ความหลากหลายทางพันธุกรรม และวิวัฒนาการของเชื้อไวรัสไข้หวัดใหญ่สุกรในประเทศไทย
ในช่วงปี ค.ศ. 2012-2015

นางสาวณัฐวัลย์ นนทเบญจวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

GENETIC DIVERSITY AND EVOLUTION OF SWINE INFLUENZA VIRUSES IN THAILAND DURING 2012-2015

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Public Health
Department of Veterinary Public Health
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2016

Thesis Title	GENETIC DIVERSITY AND EVOLUTION OF SWINE INFLUENZA VIRUSES IN THAILAND DURING 2012-2015
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Field of Study	Veterinary Public Health
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เชื้อไวรัสไข้หวัดใหญ่ ชนิด เอ เป็นสาเหตุให้เกิดโรคในระบบทางเดินหายใจทั้งในคนและสัตว์ ในสุกร เชื้อไวรัสไข้หวัดใหญ่ ชนิด H1N1 H1N2 และH3N2 มีรายงานการก่อโรคในสุกรทั่วโลก ส่วนสัตว์ปีกสามารถพบเชื้อไวรัสไข้หวัดใหญ่ชนิด H1-H16 และ N1-N9 ได้ วิทยานิพนธ์ฉบับนี้ ประกอบไปด้วยการศึกษา 3 ส่วน โดยการศึกษาในส่วนที่หนึ่ง ทำการสำรวจเชื้อไวรัสไข้หวัดใหญ่สุกร ในประเทศไทย โดยทำการเก็บตัวอย่างสวอปจมูกสุกร ตั้งแต่เดือนกันยายน พ.ศ. 2554 ถึง เดือนธันวาคม พ.ศ. 2558 จากฟาร์มสุกร จำนวน 46 ฟาร์ม ในพื้นที่ 11 จังหวัด ของประเทศไทย ผลการศึกษาพบว่ามีอุบัติการณ์ของไวรัสไข้หวัดใหญ่สุกร 3.81% (139/3,646 ตัวอย่าง) การสำรวจครั้งนี้แยกไวรัสไข้หวัดใหญ่สุกรได้ จำนวน 139 ตัว ซึ่งเป็นชนิด H1N1 (77 ตัว) H1N2 (13 ตัว) และ H3N2 (49 ตัว) นอกจากนี้ ไวรัสไข้หวัดสุกรจำนวน 12 ตัว (H1N1 จำนวน 5 ตัว H1N1 จำวนว 2 ตัว และH3N2 จำนวน 5 ตัว) ถูกเลือกมาศึกษาทางพันธุกรรมต่อ พบว่า ไวรัส H1N1 จำนวน 3 ตัว มีลักษณะทางพันธุกรรมเหมือนไวรัสไข้หวัดใหญ่สุกรที่พบในประเทศไทย ส่วนไวรัสอีก 9 ตัว เป็นไวรัสสายพันธุ์ใหม่ที่มีการแลกเปลี่ยนสารพันธุกรรมระหว่างไวรัสไข้หวัดใหญ่ H1N1 2009 และไวรัสไข้หวัดใหญ่สุกรที่พบดั้งเดิมในประเทศไทย จากการศึกษานี้ชี้ให้เห็นว่าไวรัสสายพันธุ์ใหม่นี้เป็นไวรัสกลุ่มหลักที่พบในสุกรไทย การศึกษาส่วนที่สอง เป็นการศึกษาการติดเชื้อร่วมของไวรัสไข้หวัดใหญ่สุกรมากกว่าหนึ่งสายพันธุ์ในสุกรตัวเดียว โดยทำการศึกษาในฟาร์มสุกรที่มีประวัติการพบเชื้อไวรัสไข้หวัดใหญ่สุกรมากกว่าหนึ่งชนิด จำนวน 4 ฟาร์ม ในการศึกษานี้สามารถแยกเชื้อไวรัสได้ จำนวน 18 ตัวอย่าง จากตัวอย่างทั้งหมด 145 ตัวอย่าง ซึ่งทั้ง 18 ตัวอย่าง ถูกนำไปถอดรหัสพันธุกรรมด้วยวิธี Illumina โดยผลการศึกษาพบว่ามียีนของไวรัสมากกว่าหนึ่งสายพันธุ์ผสมกันใน 3 ตัวอย่าง โดยพบทั้งยีน H1, H3, N1 และ N2 นอกจากนี้ยังพบไวรัส H3N1 ซึ่งไม่ได้เป็นไวรัสที่พบทั่วไปในสุกรไทย ผลการศึกษาชี้ให้เห็นว่าการถอดรหัสพันธุกรรมด้วยวิธี Illumina มีประสิทธิภาพในการจำแนกการติดเชื้อร่วม การศึกษาส่วนที่สาม เป็นการวิเคราะห์หาต้นกำเนิดของไวรัสไข้หวัดนกชนิดก่อโรครุนแรง สายพันธุ์ H5N2 ที่เป็นสาเหตุการระบาดในประเทศสหรัฐอเมริกา ในปี พ.ศ. 2558 โดยตัวอย่างจำนวน 46 ตัวอย่าง จากไก่งวงและสิ่งแวดล้อม ถูกนำมาถอดรหัสพันธุกรรมด้วยวิธี Illumina โดยไม่ผ่านการแยกเชื้อไวรัส ผลการวิเคราะห์หาช่วงเวลาที่เป็นจุดกำเนิดร่วมของเชื้อไวรัส (time to most recent common ancestor) แสดงให้เห็นความเป็นไปได้ 2 ทาง เกี่ยวกับการกำเนิดของเชื้อ H5N2 หนึ่ง ไวรัสเกิดมาจากการแลกเปลี่ยนยีนที่รัฐอลาสก้า สอง มีการแลกเปลี่ยนยีนระหว่างไวรัสไข้หวัดนกชนิดก่อโรคไม่รุนแรงที่พบในสหรัฐอเมริกาหลายครั้ง ก่อนมีการส่งถ่ายยีนและกำเนิดขึ้นของไวรัส H5N2 นี้ จากการศึกษาทางพันธุกรรมของไวรัสไข้หวัดใหญ่ทั้งในสุกร และสัตว์ปีก ชี้ให้เห็นว่าไวรัสวิวัฒนาการอย่างรวดเร็วอยู่ตลอดเวลา โดยเฉพาะเมื่อมีไวรัสสายพันธุ์ใหม่เข้ามาในประชากร หรือการติดเชื้อข้ามชนิดสัตว์ ดังนั้น การสำรวจเชื้อไวรัสไข้หวัดใหญ่ ชนิด เอ ควรมีการดำเนินการอย่างต่อเนื่อง เพื่อเป็นการเตรียมความพร้อมและป้องกัน การติดเชื้อไวรัสไข้หวัดใหญ่ ในมนุษย์และสัตว์ต่อไปในอนาคต

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5475413731 : MAJOR VETERINARY PUBLIC HEALTH
KEYWORDS: SWINE INFLUENZA VIRUS / SURVEILLANCE / THAILAND / ILLUMINA SEQUENCING / GENETIC DIVERSITY / REASSORTANT / MIXED-INFECTION / HIGHLY PATHOGENIC AVIAN INFLUENZA

NUTTHAWAN NONTHABENJAWAN: GENETIC DIVERSITY AND EVOLUTION OF SWINE INFLUENZA VIRUSES IN THAILAND DURING 2012-2015. ADVISOR: PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 122 pp.

Influenza A viruses cause respiratory diseases in human and animals. In pigs, swine influenza virus (SIV) subtype H1N1, H1N2 and H3N2 can infect pigs worldwide. In avian, avian influenza virus (AIV) subtype H1-H16 and N1-N9 can infect several avian species. This dissertation entitled “Genetic diversity and evolution of swine influenza viruses in Thailand during 2012-2015” contains 3 parts. Part 1, a cross-sectional surveillance of swine influenza virus (SIV) was conducted during September 2011 to December 2015 in 46 commercial swine farms in 11 provinces, Thailand. The results showed that the occurrence of SIVs in Thailand was 3.81% (139/3,646). The SIV isolates in this study could be subtyped as H1N1 (n=77), H1N2 (n=13) and H3N2 (n=49). Subsequently, twelve SIVs (H1N1; n=5, H1N2; n=2, H3N2; n=5) were subjected to for whole genome sequencing and genetic characterization. Genetic analysis revealed that three SIV-H1N1 were endemic Thai SIVs circulating in Thai pig population. On the other hand, nine SIVs were reassotant viruses between pandemic H1N1 2009 and endemic Thai SIV. This results suggested that reassortant SIVs were predominant genotypes in Thai pig population. Part 2, mix-genotype of SIVs were investigated in four commercial swine farms. In total, 145 nasal swab samples were collected and 18 samples were successfully performed virus isolation. The samples were subsequently subjected to whole genome sequencing by next-generation sequencing, illumina Miseq. The results showed that mix-genotypes could be observed in this study. For example, swine farms A, B and D infected with mix-genotypes of SIVs (H1, H3, N1 and N2). Moreover, SIV-H3N1, an uncommon subtype in Thailand, was identified in this study. This results indicated that illumina sequencing is able to discriminate mix-genotypes of SIVs. Part 3, highly pathogenic avian influenza (HPAI) H5N2 in USA in 2015 was investigated. Forty-six samples from turkeys and their environment were collected during the outbreak and directly subjected to illumina sequencing. The viruses were then analyzed to identify origins and evolutionary changes. The results showed that the time to most recent common ancestor analysis suggest two likely possibilities of reassortant HPAI-H5N2 origins: either a reassortment in Alaska area or multiple reassortments with North American low pathogenic avian influenza strains. In summary, this dissertation provided useful information that swine influenza and avian influenza are fast evolving of the viruses especially after the introduction of new genetic pool of the viruses into population or interspecies transmission. Thus, influenza A virus surveillance should be continuously conducted to promote awareness and prevention and control of influenza A virus infection in human and animals in the future.

Department: Veterinary Public Health Student's Signature .....
Field of Study: Veterinary Public Health Advisor's Signature .....
Academic Year: 2016

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest grateful thank to my advisor, Prof. Dr. Alongkorn Amonsin for the continuous support of my Ph.D. study and related research, for his excellent supervisions, constructive criticisms, immense knowledge and patience. His guidance helped me in all the time of research and writing of this thesis.

Besides my advisor, I would like to express my sincere thank to my thesis chairman and committees: Assoc. Prof. Rungthip Chuencheun, Dr. Aunyaratana Thontiravong, Dr. Taradon Luengtongkum and Prof. Dr. Thaweesak Songserm, for their insightful comments and valuable suggestions. Moreover, I would like to thanks, Prof. Dr. Srinand Sreevatsan, for his valuable advices, motivation and his kindness in supporting and sponsoring during my study at the University of Minnesota.

My sincere thanks goes to the senior students of AA lab, Dr. Manoosak Wongpatcharachai, and staffs of Center of Excellence for Emerging and Re-Emerging Infectious Diseases in Animals (CU-EIDAs) from past to present including Dr. Napawan Bunpapong and Dr. Supanat Boonyapisitsopa for relaying and sharing the cognition and experience on the laboratory and research. I would like to thanks all of my AA labmates for their kindly help and support on this work. We are a harmonized team. Additionally, I would like thanks all graduate students and staffs of the Department of Veterinary Public Health and Virus unit of the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University for their kindness, encouragement and friendship.

My sincere thanks also goes to the kindness cooperation of farmers for farm visiting and sample collection.

I would like to express my gratitude to my family for their love and supporting me throughout my life without any doubt on my effort and my chosen way.

I would like to thank Chulalongkorn University Dutsadi Phiphat Scholarship for the financial support of the Doctor of Philosophy program throughout my study period.

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LIST OF ABBREVIATIONS

AIV	Avian Influenza Virus
BEAST	Bayesian Evolutionary Analysis by Sampling Tree
BMCMC	Bayesian Markov Chain Monte Carlo method
bp	Base Pair
CDC	The Center of Disease control and Prevention
cDNA	complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
HA	Hemagglutinin, Hemagglutination
HI	Hemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
IAV	Influenza A Virus
IVPI	Intravenous pathogenicity index
LPAI	Low Pathogenic Avian Influenza
M	Matrix protein
MCC	Maximum Clade Credibility
MDCK	Madin Darby Canine Kidney
NA	Neuraminidase
NP	Nucleoprotein
NS	Nonstructural protein
OIE	The Office International des Epizooties
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate Buffered Saline
RBC	Red Blood Cell
RNA	Ribonucleic acid
rRT-PCR	Real-time Reverse-Transcription Polymerase Chain Reaction
SIV	Swine Influenza Virus
v.	version
VTM	Viral Transport Medium
WHO	World Health Organization

CHAPTER I

Introduction

1.1 Importance and Rationale

Animal influenza is one of three priorities of health issues in the tripartite alliance which in a collaboration among Food and Agricultural Organization (FAO), World Organization for Animal Health (OIE) and World Health Organization (WHO) (OIE, 2016a). This action was implemented due to the concerns of the epidemic of highly pathogenic avian influenza (HPAI) H5N1 at the beginning of the 2000s and pandemic of swine origin H1N1 in 2009.

A century ago, four influenza pandemics had been reported including Spanish flu (H1N1) in 1918, Asian flu (H2N2) in 1957, Hong Kong flu (H3N2) in 1968 and the pandemic H1N1 in 2009. The latest pandemic influenza H1N1 was first recognized in Mexico in early April 2009. In June 2009, WHO raised the pandemic alert to level 6 of 6 due to more than 70 countries reported of human cases (CDC, 2010). It has been reported that swine was suspected to be the mixing vessel to generate the pandemic viruses that responsible for the first three pandemics (Smith, et al., 2009a). Moreover, the origin of the pandemic influenza H1N1 in 2009 was also confirmed as a swine origin virus (Smith, et al., 2009b).

Swine influenza virus (SIV) causes swine influenza in pigs. SIV subtypes H1N1, H1N2 and H3N2 have been reported to infect and circulate in pig populations worldwide including Thailand. It has been known that pigs are the potential mixing vessel for influenza A viruses (IAV) due to pig posed specific viral receptors for mammal and avian influenza viruses on the respiratory epithelial cells. Thus, an epidemiological surveillance of swine influenza viruses was conducted in swine

farms in Thailand. The results revealed the occurrence and subtypes of SIV circulating in Thailand. Moreover, whole genome sequencing and genetic characterization were performed.

Swine influenza viruses can co-circulate with multiple genotypes in a swine farm. Reassortment of swine influenza viruses could be occurred. In this study, comprehensive SIV surveillance in selected swine farms were performed to reveal SIV mix-genotypes and variants. Moreover, whole genome sequencing analysis could provide information of antigenic drift and antigenic shift of the viruses, which pose a risk for human and animal health. In this study, the high performance sequencing technology, Next-Generation Sequencing (NGS), illumina Miseq platform, was applied to characterize and identify mix genotypes and variants of swine influenza viruses.

Avian influenza virus (AIV) causes respiratory disease in several avian species. The disease cause economic losses from animal productions and public health concerns from human death. Recently, in 2016, highly pathogenic avian influenza (HPAI) has been reported in poultry in more than 50 countries (OIE, 2016b). In addition, more than 700 human infections of HPAI-H5 has been reported (CDC, 2015a). It has been reported that an ancestor of HPAI-H5 was traced back to A/Goose/Guangdong/1/96 isolated in China, 1996 (Xu, et al., 1999). Currently H5 gene has been classified into clade (0-9) based on OIE nomenclature system (OIE, 2011). Recently, the later predominant clade of HPAI-H5 was clade 2.3.4.4 (Smith, et al., 2015). Since late 2014, HPAI-H5 clade 2.3.4.4 has been emerged in East Asia and South East Asia, Europe including North America (Ip, et al., 2015; Ozawa, et al., 2015; Verhagen, et al., 2015). During March to June 2015, turkey farms in Midwest area of USA were affected by HPAI-H5N2 clade 2.3.4.4 resulting in culling of more than 48 million birds. The origin of HPAI-H5N2 is a crucial information for outbreak investigation and for improving prevention and control measures.

Addressing the health and economic impacts of Influenza A virus in animal and human, swine influenza virus surveillance has been conducted to provide an insight information for future

prevention and control strategies. Moreover, HPAI-H5N2 outbreak investigation in the US has also been performed to better understanding of viral origin and transmission route. Overall, this study has provided the epidemiological information and genetic characterization of Thai SIVs and describes HPAI-H5N2 outbreak and its evolution. The achieved data will update the current SIV status and will be used to support the development of prevention and control measures.

1.2 Objectives

Due to the importance and public health concerns of animal influenza viruses this dissertation was designed to investigate the current epidemiological status of swine influenza viruses in Thailand, 2012-2015 and investigate highly pathogenic avian influenza H5N2 outbreaks in USA, 2015.

The objectives of this study were:

1. To identify the herd occurrence and subtypes of swine influenza viruses in Thailand
2. To investigate genetic diversity and evolution of swine influenza viruses by using whole genome sequencing
3. To discriminate the mix-genotypes and variants of swine influenza viruses by using Next-Generation Sequencing
4. To describe the origin and evolution of highly pathogenic avian influenza H5N2 causing outbreaks in Midwest, USA, 2015

To fulfill these objectives, this dissertation present our findings into 5 separated chapters including chapter (1) Introduction and literature reviews (2) Diversity of swine influenza viruses in Thai swine farms, 2011-2014, (3) Mix-genotype of swine influenza viruses investigated in commercial swine farms, Thailand (4) Time-space analysis of highly pathogenic avian influenza H5N2 outbreak in the US and (5) Conclusions and discussions.

1.3 Literature review

1.3.1 Morphology of Influenza A virus

Influenza A virus (IAV) is a genus that belonging to family *Orthomyxoviridae.* Other two genera of influenza viruses, which are influenza B virus and influenza C virus. Only IAV has a wide host range. It can infect avian, mammals such as dogs, cats, horses, tigers and pigs including marine mammals. IAV is an enveloped particle containing 8 gene segments of negative-sense single-strand RNA, polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M) and non-structural (NS). These genes encode at least 10 proteins, PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NS2. IAV was divided into subtypes based on two envelope proteins, HA and NA. At present, 18 HA and 11 NA subtypes were reported. Aquatic wild birds are reservoir of IAV due to they can carry HA 1-16 and NA 1-9 of IAV. H17N10 and H18N11 were isolated from bats in 2009 and 2010, respectively (Tong, et al., 2013).

The viral replication cycle starts with attachment of HA protein to viral specific receptor on host cells. Thus, HA protein plays an important role of specific host range of viruses. There are two different receptor types, avian and human receptor, which have different conformation of glycoprotein. Avian specific receptor has sialic acid linked to galactose at alpha 2,3 (SA α2,3) while human receptor presents alpha 2,6 linkage (SA α2,6). After binding to host cell, receptor-mediated endocytosis occurs and the virus enter the host cell in an endosome. Then viral and endosomal membrane fusion occurs when endosome has a low pH approximately 5 to 6. Once virus releases viral ribonucleoprotein (vRNP) in cytoplasm subsequently vRNP must enter the nucleus then staring replication. Viral RNA replication starts with the synthesis of a positive sense copy of vRNA which called complementary RNA (cRNA). cRNA serves as a template to produce vRNA then mRNAs are synthesized and exported to cytoplasm. Influenza viral mRNAs are translated by host cell translation machinery. The viral polymerase subunit proteins and NP are imported into the nucleus

to catalyst the replication and transcription of vRNA. The nuclear exports newly synthesized vRNP complexes. Viral proteins are transported to the plasma membrane then assembly and budding at the region rich in sphingolipids and cholesterol where HA, NA and M2 protein are present on apical plasma membrane (Matsuoka, et al., 2013; Samji, 2009).

IAV has changed over time via two mechanisms, antigenic drift and antigenic shift, to survive and maintain in the population. First, antigenic drift refers to point mutation. It can occur during RNA replication due to RNA polymerase has no proof reading function (Sanjuan, et al., 2010). Second, antigenic shift refers to rapid genetic change of the virus due to reassortment. Reassortment can occur when a host cell get infection with the virus more than one genotype. The progeny virus can retrieve the different source of gene segments together and become a novel reassortant virus. However, some factors give a positive pressure to the virus resulting in increasing evolution rate such as host immunity even from vaccination or natural infection, interspecies transmission, antiviral drug exposure (Chen and Chen, 2014; van de Sandt, et al., 2012). Moreover, it has been documented that, reassortment mechanism involved in the influenza A pandemics in 1957, 1968 and 2009 (Kilbourne, 2006; Smith, et al., 2009b).

Encoding proteins have their function that summarized in Table 1.1

Table 1.1 RNA segment and functional protein of IAV

RNA segment	Functional protein	Function	Reference
1 PB2 (2,341)*	PB2 (759)**	- RNA transcriptase: host cell RNA cap binding	(Lamb and Choppin, 1983)
2 PB1 (2,341)	PB1 (757)	- RNA transcriptase: elongation	(Steinhauer and Skehel, 2002)
	PB1-F2 (79 or 90)	- Proapoptotic virulence factor and proinflammatory effect	(Chen, et al., 2001)
	PB1-N40 (718)	- Detrimental to virus replication - Function has not been fully identified yet	(Wise, et al., 2009)
3 PA (2,233)	PA (716)	- RNA transcriptase, Protease activity - Phosphorylation	(Steinhauer and Skehel, 2002)
4 HA (1,778)	HA (566)	- Major surface glycoprotein - To bind host cell surface SA receptor - Fusion between virion envelope and host cell	(Lamb and Choppin, 1983; Webster, et al., 1992)
5 NP (1,565)	NP (498)	- Part of RNA transcriptase complex - RNA binding and nucleocytoplastic transport of viral RNA - Target of host cytotoxic T-cell immune respose	(Steinhauer and Skehel, 2002; Webster, et al., 1992)
6 NA (1,413)	NA (454)	- Surface glycoprotein - Neuraminidase activity: release virus from host cell	(Steinhauer and Skehel, 2002)
7 M (1,027)	M1 (252)	- Matrix protein: major component of virion - Form a shell surrounding the virion nucleocapsids - Initiate progeny virus assembly	(Steinhauer and Skehel, 2002; Webster, et al., 1992)
	M2 (97)	- Integral membrane protein - Proton channel: control pH of golgi during HA synthesis - Acidification of interior of the virion during viral uncoating	(Steinhauer and Skehel, 2002; Webster, et al., 1992)
8 NS (890)	NS1 (230)	- Non-structural protein - Regulation of mRNA splicing and translation - Anti-interferon protein: TNFα response	(Steinhauer and Skehel, 2002)
	NS2/NEP (121)	- Non-structural protein - Nucleocytoplasmic export of viral RNPs - Regulation of virus transcription and translation	(Robb, et al., 2009)

* number of nucleotide

** number of amino acid

1.3.2 Influenza A virus in pigs

Swine influenza virus (SIV) causes highly contagious disease and an economic lost in pig production industry. Moreover, there are several evidences of zoonotic and reverse zoonotic. Historically, SIV was first recognized during Spanish flu H1N1 pandemic then was successfully isolated in 1930 in United States of Americas (USA) (Shope, 1931; Webster, et al., 1992). This SIV-H1N1 was called classical lineage or North American lineage and had been a predominant genotype in North America until 1998. In 1998, the triple reassortant H3N2 containing avian lineage (PB2 and PA), human lineage (PB1, HA and NA) and swine lineage (NP, M and NS) was identified in the US (Zhou, et al., 1999). Subsequently, H1N2 was emerged by reassortant between H1N1 and H3N2 viruses in USA pig population (Karasin, et al., 2000b; Vincent, et al., 2008).

In Europe, SIV classical H1N1 was first reported around 1950 and rapidly spread to other European countries. In 1979, whole avian genotypes of influenza A H1N1 was identified in European pigs and became a predominant genotype replacing classical H1N1 (Brown, 2000). Due to an avian origin it was designated as SIV avian-like lineage or Eurasian swine lineage. In 1970s, SIV H3N2 was isolated from pigs throughout the world and genetic study revealed that this H3N2 related to human IAV from 1973 (Brown, 2000). This was the discovery human-like lineage in pig population.

Recently, three subtypes (H1N1, H1N2 and H3N2) have been endemic in pig population worldwide. In Thailand, SIV H1N1 was first isolated in 1988 (Kupradinun, et al., 1991). Genetic of Thai SIV was studied and designated as H1N1 (7+1) and H1N1 (6+2) which containing 7 and 6 gene segments of Eurasian swine lineage and 1 and 2 gene segments of classical swine lineage (Kitikoon, et al., 2011). In 2010, the first whole genome analysis of H3N2 was reported and revealed that HA3 and NA2 originated from seasonal human virus in 1995 (Lekcharoensuk, et al., 2010). After emerging of pandemic H1N1 2009 (pH1N1), the reassortant SIV have been reported worldwide. In USA, reassortant SIV-H3N2 (rH3N2) contained M gene from pH1N1 while others 7 genes (PB2, PB1,

PA, HA, NP, NA and NS) related to SIV-H3N2 that circulated in USA since 1998 (Lina, et al., 2011). This observation is different in many countries including Thailand. In Thailand, SIV-rH1N1 and SIV-rH3N2 had been reported since 2010 but Thai SIV contained the triple reassortant internal gene (TRIG) cassette (Kitikoon, et al., 2011; Poonsuk, et al., 2013). Similar results in Argentina and Japan, the viruses maintain TRIG cassette and retrieved HA and NA gene from local influenza A viruses (Kobayashi, et al., 2013; Pereda, et al., 2011).

Beside three major subtypes circulating worldwide, other subtypes originated from avian influenza viruses have been periodically reported especially H3N1, H4N6 and H9N2 (Cong, et al., 2007; Karasin, et al., 2000a; Lekcharoensuk, et al., 2006; Peiris, et al., 2001; Shin, et al., 2006) .

1.3.3 Influenza A virus in avian

Avian influenza is a highly contagious infectious disease that affects avian production industry and international trading. Avian influenza virus (AIV) is classified into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) by its virulence in chicken or predictable from amino acid sequences at HA cleavage site. Since 1996, highly pathogenic avian influenza (HPAI) H5N1 has been first identified in geese in Guangdong, China. Subsequently, HPAI-H5N1 spread worldwide then it was designated as HPAI H5 goose Guangdong (GS/GD) lineage. Recently, HPAI H5 is an endemic disease in many countries including Bangladesh, China, Egypt, India, Indonesia, and Vietnam (CDC, 2015a). Due to diversity and evolutionary of HPAI-H5 GS/GD lineage, OIE has been classified HPAI-H5 into clade (0-9). The later clade was designated in 2015 as clade 2.3.4.4. Furthermore, AIV can cause mild to severe respiratory disease or until fatal in human. Not only HPAI-H5 has ability to infect human but also H7N9 and H9N2 which are low virulence viruses in avian species but can also infect human (WHO, 2016).

In natural, aquatic wild birds are considered the main reservoir of IAV. They carry IAVs without clinical signs. It has been documented that wild bird especially in order *Anseriformes* (e.g.

duck, geese and swan) and *Charadriiformes* (e.g. snipe and gull) are the major natural reservoir of IAV (Webster, et al., 1992). Due to they have annual long distance migratory pattern this factor allow viruses transmission to other domestic or different wild bird species in gathering area for feeding, resting and breeding (Olsen, et al., 2006).

1.3.4 Standard methods for influenza A virus isolation and identification

Virus isolation and identification are the conventional technique for IAV diagnosis (OIE, 2016c). For virus isolation, there are two host systems including chicken embryonic eggs and Madin-Darby Canine Kidney (MDCK) cell lines that serve as virus hosts. The suspensions of viral transport medium (VTM) of nasal swabs from mammals or oropharyngeal and cloacal swabs from avian species were inoculated in the host system. For chicken eggs, the VTM suspension was inoculated in allantoic cavity of 9-to-11-day old embryonated eggs according to WHO/OIE recommendations. The eggs are incubated at 37 ℃ for 3-7 days when the incubation period is end the allantoic fluid of all eggs including eggs containing dead embryo were individual collected. For MDCK cell lines, the VTM suspension was inoculated to MDCK cells and incubated in 5% CO_2 at 37 ℃ for 72 hours with daily observe of cytopathic effects (CPE). Both allantoic fluid and cell culture medium suspension were tested the presence of viral particle by hemagglutination test (HA test) by using 0.5% suspension of turkey red blood cells (RBC). The negative samples for HA test should be inoculated at least one further passage while the positive samples will be subject to IAV subtype identification by using hemagglutination inhibition test (HI test) to identified the specific HA subtypes.

The conventional methods are using as a method of choice or gold standard of IAV infection diagnosis but there tend to be costly, labor intensive, time consuming and requires maintenance of antiserum stock (Alexander, 2008). The molecular technique has been developed for over the past decade to reduce time consuming of diagnosis. Reverse transcription-polymerase

chain reaction (RT-PCR) was developed for direct viral RNA detection and IAV subtyping by using specific primers (Tsukamoto, et al., 2008; Tsukamoto, et al., 2009). Moreover, one step-real time RT-PCR was developed for rapid detection by using the specific hydrolysis probe and primers (Spackman, et al., 2002). However, the nature of short time period of virus shedding may lead to fault negative results serological assay is also applied to detect antibodies producing by infected animals.

1.3.5 Serological study for influenza A virus

Serological assay is a useful method to diagnostic and monitoring SIV infection status in swine farms. Antibody detection can be done in many kinds of assay such as hemagglutination inhibition test (HI test) which is a gold standard method, serum-virus neutralization (SVN) and enzyme-linked immunosorbent assay (ELISA) (Ciacci-Zanella, et al., 2010). The HI and SVN assay use to identify the specific subtype of viral exposure. Commercial ELISA has both kind of antibody detection, specific and non-specific subtype. For screening the SIV infection, high sensitivity assay will be selected. Antibody against nucleocapsid protein detection is suitable for screening test and has a higher sensitivity than antibody against hemagglutinin detection due to NP antibody is not specific to subtypes. The sensitivity and specificity of three commercial ELISA kit (IDEXX Influenza A Ab test, IDEXX AI MultiS-Screen Ab test and IDVet ID Screen influenza A antibody competition ELISA) were evaluated and revealed that ID Screen® influenza A antibody competition ELISA (IDVet-Innovative Diagnostics, France) has a highest performance (Tse, et al., 2012). However, HI test assesses the quantity of antibody against specific HA subtype of IAV so the reference virus that using can directly affected to results. In Thailand, since the introduction of pandemic 2009 H1N1 in pig population the predominant SIV was differed from previous reports. Recently, three major subtypes has been circulated in Thai pig populations including endemic Thai H1N1, endemic Thai H3N2 and pandemic 09 H1N1. HI test results may affected by inappropriate the references viruses

however, the contemporary study demonstrated that eH1N1-2006, pH1N1-2009 and eH3N2-2009 should be selected as reference viruses for serological study and HI tests (Arunorat, et al., 2016).

1.3.6 Next-Generation Sequencing

The order of nucleic acid of biological samples contains the information for the hereditary and biochemical properties of life. The well-known nucleotide sequencing technique is Sanger sequencing or dideoxy chain-termination method by DNA polymerase during in vitro DNA replication that was developed by Frederick Sanger and colleagues in 1977 (Sanger, et al., 1977). Sanger sequencing method is a polymerase chain reaction (PCR) in a mixture containing primer, DNA polymerase, all four deoxynucleotides (dNTPs: dGTP, dATP, dTTP and dCTP) and one 2', 3' dideoxynucleotides (ddNTPs: ddGTP, ddATP, ddTTP and ddCTP) to produce strands of different length equal to the position of each base of the type that complements the type having a ddNTP present. The PCR product will visualized by electrophoresis, each ddNTP reaction a lane so the nucleotide order can be read from product length 1 base pair and so on until the longest length of PCR product. Thus, a reaction will give one coverage of nucleotide sequence. This method was wildly use until the development of new massive sequencing technique called next generation sequencing (NGS). Since first introduced to market in 2005, NGS have had a huge impact on genomic research (Morozova and Marra, 2008). The principle of NGS is thousands reaction of sequencing simultaneously occur. Now, there are many platforms available such as illumina and ion semiconductor, but all of them have the same three major steps. Roughly, first step is library preparation, this step starts with nucleic acid of interest extraction then it is sheared in desired length. The proper length of nucleic acid strands were extended by oligonucleotide adaptors. Second, template amplification, there are two methods which are emulsion base PCR and bridge amplification. Adapted nucleic strands from step one were hybridized to the complementary adaptor on the solid phase such as glass bead or glass slide. The hallmark of second step is to

amplify a clone of each nucleic strand. After amplification, start from one nucleic strand will turn to a clonal of same sequencing information. Third step is sequencing all strands from step two at the same time. Thus, one reaction will provide more than megabase of nucleotides.

