ลักษณะทางอณูชีวโมเลกุล และวิวัฒนาการสายพันธุ์ไข้หวัด (H1N1, H3N2 และ H5N1) ในประเทศไทย

นาย กมล สุวรรณการ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2552

MOLECULAR CHARACTERIZATION AND EVOLUTION OF INFLUENZA A VIRUS (H1N1, H3N2 AND H5N1) IN THAILAND

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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Thesis Title	MOLECULAR CHARACTERIZATION AND EVOLUTION OF INFLUENZA A VIRUS (H1N1, H3N2 AND H5N1) IN THAILAND
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กมล สุวรรณการ: ลักษณะทางอณูชีวโมเลกุล และวิวัฒนาการสายพันธุ์ไข้หวัด (H1N1, H3N2 และ H5N1) ในประเทศไทย (Molecular Characterization and evolution of Influenza A virus (H1N1, H3N2 and H5N1) in Thailand) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศาสตราจารย์ นายแพทย์ ยง ภู่วรวรรณ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รองศาสตราจารย์ นายสัตวแพทย์ ดร. อลงกร อมรศิลป์, 145 หน้า.

เชื้อไข้หวัดใหญ่เป็นปัญหาทางสุขภาพที่สำคัญ และส่งผลกระทบจากการระบาดอย่างกว้างขวาง ในการศึกษาครั้งนี้ได้ทำการศึกษา เชื้อไข้หวัดใหญ่ชนิดเอในคน สายพันธุ์ H1N1 และ H3N2 และ เชื้อไข้หวัดนก สายพันธุ์ H5N1 ที่จำแนกได้ในประเทศไทย โดยการศึกษานี้แบ่งออกเป็น 3 ส่วน ส่วนที่ 1 ทำการพัฒนาวิธีการตรวจเชื้อไข้หวัดใหญ่ โดยแบ่งเป็น การตรวจจำแนกชนิดเชื้อไข้หวัดชนิด A และ B และการตรวจจำแนกสายพันธุ์เชื้อไข้หวัดใหญ่ชนิด A สายพันธุ์ H1, H3 และ H5 โดยได้พัฒนาทำให้ได้เทคนิคที่มีความไว ความจำเพาะ และความถูกต้องสูง การศึกษาส่วนที่ 2 ได้ทำการศึกษาวิวัฒนาการเชิงโมเลกุลของเชื้อไข้หวัดใหญ่ในคน และ ไข้หวัดนก โดยถอดรหัสพันธุกรรมของเชื้อไข้หวัดใหญ่ในคนตามฤดูกาลที่พบในประเทศไทยในปี 2549 ถึง 2551 สายพันธุ์ H1N1 (5 ตัวอย่าง) และ H3N2 (13 ตัวอย่าง) โดยผลการวิเคราะห์ด้วย Phylogenetic analysis พบว่า HA และ NA ยีน สามารถจำแนกได้เป็นฤดูกาลการระบาด และมีความใกล้เคียงกับเชื้อไข้หวัดใหญ่ในวัคซีนที่แนะนำโดยองค์การอนามัยโลก และได้ศึกษาในส่วนของ Antigenic sites, N-linked glycosylation pattern และ selection pressure สำหรับผลการศึกษาวิวัฒนาการของเชื้อไข้หวัดนกสายพันธุ์ H5N1 ที่พบในประเทศไทย ระหว่างปี 2547 ถึง ปี 2551 พบว่า Phylogenetic analysis ของไวรัสที่อยู่ใน clade 1 นั้นสามารถจำแนกออกได้เป็น 3 lineage ได้แก่ CUK2-like, PC168-like และ PC170-like virus ในปี 2551 พบไวรัสที่เกิดการแลกเปลี่ยนสารพันธุกรรมกัน 2 lineage ระหว่าง PC-168-like และ PC170-like virus ซึ่งพบที่บริเวณภาคเหนือตอนล่าง และพบในบริเวณภาคกลางตอนบน นอกจากนี้ ปี 2551 ยังคงพบ CUK2-like virus บริเวณ ภาคเหนือตอนล่างและภาคกลางตอนบนอีกด้วย ค่า substitution rate ของไวรัสใน clade 1 ที่พบในประเทศไทยนั้นพบว่ามีค่าที่ค่อนข้างต่ำ การศึกษาในส่วนสุดท้ายส่วนที่ 3 นั้น ได้ทำการศึกษา viral quasispecies ของเชื้อไข้หวัดนก สายพันธุ์ H5N1 ในเสือ ซึ่งได้พบว่ามี quasispecies ใน PB2 protein ที่กรดอะมิโนตำแหน่ง 627 ด้วยวิธี clonal sequencing โดยพบ quasispecies ระหว่างกรดอะมิโน lysine และ glutamic acid ที่ตำแหน่งนี้ และพบเฉพาะที่ บริเวณ หลอดลมของเสือเพียงแค่ตัวเดียวเท่านั้น ในส่วนการศึกษา viral genome quesispecies โดยการใช้เทคโนโลยี high resolution sequencing นั้นยังจำเป็นต้องทำการศึกษาเพิ่มเติมต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต.....กมล สุวรรณการ.....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

#4989652920 : MAJOR BIOMEDICAL SCIENCES

KEYWORDS: Influenza A virus, Molecular evolution, H5N1, H3N2, H1N1, quasispecies

KAMOL SUWANNAKARN: MOLECULAR CHARACTERIZATION AND EVOLUTION OF INFLUENZA A VIRUS (H1N1, H3N2 AND H5N1) IN THAILAND. THESIS ADVISOR: PROF. YONG POOVORAWAN, M.D., THESIS CO-ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 145 pp.

Influenza virus infection is a major public health problem of global concerns. In this study, human influenza (H1N1 and H3N2) and avian influenza (H5N1) viruses isolated in Thailand were studied. This study consists of 3 parts. Firstly, a specific and sensitive one-step multiplex real-time RT-PCR was developed in two assays for typing influenza A and influenza B virus and subtyping H1, H3 and H5 influenza A virus. These assays have high sensitivity, high specificity and high accuracy. Second, the complete genome of seasonal influenza A virus from Thailand in 2006 to 2008 subtypes H1N1 (n=5) and H3N2 (n=13) were selected for molecular evolution analyses. Phylogenetic analysis of the HA and NA genes formed seasonal clusters and closely related to the WHO recommended vaccine strains in each season. Antigenic sites characterization, N-linked glycosylation pattern in surface gene and selection pressure were studied. Moreover, H5N1 isolated in Thailand between 2004 and 2008 were also studied on the molecular evolution. Phylogenetic analysis among Thai isolates indicated that clade 1 viruses in Thailand consist of three distinct lineages: CUK2-like, PC168-like, and PC170-like viruses. In 2008, viruses reassorted from these two lineages, PC168-like and PC170-like viruses, were initially isolated in the lower northern provinces of Thailand and subsequently spread to the upper central part of Thailand. On the other hand, CUK2-like viruses were still detected around the lower northern and the upper central part of Thailand. The substitution rate among clade 1 viruses in Thailand was low. Third, H5N1 viral quasispecies in tiger were detected at position 627 in PB2 viral protein by using clonal sequencing. Quasispecies between lysine and glutamic acid at this position just found only one tiger in trachea organ. The viral genome quasispecies perform by high resolution genomic sequencing needs further study.

