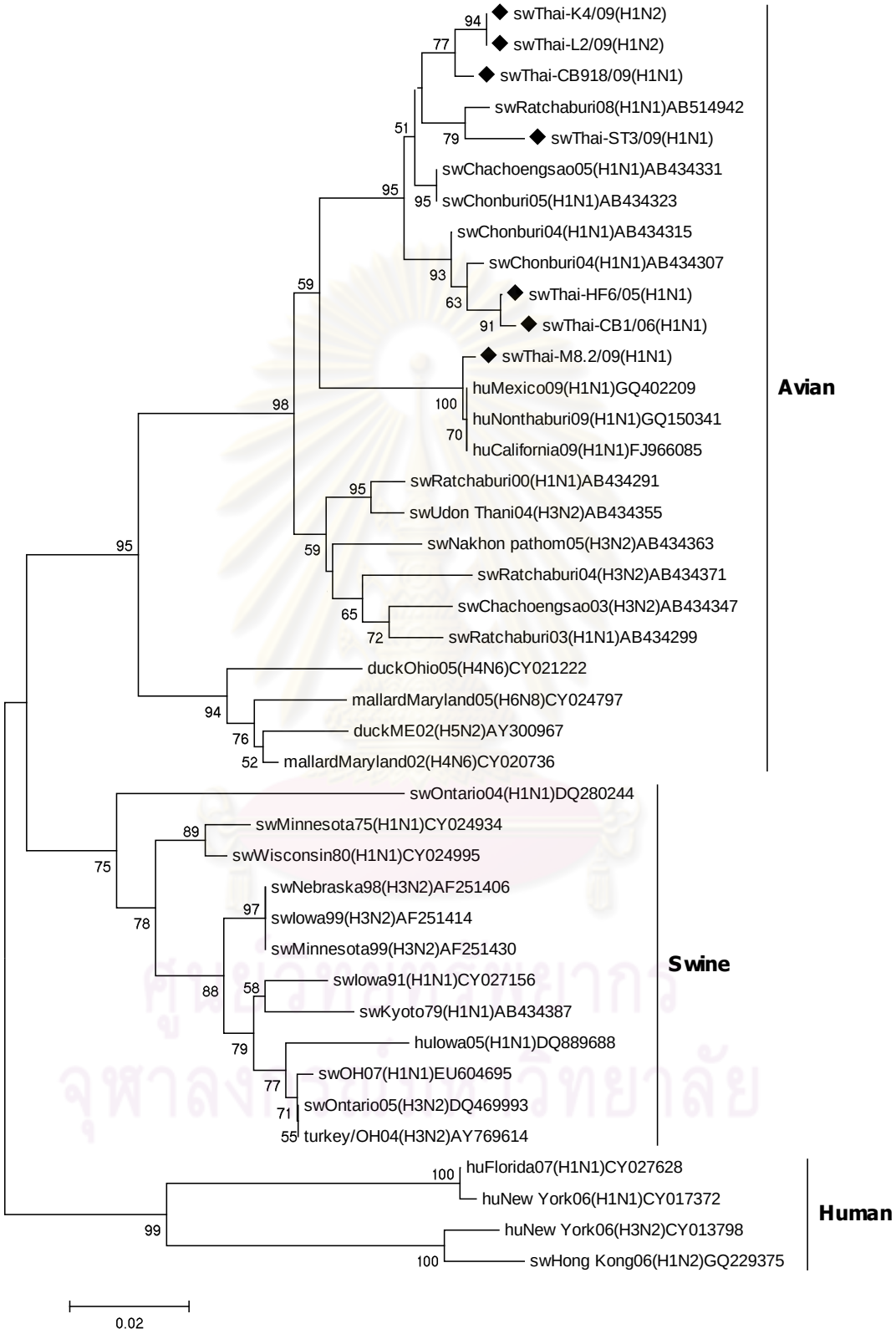

Figure 2.1(h) Phylogenetic tree of M gene

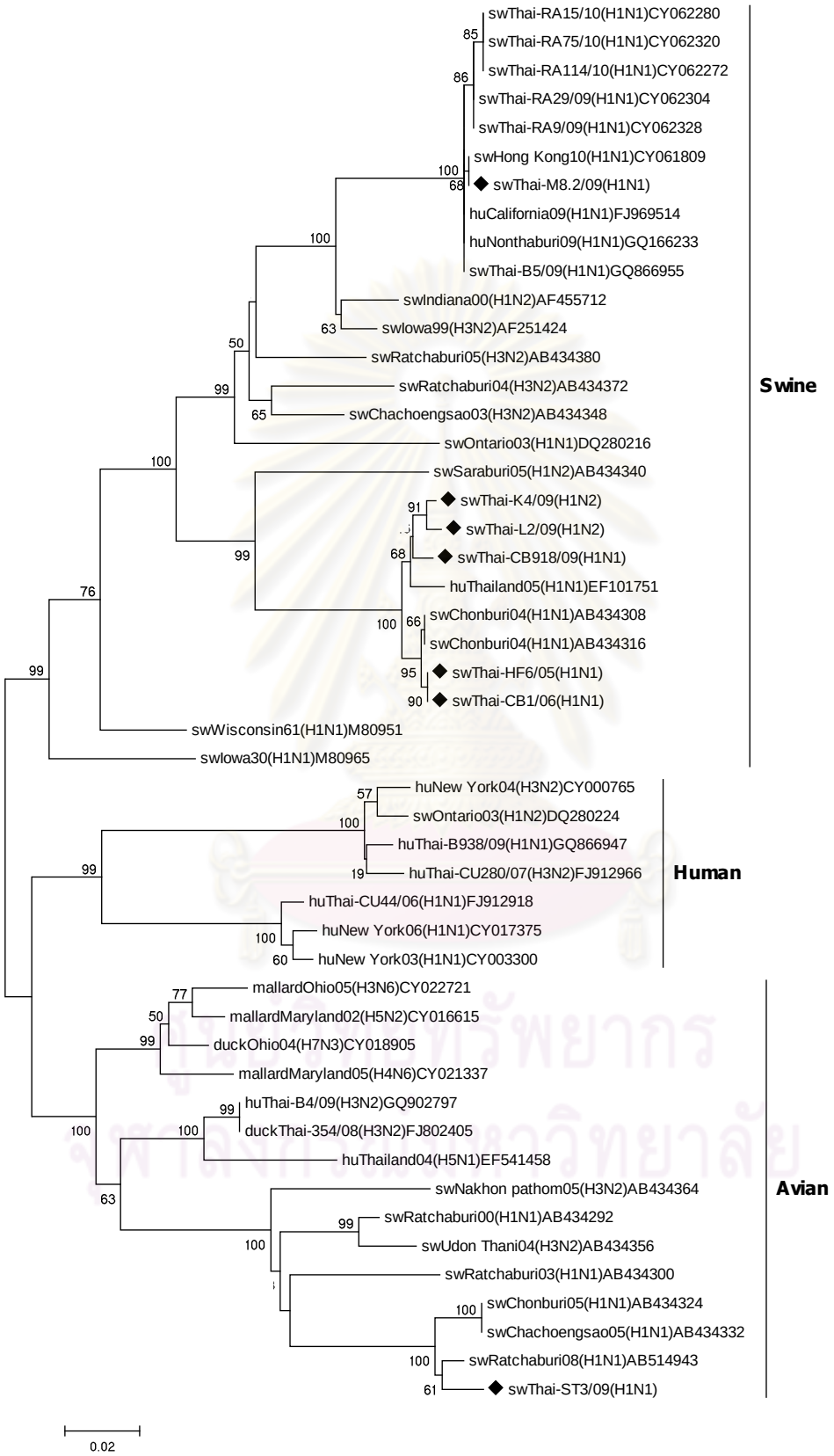

Figure 2.1(i) Phylogenetic tree of NS gene

CHAPTER 3

Serologically study of swine influenza virus subtype H1 and H3 in rabbit and serologic surveillance in the four highest pig density provinces in 2008-2009 in Thailand

3.1 Introduction

Swine influenza is an acute respiratory disease in pigs caused by type A influenza viruses. Three major subtypes including H1N1, H3N2 and H1N2 are currently circulating worldwide in the swine populations (Brown, 2000) including Thailand (Sreta et al., 2010). Swine influenza virus (SIV) infection causes acute respiratory syndrome including coughing, dyspnea, fever and prostration with rapid recovery (Sreta et al., 2009). The morbidity rate of SIV is high and may increase up to 100 % in immunologically naïve pig population whereas the mortality rate can be as low as 1%. The infected pigs will lose their weight, resulting in an increase growing period to normalize weight before going to the slaughter house (Kay et al., 1994). In addition, SIV is one of the primary pathogen of porcine respiratory disease complex (PRDC) causing major economic loss in many swine production countries (Van Reeth et al., 2001; Drolet et al., 2003; Yazawa et al., 2004). Besides causing veterinary problems, SIV is one of public health concerns on its zoonotic potential. Retrospectively, evidences of SIV isolated from humans with respiratory symptom or showed serologic evidence of SIV infection were occasionally reported (Myers et al., 2006; Myers et al., 2007; Kitikoon et al., 2010). From these reasons, the World Health Organization (WHO) and many public organizations encourages the influenza surveillance not only in humans but in all animal species, particularly in pigs (Galwankar and Clem, 2009). Retrospective and cross-sectional studies on virological and serological investigation of influenza A virus in animals are important for early recognition of outbreak threats and understanding the interspecies epidemiology. Therefore, influenza A virus investigation in pigs will not only provide the basic information of the current status in swine population but generate data for epidemic prediction and vaccine development leading to effective prevention and

control. In addition, direct detection of the viruses and/or viral genetic materials can be problematic since the duration of SIV shedding in the infected pigs is somewhat short (Olsen et al., 2000; Sreta et al., 2009). Thus, SIV surveillance study must be carefully planned as the true prevalence can be underestimated.

Hemagglutination inhibition (HI) test is a gold standard recommended by WHO, but it requires a suitable representative virus from the circulating strain used as the HI virus antigen. In general, SIV is genetically unstable. Many sub-clusters have arisen within the different H1N1 and H3N2 SIV subtypes (Ma et al., 2009). For example historic data indicated that the H1N1 SIV comprised of two major lineages; the North American- and Eurasian-swine lineages (Brown, 2000). Interestingly, in Thailand the reassortant H1N1 SIV between the North American and Eurasian lineage has been reported since swine breeders have been imported from both continents (Takemae et al., 2008; Sreta et al., 2009). It should be noted that changing of amino acid in antigenic site on HA1 regions was related to the antigenic variation of the swine H1N1 and H1N2 viruses in the United States (Vincent et al., 2009). Similarly, antigenic and genetic diversity of swine H1N1 and H1N2 viruses in Europe have been documented (Marozin et al., 2002). In addition, in countries where SIV vaccination is implemented, SIV genetic data correlation with antibody response to SIV vaccination is important and must be monitored through serological analysis.

In Thailand, no SIV vaccines are currently used in the Thai swine population. The SIV-serologic status within each farm, therefore, reflects SIV infection or historic exposure to the specific virus. Unfortunately, minimal data of SIV serological surveillance have been found in Thailand and seroprevalence of H1N1 and H3N2 SIV in 2003 was 7.9% and 20.6%, respectively (Damrongwatanapokin et al., 2003). Later in 2005, sero-positive percentage of H1N1 SIV was higher than that of H3N2 SIV (Damrongwatanapokin et al., 2006). However, serological and genetic data of Thai SIVs from 2005 to 2010 are limited except for one study reporting only 2.8% SIV positive pigs in the central and eastern Thailand by RT-PCR in 2008 (Nakharuthai et al., 2008). The low incidence of such SIV positive cases in that study is possibly due to underestimated

since SIV antigen and/or nucleic acid detection is limited due to short shedding period. As a result, SIV serological surveillance is necessary particularly in countries where SIV diagnostic laboratories are not widely established. In addition, SIV-serological data can provide an estimation of the true SIV status in swine farms.

Since HI test is considered as a flu standard test for international trade of animals by Office International des Epizooties (OIE) (Webster et al., 2002) and has been used commonly in most veterinary diagnostic laboratories. It is important that specific endemic subtypes isolated in each region or selected subtypes having a wide cross-reactivity to antibodies against different SIV strains must be used as the test antigens (Smith, 2003; Andreasen and Sasaki, 2006). Previous SIV genetic characterization in Thailand showed at least 2 clusters in each H1 and H3 SIVs (Takemae et al., 2008). Since the pandemic H1N1 2009 has been reported in a Thai swine herd (Sreta et al., 2010), it should be noted that the SIV HI test antigens have not yet been evaluated. The objectives of this study are i) to investigate cross reactivity among different field isolates of Thai H1 and H3 SIVs ii) to use the established HI test for evaluation of swine sera collected from the 4 highest pig density provinces of Thailand and iii) to compare the findings of the established HI test with the SIV commercial ELISA test kits for H1 and H3.

Basically, we hyperimmunized influenza A sero-negative rabbits with different representative Thai SIV strains (subtypes H1N1 and H3N2) and used rabbit antisera for known positive and negative control sera for the established HI test. Two commercial ELISA tests (HerdChek H1N1 and H3N2 ELISA, Idexx Laboratories, Westbrook, Maine) and the established HI test were evaluated with the swine sera collected from pigs known to have specific H1N1 and H3N2 SIVs circulating in the selected farms from the 4 highest pig population provinces of Thailand.

3.2 Materials and Methods

3.2.1 Analysis of HA gene

All 38 HA nucleotide sequences of both H1 and H3 Thai SIVs available from GenBank database were analyzed by multiple alignment and phylogeny construction using MEGA4 program. Homology analysis of amino acid sequences were evaluated

using clustalW2 (www.ebi.ac.uk/Tools/clustalW2/). The differences of HA gene were clustered based on sub-branching division and similarity. Then, we analyzed the antigenic properties of viruses belonging to each cluster available from our laboratory (figure 3.1 and table 3.1). The amino acid changes in host-restrictive or phylogenetically important regions (PIR) of HA H1 subtype (amino acid residue 96, 133 and 183) were compared with human influenza virus (A/sw/NC/00573/05; GenBank accession no. FS638306) and human-like swine H1 virus (A/sw/Minnesota/37866/1999), a classic swine lineage having cross reactivity to the swine viruses from 1930-2004 in the United State (Vincent et al., 2006; Vincent et al., 2009). In addition, the amino acid change on the antigenic site of HA1 region among H1 isolates was evaluated (Caton et al., 1982). For HA H3 subtype, 8 major potential immunodominant positions (121, 135, 144, 145, 155, 156, 157 and 188) were compared between swH3N2-05 and swH3N2-07 (Lee et al., 2007).

3.2.2 Viruses

Total six SIVs, H1 cluster I virus (A/sw/Thailand/CU-CB1/2006, swH1N1-06) , H1 cluster II viruses (A/sw/Thailand/CU-CB918/2009, swH1N1-09 and A/sw/Thailand/CU-K4/2009, swH1N2-09), H1 cluster III virus (A/sw/Thailand/CU-RA29/2009, pH1N1-09), H3 cluster I virus (A/sw/Thailand/S1/2005, swH3N2-05), and H3 cluster II virus (A/sw/Thailand/CU-CB8.4/2007, swH3N2-07) (figure 3.1 and table 3.1) were obtained from the Chulalongkorn University-Veterinary Diagnostic laboratory (CU-VDL). Viruses used for rabbit inoculation and for test antigens in HI assay were propagated in Madin-Darby canine kidney (MDCK) cells as described previously (Kitikoon et al., 2006) and stored at -80^{o}C until used.

Table 3.1 Rabbit groups were used in antiserum preparation

Group	Number of rabbits	Inocula	Code	HA character
1	5	A/sw/Thailand/CU-CB1/2006	swH1N1-06	Classic swine
2	5	A/sw/Thailand/CU-CBP18/2009	swH1N1-09	Classic swine
3	5	A/sw/Thailand/CU-RA29/2009	pH1N1-09	Classic swine
4	5	A/sw/Thailand/CU-K4/2009	swH1N2-09	Classic swine
5	5	A/sw/Thailand/S1/2005	swH3N2-05	Human-like 1970s
6	5	A/sw/Thailand/CU-CB8.4/2007	swH3N2-07	Human-like 1990s

3.2.3 Rabbit Antiserum preparation

Selected six virus isolates mentioned above were purified by sucrose gradient ultracentrifugation at 28,000 rpm for 2 hr and re-suspended in PBS. Each virus was formalin-inactivated and later used as an antigen for immunization with the ratio 1:1 of Freund's adjuvant (Sigma-Aldrich, Saint Louis, Missouri, USA) at 400 HA unit per ml (Kitikoon et al., 2009). Total 30 rabbits (H1 and H3 SIV seronegative) were tagged and divided into 6 groups with five animals in each group being intramuscularly injected with killed selected Thai SIV antigen (1 ml each) as assigned in Table 1. Each group was housed in separate cages provided with commercial food and clean water. Before starting the experiment, rabbit blood was collected and used as negative control sera. After being acclimatized for a week, those animals were immunized every 2 weeks and sera were collected every week to test for specific HI titer to the inoculated homologous virus antigen. When the HI titers raised to $\geq$ 1:160, animals were humanely sacrificed and sera were collected and stored in minus 20°C until used. In addition, the animal use protocol number for rabbits experiment is 0931049.

3.2.4 Swine serum collection

During June 2008 to May 2009, 850 pig sera were cross-sectionally collected from pigs of various age groups (gilts, sows, finishers, growers and weaning pigs) in those four provinces located in the highest pig density in Thailand (http://www.dld.go.th/ict/yearly/yearly/50stock.50html). Total 12 pig farms facing with respiratory symptoms from Ratchaburi, NakornPathom, Chonburi and Chachoengsao (figure 3.2) were obtained. All pig sera were stored in minus 20°C until used.

3.2.5 Hemagglutination inhibition (HI) test

All rabbit and pig sera were subjected to HI test. Sera subjected to test with H1N1 SIV were treated with 20% kaolin and absorbed with 50% RBC. Sera tested with H3N2 virus were treated with receptor-destroying enzyme (RDE) and absorbed with 50% chicken red blood cells (RBC) to remove non-specific inhibitors of agglutination and natural serum agglutinins. Positive SIV infection was considered when the HI titer is ≥1:40 (Olsen et al., 2002). Virus antigen used in HI test was 8 HA unit per 50 μl and 0.5% chicken RBCs was used for titer evaluation. SIV antigens used for antibody reactivity both homologous and heterologous strains in the rabbit sera included swH1N1-06, swH1N1-09, pH1N1-09, swH3N2-05, swH3N2-07 and swH1N2-09. For serologic surveillance from pig sera, four isolates (swH1N1-06, swH1N1-09, swH3N2-05 and swH3N2-07) were used as test antigens. We excluded swH1N2-09 and pH1N1-09 isolated late 2010 from this study due to the time of serum collection.

3.2.6 Enzyme-linked immunosorbent assay (ELISA)

Pig sera with known HI titers against H1N1 (swH1N1-06) and negative for H3N2 (swH3N2-05 and swH3N2-07) were evaluated by a commercial ELISA H1N1 kit (HerdChek H1N1ELISA; Idexx Laboratories, Westbrook, Maine). Similarly, sera with known HI titers against H3N2 (swH3N2-07) and negative for H1N1 (swH1N1-06) were evaluated by a commercial ELISA H3N2 kit (HerdChek H1N1ELISA; Idexx Laboratories,

Westbrook, Maine). Ninety one pig sera were tested for H1N1 and 85 were tested for H3N2 according to the protocol recommended by the manufacturer.

3.2.7 Statistical analysis

The geometric mean titers of HI test were measured and compared with the rabbit antisera groups in each respective SIV isolate using Wilcoxon rank sum analysis with normal approximation. Numbers of pig sera with an HI titer $\geq$ 1:40 were compared among the strains and provinces by chi-square analysis. The numbers of positive sera in HI test and ELISA were compared among respective SIV strains by chi-square analysis.