Both techniques have different pros and cons that were summarized in table 1.2.

Table 1.2 Comparison between Sanger sequencing and Next-Generation Sequencing

Sanger sequencing method	Next-generation sequencing
Advantages	**Advantages**
- Lowest error rate from polymerase	- Massively parallel: million fragments to be sequenced in a single run
- Long read length	- High sequencing coverage
	- Lower cost per base
Disadvantages	**Disadvantages**
- One sequence coverage at a time	- Complex procedure for sample preparation
- High cost per base	- Short read length
	- Require advance procedure for data analysis and management

Among various NGS platforms, illumine Miseq is a popular one for viral diversity study. A study showed that illumina Miseq platform was able to identify seasonal influenza H3N2, pandemic 2009 H1N1 and influenza B in the DNA library mixtures efficiently (Rutvisuttinunt, et al., 2013). Moreover, two platforms of NGS, Illumina Miseq and Ion torrent PGM, were compared the accuracy and sensitivity for the analysis of influenza virus gene diversity. The researchers concluded that the illumina MiSeq platform is better suited for detecting variant sequences (Silvie, 2015).

CHAPTER II

Diversity of swine Influenza viruses in Thai swine farms, 2011-2015

Parts of this work has been published in the topic of

Diversity of swine Influenza viruses in Thai swine farms, 2011-2014

Virus Genes April 2015, Volume 50, Issue 2, pp 221-230.

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Swine influenza virus (SIV) causes an economic loss in swine industry worldwide including Thailand. Moreover after emerging of swine origin pandemic H1N1 2009 (pH1N1), the important of SIV was raised in term of public health concerns. In this chapter, cross-sectional surveillance of SIVs was conducted in high density pig production areas of Thailand, during 2011-2015. The study provided epidemiological data and genetic characterization of contemporary Thai SIVs. In addition, significant mutations related to potential public health concerns was monitored.

2.1 Abstract

The pig is known as a “mixing vessel” for influenza A viruses. The co-circulation of multiple influenza A subtypes in pig populations can lead to novel reassortant strains. For this study, swine influenza surveillance was conducted from September 2011 to December 2015 on 46 swine farms in Thailand. In total, 139 swine influenza viruses were isolated from 3,646 nasal swabs and 12 were selected for characterization by whole genome sequencing. Our results showed that the co-circulation of swine influenza subtypes H1N1, H3N2 and H1N2 in Thai swine farms was observable throughout the 4 years of surveillance. Furthermore, we repeatedly found reassortant viruses between endemic swine influenza viruses and pandemic H1N1 2009. This observation suggests that there is significant and rapid evolution of swine influenza viruses in swine. Thus, continuous surveillance is critical for monitoring novel reassortant influenza A viruses in Thai swine populations.

Keywords: Genetic diversity, Reassortant, Swine Influenza, Surveillance, Thailand

2.2 Introduction

The influenza A virus (IAV) belongs to the family *Orthomyxoviridae*. IAVs can be classified into subtypes based on two, major surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Currently, 18 HA and 11 NA subtypes have been identified, while IAV subtypes H1N1, H1N2 and H3N2 have been reported in swine populations worldwide (Olsen, 2002; Tong, et al., 2013). In Thailand, the endemic swine influenza virus (SIV) subtype H1N1 was first reported in 1991 (Kupradinun, et al., 1991). The genetic composition of Thai endemic SIV-H1N1 (eH1N1) has been characterized as eH1N1 (6+2) and eH1N1 (7+1) (Kitikoon, et al., 2011). The genetic composition of Thai endemic SIV-H3N2 (eH3N2), however, has been characterized as of Eurasian swine lineage (PB2, PB1, PA and M), classical swine lineage (NP and NS) and of seasonal human H3N2 origin (H3 and N2) (Lekcharoensuk, et al., 2010; Takemae, et al., 2008).

In April 2009, pandemic H1N1 (pH1N1) was first reported in humans and quickly spread worldwide. It was first isolated from Thai pigs in November 2009 (Sreta, et al., 2010) and subsequent surveillance in Thailand detected a novel reassorted SIV in 2010 (Kitikoon, et al., 2011). Because of the ongoing circulations of multiple SIV lineages among Thai pigs and the evidence of viral reassortment in swine, SIV surveillance in Thailand should be a priority. This study conducted 3 years of SIV surveillance on Thai swine farms and found SIV subtypes H1N1, H1N2 and H3N2 circulating among pigs. Observations of the reassortant SIVs, rH1N1, rH1N2 and rH3N2, however, were predominant. The genetic diversity of those reassortant viruses is described herein.

2.3 Materials and Methods

2.3.1 Surveillance of Thai swine farms

Between September 2011 and February 2014, a cross-sectional SIV surveillance program was conducted at Thai swine farms located in 13 high swine-density provinces representing all parts of Thailand. In total, 3,646 nasal swab samples were obtained from 46 swine farms. The samples

were collected individually from pigs of different ages and transported within 24 hours for laboratory analysis. During transport, each sample was kept in a standard viral transport medium encased in ice. Details of samples and farm locations are shown in Table 2.1.

2.3.2 Identification and isolation of SIVs

All nasal swabs were screened for IAVs through one-step real-time RT-PCR (rRT-PCR). Viral RNAs were extracted from samples with the QIAamp Viral RNA Mini Kit (Qiagen®; Hilden, Germany). rRT-PCR was conducted by using a TaqMan probe to detect the IAV matrix (M) gene with some modification (Spackman, et al., 2002). One-step rRT-PCR was performed on a Rotor-Gene 3000 (Corbett Research; Sydney, Australia) utilizing the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen™; California, USA). Data acquisition and analysis of the rRT-PCR assay were done through the Rotor-Gene software, v.6.0.19. Samples exhibiting a Ct value of < 36 were interpreted as positive and those with a Ct value of 36-40 as suspect.

The positive rRT-PCR samples were then subjected to IAV isolation using egg inoculation and/or cell culture. For egg inoculations, we inoculated embryonated chicken eggs according to WHO recommendations (WHO, 2002). After a 72-hour incubation period, the allantoic fluid of each egg was collected and tested for hemagglutinin activity with a hemagglutination test (HA test) using a 1% suspension of chicken red blood cells. For cell culture, Madin Darby Canine Kidney (MDCK) cells were used for viral propagation. During the incubation period of 48 hours, we made daily observations for cytopathic effect (CPE) and, after incubation, collected the supernatants of CPE positives. Samples that tested positive by HA test at 4 HA units/50 µl or more and CPE positive cell supernatants were subsequently subjected to IAV confirmation by rRT-PCR for M gene detection as previously described.

2.3.3 Genetic characterization of Thai SIVs

To subtype IAVs, cDNA was synthesized using the influenza universal primer Uni12 (Hoffmann, et al., 2001) and the ImProm-II™ Reverse Transcription System (Promega; Wisconsin, USA). The cDNA served as a template for subtype identification using specific primers in our inventory for the HA and NA genes. In this study, 12 viruses were selected based on epidemiological data as representatives for whole genome sequencing. Each viral gene was amplified by using specific primers then PCR products were subjected to DNA sequencing (1st Base Laboratories Sdn Bhd, Malaysia). The nucleotide sequences of each gene were validated and assembled in SeqMan software v.5.03 (DNASTAR Inc.; Wisconsin, USA).

Phylogenetic and genetic analyses were performed by comparing each viral gene segment with reference SIV sequences available at the GenBank database. The reference nucleotide sequences that were retrieved included all geographical origins (Eurasia and North America) and three host origins (human, swine and avian) for constructing phylogenetic trees. The nucleotide sequences of each gene were aligned in Muscle v.3.6 (Edgar, 2004). The phylogenetic trees were constructed with two software: MEGA v.6.0, using the neighbor-joining algorithm with the Kimura-2 parameter model applied to 1,000 replications of bootstrap, and Mr. Bayes software, using the BMCMC with 50,000 generations and an average standard deviation of split frequencies < 0.05 (Drummond and Rambaut, 2007; Tamura, et al., 2007). To support tree topology, the bootstrap percentages and posterior probabilities were evaluated. The nucleotide sequences and deduced amino acids of each viral gene were aligned and compared in MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA). The nucleotide sequences of the Thai SIVs were submitted to the GenBank database under the accession numbers shown in Table 2.2.

2.4 Results

2.4.1 Prevalence and subtypes of SIVs in Thai swine farms

During our 4 years of SIV surveillance in Thai swine farms, 3,646 nasal swab samples were collected and examined. 268 (7.35%) were identified as IAV positive through real-time RT-PCR. Subsequently, 139 SIV isolates (51.87%) were successfully recovered from IAV positive samples by egg inoculation and/or MDCK cell culture (Table 2.1). Further identification of the 139 SIV isolates revealed the subtypes H1N1 (77; 55.40%), H1N2 (13; 9.35%) and H3N2 (49; 35.25%). These results suggest that SIV subtypes H1N1 and H3N2 were the predominant subtypes in Thai swine populations (Table 2.2).

Table 2.1 Description of samples, virus identification, virus isolation and virus subtyping

Year	# of samples	# of Farms (provinces)	rRT-PCR (% positive)	SIV isolation (% positive)	SIV subtype (# positive sample)		
					H1N1	H1N2	H3N2
2011*	174	9 (4)	28/174 (16.09%)	4/174 (2.29%)	0	1	3
2012	1219	19 (8)	109/1219 (8.94%)	28/1219 (2.30%)	12	2	14
2013	1345	32 (10)	42/1345 (3.12%)	39/1345 (2.89%)	19	0	20
2014	538	5 (6)	59/538 (10.97%)	47/538 (8.74%)	38	1	8
2015	370	3 (3)	30/370 (8.11%)	21/370 (5.68%)	8	9	4
	3646	46 (11)	268/3647 (7.35%)	139/3646 (3.81%)	77	13	49

* 2011: sample collection in September – December

Table 2. 2 Description of selected SIVs characterized in this study

Virus	Subtype	Year	Age	Type of farm*	Location	GenBank Accession No.
A/swine/Chonburi/NIAH9469/04[a]	eH1N1 (6+2)	2004	-	-	Chonburi	AB434301-08
A/Swine/Thailand/CU-S3334/12	eH1N1 (6+2)	2012	8 weeks	C	Chonburi	KJ162027-33, KJ162046
A/Swine/Thailand/CU-S3350/12	eH1N1 (6+2)	2012	6 weeks	D	Ratchaburi	KJ162034-41
A/Swine/Thailand/CU-S3406/12	eH1N1 (6+2)	2012	4 weeks	D	Ratchaburi	KJ526053-59, KM355356
A/Swine/Thailand/CU-S3629/12	rH1N1 (TRIG+2)	2012	4 weeks	B	Nakohn Pathom	KJ526067-73, KM355357
A/Swine/Thailand/CU-S3795/13	rH1N1 (TRIG+2)	2013	4 weeks	B	Nakohn Pathom	KJ526034-38, KM355358-60
A/swine/Saraburi/NIAH13021/05[b]	eH1N2	2005	-	-	Saraburi	AB434333-40
A/swine/Thailand/CU-S3073/11	rH1N2 (7+1)	2011	4 weeks	D	Ratchaburi	KJ162042-43, KM355361-66
A/Swine/Thailand/CU-S3631/12	rH1N2 (TRIG+2)	2012	4 weeks	B	Nakohn Pathom	KJ526039-44, KM355367-68
A/swine/Thailand/KU5.1/04[c]	eH3N2	2004	-	-	-	FJ561057-64
A/Swine/Thailand/CU-S3474/12	rH3N2 (TRIG+2)	2012	8 weeks	C	Chonburi	KM355369-76
A/Swine/Thailand/CU-S3673/12	rH3N2 (TRIG+2)	2012	4 weeks	D	Chonburi	KJ526061-66, KM355377-78
A/Swine/Thailand/CU-S3689/13	rH3N2 (TRIG+2)	2013	4 weeks	A	Chonburi	KJ526048-52, KM355379-81
A/Swine/Thailand/CU-S14129/13	rH3N2 (TRIG+2)	2013	4 weeks	D	Ratchaburi	KM355382-89
A/Swine/Thailand/CU-S14252/14	rH3N2 (TRIG+2)	2014	4 weeks	D	Ratchaburi	KM355390-97

* Type of farm abbreviation; A = < 50-sow herd, B = 51-200-sow herd, C = 201-500-sow herd, D = >500-sow herd

[a] Reference Thai eH1N1; A/swine/Chonburi/NIAH9469/04 [5] was included in the analysis

[b] Reference Thai eH1N2; A/swine/Saraburi/NIAH13021/05 [5] was included in the analysis

[c] Reference Thai eH3N2; A/swine/Thailand/KU5.1/04 [6] was included in the analysis

2.4.2 Genetic characteristics of Thai SIV

Out of the 139 SIV isolates, 12 were selected for whole genome sequencing based on epidemiological data such as location, influenza subtype, year of isolation, age of pig and type of swine farm (Table 2.2). The twelve SIVs characterized in this study were of the subtypes H1N1 (n=5), H1N2 (n=2), and H3N2 (n=5).

In general, the H1 gene of SIVs can be phylogenetically grouped into two, major lineages: classical and Eurasian. The classical lineage can be further divided into four sub-lineages: alpha, beta, gamma and delta. Phylogenetic analysis of the H1 gene revealed that six Thai SIVs (CU-S3334, CU-S3350, CU-S3406, CU-S3629, CU-S3795 and CU-S3631) were clustered into the alpha group of the classical lineage while only one (CU-S3073) was grouped into the pandemic cluster, which is a member of the gamma group of the classical lineage (Figure 2.1). Phylogenetic analysis of the N1 gene showed that all five Thai H1N1 SIVs belonged to the Eurasian lineage.

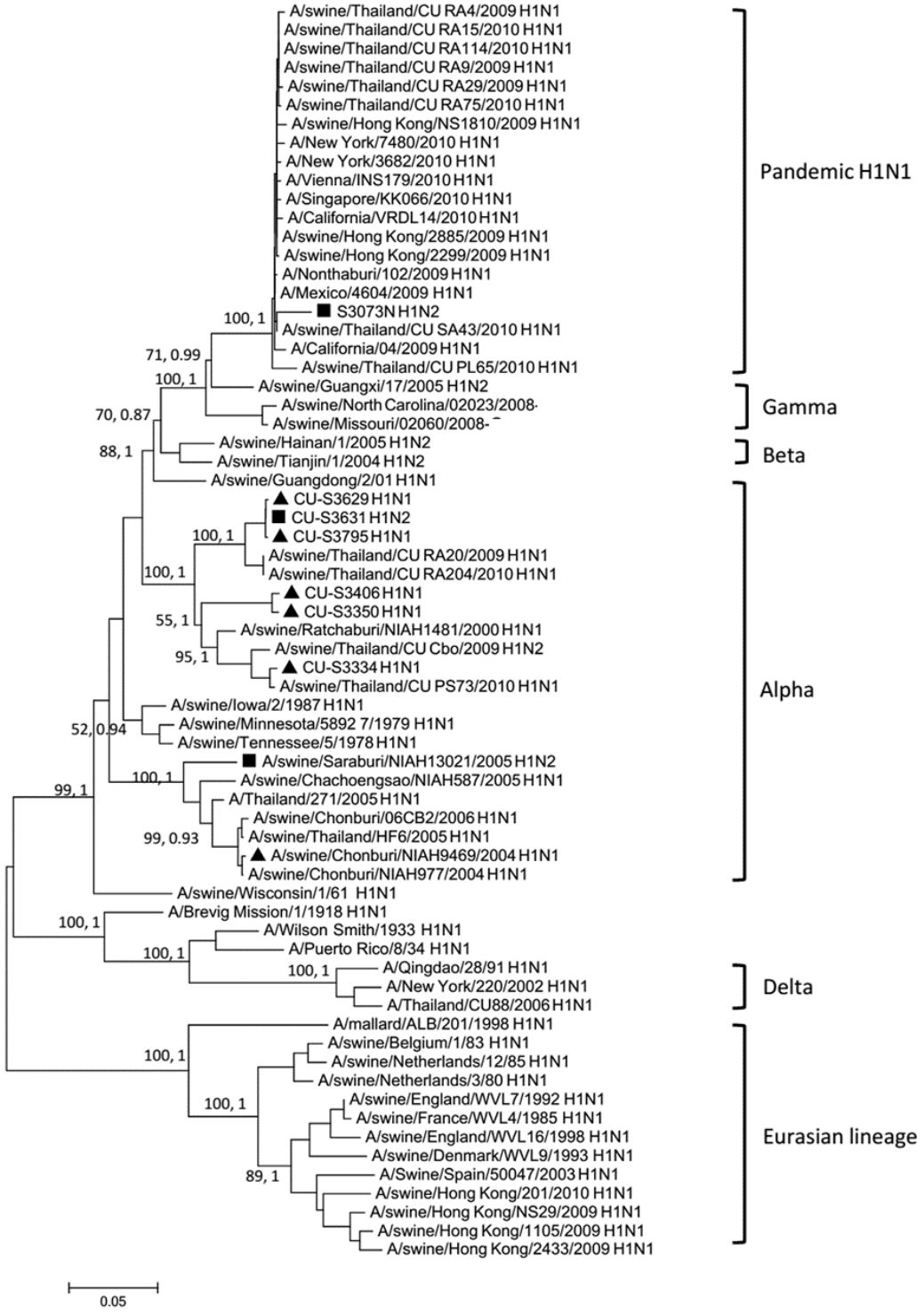

Figure 2.1 Phylogenetic analysis of the H1.

Node label shows the bootstrap percentage and posterior probabilities in parenthesis (bootstrap percentage, posterior probability). Triangle and quadrilateral indicate SIV-H1N1 and SIV-H1N2, respectively.

In general, the H3 gene of SIVs can be grouped into Ha and Hb subgroups which H3 can be evolved from either a human H3N2 strain circulating in late 1990s or human-like H3N2 swine strain circulating in early 1970s (Takemae, et al., 2008). In this study, the H3 genes of five H3N2 SIVs (CU-S3474, CU-S3673, CU-S3689, CU-S14129 and CU-S14252) were clustered into the Ha subgroup of human H3N2 lineage (Fig. 2). Similarly, seven of the N2 genes of H1N2 (CU-S3073 and CU-S3631) and H3N2 SIVs (CU-S3474, CU-S3673, CU-S3689, CU-S14129 and CU-S14252) were clustered into the human H3N2 lineage.

Overall, five, distinct genetic constellations of Thai SIVs were observed in this study: eH1N1 (6+2), rH1N1 (TRIG+2), rH1N2 (7+1), rH1N2 (TRIG+2) and rH3N2 (TRIG+2). Based on previous reports from Thailand, Thai endemic SIV-H1N1 (eH1N1) has only two genetic constellations. The first genetic constellation is eH1N1 (7+1), comprised of the H1 gene from the classical lineage and seven other genes from the Eurasian lineage. The second genetic constellation is eH1N1 (6+2) comprised of the H1 and NS genes of the classical lineage and six other genes from the Eurasian lineage (Kitikoon, et al., 2011). Both eH1N1 (7+1) and eH1N1 (6+2) were circulating among Thai swine populations until 2005. Subsequently, eH1N1 (7+1) disappeared while eH1N1 (6+2) was continuously observed until 2012. In this study, we observed both eH1N1 (6+2) (CU-S3334, CU-S3350 and CU-S3406) and reassortant H1N1 viruses (rH1N1) (CU-S3629 and CU-S3795) (Figure 2.3). The rH1N1 viruses contained the TRIG cassette of pH1N1 as well as the H1 and N1 genes of Thai endemic SIVs (TRIG+2) (Figure 2.3).

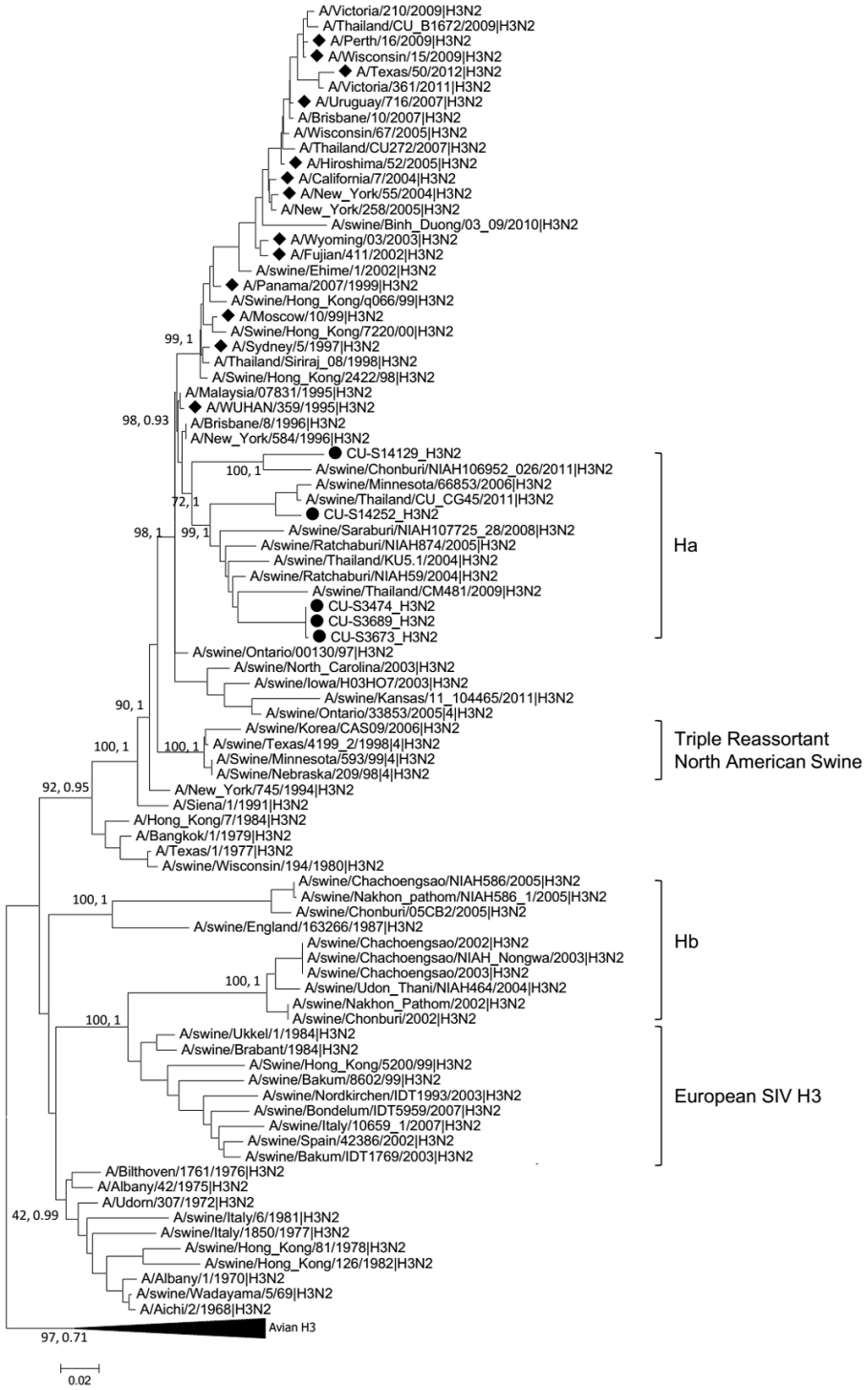

Figure 2.2 Phylogenetic analysis of the H3.

Node label shows the bootstrap percentage and posterior probabilities in parenthesis (bootstrap percentage, posterior probability). Diamond and circle indicate seasonal human vaccine strains H3N2 and SIV-H3N2 in this study, respectively.

The genetic constellation of Thai endemic SIV-H1N2 (eH1N2) in 2005 (NIAH13021) had five genes of the Eurasian lineage (PB2, PB1, PA, NP, and M), two genes of the classical lineage (H1 and NS) and an N2 gene of human origin. In this study, we observed two types of rH1N2 during 2011 and 2012. The first rH1N2 (CU-S3073) contained seven genes from pH1N1 with an N2 gene from eH1N2 and was designated as 7+1. The second rH1N2 (CU-S3631), containing the TRIG cassette and the H1 and N2 genes from eH1N2, was designated as TRIG +2 (Figure 2.3).

Thai endemic SIV-H3N2 (eH3N2) in 2004 and 2005 had two, distinct genetic constellations. The first constellation was comprised of PB2, PB1, PA and M of the Eurasian lineage, NP and NS of the classical lineage and H3 and N2 of human H3N2 origin (KU5.1). The second constellation was comprised of PB2, PB1, PA, M and NS of the Eurasian lineage, NP of the classical lineage, and H3 and N2 of human H3N2 origin (NIAH586-1). In this study, we found that rH3N2 (TRIG+2) was predominant in Thai pigs from 2011 to 2014. The viruses (CU-S3474, CU-S3673, CU-S3689, CU-S14129 and CU-S14252) contained the TRIG cassette with H3 and N2 genes from eH3N2 (Figure 2.4).

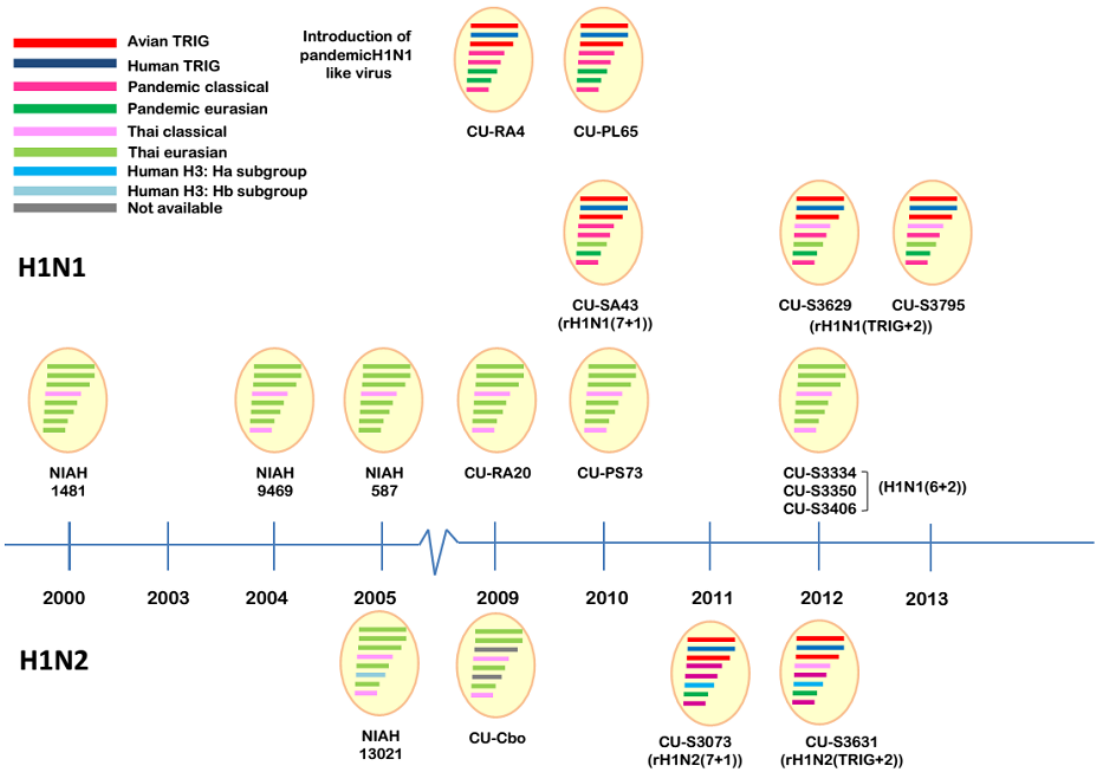

Figure 2.3 Schematic representation of the genetic constellation of Thai SIV-H1N1 and SIV-H1N2 during 2000-2013.
The oval represents viral particle and each line represents each gene segment ascending from segment 1 to segment 8, respectively. The lineages of gene segment presents in different colors.

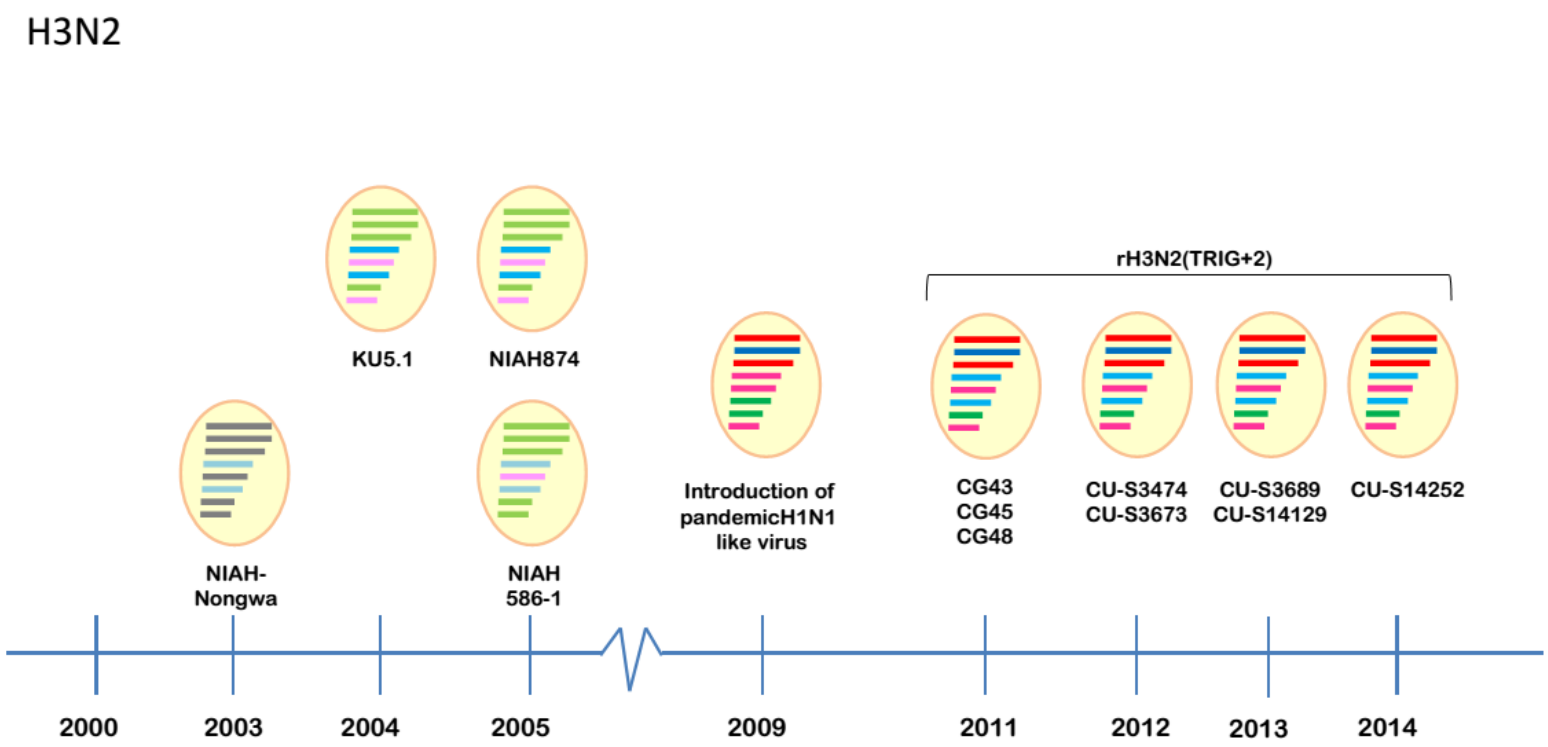

Figure 2.4 Schematic representation of the genetic constellation of SIV-H3N2 during 2003-2014.