Field of Study: Biomedical Sciences Student's Signature: Kamol Suwannakarn

Academic Year: 2009 Advisor's Signature: Yong Poovorawan

Co-Advisor's Signature: Alongkorn Amonsin

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LIST OF ABBREVIATIONS

µg	=	Microgram
µl	=	Microlitre
µM	=	Micromolar
BLAST	=	Basic Local Alignment Search Tool
bp	=	Base pair
BMCMC	=	Bayesian Markov Chain Monte Carlo
cDNA	=	Complementary deoxyribonucleic acid
Ck	=	Chicken
Dk	=	Duck
DNA	=	Deoxyribonucleic acid
dsRNA	=	Double-stranded RNA
FAO	=	Food and Agriculture Organization
FEL	=	two rate fixed effects likelihood
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
HA	=	Hemagglutinin
HPAI	=	High Pathogenic Avian Influenza
IFN	=	Interferon
LNA	=	Locked nucleic acid
LPAI	=	Low Pathogenic Avian Influenza
mM	=	Millimolar
min	=	Minute
NA	=	Neuraminidase
NJ	=	Neighbour-joining
NP	=	Nucleoprotein
NP suction	=	Nasopharyngeal suction
NS	=	Non-structural protin
nt	=	Nucleotide

LIST OF ABBREVIATIONS (CONTINUE)

OIE	=	Office International des Epizooties
PA	=	Polymerase Acidic protein
PB1	=	Polymerase Basic protein 1
PB2	=	Polymerase Basic protein 2
PCR	=	Polymerase Chain Reaction
RT	=	Reverse Transcription
RNP	=	Ribonucleoprotein
REL	=	Random effects likelihood
SLAC	=	Single likelihood ancestor counting
sstDNA	=	Single-stranded fragments nucleic acid
sec	=	Second
sscDNA	=	Single-stranded complementary deoxyribonucleic acid
vRNA	=	Viral ribonucleic acid
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Influenza virus infection is a major public health problem of global proportions, about 20% of children and 5% of adults developed symptomatic influenza A or B each year (Turner et al., 2003). It is the cause of a broad range of illnesses ranging from minor symptomatic infections to various respiratory syndromes and disorders affecting the lung, heart, brain, liver, kidneys, and muscles. Every year, in excess of 200,000 people worldwide succumb to severe respiratory illness caused by influenza A virus. The disease is responsible for 50 million illnesses and up to 47,200 deaths in the United States each year (Cox and Subbarao, 1999; Simonsen, 1997), with similar figures in Europe too. (Carrat and Valleron, 1995; Fleming et al., 1999).

The subtypes of influenza A virus were classified based on the antigenic variations of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins expressed on the surface of viral particles. All subtypes have been isolated from aquatic birds; they are considered as a reservoir of influenza A viruses for other animal species. The most common subtypes of influenza A virus that causing diseases in humans are H1N1, H2N2, and H3N2 (Harper et al., 2004).

In 1997, an outbreak of highly pathogenic avian influenza (HPAI) subtype H5N1 virus emerged and caused severe systemic disease among poultry and humans in Hong Kong. Since 2003 to early 2004, the virus has reached endemic levels among poultry in several south-east Asian countries. During 2005, H5N1 virus infected birds more than 50 countries in Europe and Africa. In addition to spread among poultry, this virus has also emerged, causing virulent disease in humans and other mammals. The World Health Organization had voiced their concerns about a potential pandemic with the imminent threat to humankind.

The haemagglutinin (HA) nucleotide sequences of the majority of H5N1 viruses circulating separated into 10 distinct phylogenetic clades (clades 0–9) (WHO/OIE/FAO

H5N1 Evolution Working Group, 2008). The reciprocal cross-reactions in antigenic tests demonstrated antigenic similarity to HA within the same genetic clade and distinguished representatives of different clades (WHO, 2006). The evolution of H5 hemagglutinin shows a notable difference from the typical evolution of HA genes from human H3 influenza viruses. The multiple H5 hemagglutinin influenza A clades continue to evolve and co-circulate in different regions and species.

During the H5N1 virus outbreak in Thailand in 2004, the outbreak also occurred in felines (tiger, leopard, and cat) (Amonsin et al., 2006; Keawcharoen et al., 2004; Thanawongnuwech et al., 2005). The affected animals had eaten an infectious avian and had probable horizontal transmission. Virology and pathology assay demonstrated virus not only in the respiratory tract but also in multiple extra-respiratory tissues, digestive tract, brain and heart. The studies in felines demonstrate that H5N1 virus infection causes systemic disease and can spread within and between mammalian hosts. The genetics adaptation may lead to the extended host range of H5N1 virus. During viral replication, the complex and dynamic distribution of variants termed viral quasispecies which play a role in the genetics adaptability of viruses to changing in environments. The production of quasispecies leads to adaptability of virus which may alter their virulence and tissue tropism.

In the past few years, the various additional subtypes of influenza A virus including H5N1 have been reported not only in poultry but also proven highly pathogenic and fatal to mammalian species including human. So, the early and rapid identification of infectious strains and subtypes in each patient are required for treatment as well as for prevention of widespread transmission. And the understanding of the molecular evolutionary of whole genome sequencing in human and avian influenza was important for further improve vaccine strain selection and potentially make influenza virus evolution more predictable.

Research question

1. How to rapid identify the typing of influenza A virus and subtyping of H1, H3 and H5.
2. What is the pattern of molecular evolution of human H1N1, H3N2 and avian H5N1 influenza virus in Thailand?
3. Does it have the H5N1 viral quasispecies in mammals?

Objective

1. To develop the rapid assay for early detection Influenza A and B and subtype H1, H3 and H5.
2. To investigate and study the molecular evolution of H1N1, H3N2 and H5N1 influenza A virus in Thailand by using the whole genome sequences.
3. To investigate of the H5N1 viral quasispecies and tissue tropism in mammal.

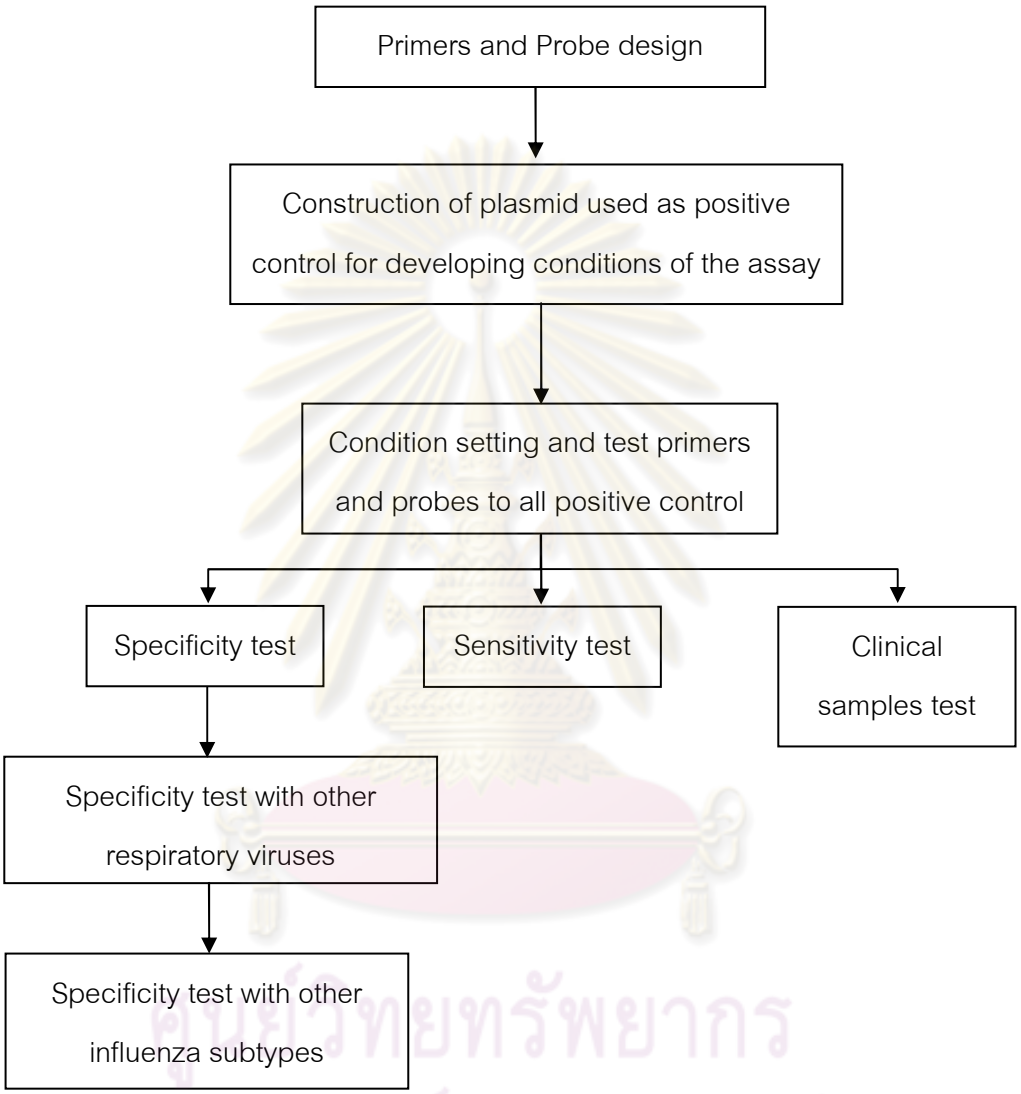
Key words

influenza A virus, molecular evolution, H5N1, H3N2, H1N1, subtypes, quasispecies.