3.3 Results

3.3.1 Analysis of HA gene

Based on phylogenic analysis, five clusters of Thai SIVs could be divided, three of the H1 viruses (H1 cluster I, II and III) and two of the H3 viruses (H3 cluster I and II) (figure 3.1). One selected virus in each cluster was studied on its antigenic property except for the H1 cluster II using both swH1N1-09 and swH1N2-09. For homology analysis of four H1 viruses, there was about 82.2% to 86.7% homology to each other except for swH1N1-09 having 96.4% homology to swH1N2-09. Similarly, swH3N2-05 HA gene had 83.6% homology to the swH3N2-07.

For amino acid substitution, the HA1 amino acid sequences were compared and showed important residues of the H1 and H3 strains. The amino acid residue 133, 96 and 183 are characterized as host-restrictive residues or PIR of human H1 influenza virus. All four swH1 (swH1N1-06, swH1N1-09, swH1N2-09 and pH1N1-09) were compared to the A/sw/NC/00573/2005, a human-like swine isolate and A/sw/Minnesota/37866/1999, a classic swine lineage showing serum cross reactivity to the swine viruses from 1930-2004 in the United State (table 3.2). Among four swine H1 in this study, the PIR was similar except for the pH1N1-09 at residue 96. The amino acid residue 96 of the swH1N1-06, swH1N1-09 and swH1N2-09 is Threonine similar to A/sw/NC/00573/2005 but pH1N1-09 contains Isoleucine similar to A/sw/Minnesota/37866/1999. The amino acid residue 133 and 183 were similar to

A/sw/Minnesota/37866/1999, Valine and Proline, respectively. In addition, amino acid change on antigenic site (Ca, Cb, Sa and Sb) of HA1 region was evaluated in table 3.3. Total 19 amino acid residues were different among four H1 viruses. There were four residues similar in three H1 viruses (swH1N1-09, swpH1N1-09 and swH1N2-09) but different from swH1N1-06 (Ca141, Cb73, Sa162 and Sa155). For HA H3 subtype, the 8 major potential immunodominant positions (121, 135, 144, 145, 155, 156, 157 and 188) were compared (table 3.4). Seven amino acid positions were different and only an amino acid on residue 156 was similar in swH3N2-05 and swH3N2-07.

3.3.2 HI assay

For antigenic properties using rabbit antisera, HI titers of each group of the studied rabbit reached $\geq$ 1:160 against the homologous virus within 5 weeks post inoculation. Antisera from those rabbits injected with H1 antigen had no antibody titers to the other two H3 subtypes and vice versa. The HI geometric mean titers in each group against H1 and H3 antigens are shown in table 3.5 and 3.6, respectively. Rabbit antisera against swH1N1-09, pH1N1-09 and swH1N2-09 isolates had high cross-reactive HI titers (>1:160) against swH1N1-06. Interestingly, swH1N1-06 antiserum had no cross-reactivity against current H1 isolates. Similarly, swH3N2-05 and swH3N2-07 antisera had no cross reactivity to the heterologous viral antigen. These findings indicated that swH1N1-06 is a suitable representative isolate used as the HI test antigens for H1 SIV specific antibody detection in the Thai swine population. In contrast, both swH3N2-05 and swH3N2-07 should be used as the HI test antigens for H3 SIV specific antibody detection.

Total 850 field pig sera from 12 farms were evaluated for HI test using the swH1N1-06, swH3N2-05 or swH3N2-07 as the representative Thai SIV antigens. In addition, the current isolate (swH1N1-09) was also performed for the sero-prevalence of the previous pig sera. The findings demonstrated that SIV sero-status in pigs during 2008-2009 was sero-positive to both H1 and H3 viruses (figure 3.3). The number of pigs

having sero-positive to swH3N2-07 were highest compared to swH1N1-06, swH1N1-09 and swH3N2-05 (85.4, 50.1, 18.6 and 15.8%), respectively.

3.3.3 Commercial ELISA evaluation

A total of 91 pig sera were tested with the HerdChek H1N1 ELISA kit and compared to HI test using swH1N1-06 virus antigen. The two-by-two table of those tests was constructed and evaluated of sensitivity (89.36%), specificity (77.27%), false negative (8.5%) and false positive (22.72%).

Similarly, a two-by-two table of the HerdChek ELISA H3N2 and swH3N2-05 HI test showed sensitivity (70.83%), specificity (91.89%), false negative (29.16%) and false positive (8.11%) values

3.4 Discussion

In general, SIV genetic mutation normally causes by point mutation, recombination and introduction of new viruses into the local area. Unlike North American and European SIVs, Thai SIVs contain mixed genomes from both North American and European lineages. In the United States, swine H1 had been divided into three clusters, classic H1N1, reassortment H1N1 and H1N2-like (Vincent et al., 2006; Ma et al., 2010). Interestingly, genetic characterization of HA gene of Thai SIV subtype H1 belongs to classic swine similar to the classic H1N1 clusters of the North American swine lineage (Takemae et al., 2008; Sreta et al., 2009) but different from the original source. Recently, Thai SIV subtype H1 could be divided into three clusters (cluster I, II and III) by homology and phylogeny analysis of HA gene (Takemae et al., 2008; Sreta et al., 2009; Sreta et al., 2010). Therefore, whole genome characterization of previous and current Thai SIVs is necessary since it could provide the essential epidemiologic data and disease preparedness. Currently in Thailand, there are available 16 isolates (6 H1N1, 9 H3N2 and one H1N2) in 2000-2005 and 8 H1N1 in 2009-2010 whole genome (Takemae et al., 2008; Lekcharoensuk et al., 2010; Sreta et al., 2010) and 38 HA genes, which can be divided into five clusters. However, the internal genes of 14 SIV isolates are

unavailable. Those data needs for reassortment detection and evolution since each gene segment possible from different origins. For epidemiology study, all 8 gene segments must be evaluated. In addition, HI test required the specific virus for the test antigen. Both direct and indirect diagnostic tools need the genetic data compatible with the circulating SIVs in local area since SIVs from the United Stage and Europe are genetically and antigenically different from Thailand.

Moreover, we evaluated the antigenic properties using rabbit antisera by HI assay. To elucidate H1 cross reactivity, the represent isolate of subtype H1N1 in each cluster and the recent H1N2 isolate were selected for evaluation of HI titers using known rabbit antisera. The results showed that sera from the previous swH1N1-06 (H1 cluster I) inoculation had no cross antibody reactivity to all three present swH1 in 2009 (H1 cluster II and III) viruses. However, all three present swH1 rabbit sera showed cross reactivity to the previous swH1N1-06. For routine sero-surveillance using HI test, the specific endemic subtypes isolated in the local area or having a broad cross-reactivity must be used to obtain the accurate results (Smith, 2003; Andreasen and Sasaki, 2006). In this study, swH1N1-06 could be used as the representative virus for Thai SIV subtype H1 HI test since it showed one way cross reactivity to other H1 viruses. For H3N2 subtype, the rabbit sera showed no cross antibody reactivity in either selected H3N2 isolate. In addition, both swH3N2-05 and swH3N2-07 viruses were isolated from the same farm in 2005 and 2007, respectively. However, the swine serum during the studied period showed different sero-profiles with the high sero-prevalence for swH3N2-07. This result suggests that the virus could have genetic mutation within 2 years and the represent virus antigens used for HI test, therefore, should be evaluated at least every 2 years as well as genetic monitoring.

During June 2008 to May 2009, serologic surveillance in this study showed that all four selected provinces in Thailand had higher H3N2 sero-positive percentages than those of the H1N1 subtype (swH3N2-07 >70% and swH1N1-06 >40%). It should be noted that the pandemic H1N1 2009 virus did transmitted to the Thai pigs in late 2009 (Sreta et al., 2010) and the current sero-profile of the Thai SIVs could possibly be

different from this study. We strongly suggest continuous monitoring SIV status on both virology and serology.

Based on the farm history, all 12 selected pig farms in this study showed respiratory problems concurrently with the presence of PRRSV and PCV2. However, we did not evaluate the economic loss from those pathogens since the production parameters did not obtain. In addition, economic impact of Thai SIVs should be evaluated if the virus could play a major role in PRDC. Importantly, SIV has gained significant attention because of its public health concern, particularly, after the emerging of pandemic H1N1 2009. Moreover, this swine origin pandemic flu showed continuing reassortment with the endemic SIV (Vijaykrishna et al., 2010). Similarly, we found co-circulation of both pandemic H1N1 and endemic Thai swine H1N1 in the same farm fortunately with no reassortment (Sreta et al., 2010). As a result, a novel pandemic virus can possibly occur without noticing. It should be noted that SIV-infected pigs may show only acute mild respiratory distress and may not be noticed by farmers or veterinarians. In addition, human to pig transmission or vice versa may unnoticingly occur. Our study demonstrated a great benefit of the HI test which could be useful for interspecies transmission surveillance.

Importantly, countries lacking genetic information using the SIV test antigens from other countries should be considered since the genetic variation of SIV might not suitable for the surveillance of the endemic SIVs. In this study, we compared the commercial ELISA test kits with the established HI test. The results showed that the commercial ELISA H1N1 kit had 89.36% sensitivity and 77.27% specificity. The high sensitivity related to the antigens used in both tests sharing the same origin of swine H1, classical swine H1 (Takemae et al., 2008; Sreta et al., 2009). However, the swine H3 viruses found in both the United State and Thailand are human-like but both tests yielded low sensitivity (70.83%). Therefore, evaluation of the commercial test kits from other countries is necessary for the real epidemiology data due to the genetic differences of the circulating viruses.

In general, nucleotide sequences encode the amino acid sequences mostly related to protein function. The PIRs suggesting possible host adaptation (Vincent et al., 2009) was located at the amino acid residue 96 of the HA H1 gene. The change at the amino acid residue 96 from Threonine ⟶ Isoleucine of the pH1N1-09 in year 2009 was different from other swine H1 viruses in 2006-2009. The pH1N1-09 virus found in pigs in this study is similar to the pandemic H1N1 2009 that emerged and spread worldwide in humans and well adapted in both human and swine populations (Dawood et al., 2009; Pasma and Joseph, 2010). In addition, the endemic swine H1N1 virus had been reported in one human case in 2005 in Thailand (Komadina et al., 2007). Moreover, rabbit serum inoculated with the pH1N1-09 showed no cross antibody reactivity to other Thai swine H1N1 viruses. Hence, the HI test using pH1N1-09 virus antigen could be applied for the pandemic H1N1 2009 infected pig detection retrospectively. For HA H3 subtype, 7 of 8 major potential immunodominant positions were different between the swH3N2-05 and swH3N2-07 (Lee et al., 2007) and they showed no cross reactivity. Again, the swine H1 viruses have similar PIRs except for the pH1N1-09. In addition, swH1N1-06 virus showed one way cross reactivity to other swine H1 viruses. Those results could possibly be from the change of four amino acid residues on the antigenic site of HA1 region (Ca141, Cb73, Sa162 and Sa155). Although, changing amino acid residue may have less relation to protein function, many factors related to binding ability of antibody and antigen such as protein sequences and conformation may have the impact on the HI test (Suzuki and Nei, 2002).

Table 3.2 Amino acid changes in host-residues or phylogenetically important regions (PIR) of H 1 subtype.

Residue*	PIR	NC05	swMN99	swH1N1-06	swH1N1-09	swH1N2-09	pH1N1-09
133	-	S	V	V	V	V	V
96	D	T	I	T	T	T	I
183	I	P	P	P	P	P	P

*H1 numbering with being the first amino acid after the signal sequence

NC05 is A/sw/NC/00573/2005 H1N1 (accession no. FJ638306).

swMN99 is A/sw/Minnesota/37866/1999 H1N1 (accession no. EU139827).

Table 3.3 Amino acid change on antigenic site of HA1 region of swine H1N1 viruses

Antigenic site	Ca							Cb					Sa				Sb
Amino acid residue	138	141	142	168	170	205	222	69	71	72	73	74	121	155	160	162	186
swH1N1-06	Y	T	N	N	K	R	G	L	F	A	V	N	S	E	K	R	T
swH1N1-09	Y	A	N	N	K	T	N	L	F	K	A	N	N	G	K	S	T
pH1N1-09	H	A	K	D	G	R	D	S	S	T	A	S	S	G	K	S	A
swH1N2-09	Y	A	N	N	K	T	D	L	F	K	A	N	N	G	R	S	T

Table 3.4 The 8 major potential immunodominant positions of both swH3N2-05 and swH3N2-07.

Position	121	135	144	145	155	156	157	188
swH3N2-05	I	G	D	N	Y	K	S	N
swH3N2-07	T	T	V	K	H	K	L	D

Table 3.5 Group geometric means of HI titers were evaluated against 3 H1N1 and one H1N2 influenza challenge antigens used in this study.

		Test antigen			
Group	Serum	**swH1N1-06**	**swH1N1-09**	**pH1N1-09**	**swH1N2-09**
1	swH1N1-06	**367.58**[a]	0	0	0
2	swH1N1-09	211.12[a]	**183.79**[a]	30.31[a]	139.29[a]
3	pH1N1-09	211.12[a]	91.90[b]	**320.00**[b]	91.90[b]
4	swH1N2-09	320.00[a]	367.58[a]	139.29[b]	**557.15**[a]

[a, b] Statistically significantly different ($p<0.05$) in each rolls

SIV isolates were tested as antigens and HI titers of homologous virus/sera are in bold.

Table 3.6 Group geometric mean of HI titers was evaluated against 2 H3N2 influenza challenge antigens used in study.

		Test antigen	
Group	Serum	**swH3N2-05**	**swH3N2-07**
5	swH3N2-05	**160.00**	0
6	swH3N2-07	0	**844.49**

SIV isolates were tested as antigens and HI titers of homologous virus/sera are in bold.

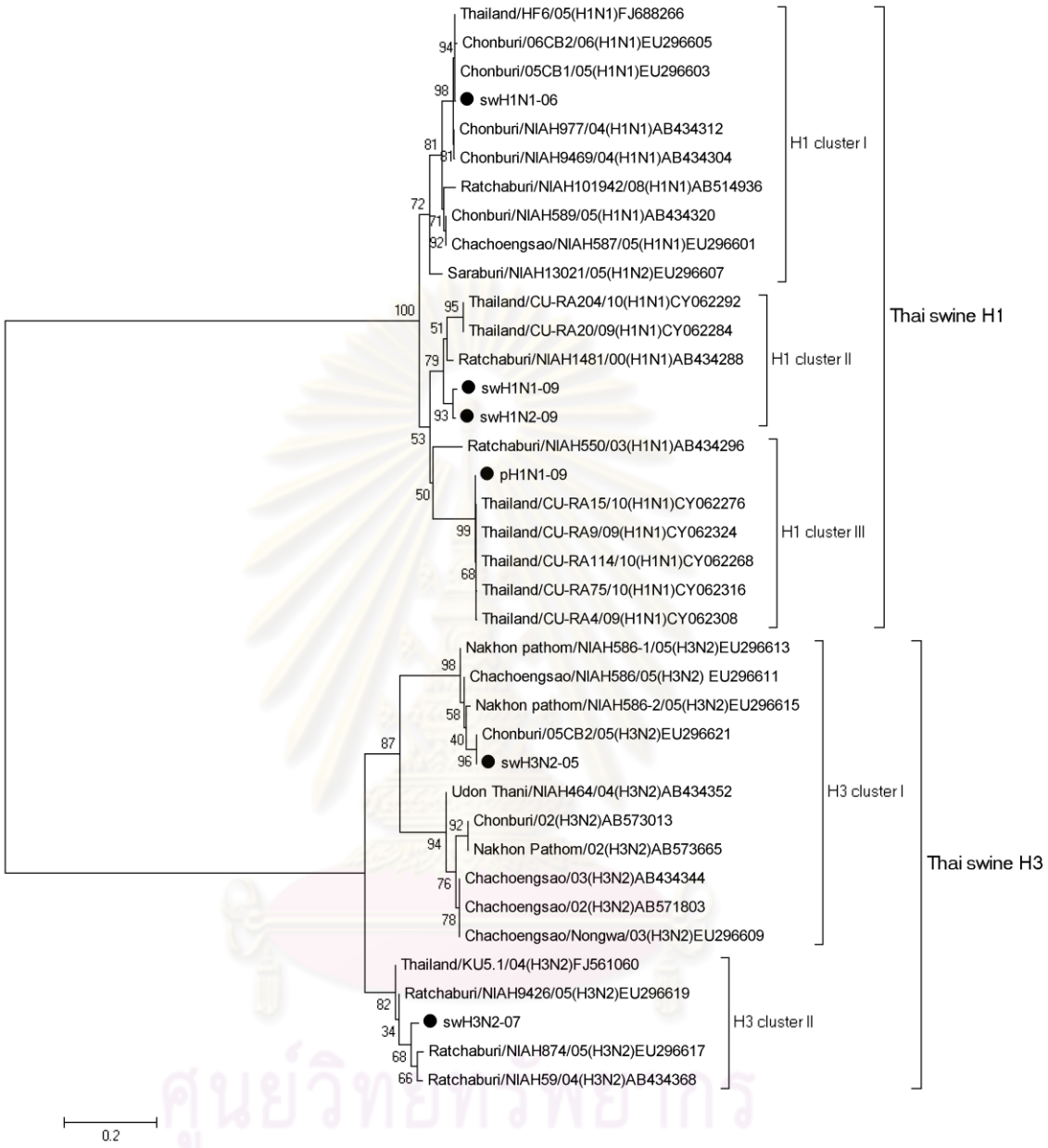

Figure 3.1 Phylogenetic analysis of the nucleotide sequence of the HA gene of Thai SIVs that summated to the GenBank database and the isolates evaluated antigenic property in the study (*filled circle*). The tree was constructed by the neighbor-joining method and bootstrapped 1,000 replicates. The bootstrap numbers show on each node.

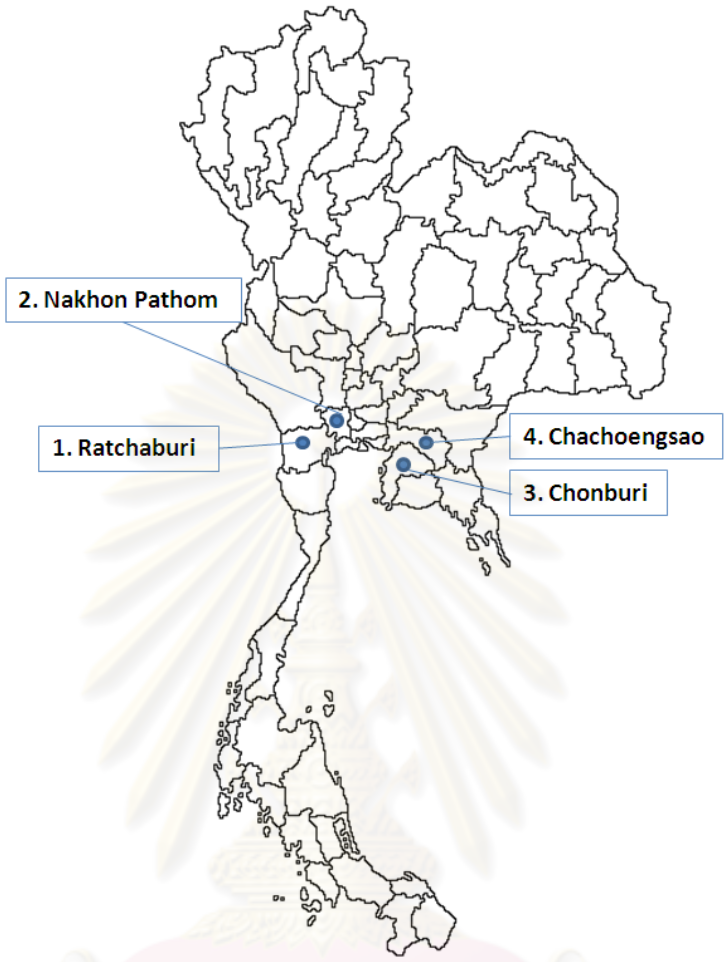

Figure 3.2 Thailand map show the four provinces that had high pig density in Thailand 2008. Those provinces include two from the central (Ratchaburi and Nakhon-Pathom) and two from the eastern (Chonburi and Chachoengsao).