The oval represents viral particle and each line in oval represents each gene segment ascending from segment 1 to segment 8. The lineages of gene segment presents in different colors.

Genetic analyses of SIVs characterized in this study are shown in Tables 2.3 and 2.4. The seven SIV-H1 genes characterized in this study were compared with four reference viruses: eH1N1 (NIAH9469), pH1N1 (CA/04 and CU-RA4) and eH1N2 (NIAH13021). Our results showed that the H1 viruses were divided into either pandemic (P) or alpha (α) clusters. H1 SIVs of the alpha cluster exhibit high amino acid diversity at five antigenic sites: Sa, Sb, Ca1, Ca2, Cb. In contrast, only one amino acid change at the Sa antigenic site (G158E) was observed in H1 SIVs of the pandemic cluster. Analysis of the receptor binding site showed that recent (2011-2014) Thai H1 SIVs contained aspartic acid (D) at positions 190 and 225, indicating preferential binding to the SA α2,6 receptor. In contrast, older (2004-2005) Thai H1 SIVs contained glycine (G) at position 225 (Table 2.3).

Five H3 SIVs were compared with two reference viruses: eH3N2 (KU5.1) and the human H3N2 vaccine strain (Wuhan/359). Three (CU-S3474, CU-S3673 and CU-S3689) had no amino acid changes at five antigenic sites although the viruses were isolated from different farms. One H3 SIV (CU-S14129) had amino acid changes at four antigenic sites: A, B, C and E. This observation corresponded well with the phylogenetic analysis result in which CU-S14129 was clustered away from the main group. In analyses of the receptor binding site, all H3 SIVs had isoleucine (I) at position 226 and serine (S) at position 228, which is similar to the reference H3 viruses and indicates Thai H3 SIVs prefer to bind to the SA α2,6 receptor.

Table 2.3 Genetic analysis of the H1 gene of Thai SIVs in this study

Viruses	Subtype	HA cluster	Amino acid sequence alignment of H1 gene													GenBank accession no.
			Antigenic site											Receptor binding site		HA cleavage site	
			Sa			Sb		Ca1			Ca2		Cb				
			128-129*	156-160*	162-167*	187-198*	169-173*	206-208*	238-240*	140-145*	224-225*	78-83*		190*	225*	325-333*	
A/swine/Chonburi/NIAH9469/04	eH1N1	α[a]	PN	KKGNS	PKLRKA	TNTDQQSLYQNA	VNNKK	GSS	EPG	PYAGTN	RG	LFAVNS		D	G	PSIQSRGLF	AB434304
A/California/04/09	pH1N1	P[b]	PN	KKGNS	PKLSKS	TSADQQSIYQNA	INDKG	GSS	EPG	PHAGAK	RD	LSTASS		D	D	PSIQSRGLF	GQ280797
A/swine/Thailand/CU-RA4/09	pH1N1	P	PN	KKGNS	PKLSKS	TSADQQSLYQNA	INDKG	GTS	EPG	PHAGAK	RD	LSTASS		D	D	PSIQSRGLF	CY062308
A/Swine/Thailand/CU-S3334/12	eH1N1	α	PN	KKGNS	PKLSKS	TSTDQQSLYQNA	VNNKK	SSS	EPG	HHAGAK	RD	LFKANS		D	D	PSIQSRGLF	KJ162030
A/Swine/Thailand/CU-S3350/12	eH1N1	α	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS		D	D	PSIQSRGLF	KJ162037
A/Swine/Thailand/CU-S3406/12	eH1N1	α	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS		D	D	PSIQSRGLF	KM355356
A/Swine/Thailand/CU-S3629/12	rH1N1	α	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS		D	D	PSIQSRGLF	KJ526069
A/Swine/Thailand/CU-S3795/13	rH1N1	α	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS		D	D	PSIQSRGLF	KM355360
A/swine/Saraburi/NIAH13021/05	eH1N2	α	PD	KKGNS	PKLSKS	TDTDQQSLYQNV	VNDKK	GSS	EPG	PYAGTN	RG	LFEVNS		D	G	PSIQSRGLF	AB434336
A/swine/Thailand/CU-S3073/11	rH1N2	P	PN	KKENS	PKLSKS	TSADQQSIYQNA	INDKG	GSS	EPG	PHAGAK	RD	LSTASS		D	D	PSIQSRGLF	KJ162042
A/Swine/Thailand/CU-S3631/12	rH1N2	α	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS		D	D	PSIQSRGLF	KM355368

* H3 numbering

[a] Alpha group of classical lineage

[b] Pandemic cluster of classical lineage

Table 2.4 Genetic analysis of the H3 gene of Thai SIVs in this study

Virus	Subtype	Amino acid sequence alignment of H3 gene									Genbank Accession No.
		Antigenic site							Receptor binding site		
		A		B	C	D	E				
		140-146*	156-161*	189-199*	277-282*	205-221*	171-175*	243-249*	226*	228*	
A/Wuhan/359/1995	hH3N2	KRGSVKS	KLEYKY	SDQTSIYVQAS	CNSECI	STKRSQQTVIPNIGSRP	NDKFD	LLINSYG	I	S	JX518888
A/swine/Thailand/KU5.1/2004	eH3N2	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGSRP	NDKFD	LLINSTG	I	S	FJ561060
A/Swine/Thailand/CU-S3474/12	rH3N2	KRGSVKS	KLDYKY	NNQTNLYVQAS	CNYGCI	STKRSQQTVIPNIGSRP	NDKFN	LLINSTG	I	S	KJ526029
A/Swine/Thailand/CU-S3673/12	rH3N2	KRGSVKS	KLDYKY	NNQTNLYVQAS	CNYGCI	STKRSQQTVIPNIGSRP	NDKFN	LLINSTG	I	S	KJ526062
A/Swine/Thailand/CU-S3689/13	rH3N2	KRGSVKS	KLDYKY	NNQTNLYVQAS	CNYGCI	STKRSQQTVIPNIGSRP	NDKFN	LLINSTG	I	S	KJ526048
A/Swine/Thailand/CU-S14129/13	rH3N2	KRGYVNS	QSGHKY	SDQTSLYVQAS	CNSECV	STKRSQQTVIPNIGSRP	NEKFD	LLINSTG	I	S	KM355385
A/Swine/Thailand/CU-S14252/13	rH3N2	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	I	S	KM355393

* H3 numbering

Genetic analysis of the NA gene on Oseltamivir-resistance related to E119V, H275Y, R293K and N295S on N1 and N146K, S219T, A272V and 245-248 deletion on N2 (Arias, et al., 2009). The results showed that all SIVs in this study had an amino acid referred to Oseltamivir susceptibility. Genetic analysis of the N2 showed that all five Thai H3N2 SIVs contained valine (V) at position 275 (Data not shown) that may increase SA α2,6 receptor specificity (Kobasa, et al., 1999). Moreover, analysis of virulence determinants on PB2 (E627K and N701D) and NS1 (E92D) (Neumann, et al., 2009) showed no significant amino acid changes in particular positions.

2.5 Discussions

From 2011 to 2015, our SIV surveillance revealed that reassortant SIVs are a dominant subtype circulating among Thai pigs. Previous studies, however, reported that pH1N1 was a major SIV subtype between 2010 and 2011 (Charoenvisal, et al., 2013; Hiromoto, et al., 2012). In Thailand, the first reassortant SIV between pH1N1 and Thai SIVs was reported in 2010 (CU-SA43) with a genetic constellation of seven genes from pH1N1 and an N1 gene from eH1N1 (Kitikoon, et al., 2011). In this study, we reported that novel rH1N1 (TRIG+2) has become a dominant variant of SIV-H1N1. We identified rH1N2 (7+1) (CU-S3073) in October 2011 and rH1N2 (TRIG+2) (CU-S3631) in December 2012. Similar results have been reported from Argentina and Japan. In Argentina, rH1N1 and rH1N2 containing the TRIG cassette and human-like H1 and N1/N2 were reported between 2009 and 2010 (Pereda, et al., 2011). In Japan, rH1N2 containing seven genes from pH1N1 and an N2 from Japanese SIV was reported in 2012 (Kobayashi, et al., 2013). For SIV-H3N2, rH3N2 (TRIG+2) was a dominant SIV subtype in late 2011 in Thailand (Charoenvisal, et al., 2013). This corresponds with our finding that eH3N2 disappeared from Thai swine populations in 2011 and rH3N2 (TRIG+2) has dominated since. These observations indicate that Thai SIVs, after the introduction of pH1N1, have evolved by maintaining the TRIG cassette and retrieving other genes from endemic SIVs in their virus gene pools. This confirms the hypothesis that the TRIG cassette has a very high potential for viral

infection, replication and transmission within pig populations. The TRIG cassette in the virus particle is very compatible and the virus changes its surface proteins for escaping host immunity (Vincent, et al., 2008). In contrast, rH3N2 was reported in the USA with a different constellation: PB2, PB1, PA, HA, NP, NA and NS relate to TRIG SIV-H3N2 and carry M from pH1N1 (Lina, et al., 2011).

It should be noted that SIV-H1N1 and SIV-H1N2 took approximately 3 years (November 2009-December 2012) for adaptation to the TRIG+2 constellation. During this period, we observed both rH1N1 (7+1) and rH1N2 (7+1) constellations. In contrast, SIV-H3N2 took a shorter time (15 months; November 2009-February 2011) to settle its genetic constellation. This evidence supports the theory that the pig is a mixing vessel for novel viruses which could potentially exhibit high virulence or cause pandemics. Strict biosecurity is therefore an important measure to reduce the chance of new genetic material being introduced into swine populations.

Genetic analyses of all Thai SIV subtypes showed amino acids with preferential binding to the SA α2,6 receptor. All Thai SIV-H3N2 isolates had isoleucine (I226) instead of leucine (L226) in the HA1 region. This unique amino acid residue was observed in human H3N2 from China and Japan in 1994 and 1995, indicating the potential risk for human infection with Thai SIV-H3N2 (Lindstrom, et al., 1996). This observation supports the idea that the Ha subgroup of H3 originated during the late 1990s from human H3N2 to become a dominant cluster. Based on our observation that no significant amino acid mutations occurred, it should be noted that influenza vaccination was incomprehensive practices in Thai swine farms.

In summary, the reassortant SIVs have become predominant among SIVs circulating in Thai pig populations since the introduction of pH1N1 2009. This observation suggests that there was a significant diversity and rapid evolution of Thai SIVs during the past 4 to 5 years. Further swine

influenza surveillance is critical for monitoring the novel reassortant SIVs in Thai swine populations and their potential to spread to humans.

CHAPTER III

Mix-genotypes of swine influenza virus in commercial swine farms

Manuscript in preparation

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In Thailand, co-circulation of multiple swine influenza virus (SIV) subtypes in commercial swine farms has been documented. This situation promoted virus reassortment and evolution. In this study, mix-genotype was investigated by applying next generation sequencing, illumina Miseq platform. Illumina sequencing provides high depth of sequencing coverage which able to present mix-genotype in a sample. In this study, eighteen isolated SIVs from four commercial swine farms were subjected to illumina sequencing to monitor the mix-genotype and novel strain of SIVs. Moreover, genetic traits responsible for increased host range and virulence in mammal were investigated. In conclusion, the results from this study could provide new insight information of SIVs evolution in Thailand by applying illumina sequencing.

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3.1 Abstract

Swine influenza is a highly contagious and an important zoonotic disease. Co-circulation of multiple strains of swine influenza viruses (SIV) in pigs can promote reassortant SIVs and probably novel SIV strains. In this study, we monitored mixed-infection of multiple SIVs subtypes in pigs by applying illumina sequencing to discriminate SIV genotypes. One hundred and forty-five nasal swab samples were collected from four commercial swine farms. In total, eighteen SIVs were isolated and subjected to illumina sequencing for whole genome sequences. Our results showed that two nasal swab samples contained mixed-SIVs of HA1, HA3, NA1 and NA2. Moreover, one nasal swab sample contained SIVs of HA3, NA1 and NA2. Our results revealed that illumina sequencing could be used to discriminate mixed-SIV genotypes. In this study, novel reassortant SIV, H3N1 was identified in Thai pigs. Moreover, E627K mutation was first observed in Thai SIVs. Thus, evidences of reassortant H3N1 and E627K indicated SIVs evolution and required routine influenza surveillance in pigs.

Keywords: Illumina sequencing, Mix-genotype, Swine Influenza Virus, Thailand

3.2 Introduction

Swine influenza is an important zoonotic disease caused by influenza A virus (IAV). Influenza A virus is an enveloped RNA virus, which belongs to the family *Orthomyxoviridae.* The virus contains eight gene segments which are PB2, PB1, PA, HA, NP, NA, M and NS. Viral evolution mechanism, antigenic drift, mutation could occur due to lack of proofreading function of RNA polymerase during viral RNA replication. Another evolution mechanism, antigenic shift, the host cell is infected by two or more different viral strains and their genome exchanging during viral replication. Pig is a potential host to generate the new IAV because it susceptible to both avian and human viruses.

Swine influenza virus (SIV) was first reported in 1931 in the United States and its common ancestor was traced back to pandemic H1N1 in 1918 (Shope, 1931; Taubenberger and Morens, 2006). This SIV lineage was designated as classical swine lineage or North American swine lineage by geographic. Classical H1N1 became a predominant SIV in the U.S. until 1998 when H3N2 was generated via reassortment (Karasin, et al., 2000b). This H3N2 contains human gene segments (PB1, HA and NA), avian gene segments (PB2 and PA) and classical swine gene segments (NP, M and NS) (Olsen, 2002). Around 1950, classical H1N1 had been spread worldwide including Europe but approximately 15 years later it was replaced by H1N1 viruses of avian origin that entered to European pig population in 1976 (Brown, 2000; Pensaert, et al., 1981). This gene pool was designated as avian-like H1N1 lineage or Eurasian swine lineage by geographic. The avian-like H1N1 was successfully adapted to cross species transmission and maintained in pig populations. Subsequently, H1N2 was emerged by reassortment between avian-like H1N1 and human-like H3N2 in 1987 (Gourreau, et al., 1994). Beside major three subtypes of H1N1, H1N2 and H3N2 those circulate in pig population worldwide, there are H9N2, H5N6, H4N6 and H3N1 has been reported

periodically (Abe, et al., 2015; Cong, et al., 2007; Karasin, et al., 2000c; Lekcharoensuk, et al., 2006; Li, et al., 2015; Peiris, et al., 2001; Shin, et al., 2006; Tsai and Pan, 2003)

In Thailand, all three subtypes of SIV-H3N2, H1N1 and H1N1 has been reported since 1978, 1988 and 2005, respectively (Kupradinun, et al., 1991; Nerome, et al., 1981; Takemae, et al., 2008). According to genetically study of Thai SIV, the constellation of Thai SIV-H1N1 comprised of classical (HA and NS) and Eurasian swine lineage (PB2, PB1, PA, NP, NA and M) (Kitikoon, et al., 2011). The genetic constellation of Thai SIV-H3N2 was characterized and revealed that HA3 and NA2 were originated from seasonal human H3N2 and especially related to A/Wuhan/359/1995 strain (Lekcharoensuk, et al., 2010). Thai H1N2 constellation is a reassortant between Thai H1N1 and H3N2 so it contains human gene segment (NA), classical swine gene segments (HA and NS) and Eurasian swine gene segments (PB2, PB1, PA, NP and M) (Nonthabenjawan, et al., 2015). After the introduction of triple reassortment pandemic 2009 H1N1 (p09 H1N1), reassortant Thai SIV of all three subtypes, H1N1, H1N2 and H3N2, have been reported and become predominant strains in Thai pig populations (Charoenvisal, et al., 2013; Hiromoto, et al., 2012; Sreta, et al., 2009).

In this study, we applied illumina sequencing to discriminate the mixed-genotypes and to monitor novel viral genetic constellation of SIVs isolated from commercial swine farms in Thailand.

3.3 Materials and Methods

3.3.1 Swine farms selection and sample collection

In this study, four commercial swine farms were selected based on their history of multiple SIV subtypes co-circulation in a farm. Sample collection was conducted during January 2012 – December 2013.

One hundred forty five nasal swab samples were collected from pigs of different ages including gilts, sows, suckling piglets and weaning piglets. In this study, sample collection was targeted on clinical pigs with flu-like symptoms such as cough, abdominal breath and present of

nasal discharge. Then the swab was placed into a viral transport media tube (Minimum Essential Medium: MEM). Nasal swab sample was embedded in the ice and then transported to the laboratory within 24 hours.

3.3.2 Swine influenza virus detection and Isolation

All nasal swab samples were extracted RNA by using QIAmp RNA mini kit (Qiagen; Hilden, Germany) according to manufacturer's instruction. Influenza A virus detection was conducted by using real time RT-PCR on a Rotor-Gene 3000 (Corbett Research; Sydney, Australia) utilizing the SuperScript™III Platinum® One-Step Quantitative RT-PCR System (Invitrogen™; California, USA) with 0.4 μM final primer and probe concentration (Spackman, et al., 2002).

The positive samples from rRT-PCR were continued to virus isolation by egg inoculation and MDCK cell culture. For egg inoculations, we inoculated embryonated chicken eggs according to WHO recommendations (WHO, 2002). After a 72-hour incubation period, the allantoic fluid of each egg was collected and tested for hemagglutinin activity with a hemagglutination test (HA test) using a 0.5% suspension of turkey red blood cells. For cell culture, Madin Darby Canine Kidney (MDCK) cells were used for viral propagation. During the incubation period of 72 hours, we made daily observations for cytopathic effect (CPE) and, after incubation, collected the supernatants of CPE positives. Samples that tested positive by HA test at 4 HA units/50 μl or more and CPE positive cell supernatants were subsequently subjected to IAV confirmation by rRT-PCR for M gene detection as previously described.

3.3.3 Whole genome sequencing

Viral RNA of passage one allantoic fluid and cell culture supernatant (n=18) were extracted by using QIAmp RNA mini kit (Hilden, Germany) followed manufacturer's instruction. RNA was used as a template to amplify eight segments of virus by one-step RT-PCR as described (Zhou, et al., 2009). Briefly, SuperScript® III RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen™;

CA, USA) was used with 1.6 mM and 0.2 μM final concentration of magnesium and primers, respectively. PCR products were imaged by electrophoresis in 1.5% agarose gel and then purified by QIAquick PCR Purification kit (Hilden, Germany). Purified PCR products were submitted to University of Minnesota Genomics Center (UMGC) for illumina paired-end 250 cycles sequencing by using Nextera XT DNA kit (California, USA) to generate multiplexed paired-end sequencing libraries. The adapter contaminations and low quality base at both extremities were removed from the raw reads. Reads that passed quality control were assembled by de-novo assembly method. Base on de-novo assembly results the influenza reference strains will be selected then used for mapping all reads to references. Then whole genome sequences were extracted via CLC genomics workbench module available on Minnesota Supercomputing Institute (MSI) resources at the University of Minnesota.

3.3.4 Phylogenetic analysis

The reference nucleotide sequences were obtained from Influenza Research Database (http://www.fludb.org/). Reference sequences were selected to represent pandemic, Eurasian and classical swine lineage especially Thai IAV-S isolates. Reference and sample sequences from the current study were aligned using Muscle v.3.6 (Edgar, 2004) subsequently any extra sequences beyond and after start and stop codon were trimmed. Maximum clade credibility (MCC) tree of each gene segments were generated by BEAST 1.8 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. Strict clock model with coalescent constant population and HKY with gamma 4 substitution was used as model parameters (Drummond, et al., 2002; Drummond, et al., 2012) and Bayesian MCMC chain lengths were ten million generations with sampling every 1,000 generations and the effective sample size (ESS) value was assessed by using Tracer (v1.6.0) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) (Rambaut, et al., 2014). Every gene segment analysis had ESS value greater than 200 to suggesting minimal standard

error. The resulting tree of each iteration was summarized for a representative clustering pattern by using a tree annotator with 10% discarding of the chains as burn-in and the maximum clade credibility tree was visualized with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).

3.4 Results

In this study, eighteen swine influenza viruses out of one hundred and forty-five nasal swab samples were successfully identified. Subtyping of SIVs by RT-PCR showed that samples from farm A (n=1) and D (n=1) could be subtyped as HA1, HA3, NA1 and NA2 and sample of farm C could be subtyped as HA3, NA1 and NA2. Other fifteen samples were subtyped as H1N1 (n=13; farm A=6, farm B=2, farm C=1 and farm D=4) and H1N2 (n=2 from farm C) (Table 3.1). Thus we interested to further investigate the samples with mixed SIV genotypes by illumina sequencing.

Table 3.1 Number of nasal swab samples and subtyping results by RT-PCR

Farm	# nasal swab	# recovery virus	Sample No.	Subtyping results*			
				HA1	HA3	NA1	NA2
A	60	7	A-S1	+	-	+	-
			A-S2	+	+	+	+
			A-S3	+	-	+	-
			A-S4	+	-	+	-
			A-S5	+	-	+	-
			A-S6	+	-	+	-
			A-S7	+	-	+	-
B	30	4	B-S8	+	-	+	-
			B-S9	+	-	-	+
			B-S10	+	-	-	+
			B-S11	+	-	+	-
C	30	2	C-S12	-	+	+	+
			C-S13	+	-	+	-
D	25	5	D-S14	-	+	-	+
			D-S15	+	+	+	+
			D-S16	+	-	+	-
			D-S17	+	-	+	-
			D-S18	+	-	+	-
Total	145	18	-	16	3	15	6

Note: * Subtyping results from RT-PCR with specific primers for each subtype results

3.4.1 Map reads to reference and whole genome sequences extraction

Eighteen samples were mapped to a set of references (n=18 segments) comprising pandemic H1N1 2009 (8 gene segments), endemic Thai SIV-H1N1 (8 gene segments), HA3 and NA2 of endemic Thai SIV-H3N2. The results of map reads to reference were shown in Table 3.2. For example, sample A-S1 had 2,026,201 reads mapped to all references. Number of reads map to each gene segments demonstrated that majority of sequence reads mapped to endemic SIV-H1N1 (eH1N1) however, less than 1.00% of sequence reads were mapped to pandemic genotype references in NP, M and NS. Non-specific mapped could happened due to matching of short reads so this number of mapped reads was not significant. In conclusion, A-S1 presented a single genotype in a sample. While sample A-S2 had 3,229,281 reads mapped to all references. Sequence reads of A-S2 mapped against all segment of references in difference number of reads for example in total 43,436 reads mapped to PB2 but 15,347 (15,347/43,436; 35.33%) reads mapped to pandemic genotype and 28,089 (28,089/43,436; 64.67%) mapped to endemic genotype. The majority of reads mapped to endemic genotypes so it was named as a background genotype (BG) while pandemic genotype was called mix genotype (MG). The rested samples of farm A (A-S3 – A-S7) showed SG of eH1N1. Moreover, mix genotype was observed in sample C-S12 and D-S15. C-S12 had NA1 gene as a mix genotype which 6.44% of sequence reads mapped against NA1 segment while 93.56% of reads mapped to NA2 segment. D-S15 had HA3 and NA2 gene as a mix genotype with 9.92% and 6.42% of sequence reads mapped against HA3 and NA2 segment, respectively. While 90.08% and 93.58% of reads mapped to HA1 and NA1 segment, respectively (Table 3.2).

Table 3.2 Numbers of sequence reads mapped to references

Sample	All	No. reads mapped to references															
		PB2		PB1		PA		HA		NP		NA		M		NS	
	references	pdm*	en**	pdm	en	pdm	en	HA1	HA3	pdm	en	NA1	NA2	pdm	en	pdm	en
A-S1	2,026,201	0	29,067	0	20,590	0	34,342	175,910	0	412 (0.11%)	389,874 (99.89%)	236,232	0	2,635 (0.38%)	689,879 (99.62%)	443 (0.10%)	446,817 (99.90%)
A-S2	2,608,281	**15,347 (35.33%)**	**28,089 (64.67%)**	**16,642 (39.17%)**	**25,844 (60.83%)**	**11,247 (38.05%)**	**18,309 (61.95%)**	**147,270 (62.10%)**	**89,872 (37.90%)**	**108,404 (28.54%)**	**271,415 (71.46%)**	**69,553 (65.35%)**	**36,884 (34.65%)**	**320,345 (30.52%)**	**729,193 (69.48%)**	**290,425 (40.28%)**	**430,442 (59.71%)**
A-S3	2,272,860	60 (0.05%)	121,505 (99.95%)	34 (0.17%)	19,679 (99.83%)	188 (0.35%)	53,342 (99.65%)	81,344	0	91 (0.04%)	238,238 (99.96%)	76,028	0	6,973 (0.46%)	1,516,947 (99.54%)	287 (0.18%)	158,144 (99.82%)
A-S4	2,215,303	31 (0.26%)	11,807 (99.74%)	32 (0.21%)	15,017 (99.79%)	89 (0.62%)	14,243 (99.38%)	350,979	0	475 (0.10%)	463,633 (99.90%)	347,301	0	2,376 (0.44%)	538,268 (99.56%)	584 (0.12%)	470,468 (99.88%)
A-S5	2,195,002	68 (0.39%)	17,558 (99.61%)	28 (0.22%)	12,481 (99.78%)	118 (0.36%)	32,396 (99.64%)	387,625	0	373 (0.16%)	238,894 (99.84%)	293,911	0	2,578 (0.49%)	525,956 (99.51%)	636 (0.09%)	682,380 (99.91%)
A-S6	2,187,900	39 (0.33%)	11,908 (99.67%)	39 (0.31%)	12,504 (99.69%)	80 (0.69%)	11,492 (99.31%)	304,854	0	405 (0.09%)	429,633 (99.91%)	312,526	0	2,226 (0.37%)	603,274 (99.63%)	475 (0.10%)	498,445 (99.90%)
A-S7	1,864,272	9 (0.09%)	10,070 (99.91%)	11 (0.27%)	4,132 (99.73%)	30 (0.41%)	7,253 (99.59%)	98,005	0	104 (0.08%)	123,483 (99.92%)	66,567	0	4,402 (0.33%)	1,313,972 (99.67%)	202 (0.09%)	236,032 (99.91%)
B-S8	1,773,593	6,980 (99.97%)	2 (0.03%)	2,764 (99.93%)	2 (0.07%)	17,561 (99.97%)	5 (0.03%)	53,157	0	346,710 (99.99%)	20 (0.01%)	305,316	0	711,172 (99.92%)	564 (0.08%)	329,300 (99.99%)	40 (0.01%)
B-S9	1,984,312	16,246 (99.96%)	7 (0.04%)	6,414 (99.94%)	4 (0.06%)	28,775 (99.98%)	5 (0.02%)	32,741	0	384,516 (99.99%)	47 (0.01%)	0	74,425	1,204,166 (99.77%)	2,828 (0.23%)	233,840 (99.98%)	43 (0.02%)
B-S10	1,484,595	7,317 (99.89%)	8 (0.11%)	5,202 (99.94%)	3 (0.06%)	25,258 (99.96%)	10 (0.04%)	151,962	0	391,317 (99.98%)	84 (0.02%)	0	68,127	667,295 (99.71%)	1,964 (0.29%)	165,850 (99.97%)	52 (0.03%)
B-S11	1,799,466	4,122	0	5,454 (99.98%)	1 (0.02%)	8,930 (99.93%)	6 (0.07%)	77,103	0	265,049 (99.99%)	29 (0.01%)	258,368	0	710,540 (99.92%)	589 (0.08%)	469,218 (99.99%)	57 (0.01%)
C-S12	1,868,106	162,488 (99.99%)	9 (0.01%)	67,309 (99.99%)	8 (0.01%)	180,563 (99.99%)	28 (0.02%)	0	153,059	348,947 (99.99%)	41 (0.01%)	**8,357 6.44%**	**121,428 93.56%**	512,900 (99.86%)	730 (0.14%)	312,169 (99.98%)	70 (0.02%)
C-S13	1,441,651	101,956 (99.99%)	7 (0.01%)	96,954 (99.99%)	6 (0.01%)	108,000 (99.99%)	9 (0.01%)	116,089	0	212,738 (99.99%)	23 (0.01%)	249,981	0	310,059 (99.90%)	323 (0.10%)	245,449 (99.98%)	57 (0.02%)
D-S14	2,109,786	96,051 (99.94%)	55 (0.06%)	24,441 (99.92%)	19 (0.08%)	361,369 (100.00%)	17 (0.00%)	0	60,971	415,882 (99.95%)	211 (0.05%)	0	36,193	980,641 (99.84%)	1,595 (0.16%)	132,104 (99.82%)	237 (0.18%)
D-S15	1,769,274	100,977 (99.97%)	33 (0.03%)	93,411 (99.99%)	13 (0.01%)	131,756 (99.96%)	52 (0.04%)	**87,818 90.08%**	**9,668 9.92%**	296,481 (99.93%)	193 (0.07%)	**276,094 93.58%**	**18,930 6.42%**	429,474 (99.85%)	643 (0.15%)	323,523 (99.94%)	208 (0.06%)
D-S16	1,689,792	108,294 (100.00%)	4 (0.00%)	114,547 (100.00%)	3 (0.00%)	120,032 (99.99%)	8 (0.01%)	198,232	0	239,847 (99.99%)	32 (0.01%)	275,985	0	330,429 (99.87%)	417 (0.13%)	301,914 (99.98%)	48 (0.02%)
D-S17	2,131,174	78,944 (99.99%)	7 (0.01%)	24,778 (99.99%)	3 (0.01%)	445,889 (100.00%)	18 (0.00%)	13,919	0	472,451 (99.99%)	36 (0.01%)	106,211	0	814,383 (99.88%)	944 (0.12%)	173,530 (99.96%)	61 (0.04%)
D-S18	1,861,981	78,634 (99.98%)	13 (0.02%)	49,364 (99.99%)	6 (0.01%)	355,730 (99.99%)	27 (0.01%)	58,244	0	148,938 (99.97%)	45 (0.03%)	111,657	0	842,297 (99.82%)	1,496 (0.18%)	215,459 (99.97%)	71 (0.03%)

Note: * pdm stands for pandemic genotype reference, ** en stands for endemic genotype reference

3.4.2 Phylogenetic and Genetic analysis of Thai SIVs

Phylogenetic analysis of HA1 gene showed that Thai H1-SIVs (n=16) were clustered into pandemic (n=1) and classical swine lineage (n=15). Moreover within classical swine lineage, SIVs formed 2 subcluster; Alpha-a (H1-SIVs of farm A) and Alpha-b (H1-SIVs from farm B, C and D) (Figure 3.1). Percentage of nucleotide similarities analyzed by Megalign program (DNAstar Inc., Madison, WI, USA) showed 99.85-99.90% similarities within Alpha-a subcluster and 98.93-99.91% within Alpha-b, while percentage of nucleotide similarities between clusters is 91.07-91.73% (Figure 3.1). Thai H3-SIVs (n=4) were grouped into seasonal human lineage and related to A/Wuhan/359/1995 ancestral (Figure 3.2).

Phylogenetic analysis of NA1 gene of Thai N1-SIVs (n=15) showed that all N1 were grouped into Eurasian swine lineage. However, N1 genes from 4 farms were grouped into 2 subclusters (A and B). Subcluster A composed of NA1 gene from farm A with high similarity (99.70 - 100.00%). Subcluster B combined with N1 gene from farm B, C and D with 99.10% - 100.00% nucleotide similarity. The nucleotide similarity between subcluster A and B was 91.80% -92.33% (Figure 3.3).