Conceptual Framework

Part 1

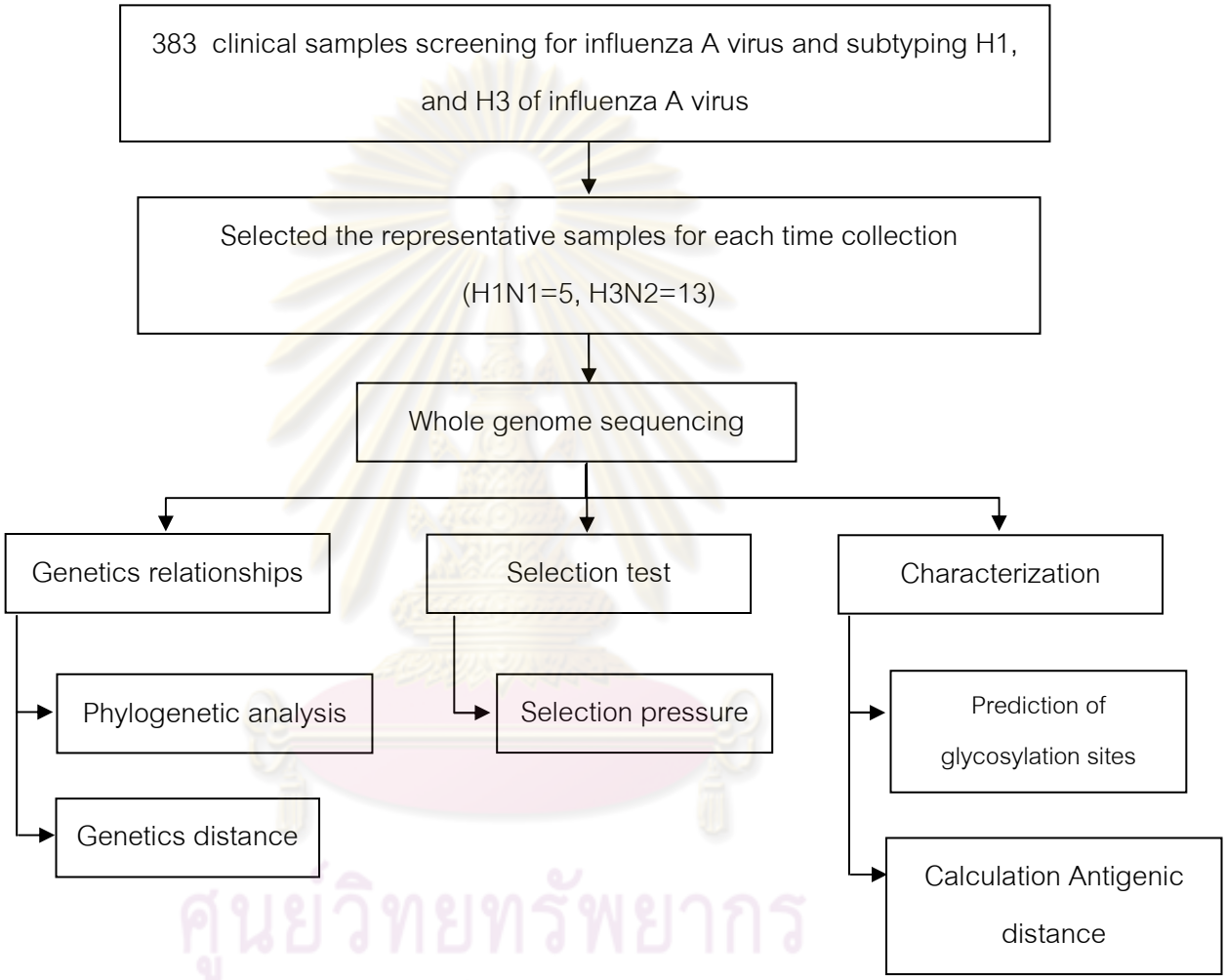
Objective: To develop the rapid assay for early detection Influenza A and B and subtype H1, H3 and H5.