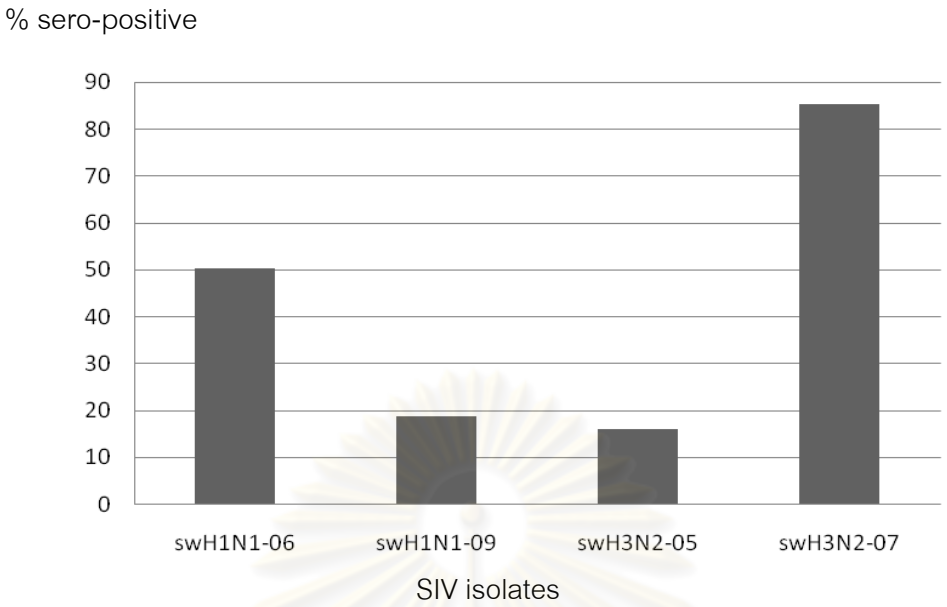

Figure 3.3 The percent of sero-positive HI test against variant four SIV isolates. The pig sera were collected from pigs of various age groups (gilts, sows, finishing, growing and weaning pigs), during June 2008 to May 2009 and N=850. The SIV sero-positive was HI titer ≥ 1:40.

CHAPTER 4

Serological evidence of pig-to-human influenza virus transmission on Thai swine farms

4.1 Introduction

Occasionally, swine influenza virus (SIV) transmission to humans, and vice versa, has been documented (Katsuda et al., 1995; Karasin et al., 2006). Previously documented SIV-infected human cases were nonfatal and human-to-human transmission was rare (Myers et al., 2007). In addition, a large number of cases involved swine farmers, people who lived on swine farms or were in close contact with pigs. At present, three SIV subtypes, H1N1, H1N2 and H3N2 are commonly found in swine throughout the world (Easterday and Van Reeth, 1999). The first subtype isolated was named “classical-swine” influenza virus as all eight gene segments were of swine origin. Classical swine H1N1 (cH1N1) was known as the dominant virus in North American swine populations for over 60 years (Easterday and Van Reeth, 1999). Recent data indicated that the HA1 genes of classical-swine subtypes could now be grouped into three separate clusters, swine H1-alpha (swH1α), swine H1-beta (swH1β) and swine H1-gamma (swH1γ) (Vincent et al., 2009). The HA1 gene of swH1γ is known as the progenitor of the pandemic H1N1 2009 (pH1N1) viruses (Kingsford et al., 2009). In contrast, H1N1 virus originally introduced into the European pig population is often referred to as “avian-like” virus since it contains entire avian genes that are genetically distinct from the cH1N1 viruses. Currently, European H1N1 isolates contain an HA1 segment from both human and avian lineages while the remaining gene segments are still of avian origin (Zell et al., 2008). Thai H1N1 SIV contains surface HA1 and NA1 antigens from the North American (classical swine)- and Eurasian (avian-like)- swine lineages, respectively (Takemae et al., 2008; Sreta et al., 2009). Notably, this feature is uniquely shared among the pH1N1 viruses that emerged in eary 2009 (Kingsford et al., 2009).

Swine production system in Thailand is different from the systems in North America and Europe. The production size in Thailand rages from large industrialized farms (>1000 pigs) to backyard farms (<50 pigs). Pig housing in Thailand also consists of both closed housing or evaporation cooling system and open housing where natural air flow ventilates the units. Normally, Thai swine farm owners and farm workers including their spouses and children live on the farm. Such an environment provides an excellent human-animal interface for influenza virus cross-species transmission. The aim of this study was to investigate the serological evidence of influenza virus interspecies transmission among Thai swine workers and pigs on the farm. Tests for hemagglutination-inhibition (HI) antibodies against the Thai swine H1N1 viruses (swH1N1 and swH1N2) isolated from both farms and representative Thai human H1N1 (huH1N1) and pH1N1 were performed on both human and swine populations. To evaluate the genetic diversity of the viruses utilized as HI test antigen, the HA1 genes of swH1N1 and swH1N2 viruses were sequenced followed by phylogenetic analysis adding huH1N1, pH1N1 and other reference H1 influenza viruses form GenBank.

4.2 Materials and methods

4.2.1 Study population

4.2.1.1 Human population

From 2008 to early 2009, sera from 78 swine-exposed participants were collected from two large scale commercial farms in the central-eastern region of Thailand. Subjects were farm owners (n = 2), pig handlers (n = 52), veterinarians (n = 8), farm cleaners (n = 8) and people working in the farm office (n = 8) with their age ranging from 18 to 59 years (50% males and 50% females). Sixty negative control subjects with no history of swine exposure (verified by personal interview) were voluntarily recruited from the Blood centre and hospital in the central-eastern region of Thailand. The non-swine-exposed control sera were obtained from 50% males and 50% females with their age ranging from 19 to 60 years. During the time of investigation all subjects were healthy with no influenza-like illness. The study had been approved by the institutional review board (137/2007record#400/49).

4.2.1.2 Swine population

Eighty-five pig serum samples were collected from farms A and B, 46 and 39 samples, respectively. Both farms maintained an open housing system. Swine serum was randomly sampled cross-sectionally from different age groups including gilts, sows, weaning and growing pigs.

4.2.2 Influenza viruses for hemagglutination-inhibition (HI) test

Influenza viruses used as HI test antigens included two swine and human influenza viruses each. The swine viruses, H1N1 (A/Swine/Thailand/CU-CB1/06; swH1N1) and H1N2 (A/Swine/Thailand/CU-CHK4/09; swH1N2) were isolated from the lungs of pigs during an outbreak of widespread illness among nursery pigs from farm A and B, respectively. Both isolates were propagated in MDCK cells as described previously (Kitikoon et al., 2006). The human viruses, Thai seasonal H1N1 (A/Thailand/CU41/06; huH1N1) and pandemic H1N1 2009 (A/Nonthaburi/102/09; pH1N1), accession numbers EU021246 and GQ150342, respectively were kindly provided by Professor Y. Poovorawan (Chulalongkorn University, Thailand).

4.2.3 Hemagglutination inhibition (HI) test

Swine and human serum samples were pretreated with 20% kaolin and receptor-destroying enzyme (Denka Seiken Co., Ltd., Tokyo, Japan) and influenza-specific antibody detection was performed with a standard HI assay (Yoon et al., 2004). Serum only controls along with positive and negative serum controls (from influenza A seronegative rabbits hyperimmunized with swH1N1, swH1N2 and pH1N1 HI test antigens) were included with each set of samples tested. HI assays on swH1N1, swH1N2 and huH1N1 viruses were performed using 0.5% chicken RBC in phosphate buffered saline (PBS). Assays on pH1N1 virus were performed with 0.5% turkey RBC in PBS. Samples with HI titers≥1:40 were considered positive evidence to previous exposure (Olsen et al., 2002).

4.2.4 HA1 gene analysis of the Thai swine H1 viruses

Viral RNA was extracted from swH1N1 and swH1N2 using the NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) and cDNA were synthesized using universal primer (Uni12 primer 5'-AGCAAAAGCAGG-3') and Omniscript RT kit (Qiagen, USA) followed by hemagglutinin (HA) gene amplification as described previously (Hoffmann et al., 2001). Complete HA gene sequencing was carried out by 1st BASE Company and analyzyed by Bioedit Sequence Alignment Editor V.7.0.5.3. Sequences were submitted to GenBank [accession number, GU454848 (swH1N1) and Gu454849 (swH1N2)]. The phylogenetic tree was constructed using MEGA4 with neighbor-joining methad and 1000 bootatrap replicates (Saitou and Nei, 1987).

4.2.5 Statistical analysis

The differences between each group's HI geometric mean titers were measured using Wilcoxon/Kruskal-Wallis test or Rank sum test (JMP 5.1 Software, SAS Institute, Cary, NC). HI test results were also evaluated as dichotomous outcomes with HI titers ≥ 1:40 considered to be previously exposed to virus antigen. The association of occupational risks was then examined for statistical significance by Chi-square or two-sided Fisher's exact analysis. Analysis was performed using Open Source Epidemiologic Statistics for Public Health, Version 2.3 (Dean et al., 2009).

4.3 Results

4.3.1 Influenza virus antibodies in human population

Fifty and 92% of swine-exposed workers from A and B, respectively had developed antibodies against the SIV isolate circulating on the corresponding farm (table 4.1). The SIV antibody positive participants included 2 farm owners (2/2, 100%), 46 pig handlers (46/52, 88.5%), 4 veterinarians (4/8, 50%), 5 farm cleaners (5/5, 62.5%) and 2 people working in the farm office (2/8, 25%). The participants from farms A and B seropositive to Thai huH1N1 amounted to 14.3 and 42%, respectively. However, none were seropositive to pH1N1. The difference in numbers of antibody positive samples

between the farm workers and non-exposed controls was statistically significant ($p < 0.001$). When compared to the non-exposed control subjects, swine-exposed participants had increased odds of elevated antibody levels to SIV [swH1N1 odds ratio (OR) 42.63, 95% confidence interval (CI), 14.65-124 and swH1N2 58, 95% CI 13.12-256.3].

Table 4.1 Hemagglutination inhibition titers and percentage of seropositive serum samples from farm and control humans against the Thai swine and human influenza viruses and pandemic H1N1 2009 virus.

Test antigens	Farm A (*n* = 28)		Farm B (*n* = 50)		Control participants (*n* = 60)	
	HI titer	% (*n*)	HI titer	% (*n*)	HI titer	% (*n*)
swH1N1	40[b]	50 (14)	160[c]	96 (48)	10[a]	5 (3)
swH1N2	20[b]	21.4 (6)	80[c]	92 (46)	10[a]	3.3 (2)
huH1N1	10	14.3 (4)	20	42 (21)	5	1.7 (1)
pH1N1	10	0 (0)	10	0 (0)	5	0 (0)

Mean titers with different exponential letters within a row are statistically significant ($p > 0.0001$). HI titer $\geq$ 40 are considered seropositve and indicate previous infection.
swH1N1 = A/Sw/Thailand/CU-CB1/06 (H1N1) isolated from pigs in farm A.
swH1N2 = A/Sw/Thailand/CU-CHK4/09 (H1N2) isolated from pigs in farm B.
huH1N1 = A/Thailand/CU41/06 (H1N1) pH1N1 = A/Nonthaburi/102/09 (H1N1).

4.3.2 Influenza virus antibodies in swine population

Overall, pigs from all age groups from both farms were found seropositive to both swH1N1 and swH1N2 viruses except for gilts on farm B proving seronegatative to

swH1N1 virus. No huH1N1- or pH1N1- specific HI antibodies were detected in the studied pig population.

4.3.3 HA1 sequence analysis of Thai swine H1 viruses

Phylogenetic analysis based on the HA genes of swine H1 viruses revealed that the viruses grouped in the classical swine lineage cluster swH1α as two separate clades (classical swine a (Cla) and Clb) (table 4.2 and figure 4.1). Data indicated that swH1N1 and swH1N2 differed by less than 3.2% and 4.2% from the representative strain of Clb and Cla, respectively (table 4.2). Genetic analysis has shown that the HA1 genes of all Thai H1N1 human influenza viruses isolated prior to2009 (with the exception of A/Thailand/271/05 (H1N1)) have HA genes of human origin and are distinct from the Thai swine H1 viruses (less than 80% similarity). The HA gene of huH1N1 differed by less than 3.3% at the nucleotide level when compared to other Thai human H1N1 (isolated during 2006-2008) and human H1N1 strains incorporated into the human influenza vaccines during 2007-2009 (table 4.2). These Thai seasonal human H1N1 isolates shared 98.9% amino acid homology of the HA protein indicating antigen similarity between seasonal human H1N1 viruses isolated in Thailand during 2006-2008.

4.4 Discussion

Previous genetic analysis of Thai swine H1 viruses (subtypes, H1N1 and H1N2) has demonstrated that Thai HA1 genes isolated in the course of 2000-2005 were derived from the North American classical-swine lineage and are separated into 2 subclusters (Cla and Clb) (Takemae et al., 2008). The HA1 genes of the Thai swine H1 viruses isolated in 2006 and 2009 were largely homologous to the previous Thai swine H1 viruses. In addition, recent isolates, swH1N2 and swH1N1 were place into 2 separate subclusters Cla and Clb, respectively, similar to older isolates from the same provinces (Takemae et al., 2008). Phylogenetically, both old and new Thai swine H1 viruses were genetically distinct from the Thai seasonal human (huH1N1) viruses (2006-2008). Human H1N1 vaccine strains (vacH1N1) included into the 2007-2009 human influenza vaccines and pH1N1 viruses. The Thai huH1N1 viruses, however, grouped

with A/Swine/Minnesota/07002083/07 virus which was isolated from pigs in North America and previously grouped with a new cluster, swH1δ consisting of HA1 genes of human origin (Vincent et al., 2009)

The lack of control antibodies against the huH1N1 HI test antigens has been a limitation to our study. However, recent research has reported minimal cross-reactivity between HI antibodies raised against swH1α virus and antibodies raised against swH1δ and pH1N1 viruses and vice versa (Vincent et al., 2009). HI antibodies against swH1n1 and swH1N2 viruses from pigs on both farms indicated the endemic nature of both viruses. The studied pigs were seronegative to huH1N1 and pH1N1 indicating no human-to-swine influenza virus transmission during the investigation period. Importantly, swine workers infected with seasonal huH1N1 viruses should neither work nor have contact with pigs in order to lower the potential of human-to-pig viral transmission. The timing of this investigation (2008 to early 2009) was prior to the emergence of pH1N1 in the human population (April 2009) confirming the absence of pH1N1 virus at least on these pig farms.

In contrast, serological analysis of human sera clearly indicated swine-to-human virus transmission having occurred on both farms. Almost all swine-exposed participants (except for one) in this study had no history of human influenza virus vaccination, therefore, confounding cross-reactive human vaccine H1-antisera to Thai swine H1 viruses are limited. Base on phylogenetic analysis, confounding cross-reactive human sera with Thai huH1N1-antibodies to the Thai swine H1 viruses are also less likely. Former studies in the U.S. have listed swine exposure as an important risk factor for humans to contract SIV infection and increased odds of elevated SIV-antibody titers in titers in such human populations were detected (Olsen et al., 2002; Gray et al., 2007). The increased odds of elevated SIV-specific antibodies detected among the Thai swine-exposed workers (swH1N1 OR 42.6 and swH1N2 OR 58) were close to the findings described by another study (OR 54.9) conducted on North American swine workers (Gray et al., 2007). The distinctive cluster among the HA1 genes of contemporary Thai

swine H1 viruses and Thai huH1N1 and vacH1N1 viruses suggests that Thai swine-exposed workers might not have been protected against the Thai swine H1 viruses ever if they had been vaccinated with the previously available human influenza vaccines. To date, the pH1N1 strain has been included into the 2010 human influenza vaccines. However, cross-protection provided by the vaccine to the contemporary Thai swine H1 viruses requires further investigation. Swine-exposed populations as the frontline of contracting SIV, particularly in Thailand should be concerned since (1) swine influenza vaccination is not performed on Thai swine farms; (2) clinical signs in pigs are hard to identify on endemic farms which renders personal protection management difficult; and (3) most pig handlers are uneducated migrant workers from neighboring countries. Therefore, continuous monitoring for SIV infection, education on self-protection and additional research aimed at characterizing specific risk factors unique to the populations are required to help implement future influenza control programs.

Table 4.2 Percent homology of the HA genes of swH1N2, swH1N1, huH1N1 and pH1N1 compared to reference swine and human H1 viruses available at the GenBank database

Species of origin (cluster)	Strain	Designation	% nucleotide homology		
			swH1N2	swH1N1	huH1N1
Swine (swH1α)	A/Sw/Thailand/CU-CHK4/09(H1N2)	swH1N2	100.0	85.8	71.1
	A/Sw/Thailand/CU-CB1/06 (H1N1)	swH1N1	85.8	**100.0**	72.5
	A/Sw/Ratchaburi/NIAH1481/00(H1N1)	swRat00[a]	96.8	86.2	72.0
	A/Sw/Chonburi/NIAH589/05(H1N1)	swChon[b]	86.5	95.8	72.2
Swine (swH1γ)	A/Sw/Indiana/P12439/00(H1N2)	swIN00	87.9	86.1	69.7
	A/Nonthaburi/102/09(H1N1)	pH1N1	85.5	83.4	69.6
	A/Thailand/CU-B5/09(H1N1)	pH1N1	85.6	83.4	69.3
Thai Human Seasonal	A/Thailand/CU41/06(H1N1)	huH1N1	71.1	72.5	**100.0**
	A/Thailand/500/07(H1N1)	huH1N1/07	79.2	77.7	96.7
	A/Thailand/669/08(H1N1)	huH1N1/08	78.4	77.7	97.3
Human H1N1 Vaccine	A/New Caledonia/20/99(H1N1)	Vaccine 2007	73.3	74.1	97.5
	A/Solomon Islands/3/06(H1N1)	Vaccine 2008	69.6	71.4	99.1
	A/Brisbane/59/07(H1N1)	Vaccine 2009	72.8	73.2	97.9

[a] Representative strain of Thai swine H1 classical swine a (Cla) (Takemae et al., 2008).

[b] Representative strain of Thai swine H1 classical swine b (Clb) (Takemae et al., 2008).