Phylogenetic analysis of NA2 gene of Thai N2-SIVs (n=6) were grouped into seasonal human linage with three distinct sub-linages including WH-a, WH-b and SN-c. It is noted that both WH-a and WH-b SIVs were originated from A/Wuhan/359/1995 (A/WH/95; H3N2) similar to previous reports in Thai SIV (Lekcharoensuk, et al., 2010). WH-a comprised of Thai SIVs strain B-S9, B-S10 and early Thai SIVs since 2004, while WH-b included Thai SIVs strain C-S12_BG, D-S14, D-S15_MS and recent Thai SIVs 2011. From our observation, it is the first to report of Thai SIVs clustered into SN-c sublineage. The SN-c sublineage included a seasonal human vaccine strain A/Sydney/5/1997 (A/SN/97; H3N2) (Figure 3.4).

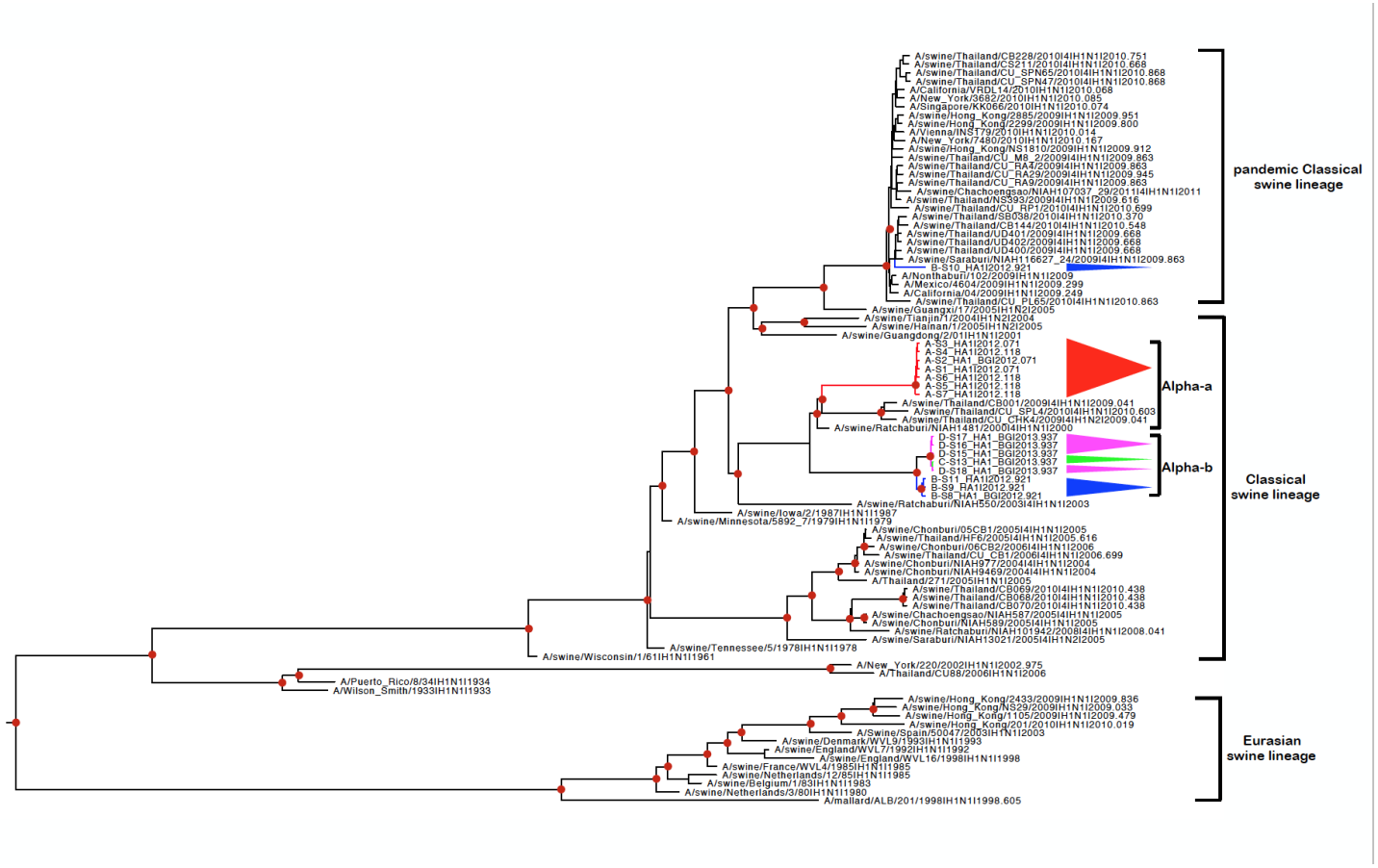

Figure 3.1 HA1 phylogenetic tree.

Red, blue, green and pink triangle signs after taxa name represent SIV-H1 from farm A, B, C and D, respectively. Red dot at each node represents posterior probability higher than 0.7.

Figure 3.2 HA3 phylogenetic tree.

Red, green and pink triangle signs after taxa name represent SIV-H1 from farm A, C and D, respectively. Red dot at each node represents posterior probability higher than 0.7.

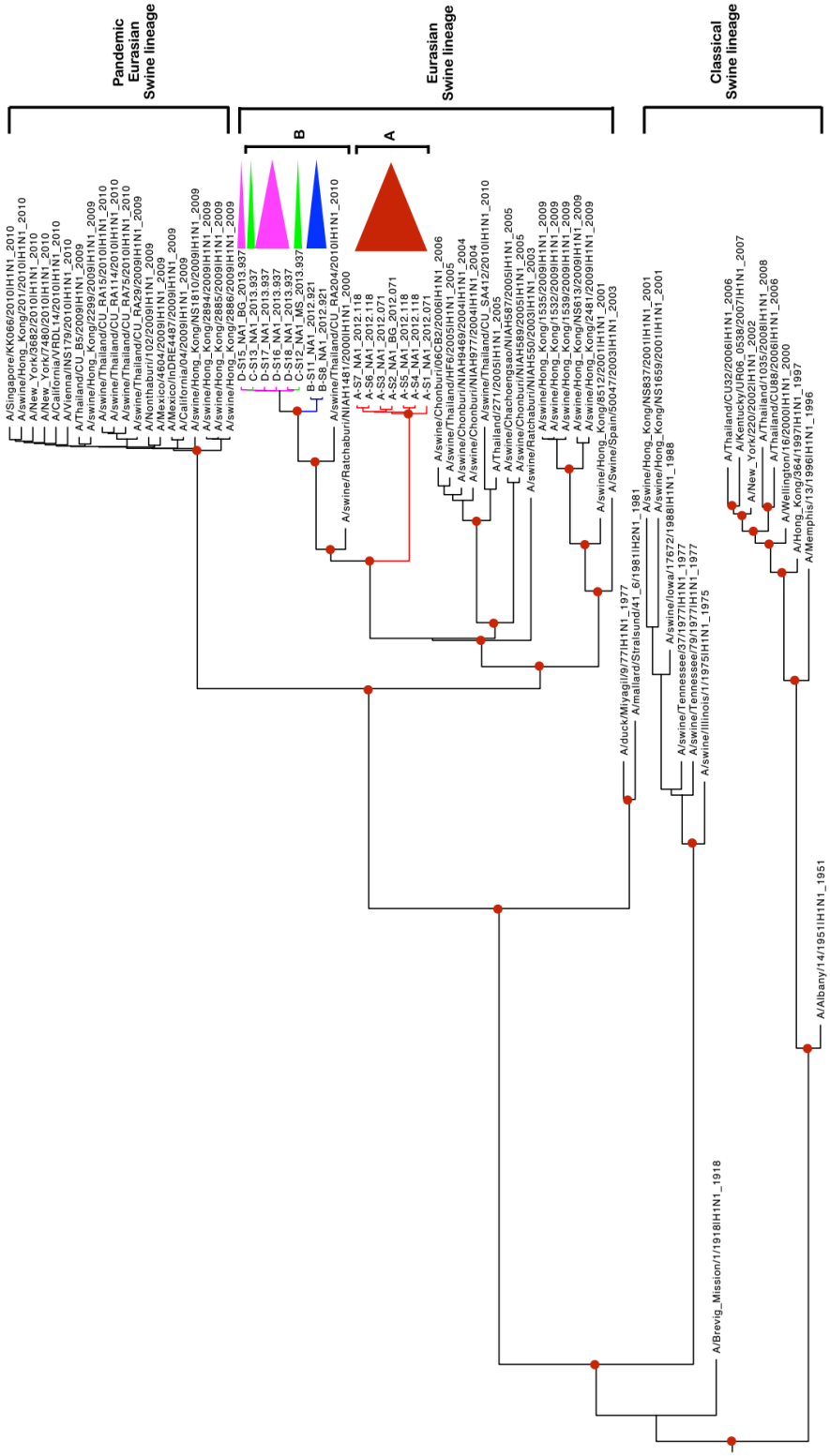

Figure 3.3 NA1 phylogenetic tree.

Red, blue, green and pink triangle signs after taxa name represent IAV-S from farm A, B, C and D, respectively. Red dot at each node represents posterior probability higher than 0.7.

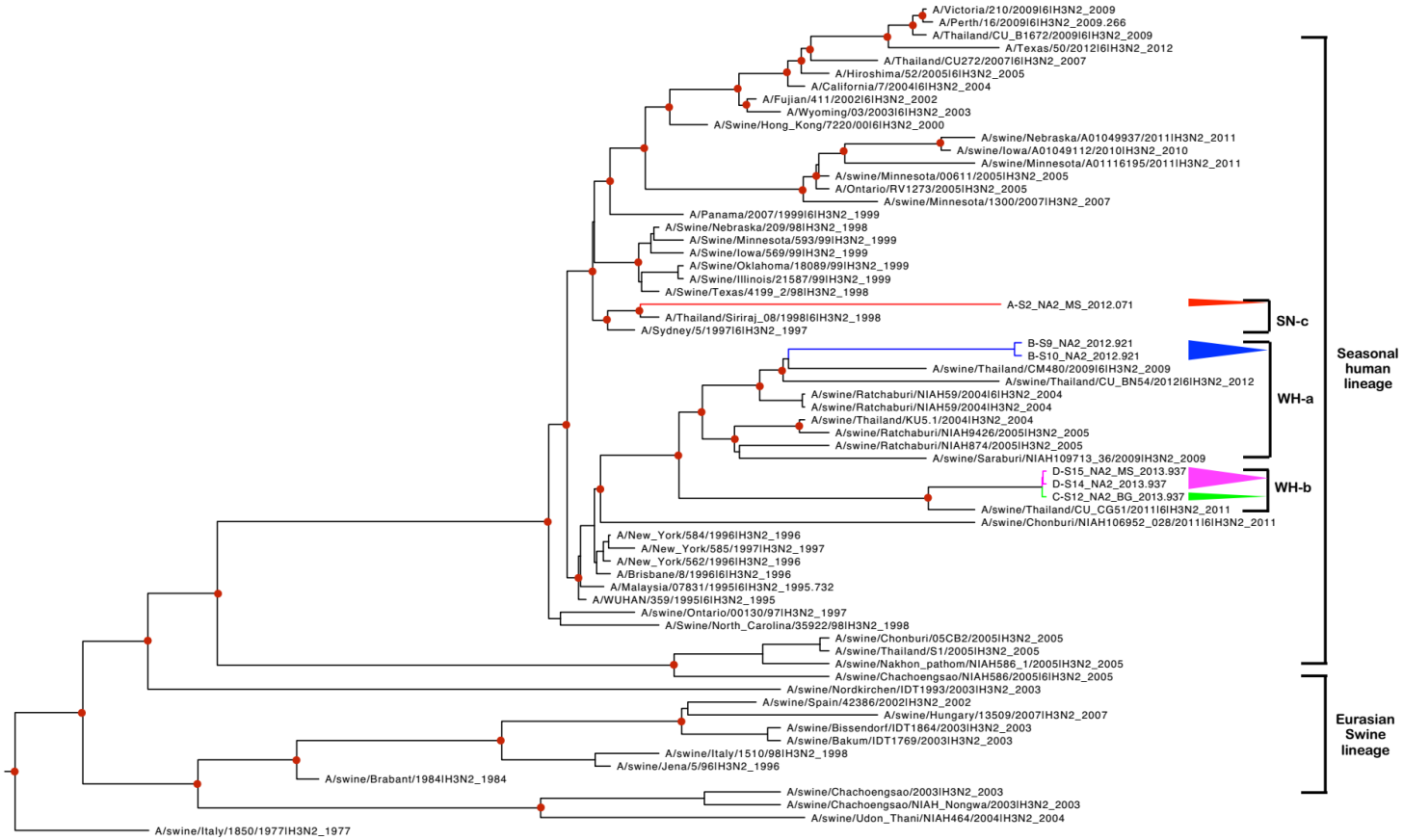

Figure 3.4 NA2 phylogenetic tree.

Red, blue, green and pink branch represent IAV-S from farm A, B, C and D, respectively. Red dot at each node represents posterior probability higher than 0.7.

Comparison of five antigenic sites (Sa, Sb, Ca1, Ca2 and Cb) of HA1 Thai SIVs in this study demonstrated that 10 out of 50 positions of antigenic site contained unique amino acid between subclusters, alpha-A and alpha-B (Table 3.3). While only 3 out of 58 positions of antigenic site on HA protein showed diversity between HA3 Thai SIVs in this study (Table 3.4). Genetic analysis of Thai SIVs showed that receptor binding site of Thai H1-SIVs pose D190 and D225, and Thai H3-SIVs are I226 and S228 suggesting preferential binding to human receptor (Table 3.3 and 3.4). Internal genes of Thai SIVs were also investigated at the amino acids for virulence determinants. For example, eighteen internal gene of Thai SIVs in this study contain E627 in PB2, while one Thai SIV (B-S10) pose E627K substitution. This observation is the first to report of Thai SIVs containing 627K (B-S10) suggesting increase viral replication and virulence in mammal cells (Neumann, et al., 2009). For PB2 at position 701, seven Thai SIVs from farm A contain N701 while one mix genotype (A-S2_MG) and the rest SIVs from other farms pose 701D indicating more virulence of the viruses. For NS at position 92, all nineteen NS gene pose 92D showed significant amino acid substitution related to increase viral virulence (Table 3.5) (Schrauwen and Fouchier, 2014; Taubenberger and Kash, 2010). Genetic analysis of antiviral resistance determinant showed that all Thai SIVs pose amantadine resistant amino acids (M2; S31N) (Table 3.5) but not Oseltamivir resistant (NA1; E119V, H275Y, R293K, N295S and NA2; N146K, S219T, A272V and 245–248 deletion) (Table 3.6) (Arias, et al., 2009).

Table 3.3 Genetic analysis of the H1 gene of Thai SIVs in this study

Viruses	Subtype	HA cluster	Amino acid sequence alignment of H1 gene													GenBank accession no.
			Antigenic site										Receptor binding site		HA cleavage site	
			Sa		Sb		Ca1			Ca2		Cb				
			128-129*	156-160*	162-167*	187-198*	169-173*	206-208*	238-240*	140-145*	224-225*	78-83*	190*	225*	325-333*	
A/sw/Chonburi/NIAH9469/04	eH1N1	α	PN	KKGNS	PKLRKA	TNTDQQSLYQNA	VNNKK	GSS	EPG	PYAGTN	RG	LFAVNS	D	G	PSIQSRGLF	AB434304
A/California/04/09	pH1N1	P[a]	PN	KKGNS	PKLSKS	TSADQQSIYQNA	INDKG	GSS	EPG	PHAGAK	RD	LSTASS	D	D	PSIQSRGLF	GQ280797
A-S1	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
A-S2_BG	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
A-S3	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
A-S4	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
A-S5	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
A-S6	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
A-S7	eH1N1	α-a	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	
B-S8	rH1N1	α-b	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS	D	D	PSIQSRGLF	
B-S9	rH1N2	α-b	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS	D	D	PSIQSRGLF	
B-S10	rH1N2	P	PN	KKGNS	PKLSKS	TSADQQSLYQNA	INDKG	GSS	EPG	PHAGAK	RD	LSSASS	D	D	PSIQSRGLF	
B-S11	rH1N1	α-b	PN	KKENS	PKISKS	TSADQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFRANS	D	D	PSIQSRGLF	
C-S13	rH1N1	α-b	PN	KKGNS	PKLSKS	TSADQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFRANS	D	D	PSIQSRGLF	
D-S15_BG	rH1N1	α-b	PN	KKGNS	PKLSKS	TSADQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFRANS	D	D	PSIQSRGLF	
D-S16	rH1N1	α-b	PN	KKGNS	PKLSKS	TSADQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFRANS	D	D	PSIQSRGLF	
D-S17	rH1N1	α-b	PN	KKGNS	PKLSKS	TSADQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFRANS	D	D	PSIQSRGLF	
D-S18	rH1N1	α-b	PN	KKGNS	PKLSKS	TSADQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFRANS	D	D	PSIQSRGLF	

Note: * H3 numbering, [a] pandemic lineage

Table 3.4 Genetic analysis of the H3 gene of Thai swine influenza viruses in this study

Virus	Subtype	Amino acid sequence alignment of H3 gene									Genbank Accession No.
		Antigenic site							Receptor binding site		
		A		B	C	D	E				
		140-146*	156-161*	189-199*	277-282*	205-221*	171-175*	243-249*	226*	228*	
A/Wuhan/359/1995**	hH3N2	KRGSVKS	KLEYKY	SDQTSIYVQAS	CNSECI	STKRSQQTVIPNIGSRP	NDKFD	LLINSYG	I	S	JX518888
A/swine/Thailand/KU5.1/2004	eH3N2	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGSRP	NDKFD	LLINSTG	I	S	FJ561060
A-S2_MG	rH3N2	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSYG	I	S	
C-S12_BG	rH3N2	KRGSVKS	KSEYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	I	S	
D-S14	rH3N2	KRGSVKS	KSEYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSYG	I	S	
D-S15_MG	rH3N2	KRGSVKS	KSEYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	I	S	

Note: * H3 numbering, ** Human seasonal Vaccine strain

Table 3.5 Genetic analysis of signature mutation on PB2, M and NS gene

Sample	PB2		NS	M
	E627K	N701D	E92D	S31N
A-S1	E	N	D	N
A-S2_BG	E	N	D	N
A-S2_MG	E	D	D	N
A-S3	E	N	D	N
A-S4	E	N	D	N
A-S5	E	N	D	N
A-S6	E	N	D	N
A-S7	E	N	D	N
B-S8	E	D	D	N
B-S9	E	D	D	N
B-S10	K	D	D	N
B-S11	E	D	D	N
C-S12	E	D	D	N
C-S13	E	D	D	N
D-S14	E	D	D	N
D-S15	E	D	D	N
D-S16	E	D	D	N
D-S17	E	D	D	N
D-S18	E	D	D	N

Table 3.6 Genetic analysis of oseltamivir resistance related position on NA gene

Sample	NA subtype	NA1				NA2			
		E119V	H275Y	R293K	N295S	N146K	S219T	A272V	245–248 deletion
A-S1	NA1	E	H	R	N				
A-S2_BG	NA1	E	H	R	N				
A-S2_MG	NA2					N	S	A	No
A-S3	NA1	E	H	R	N				
A-S4	NA1	E	H	R	N				
A-S5	NA1	E	H	R	N				
A-S6	NA1	E	H	R	N				
A-S7	NA1	E	H	R	N				
B-S8	NA1	E	H	R	N				
B-S9	NA2					N	S	A	No
B-S10	NA2					N	S	A	No
B-S11	NA1	E	H	R	N				
C-S12_BG	NA2					N	S	A	No
C-S13	NA1	E	H	R	N				
D-S14	NA2					N	S	A	No
D-S15_BG	NA1	E	H	R	N				
D-S15_MG	NA2					N	S	A	No
D-S16	NA1	E	H	R	N				
D-S17	NA1	E	H	R	N				
D-S18	NA1	E	H	R	N				

Note: Grey box represents unavailable data

3.4.3 Mix-genotypes of SIVS in Thai swine farms

Genetic constellations of Thai SIVs in this study are shown in Figure 3.5. Mixed-infection of SIVs was observed in in farm A, C and D. For example, in Farm A, dominant subtype was endemic H1N1 (eH1N1). eH1N1 comprise of genes from Eurasian swine lineage (PB2, PB1, PA, NP, NA1 and M) and classical swine lineage (HA1 and NS). While, one SIV (A-S2) showed mix genotype including eH1N1 as background genotype and rH3N2 as a mixed genotype. This mixed genotype (rH3N2) comprise of HA3 and NA2 from eH3N2 and internal genes from pH1N1 (rH3N2-TRIG+2). In farm C, one SIV (C-S12) showed mix genotype including rH3N2 as background genotype and rH3N1 as a mixed genotype. In farm D, one SIV (D-S15) showed mix genotype of rH1N1 as background genotype and rH3N2 as a mixed genotype. In farm B, multiple single genotype were found including rH1N1, (B-S8 and B-S11), rH1N2 (B-S9) and rH1N2 (B-S10).

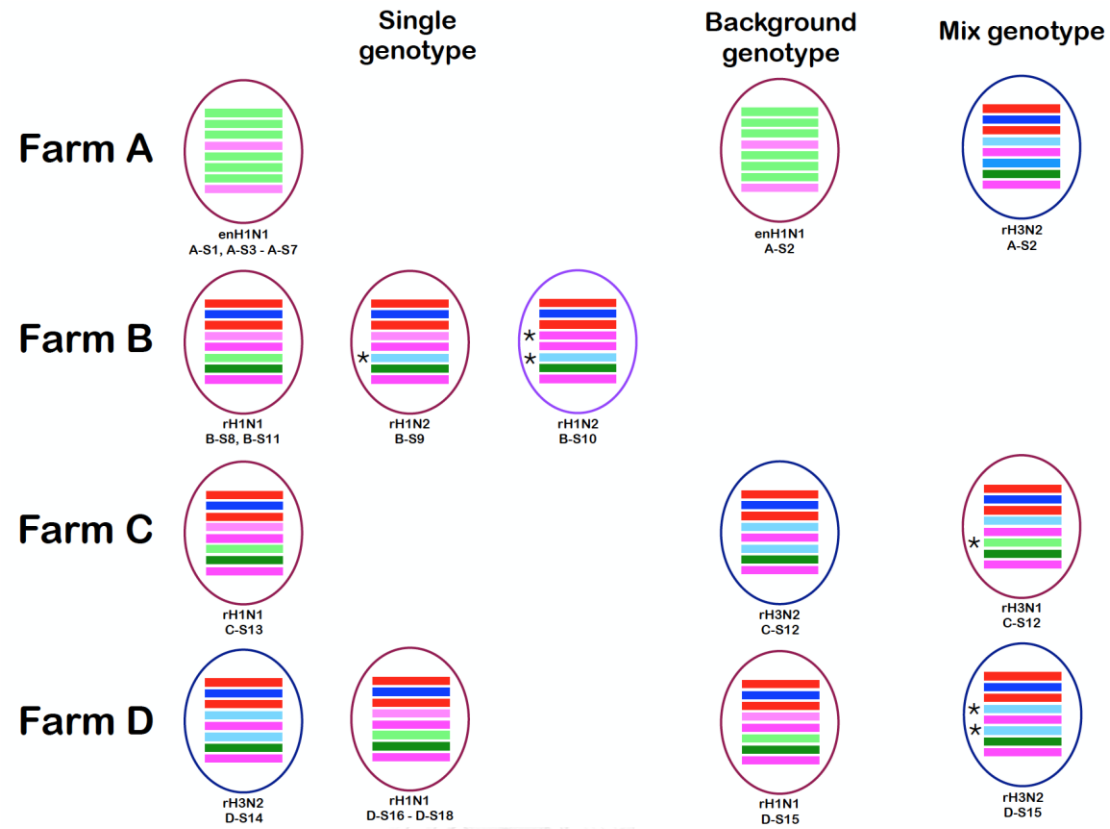

Figure 3.5 The schematic of genetic constellation of SIV in this study.
Each oval and bar inside represents a viral particle and viral gene segment from above to below is segment 1 to 8. The different color of each bar represent different gene lineage; red, dark blue, magenta, dark green, light blue, pink and green represent avian TRIG, human TRIG, pandemic classical swine, pandemic Eurasian swine, human seasonal lineage, classical swine lineage and Eurasian swine lineage, respectively.

3.5 Discussions

Multiple SIV subtypes in swine farms can promote reassortant viruses. In this study, all swine farms presented co-circulation of multiple SIV subtypes. We were applying illumina sequencing to monitor the reassortant and/or mixed-genotypes in swine farms. Our results showed that mix-genotypes of SIVs were observed in this study.

In this study, the finding of mix genotype was confirmed by the presence of significant number of sequence reads mapped to reference gene (6.42% – 40.28%). The presenting of mix genotypes of influenza A virus in one sample can refer to mixed-infection. As demonstration in other study, the researchers reported mixed-infection of different HA subtypes in two samples, a bald eagle isolate and a cloacal swab showed evidence of mixed infections with two (H1 and H2) and three (H1, H3, and H4) HA subtypes, respectively (Ramakrishnan, et al., 2009).

Mixed-infection of multiple SIV strains in one host was also evidenced by serological surveillance using hemagglutination inhibition (HI) assay. For example, in Poland, 0.65% - 12.3% of serum samples showed HI positive against more than two or three SIV subtypes (H1N1+H1N2, H1N1+H3N2, H1N2+H3N2, H1N1+H1N2+H3N2). In Thailand the evidence of dual SIV infection (26.18%) was reported including dual and triple infections between eH1N1 and pH1N1 viruses (19.56%), eH1N1 and H3N2 viruses (3.47%) and pH1N1, H3N2 viruses (3.15%) and eH1N1, pH1N1 and H3N2 viruses (8.20%) (Chanvatik, 2014). In human, mixed-infection between IAV-H3N2 and pH1N1 was reported in a 3-year-old male and reassortant IAV-H3N2 was isolated which contained a pH1N1 NS1 gene fragment (Rith, et al., 2015).

Another finding, SIV subtype H3N1 was reported in Thai pig population. In this study, mix-genotype (C-S12; rH3N2 and rH3N1) was confirmed by illumina sequencing result with 6.44% of sequence reads mapped against NA1 gene while the rested 93.56% sequence reads mapped to NA2. It is noted that partial gene of H3N1-SIV has been reported in Thai pigs by plaque purification

techniques (Abe, et al., 2015). In Thailand, although SIV-H1N1 and SIV-H3N2 has been reported for decade in Thai pig population, there is no evidence of reassortant H3N1. Moreover, since 1992 SIV-H3N1 has periodically reported in the US, Taiwan and Korea which were the result from reassortment between turkey H3N2 and SIV-H1N1 (in the US), classical H1N1 and SIV-H3N2 (in Taiwan) and human seasonal H3 and endemic SIV (in Korea) (Lekcharoensuk, et al., 2006; Shin, et al., 2006; Tsai and Pan, 2003).

Our HA1 and NA1 phylogenetic study demonstrated that in Thai pig population there are 2 subclusters of endemic HA1 and NA1 which HA1-Alpha-b and NA1-subcluster b were a recent predominant genotype. While, SIV-HA3 were clustered with A/WH/95 as previously reports which referred to less diversity (Lekcharoensuk, et al., 2010; Takemae, et al., 2008). Diversity of HA protein can affected to sensitivity and specificity of diagnostic assays especially hemagglutination inhibition (HI) assay or serum neutralizing assay, which are the gold standard of serological study. Thus, reference viral strains of the assay should be reviewed to update current methodology. In addition, antigenic analysis of Thai SIV should be further investigated for better understanding of relation between gene characteristic and viral properties. Interestingly, we first identified NA2 genotype related to A/SN/97 in Thailand which this ancestral genotype was reported in Hong Kong however HA3 and NA2 originating from A/WH/59 still the predominant strain in Thai pigs (Peiris, et al., 2001).

Additionally, here we reported the first E627K identification on PB2 in Thai SIV same as previously reports in China (Liu, et al., 2012). Fortunately, amino acid substitution relates to oseltamivir resistance were not found in this study however, the monitoring of genetic evolution should be continuously performed.

We demonstrated that illumine sequencing provide whole genome sequences and capable to monitor the reassortment event in swine farms. The results from this study could provide new insight information of SIV evolution in Thailand.

CHAPTER IV

Time-space analysis of highly pathogenic avian influenza H5N2 outbreak in the US

This study has been done at the University of Minnesota as part of research collaboration in abroad of the Chulalongkorn University Dutsadi Phiphat Scholarship.

Parts of this work has been published in

Virology Journal. August 30, 2016; 13(1):147.

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In 1996, highly pathogenic avian influenza (HPAI) H5N1 goose Guangdong (GS/GD) lineage was emerged and had been disappeared for 5 years. In 2003, re-emerging of HPAI H5 GS/GD lineage was reported and spread worldwide. Since then, the H5 gene has continued to evolve and has been classified into clades from 0-9 by OIE nomenclature system. In 2014, HPAI H5N8 has been reported in East Asia then dispersed to South East Asia, Europe, Middle East and North America. Later on in 2015, this HPAI H5 was identified as a novel clade, 2.3.4.4. H5 clade 2.3.4.4 was reported in Canada and West coast states, USA in late 2014. After that during March to June 2015, HPAI H5N2 clade 2.3.4.4 had been outbreak in turkey farms in Midwest, USA and affected more than 48 million birds. In this study, swab samples from turkeys and environmental samples were obtained from affected farms in different point of time. To describe origin and evolution of viruses, illumina sequencing was used to achieve whole genome sequence data and Bayesian Evolution Analysis Sampling Tree (BEAST) analysis was applied to obtain time to most recent common ancestor and substitution rate of viruses.

4.1 Abstract

In early 2015, highly pathogenic avian influenza H5N2 caused outbreaks in commercial poultry farms in Minnesota and neighboring states where more than 48 million birds were affected. To date, the origin and transmission pathways of HPAI H5N2 have not been conclusively established. In this study, we analyzed forty-six samples from turkeys and their environment that were collected at different time-points of the outbreak to identify origins and within outbreak evolutionary changes. We performed de-novo whole genome sequencing from primary samples and the most recent common ancestors of the PB2, PA, HA5, M and NS segments were traced back to Japanese HPAI H5N8 isolates. These segments appeared to have diverged from the ancestor around June and November 2014. The time to most recent common ancestor analysis for PB1, NP and NA2 segments suggest two likely possibilities of reassortant HPAI H5N2 origin - either a reassortment in Alaska area or multiple reassortments with North American low pathogenic avian influenza strains, before the HPAI H5N2 outbreak strain emerged. Within the outbreak, viruses clustered into two and three subgroups suggesting high substitution rates of $0.702x10^{-2}$-$1.665x10^{-2}$ (subs/site/year), over the 5-month outbreak period. Data are suggestive of a fast evolving HPAI strain within an outbreak that should be taken into consideration in developing appropriate control strategies in the future.

Keywords: Evolutionary, H5N2, Highly pathogenic avian influenza, Minnesota

4.2 Introduction

Avian influenza virus (AIV) is an enveloped virus that contains eight negative sense single strand RNA segments which encode at least ten functional proteins (Lamb and Krug, 1996). Hemagglutinin (HA) and neuraminidase (NA) are surface proteins that are used to classify AIV into subtypes. At present, 16 HA and 9 NA subtypes have been identified in aquatic wild birds which are a natural reservoir of these viruses (Webster, et al., 1992). AIV can be divided into low pathogenic avian influenza viruses (LPAI) and highly pathogenic avian influenza viruses (HPAI) by its ability to cause disease in poultry which is identified by intravenous pathogenicity index (IVPI) test or by its possession of poly-basic amino acid feature at the HA cleavage site (Horimoto and Kawaoka, 1994)

Outbreaks HPAI H5 in Guangdong, China in 1996 have likely origins from goose Guangdong lineage HPAI viruses (World Health Organization Global Influenza Program Surveillance, 2005). Since 1997, HPAI H5N1 dispersed in more than 50 countries in Africa, Asia, Europe, and the Middle East. In 2003, a re-emergence of HPAI H5N1 in China and South East Asia was reported. Since its emergence, the H5 gene has continued to evolve and has been arbitrarily classified in the OIE nomenclature system into clades identified from 0-9 (OIE, 2011). The latest clade, 2.3.4.4, was designated in January 2015 and replaced the provisional clade 2.3.4.6. The first isolation of H5 clade 2.3.4.4 viruses was from domestic mallard ducks (*Anas platyrhynchos*) in China in 2008 (Gu, et al., 2011). The H5 clade 2.3.4.4 has demonstrated an ability to reassort with multiple neuraminidase subtypes including N1, N2, N3, N5, N6 or N8 (Ip, et al., 2015).