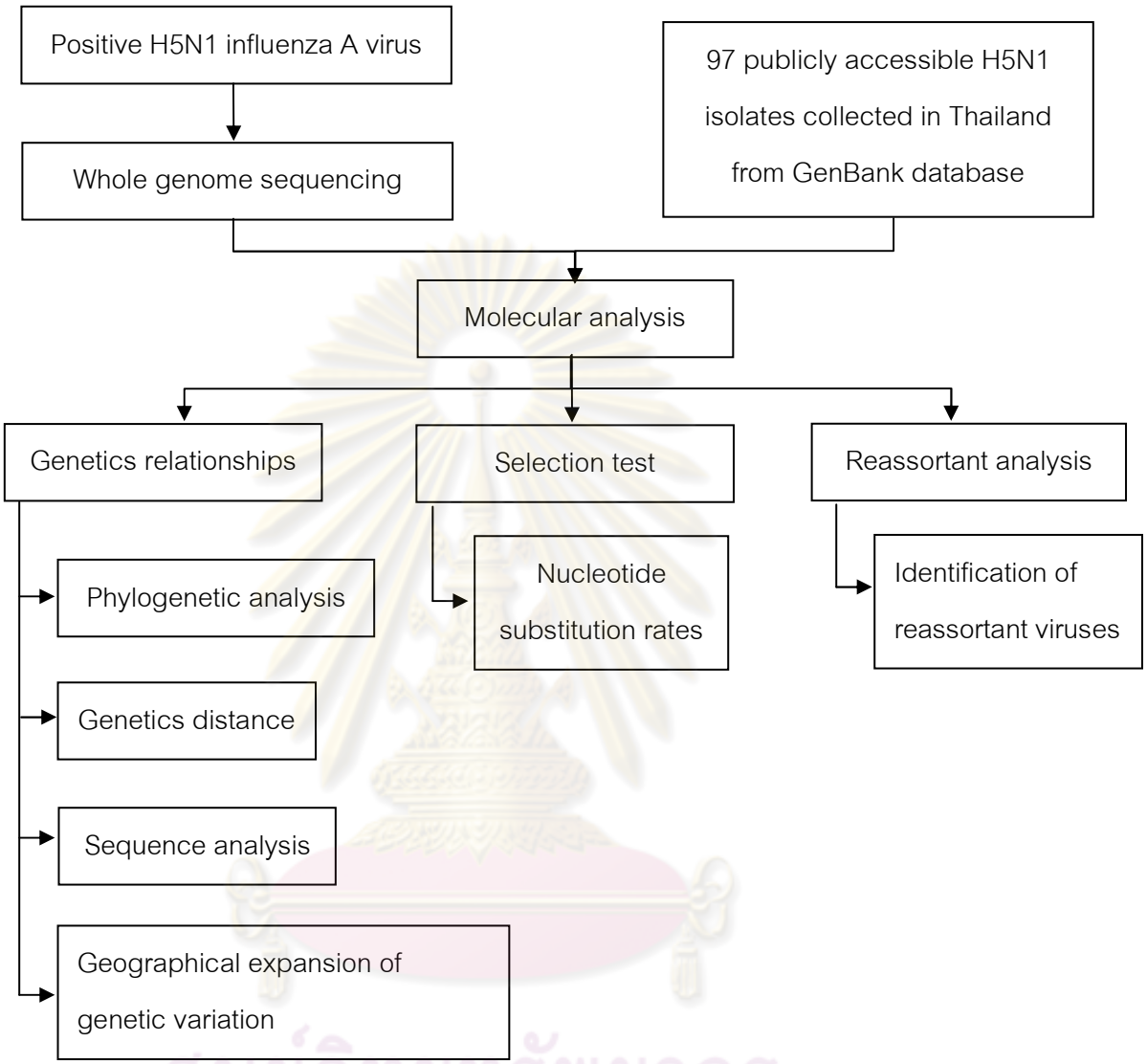
Part 2

Objective: To investigate and study the molecular evolution of H1N1, H3N2 and H5N1 influenza A virus in Thailand by using the whole genome sequences.

Part 2.1: Molecular evolution of human influenza (H1N1 and H3N2) in Thailand, 2006-2008



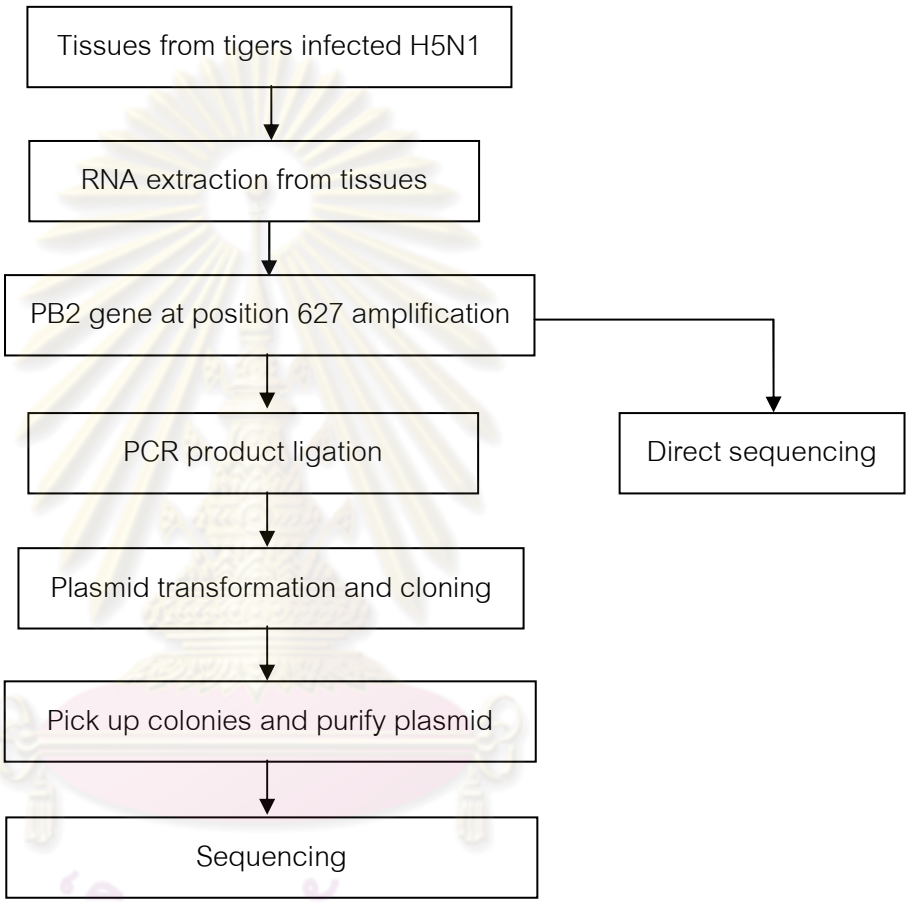
Part 2.2: Molecular evolution of H5N1 in Thailand between 2004 and 2008



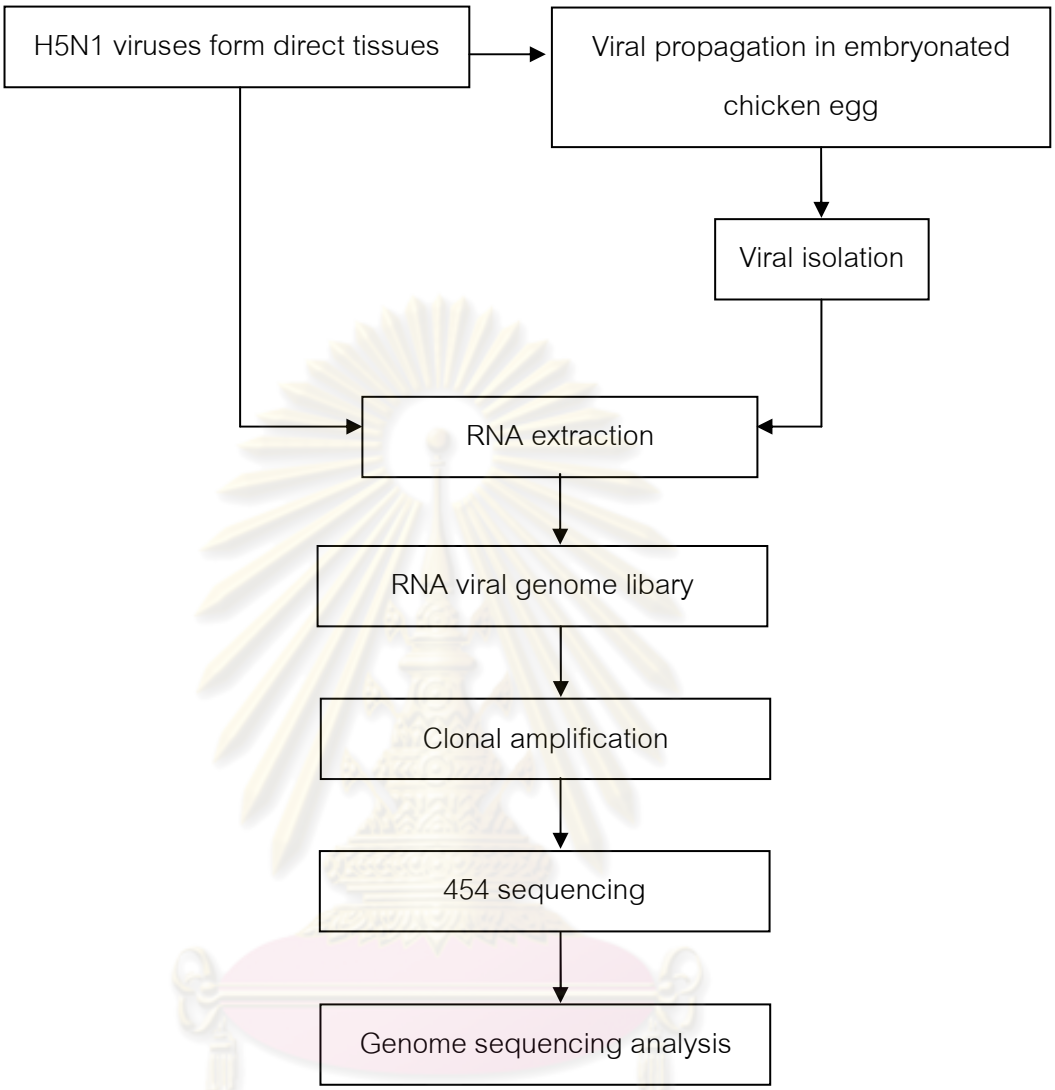
Part 3

Objective: To investigate the viral quasispecies and viral tissue tropism of H5N1 in mammal.