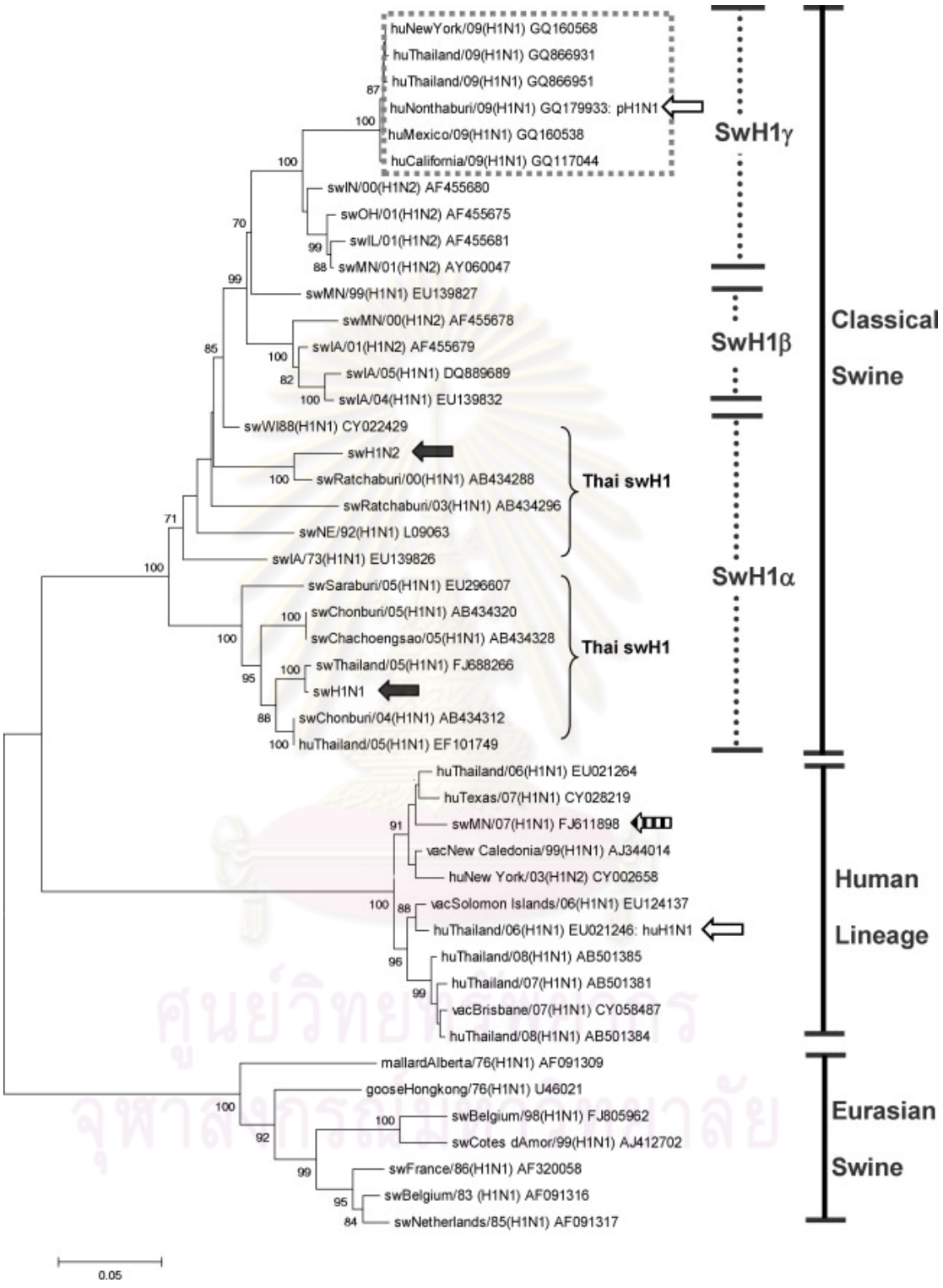

Figure 4.1 Phylogenetic tree for the HA1 segment based on nucleotide sequence of the Thai swine (swH1N1 and swH1N2) and human (huH1N1 and pH1N1) influenza viruses and other sequences available at GenBank. Three clusters of related classical swine H1

viruses, swH1α (classical swine H1N1), swH1β (reassorted H1N1-like) and swH1γ (H1N2-like) are shown (Vincent et al., 2009). The black arrow indicates the Thai swine H1 isolates sequenced and analyzed in this study. The open arrow indicates human viruses used as hemagglutination-inhibition (HI) test antigen. The striped arrow indicates the representative human-like virus isolated in 2007 from pigs in North America that grouped with swH1δ (human-like H1). Isolates in the dotted square box are the pandemic H1N1 2009 viruses. The reference influenza viruses used for the analysis are host abbreviated (*sw* swine; *hu* human; *vac* human influenza vaccine) followed by place and year of origin, subtype and GenBank accession number. The length of the horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes.

CHAPTER 5

Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand

5.1 Introduction

In April 2009, a novel swine origin influenza A H1N1 virus now referred to as pandemic (H1N1) 2009 virus, emerged in human in Mexico and the United States and spread worldwide (Dawood et al., 2009). In May 2009, pandemic (H1N1) 2009 was confirmed in 2 patients in Thailand who had a history of travel to Mexico. Shortly after emergence of this virus, reports of transmission from humans to pigs on pig farms were documented (Pasma and Joseph, 2009; Pereda et al., 2009). Human-to-pig transmission of this virus was reported in Thailand on December 17, 2009 (www.dld.go.th/dcontrol/Alert/Ah1n1/H1N1%20update22_12_2009.pdf). Pigs showed mild respiratory signs; only 1 pandemic (H1N1) 2009 virus was isolated from 80 nasal swab specimens.

Swine influenza virus (SIV) was reported in Thailand since 1981 (Kanai et el., 1981). All 3 subtypes (H1N1, H3N2 and H1N2) of this virus are circulating in Thailand (Takemae et al., 2008). A recent pathogenesis study demonstrated that subtype H1N1 induces typical SIV-like illness and slightly more severe gross lesions than illness induced by subtype H3N2 (Sreta et al., 2009). Genetic data indicate that SIV (H1N1) in Thailand differs from pandemic (H1N1) 2009 virus. SIV (H1N1) in Thailand contains surface proteins of influenza viruses from North America and Eurasia, which are also found in pandemic (H1N1) 2009 virus; SIV (H1N1) in Thailand contains internal proteins of viruses from Eurasia; and pandemic (H1N1) 2009 viruses contain swine, human, and avian virus gene segments (Takemae et al., 2008; Kingsford et al., 2009).

We report an outbreak of infection with pandemic (H1N1) 2009 virus during November 2009-March 2010 on a commercial pig farm in Thailand. The outbreak presumably resulted from human-to-pig transmission because 1of the workers on this farm had influenza-like clinical signs at the beginning of the outbreak. Infection in this

worker was not confirmed because he quit his job on the farm after the start of the outbreak and could not be located.

5.2 The Study

In early November 2009, a small commercial pig farm in central Thailand reported respiratory problems in pigs (morbidity rate 50%, mortality rate 10%) in nursery pigs. The farm contained 3,235 pigs (700 sows, 35 boars, 1,000 piglets, 1,000 nursery and 500 finishing pigs). It has a conventional open-house production system in which both sides of the unit have natural air flow ventilation. The farm also has continuous nursery herd flow in which new pigs are continuously added when they are old enough. This process results in pigs of different ages being in the same unit. Sick pigs had clinical signs (fever, cough, nasal discharge, edematous eyelids, and conjunctivitis) of infection.

Nasal swabs from 20 nursery pigs (4 – 9 week old pigs) were submitted to Chulalongkorn University Veterinary Diagnostic Laboratory. All samples were positive for porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus (these viruses are major cause of swine respiratory disease), and 2 samples were positive for influenza A virus by reverse transcription-PCR (RT-PCR) with primers for each specific pathogen (Kim and Chae 2004; Payungporn et al., 2004; Thanawongnuwech et al., 2004).

Because respiratory problems in the nursery pigs continued, nasal swabs specimens from 20 nursery pigs and finishing pigs, gilts (young females), and sows (10 per group) with clinical signs were submitted to the diagnostic laboratory by the end of December 2009. Two samples from the nursery pigs were positive for influenza virus A (H1N1) by multiplex RT-PCR (Choi et al., 2002). Both samples were subjected to virus isolation in MDCK cells (Kitikoon et al., 2006) and designated RA20 and RA29 (table 5.1). Genome characterization identified RA20 as SIV and RA29 as pandemic (H1N1) 2009 virus (table 5.2 and figure 5.1). SIV-positive nasal swabs obtained in November were then characterized. Results showed that isolates RA4 and RA9 were pandemic (H1N1) virus, which indicated that pigs on the farm were infected with this virus.

Pandemic (H1N1) 2009 investigation on the farm included clinical surveillance and sample collection from sick and contact pigs and closely monitoring of swine workers and farm pets for influenza-like illness. Nasal swab specimens were obtained from pigs on January 17, 2010, January 30, 2010, and March 9, 2010. Because initial laboratory finding indicated that the outbreak involved the nursery herd, weaned pigs Following the Food and Agriculture Organization of the United Nations (www.fao.org) sample collection recommendations, we obtained 20 nasal swabs from pigs with SIV-like illness. In addition, nasal swab specimens (n = 10 per group) were collected form gilts, sows, and finishing pigs to test for pandemic (H1N1) 2009 virus, although no clinical signs were observed in any pigs from these age groups. All SIV-positive samples were subjected to virus isolation (Kitikoon et al., 2006), virus subtyping by multiplex RT-PCR (Choi et al., 2002) and whole genome sequencing of subtype H1N1 viruses (Hoffmann et al., 2001). Of 175 samples obtained during December 26, 2009-March 9, 2010, fifteen swab specimens from nursery pigs with clinical signs were positive for influenza (H1N1) 2009 virus; 8 viruses were characterized. No other SIV subtypes were found. On March 9, approximately 1 month after implementing the change in handling of pigs, no pigs showed respiratory signs and 34 nasal swab specimens were negative for influenza virus.

Gene sequences were compared for corresponding genes of other influenza virus strains obtained from GenBank by using the MegAlign program (DNASTAR, Madison, WI, USA). Phylogenetic trees were constructed by MEGA4 (www.megasoftware.net/) and the neighbor-joining method with 1,000 bootstrap replicates. Whole genome analysis showed that contemporary SIV (H1N1) and pandemic (H1N1) 2009 virus were concurrently circulating in the nursery herd (table 5.2 and figure 5.1). On the basis of virus hemagglutinin 1 gene grouping (Vincent et al., 2009), our findings showed that the newly isolated SIV (H1N1) from Thailand are grouped in classical swine cluster with other SIV (H1N1) isolates (figure 5.2 and 5.3). There was no evidence of gene reassortment between SIV (H1N1) and pandemic (H1N1) 2009 virus during the investigation (table 5.2).

To test for evidence of pandemic (H1N1)2009 virus interspecies transmission, we obtained serum samples on January 17, 2010, from 40 pigs in 8 age groups (5/group), 15 workers, and 4 farm pets (3 dogs and 1 cat). Samples were subjected to hemagglutination-inhibition (HI) testing with SIV (H1N1) and pandemic (H1N1) virus antigens (Kitikoon et al., 2006).

Control rabbit antibodies against SIV (H1N1) viruses did not cross-react with pandemic (H1N1) 2009 virus. Serological results showed that only 2 (9.5%) of 21 test samples from the nursery group had positive HI titers for pandemic (H1N1) 2009 virus and 8 (38%) of 21 had positive HI titers for SIV (H1N1) virus (figure 5.1). For pigs in other age groups, 11 (55%) of 20 had positive HI titers for pandemic (H1N1) 2009 virus and 14 (70%) of 21 had positive HI titers for SIV (H1N1) by HI test. No human cases of co-infection were observed. We found no evidence of pandemic (H1N1) 2009 virus interspecies transmission from pigs to human or to farm pets.

5.3 Conclusion

Consistent with findings of previous reports (Pasma and Joseph, 2009; Pereda et al., 2009) our findings demonstrated that young pigs are susceptible to infection with pandemic (H1N1) 2009 virus. Infection in pigs substantiates the hypothesis that the clinical outcome caused by infection with pandemic (H1N1) 2009 virus differs from that of infection with SIV (H1N1), which currently circulates in pigs in Thailand. Serological results demonstrated that uninfected populations are susceptible to infection with pandemic (H1N1) 2009 virus. Results of genome analysis did not show gene reassortment between the 2 different influenza (H1N1) viruses. However, a previous report showed that reassortment of influenza virus genes occurs in pigs (Castrucci et al., 1993). Continued monitoring, characterization of SIVs, and serologic surveillance of pigs are necessary for future influenza pandemic preparedness.

Table 5.1 H1N1 swine influenza virus collection date and GenBank accession number of each gene segment

H1N1 virus isolate	Collection date	Identification	Study designation	GenBank Accession# (segment 1-8)
A/sw/Thailand/CU-RA4/2009	Nov 6, 2009	Pandemic 2009	RA4	CY062305-CY062312
A/sw/Thailand/CU-RA9/2009	Nov 6, 2009	Pandemic 2009	RA9	CY062321-CY062328
A/sw/Thailand/CU-RA20/2009	Dec 26, 2009	Thai SIV	RA20	CY062281-CY062288
A/sw/Thailand/CU-RA29/2009	Dec 26, 2009	Pandemic 2009	RA29	CY062297-CY062304
A/sw/Thailand/CU-RA114/2010	Jan 17, 2010	Pandemic 2009	RA114	CY062265-CY062272
A/sw/Thailand/CU-RA204/2010	Jan 17, 2010	Thai SIV	RA204	CY062289-CY062296
A/sw/Thailand/CU-RA15/2010	Jan 30, 2010	Pandemic 2009	RA15	CY062273-CY062280
A/sw/Thailand/CU-RA75/2010	Jan 30, 2010	Pandemic 2009	RA75	CY062313-CY062320

Table 5.2 Gene origin of each SIV RNA segment and %homology of each gene segment compared to pandemic (H1N1) 2009

H1N1 virus	PB2 (1-2229)†	PA (1-2153)	NA (1-1347)	M (1-982)	HA (1-1698)	NS (1-778)	NP (1-1443)	PB1 (1-2153)
Pandemic‡	Avian-TRIG*		Eurasian-swine		Classical-swine			Human-TRIG
Thai swine‡‡	Eurasian-swine				Classical-swine		Eurasian-swine	
RA4	Avian-TRIG		Eurasian-swine		Classical-swine			Human-TRIG
RA9	Avian-TRIG		Eurasian-swine		Classical-swine			Human-TRIG
RA20*	Eurasian-swine				Classical-swine		Eurasian-swine	
	(83.1%)	(85.1%)	(89.5%)	(94.2%)	(86.4%)	(90.8%)	(82.4%)	(85.1%)
RA29	Avian-TRIG		Eurasian-swine		Classical-swine			Human-TRIG
RA114	Avian-TRIG		Eurasian-swine		Classical-swine			Human-TRIG
RA204**	Eurasian-swine				Classical-swine		Eurasian-swine	
	(83.2%)	(85.2%)	(89.5%)	(94.2%)	(86.8%)	(90.8%)	(82.3%)	(85.1%)
RA15	Avian-TRIG		Eurasian-swine		Classical-swine			Human-TRIG
RA75	Avian-TRIG		Eurasian-swine		Classical-swine			Human-TRIG

NOTE All SIV isolates except for RA20 and RA204 have > 99% homology to the corresponding genes of A/Nonthaburi/102/2009

† nucleotide position compared

‡ Pandemic H1N1 virus = A/Nonthaburi/102/2009 (H1N1)

‡‡ Thai swine H1N1 virus = A/sw/Ratchaburi/NIAH1481/2000 (H1N1)

* TRIG = Triple Reassorted Internal Gene

** percent homology of RA20 and RA204 compared to the corresponding genes of A/Nonthaburi/102/2009

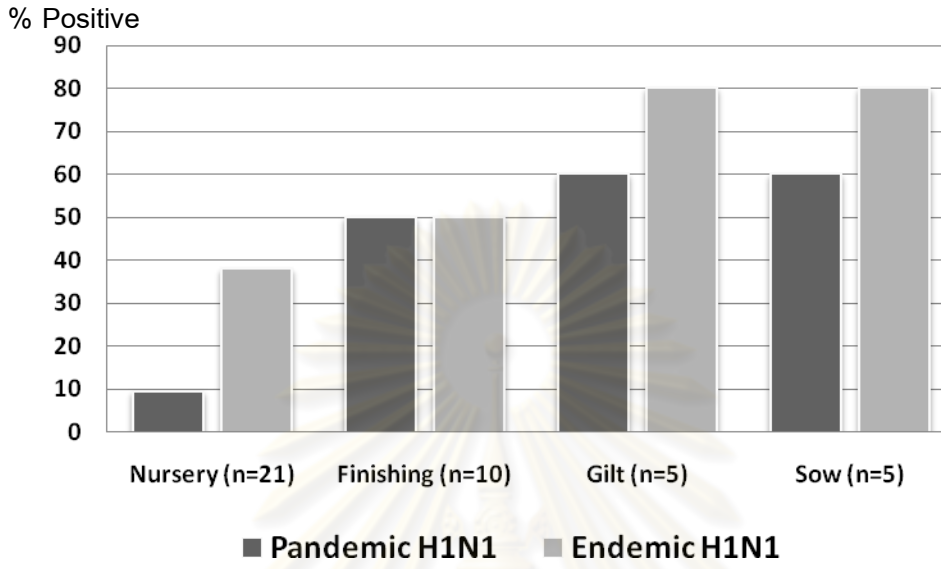

Figure 5.1 Percentage of pigs positive to pandemic H1N1 and endemic H1N1 virus antibodies detected by hemagglutination-inhibition (HI) test. Serum samples were collected from different pig age groups on January 2010. Samples are positive when HI titer $\geq$ 40.