In January 2014, HPAI H5N8 caused outbreaks in 161 commercial poultry flocks in South Korea and led to culling of 14 million birds (Jeong, et al., 2014). In early November 2014, HPAI H5N8 was detected in a turkey flock in Germany followed by an outbreak in a duck farm in England and a chicken farm in the Netherlands. Wild birds may play an important role in generating novel

reassortant subtypes of clade 2.3.4.4 and carrying viruses across continents (Verhagen, et al., 2015). In late November 2014, HPAI H5N2 caused outbreaks in turkey and broiler breeder flock in British Columbia, Canada. In the United States, the first case of HPAI H5 was detected in a captive gyrfalcon (*Falco rusticolus*) in Washington in early December and subsequently the first detection of the reassortant HPAI H5N2 was reported in a northern pintail duck (*Anas acuta*) in Washington. In December 2014 and January 2015, HPAI H5 viruses associated with various NA subtypes were detected in backyard poultry flocks in Oregon and Washington, USA (USDA, 2014). In January 2015, an HPAI H5N8 virus was detected in a commercial turkey flock in California (USDA, 2015). On March 2nd, 2015, the first case of HPAI H5N2 in the Midwestern USA was confirmed in Pope County, Minnesota. On June 17th, 2015, last confirmed case in this outbreak was reported in Iowa. In the Midwest region, more than 200 confirmed cases were reported and more than 48 million birds were affected (APHIS, 2015).

In this study, we investigated the origin and within outbreak evolution of this new emergent HPAI H5N2 that caused outbreaks in Midwestern states.

4.3 Methods

4.3.1 Sample Collection

Samples were collected from turkeys and the environment at locations involved in outbreaks of HP clade 2.3.4.4 H5N2. Sampling was performed over a 106-day period during the outbreak. A total of 46 oropharyngeal swab or cloacal swab or environmental samples were collected. Samples were divided into three phases representing an approximate 35-day interval each - early phase (March 4th - April 7th) [*n* = 18], mid phase (April 8th - May 12th) [*n* = 19] and late phase (May 13th - June 17th) [*n* = 9].

All samples were identified by RT-PCR testing water and bird samples from all barns of turkey flocks to identify infected samples per established methods (Spackman, et al., 2003). All

RT-PCR positive samples (water in infected barns, tracheal and cloacal swabs from birds or air) collected at early, mid and late phases of the outbreak were genome sequenced directly from primary samples (Table 4.1).

4.3.2 Whole genome sequencing

Forty-six samples from turkeys, drinker biofilm, air and environment submitted to the University of Minnesota Mid Central Research and Outreach Center (Willmar, MN) were used in this study. RNA was extracted with the MagMAX™-96 Viral RNA Isolation Kit (Ambion) using a magnetic particle processor (Kingfisher, model 700) according to manufacturer's instructions. All RNA samples were tested by RT-PCR for matrix gene as described (Spackman, et al., 2003). The positive RNA samples were subjected to amplify all eight segments of virus simultaneously using a one-step RT-PCR as described (Zhou, et al., 2009)]. Briefly, SuperScript® III one-Step RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen™; CA, USA) was used with 1.6 mM and 0.2 µM final concentration of Magnesium and primers, respectively. PCR products were imaged by electrophoresis in 1.5% agarose gel and subsequently purified by QIAquick PCR Purification Kit (Qiagen®; Hilden, Germany). Purified PCR products were submitted to University of Minnesota Genomics Center (UMGC) for illumina paired-end 250 cycles sequencing by using Nextera XT DNA kit for library generation. Sequences were assembled by mapping all reads to a reference and whole- or partial-genome sequences were extracted via CLC genomics workbench module available on Minnesota supercomputing institute (MSI) resources at the University of Minnesota.

4.3.3 Phylogenetic analysis

The reference nucleotide sequences were obtained from Influenza Research Database (http://www.fludb.org/) and GISAID (http://gisaid.org/) in May 2016. Reference sequences were selected to represent previous and recent avian influenza strains from varying geographic areas including North America and Eurasia. The following approach was applied to select reference

sequences: 1) include an ancestral strain of clade 2.3.4; 2) use of sequences of isolates selected from 3 well characterized H5 (2.3.4.4 lineage) outbreaks in Asia, Europe and North America; and 3) use of a double selection criteria to identify the top 30 hits to the current outbreak isolates by BLAST and develop a tree. Subsequently, these closely related sequences were reanalyzed by BLAST to expand the reference database and used to reconstruct phylogeny. LPAI North American strains that were isolated during 2012-2015 were also included. Reference and sample sequences from the current study were aligned using Muscle v.3.6 (Edgar, 2004) subsequently any extra sequences beyond and after start and stop codon were trimmed. Maximum clade credibility (MCC) tree of each gene segments were generated by BEAST 1.8 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. Strict clock model with coalescent constant population and HKY with gamma 4 substitution was used as model parameters (Drummond, et al., 2002; Drummond, et al., 2012)

Mean substitution rate was estimated by Bayesian coalescent with constant population size (Drummond, et al., 2002) and strict clock model was applied. The Bayesian MCMC chain lengths were ten million generations with sampling every 10,000 generations and the effective sample size (ESS) value was assessed by using Tracer (v1.6.0) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).

4.4 Results

First case of this series of outbreaks in the Midwest area was confirmed on March 4th, 2015 in Pope County, Minnesota and last case on June 17th, 2015 in Iowa (APHIS, 2015). A 106-day period was divided into three phases representing an approximate 35-day interval - early phase (March 4th - April 7th), mid phase (April 8th - May 12th) and late phase (May 13th - June 17th). Eighteen, nineteen and nine samples were collected during early, mid and late phases of the outbreak, respectively (Table 4.1).

Table 4.1 Details of samples in this study

Sample No.	Sample type	Collection date	Phase of outbreak	Location	Sequencing Data (Segment)								Accession No.
					1	2	3	4	5	6	7	8	
E1	Envi	2/27/2015	Early	Minnesota									KY341927 – 34
E2	Envi	2/27/2015	Early	Minnesota									KY341935 - 42
E3	TS	3/4/2015	Early	Minnesota									KY341943 - 50
E4	TS	3/4/2015	Early	Minnesota									KY341951 – 58
E5	Envi	3/7/2015	Early	Minnesota									KY341959 – 66
E6	TS	3/7/2015	Early	Minnesota									KY341967 – 74
E7	Envi	3/24/2015	Early	Minnesota									KY341975 - 82
E8	Envi	3/24/2015	Early	Minnesota									KY341983 - 90
E9	Envi	3/26/2015	Early	Minnesota									KY341991 – 98
E10	Envi	3/26/2015	Early	Minnesota									KY341999-2006
E11	TS	3/28/2015	Early	Minnesota									KY342007 – 14
E12	CS	3/28/2015	Early	Minnesota									KY342015 – 22
E13	TS	3/28/2015	Early	Minnesota									KY342023 – 30
E14	CS	3/28/2015	Early	Minnesota									KY342031 – 38
E15	Envi	4/3/2015	Early	Minnesota									KY342039 – 46
E16	Envi	4/3/2015	Early	Minnesota									KY342047 – 54
E17	TS	4/3/2015	Early	Minnesota									KY342055 – 62
E18	TS	4/3/2015	Early	Minnesota									KY342063 – 70
M1	Envi	4/23/2015	Mid	Minnesota									KY342071 – 78
M2	Envi	4/23/2015	Mid	Minnesota									KY342079 – 86
M3	Envi	4/23/2015	Mid	Minnesota									KY342087 – 94
M4	Envi	4/23/2015	Mid	Minnesota									KY342095-102
M5	Envi	4/23/2015	Mid	Minnesota									KY342103 – 10
M6	Envi	4/23/2015	Mid	Minnesota									KY342111 – 18

Sample No.	Sample type	Collection date	Phase of outbreak	Location	Sequencing Data (Segment) 1 2 3 4 5 6 7 8	Accession No.
M7	Air	4/24/2015	Mid	Minnesota		KY342119 – 26
M8	Air	4/24/2015	Mid	Minnesota		KY342127 – 34
M9	Air	4/27/2015	Mid	Minnesota		KY342135 – 42
M10	Air	4/27/2015	Mid	Minnesota		KY342143 – 50
M11	Envi	4/28/2015	Mid	Minnesota		KY342151 – 58
M12	Envi	4/28/2015	Mid	Minnesota		KY342159 – 66
M13	Envi	4/28/2015	Mid	Minnesota		KY342167 – 74
M14	TS	4/28/2015	Mid	Minnesota		KY342175 – 82
M15	TS	4/28/2015	Mid	Minnesota		KY342183 – 90
M16	TS	4/28/2015	Mid	Minnesota		KY342191 – 98
M17	TS	4/28/2015	Mid	Minnesota		KY342199-206
M18	Air	4/28/2015	Mid	Minnesota		KY342207 – 10
M19	Air	4/28/2015	Mid	Minnesota		KY342211 – 14
L1	Air	5/12/2015	Late	Iowa		KY342215 – 18
L2	Air	5/12/2015	Late	Iowa		KY342219 – 24
L3	Air	5/22/2015	Late	Nebraska		KY342225 – 31
L4	Air	5/23/2015	Late	Nebraska		KY342232 – 39
L5	Air	5/23/2015	Late	Nebraska		KY342240 – 47
L6	Air	5/23/2015	Late	Nebraska		KY342248 – 55
L7	Envi	5/23/2015	Late	Nebraska		KY342256 – 57
L8	Envi	5/23/2015	Late	Nebraska		KY342258 – 65
L9	Envi	5/23/2015	Late	Nebraska		KY342266 – 73

Note: White and grey box indicates available and unavailable sequencing data on each segment, respectively. TS, CS and Envi stand for tracheal swab, cloacal swab and environmental sample, respectively.

4.4.1 Origin of HPAI H5N2

Forty samples were successfully whole genome sequenced and six provided whole segment sequences of some gene segments as shown in Table 4.1. Phylogenetic analysis of HPAI H5N2 (HPAI H5N2 EA/NA) showed that it was a reassortant between Eurasian (EA) HPAI H5N8 and North American (NAm) LPAI. HPAI H5N2 EA/NA genetic constellation is composed of five gene segments (PB2, PA, HA, M and NS) from EA HPAI H5N8 and the remaining three segments (PB1, NP and NA) from NAm LPAI (Figure 4.1-4.8).

Time to most recent common ancestor (TMRCA) analysis showed that EA HPAI H5N8 likely evolved from an AIV H5 clade 2.3.4.4 (China) between April 2008 and January 2011 (2008.297-2010.255). Longer-term analysis of HPAI H5N2 EA/NAm, TMRCA analysis indicated that PB2, PA, HA5, M and NS segments diverged from EA HPAI H5N8 (Japan) strain, around June and November 2014 (2014.438-2014.900). PB1 was closely related to non-H5 LPAI North American strain isolated from Alaska (blue branch) likely diverged from these isolates around June 2012 and June 2013 (2012.453-2013.438, mean TMRCA 2012.950). NP appears to have diverged from A/American green-winged teal/Alaska/472/2014 (A/AGWT/AK/472/14; pink branch) an LPAI H5N2 North American strain, during September 2012 and December 2013 (2012.721-2013.941, mean TMRCA 2013.358). NA2 ancestry traced back to H5N2 that was isolated from Alaska during flu season 2014-2015 (pink and green branches) and reassortment likely occurred around December 2012 and November 2013 (2012.993-2013.824, mean TMRCA 2013.400). TMRCA of all gene segments are summarized in Figure 4.9. The TMRCA format (E.g., 2006.272) was calculated using the formula: collection date divided by the number days in a year. For example, January 10 2006 is day 10^{th} of the year; TMRCA = (1/365)*10 = 0.027 in the year 2006 = 2006.027.

4.4.2 Within outbreak evolution

The mean substitution rate of all segments over the 106-day interval of this study was estimated to be between 0.702-1.665 x 10^{-2}. The NS gene segment was the most divergent segment while NP gene segment was the most conserved segment (Table 4.2). Bayesian Coalescent analysis showed that every gene segment of samples in this study formed three clades (I, II and III) except M and NS segment that form two major clades (I and II) (Figure 4.1-4.8). Members in subgroup I of PB2, PB1, PA, HA5, NP and NA2 were consistent in terms of clustering by time within the outbreak period with some minor variations. For example two samples (E15 and E16) fell into cluster II for M while the same samples clustered in clade I for NS. The HA5 segment of Korean H5N8 clustered within groups A and B (Jeong, et al., 2014). Group A is a predominant cluster and appears to have dispersed to other continents. In addition, this cluster was subdivided into 3 clades (A1, A2 and A3) (Ozawa, et al., 2015). HPAI EA/NAm H5N2 belongs to the A2 subgroup which same subgroup as the two isolates H5N8 (Japan) and HPAI H5N2 (Canada) lineages (Figure 4.4). These results of high polymorphism rate and diversification of gene segments over the course of the epidemic indicate rapid evolution of viruses within this outbreak. These analyses assume evolution from a single introduction. Thus, that this high rate of change could likely be explained by multiple introductions cannot be ruled out.

Table 4.2 Mean nucleotide substitution rate of the H5N2 epidemic

Gene segment	Mean Substitution rate ($x10^{-2}$)	Substitution rate 95% HPD ($x10^{-2}$)
PB2	1.079	0.526-1.710
PB1	1.160	0.620-1.760
PA	0.712	0.332-1.150
HA	1.290	0.670-2.000
NP	0.702	0.261-1.240
NA	1.413	0.644-2.310
M	1.329	0.315-2.540
NS	1.665	0.595-2.790

Figure 4.1-4.8 Time-scaled Bayesian maximum clade credibility (MCC) tree inferred for the PB2 (4.1), PB1 (4.2), PA (4.3), HA5 (4.4), NP (4.5), NA2 (4.6), M (4.7) and NS (4.8). Trees were generated by Bayesian Markov Chain Monte Carlo algorithm in Bayesian evolutionary analysis by sampling trees. The TMRCAs representing the estimated timing of viral divergence from their ancestor are provided in parentheses. Red dot at each node represents the posterior probability above 0.7. A/AGWT/AK/472/14, group of A/mallard/SAK/14 and non-H5 LPAI NAm strain were labeled by pink, green and blue branches. Clustering is shown with reference strains of recent ancestry and within outbreak viruses clearly cluster in 2 or 3 clades separated by 35-day intervals.