Quasispecies in viral PB2 gene by clonal sequencing



Quasispecies in viral whole genome by high resolution sequencing



CHAPTER II

REVIEW AND RELATED LITERATURES

Influenza, commonly known as the flu, is an infectious disease that affects birds and mammals caused by RNA viruses. The name influenza comes from the Italian: influenza, meaning "influence", (Latin: influentia). Influenza is a highly infectious disease that affects the respiratory tract. Typically, influenza is transmitted from infected mammals through the air by coughs or sneezes, creating aerosols containing the virus, and from infected birds through their droppings. Influenza can also be transmitted by saliva, nasal secretions, feces and blood. Infections also occur through contact with these body fluids or with contaminated surfaces. Flu viruses can remain infectious for about one week at human body temperature, over 30 days at 0 °C, and for much longer periods at very low temperatures. Most influenza strains can be inactivated easily by disinfectants and detergents (Suarez et al., 2003). Flu spreads around the world in seasonal epidemics, resulting in the deaths of millions in pandemic years and hundreds of thousands annually in non-pandemic years.

The influenza virus classification

Influenza viruses are RNA viruses belong to the Orthomyxoviridae family. Three types of influenza virus are classified, influenza A, B and C. The antigenic differences in nucleoprotein (NP) and matrix (M) proteins are used to distinguish the influenza A, B and C viruses. Influenza A viruses infect multiple species of mammals, including humans, horses, pigs, ferrets and birds. Influenza B viruses infect mostly humans. They are quite common, but clinical disease is usually less severe than influenza A (Shaw et al., 2002). Influenza C viruses have been identified in humans. They are rare and usually produce mild or no clinical symptoms.

Influenza A viruses are considered the most virulent group. Influenza A viruses are further divided into subtypes based on the antigenicity of their two surface antigens hemagglutinin (HA) and neuraminidase (NA). Currently, there are sixteen

recognized HA subtype (H1-H16) and nine NA subtypes (N1-N9) (Fouchier et al., 2005a). All these subtypes are present in aquatic birds and ducks which are the natural hosts and reservoirs of influenza viruses in nature. The host range of different influenza viruses are specified by different forms of sialic acid presented on the cell glycoproteins. Avian viruses preferentially bind to avian cells while human viruses preferentially bind human cell receptors. However, porcine cells possess both human and avian type sialic acids on their surface and are susceptible to infection with both avian and human influenza viruses. Therefore, pigs are regarded as the mixing vessel where avian and human influenza viruses can reassort to generate novel pandemic strains of influenza (Wright et al., 1992)

Nomenclature of influenza virus

The guidelines for nomenclature of influenza viruses by the World Health Organization (WHO) are as follows (Smorodintsev et al., 1982). First, the type of virus is designated (A, B or C), then the host origin (except for human), followed by geographical site of isolation, strain number and years of isolation and each is separated by slashes. Influenza A viruses the HA and NA subtypes are put in parenthesis. Examples of the nomenclature are A/Moscow/10/99 (H3N2), B/Hong Kong/330/2001.

Morphology and viral genome of the Influenza virus

The virion of influenza virus is pleomorphic, the envelope can occur in spherical and filamentous forms. In general the virion morphology is spherical particle 50 to 120 nm in diameter, or filamentous virions 20 nm in diameter and 200 to 300 (-3000) nm long.

The most striking feature of influenza A virion is a layer of about 500 spikes radiating outward (10 to 14 nm) from the lipid envelope. These spikes are of two types: rod-shaped spikes of HA and mushroom-shaped spikes of NA. Influenza A, B and C viruses also encode another integral membrane protein, the M2, NB and CM2 proteins,

respectively. The viral matrix protein 1 (M1) is thought to underlie the lipid bilayer and to associate with the ribonucleoprotein core of the virus.

Inside the virus the ribonucleoprotein (RNP) structures, which contain eight different segments of single-stranded RNA. The RNPs consist of four protein species and RNA. NP is the predominant protein subunit of the nucleocapsid and coats the RNA. Associated with the RNPs is the RNA-dependent RNA polymerase complex consisting of the three polymerase proteins, PB1, PB2 and PA (Inglis et al., 1976). The polymerase proteins in the RNP complex carry out the cap-binding, endonuclease, RNA synthesis and polyadenylation reactions (Li et al., 1998).

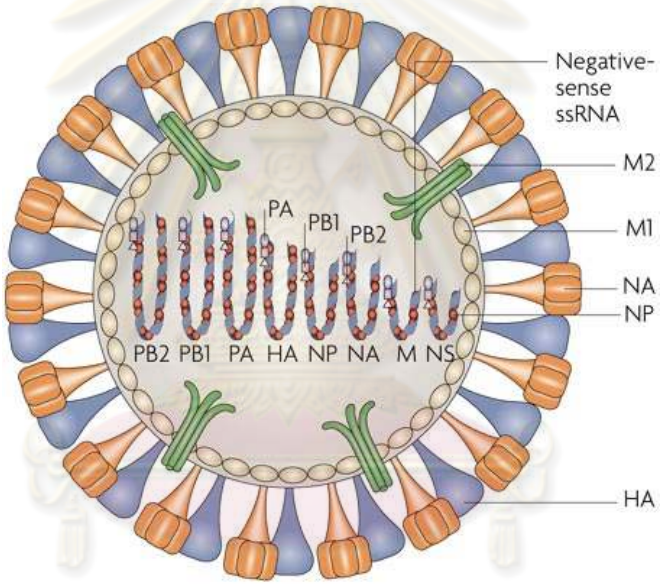


Figure 1 The virion structure of influenza A virus. Inside virion consist of the genome of influenza A virus which composed of eight genomic segments. (Nelson and Holmes, 2007)

The NS2 protein forms an association with the M1 protein (Richardson and Akkina, 1991), which is thought to be an essential interaction in the virus life cycle for export of the RNP complex from the nucleus (O'Neill et al., 1998). It has been suggested that NS2 be renamed NEP for nuclear export protein.

The genome of influenza A virus contain eight segments of single stranded RNA (ssRNA). The gene assignment for influenza A virus is as follows: RNA segment 1 codes for PB2, 2 for PB1, 3 for PA, 4 for HA, 5 for NP, 6 for NA, 7 for M1 and M2 and 8 for NS1 and NS2.

PB2 polymerase is encoded by RNA segment 1. It is a member of the protein complex providing viral RNA-dependent RNA polymerase activity. It is known to function during initiation of viral mRNA transcription as the protein, which recognizes and binds the 5' cap structures of host cell mRNAs for use as viral mRNA transcription primers.

PB1 polymerase is encoded by RNA segment 2. It functions in the RNA polymerase complex as the protein responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis. The PB1 gene of most avian and human influenza A viruses encodes a second protein, PB1-F2, that is expressed from the +1 reading frame (Chen et al., 2001). PB1-F2 induces apoptosis, probably by interaction with two mitochondrial proteins.

PA polymerase is encoded by RNA segment 3. It is a member of the RNA-dependent RNA polymerase complex along with PB1 and PB2, but its role in viral RNA synthesis is unknown. There is evidence for possible roles as a protein kinase or as a helix-unwinding protein.

Hemagglutinin (HA) protein is an integral membrane protein and the major surface antigen of the influenza virus virion. It is responsible for binding of virions to host cell receptors and for fusion between the virion envelope and the host cell. HA is encoded by RNA segment 4. HA is the major target of the host immune response. HA monomers are synthesized as precursors that are then glycosylated and cleaved into two smaller polypeptides: the HA1 and HA2 subunits (uncleaved HA is called HA0), connected by disulfide linkages.

Nucleoprotein (NP) is encoded by RNA segment 5. It is phosphorylated; the pattern of phosphorylation is host cell dependent and may be related to viral host range restriction. NP is also a major target of the host cytotoxic T-cell immune response.