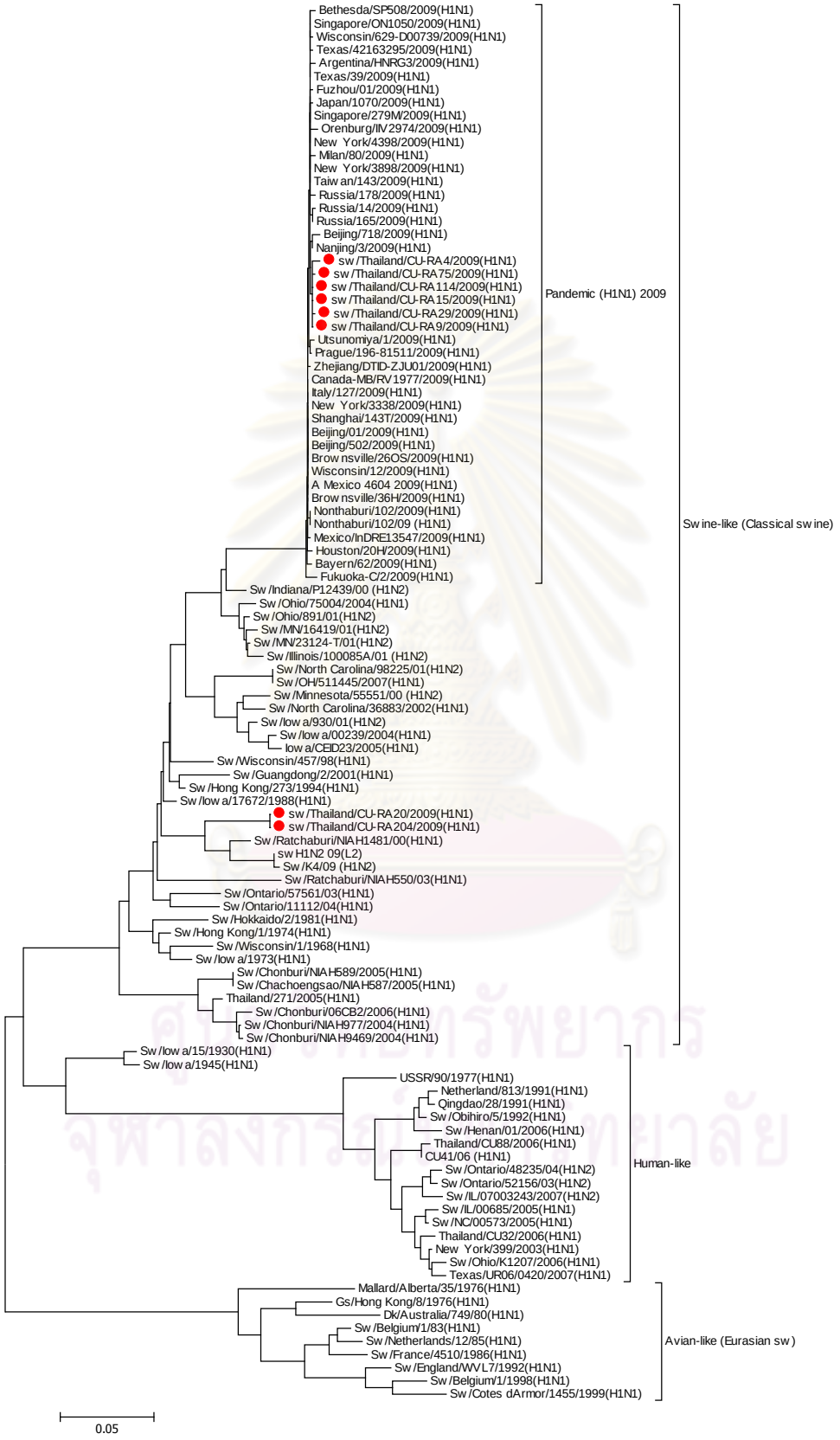

Figure 5.2 Phylogenetic analysis of HA genes

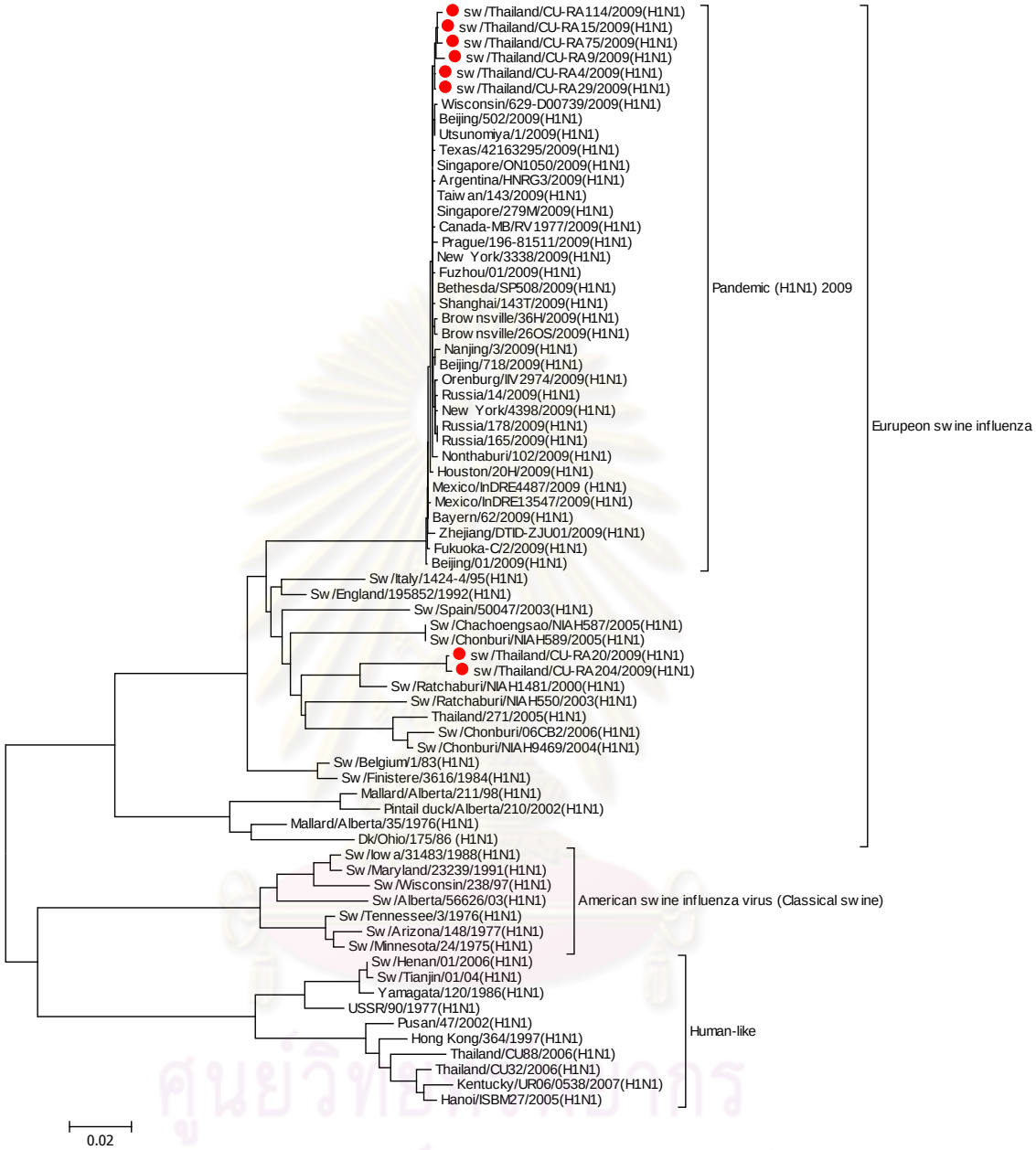

Figure 5.3 Phylogenetic analysis of NA genes

CHAPTER 6

CONCLUSION

The investigations described in this dissertation were conducted with three main objectives. The first objective was to establish an appropriate HI test for SIV infection in Thailand. The second objective was to study the seroprevalence of SIV in pigs from June 2008 to May 2009 from swine farms in the highest pig density provinces in Thailand. The third objective was to genetically characterize the current Thai SIV isolates from 2005 to 2010. For the first objective, we established the HI test for swine H1 and H3 subtypes using selected six Thai SIV isolates as antigens. The rabbit antisera were used in HI test with the homologous and heterologous viruses for cross antibody reaction before selecting the represent viruses as suitable antigens for the established HI test in Thailand. Based on the results from the second objective, HI assay was used for evaluation of Thai SIV sero-status using the selected virus isolates and interspecies transmission on Thai swine farms was investigated using homologous and heterologous virus antigens. For the third objective, we characterized viral whole genome of 15 current Thai SIV isolates. In addition, SIV monitoring on an outbreak farm of pandemic (H1N1) 2009 virus was investigated for prevention and control using virological and serological studies as well as genetic characterization.

The first objective was to establish an appropriate HI test for SIV infection in Thailand. For serologic diagnostic tool, Hemagglutination inhibition (HI) test is a gold standard for cases infected with influenza A virus, recommended by WHO. The test is specific for antibody detection of HA subtypes and it requires a suitable representative virus from the circulating strain used as the HI virus antigen. In general, SIV is genetically unstable. Many sub-clusters have arisen within the different H1N1 and H3N2 SIV subtypes (Ma et al., 2010). In Thailand, total 38 HA nucleotide sequences of both H1 and H3 SIVs could be divided into five clusters containing three of the H1 viruses (H1 cluster I, II and III) and two of the H3 viruses (H3 cluster I and II). To establish an appropriate HI test for SIV infection in Thailand, antigenic properties were analyzed using hyper-immune rabbit sera. Then, we selected swH1N1-06 as a reference H1

antigen for swine H1 virus antibody detection. For diagnosis of Thai swine H3 virus infection, both swH3N2-05 and swH3N2-07 viruses must be included since they did not have cross antibody reactivity. Moreover, the genetic characterization monitoring is necessary for HI assay evaluation since phenotypic characterization relates to genetic and other factors. We suggest that the SIV antigen in the HI test should be genetically evaluated at least every two years.

The second objective was to study the seroprevalence of SIV in pigs from June 2008 to May 2009 from swine farms in the four highest pig density provinces in Thailand. During June 2008 to May 2009, serologic surveillance in this study showed that all four selected provinces in Thailand had higher H3N2 sero-positive percentages than those of the H1N1 subtype (swH3N2-07 >70% and swH1N1-06 >40%). It should be noted that the pandemic H1N1 2009 virus transmitted to the Thai pig population in late 2009 and sero-profile of the Thai SIVs could possibly be different from this study (Sreta et al., 2010). Moreover, the result showed that sero-positive percentage of SIV in this study was higher than previous reports in different provinces (Damrongwatanapakin et al., 2003; 2006; Nakharuthai et al., 2008). The infromaton possibly demonstrated that SIV circulation in Thailand were dynamic. We strongly suggest continuous monitoring SIV status on both virology and serology. Based on the farm history, all selected pig farms in this study showed respiratory problems concurrently with the presence of other respiratory pathogens including PRRSV and PCV2. However, we did not evaluate the economic loss from porcine respiratory disease complex (PRDC) since the production parameters did not obtain and were not included in the objective. Economic impact of Thai SIVs should be further evaluated if the virus could play a major role in PRDC.

In addition, swine-to-human influenza transmission of Thai swine farms was demonstrated that swine-exposure is one of the risk factors since swine-exposed participants had significant higher percentages of sero-positive results than the non-swine-exposed group (odds ratio > 40, 95% confidence interval). In Thailand, swine production systems mostly are open housing of growing to finishing pig or a continuous flow system which may increase a chance of interaction of humans, pigs, farm pets and

other animals. Future investigation of specific risk factors of swine-to-human influenza transmission on Thai swine farm should be performed. Also, swine-exposed populations as the frontline of contacting SIV, particularly in Thailand should be concerned since (1) swine influenza vaccination is not performed on Thai swine farms; (2) clinical signs in pigs are hard to identify on endemic farms which renders personal protection management difficulty; and (3) most pig handlers are uneducated migrant workers from neighboring countries. Therefore, continuous monitoring for SIV infection, education on self-protection and additional research aimed at characterizing specific risk factors unique to the populations are required to help implement future influenza control programs.

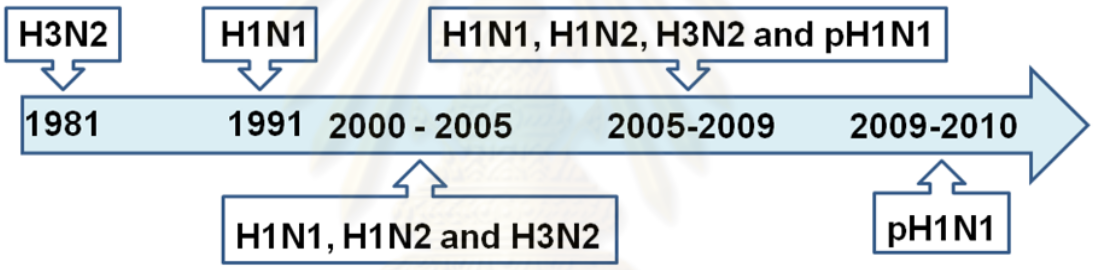

Figure 6.1 Chronologically evidences of SIV in Thailand since 1981 to 2010

The third objective was to genetically characterize the current Thai SIV isolates. Total seven H1N1 and H1N2 viruses from year 2005 to 2009 were characterized. Based on phylogenetic analysis, Thai swine H1 viruses originated from classical swine H1 were divided into three clusters; I, II and III and mostly similar to swine H1α in the North American lineage. However, the N1 NA gene belongs to the Eurasian lineage. Genetic data indicate that SIV (H1N1) in Thailand differs from pandemic (H1N1) 2009 virus. SIV (H1N1) in Thailand contains surface proteins of influenza viruses from North America and Eurasia, which are also found in the pandemic (H1N1) 2009 virus. Thai SIV (H1N1) contains internal proteins of viruses from Eurasia whereas the pandemic (H1N1) 2009 viruses contain swine, human and avian virus gene segments (Takemae et al., 2008;

Kingsford et al., 2009). Moreover, a novel reassortant H1N2 virus is mixed with swine, human and avian origins based on its H1 and NS genes belonging to classical swine lineage, N2 gene belonging to human-like 1990s and the remaining internal genes belonging to Eurasian swine lineage or an avian origin (figure 6.2). Furthermore, SIV mutation in Thailand depends on chronological and geographical factors. Chronologically, the nucleotide of swine H1 viruses in 2009 had changed showing only 86.7% homology to the previous 2005-2006 viruses. For geographic areas, we found that SIVs from the eastern part of Thailand, Chonburi province showed different genetic diversity from SIVs in the central part, Saraburi province in the same year 2009. For example, in both areas the H1 HA gene tends to have separated evolution line, resulting in the viral antigenic differences which may fail the host immune system of naïve pigs to recognize the mutated virus. Thus, moving the infected pig into the naïve swine population may cause the epizootic outbreak. Control program depends on biosecurity and avoiding the introduction of carrier animals. Quarantine and acclimation should be done when introducing replacement animals.

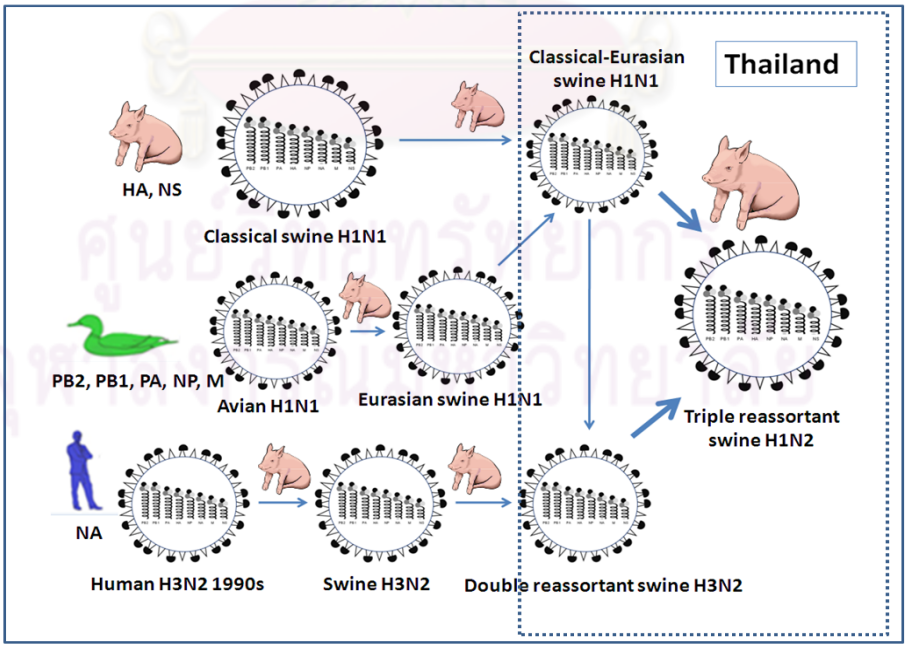

Figure 6.2 Novel triple reassortant swine H1N2 occurred in Thailand as a result of reassortment between human, avian and swine origins.

From the third objectives, sample collection, virological and serological (HI test) studies, and genetic characterization were evaluated for SIV surveillance in Thailand. Then, those tools were used for SIV monitoring in an outbreak farm together with the control program since we could isolate the pandemic H1N1 2009 virus from a commercial farm at that time of the virus emerging in humans. Accordingly, genetic monitoring and virologic and serologic studies of the pandemic H1N1 2009 virus from pigs, humans, and farm pets in the commercial farm were investigated. Total 8 H1N1 viruses from a commercial farm in the central part of Thailand demonstrated co-infection of pandemic and endemic H1N1 SIVs. The SIV outbreak lasted for 64 days and no genetic reassortment between the two different strains was demonstrated. However, co-circulation of both H1N1 viruses probably can lead to produce a novel reassorted virus or only a predominant virus continues to be endemic. Therefore, continuous genetic monitoring in the pig farm is necessary for the outbreak threat and pandemic preparedness.

In conclusion, this study indicated that swine H1N1, pandemic H1N1, human-like H3N2 and reassortant H1N2 influenza viruses have been circulating in Thai pig population. Moreover, the combination of “classical-Eurasian swine pattern” occurred and the TRIG cassette was evidently introduced with the pandemic H1N1 2009 virus into the Thai pig population. As the result, SIVs genetic variation tends to increase and novel reassortment viruses could occur as well as swine-to-human transmission or vice versa. Genetic characterization of SIVs after the introduction of the pandemic H1N1 2009 into the Thai pig population should continuously be monitoring.

Furthermore, at least 3 virus antigens, swH1N1-06 (H1 cluster I), swH3N2-05 (H3 cluster I) and swH3N2-07 (H3 cluster II), used for HI test should be included in the standard test currently. Those three virus antigens are suitable for indirect antibody detection of SIV infection in Thailand during 2009. The SIV antigens in the HI test should be evaluated at least every two years together with genetic monitoring. On June 2008 to May 2009, percent sero-positive of swH3N2-07 was higher than those of swH1N1-06, swH1N1-09 and swH3N2-05, respectively. However, it could be predicted that Thai SIV

status has changed currently since, on November 2009, the pandemic H1N1 2009 virus was isolated from pigs in Thailand. Therefore, monitoring of SIV sero-prevalence should be continuous for evaluation of the true SIV status. In addition, the antigenic property study found that the control rabbit antibodies against SIV (H1N1) viruses did not cross-react with the pandemic (H1N1) 2009 virus. So, serological surveillance should be continuously performed and sero-status of the pandemic (H1N1) 2009 virus should also be investigated. Hence, the true SIV status can be helpful for the prevention and control program of influenza virus in both swine and humans.

Overall, our results provide the picture of SIV epidemiology in Thailand. The genetic characterization showed that Thai swine H1 viruses were rather stable and showed only a few nucleotide changes due to genetic drift different from what happening in the United State and European countries. Until late 2009, Thai SIVs have undergone increased genetic variation since the introduction of the triple reassorted internal gene (TRIG) cassette that could play a role in interspecies transmission. The TRIG cassette introducing with the pandemic H1N1 virus may increase the chance of genetic reassortant among the endemic viruses producing a novel reassorted virus. Monitoring of virus genetic variation may provide a better understanding of SIV epidemiology and preparing for the outbreak treats in both swine and human population.

We suggest performing the pathogenesis study of the current H1N1, a novel reassortant H1N2 and the pandemic H1N1 2009 viruses isolated from clinical sick pigs in Thailand since changing on antigenic sites, receptor binding sites and other virulent factors may make SIV-infected pigs unrecognizable or more severity than the previous study. For the best prevention and control program of swine influenza virus on pig farms, good management and biosecurity avoiding the introduction of carrier animals are important. Since, introduction of new animals into the herd may increase the risk of introducing the new virus strains into the herd. Quarantine and acclimatization should be done properly in order to reduce the risk of the introducing new viruses. Also, continued monitoring and characterizing of SIVs as well as serological surveillance in the pig

population and the risk of human population are necessary and can provide important data for influenza pandemic preparedness in both human and other animals. In addition, specific Thai SIV diagnosis manual, suitable protocol for prevention and control, and community response when facing with the disease outbreak should be performed and rehearsed routinely. Most importantly, education on self prevention of cross transmission between pigs and humans should be informed, particularly people working with pigs. Influenza vaccination should be done in those risk populations including veterinarians, farm owners, farm workers and slaughter-house workers etc. in order to reduce the risk of reassortment. In addition, people with flu-like symptoms should avoid contacting with pigs or other animals.

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APPENDICES

APPENDIX A

Criteria of sample collection

1. Nasal swabs and lung tissues

- submitted to Chulalongkorn veterinary laboratory diagnosis office will be included in the study.

- nasal swabs will be collected from swine farms that have history or currently have respiratory problems.

- at least 20 nasal swabs/time

2. Serum samples

- were collected from year 2008 to 2009

- from pigs in four provinces of Thailand that have high pig density including Ratchaburi, Nakhon-Pathom, Chonburi and Chachoengsao.

- farms with $\geq$ 500 pigs

- from weaners, finishers, gilts and sows (at least 5 pigs in each age group).

- at least twice from each farm in each province.

3. Sample collection forms as Form A. 1a and A3

Form A.1a
(modified from FAO form A.1)

Date of visit……

Contact details of the staff person visiting the pig farm

Name .....

Telephone.....E-mail address (if available) .....

Preliminary data

(1) Province .....

(2) District .....