Figure 4.1 Time-scaled Bayesian MCC tree inferred for the PB2

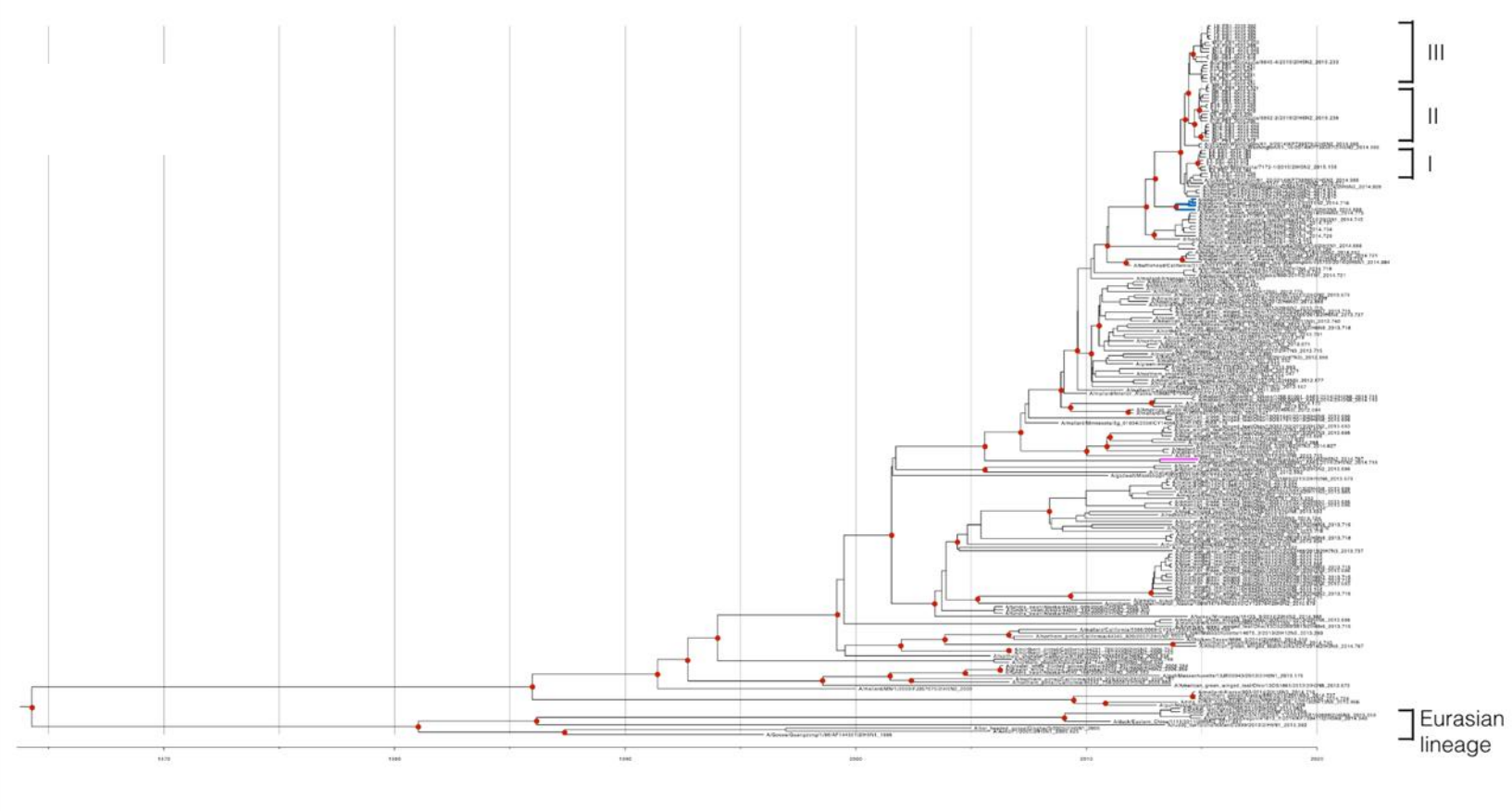

Figure 4.2 Time-scaled Bayesian MCC tree inferred for the PB1

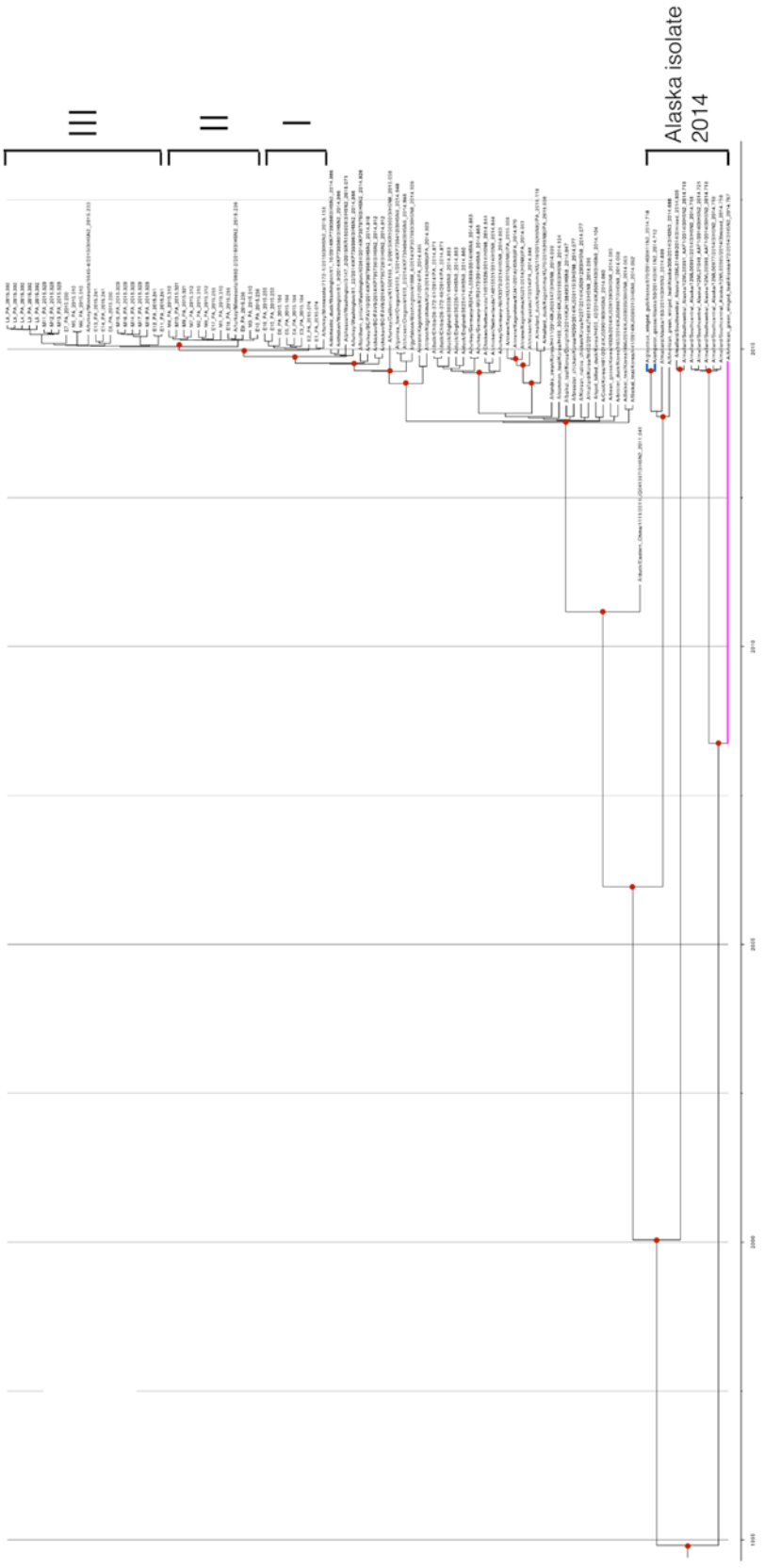

Figure 4.3 Time-scaled Bayesian MCC tree inferred for the PA

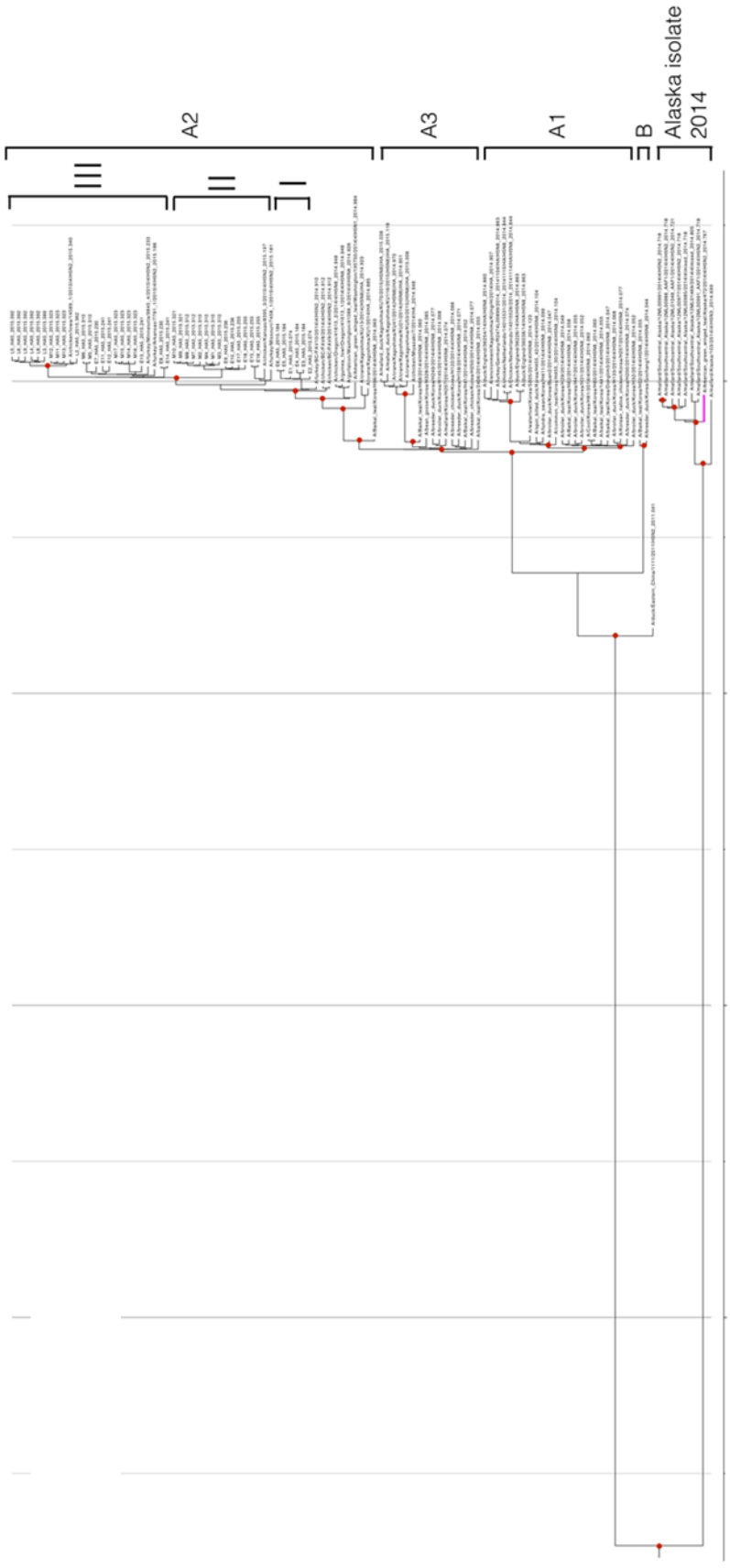

Figure 4.4 Time-scaled Bayesian MCC tree inferred for the HA5

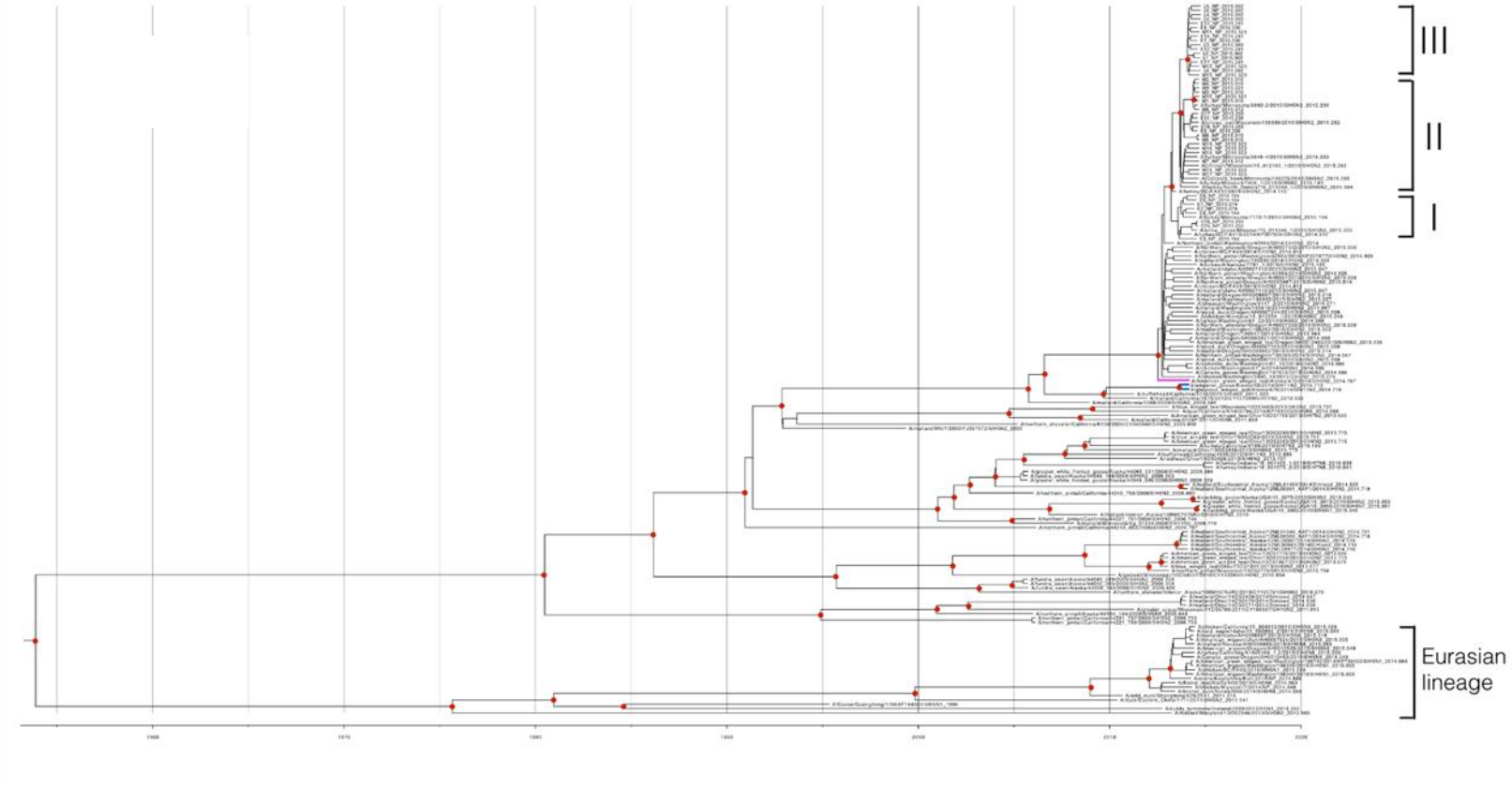

Figure 4.5 Time-scaled Bayesian MCC tree inferred for the NP

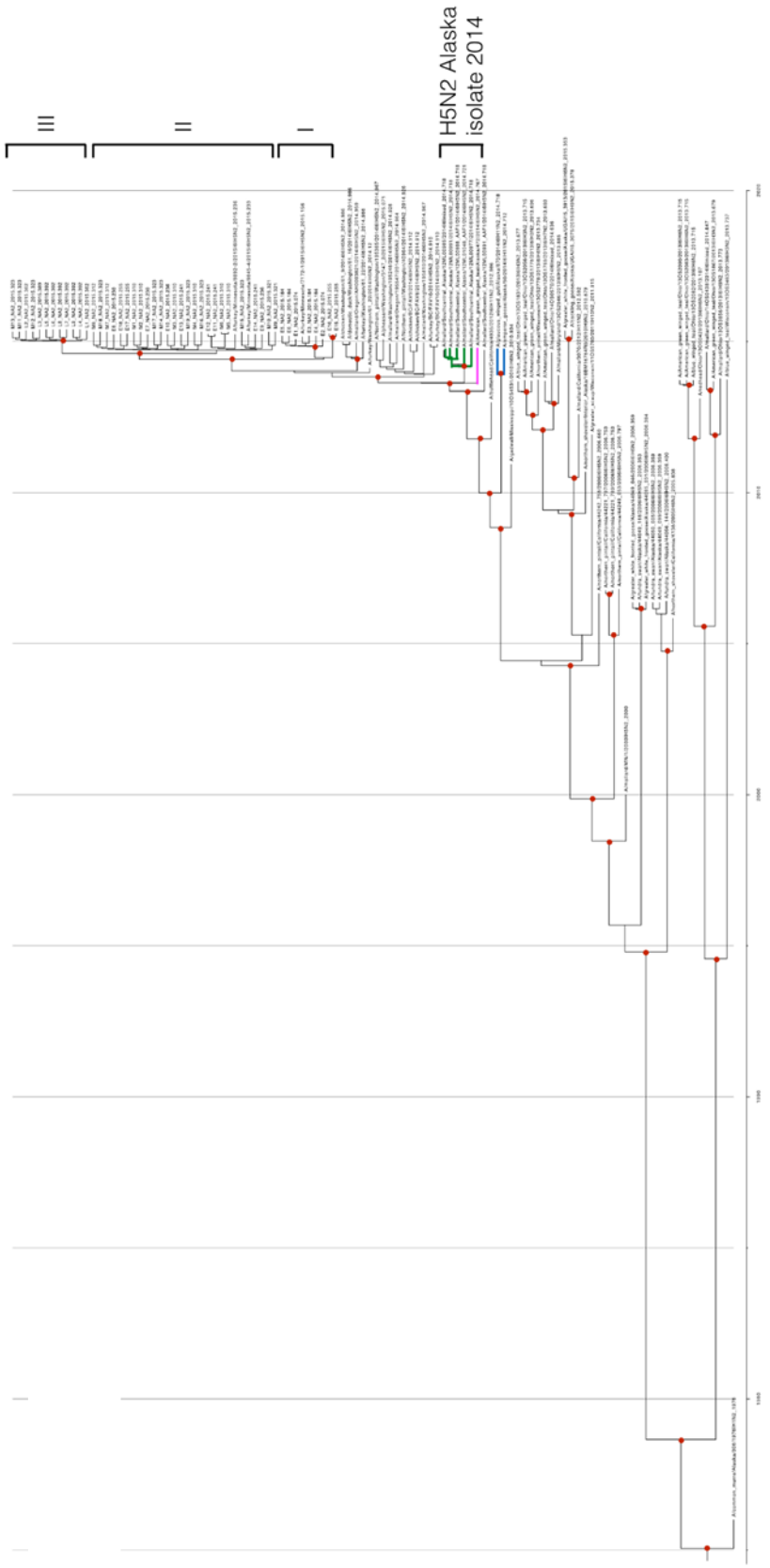

Figure 4.6 Time-scaled Bayesian MCC tree inferred for the NA2

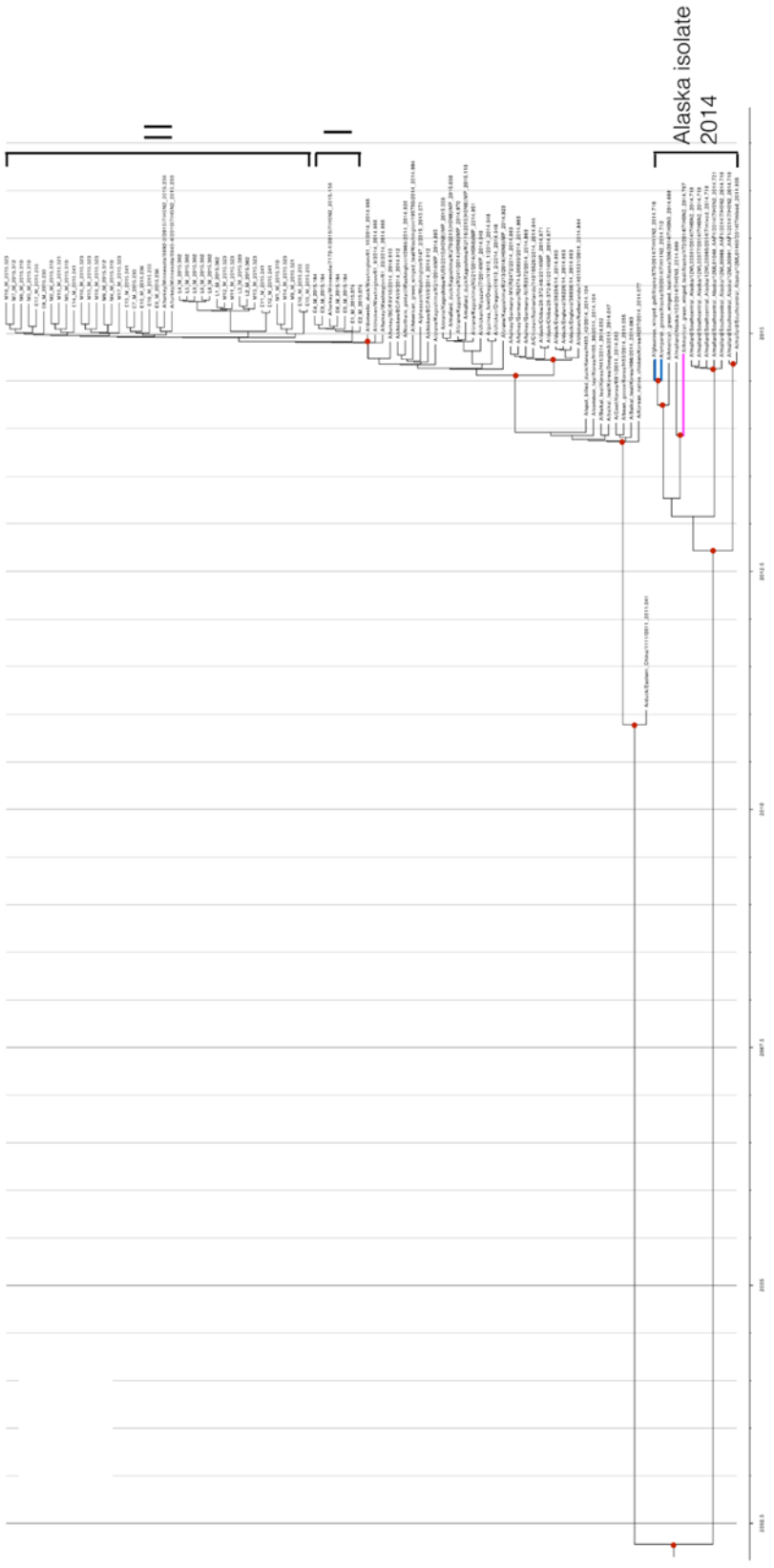

Figure 4.7 Time-scaled Bayesian MCC tree inferred for the M

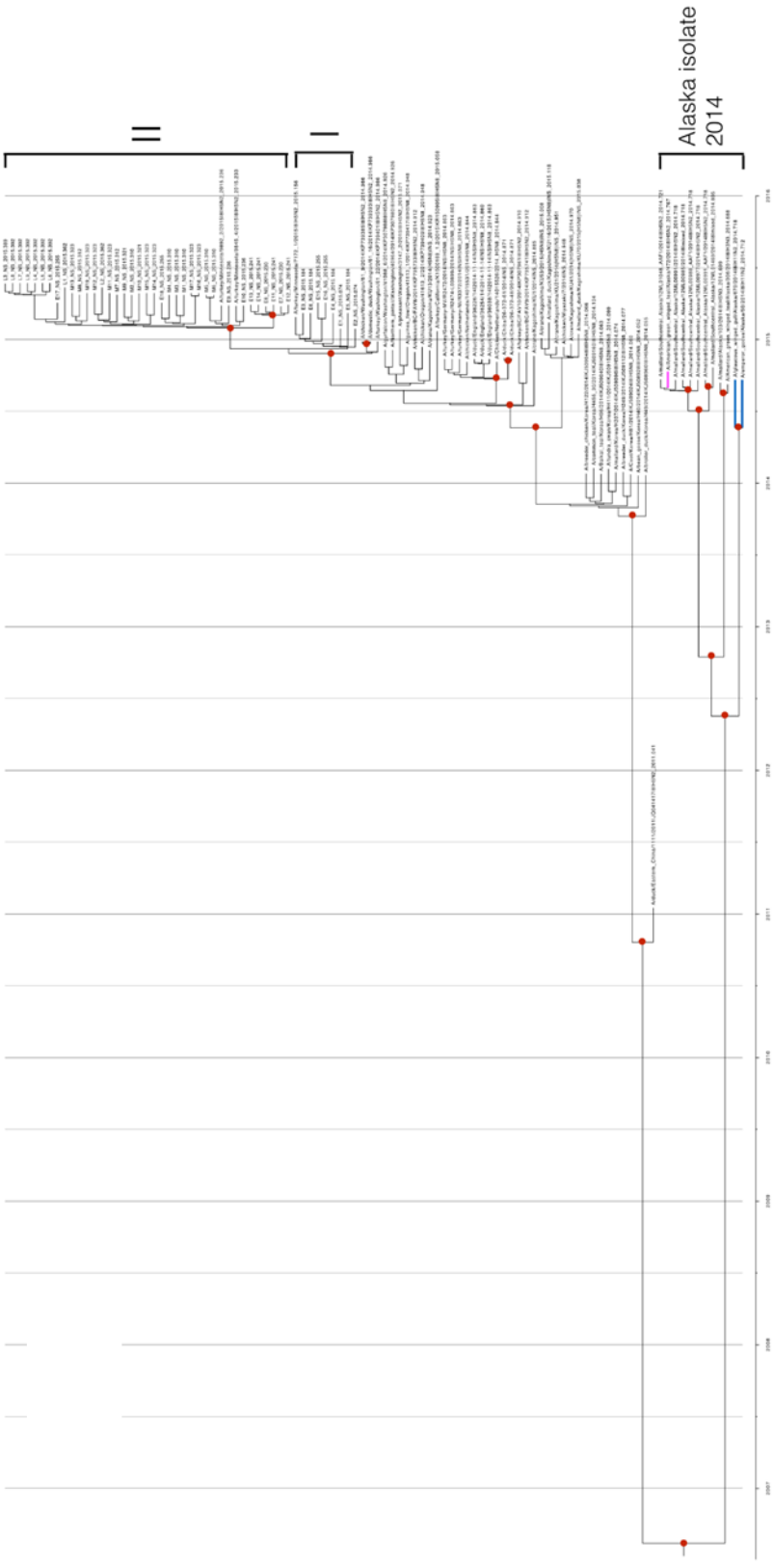

Figure 4.8 Time-scaled Bayesian MCC tree inferred for the NS

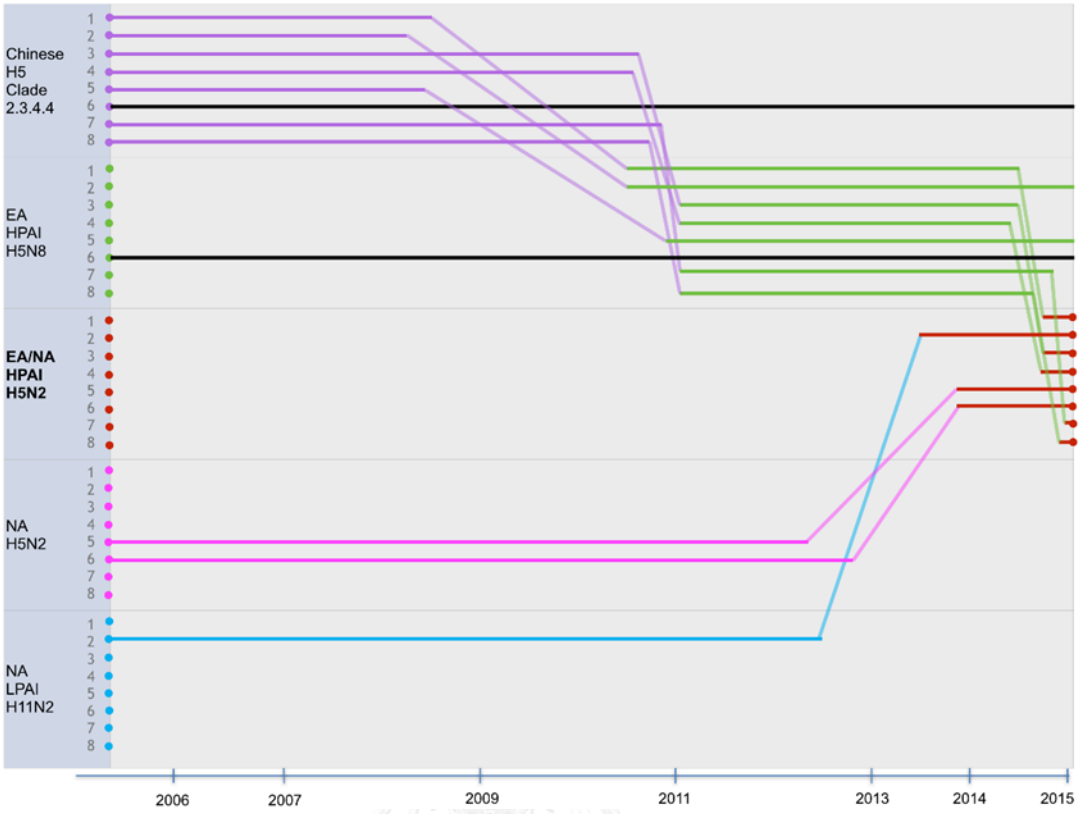

Figure 4.9 Reconstruction of the genetic constellation of reassortment events leading up to the emergence of EA/NA H5N2.

The eight genomic segments are represented as parallel lines in descending order of segment 1 to 8. Each colored line represent transmission pathways of influenza genes from different ancestor: Chinese H5 Clade 2.3.4.4 (purple), Eurasian HPAI H5N8 (green), Eurasian/North American HPAI H5N2 (red), LPAI H5 North American strain (pink) and non-H5 LPAI North American strain (blue). Black line represents the different NA subtype that was not included in the analysis. Slopes lines represent divergent dates of each segment at 95% highest posterior density interval.

4.4.3 Genetic analysis for amino acid variability

Genetic analysis of the HA5 segment indicated multiple basic amino acid at HA cleavage site. Samples from early and mid phase of the outbreak contain the motif P**L**RERRRKR/GLF that is a characteristic of H5 clade 2.3.4, while samples collected during late phases of the outbreak carried the P**Q**RERRRKR/GLF motif Glutamine (Q) is the major amino acid of other H5 clades (OFFLU, 2015). Amino acid substitutions were analyzed by comparing with A/Northern pintail/Washington/ 40964/2014 (A/NP/WA/14), the first isolate of HPAI H5N2 EA/NA in the US. At position 141 (H5 numbering system) on the HA gene, A/NP/WA/14 and sub-cluster I contained serine (S) while seventeen samples, sub-cluster II and III, contain proline (P). S141P mutations are related to surface protein stability and reduced host immune response (Cattoli, et al., 2011). The deduced amino acid sequence of the HA stalk region of all samples were identical.

All samples contained S at position 31 on M2 that has been shown to confer reduced susceptibility to amantadine and rimantadine antiviral drugs. However, analysis of resistance to neuraminidase inhibitors (NAI) at position 119, 151, 222, 224, 276, 292 and 371 on NA2 gene segment (Eshaghi, et al., 2014) showed that all samples in this study contain the conserved amino acid at every position which are expected to maintain NAI susceptibility. The E627K and D701N substitutions in the PB2 gene segment that are expected to increase virulence in mammals were examined. The results showed that all samples contain E and D in respective amino acid sites suggesting low mammalian virulence in this group of viruses.

4.5 Discussions

A study addressing the spread of HPAI H5N8 from South Korea to Europe and Japan have suggested that these outbreaks originated from a single source population (Dalby and Iqbal, 2015). Dispersion of the virus to different geographic localities coincides with wild bird migratory seasons strongly suggesting their important role in the transmission of this pathogen and has been suggested

as a mechanism by which H5N8 entered South Korea (Hill, et al., 2015). The most likely flyway involved in the recent spread of HPAI EA H5N8 to Japan was the East Asian Australian (EAA) Flyway (Dalby and Iqbal, 2015). The EAA flyway extends from north-eastern Asia and western Alaska to the southern extent of Australia and New Zealand and Mongolia, western China and eastern India (Bamford, et al., 2008). On the North America continent, Alaska is an area where there is major overlap between migratory birds from the EAA and Pacific Flyways (Bamford, et al., 2008) but there exists no evidence of HP H5 clade 2.3.4.4 in Alaska. The existence of PB1, NP and NA2 ancestor that related to HP H5N2 in Alaska arise the possibility that HPAI H5N2 EA/NAm originated in a location that coincides with Alaska and subsequently spread to British Columbia, Canada and Washington, USA. However, the mechanism and timing of viral transfer from the Pacific Flyway to the Mississippi Flyway in NAm has not been established.

The ancestral strain and the overlapping of interval between TMRCA estimates of PB1, NP and NA2 suggest two hypothesis of HPAI EA H5N2 origin. First, it is possible that reassortant event occurred in Alaska. This finding is concordant with the report of avian influenza surveillance in Alaska during spring and summer 2015 that found reassortant LPAI between EA and NAm (Ramey, et al., 2016b). Second, the outbreak viral genome constellation likely occurred via multiple reassortment events with an LPAI before addition of genes of HPAI EA H5N2 origin due to the earlier divergent time of PB1, NP and NA2. Furthermore, HA5 clade 2.3.4.4 has the potential to reassort with multiple NA subtypes including NA1, NA2, NA5, NA6 and NA8 (Torchetti, et al., 2015). For example, reassortant H5N1 (EA/NAm H5N1) containing PB2, HA5, NP and M from EA H5N8 and the rest of the gene segments (PB1, PA, NA1 and NS) from AIV North American lineage was detected in a green wing teal and an American wigeon in December 2014, with no subsequent case reports. This finding indicates that EA/NAm H5N1 was likely unable to persist in the population while

EA/NAm H5N2 provided a compatible constellation for maintenance. Alternately, this may indicate that there was insufficient surveillance to detect the fate of that reassortment.

The nucleotide substitution rate of all segments within the outbreak are greater than $5.000x10^{-3}$ substitution/site/year which are higher than the rates estimated in long term analysis (Vijaykrishna, et al., 2008). These results indicated that viruses were evolving rapidly during the current outbreak. Host jump from wild birds to domestic poultry including turkeys and chickens is a factor that provoked this rapid evolution. Therefore, identification of bridge of interspecies transmission is an important process to prevent the further outbreaks. While the HA gene had an extremely high substitution rate, the HA stalk region of all samples were identical at the amino acid level. The HA stalk region, PA and NP proteins were highly conserved, opening avenues for a universal subunit vaccine concept and development.

At present, no human case has been reported due to HPAI H5N2 EA/NAm and it is considered as low health risk to public health (CDC, 2015b). However, three human cases with severe respiratory disease caused by HPAI H5N6 HA5 clade 2.3.4.4 have been reported in China (Pan, et al., 2015). These cases confirmed that HA5 clade 2.3.4.4 is capable of causing human infection and thus ongoing monitoring of viral evolution should be performed.

Wild bird surveillance in United States has been performed annually during September and November, which is a migratory season and only fecal or cloacal swabs are traditionally collected. After HPAI H5 clade 2.3.4.4 was detected in December 2014, USDA/APHIS wildlife services responded by enhancing surveillance in Pacific Flyway and detected more viruses. Furthermore, multiple introductions of this clade of viruses cannot be ruled out. Therefore, there is a need for more comprehensive wild bird surveillance effort to address a complex wild-domestic animal-human interface to capture variations in subtype specific organ predilection and varying patterns of shedding by different host species.

CHAPTER V

Conclusions and discussions

Influenza is an important zoonotic disease. There are strong evidences that both avian and swine influenza viruses have a potential to threaten public health concern and cause outbreaks in human. For swine influenza virus (SIV), this dissertation was conducted to identify the occurrence of SIVs in Thai swine farms and investigate genetic diversity and evolution of Thai SIVs by whole genome sequencing. Moreover, next-generation sequencing, illumina Miseq platform, was applied to discriminate the mix-genotypes of SIVs. The information from our studies helped better understanding of current situation of SIVs in pigs in Thailand and can be used to develop appropriate prevention and control measures. For avian influenza virus (AIV), this dissertation was also conducted to investigate HPAI-H5N2 outbreak in USA. Data are suggestive of an important role of migratory birds and a fast evolving HPAI strains within an outbreak. Thus, comprehensive wild bird surveillance should be taken into consideration in developing appropriate control strategies in the future.

In chapter 2, the first phase of this dissertation, the recent occurrence of SIVs in swine farms in Thailand was investigated. During 2012-2015, the occurrence of SIVs in Thai pigs was 3.81% (139/3,646) by real-time RT-PCR. Our results indicated that swine influenza is an endemic disease in pigs in Thailand. This chapter also included the second objective of this dissertation which genetic characterization of SIVs was performed by whole genome sequencing. The results revealed predominant subtypes of Thai SIVs including reassortant H1N1 and reassortant H3N2 which containing TRIG and endemic HA and NA genes. It is noted that, there were no significant mutations related to virulence and resistant to antiviral drugs of the viruses in this study. This observation

suggested that there was a significant diversity and rapid evolution of Thai SIVs. Based on the results from this study, further swine influenza surveillance is critical for monitoring novel reassortant SIVs in Thai swine populations and their potential to spread to humans.

In chapter 3, mix-genotypes of SIVs in Thai swine farms was investigated. Nasal swab samples were collected from 4 commercial swine farms (A, B, C and D) with a history of multiple subtypes of SIVs during 2012-2013. Eighteen samples (18/145, 12.41%) were influenza positive by virus isolation and then further investigated by illumina sequencing to confirm mix-genotypes. One sample (A-S2) showed mix-genotypes between endemic Thai SIV-H1N1 and rH3N2 containing TRIG. One sample (D-S15) posed mix-genotypes between rH1N1 and rH3N2 both containing TRIG. It is interesting to note that one sample (C-S12) had unique subtype, rH3N1, which is an uncommon subtype in Thailand. The results also showed that Thai SIV (B-S10) posed NA2 closely related to new common ancestor in Thailand, A/Sydney/5 /1997. The results also revealed the first report of significant amino acid mutations (PB2-627K) related to virulence of Thai SIVs. Based on the results from this study, Illumina sequencing is able to provide comprehensive data of whole genome sequences and to monitor the reassortment event of SIVs in swine farms. The results from this study provided new insight information of SIV evolution in Thailand.

In chapter 4, highly pathogenic avian influenza (HPAI) subtype H5N2 in USA was investigated. This study provided strong evidences that migratory bird play an important role of cross-continent transmission of the viruses. The HPAI-H5N2 origin was speculated in Alaska where overlapping of migratory flyways, gathering of wild and domestic birds (Ramey, et al., 2016a). In addition, mean substitutional rate of 8 gene segments during the course of outbreak was estimated to be between 0.702 – 1.655 x 10^{-2} which greater than 5.000x10-3 substitution/site/year. This was higher than the rates estimated in long term analysis (Vijaykrishna, et al., 2008). In Thailand, there is no reports of HPAI for more than a decade, however active surveillance in live bird markets in

Thailand reported various LPAI subtypes in poultry (H4N6, H4N9 and H7N6), free grazing ducks (H1N3 and H1N9) and wild birds (H12N1) (Chaiyawong, et al., 2016; Jairak, et al., 2016; Wisedchanwet, et al., 2011; Wongphatcharachai, et al., 2012). Moreover, emerging of H5 clade 2.3.4.4 in East Asia and neighboring countries should not be ignored (Claes, et al., 2016). Local and transboundary animal movements should be focused for avian influenza prevention and control in the country. Thus monitoring and surveillance for avian influenza should be routinely implemented.

In conclusion, this dissertation provided the benefits of routine surveillance program of animal influenza. Moreover, information sharing about animal influenza status should be encouraged among stakeholders including farmers, vet practitioners, local authorities and consumers.

From this study, the recommendations for animal influenza prevention and control have been proposed following:

Swine influenza

- Farm workers, owners, vet practitioners and those who contact with pigs should be received seasonal human flu vaccination to reduce the risk of influenza infection from and/or to farms.
- Genetic diversity of swine influenza viruses may affected the sensitivity and specificity of diagnostic tests. Thus the diagnostic protocols should be regularly reviewed to update the reference viruses in use.
- Swine influenza surveillance should be continuously conducted to provide an early detection of virus evolution. Rapid evolution of the SIVs may rising the risk for animal and/or human health.

- Information sharing should be encouraged to all level of relevant persons to bring awareness of SIVs infection in animals and human.

Avian influenza: USA outbreak relay learning and recommendations to Thailand

- In USA, migratory wilds birds play an important role on avian influenza transmission. In contrast, Thailand has low prevalence of AIV in wild birds. Thus, wild birds may not a potential source of outbreak in Thailand.
- In Thailand, no HPAI-H5 has been reported for almost ten years however outbreak in neighboring countries has occasionally reports. Therefore, transboundary animal movements could play an important role of transmission and rigorous enforcement should be implement.
- The successful of HPAI-H5 investigation is influenced by data richness and accessibility. So active surveillance should be routinely conducted in high risk areas of avian influenza and data sharing should be concern.

Further studies

- Investigation of phenotypic characterization of novel SIVs
- Development of rapid diagnostic test kit using in field setting

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APPENDIX

APPENDIX A

Criteria of sample collection from pigs

1. Criteria of pig farm selection

In cross-sectional swine influenza surveillance

- Commercial pig farm locate in top ten pig production provinces
- Farm owner cooperate in the study

In serial cross-sectional swine influenza study

- The commercial pig farm that has a history of multiple subtypes of swine influenza virus co-circulation.
- Farm owner cooperate in the study

2. Sample collection from pigs

- Pigs with respiratory symptoms such as cough, abdominal breath, presence of serious nasal and ocular discharge.
- Each pig was collected two kinds of sample; nasal swab and blood sample.
    - The nasal swab was performed by using a sterile rayon-tipped swabs (Puritan®, USA) inserted into the pig's nostril in the dorsomedial direction. Then the swab will be placed into the 2 ml. viral transportation medium and packed in ice for transportation. The samples were transported to the laboratory at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University within 24 hours.
    - The blood sample of approximately 5 ml. was collected via the jugular vein.

APPENDIX B

Nucleotide sequences

Table A1 Collection of nucleotide sequences related to this dissertation

#	Strain name	Gene segment								Genbank Accession number
		1	2	3	4	5	6	7	8	
1	A/swine/Thailand/CU-S3073N/2011									KJ162042 – 43 KM355361 - 66
2	A/swine/Thailand/CU-S3334N/2012									KJ162027 – 33 KJ162045 - 46
3	A/swine/Thailand/CU-S3350N/2012									KJ162034 - 41
4	A/swine/Thailand/CU-S3406N/2012									KJ526053 - 59 KM355356
5	A/swine/Thailand/CU-S3474N/2012									KM355369 - 76
6	A/swine/Thailand/CU-S3629N/2012									KJ526067 - 73 KM355357
7	A/swine/Thailand/CU-S3631N/2012									KJ526039 - 44 KM355367 - 68
8	A/swine/Thailand/CU-S3673N/2012									KJ526061 – 66 KM355377 – 78
9	A/swine/Thailand/CU-S3689N/2013									KJ526048 - 52 KM355379 - 81
10	A/swine/Thailand/CU-S3795N/2013									KJ526034 - 38 KM355358 - 60
11	A/swine/Thailand/CU-S14129N/2013									KM355382 - 89
12	A/swine/Thailand/CU-S14252N/2014									KM355390 - 97
13	A/swine/Thailand/A-S1/20									
14	A/swine/Thailand/A-S2_BG/20									
15	A/swine/Thailand/A-S2_MG/20									
16	A/swine/Thailand/A-S3/20									
17	A/swine/Thailand/A-S4/20									
18	A/swine/Thailand/A-S5/20									
19	A/swine/Thailand/A-S7/20									
20	A/swine/Thailand/B-S8/20									
21	A/swine/Thailand/B-S9/20									
22	A/swine/Thailand/B-S10/20									
23	A/swine/Thailand/B-S11/20									

#	Strain name	Gene segment 1 2 3 4 5 6 7 8	Genbank Accession number
24	A/swine/Thailand/B-S12_BG/20		
25	A/swine/Thailand/B-S12_MG/20		
26	A/swine/Thailand/C-S13/20		
27	A/swine/Thailand/C-S14/20		
28	A/swine/Thailand/D-S15_BG/20		
29	A/swine/Thailand/D-S15_MG/20		
30	A/swine/Thailand/D-S16/20		
31	A/swine/Thailand/D-S17/20		
32	A/swine/Thailand/D-S18/20		
33	A/environment/Minnesota/E1/2015		KY341927 – 34
34	A/environment/Minnesota/E2/2015		KY341935 - 42
35	A/turkey/Minnesota/E3/2015		KY341943 - 50
36	A/turkey/Minnesota/E4/2015		KY341951 – 58
37	A/environment/Minnesota/E5/2015		KY341959 – 66
38	A/turkey/Minnesota/E6/2015		KY341967 – 74
39	A/environment/Minnesota/E7/2015		KY341975 - 82
40	A/environment/Minnesota/E8/2015		KY341983 - 90
41	A/environment/Minnesota/E9/2015		KY341991 – 98
42	A/environment/Minnesota/E10/2015		KY341999 – 342006
43	A/turkey/Minnesota/E11/2015		KY342007 – 14
44	A/turkey/Minnesota/E12/2015		KY342015 – 22
45	A/turkey/Minnesota/E13/2015		KY342023 – 30
46	A/turkey/Minnesota/E14/2015		KY342031 – 38
47	A/environment/Minnesota/E15/2015		KY342039 – 46
48	A/environment/Minnesota/E16/2015		KY342047 – 54
49	A/turkey/Minnesota/E17/2015		KY342055 – 62
50	A/turkey/Minnesota/E18/2015		KY342063 – 70
51	A/environment/Minnesota/M1/2015		KY342071 – 78
52	A/environment/Minnesota/M2/2015		KY342079 – 86
53	A/environment/Minnesota/M3/2015		KY342087 – 94
54	A/environment/Minnesota/M4/2015		KY342095 – 342102
55	A/environment/Minnesota/M5/2015		KY342103 – 10
56	A/environment/Minnesota/M6/2015		KY342111 – 18
57	A/environment/Minnesota/M7/2015		KY342119 – 26
58	A/environment/Minnesota/M8/2015		KY342127 – 34
59	A/environment/Minnesota/M9/2015		KY342135 – 42
60	A/environment/Minnesota/M10/2015		KY342143 – 50
61	A/environment/Minnesota/M11/2015		KY342151 – 58

#	Strain name	Gene segment								Genbank Accession number
		1	2	3	4	5	6	7	8	
62	A/environment/Minnesota/M12/2015									KY342159 – 66
63	A/environment/Minnesota/M13/2015									KY342167 – 74
64	A/turkey/Minnesota/M14/2015									KY342175 – 82
65	A/turkey/Minnesota/M15/2015									KY342183 – 90
66	A/turkey/Minnesota/M16/2015									KY342191 – 98
67	A/turkey/Minnesota/M17/2015									KY342199 – 342206
68	A/environment/Minnesota/M18/2015									KY342207 – 10
69	A/environment/Minnesota/M19/2015									KY342211 – 14
70	A/environment/Minnesota/L1/2015									KY342215 – 18
71	A/environment/Minnesota/L2/2015									KY342219 – 24
72	A/environment/Minnesota/L3/2015									KY342225 – 31
73	A/environment/Minnesota/L4/2015									KY342232 – 39
74	A/environment/Minnesota/L5/2015									KY342240 – 47
75	A/environment/Minnesota/L6/2015									KY342248 – 55
76	A/environment/Minnesota/L7/2015									KY342256 – 57
77	A/environment/Minnesota/L8/2015									KY342258 – 65
78	A/environment/Minnesota/L9/2015									KY342266 – 73

Note: White and grey boxes indicate available and unavailable sequencing data on each segment, respectively.

APPENDIX C

Single letter code for amino acids

A	Alanine	Ala
C	Cysteine	Cys
D	Aspartic acid	Asp
E	Glutamic acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

APPENDIX D

Genetic diversity of swine influenza viruses in Thai swine farms, 2011-2014

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Keywords: Genetic diversity, Swine Influenza, Surveillance, Thailand

Virus Genes (2015) 50:221–230
DOI 10.1007/s11262-014-1153-x

Genetic diversity of swine influenza viruses in Thai swine farms, 2011–2014

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Received: 19 August 2014 / Accepted: 2 December 2014 / Published online: 12 December 2014

Abstract The pig is known as a "mixing vessel" for influenza A viruses. The co-circulation of multiple influenza A subtypes in pig populations can lead to novel reassortant strains. For this study, swine influenza surveillance was conducted from September 2011 to February 2014 on 46 swine farms in Thailand. In total, 78 swine influenza viruses were isolated from 2,821 nasal swabs, and 12 were selected for characterization by whole genome sequencing. Our results showed that the co-circulation of swine influenza subtypes H1N1, H3N2, and H1N2 in Thai swine farms was observable throughout the 3 years of surveillance. Furthermore, we repeatedly found reassortant viruses between endemic swine influenza viruses and pandemic H1N1 2009. This observation suggests that there is significant and rapid evolution of swine influenza viruses in swine. Thus, continuous surveillance is critical for monitoring novel reassortant influenza A viruses in Thai swine populations.