Neuraminidase (NA) encoded by RNA segment 6, is also an integral membrane glycoprotein and a second major surface antigen of the virion. NA cleaves terminal sialic acid from glycoproteins or glycolipids. Thus, it functions to free virus particles from host cell receptors, to permit progeny virions to escape from the cell in which they arose, and so facilitate virus spread. Like HA, NA is highly mutable with variant selection partly in response to host immune pressure.

Influenza virus RNA segment 7 is bicistronic, encoding both M1 and M2 proteins. Colinear transcription of segment 7 yields mRNA for the matrix protein. This is the most abundant protein in the influenza virus virion. Matrix protein forms a shell surrounding the virion nucleocapsids, underneath the virion envelope. It has no known enzymatic activity, although it has been speculated to play an important role in initiating progeny virus assembly.

The mRNA for M2 is also transcribed from RNA segment 7. It is derived from the colinear (M1) transcript by splicing. M2 is an integral membrane protein, whose membrane-spanning domain also serves as a signal for transport to the cell surface. It is believed to act as a proton channel to control the pH of the Golgi during HA synthesis and to allow acidification of the interior of the virion during virus uncoating.

RNA segment 8 encodes the two nonstructural proteins 1 (NS1) and 2 (NS2). NS1 mRNA is colinear with the vRNA, whereas NS2 mRNA is derived by splicing. Both proteins play roles in virus replication, but those roles have not been fully defined. NS2 appears to modulate the synthesis of NS.

Life cycle

After attach to sialic acid-containing receptors on the membrane surface, the virus enters the cell by receptor mediated endocytosis. A low pH in the endosome induces a conformational change in HA (Bullough et al., 1994), resulting in membrane fusion between the viral envelope and the endosomal membrane. Within the endosome, the M2 proton channel exposes the viral core to low pH, resulting in dissociation of M1 from RNP and leading to a release of RNP to the cytoplasm. RNP is then transported to

the nucleus, most probably by nuclear localization signals in proteins composed of the RNP complex.

The mechanism of viral RNA (vRNA) transcription is unique. The 5' cap from cellular mRNAs is cleaved by a viral endonuclease and used as a primer for transcription by the viral transcriptase (Krug et al.,1979; Plotch et al., 1981). Six of eight RNA segments are transcribed into mRNAs and translated into HA, NA, NP, PB1, PB2 and PA. Two RNA segments are each transcribed to mRNAs by splicing. The mRNA of M and NS genes are translated in different reading frames, generate M1 and M2 proteins and NS1 and NS2 protein, respectively.

A full-length complementary RNA (cRNA), a positive-sense copy of the vRNA, is first made and this in turn is used as a template to produce more vRNA. The viral proteins are expressed and processed and eventually assemble with vRNAs at budding sites within the host cell membrane. The viral protein complexes and ribonucleoproteins are assembled into viral particles and bud from the host cell, enveloped in the host cell's membrane.

At the late of infection, the translation products are M1, HA and NA. HA and NA are glycosylated in the rough endoplasmic reticulum, further processed in the Golgi apparatus, and then transported to the cell surface, where they integrate into the cell membrane. Nuclear localization of M1 and NS2 proteins is essential for the migration of RNP out of the nucleus for assembly into progeny viral particles in the cytoplasm (Martin and Helenius, 1991; O'Neill et al., 1998). The RNP-M1 complex presumably interacts with M1 proteins that are associated with the plasma membrane and then buds outward through the cell membrane, enclosing itself within a bubble of membrane (envelop) studded with both the HA and NA glycoproteins.

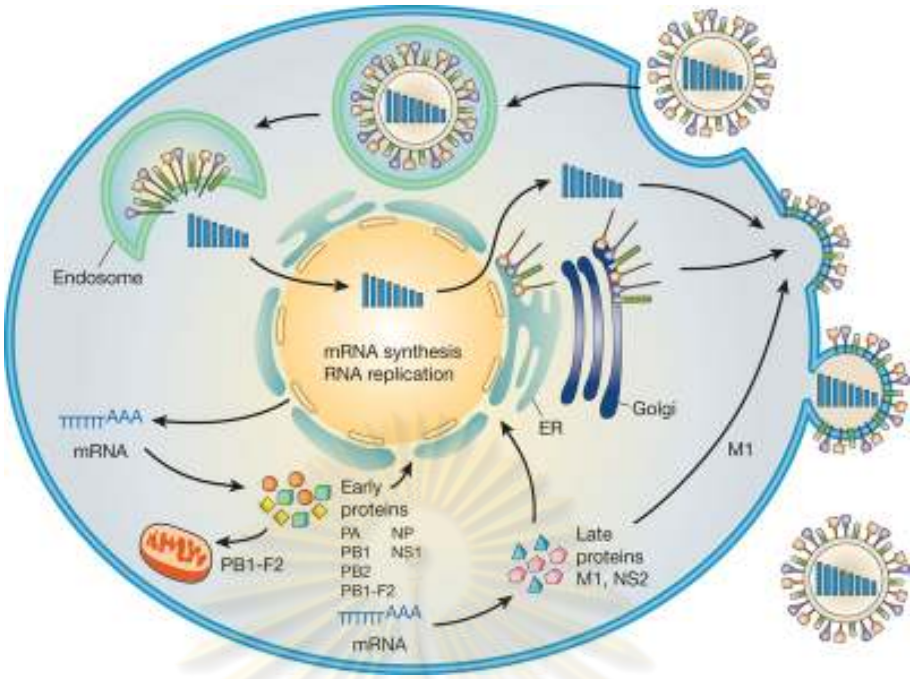


Figure 2 The life cycle of influenza A virus. (Neumann, Noda and Kawaoka. 2009)

Antigenic Variation

The antigenicity of influenza virus viruses changes gradually by protein mutation (antigenic drift) or drastically by genetic reassortment (antigenic drift) (Murphy and Webster, 1996). Immunological pressure on HA and NA is thought to drive antigenic drift. As in all RNA viruses, mutations in influenza occur frequently because the virus' RNA polymerase has no proofreading mechanism, providing a strong source of mutations. Such changes in the antigenicity of human influenza virus necessitate the replacement of vaccine strains every several years. Antigenic shift is caused by either direct transmission of nonhuman influenza viruses to human or the reassortment of gene from two different influenza viruses that have infected a single cell (Webster et al., 1982). Theoretically, more than 256 different combinations of RNA can be produced from the shuffling of the eight different genomic segments of the virus. Genetic reassortment is well documented both in vitro and in vivo under laboratory condition (Webster and Laver, 1975). More importantly, mixed infections occur relatively frequently in nature and can lead to genetic reassortment (Bean et al., 1980; Hinshaw, et al., 1980; Young and Palese, 1979). Reemergence of a previously circulating virus is another mechanism by which antigenic shift can occur.