(3) Town/village .....

(4) Owner of farm.....

(5) Telephone (if available) .....

(6) Latitude/Longitude of the farm (if available)

Lat..... Long .....

(7) **Type of pig production system:**

[ ] large/industrialized [ ] medium/commercial [ ] small scale [ ] backyard

(> 500) (200-500) (51-199) (≤ 50)

[ ] free ranging [ ] closed housing (Evaporation cooling system) [ ] Open house

(8) **Type of nursery production system:**

[ ] all in all out [ ] continuous

(9) **Total number of pigs present in the farm:** .....

____ piglets ___ weaners ____ growers/finishers ____ sows ____ boars

(10) **Indicate if human cases were present at the time of swine sample collection:**

[ ] swine-workers [ ] farm-residence [ ] veterinarian [ ] other.....

(11) **Indicate if other PRDC pathogen is present at the time of investigation:**

[ ] PRRSV [ ] PCV2 [ ] *Mycoplasma hyopneumoniae* [ ] None [ ] do not know

(12) **If the investigation is undertaken in relation with human cases please indicate if pigs in the farm is categorized as:**

[ ] C1 (clinical disease ongoing) [ ] C2 (clinical disease in past 30 days) [ ] C3 (history of clinical disease >30 days)

Form A.3 Date .....

Sample record sheet

			Type of animal					Sex		Status			
Number	Animal ID or Pen ID	Age	Breeding adult	Suckling piglet	Weaner (<30 Kg)	Fattener (>30 Kg)	Approximate weight in Kg	M	F	Healthy	Sick	Dead	Comments

Recorded by .....

APPENDIX B

Protocol for genetic characterization

1. Primers used in this study

Primer name	Sequence (5' – 3')
Uni 12	AGC AAA AGC AGG
PB2 hoffman Forward	TAT TGG TCT CAG GGA GCG AAA GCA GGT C
PB2 hoffman Reverse	ATA TGG TCT CGT ATT AGT AGA AAC AAG GTC GTT T
PB1 hoffman Forward	TAT TCG TCT CAG GGA GCG AAA GCA GGC A
PB1 hoffman Reverse	ATA TCG TCT CGT ATT AGT AGA AAC AAG GCA TTT
PA hoffman Forward	TAT TCG TCT CAG GGA GCG AAA GCA GGT AC
PA hoffman Reverse	ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA CTT
NPhoffman Forward	:5' TAT TCG TCT CAG GGA GCA AAA GCA GGG TA 3'
NPhoffman Reverse	5' ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT ATT TTT 3'
M hoffman Forward	5' TAT TCG TCT CAG GGA GCA AAA GCA GGT AG 3'
M hoffman Reverse	5' ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT 3'
NS hoffman Forward	5' TAT TCG TCT CAG GGA GCA AAA GCA GGG TG 3'
NS Hoffman Reverse	5' ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TT 3'
H1-1.1	AAG CAA AAG CAG GGA AAA TAA
H1-1770R	CAA GGG TGT TTT TTC TCA TGT CTC
H1F Choi	GGG ACA TGT TAC CCA GGA GAT
H1R Choi	GCA TTG TAT GTC CAA ATA TCC A
H1-209F	CAT-GGT-CTT-ACA-TTG-TGG-AAA-C
H1-634F	TAC-CAG-AAT-GCA-GAT-GCC-TAT-GTT-TTT
H1-789R	GCC-ACT-AGA-TTT-CCA-GTT-GCT-T
H1-1096R	ACC-ATC-CAT-CTA-TCA-TTC-CT
NAF	AGC AAA AGC AGG AGT TTA AAA TG
NAR	AGT AGA AAC AAG GAG TTT TTT
NA260R	CAG-GGC-AAA-GAG-AGG-AAT-TG
NAF1-1	AGC-AAA-AGC-AGG-AGT-TCA-AA
N1F Choi	GGT TCC AAA GGA GAC ATT TTT G
N1R Choi	CTA TCC AAA CAC CAT TGC CAT A
N2F Choi	TGC GAT CCT GAC AAG TGT TAT C

Primers used in this study (continue)

Primer name	Sequence (5' – 3')
N2R Choi	CAG ACA CAT CTG ACA CCA GGA T
PB2F	CTC GAG CAA AAG CAG GTC AA
PB2-1230R	CAA TCC TCY TGT GAA AAH ACC AT
PB2-960F	CAT ATG CAA RGC DGC AAT GGG
PB2R	AGT AGA AAC AAG GTC GTT TTT AAA C
PB1F	AGC AAA AGC AGG CAA ACC AT
PB1-1250R	ATR TTG AAC ATG CCC ATC ATC AT
PB1-1050F	ATA ATG TTC TCA AAC AAA ATG GC
PB1R	AGT AGA AAC AAG GCA TTT TTT CAT
PAF	CTC GAG CAA AAG CAG GTA CTG AT
PA-750R	ATT TGR GAA AGC TTG CCC TCA A
PA-610F	GAG GCG ARG AGR CAA TTG AAG A
PAR	AGT AGA AAC AAG GTA CTT TTT TGG AC

2. **Protocol for Genetic Characterization of NP, M & NS gene**

RNA extraction

↓

cDNA with Uni12 primer AGC AAA AGC AGG

↓

PCR with specific primer from Hoffman

PCR Condition 94C 4min, 30 cycles of 94C20s-58C30s-72C7min, 72C 7 min

NPhoffman Forward : **NP**hoffman Reverse

M hoffman Forward : **M** hoffman Reverse

NS hoffman Forward : **NS** Hoffman Reverse

↓

Use the PCR product of each gene to PCR with specific internal primers

NPF5'/**NP**R1 613 bp and **NP**F2/**NP**R3 1087 bp

NP gene with condition 94C 3min, 35 cycles of 94C30s-45C30s-72C1.30min, 72C 7 min

M71F/**M**71R 603 bp and **M**72F/**M**72R 765 bp

NS81F/**NS**81R 588 bp and **NS**82F/**NS**82R 737 bp

M and **NS** gene with condition 94C 3min, 35 cycles of 94C30s-58C1min-72C1min, 72C 7 min

↓

RUN GEL, Cut GEL and Purified

Sent to sequencing company

3. Protocol for Genetic Characterization of PB2, PB1 and PA genes

RNA extraction

↓

RT-PCR (Promega) with specific primers

Condition 48C 45min, 94C 2min, 35 cycles of 94C20s-55C30s-72C1.30min, 72C 10 min

PB2F : PB2-1230R

PB2-960F: PB2R

PB1F : PB1-1250R

PB1-1050F: PB1R

PAF : PA-750R

PA-610F: PAR

↓

RUN GEL, Cut GEL and Purified

Sent to sequencing company

APPENDIX C

Nucleotide sequences

A/swine/Thailand/HF6/2005 (swH1N1-05)

Hemagglutinin (HA) gene 1732 nucleotides; GenBank: FJ688266

AAGCAACCGAAATGAAAGCAATACTACTAATCTTGCTATGCACACTTGCAGCAGCAAATGCAGACACAC
TGTGTATAGGTTATCATGCAAATAATTCAACTGACACTGTTGATACAGTATTAGAAAAGAATGTTACAG
TAACACACTCTGTTAACCTTTTAGAAGACAGACACAATGGAAAACTATGTAACCTAAGGGGGAAGGCCC
CACTGCATTTGGGTAAATGTAACATTGCCGGATGGCTCCTAGGAAACCCAGAGTGCGAATTACTATTTG
CAGTAAACTCATGGTCTTACATTGTGGAAACATCGAACTCAGACAATGGGACATGTTACCCAGGAGATT
TCACCAGTTATGAAGAGCTAAGAGAACAATTGAGCTCAGTGTCATCATTTGAAAGATTCGAAATATTCC
CCAAAGCAAGCTCTTGGCCCAACCATGAAACAAACAGAGGTGTAACGGCAGCATGCCCTTATGCTGGAA
CAAACGGCTTCTACAGGAATTTGATATGGCTAGTAAAAAAGGGAAACTCATATCCAAAACTCAGAAAAT
CCTATGTTAATAATAAGAAGAAGGAAGTCCTTGTACTATGGGGCATCCACCATCCACCCACCAATACTG
ATCAACAAAGTCTCTACCAGAATGCAGATGCCTATGTTTTTGTGGGATCATCAAAATATAGCAGGAAAT
TCAAACCAGAAATAGCAGAAAGACCCAAGGTGAGGGGTCAAGCAGGAAGAATGAACTATTATTGGACAT
TAATAGAGCCTGGAGACACAATAACATTTGAAGCAACTGGAAATCTAGTGGCACCAAGATATGCTTTTG
CAATGAATAGAGGTCCTGTATCGGGTATCATAACATCGGATGCACCAGTCCATGACTGTAATGCGACTT
GTCAAACACCCAAGGGTGCCATAAACACCAGCCTCCCATTTCAGAATATTCATCCAATCACTATTGGAG
AATGTCCCAAATATGTCAAAAGCACAAGACTAAGAATGGCTACAGGATTAAGAAATATCCCTTCTATTC
AATCTAGAGGTCTGTTTGGGGCCATTGCTGGTTTCATTGAAGGAGGATGGACAGGGATGATAGATGGGT
GGTACGGTTATCATCATCAGAATGGACAAGGATCAGGATATGCAGCGGACCAAAAGAGCACACAGAATG
CCATCGATAGGATAACTAACAAGGTAAATTCTGTTATTGAAAAGATGAACATACAATTCACAGCAGTGG
GTAAAGAATTTAACCACTTAGAAAGAAGAATAGAAAACTTAAACAAAAAGGTTGATGATGGATTTTTGG
ATGTTTGGACATACAATGCCGAACTGTTAGTCCTATTGGAAAATGAGAGAACTTTGGATTTCCATGACT
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GCTGCTTTGAATTCTATCACAAATGTGATGACACATGCATGGAGAGCATCAAAAATGGGACTTACAATT
ACCCCAAATACTCGGAAGAATCAAAACTAAACAGAGAGGAAATAGATGGAGTAAAACTGGAATCAACAA
GGATTTACCAGATTTTGGCGATCTATTCAACTGCCGCCAGTTCACTGGTACTGTTGGTCTCCCTGGGGG
CAATCGGTTTCTGGATGTGCTCCAATGGGTCTTTGCAGTGCAGAATATGTATTAAAAACTAAGATTTCA
GAGACAT

Neuraminidase (NA) gene 1409 nucleotides; GenBank: FJ688267

ATGAATCCAAACCAGAAGATAATAACTATTGGTTCGGTCTGTGTGACAATTGGGATAGCAAGCTTGATA
CTACAAATTGGAAACATAATCTCAATATGGATTAGCCATTCAATTCAAATTGGAAATCAAAACCAGCCT
GAAACATGCAACCGAAGCGTCATCACTTACGAAAACAACACTTGGGTAAATCAGACATTTGTTAACATC
AACAACACCAATTTTGTTGCTGAACAGGCAATAGTCTCAGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAAGATAATAGTGTAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAGCCTTTCATCTCATGTTCTCACTTGGAATGCAGAACTTTCTTTTTGACTCAAGGTGCC
CTGCTGAATGACAAACATTCTAATGGAACCATTAAAGACAGAAGCCCATATAGAACCCTGATGAGCTGT
CTTGTTGGTGAGGTTCCTTCTCCATACAACTCAAGGTTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGC
CATGATGGCATCAGTTGGCTGACAATTGGAATTTCTGGCCCAGACAATGAAGCAGTGGCTGTGCTGAAA
TACAATGGCATAATAACAGACACCATCAAGAGTTGGAGAAAAAACATATTAAGGACACAAGAATCTGAA
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GAGGAATGCTCCTGTTATCCTGAGTCTGGCAAAATCACATGTGTATGCAGGGATAATTGGCATGGCTCG
AATCGACCGTGGGTGTCTTTCAATCAGAATCTGGAGTATCAAATAGGATACATATGCAGTGGAATTTTC
GGAGACAATCCGCGCCCTAATGATAGAACAGGCAGTTGCGGTCCTGTATCTTCTAATGGAGCAAATGGG
GTGAAAGGATTTTCATTTAAATACGGCAACGGTGTTTGGATAGGAAGAACCAAGAGCACTAGTTTAAGG
AGCGGTTTTGAGATGATCTGGGATCCAAACGGGTGGACAGGAACTGACAATAACTTCTCCGCAAAGCAA
GATATCATAGGAATAAATGATTGGTCAGGATACAGCGGGAGTTTTGTTCAGCATCCAGAACTGACTGGA

CTGGATTGTATGAGACCTTGCTTTTGGGTTGAACTTATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGTGGGAGCAGCATATCCTTTTGTGGTGTGAATAGCGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTAACTATGACAAGTA

A/swine/Thailand/CU-CB1/2006 (swH1N1-06)

HA gene 1636 nucleotides; GenBank: GU454848

TAGGTTATCATGCAAATAATTCAACTGACACTGTTGATACAGTATTAGAAAAGAATGTTACAGTAACAC
ACTCTGTTAACCTTTTAGAAGACAGACACAATGGAAAACTATGTAACCTAAGGGGGAAGGCCCCACTGC
ATTTGGGTAAATGTAACATTGCCGGATGGCTCCTAGGAAACCCAGAGTGCGAATTACTATTTGCAGTAA
ACTCATGGTCTTACATTGTGGAAACATCGAACTCAGACAATGGGACATGTTACCCAGGAGATTTCACCA
GTTATGAAGAGCTAAGAGAACAATTGAGCTCAGTGTCATCATTTGAAAGATTCGAAATATTCCCCAAAG
CAAGCTCTTGGCCCAACCATGAAACAAACAGAGGTGTAACGGCAGCATGCCCTTATGCTGGAACAAACG
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AATACTCGGAAGAATCAAAACTAAACAGAGAGGAAATAGATGGAGTAAAACTGGAGTCAACAAGGATTT
ACCAGATTTTGGCGATCTATTCAACTGCCGCCAGTTCACTGGTACTGTTGGTCTCCCTGGGGGCAATCG
GTTTCTGGATGTGCTCCAATGGGTCTTTGCAGTGCAGAATATGTATTAA

NA gene 1415 nucleotides

ATGAATCCAAACCAGAAGATAATAACCATTGGTTCGGTCTGTGTGACAATTGGGATAGCAAGCTTGATA
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GAAACATGCAACCAAAGCGTCATCACTTACGAAAACAACACTTGGGTAAATCAGACATTTGTTAACATC
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CCTGTTAGTGGATGGGCTATATACAGTAAAGATAATAGTGTAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAGCCTTTCATCTCATGTTCTCACTTGGAATGCAGGACTTTCTTTTTGACTCAAGGTGCC
CTGCTGAATGACAAACATTCTAATGGAACCATTAAAGACAGAAGCCCATATAGAACCCTGATGAGCTGT
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GATATCATAGGAATAAATGATTGGTCAGGATACAGCGGGAGTTTTGTTCAGCATCCAGAACTGACTGGA
CTGGATTGTATGAGACCTTGCTTTTGGGTTGAACTTATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGTGGGAGCAGCATATCCTTTTGTGGTGTGAATAGCGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTACTATTGACAAGTAATTTGTTCGAAAAAACTCCTTGTTTCTACTGGA

A/swine/Thailand/CU-CB918/2009 (swH1N1-09)

HA gene 1694 nucleotides

ATGaagGCATTACTATTAGTCTTGCAATGTATACTTACAGCCGCAAGTGCAGACACATTATGTATAGGT
TACCATGCAAATAATTCAACTGACACAGTTGATACAGTACTAGAAAGGAATGTAACAGTAACACACTCT
GTGAACCTTCTGGAAGACAGACACAACGGGAAATTATGTAAGCTGAAAGGGATAGCCCCATTGCATTTG
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TGGTCTTACATTGTGGAAACATCTTACTCAGACAATGGGACATGTTATCCAGGGGATTTCACCAATTAT
GAAGAGTTGAGAGAGCAGTTGAGCTCAGTGTCATCATTTGAAAGGTTTGAGATGTTCCCCAAAGCAAAT
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TACAGAAACTTAaTATGGCTGGTAAAAAAAGGAAATTCATATCCAAAACTCAGCAAATCCTATGTCAAC
aATAAgAAAAAAgAGGTCCTCgTGCTATGGggCATTCacCACCCATCTACCAGTACTGACCAACAAAGT
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GGAGACACAATAACATTTGAAGCAACTGGAAATCTAGTGGCACCAAGTTACGCATTCGCAATGAAAAGA
GATTCTGGATCTGGTATTATCATGTCAGACACACCAATCCACGATTGTAACACAACTTGTCAAACACCT
AAAGGTGCTATAAACACCAGCCTCCCATTTCAAAATGTACATCCAGTCACAATTGGGGAATGTCCAAAA
TATGTCAAAAGCACAAAATTAAGAATGGCTACAGGACTAAGGAACATCCCGTCTATTCAATCCAGAGGT
CTGTTTGGAGCCATTGCTGGCTTTATTGAAGGGGGATGGACAGGAATGATAGATGGATGGTACGGTTAT
CACCATCATAATGAGCAAGGATCAGGATATGCAGCTGACCGAAAGAGCACACAGAATGCCATTGACGGG
ATCACTAACAAAGTGAACTCAATCATTGAAAAAATGAACATACAATTCACAGCAGTAGGTAAAGAATTC
AGCCATCTGGAAAAAAGGGTAGAGAATTTAAACAAAAAAGTTGAGGATGGTTTTCTAGATGTTTGGACT
TACAATGCCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGATTATCATGACTCAAATGTGAAA
ACCGTATATGAGAAAGTAAGAAGCCAGCTGAAAAACAATGCCAAAGAAATTGGAAATGGATGTTTTGAA
TTTTACCACAAATGTGATGACTCATGCATGGAGAGCATTAAAAATGAGACTTATGACTATTCGAAGTAT
TCAGAAGAATCAAGATTAAACAGAGAGAAAATAGATGGAGTGAAATTGGAATCAACACAAATTTATCAG
ATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACTGTTAGTCTCCCTGGGGGCAATCAGTTTC
TGGATGTGCTCCAATGGGTCTTTACAGTGCAGAATATG

NA gene 1460 nucleotides

TAGCAAAAGCAGGAGTTTAAAATGAATCCAAATCAGAAGATAATAATCATTGGTTCAGTTTGTATGACG
ATTGGAATAGCCAGCTTGATATTACAAATTGGGAACATAATCTCAGTATGGATTAGCCATTCAATTCAA
ATTAGGAATAAAAATCAGCCTGAAACATGCAACCAAAGCATCATTACCTACGAAAACAACACTTGGGTA
AACCAGACATATGTTAACATCAGCAACACCAATTTTGTTGCTGAACAGGCAGTAGTTTCAGTGAAATTA
GCAGGCAATTCCTCTCTTTGCCCTGTTAGTGGATGGGCTAAAAACAGTAAAGATAATAGTGTAAGAATT
GGTTCCAAGGGGGATGTGTTTGTCATAAGAGAGCCATTCATCTCATGCTCTCATTTGGAGTGCAGAACC
TTCTTCTTGACTCAAGGTGCTCTTTTGAATGACAAACATTCTAATGGAACAATTAAGGACAGAAGCCCA
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GCTTGGTCAGCAAGTGCTTGCCATAATGGTACCAGTTGGCTAACAATTGGAATTTCTGGCCCAGACAAT
GGGGCAGTGGCTGTGCTGAAATACAATGGAATAATAACAGACACTATAAAGAGTTGGAGAAAAAACATA
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AGGGATAATTGGCATGGCTCGAATCGACCGTGGGTGTCTTTCAATCAGAATCTGGAGTATCAAATAGGG
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TCTTCTAATGGAGCAAATGGGGTAAAAGGATTTTCGTTTAAATACGGCAATGGTGTTTGGATAGGAAGA
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AACAACTTCTCATTAAAGCAAGATATCATAGGGATAACTGATTGGTCAGGATACAGCGGGAGTTTTGTC
CAGCATCCAGAACTAACCGGACTGGACTGTATGAGACCTTGCTTCTGGGTTGAACTAATCAGAGGAAGA
CCTAAAGAGAACACAATCTGGACTAGTGGGAGCAGCATATCCTTTTGTGGGGTGGACAGTGACACTGTG
GGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTTACTGTTGACAAGTAATTTGTTCAAAAAAACTCC
TTGTTTCTACT