Keywords Genetic diversity · Reassortant · Swine influenza · Surveillance · Thailand

Introduction

The influenza A virus (IAV) belongs to the family *Orthomyxoviridae*. IAVs can be classified into subtypes based on two major surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Currently, 18 HA and 11 NA subtypes have been identified [1], while IAV subtypes H1N1, H1N2, and H3N2 have been reported in swine populations worldwide [2]. In Thailand, the endemic swine influenza virus (SIV) subtype H1N1 was first reported in 1991 [3]. The genetic composition of Thai endemic SIV-H1N1 (eH1N1) has been characterized as eH1N1 (6 + 2) and eH1N1 (7 + 1) [4]. The genetic composition of Thai endemic SIV-H3N2 (eH3N2), however, has been

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characterized as of Eurasian swine lineage (PB2, PB1, PA, and M), classical swine lineage (NP and NS), and of seasonal human H3N2 origin (H3 and N2) [5, 6].

In April 2009, pandemic H1N1 (pH1N1) was first reported in humans and quickly spread worldwide. It was first isolated from Thai pigs in November 2009 [7], and subsequent surveillance in Thailand detected a novel reassorted SIV in 2010 [4]. Because of the ongoing circulations of multiple SIV lineages among Thai pigs and the evidence of viral reassortment in swine, SIV surveillance in Thailand should be a priority. This study conducted 3 years of SIV surveillance on Thai swine farms and found SIV subtypes H1N1, H1N2, and H3N2 circulating among pigs. Observations of the reassortant SIVs, rH1N1, rH1N2, and rH3N2, however, were predominant. The genetic diversity of those reassortant viruses is described herein.

Materials and methods

Surveillance of Thai swine farms

Between September 2011 and February 2014, a cross-sectional SIV surveillance program was conducted at Thai swine farms located in 13 high swine density provinces representing all parts of Thailand. In total, 2,821 nasal swab samples were obtained from 46 swine farms. The samples were collected individually from pigs of different ages and transported within 24 h for laboratory analysis. During transport, each sample was kept in a standard viral transport medium encased in ice. Details of samples and farm locations are shown in Table 1.

Identification and isolation of SIVs

All nasal swabs were screened for IAVs through one-step real-time RT-PCR (rRT-PCR). Viral RNAs were extracted from samples with the QIAamp Viral RNA Mini Kit (Qiagen®; Hilden, Germany). rRT-PCR was conducted using a TaqMan probe to detect the IAV matrix (M) gene with some modification [8]. One-step rRT-PCR was performed on a Rotor-Gene 3000 (Corbett Research; Sydney, Australia) utilizing the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen™; California, USA). Data acquisition and analysis of the rRT-PCR assay were done through the Rotor-Gene software, v.6.0.19. Samples exhibiting a Ct value of <36 were interpreted as positive and those with a Ct value of 36–40 as suspect.

The positive rRT-PCR samples were then subjected to IAV isolation using egg inoculation and/or cell culture. For egg inoculations, we inoculated embryonated chicken eggs according to WHO recommendations [9]. After a 72-h incubation period, the allantoic fluid of each egg was collected and tested for hemagglutinin activity with a hemagglutination test (HA test) using a 1 % suspension of chicken red blood cells. For cell culture, Madin Darby Canine Kidney (MDCK) cells were used for viral propagation. During the incubation period of 72 h, we made daily observations for cytopathic effect (CPE) and, after incubation, collected the supernatants of CPE positives. Samples that tested positive by HA test at 4 HA units/50 µl or more and CPE positive cell supernatants were subsequently subjected to IAV confirmation by rRT-PCR for M gene detection as previously described.

Genetic characterization of Thai SIVs

To subtype IAVs, cDNA was synthesized using the influenza universal primer Uni12 [10] and the ImProm-II™ Reverse Transcription System (Promega; Wisconsin, USA). The cDNA served as a template for subtype identification using specific primers in our inventory for the HA and NA genes. In this study, 12 viruses were selected based on epidemiological data as representatives for whole

Table 1 Description of samples, virus identification, virus isolation, and virus subtyping

Year	# of samples	# of farms (provinces)	rRT-PCR (% positive)	SIV isolation (% positive)	SIV subtype (# positive sample)		
					H1N1	H1N2	H3N2
2011[a]	174	9 (4)	28/174 (16.09 %)	4/174 (2.29 %)	0	1	3
2012	1,219	19 (8)	109/1,219 (8.94 %)	28/1,219 (2.30 %)	12	2	14
2013	1,345	32 (10)	42/1,345 (3.12 %)	39/1,345 (2.89 %)	19	0	20
2014[b]	83	3 (3)	9/83 (10.84 %)	7/83 (8.43 %)	5	0	2
	2,821		188/2,821 (6.66 %)	78/2,821 (2.76 %)	36	3	39

[a] 2011: sample collection in September–December

[b] 2014: sample collection in January–February

Table 2 Description of selected SIVs characterized in this study

Virus	Subtype	Year	Age (weeks)	Type of farm[a]	Location	GenBank Accession No.
A/Swine/Chonburi/NIAH9469/04[b]	eH1N1 (6 + 2)	2004	–	–	Chonburi	AB434301-08
A/Swine/Thailand/CU-S3334/12	eH1N1 (6 + 2)	2012	8	C	Chonburi	KJ162027-33, KJ162046
A/Swine/Thailand/CU-S3350/12	eH1N1 (6 + 2)	2012	6	D	Ratchaburi	KJ162034-41
A/Swine/Thailand/CU-S3406/12	eH1N1 (6 + 2)	2012	4	D	Ratchaburi	KJ526053-59, KM355356
A/Swine/Thailand/CU-S3629/12	rH1N1 (TRIG + 2)	2012	4	B	Nakhon Pathom	KJ526067-73, KM355357
A/Swine/Thailand/CU-S3795/13	rH1N1 (TRIG + 2)	2013	4	B	Nakhon Pathom	KJ526034-38, KM355358-60
A/Swine/Saraburi/NIAH13021/05[c]	eH1N2	2005	–	–	Saraburi	AB434333-40
A/Swine/Thailand/CU-S3073/11	rH1N2 (7 + 1)	2011	4	D	Ratchaburi	KJ162042-43, KM355361-66
A/Swine/Thailand/CU-S3631/12	rH1N2 (TRIG + 2)	2012	4	B	Nakhon Pathom	KJ526039-44, KM355367-68
A/Swine/Thailand/KU5.1/04[d]	eH3N2	2004	–	–	–	FJ561057-64
A/Swine/Thailand/CU-S3474/12	rH3N2 (TRIG + 2)	2012	8	C	Chonburi	KM355369-76
A/Swine/Thailand/CU-S3673/12	rH3N2 (TRIG + 2)	2012	4	D	Chonburi	KJ526061-66, KM355377-78
A/Swine/Thailand/CU-S3689/13	rH3N2 (TRIG + 2)	2013	4	A	Chonburi	KJ526048-52 KM355379-81
A/Swine/Thailand/CU-S14129/13	rH3N2 (TRIG + 2)	2013	4	D	Ratchaburi	KM355382-89
A/Swine/Thailand/CU-S14252/14	rH3N2 (TRIG + 2)	2014	4	D	Ratchaburi	KM355390-97

[a] Type of farm abbreviation; A = <50-sow herd, B = 51–200-sow herd, C = 201–500-sow herd, D = >500-sow herd

[b] Reference Thai eH1N1; A/swine/Chonburi/NIAH9469/04 [5] was included in the analysis

[c] Reference Thai eH1N2; A/swine/Saraburi/NIAH13021/05 [5] was included in the analysis

[d] Reference Thai eH3N2; A/swine/Thailand/KU5.1/04 [6] was included in the analysis

genome sequencing. Each viral gene was amplified using specific primers, and then PCR products were subjected to DNA sequencing (1st Base Laboratories Sdn Bhd, Malaysia). The nucleotide sequences of each gene were validated and assembled in SeqMan software v.5.03 (DNASTAR Inc.; Wisconsin, USA).

Phylogenetic and genetic analyses were performed by comparing each viral gene segment with reference SIV sequences available at the GenBank database. The reference nucleotide sequences that were retrieved included all geographical origins (Eurasia and North America) and three host origins (human, swine, and avian) for constructing phylogenetic trees. The nucleotide sequences of each gene were aligned in Muscle v.3.6 [11]. The phylogenetic trees were constructed with two software: MEGA v.6.0, using the neighbor-joining algorithm with the Kimura-2 parameter model applied to 1,000 replications of bootstrap, and BEAST software, using the BMCMC with 1,000,000 generations and an average standard deviation of split frequencies <0.05 [12, 13]. To support tree topology, the bootstrap percentages and posterior probabilities were evaluated. The nucleotide sequences and deduced amino acids of each viral gene were aligned and compared in MegAlign software v.5.03 (DNASTAR Inc.; Wisconsin, USA). The nucleotide sequences of the Thai SIVs were submitted to the GenBank database under the accession numbers shown in Table 2.

Results

Prevalence and subtypes of SIVs in Thai swine farms

During our 3 years of SIV surveillance in Thai swine farms, 2,821 nasal swab samples were collected and examined. 188 (6.66 %) were identified as IAV positive through rRT-PCR. Subsequently, 78 SIV isolates (41.5 %) were successfully recovered from IAV-positive samples by egg inoculation and/or MDCK cell culture (Table 2). Further identification of the 78 SIV isolates revealed the subtypes H1N1 (36; 46.15 %), H1N2 (3; 3.85 %), and H3N2 (39; 50.00 %). These results suggest that SIV subtypes H1N1 and H3N2 were the predominant subtypes in Thai swine populations (Table 1).

Genetic characteristics of Thai SIV

Out of the 78 SIV isolates, 12 were selected for whole genome sequencing based on epidemiological data such as location, influenza subtype, year of isolation, age of pig, and type of swine farm (Table 2). The twelve SIVs characterized in this study were of the subtypes H1N1 ($n = 5$), H1N2 ($n = 2$), and H3N2 ($n = 5$).

In general, the H1 gene of SIVs can be phylogenetically grouped into two major lineages: classical and Eurasian. The classical lineage can be further divided into four

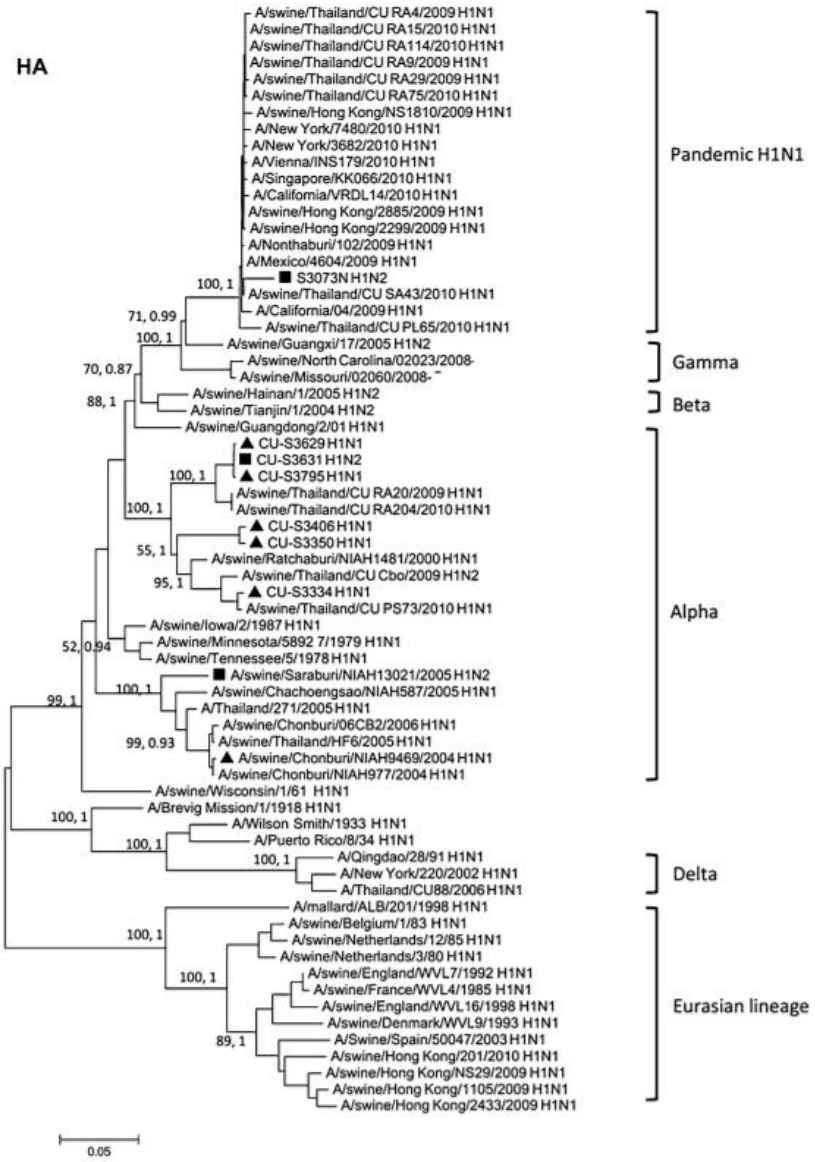

Fig. 1 Phylogenetic analysis of the H1. The phylogenetic tree was constructed with the neighbor-joining algorithm and the Kimura-2 parameter model applied to 1,000 replications of bootstrap and with the BMCMC. Node label shows the bootstrap percentage and posterior probabilities in *parenthesis* (bootstrap percentage, posterior probability). *Triangle* and *quadrilateral* indicate SIV-H1N1 and SIV-H1N2, respectively

sub-lineages: alpha, beta, gamma, and delta [14]. Phylogenetic analysis of the H1 gene revealed that six Thai SIVs (CU-S3334, CU-S3350, CU-S3406, CU-S3629, CU-S3795, and CU-S3631) were clustered into the alpha group of the classical lineage, while only one (CU-S3073) was grouped into the pandemic cluster, which is a member of the gamma group of the classical lineage (Fig. 1). Phylogenetic analysis of the N1 gene showed that all five Thai H1N1 SIVs belonged to the Eurasian lineage.

In general, the H3 gene of SIVs can be grouped into Ha and Hb subgroups in which H3 can be evolved from either a human H3N2 strain circulating in late 1990s or human-like H3N2 swine strain circulating in early 1970s [5]. In this study, the H3 genes of five H3N2 SIVs (CU-S3474, CU-S3673, CU-S3689, CU-S14129, and CU-S14252) were clustered into the Ha subgroup of human H3N2 lineage (Fig. 2). Similarly, seven of the N2 genes of H1N2 (CU-S3073 and CU-S3631) and H3N2 SIVs (CU-S3474,

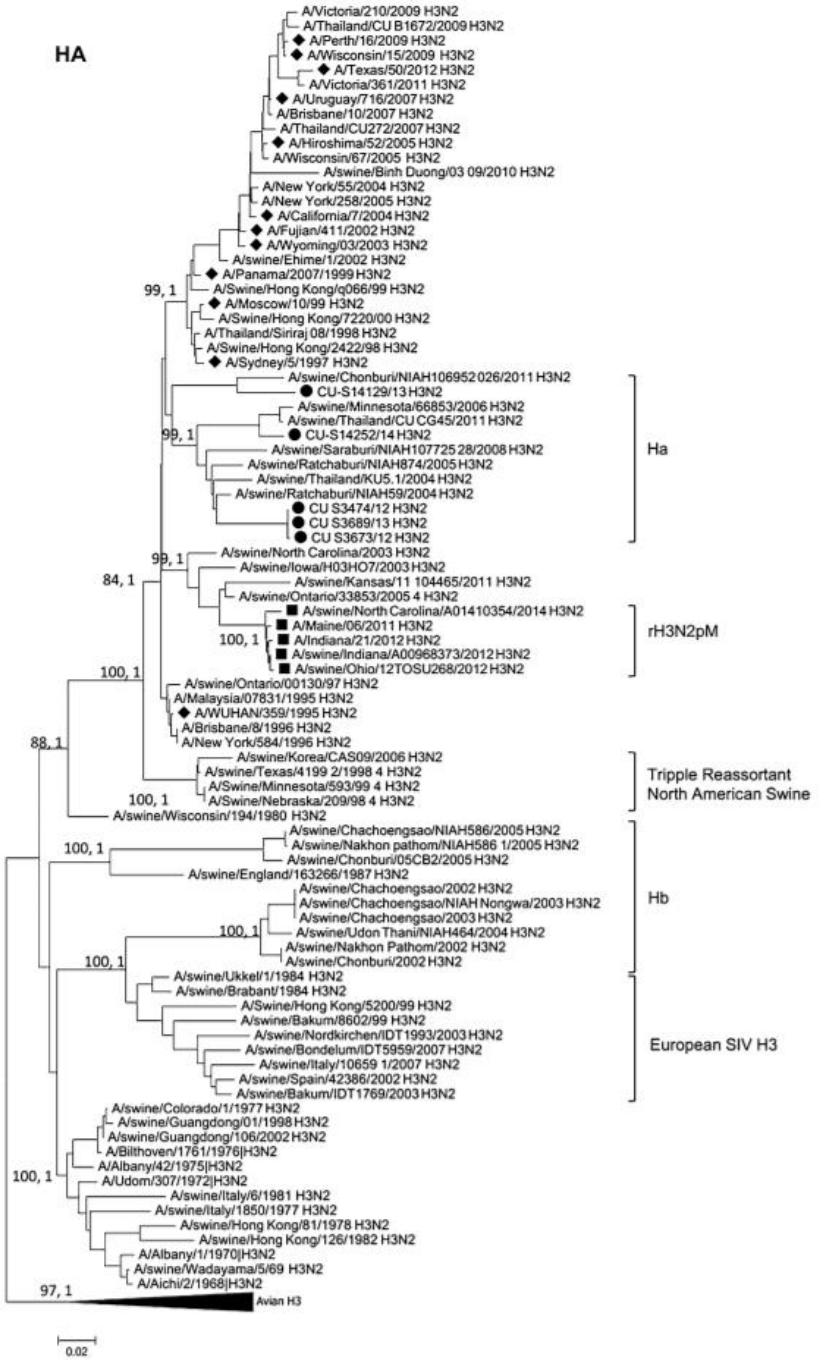

Fig. 2 Phylogenetic analysis of the H3. The phylogenetic tree was constructed with the neighbor-joining algorithm and the Kimura-2 parameter model applied to 1,000 replications of bootstrap and with the BMCMC. Node label shows the bootstrap percentage and posterior probabilities in *parenthesis* (bootstrap percentage, posterior probability). *Diamond*, *circle*, and *squares* indicate seasonal human vaccine strains H3N2, SIV-H3N2 in this study, and H3N2pM, respectively

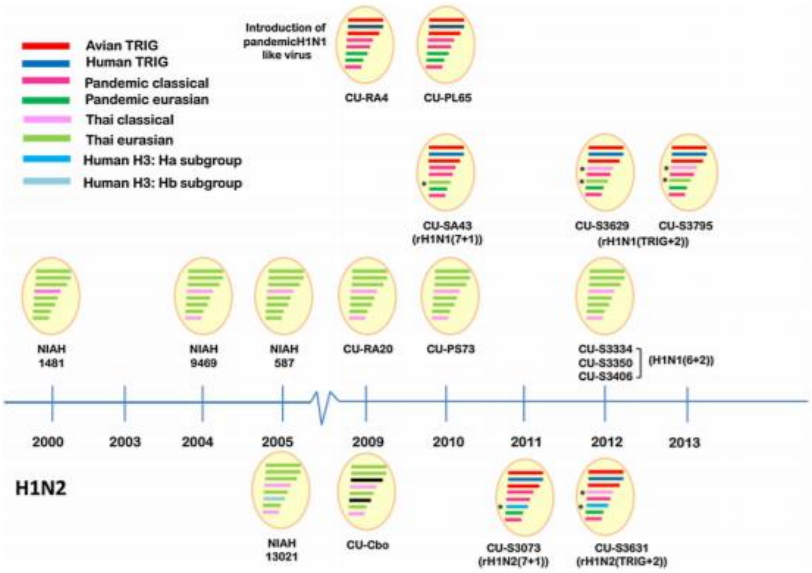

Fig. 3 Schematic representation of the genetic constellation of Thai SIV-H1N1 and SIV-H1N2 during 2000–2013. The *oval* represents viral particle, and each *line* represents each gene segment ascending from segment 1 to segment 8, respectively. The lineages of gene segment present in different *colors*. The reassortant segments are emphasized by shape outline (Color figure online)

CU-S3673, CU-S3689, CU-S14129, and CU-S14252) were clustered into the human H3N2 lineage.

Overall, five, distinct genetic constellations of Thai SIVs were observed in this study: eH1N1 (6 + 2), rH1N1 (TRIG + 2), rH1N2 (7 + 1), rH1N2 (TRIG + 2), and rH3N2 (TRIG + 2). Based on previous reports from Thailand, Thai endemic SIV-H1N1 (eH1N1) has only two genetic constellations. The first genetic constellation is eH1N1 (7 + 1), comprised the H1 gene from the classical lineage and seven other genes from the Eurasian lineage. The second genetic constellation is eH1N1 (6 + 2), comprised the H1 and NS genes of the classical lineage and six other genes from the Eurasian lineage [4]. Both eH1N1 (7 + 1) and eH1N1 (6 + 2) were circulating among Thai swine populations until 2005. Subsequently, eH1N1 (7 + 1) disappeared, while eH1N1 (6 + 2) was continuously observed until 2012. In this study, we observed both eH1N1 (6 + 2) (CU-S3334, CU-S3350, and CU-S3406) and reassortant H1N1 viruses (rH1N1) (CU-S3629 and CU-S3795) (Fig. 3). The rH1N1 viruses contained the TRIG cassette of pH1N1 as well as the H1 and N1 genes of Thai endemic SIVs (TRIG + 2) (Fig. 3).

The genetic constellation of Thai endemic SIV-H1N2 (eH1N2) in 2005 (NIAH13021) had five genes of the Eurasian lineage (PB2, PB1, PA, NP, and M), two genes of the classical lineage (H1 and NS), and an N2 gene of human origin. In this study, we observed two types of rH1N2 during 2011 and 2012. The first rH1N2 (CU-S3073) contained seven genes from pH1N1 with an N2 gene from eH1N2 and was designated as 7 + 1. The second rH1N2 (CU-S3631), containing the TRIG cassette and the H1 and N2 genes from eH1N2, was designated as TRIG +2 (Fig. 3).

Thai endemic SIV-H3N2 (eH3N2) in 2004 and 2005 had two distinct genetic constellations. The first constellation comprised PB2, PB1, PA and M of the Eurasian lineage, NP and NS of the classical lineage and H3 and N2 of human H3N2 origin (KU5.1). The second constellation comprised PB2, PB1, PA, M, and NS of the Eurasian lineage, NP of the classical lineage, and H3 and N2 of human H3N2 origin (NIAH586-1). In this study, we found that rH3N2 (TRIG + 2) was predominant in Thai pigs from 2011 to 2014. The viruses (CU-S3474, CU-S3673, CU-S3689, CU-S14129, and CU-S14252) contained the TRIG cassette with H3 and N2 genes from eH3N2 (Fig. 4).

Genetic analyses of SIVs characterized in this study are shown in Tables 3 and 4. The seven SIV H1 genes characterized in this study were compared with four reference viruses: eH1N1 (NIAH9469), pH1N1 (CA/04 and CU-RA4), and eH1N2 (NIAH13021). Our results showed that the H1 viruses were divided into either pandemic (P) or alpha (α) clusters. H1 SIVs of the alpha cluster exhibit high amino acid diversity at five antigenic sites: Sa, Sb, Ca1, Ca2, and Cb. In contrast, only one amino acid change at the Sa antigenic site (G158E) was observed in H1 SIVs of the pandemic cluster. Analysis of the receptor binding site showed that recent (2011–2014) Thai H1 SIVs contained aspartic acid (D) at positions 190 and 225, indicating preferential binding to the SA α2,6 receptor. In contrast, older (2004–2005) Thai H1 SIVs contained glycine (G) at position 225 (Table 3).

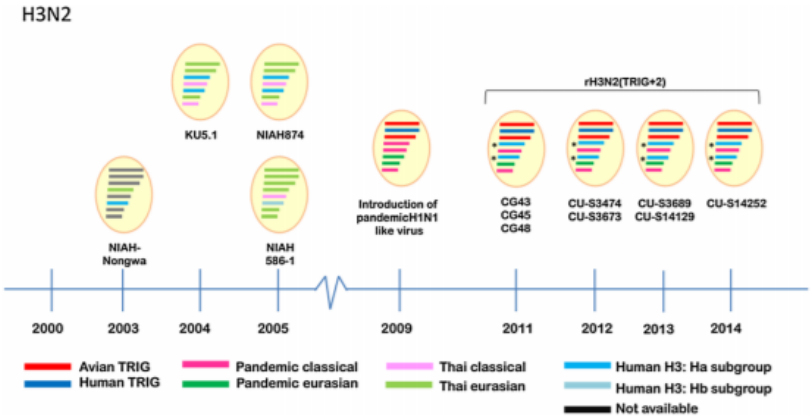

Fig. 4 Schematic representation of the genetic constellation of SIV-H3N2 during 2003–2014. The *oval* represents viral particle and each *line* in *oval* represents each gene segment ascending from segment 1 to segment 8. The lineages of gene segment present in different *colors*. The reassortant segments are emphasized by shape outline (Color figure online)

Five H3 SIVs were compared with two reference viruses: eH3N2 (KU5.1) and the human H3N2 vaccine strain (Wuhan/359). Three (CU-S3474, CU-S3673, and CU-S3689) had no amino acid changes at five antigenic sites, although the viruses were isolated from different farms. One H3 SIV (CU-S14129) had amino acid changes at four antigenic sites: A, B, C, and E. This observation corresponded well with the phylogenetic analysis result in which CU-S14129 was clustered away from the main group. In analyses of the receptor binding site, all H3 SIVs had isoleucine (I) at position 226 and serine (S) at position 228, which is similar to the reference H3 viruses and indicates Thai H3 SIVs prefer to bind to the SA α2,6 receptor.

Genetic analysis of the NA gene on Oseltamivir resistance related to E119V, H275Y, R293K, and N295S on N1 and N146K, S219T, A272V, and 245–248 deletion on N2 [15]. The results showed that all SIVs in this study had an amino acid referred to Oseltamivir susceptibility. Genetic analysis of the N2 showed that all five Thai H3N2 SIVs contained valine (V) at position 275 (Data not shown) that may increase SA α2,6 receptor specificity [16]. Moreover, analysis of virulence determinants on PB2 (E627K and N701D) and NS1 (E92D) [17] showed no significant amino acid changes in particular positions.

Discussion

From 2011 to 2014, our SIV surveillance revealed that reassortant SIVs are a dominant subtype circulating among Thai pigs. Previous studies, however, reported that pH1N1 was a major SIV subtype between 2010 and 2011 [18, 19]. In Thailand, the first reassortant SIV between pH1N1 and Thai SIVs was reported in 2010 (CU-SA43) with a genetic constellation of seven genes from pH1N1 and an N1 gene from eH1N1 [4]. In this study, we reported that novel rH1N1 (TRIG + 2) has become a dominant variant of SIV-H1N1. We identified rH1N2 (7 + 1) (CU-S3073) in October 2011 and rH1N2 (TRIG + 2) (CU-S3631) in December 2012. Similar results have been reported from Argentina and Japan. In Argentina, rH1N1 and rH1N2 containing the TRIG cassette and human-like H1 and N1/N2 were reported between 2009 and 2010 [20]. In Japan, rH1N2 containing seven genes from pH1N1 and an N2 from Japanese SIV was reported in 2012 [21]. For SIV-H3N2, rH3N2 (TRIG + 2) was a dominant SIV subtype in late 2011 in Thailand [18]. This corresponds with our finding that eH3N2 disappeared from Thai swine populations in 2011, and rH3N2 (TRIG + 2) has dominated since. These observations indicate that Thai SIVs, after the introduction of pH1N1, have evolved by maintaining the TRIG cassette and retrieving other genes from endemic SIVs in their virus gene pools. This confirms the hypothesis that the TRIG cassette has a very high potential for viral infection, replication, and transmission within pig populations. The TRIG cassette in the virus particle is very compatible, and the virus changes its surface proteins for escaping host immunity [22]. In contrast, rH3N2pM was reported in the USA with a different constellation: PB2, PB1, PA, HA, NP, NA, and NS relate to TRIG SIV-H3N2 and carry M from pH1N1 [23].