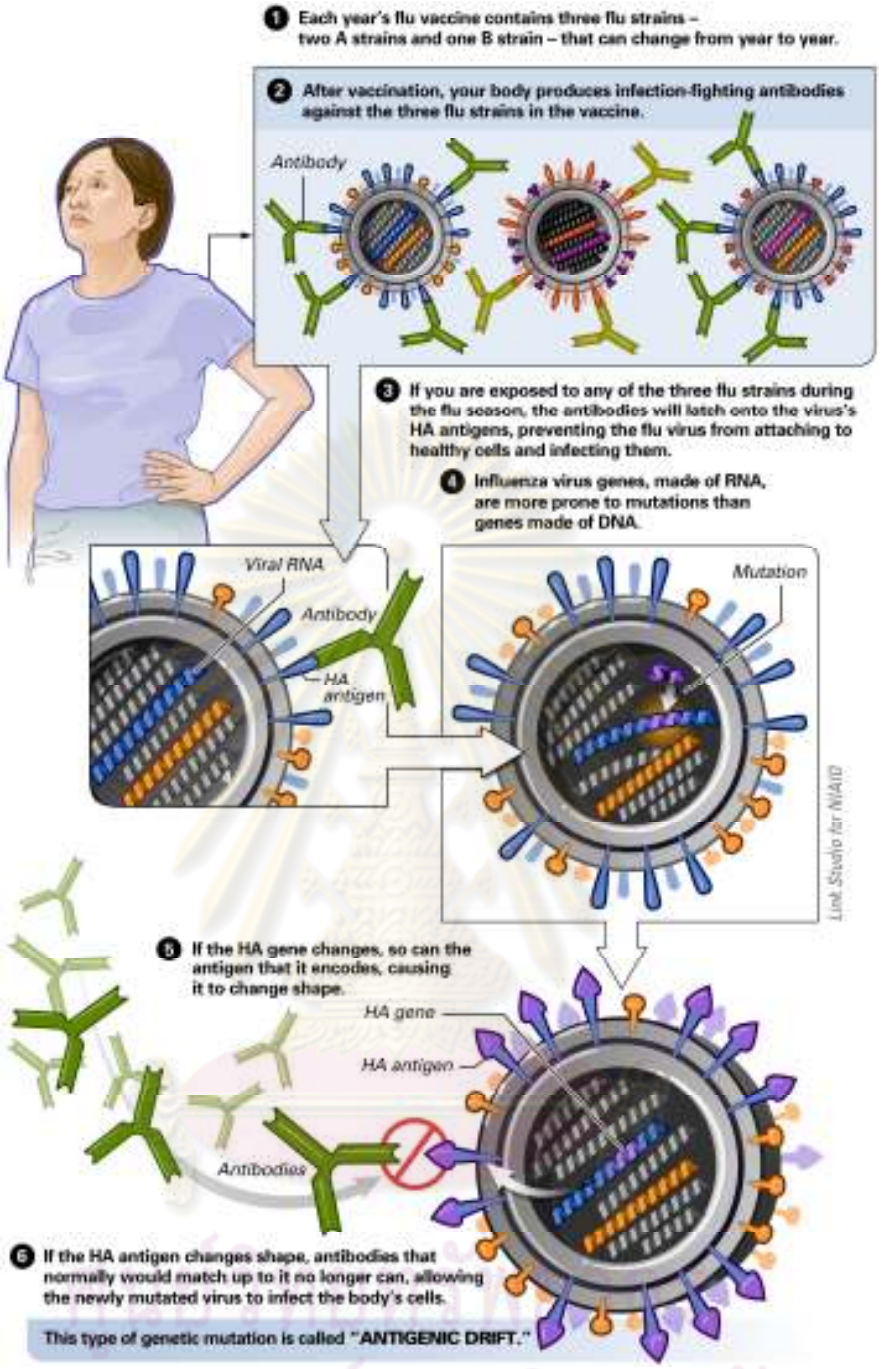


Figure 3 Antigenic drift. A mutation of the genes that code for the flu virus's surface antigens, enables a virus to escape or bypass the body's defenses. (National Institute of Allergy and Infectious Disease[NIAH], 2006: online)

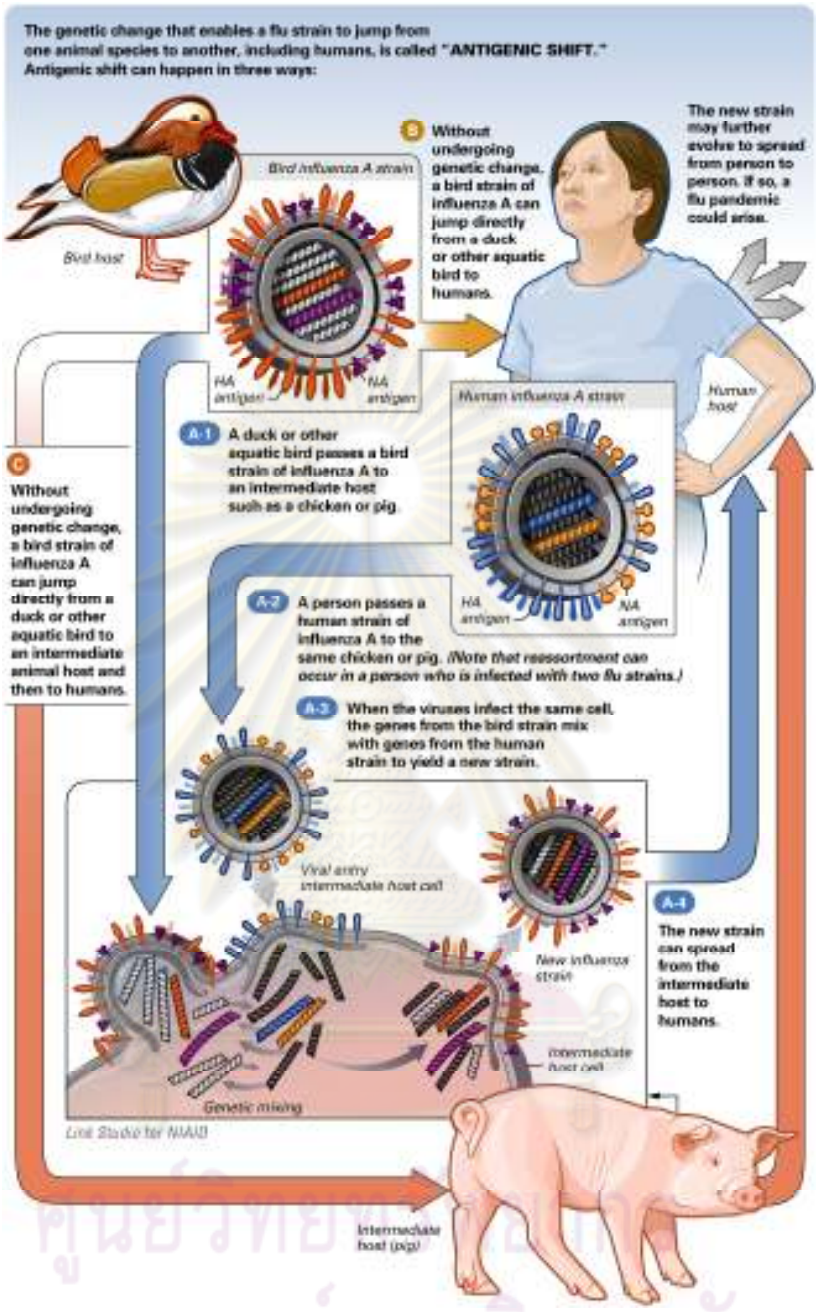


Figure 4 Antigenic Shift. The genetic change that enables a flu strain to jump from one animal species to another, including humans. (NIAH, 2006: online)

Emerging of influenza

In 1918-1919, the Spanish influenza caused by H1N1 influenza virus was responsible for the deaths of at least 40 million people (Johnson and Mueller, 2000). Although its clinical symptoms and pathological manifestations were largely confined to the respiratory tract (Reid and Taubenberger, 2003), almost 50% of deaths (the case mortality rate in the USA averaged 2.5%) occurred in an unusually young age group, 20–40-year-olds (Simonsen et al, 1998). The biological properties of the virus responsible for Spanish influenza have not been studied, owing to the lack of viral isolates. Phylogenetic and seroarcheological studies suggest that all gene of the 1918 pandemic strain were an unusual avian precursor (Reid et al., 2004), although sequence data to support this notion were lacking until 1997, when Taubenberger et al. (Taubenberger et al., 1997), using reverse transcription-PCR, succeeded in obtaining nucleotide sequences of this pandemic virus from a formalin-fixed, paraffin-embedded, lung tissue sample from an influenza victim. The three-dimensional structure of the HA of the 1918 virus indicates that, despite retaining the residues in the host-receptor-binding site that are characteristic of an avian precursor HA (Gamblin et al., 2004; Stevens et al., 2004), the 1918 virus HA could bind human cell-surface receptors, an observation that is consistent with receptor-binding assays carried out with a recombinant virus carrying the HA from the 1918 virus (Kobasa et al, 2004). Recent studies with viruses generated by reverse genetics that carry genes derived from the 1918 virus indicate that the HA of this virus had a role in its increased virulence in a mouse model (Kobasa et al., 2004; Tumpey et al., 2004; Kash et al., 2004).