A/Swine/Thailand/CU-ST3/2009 (swH1N1-09)

HA gene 1357 nucleotides

AAGCAAAAGCAGGGAAAATAAAAGCAACCGCAATGAAAGCAATACTATTAGTCTTGCTATGTACATTTG
CCGCAGCAAATGCAGACACACTATGCGTAGGCTATCATGCAAATAACTCAACTGACACTGTTGATACCA
TATTAGAAAAGAATGTCACAGTAACACACTCTGTTAACCTTTTAGAAGACAGACACAATGGGAAGCTAT
GTAACCTAATGGGAATGGCCCCACTGCATTTGGGCAAATGTAACATTGCCGGATGGCTCCTGGGAAACC
CAGAGTGCGAATTATTATTTGCAATAAACTCATGGTCTTACATTGTGGAAACGTCAAACTCAGACAATG
GGACATGTTACCCAGGAGATTTCACCAATTATGAAGAGCTAAGAGAACAATTGGGCTCAGTATCATCAT
TTGAAAGATTCGAGATATTCCCCAAAGAAAGTTCATGGCCCAACCATGAGACAAACAGAGGTGTAACGG
CAGCATGCCCTTATGCTGGAGCAAACAGCTTCTACAGGAATCTGATATGGCTAGTAAAGAAGGGGAACT
CATATCCAAAGCTCAGCAAATCCTATGTTAATAATAAGAAGAAAGAAGTCCTTGTGTTATGGGGCATCC
ACCATCCACCCACCAATACTGATCAACAAAGTCTCTACCAGAATGCAGATGCCTATGTTTTTGTGGGGT
CATCAAAGTATAACAGGAAATTCAAACCAGAAATAGCTGAGAGACCCAAGGTGAGGGGTCAAGCAGGAA
GAATGGACTATTACTGGACATTAGTGGAGCCTGGAGACACAATAACATTTGAAGCAACTGGAAATCTAG
TGGCACCAAGATATGCTTTTGCAATGAATAGAGGTCCCGGGTCTGGTATCATGACATCGGATGTACCAG
TCCATGATTGCAATGCAACCTGCCAAACACCCAAGGGGGCCATAAACACCAGCCTTCCATTTCAGAATA
TACATCCAATCTCTATTGGAGAATGTCCAAAATATGTCAAAAGCACAAGACTAAGAATGGCTACAGGAC
TGAGGAATATACCTTCTATTCAATCTAGAGGTCTGTTCGGAGCCATTGCTGGCTTTATTGAGGGAGGAT
GGACAGGAATGATAGATGGGTGGTACGGCTATCATCACCAGAATGGACAAGGATCAGGATATGCAGCGG
ACCAAAAGAGCACACAGAATGCCATCGATAGGATCACTAACAAGGTAAATTCTGTTATTGAAAAAATGA
ACATACAGTTCACAGCAGTGGGTAAAGAATTCAACCACTTGGAAAAAAGAATAGAGAACTtGAaCAAAA
aagGTTGATGATGGCTTTCTGGATATTTGGACATTACAATGCAA

NA gene 1406 nucleotides

ATGAATCCAAACCAGAAGATAATAAcCATTGGTTCAGTCTGTGTGACGATTGGAATAGCCAACTTGATA
TTACAAATTGGAAACATAATCTCAATATGGATTAGCCGTTCAACTCAAGTTGGGAATCAAAATCAGACT
GAAGCATGCAACCAAAGAGTCATTACTTACGAAAACAATACTTGGGTAAATCAGACATATGTTAACATC
AGCAACATCAATTTTGTTGCTAAACAGGCAGTAGTTTCAATGAAATTAGCGGGAAATTCCTCTCTTTGC
CCTGTTAGTGGGTGGGCTATATATAGTAAGGATAATAGTGTAAGAATTGGTTCCAAGGGGGACATGTTT
GTAATAAGAGAACCATTCATCTCATGCTCTAACTTTGAATGCAGAAACTTCTTCTTGACTCAGGGTGCC
CTGCTGAATGACAAGCACTCTAATGGAACCATTAAAGACAGAAGCCCATATAGAACACTGATGAGCTGT
CCTATTGGTGAAGTTCCTTCCCCATATAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCCTGC
CATGATGGCACCAGTTGGCTGACAATCGGAATTTCTGGCCCAGACAACGGGGCAGTGGCTGTGCTGAAA
TACAATGGAATAATAACAGACACCATCAAAAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAA
TGTGCATGTATGGATGGTTCTTGTTTTACTTTAATGACCGATGGACCTAGTGATGGGCAGGCCTCATAT
AAAATCTTCAAAATAGAAAAGGGAAAAATAGTCAAATCAGTCGAGTTGAATGCCCCCAATTATCACTAC
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GGAGACAATCCGCGCCCAAATGATAGAACAGGCAGTTGCGGTCCAGTATCTTCCAATGGAGCAAATGGA
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AGCGGGTTTGAGATGATTTGGGACCCAAACGGATGGACCGAAACTGACAATAACTTCTCAGTAAAGCAA
GATATCATAGGAATAACTGATTGGTCAGGATACAGCGGGAGTTTTGTTCAGCACCCAGAACTGACCGGG
ATGGACTGTATGAGACCTTGCTTTTGGGTTGAACTAATCAGAGGGCGACCAAAAGAGAACACAATCTGG
ACTAGTGGGAGCAGCATATCCTTTTGTGGTGTGAATAGCGACACTGTAGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTACTATTGACAA

A/Swine/Thailand/CU-M 8.2/2009 (sw H1N1-09)

HA gene 1732 nucleotides

GCAAAAGCAGGGGAAAACAAAAGCAACAAAAATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGC
AACCGCAAATGCAGACACATTATGTATAGGTTATCATGCGAACAATTCAACAGACACTGTAGACACAGT
ACTAGAAAAAAATGTAACAGTAACACACTCTGTTAACCTTCTAGAAGACAAACATAACGGGAAACTATG
CAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCC
AGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTATATTGTGGAAACATCTAGTTCAGACAATGG
AACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTGAGCTCAGTGTCATCATT
TGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGC
AGCATGTCCTCATGCTGGAGCGAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTC
ATACCCAAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCA
CCATCCATCTACTAGTGCTGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGAC
ATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAG
AATGAACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGT
GGTACCGAGATATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGT
CCACGATTGCAATACAACTTGTCAGACACCCAAGGGTGCGATAAACACCAGCCTCCCCTTTCAGAATAT
ACATCCGATCACAATTGGAAAATGTCCAAAATATGTAAAAAGCACAAAATTGAGACTGGCCACAGGACT
GAGGAATGTCCCGTCTATTCAATCTAGAGGCCTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTG
GACAGGGATGGTAGATGGATGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGA
CCTGAAGAGCACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAA
TACACAGTTCACAGCAGTAGGTAAAGAGTTCAATCACCTGGAAAAAAGAATAGAGAATTTAAATAAAAA
AGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGGTTCTATTGGAAAATGAAAG
AACTTTGGACTACCACGATTCAAATaTGAAGAACTTATATGAAAAGGTAAGAAGCCAGTTAAAAAACAA
TGCCAAGGAAATTGGAAACGGCTGCTTTGAATTTTACCACAAATGCGATAACACGTGCATGGAAAGTGT
CAAAAATGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGG
GGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGT
ACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATATG
TATTtAA

NA gene 1391 nucleotides

AAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGTTAACTTAATATTACAAATTGGAA
ACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGGGAATCAAAATCAGATTGAAACATGCAATC
AAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATCAGCAACACCAACA
TTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGCCCTGTTAATGGAT
GGGCTATATACAGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTTGTCATAAGGGAAC
CATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCCTTGCTAAATGACA
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ATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGTACAACGGCATAA
TAACAGACACTATCAAGAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAATGTGCATGTGTAA
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TAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTATGAGGAATGCTCCT
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TGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGACAATCCAC
GCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGAGTAAAAGGATTTT
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TGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAAGATATCGTAGGAA
TAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGGCTGGATTGTATAA
GACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCA
GCATATCCTTTTGTGGTGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCAT
TTACCATTGAC

A/Swine/Thailand/CU-CHL2/2009 (swH1N2-09)

HA gene1583 nucleotides

AGAATGTAACAGCCCCACACTCTGTGAACCTTCTGGAAGACAAACACCCCGGGAAATTATGTCAGCTGA
AAGGCATAGCCCCATTGCATTTGGGTAAATGTAACATTGCTAGATGACTCCTGGGGAACCCAGAATGTG
AATTACTATTCAAAGCAAACTCATGGTCTTACATTGTGGAAACATCTTACTCAGACAATGGGACATGTT
ATCCAGGGGATTTCACCAATTACGAAGAGTTGAGAGAGCAGTTGAGCTCAGTGTCATCATTTGAAAGGT
TTGAGATGTTCCCCAAAGCAAATTCATGGCCCAATCATGAAACAAACAAAGGTGTGACGGCAGCATGCC
CTTATGCTGGAGCAAACAGCTTCTACAGAAACTTAATATGGCTGGTAAAAAAAGGAAATTCATATCCAA
AGCTCAGCAAATCCTATGTCAACAATAAGAAAAAAGAGGTCCTTGTGCTATGGGGCATTCACCACCCAT
CTACCAGTACTGATCAACAAAGTCTCTACCAGAATGCAGATGCCTATGTTTTTGTGAGCTCATCAACAT
ACAACAAGAGATTCAAGCCAGAAATAGGAACAAGACCAAAGGTGAGAGATCAATCAGGGAGAATGAATT
ATTACTGGACAATAGTAGAGCCTGGAGACACAATAACATTTGAAGCAACTGGAAATCTAGTGGCACCAA
GATACGCATTCGCAATGAAAAGAGATTCTGGATCTGGTATTATCATGTCAGACACACCAGTCCACGATT
GTAACACAACTTGTCAAACACCTAAAGGTGCTATAAACACCAGCCTCCCATTTCAAAATGTACATCCAG
TCACAATTGGGGAATGTCCAAAATATGTCAAAAGCACAAAATTAAGAATGGCTACAGGACTAAGGAACA
TCCCGTCTATTCAATCCAGAGGTCTGTTTGGAGCCATTGCTGGCTTTATTGAAGGAGGATGGACAGGAA
TGATAGATGGATGGTACGGTTATCACCATCAGAATGAGCAAGGATCAGGATATGCAGCTGACCGAAAGA
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ATTATCATGACTCAAATGTGAAAAACCTATATGAGAAAGTAAGAAGCCAGCTGAAAAACAATGCCAAAG
AAATTGGAAATGGATGCTTTGAATTTTACCACAAATGTGATGACTCATGCATGGAGAGCATTAAAAATG
AGACTTATGACTATTCGAAGTATTCAGAAGAATCAAGATTAAACAGAGAGAAAATAGATGGAGTGAAAT
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TCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCCAATGGGTCTTTACAGTGCAGAATATGTATTTTAA
ATTAGGATTTCAGAGAAATGAGAAACCCCCCCCCTGTTTTCTACTATAAAAAAT

NA gene 1388 nucleotides

ATGAATCCAAATCAAAAGATAATAACKATTGGCTCTGTTTCtCTCACTATTGCCACAACATGCTTCTTC
ATGCAAATCGCCATCCTAGTAATTACTGTGACATTACATTTCAAACACTATGAATGCAACTCCTCTCCA
AATAATCAAGTAGTGCTGTGTGAACCAACAATAATAGAAAGAAACATAACAGAGATAGTATATCTAACC
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AAAATTACAGGATTTGCACCTTTTTCTAAAGACAATTCAATCCGGCTTTCCGCTGGTGGGGACATTTGG
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ACACTAAACAACAAGCACTCAAATGACACCGTACATGATAGGACCCCTTATCGAACCCTATTGATGAAT
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TGGACCTCAAACAGTATTATTGTGTTTTGTGGCACATCGGGTACATATGGAACAGGCTCATGGCCTGAT
GGGGCGGA

A/sw/Thailand/CU-CHK4/2009 (swH1N2-09)

HA gene 1735 nucleotides

TAAGCAAAAAGCAGGGAAAATAAAAGAAGCCAAAATGAAGGCaATACTATTAGTCTTGCAATGTATACT
TACAGCCGCAAATGCAGACACATTATGTATAGGTTACCATGCAAATAATTCAACTGACACAGTTGATAC
AGTATTAGAAAAGAATGTAACAGTAACACACTCTGTGAACCTTCTGGAAGACAGACACAACGGGAAATT
ATGTAAGCTGAAAGGGATAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGATGGCTCCTGGGGAA
CCCAGAATGTGAATTACTATTCAAAGCAAACTCATGGTCTTACATTGTGGAAACATCTTACTCAGACAA
TGGGACATGTTATCCAGGGGATTTCACCAATTACGAAGAGTTGAGAGAGCAGTTGAGCTCAGTGTCATC
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GGCAGCATGCCCTTATGCTGGAGCAAACAGCTTCTACAGAAACTTAATATGGCTGGTAAAAAAAGGAAA
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GAGAATGAATTATTACTGGACAATAGTAGAGCCTGGAGACACAATAACATTTGAAGCAACTGGAAATCT
AGTGGCACCAAGATACGCATTCGCAATGAAAAGAGATTCTGGATCTGGTATTATCATGTCAGACACACC
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AAGAACTTTGGATTATCATGACTCAAATGTGAAAAACCTATATGAGAAAGTAAGAAGCCAGCTGAAAAA
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CATTAAAAATGAGACTTATGACTATTCGAAGTATTCAGAAGAATCAAGATTAAACAGAGAGAAAATAGA
TGGAGTGAAATTGGAATCAACACAAATTTATCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATT
GGTATTGTTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCCAATGGGTCTTTACAGTGCAGAAT
ATGTATTTAA

NA gene 1410 nucleotides

ATGAATCCAAaTcGAAAGATTATAACCATTGGCTCTGTTTCTCTCACTATTGCCACAACATGCTTCTTC
ATGCAAATCGCCATCCTAGTAATTACTGTGACATTACATTTCAAACACTATGAATGCAACTCCTCTCCA
AATAATCAAGTAGTGCTGTGTGAACCAACAATAATAGAAAGAAACATAACAGAGATAGTATATCTAACC
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ACACTAAACAACAAGCACTCAAATGACACCGTACATGATAGGACCCCTTATCGAACCCTATTGATGAAT
GAGTTGGGTGTTCCATTTCATTTGGGAACCAAGCAAGTGTGCATAGCATGGTCCAGCTCAAGTTGTCAC
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AAAAGCTGCATCAATCGGTGCTTTTATGTGGAGTTGATAAGGGGAAGGAAACAGGAAACTGAAGTCTGG
TGGACCTCAAACAGTATTATTGTGTTTTGTGGCACATCGGGTACATATGGAACAGGCTCATGGCCTGAT
GGGGCGGACATCAATCTCATGCCTGTGTAA

A/swine/Thailand/CU-RA114/2010(H1N1)

HA gene 1678 Nucleotides; GenBank: CY062268

GTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGCGAACAATTCA
ACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCTGTTAACCTTCTAGAAGAC
AAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCT
GGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAA
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TCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGG
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AACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATTCA
ACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGG
TCTCTACAGTGTAGAATATGTA

NA gene 1440 nucleotides; GenBank: CY062270

ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATA
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AGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCC
TTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCCTAATGAGCTGT
CCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGT
CATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAG
TACAACGGCATAATAACAGACACTATCAAAAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAA
TGTGCATGTGTAAATGGTTCCTGCTTTACTGTAATGACCGATGGACCAAGTGATGGACAGGCCTCATAC
AAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTAT
GAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCG
AATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTC
GGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGA
GTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGA
AACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAA
GATATTGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGG
CTGGATTGTATAAGACCTTGCTTTTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGCGGGAGCAGCATATCCTTTTGTGGTGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTACCATTGACAAGTAATTTGTTCAAAAAAACTCCTTGTTTTCTACT

A/swine/Thailand/CU-RA15/2010(H1N1)

HA gene 1676 Nucleotides; GenBank: CY062276

CTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGCGAACAATTCAACA
GACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCTGTTAACCTTCTAGAAGACAAG
CATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGC
TGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACA
TCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCAATTG
AGCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGGCCCAATCATGACTCG
AACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAAAATTTAATATGGCTA
GTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTC
GTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAACAAAGTCTCTATCAGAATGCAGATGCA
TATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAGACCCAAAGTG
AGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCGGGAGACAAAATAACATTCGAA
GCAACTGGAAATCTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAGATGCTGGATCTGGTATTATC
ATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCAAGGGTGCTATAAACACCAGC
CTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTAAAAAGCACAAAATTG
AGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGAGGCCTATTTGGGGCCATTGCCGGT
TTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGGTGGTACGGTTATCACCATCAAAATGAGCAGGGG
TCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCT
GTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATA
GAGAATTTAAATAAAAAAATTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGTTGGTT
CTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTATATGAAAAGGTAAGA
AGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAATTTTACCACAAATGCGATAAC
ACGTGCATGGAAAGTGTCAAAAACGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTAAAC
AGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATTCAACT
GTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCTAATGGGTCT
CTACAGTGTAGAATATGTAT

NA gene 1412 nucleotides; GenBank: CY062278

ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATA
TTACAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGGGAATCAAAATCAGATT
GAAACATGCAATCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATC
AGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCC
TTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCCTAATGAGCTGT
CCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGT
CATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAG
TACAACGGCATAATAACAGACACTATCAAAAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAA
TGTGCATGTGTAAATGGTTCCTGCTTTACTGTAATGACCGATGGACCAAGTGATGGACAGGCCTCATAC
AAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTAT
GAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCG
AATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTC
GGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGA
GTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGA
AACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAA
GATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGG
CTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGCGGGAGCAGCATATCCTTTTGTGGTGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTACCATTGACAAGTAATT

A/swine/Thailand/CU-RA20/2009 (H1N1)