It should be noted that SIV-H1N1 and SIV-H1N2 took approximately 3 years (November 2009 to December 2012) for adaptation to the TRIG + 2 constellation. During this period, we observed both rH1N1 (7 + 1) and rH1N2 (7 + 1) constellations. In contrast, SIV-H3N2 took a shorter time (15 months; November 2009 to February 2011) to settle its genetic constellation. This evidence supports the theory that the pig is a mixing vessel for novel viruses which could potentially exhibit high virulence or cause pandemics. Strict biosecurity is therefore an

Table 3 Genetic analysis of the H1 gene of Thai SIVs in this study

Viruses	Subtype	HA cluster	Amino acid sequence alignment of H1 gene													GenBank Accession No.
			Antigenic site										Receptor binding site		HA cleavage site	
			Sa			Sb	Ca1			Ca2		Cb	190[a]	225[a]	325–333[a]	
			128–129[a]	156–160[a]	162–167[a]	187–198[a]	169–173[a]	206–208[a]	238–240[a]	140–145[a]	224–225[a]	78-83[a]				
A/Swine/Chonburi/NIAH9469/04	eH1N1	α[b]	PN	KKGNS	PKLRKA	TNTDQQSLYQNA	VNNKK	GSS	EPG	PYAGTN	RG	LFAVNS	D	G	PSIQSRGLF	AB434304
A/California/04/09	pH1N1	P[c]	PN	KKGNS	PKLSKS	TSADQQSIYQNA	INDKG	GSS	EPG	PHAGAK	RD	LSTASS	D	D	PSIQSRGLF	GQ280797
A/Swine/Thailand/CU-RA4/09	pH1N1	P	PN	KKGNS	PKLSKS	TSADQQSLYQNA	INDKG	GTS	EPG	PHAGAK	RD	LSTASS	D	D	PSIQSRGLF	CY062308
A/Swine/Thailand/CU-S3334/12	eH1N1	α	PN	KKGNS	PKLSKS	TSTDQQSLYQNA	VNNKK	SSS	EPG	HHAGAK	RD	LFKANS	D	D	PSIQSRGLF	KJ162030
A/Swine/Thailand/CU-S3350/12	eH1N1	α	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	KJ162037
A/Swine/Thailand/CU-S3406/12	eH1N1	α	PN	KKANS	PKLSKS	TITDQQSLYQNT	LNNKK	SSS	EPG	PHAGAN	RD	LFRANS	D	D	PSIQSRGLF	KM355356
A/Swine/Thailand/CU-S3629/12	rH1N1	α	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS	D	D	PSIQSRGLF	KJ526069
A/Swine/Thailand/CU-S3795/13	rH1N1	α	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS	D	D	PSIQSRGLF	KM355360
A/Swine/Saraburi/NIAH13021/05	eH1N2	α	PD	KKGNS	PKLSKS	TDTDQQSLYQNV	VNDKK	GSS	EPG	PYAGTN	RG	LFEVNS	D	G	PSIQSRGLF	AB434336
A/Swine/Thailand/CU-S3073/11	rH1N2	P	PN	KKENS	PKLSKS	TSADQQSIYQNA	INDKG	GSS	EPG	PHAGAK	RD	LSTASS	D	D	PSIQSRGLF	KJ162042

Table 3 continued

Viruses	Subtype	HA cluster	Amino acid sequence alignment of H1 gene													GenBank Accession No.
			Antigenic site										Receptor binding site		HA cleavage site	
			Sa			Sb	Ca1			Ca2		Cb	190[a]	225[a]	325–333[a]	
			128–129[a]	156–160[a]	162–167[a]	187–198[a]	169–173[a]	206–208[a]	238–240[a]	140–145[a]	224–225[a]	78-83[a]				
A/Swine/Thailand/CU-S3631/12	rH1N2	α	PN	KKENS	PKISKS	TSNDQQSLYQNA	FNNKG	SSS	KPG	PYAGAN	RD	LFNANS	D	D	PSIQSRGLF	KM355368

[a] H3 numbering

[b] Alpha group of classical lineage

[c] Pandemic cluster of classical lineage

Table 4 Genetic analysis of the H3 gene of Thai SIVs in this study

Virus	Subtype	Amino acid sequence alignment of H3 gene									Genbank Accession No.
		Antigenic site							Receptor binding site		
		A	B		C	D	E				
		140–146[a]	156–161[a]	189–199[a]	277–282[a]	205–221[a]	171–175[a]	243–249[a]	226[a]	228[a]	
A/Wuhan/359/1995	hH3N2	KRGSVKS	KLEYKY	SDQTSIYVQAS	CNSECI	STKRSQQTVIPNIGSRP	NDKFD	LLINSYG	I	S	JX518888
A/Swine/Thailand/KU5.1/2004	eH3N2	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGSRP	NDKFD	LLINSTG	I	S	FJ561060
A/Swine/Thailand/CU-S3474/12	rH3N2	KRGSVKS	KLDYKY	NNQTNLYVQAS	CNYGCI	STKRSQQTVIPNIGSRP	NDKFN	LLINSTG	I	S	KJ526029
A/Swine/Thailand/CU-S3673/12	rH3N2	KRGSVKS	KLDYKY	NNQTNLYVQAS	CNYGCI	STKRSQQTVIPNIGSRP	NDKFN	LLINSTG	I	S	KJ526062
A/Swine/Thailand/CU-S3689/13	rH3N2	KRGSVKS	KLDYKY	NNQTNLYVQAS	CNYGCI	STKRSQQTVIPNIGSRP	NDKFN	LLINSTG	I	S	KJ526048
A/Swine/Thailand/CU-S14129/13	rH3N2	KRGYVNS	QSGHKY	SDQTSLYVQAS	CNSECV	STKRSQQTVIPNIGSRP	NEKFD	LLINSTG	I	S	KM355385
A/Swine/Thailand/CU-S14252/13	rH3N2	KRGSVKS	KLDYKY	SDQTNLYVQAS	CNSECI	STKRSQQTVIPNIGFRP	NDKFD	LLINSTG	I	S	KM355393

[a] H3 numbering

important measure to reduce the chance of new genetic material being introduced into swine populations.

Genetic analyses of all Thai SIV subtypes showed amino acids with preferential binding to the SA α2,6 receptor. All Thai SIV-H3N2 isolates had isoleucine (I226) instead of leucine (L226) in the HA1 region. This unique amino acid residue was observed in human H3N2 from China and Japan in 1994 and 1995, indicating the potential risk for human infection with Thai SIV-H3N2 [24]. This observation supports the idea that the Ha subgroup of H3 originated during the late 1990s from human H3N2 to become a dominant cluster. Based on our observation that no significant amino acid mutations occurred, it should be noted that influenza vaccination was incomprehensive practices in Thai swine farms.

In summary, the reassortant SIVs have become predominant among SIVs circulating in Thai pig populations since the introduction of pH1N1 2009. This observation suggests that there was a significant diversity and rapid evolution of Thai SIVs during the past 3–4 years. Further swine influenza surveillance is critical for monitoring the novel reassortant SIVs in Thai swine populations and their potential to spread to humans.

Acknowledgments This research was supported, in part, by the National Research University Project of the Office of Higher Education Commission (WCU005-HR57), the Ratchadapiseksomphot Endowment Fund of Chulalongkorn University (RES560530129-HR), and the National Research Council of Thailand. The authors would like to thank Chulalongkorn University for its financial support to the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals and for a Dutsadi Phiphat scholarship to NN. We also would like to thank the Thailand Research Fund for its financial support to the TRF Senior Scholar to AA (RTA5780006).

Conflict of interest The author(s) declare that they have no competing interests.

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APPENDIX E

Time-space analysis of highly pathogenic avian influenza H5N2 outbreak in the US

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Keywords: Evolutionary, H5N2, Highly Pathogenic Avian Influenza, Minnesota

Nonthabenjawan et al. Virology Journal (2016) 13:147
DOI 10.1186/s12985-016-0605-4

Virology Journal

RESEARCH Open Access

Time-space analysis of highly pathogenic avian influenza H5N2 outbreak in the US

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Abstract

Background: In early 2015, highly pathogenic avian influenza H5N2 caused outbreaks in commercial poultry farms in Minnesota and neighboring states where more than 48 million birds were affected. To date, the origin and transmission pathways of HPAI H5N2 have not been conclusively established.

Methods: In this study, we analyzed forty-six samples from turkeys and their environment that were collected at different time-points of the outbreak to identify origins and within outbreak evolutionary changes. We performed de-novo whole genome sequencing from primary samples and the most recent common ancestors of the PB2, PA, HA5, M and NS segments were traced back to Japanese HPAI H5N8 isolates. These segments appeared to have diverged from the ancestor around June and November 2014.

Results: The time to most recent common ancestor analysis for PB1, NP and NA2 segments suggest two likely possibilities of reassortant HPAI H5N2 origin - either a reassortment in Alaska area or multiple reassortments with North American low pathogenic avian influenza strains, before the HPAI H5N2 outbreak strain emerged. Within the outbreak, viruses clustered into two and three subgroups suggesting high substitution rates of 0.702x10-2 - 1.665x10-2 (subs/site/year), over the 5-month outbreak period.

Conclusions: Data are suggestive of a fast evolving HPAI strain within an outbreak that should be taken into consideration in developing appropriate control strategies in the future.

Keywords: Evolutionary, H5N2, Highly pathogenic avian influenza, Minnesota

Background

Avian influenza virus (AIV) is an enveloped virus that contains eight negative sense single strand RNA segments which encode at least ten functional proteins [1]. Hemagglutinin (HA) and neuraminidase (NA) are surface proteins that are used to classify AIV into subtypes. At present, 16 HA and 9 NA subtypes have been identified in aquatic wild birds which are a natural reservoir of these viruses [2]. AIV can be divided into low pathogenic avian influenza viruses (LPAI) and highly pathogenic avian influenza viruses (HPAI) by its ability to cause disease in poultry which is identified by intravenous pathogenicity index (IVPI) test or by it's possession of poly-basic amino acid feature at the HA cleavage site [3].

Outbreaks HPAI H5 in Guangdong, China in 1996 have likely origins from goose Guangdong lineage HPAI viruses [4]. Since 1997, HPAI H5N1 dispersed in more than 50 countries in Africa, Asia, Europe, and the Middle East. In 2003, a re-emergence of HPAI H5N1 in China and South East Asia was reported. Since its emergence, the H5 gene has continued to evolve and has been arbitrarily classified in the OIE nomenclature system into clades identified from 0 to 9 [5]. The latest clade, 2.3.4.4, was designated in January 2015 and replaced the provisional clade 2.3.4.6. The first isolation of H5 clade 2.3.4.4 viruses was from domestic mallard ducks (*Anas platyrhynchos*) in China in 2008 [6]. The H5 clade 2.3.4.4 has demonstrated an ability to reassort with multiple neuraminidase subtypes including N1, N2, N3, N5, N6 or N8 [7].

In January 2014, HPAI H5N8 caused outbreaks in 161 commercial poultry flocks in South Korea and led to culling of 14,000,000 birds [8]. In early November 2014,

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HPAI H5N8 was detected in a turkey flock in Germany followed by an outbreak in a duck farm in England and a chicken farm in the Netherlands. Wild birds may play an important role in generating novel reassortant subtypes of clade 2.3.4.4 and carrying viruses across continents [9]. In late November 2014, HPAI H5N2 caused outbreaks in turkey and broiler breeder flock in British Columbia, Canada. In the United States, the first case of HPAI H5 was detected in a captive gyrfalcon (*Falco rusticolus*) in Washington in early December and subsequently the first detection of the reassortant HPAI H5N2 was reported in a northern pintail duck (*Anas acuta*) in Washington. In December 2014 and January 2015, HPAI H5 viruses associated with various NA subtypes were detected in backyard poultry flocks in Oregon and Washington, USA [10]. In January 2015, an HPAI H5N8 virus was detected in a commercial turkey flock in California [11]. On March 2nd, 2015, the first case of HPAI H5N2 in the Midwestern USA was confirmed in Pope County, Minnesota. On June 17th, 2015, last confirmed case in this outbreak was reported in Iowa. In the Midwest region, more than 200 confirmed cases were reported and more than 48,000,000 birds were affected [12].

In this study, we investigated the origin and within outbreak evolution of this new emergent HPAI H5N2 that caused outbreaks in Midwestern states.

Methods

Sample collection

Samples were collected from turkeys and the environment at locations involved in outbreaks of HP clade 2.3.4.4 H5N2. Sampling was performed over a 106-day period during the outbreak. A total of 46 tissue or oropharyngeal or environmental samples were collected. Samples were divided into three phases representing an approximate 35-day interval each - early phase (March 4th–April 7th) [$n = 18$], mid phase (April 8th–May 12th) [$n = 19$] and late phase (May 13th–June 17th) [$n = 9$].

All samples were identified by RT-PCR testing water and bird samples from all barns of turkey flocks to identify infected samples per established methods [13]. All RT-PCR positive samples (water in infected barns, tracheal and cloacal swabs from birds or air) collected at early, mid and late phases of the outbreak were genome sequenced directly from primary samples (Table 1).

Whole genome sequencing

Forty-six samples from turkeys, drinker biofilm, air and environment submitted to the University of Minnesota Mid Central Research and Outreach Center (Willmar, MN) were used in this study. RNA was extracted with the MagMAX™-96 Viral RNA Isolation Kit (Ambion) using a magnetic particle processor (Kingfisher, model 700) according to manufacturer's instructions. All RNA samples were tested by RT-PCR for matrix gene as described [13]. The positive RNA samples were subjected to amplify all eight segments of virus simultaneously using a one-step RT-PCR as described [14]. Briefly, SuperScript® III one-Step RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen™; CA, USA) was used with 1.6 mM and 0.2 μM final concentration of Magnesium and primers, respectively. PCR products were imaged by electrophoresis in 1.5 % agarose gel and subsequently purified by QIAquick PCR Purification Kit (Qiagen®; Hilden, Germany). Purified PCR products were submitted to University of Minnesota Genomics Center (UMGC) for illumina paired-end 250 cycles sequencing by using Nextera XT DNA kit for library generation. Sequences were assembled by mapping all reads to a reference and whole- or partial-genome sequences were extracted via CLC genomics workbench module available on Minnesota supercomputing institute (MSI) resources at the University of Minnesota.

Phylogenetic analysis

The reference nucleotide sequences were obtained from Influenza Research Database (http://www.fludb.org/) and GISAID (http://gisaid.org/) in May 2016. Reference sequences were selected to represent previous and recent avian influenza strains from varying geographic areas including North America and Eurasia. The following approach was applied to select reference sequences: 1) include an ancestral strain of clade 2.3.4; 2) use of sequences of isolates selected from 3 well characterized H5 (2.3.4.4 lineage) outbreaks in Asia, Europe and North America; and 3) use of a double selection criteria to identify the top 30 hits to the current outbreak isolates by BLAST and develop a tree. Subsequently, these closely related sequences were reanalyzed by BLAST to expand the reference database and used to reconstruct phylogeny. LPAI North American strains that were isolated during 2012–2015 were also included. Reference and sample sequences from the current study were aligned using Muscle v.3.6 [15] subsequently any extra sequences beyond and after start and stop codon were trimmed. Maximum clade credibility (MCC) tree of each gene segments were generated by BEAST 1.8 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. Strict clock model with coalescent constant population and HKY with gamma 4 substitution was used as model parameters [16, 17].

Mean substitution rate was estimated by Bayesian coalescent with constant population size [16] and strict clock model was applied. The Bayesian MCMC chain lengths were 10,000,000 generations with sampling every 10,000 generations and the effective sample size (ESS) value was assessed by using Tracer (v1.6.0) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) [18]. Every gene segment analysis had ESS value greater than 200 to suggesting minimal standard error. The resulting tree of each iteration was summarized for a

Table 1 Details of samples in this study

Sample No.	Sample type	Collection date	Phase of outbreak	Location	Sequencing Data (Segment)								Accession No.
					1	2	3	4	5	6	7	8	
E1	Envir	2/27/2015	Early	Minnesota									
E2	Envir	2/27/2015	Early	Minnesota									
E3	TS	3/4/2015	Early	Minnesota									
E4	TS	3/4/2015	Early	Minnesota									
E5	Envir	3/7/2015	Early	Minnesota									
E6	TS	3/7/2015	Early	Minnesota									
E7	Envir	3/24/2015	Early	Minnesota									
E8	Envir	3/24/2015	Early	Minnesota									
E9	Envir	3/26/2015	Early	Minnesota									
E10	Envir	3/26/2015	Early	Minnesota									
E11	TS	3/28/2015	Early										
E12	CS	3/28/2015	Early										
E13	TS	3/28/2015	Early										
E14	CS	3/28/2015	Early										
E15	Envir	4/3/2015	Early	Minnesota									
E16	Envir	4/3/2015	Early	Minnesota									
E17	TS	4/3/2015	Early	Minnesota									
E18	TS	4/3/2015	Early	Minnesota									
M1	Envir	4/23/2015	Mid										
M2	Envir	4/23/2015	Mid										
M3	Envir	4/23/2015	Mid										
M4	Envir	4/23/2015	Mid										
M5	Envir	4/23/2015	Mid										
M6	Envir	4/23/2015	Mid										
M7	Air	4/24/2015	Mid	Minnesota									

Sample No.	Sample type	Collection date	Phase of outbreak	Location	Sequencing Data (Segment)								Accession No.
					1	2	3	4	5	6	7	8	
M8	Air	4/24/2015	Mid	Minnesota									
M9	Air	4/27/2015	Mid	Minnesota									
M10	Air	4/27/2015	Mid	Minnesota									
M11	Envir	4/28/2015	Mid										
M12	Envir	4/28/2015	Mid										
M13	Envir	4/28/2015	Mid										
M14	TS	4/28/2015	Mid										
M15	TS	4/28/2015	Mid										
M16	TS	4/28/2015	Mid										
M17	TS	4/28/2015	Mid										
M18	Air	4/28/2015	Mid	Minnesota									
M19	Air	4/28/2015	Mid	Minnesota									
L1	Air	5/12/2015	Late	Iowa									
L2	Air	5/12/2015	Late	Iowa									
L3	Air	5/22/2015	Late	Nebraska									
L4	Air	5/23/2015	Late	Nebraska									
L5	Air	5/23/2015	Late	Nebraska									
L6	Air	5/23/2015	Late	Nebraska									
L7	Envir	5/23/2015	Late	Nebraska									
L8	Envir	5/23/2015	Late	Nebraska									
L9	Envir	5/23/2015	Late	Nebraska									

Note: White and grey box indicates available and unavailable sequencing data on each segment, respectively. TS, CS and Envi stand for tracheal swab, cloacal swab and environmental sample, respectively

representative clustering pattern by using a tree annotator with 10 % discarding of the chains as burn-in and the resulting maximum clade credibility tree was visualized with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).

Results

First case of this series of outbreaks in the Midwest area was confirmed on March 4th, 2015 in Pope county, Minnesota and last case on June 17th, 2015 in Iowa [12]. A 106-day period was divided into three phases representing an approximate 35-day interval - early phase (March 4th–April 7th), mid phase (April 8th–May 12th) and late phase (May 13th–June 17th). Eighteen, nineteen and nine samples were collected during early, mid and late phases of the outbreak, respectively (Table 1).

Origin of HPAI H5N2

Forty samples were successfully whole genome sequenced and six provided whole segment sequences of some gene segments as shown in Table 1. Phylogenetic analysis of HPAI H5N2 (HPAI H5N2 EA/NA) showed that it was a reassortant between Eurasian (EA) HPAI H5N8 and North American (NAm) LPAI. HPAI H5N2 EA/NA genetic constellation is composed of five gene segments (PB2, PA, HA, M and NS) from EA HPAI H5N8 and the remaining three segments (PB1, NP and NA) from NAm LPAI (Fig. 1a, b, c and Additional file 1a–e).

Time to most recent common ancestor (TMRCA) analysis showed that EA HPAI H5N8 likely evolved from an AIV H5 clade 2.3.4.4 (China) between April 2008 and January 2011 (2008.297–2010.255). Longer-term analysis of HPAI H5N2 EA/NAm, TMRCA analysis indicated that PB2, PA, HA5, M and NS segments diverged from EA HPAI H5N8 (Japan) strain, around June and November 2014 (2014.438–2014.900). PB1 was closely related to non-H5 LPAI North American strain isolated from Alaska (blue branch) likely diverged from these isolates around June 2012 and June 2013 (2012.453–2013.438, mean TMRCA 2012.950). NP appears to have diverged from A/American green-winged teal/Alaska/472/2014 (A/AGWT/AK/472/14; pink branch) an LPAI H5N2 North American strain, during September 2012 and December 2013 (2012.721–2013.941, mean TMRCA 2013.358). NA2 ancestry traced back to H5N2 that was isolated from Alaska during flu season 2014–2015 (pink and green branches) and reassortment likely occurred around December 2012 and November 2013 (2012.993–2013.824, mean TMRCA 2013.400). TMRCA of all gene segments are summarized in Fig. 2. The TMRCA format (E.g., 2006.272) was calculated using the formula: collection date divided by the number days in a year. For example, January 10 2006 is day 10th of the year; TMRCA = (1/365)*10 = 0.027 in the year 2006 = 2006.027.

Within outbreak evolution

The mean substitution rate of all segments over the 106-day interval of this study was estimated to be between 0.702 and 1.665×10^{-2}. The NS gene segment was the most divergent segment while NP gene segment was the most conserved segment (Table 2). Bayesian Coalescent analysis showed that every gene segment of samples in this study formed three clades (I, II and III) except M and NS segment that form two major clades (I and II) (Fig. 1a–c and Additional file 1a–e). Members in subgroup I of PB2, PB1, PA, HA5, NP and NA2 were consistent in terms of clustering by time within the outbreak period with some minor variations. For example two samples (E15 and E16) fell into cluster II for M while the same samples clustered in clade I for NS. The HA5 segment of Korean H5N8 clustered within groups A and B [8]. Group A is a predominant cluster and appears to have dispersed to other continents. In addition, this cluster was subdivided into 3 clades (A1, A2 and A3) [19]. HPAI EA/NAm H5N2 belongs to the A2 subgroup which same subgroup as the two isolates H5N8 (Japan) and HPAI H5N2 (Canada) lineages (Fig. 1a). These results of high polymorphism rate and diversification of gene segments over the course of the epidemic indicate rapid evolution of viruses within this outbreak. These analyses assume evolution from a single introduction. Thus, that this high rate of change could likely be explained by multiple introductions cannot be ruled out.

Genetic analysis for amino acid variability

Genetic analysis of the HA5 segment indicated multiple basic amino acid at HA cleavage site. Samples from early and mid phase of the outbreak contain the motif PLRERRRKR/GLF that is a characteristic of H5 clade 2.3.4, while samples collected during late phases of the outbreak carried the PQRERRRKR/GLF motif Glutamine (Q) is the major amino acid of other H5 clades [20]. Amino acid substitutions were analyzed by comparing with A/Northern pintail/Washington/40964/2014 (A/NP/WA/14), the first isolate of HPAI H5N2 EA/NA in the US. At position 141 (H5 numbering system) on the HA gene, A/NP/WA/14 and sub-cluster I contained serine (S) while 17 samples, sub-cluster II and III, contain proline (P). S141P mutations are related to surface protein stability and reduced host immune response [21]. The deduced amino acid sequence of the HA stalk region of all samples were identical.

All samples contained S at position 31 on M2 that has been shown to confer reduced susceptibility to amantadine and rimantadine antiviral drugs. However, analysis of resistance to neuraminidase inhibitors (NAI) at position 119, 151, 222, 224, 276, 292 and 371 on NA2 gene segment [22] showed that all samples in this study contain the conserved amino acid at every position which

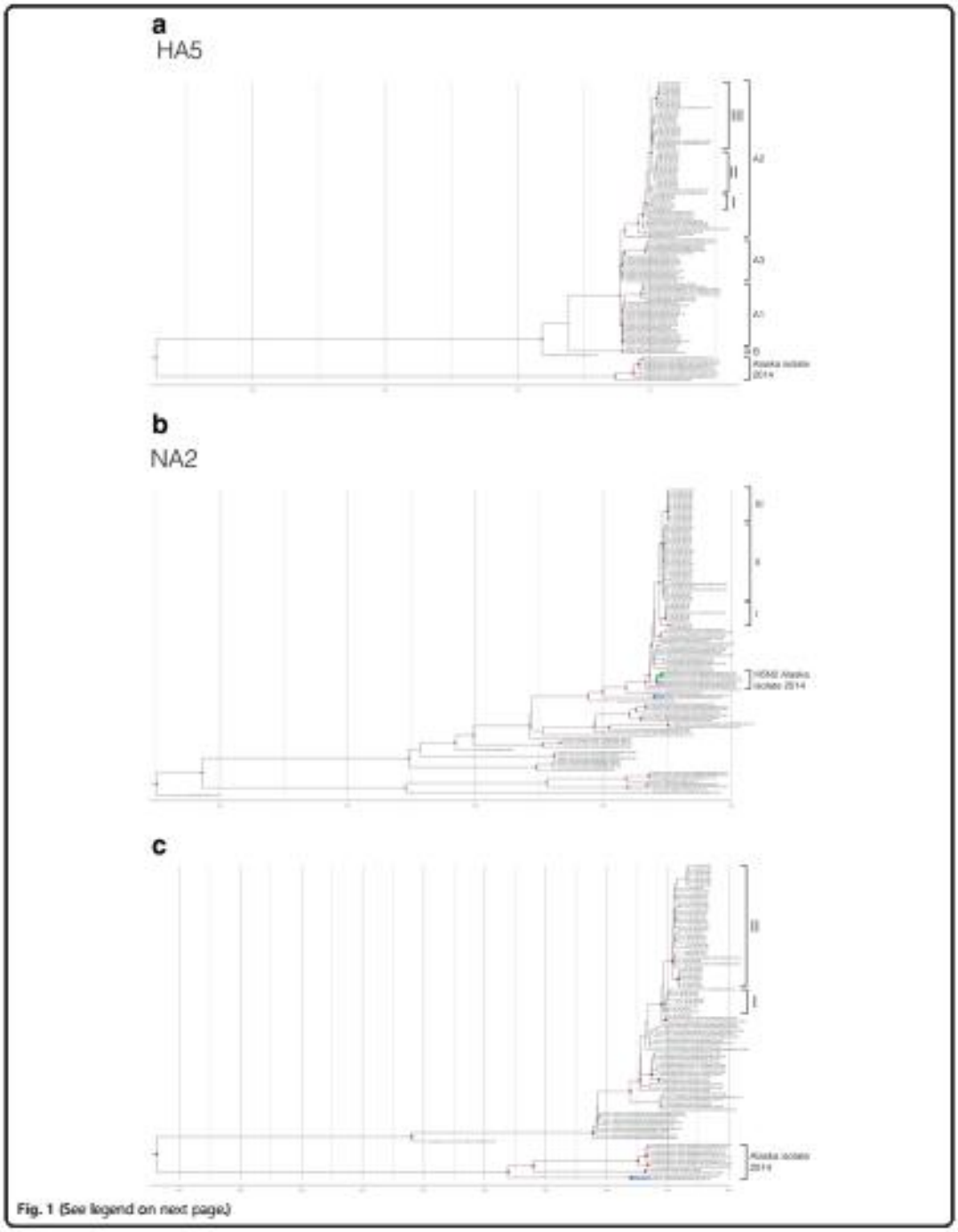

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Time-scaled Bayesian maximum clade credibility tree inferred for the HA5 **a**, NA2 **b** and NS gene **c**. Trees were generated by Bayesian Markov Chain Monte Carlo algorithm in Bayesian evolutionary analysis by sampling trees. The TMRCAs representing the estimated timing of viral divergence from their ancestor are provided in parentheses. Red dot at each node represents the posterior probability above 0.7. A/AGWT/AK/472/14, group of A/mallard/SAK/14 and non-H5 LPAI NAm strain were labeled by pink, green and blue branch. Clustering is shown with reference strains of recent ancestry and within outbreak viruses clearly cluster in 2 or 3 clades separated by 35-day intervals. The TMRCA

are expected to maintain NAI susceptibility. The E627K and D701N substitutions in the PB2 gene segment that are expected to increase virulence in mammals were examined. The results showed that all samples contain E and D in respective amino acid sites suggesting low mammalian virulence in this group of viruses.

Discussion

A study addressing the spread of HPAI H5N8 from South Korea to Europe and Japan have suggested that these outbreaks originated from a single source population [23]. Dispersion of the virus to different geographic localities coincides with wild bird migratory seasons strongly suggesting their important role in the transmission of this pathogen and has been suggested as a mechanism by which H5N8 entered South Korea [24]. The most likely flyway involved in the recent spread of HPAI EA H5N8 to Japan was the East Asian Australian (EAA) Flyway [23]. The EAA flyway extends from north-eastern Asia and western Alaska to the southern extent of Australia and New Zealand and Mongolia, western China and eastern India [25]. On the North America continent, Alaska is an area where there is major overlap between migratory birds from the EAA and Pacific Flyways [25] but there exists no evidence of HP H5 clade 2.3.4.4 in Alaska. The existence of PB1, NP and NA2 ancestor that related to HP H5N2 in Alaska arise the possibility that HPAI H5N2 EA/NA originated in a location that coincides with Alaska and subsequently spread to British Columbia, Canada and Washington, USA. However, the mechanism and timing of viral transfer from the Pacific Flyway to the Mississippi Flyway in NA has not been established.

The ancestral strain and the overlapping of interval between TMRCA estimates of PB1, NP and NA2 suggest two hypothesis of HPAI EA H5N2 origin. First, it is possible that reassortant event occurred in Alaska. This finding is concordant with the report of avian influenza surveillance in Alaska during spring and summer 2015 that found reassortant LPAI between EA and NAm [26]. Second, the outbreak viral genome constellation likely occurred via multiple reassortment events with an LPAI before addition of genes of HPAI EA H5N2 origin due to the earlier divergence time of PB1, NP and NA2. Furthermore, HA5 clade 2.3.4.4 has the potential to reassort

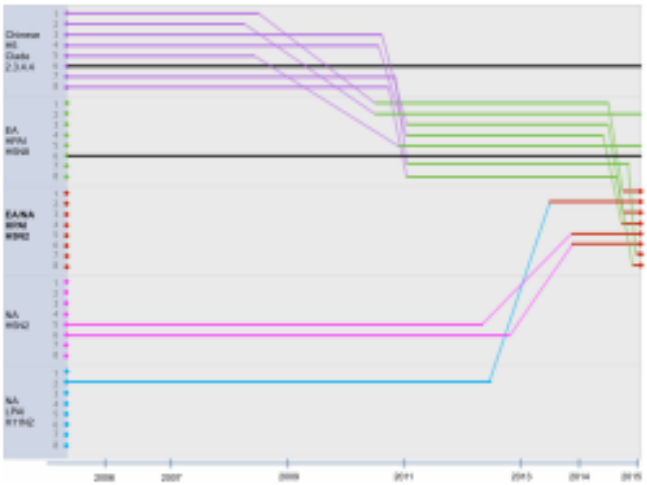

Fig. 2 Reconstruction of the genetic constellation of reassortment events leading up to the emergence of EA/NA H5N2. The eight genomic segments are represented as parallel lines in descending order of segment 1 to 8. Each colored line represent transmission pathways of influenza genes from different ancestor: Chinese H5 Clade 2.3.4.4 (purple), Eurasian HPAI H5N8 (green), Eurasian/North American HPAI H5N2 (red), LPAI H5 North American strain (pink) and non-H5 LPAI North American strain (blue). Black line represents the different NA subtype that was not included in the analysis. Slopes lines represent divergent dates of each segment at 95 % highest posterior density interval

Table 2 Mean nucleotide substitution rate of the H5N2 epidemic

	Mean Substitution rate ($\times 10^{-3}$)	Substitution rate 95 % HPD ($\times 10^{-3}$)
PB2	1.079	0.526–1.710
PB1	1.160	0.620–1.760
PA	0.712	0.332–1.150
HA	1.290	0.670–2.000
NP	0.702	0.261–1.240
NA	1.413	0.644–2.310
M	1.329	0.315–2.540
NS	1.665	0.595–2.790

with multiple NA subtypes including NA1, NA2, NA5, NA6 and NA8 [27]. For example, reassortant H5N1 (EA/NA H5N1) containing PB2, HA5, NP and M from EA H5N8 and the rest of the gene segments (PB1, PA, NA1 and NS) from AIV North American lineage was detected in a green wing teal and an American wigeon in December 2014, with no subsequent case reports. This finding indicates that EA/NA H5N1 was likely unable to persist in the population while EA/NA H5N2 provided a compatible constellation for maintenance. Alternately, this may indicate that there was insufficient surveillance to detect the fate of that reassortment.

The nucleotide substitution rate of all segments within the outbreak are greater than 5.000×10^{-3} substitution/site/year which are higher than the rates estimated in long term analysis [28]. These results indicated that viruses were evolving rapidly during the current outbreak. Host jump from wild birds to domestic poultry including turkeys and chickens is a factor that provoked this rapid evolution. Therefore, identification of bridge of interspecies transmission is an important process to prevent the further outbreaks. While the HA gene had an extremely high substitution rate, the HA stalk region of all samples were identical at the amino acid level. The HA stalk region, PA and NP proteins were highly conserved, opening avenues for a universal subunit vaccine concept and development.

At present, no human case has been reported due to HPAI H5N2 EA/NA and it is considered as low health risk to public health [29]. However, three human cases with severe respiratory disease caused by HPAI H5N6 HA5 clade 2.3.4.4 have been reported in China [30]. These cases confirmed that HA5 clade 2.3.4.4 is capable of causing human infection and thus ongoing monitoring of viral evolution should be performed.

Conclusion

Wild bird surveillance in United States has been performed annually during September and November, which is a migratory season and only fecal or cloacal swabs are traditionally collected. After HPAI H5 clade 2.3.4.4 was detected in December 2014, USDA/APHIS wildlife services responded by enhancing surveillance in Pacific Flyway and detected more viruses. Furthermore, multiple introductions of this clade of viruses cannot be ruled out. Therefore, there is a need for more comprehensive wild bird surveillance effort to address a complex wild-domestic animal-human interface to capture variations in subtype specific organ predilection and varying patterns of shedding by different host species.

Additional file

Additional file 1: Alignment and phylogeny of PB2 segment sequences also captures the 3 stages in evolution amon the outbreak isolates. (ZIP 2258 kb)

Acknowledgements

The authors would like to thank Chulalongkorn University for a Dutsadi Phiphat scholarship to NN and TRF senior scholar to AA (RTA5680006). We also thank Drs. Torremorell, Murtaugh, Marthaler and Mor for samples and critical discussions on our analysis. This project was funded by Agricultural Experiment Station grants expended as Rapid Agricultural Response fund awarded to CC.

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Received: 28 March 2016 Accepted: 23 August 2016
Published online: 30 August 2016

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