HA gene 1683 nucletides; GenBank: CY062284

TTAGTCTTGCAATGTATGCTTACAGCCGCAAATGCAGACACATTATGTATAGGATACCATGCAAATAAT
TCAACTGACACAGTTGATACGGTATTAGAAAAGAATGTAACAGTAACACACTCTGTCAACCTTCTGGAA
GACAGACACAATGGGAAATTATGTAAACTGAGAGGGATAGCCCCATTGCATTTAGGTAAATGTAACATT
GCTGGATGGCTCCTGGGGAACCCAGAATGTGAATTACTACTTAATGCTAACTCATGGTCTTACATTGTG
GAAACATCTAACTCAGACAATGGGACTTGTTACCCAGGAGATTTCATTAATTACGAAGAATTGAGAGAA
CAATTGAGCTCAGTATCATCATTTGAAAGATTTGAGATATTCCCCAAAGCAAATTCATGGCCTAATCAT
GAAACGAACAAAGGTGTAACAGCAGCATGTCCTTATGCTGGAGCAAACAGCTTCTACAGAAATTTAATA
TGGCTGGTAAAGAAAGAAAATTCATACCCAAAGCTCAGCAAATCCTATGTTAACAATAAGAAGAAGGAG
GTCCTCGTGCTATGGGGCATTCATCATCCACCTACCAGTAACGACCAACAAAGTCTCTACCAGAATGCA
GATGCCTATGTTTTTGTGGGCTCATCTACATACAGCAAGAGGTTCAAGCCAGAAATAGGAACAAGACCC
AAAGTGAGAGATCAGGCAGGGAGAATGAATTATTACTGGACAATAGTAAAGCCTGGAGACACAATAACA
TTCGAATCAACTGGAAATCTAGTGGCACCAAGATATGCCTTCGCAATGAAAAGAGATTATGGATCTGGT
ATTATCATGTCAGATACACCAGTCCACGATTGCAACACGACTTGTCAAACACCTAAAGGTGCTATAAAC
ACCAGCCTCCCATTCCAGAATGTACATCCAGTCACAATTGGGAAATGTCCAAAATATGTCAAAAGTGAA
AAATTGAGAATGGCCACAGGACTAAGAAACATCCCGTCTATTCAATCCAGAGGTCTGTTTGGGGCCATT
GCTGGTTTTATTGAAGGAGGATGGACAGGAATGGTAGATGGATGGTACGGTTATCACCATCAAAATGAT
CAAGGATCAGGATATGCAGCCGACCGTAGGAGCACACAGAATGCCATTGACGGAATCACTAACAAAGTG
AATTCAATTATTGACAAAATGAACACACAATTCACAGCAGTGGGTAAAGAATTCAATCACCTGGAAAAA
AGAGTAGAAAATTTAAACAAAAAAGTCGACGATGGTTTTCTAGATGTTTGGACTTACAATGCCGAACTG
TTGGTTCTATTGGAAAATGAAAGAACTTTGGATTATCATGACTCAAATGTGAAGAACCTATATGAGAAA
GTAAGAAGCCAGCTGAAAAACAATGCCAAGGAGATTGGAAATGGATGTTTTGAATTTTATCACAAATGT
GATGACTCGTGCATGGAGAGCATCAAAAATGAAACTTATGATTACTCAAAATATTCAGAAGAATCAAAA
TTGAGTAGAGAGAAAATAGATGGGGTAAAATTGGAATCAACACAAATTTACCAGATTCTGGCGATCTAT
TCAACTGTCGCCAGCTCATTGGTACTGTTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCCAAT
GGGTCTTTACAGTGCAGAATATGTATT

NA gene 1396 nucleotides; GenBank: CY062286

ATGAATTCAAATCAGAAGATAATAGTCATTGGTTCGGTCAGTATGACAGTTGGAATAGTTAGCTTGATG
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GAAACATGCAACCGAAGCGTGATTGCTTACGAGAACAACACTTGGATAAACCAGACATATGTTAATATC
AGCAACATCAATTTTGATGCTAAACAGGCAGCAATTTCAGCGAGATTAGCGGGCAATTCCTCTCTCTGC
CCCGTTAGTGGATGGGCTATATACAGTAAAGACAATAGTGTAAGAATCGGTTCCAAAGGGGACGTGTTT
GTCATAAGAGAGCCATTCATTTCATGCTCCCACTTGGAATGCAGAACCTTCTTCTTGACTCAAGGTGCC
CTGCTAAATGACAAACATTCTAATGGAACCATTAAAGACAGAAGCCCATATCGGACCCTGATGAGCTGT
CTTGTCGGTGAAGTTCCTTCTCCATACAACTCAAGGTTTGAGTCAGTCGCCTGGTCAGCAAGTGCTTGC
CATGATGGTACCAGTTGGCTGACAATTGGAATTTCTGGTCCAGATAACGGGGCAGTGGCTGTACTGAAA
TACAATGGTATAATAACAGACACTATCAAAAGTTGGAGAAAAAACATATTGAGAACACAAGAGTCTGAA
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AAGATCTTCAAGATAGAAAGGGGGAAAGTAGTCAAATCAGTCGAATTGAATGCCCCCAATTATCACTAC
AAAGAATGCTCCTGTTATCCTGACTCTGGTGAAATCATATGTGTATGCAGGGATAATTGGCATGGCTCG
AACCGACCATGGGTGTCTTTCAATCAGAATCTGGAGTATCAAATAGGATACATATGCAGTGGGGTTTTC
GGAGACAATCCGCGCCCTAATGATAGAATGGGCAGTTGCAGTCCAGTATCCTCTAATGGAGCAAATGGG
GTAAAAGGATTTTCATTTAAATATGGCAATGGTGTTTGGATAGGGAGAACAAAAAGCACTAGTTCAAGG
AGCGGTTTTGAGATGATTTGGGATCCAAACGGGTGGACCGGAACAGACAATAACTTCTCAGTAAAGCAA
GATATTATAGGATTAACTGATTGGTCAGGATACAGCGGGAGTTTTGTTCAGCATCCAGAACTGACTGGA
CTGGATTGCATGAGACCTTGCTTTTGGGTTGAACTAATCAGAGGGCGGCCCAAGGAGAATACAATCTGG
ACTAGTGGGAGCAGCATATCCTTTTGTGGTGTGAATAGCGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGCTGCCATTAC

A/swine/Thailand/CU-RA204/2010 (H1N1)

HA gene 1671 nucleotides; GenBank: CY062292

TTAGTCTTGCAATGTATGCTTACAGCCGCAAATGCAGACACATTATGTATAGGATACCATGCAAATAAT
TCAACTGACACAGTTGATACGGTATTAGAAAAGAATGTAACAGTAACACACTCTGTCAACCTTCTGGAA
GACAGACACAATGGGAAATTATGTAAACTGAGAGGGATAGCCCCATTGCATTTAGGTAAATGTAACATT
GCTGGATGGCTCCTGGGGAACCCAGAATGTGAATTACTACTTAATGCTAACTCATGGTCTTACATTGTG
GAAACATCTAACTCAGACAATGGGACTTGTTACCCAGGAGATTTCATTAATTACGAAGAATTGAGAGAA
CAATTGAGCTCAGTATCATCATTTGAAAGATTTGAGATATTCCCCAAAGCAAATTCATGGCCTAATCAT
GAAACGAACAAAGGTGTAACAGCAGCATGTCCTTATGCTGGAGCAAACAGCTTCTACAGAAATTTAATA
TGGCTGGTAAAGAAAGAAAATTCATACCCAAAGCTCAGCAAATCCTATGTTAACAATAAGAAGAAGGAG
GTCCTCGTGCTATGGGGCATTCATCATCCACCTACCAGTAACGACCAACAAAGTCTCTACCAGAATGCA
GATGCCTATGTTTTTGTGGGCTCATCTACATACAGCAAGAGGTTCAAGCCAGAAATAGGAACAAGACCC
AAAGTGAGAGATCAGGCAGGGAGAATGAATTATTACTGGACAATAGTAAAGCCTGGAGACACAATAACA
TTCGAATCAACTGGAAATCTAGTGGCACCAAGATATGCCTTCGCAATGAAAAGAGATTATGGATCTGGT
ATTATCATGTCAGATACACCAGTCCACGATTGCAACACGACTTGTCAAACACCTAAAGGTGCTATAAAC
ACCAGCCTCCCATTCCAGAATGTACATCCAGTCACAATTGGGAAATGTCCAAAATATGTCAAAAGTGAA
AAATTGAGAATGGCCACAGGACTAAGAAACATCCCGTCTATTCAATCCAGAGGTCTGTTTGGGGCCATT
GCTGGTTTTATTGAAGGAGGATGGACAGGAATGGTAGATGGATGGTACGGTTATCACCATCAAAATGAT
CAAGGATCAGGATATGCAGCCGACCGTAGGAGCACACAGAATGCCATTGACGGAATCACTAACAAAGTG
AATTCAATTATTGACAAAATGAACACACAATTCACAGCAGTGGGTAAAGAATTCAATCACCTGGAAAAA
AGAGTAGAAAATTTAAACAAAAAAGTCGACGATGGTTTTCTAGATGTTTGGACTTACAATGCCGAACTG
TTGGTTCTATTGGAAAATGAAAGAACTTTGGATTATCATGACTCAAATGTGAAGAACCTATATGAGAAA
GTAAGAAGCCAGCTGAAAAACAATGCCAAGGAGATTGGAAATGGATGTTTTGAATTTTATCACAAATGT
GATGACTCGTGCATGGAGAGCATCAAAAATGAAACTTATGATTACTCAAAATATTCAGAAGAATCAAAA
TTGAGTAGAGAGAAAATAGATGGGGTAAAATTGGAATCAACACAAATTTACCAGATTCTGGCGATCTAT
TCAACTGTCGCCAGCTCATTGGTACTGTTAGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGCTCCAAT
GGGTCTTTACAGTGC

NA gene 1412 nucleotide; GenBank: CY062294

ATGAATTCAAATCAGAAGATAATAGTCATTGGTTCGGTCAGTATGACAGTTGGAATAGTTAGCTTGATG
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GAAACATGCAACCGAAGCGTGATTGCTTACGAGAACAACACTTGGATAAACCAGACATATGTTAATATC
AGCAACATCAATTTTGATGCTAAACAGGCAGCAATTTCAGCGAGATTAGCGGGCAATTCCTCTCTCTGC
CCCGTTAGTGGATGGGCTATATACAGTAAAGACAATAGTGTAAGAATCGGTTTCAAAGGGGACGTGTTT
GTCATAAGAGAGCCATTCATTTCATGCTCCCACTTGGAATGCAGAACCTTCTTCTTGACTCAAGGTGCC
CTGCTAAATGACAAACATTCTAATGGAACCATTAAAGACAGAAGCCCATATCGGACCCTGATGAGCTGT
CTTGTCGGTGAAGTTCCTTCTCCATACAACTCAAGGTTTGAGTCAGTCGCCTGGTCAGCAAGTGCTTGC
CATGATGGTACCAGTTGGCTGACAATTGGAATTTCTGGTCCAGATAACGGGGCAGTGGCTGTACTGAAA
TACAATGGTATAATAACAGACACTATCAAAAGTTGGAGAAAAAACATATTGAGAACACAAGAGTCTGAA
TGTGCATGTGTGAATGGTTCTTGCTTTACTGTGATGACCGATGGACCTAGTAATGGGCAGGCCTCATAC
AAGATCTTCAAGATAGAAAGGGGGAAAGTAGTCAAATCAGTCGAATTGAATGCCCCCAATTATCACTAC
GAAGAATGCTCCTGTTATCCTGACTCTGGTGAAATCATATGTGTATGCAGGGATAATTGGCATGGCTCG
AACCGACCATGGGTGTCTTTCAATCAGAATCTGGAGTATCAAATAGGATACATATGCAGTGGGGTTTTC
GGAGACAATCCGCGCCCTAATGATAGAATGGGCAGTTGCAGTCCAGTATCCTCTAATGGAGCAAATGGG
GTAAAAGGATTTTCATTTAAATATGGCAATGGTGTTTGGATAGGGAGAACAAAAAGCACTAGTTCAAGG
AGCGGTTTTGAGATGATTTGGGATCCAAACGGGTGGACCGGAACAGACAATAACTTCTCAGTAAAGCAA
GATATTATAGGATTAACTGATTGGTCAGGATACAGCGGGAGTTTTGTTCAGCATCCAGAACTGACTGGA
CTGGATTGCATGAGACCTTGCTTTTGGGTTGAACTAATCAGAGGGCGGCCCAAGGAGAATACAATCTGG
ACTAGTGGGAGCAGCATATCCTTTTGTGGTGTGAATAGCGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGCTGCCATTTACTGTTGACAAGTAATT

A/swine/Thailand/CU-RA29/2009(H1N1)

HA gene 1702 nucleotides; GenBank: CY062300

ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGT
TATCATGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCT
GTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTG
GGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCA
TGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTAT
GAGGAGCTAAGAGAACAATTGAGCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGT
TCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTC
TACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAAT
GATAAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAACAAAGT
CTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAA
ATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCG
GGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCGCAATGGAAAGA
GATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCC
AAGGGTGCTATAAACACCAGCCTCCCATTTCAAAATATACATCCGATCACAATTGGAAAATGTCCAAAA
TATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGAGGC
CTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGATGGTACGGTTAT
CACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACGAG
ATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTC
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TACAATGCCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAG
AACTTATATGAAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAACGGGACTTATGACTACCCAAAATAC
TCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGAGTAAAGCTGGAATCAACAAGGATTTACCAG
ATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTC
TGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATATGTATTTAAC

NA gene 1406 nucleotides; GenBank: CY062302

ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATA
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GAAACATGCAATCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATC
AGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCC
TTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCCTAATGAGCTGT
CCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGT
CATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAG
TACAACGGCATAATAACAGACACTATCAAAAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAA
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AAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTAT
GAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCG
AATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTC
GGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGA
GTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGA
AACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAA
GATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGG
CTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGCGGGAGCAGCATATCCTTTTGTGGTGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTACCATTGACAA

A/swine/Thailand/CU-RA4/2009 (H1N1)

HA gene 1703 nucleotides; GenBank: CY062308

ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGT
TATCATGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCT
GTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTG
GGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCA
TGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTAT
GAGGAGCTAAGAGAGCAATTGAGCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGT
TCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTC
TACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAAT
GATAAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAACAAAGT
CTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAA
ATAGCAATAAGACCCAAAGTGAGGGATCGAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCG
GGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCGCAATGGAAAGA
GATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCC
AAGGGTGCTATAAACACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAA
TATGTAAAAAGCACAAAATTGAGACTAACCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGAGGC
CTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGGTGGTACGGTTAT
CACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACGAG
ATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACATTTCACAGCAGTAGGTAAAGAGTTC
AACCACCTGGAAAAAAGAATAGAGAATTTAAATAAAAAAATTGATGATGGTTTCCTGGACATTTGGACT
TACAATGCCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAG
AACTTATATGAAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAACGGGACTTATGACTACCCAAAATAC
TCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAG
ATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTC
TGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATATGTATTTAACA

NA gene 1347 nucleotides; GenBank: CY062310

ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATA
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GAAACATGCAATCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATC
AGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCC
TTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCCTAATGAGCTGT
CCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGT
CATGATGGCATCAATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAG
TACAACGGCATAATAACAGACACTATCAAAAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAA
TGTGCATGTGTAAATGGTTCTTGCTTTACTGTAATGACCGATGGACCAAGTGATGGACAGGCCTCATAC
AAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTAT
GAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCG
AATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTC
GGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGGAGCAAATGGA
GTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAACTAAAAGCATTAGTTCAAGA
AACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAA
GATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGG
CTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGCGGGAGCAGCATATCCTTTTGTGGTGTAAAC

A/swine/Thailand/CU-RA75/2010(H1N1)

HA gene1692 nucleotides; GenBank: CY062316

GCAATACTAGTAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCAT
GCGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCTGTTAAC
CTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAA
TGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCC
TACATTGTGGAAACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTATGAGGAG
CTAAGAGAGCAATTGAGCTCAGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATGG
CCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTCTACAAA
AATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAATGATAAA
GGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTACTGCTGACCAACAAAGTCTCTAT
CAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCA
ATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCGGGAGAC
AAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCGCAATGGAAAGAGATGCT
GGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCAAGGGT
GCTATAAACACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTA
AAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGAGGCCTATTT
GGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGGTGGTACGGTTATCACCAT
CAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACGAGATTACT
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CTGGAAAAAAGAATAGAGAATTTAAATAAAAAAATTGATGATGGTTTCCTGGACATTTGGACTTACAAT
GCCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAGAACTTA
TATGAAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAATTTTAC
CACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAACGGGACTTATGACTACCCAAAATACTCAGAG
GAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTG
GCGATCTATTCAACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTCTGGATG
TGCTCTAATGGGTCTCTACAGTGTAGAATATGTATT

NA gene 1411 nucleotides; GenBank: CY062318

ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATA
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GAAACATGCAATCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATC
AGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAAGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCC
TTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCCTAATGAGCTGT
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TACAACGGCATAATAACAGACACTATCAAAAGTTGGAGAAACAATATATTGAGAACACAAGAGTCTGAA
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AAGATCTTCAGAATAGAAAAGGGAAAGATAGTCAAATCAGTCGAAATGAATGCCCCTAATTATCACTAT
GAGGAATGCTCCTGTTATCCTGATTCTAGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCG
AATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTC
GGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTAGCGTCTAATGGAGCAAATGGA
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AACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAA
GATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGG
CTGGATTGTATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGCGGGAGCAGCATATCCTTTTGTGGTGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTTACCATTGACAAGTAAT

A/swine/Thailand/CU-RA9/2009(H1N1)

HA gene 1702 nucleotides; GenBank: CY062324

ATGAAGGCAATACTAGTAGTTCTGCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGT
TATCATGCGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACTCT
GTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTG
GGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGCTCA
TGGTCCTACATTGTGGAAACATCTAGTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTAT
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TCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAAAAAGCTTC
TACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAGCTCAGCAAATCCTACATTAAT
GATAAAGGGAAAGAAGTCCTCGTGCTATGGGGCATTCACCATCCATCTACTAGTGCTGACCAACAAAGT
CTCTATCAGAATGCAGATGCATATGTTTTTGTGGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAA
ATAGCAATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCG
GGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCGCAATGGAAAGA
GATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCC
AAGGGTGCTATAAACACCAGCCTCCCATTTCAGAATATACATCCGATCACAATTGGAAAATGTCCAAAA
TATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTATTCAATCTAGAGGC
CTATTTGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGATGGTACGGTTAT
CACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAAGAGCACACAGAATGCCATTGACGAG
ATTACTAACAAAGTAAATTCTGTTATTGAAAAGATGAATACACAGTTCACAGCAGTAGGTAAAGAGTTC
AACCACCTGGAAAAAAGAATAGAAAATTTAAATAAAAAAATTGATGATGGTTTCCTGGACATTTGGACT
TACAATGCCGAACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGAAG
AACTTATATGAAAAGGTAAGAAGCCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTTGAA
TTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAAAAATGGGACTTATGACTACCCAAAATAC
TCAGAGGAAGCAAAATTAAACAGAGAAGAAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAG
ATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGTTTC
TGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATATGTATTTAAC

NA gene 1397 nucleotides; GenBank: CY062326

ATGAATCCAAACCAAAAGATAATAACCATTGGTTCGGTCTGTATGACAATTGGAATGGCTAACTTAATA
TTACAAATTGGAAACATAATCTCAATATGGATTAGCCACTCAATTCAACTTGGGAATCAAAATCAGATT
GAAACATGCAATCAAAGCGTCATTACTTATGAAAACAACACTTGGGTAAATCAGACATATGTTAACATC
AGCAACACCAACTTTGCTGCTGGACAGTCAGTGGTTTCCGTGAAATTAGCGGGCAATTCCTCTCTCTGC
CCTGTTAGTGGATGGGCTATATACAGTAAGGACAACAGTATAAGAATCGGTTCCAAGGGGGATGTGTTT
GTCATAAGGGAACCATTCATATCATGCTCCCCCTTGGAATGCAGAACCTTCTTCTTGACTCAAGGGGCC
TTGCTAAATGACAAACATTCCAATGGAACCATTAAAGACAGGAGCCCATATCGAACCCTAATGAGCTGT
CCTATTGGTGAAGTTCCCTCTCCATACAACTCAAGATTTGAGTCAGTCGCTTGGTCAGCAAGTGCTTGT
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AATCGACCGTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTC
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AACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGGGACAGACAATAACTTCTCAATAAAGCAA
GATATCGTAGGAATAAATGAGTGGTCAGGATATAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGG
CTGGATTGCATAAGACCTTGCTTCTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGG
ACTAGCGGGAGCAGCATTTCCTTTTGTGGTGTAAACAGTGACACTGTGGGTTGGTCTTGGCCAGACGGT
GCTGAGTGCCATTACCA

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ACADEMIC PUBLICATION

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