The human pandemic viruses, such as those responsible for Asian influenza (H2N2) in 1957 and Hong Kong influenza (H3N2) in 1968, share two defining features: first emerged in southeastern Asia (Cockburn et al., 1969) and they were antigenically distinct from the influenza viruses then circulating in human. Genetic studies, together with biochemical analyses, indicated that the Asian and Hong Kong pandemic strains were generated by reassortment between human and avian viral genes (Figure 3). The 1957 virus consisted of HA (H2), NA (N2) and PB1, from an avian virus, with the other gene segments derived from a previously circulating human virus (Scholtissek et al., 1978; Kawaoka et al., 1989). The pandemic H3N2 virus indentified in Hong Kong 1968

was reassortant with avian PB1 and HA (H3) genes and six other genes from a human H2N2 (Scholtissek et al., 1978; Kawaoka et al., 1989). The acquisition of avian surface antigens allowed these viruses to circumvent the human immune response. Why both of these pandemic strains also carried the gene that encodes PB1 from an avian virus remains unclear. In view of a recently identified North American swine virus, a triple reassortant that combined swine, human and avian viral genes and possessed the genes that encode PB1 and HA from the same human virus (Zhou et al., 1999), one could argue that the genes that encode HA and PB1 interact functionally, either at a protein or nucleic acid level, in ways that enhance the replicative ability of recombinant viruses. Although many people died during these pandemics, the causative viruses do not seem to be extraordinarily pathogenic, unlike the1918 strain. The increased death rate associated with these viruses was probably a result of the lack of immunity to these viruses in human populations.

The causative agent of the Russian influenza pandemic in 1977 was essentially identical to viruses circulating among humans in the 1950 (Figure 5) (Nakajima et al., 1978). Given that influenza viruses continually evolve in animals, it is highly unlikely that this virus was maintained in an animal host for over 20 years without changes. Thus, one logical conclusion is that the virus was maintained in a freezer until it somehow was introduced into human populations. The relatively low death rate in this pandemic can be attributed to the immunity of persons over 20 years of age who had been infected with the virus when it circulated earlier in the country.

Purely avian influenza viruses, including the H5N1, H9N2 and H7N7 subtypes, have been directly transmitted to humans. In 1997, the outbreak of influenza H5N1 among chicken and poultry markets and on farms in Hong Kong, 17 additional human cases were identified, 5 of which were fatal (Chan, 2002; Yuen et al., 1998). The outbreak was contained after the slaughtering of all 1.5 million chickens in Hong Kong. In response to the outbreak, influenza surveillance in poultry was intensified permitting early recognition of other outbreaks of avian influenza in 2001 and 2002. In December 2003, an outbreak of highly pathogenic H5N1 virus was identified among poultry in the Republic of Korea (Lee et al., 2005). Subsequently, outbreaks by H5N1 highly

pathogenic avian influenza (HPAI) viruses were reported among poultry in Thailand, Viet Nam, Japan, China, Cambodia, Laos, Malaysia, and Indonesia. The occurrence of the H5N1 virus was wild spread throughout Africa and Europe.

In March and early April 2009, a new swine-origin influenza A (H1N1) virus (S-OIV) emerged in Mexico and the United States (CDC, 2009). During the first few weeks of surveillance, the virus spread worldwide by human-to-human transmission, causing the World Health Organization to raise its pandemic alert to level 6.

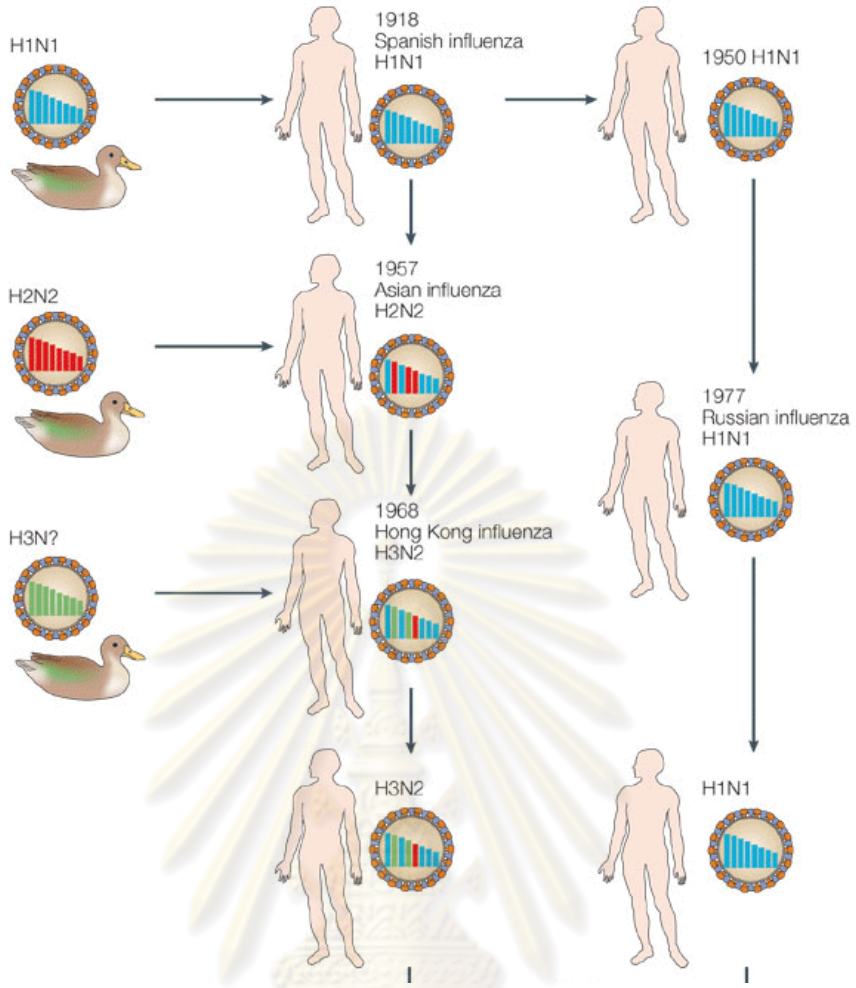


Figure 5 Possible origins of pandemic influenza viruses. Phylogenetic studies suggest that an avian influenza virus was transmitted to humans, leading to the 1918 pandemic. A reassortant virus possessing its PB1, HA and NA gene from avian virus, with the remainder coming from an H1N1 human virus, caused the Asian pandemic of 1957. The H1N1 virus subsequently disappeared from human. In 1968, a reassortant possessing its PB1 and HA gene from an avian virus and the remainder from an H2N2 human virus emerged, followed by the disappearance of the H2N2 virus. In 1977, a virus genetically almost identical to those circulating in humans in 1950 appeared and spread among children and young adults. The H1N1 and H3N2 viruses are now cocirculating in human. Since 1997, purely avian influenza viruses, including H5N1, H7N7 and H9N2 subtypes, have been directly transmitted to humans. (Horimoto and Kawaoka, 2005)

Host restriction and pathogenesis

Influenza A viruses possess eight negative-sense single-stranded RNA segments as their genome, encoding 11 proteins. However, with respect to host range restriction and pathogenicity, direct experimental evidence for these properties exists for viral proteins.

The HA protein has an important role in expressing high pathogenicity in many animal species. It mediates the binding of the virus to host cells, and the subsequent fusion of the viral and endosomal membranes for vRNP release into the cytoplasm. Influenza virus host specificity can be explained in part by the difference in receptor-binding specificity for human and avian influenza viruses. Human influenza viruses preferentially bind to sialyloligosaccharides terminated by *N*-acetylsialic acid linked to galactose by the α2,6 linkage (NeuAcα2,6Gal). This preference is matched by NeuAcα2,6Gal on epithelial cells in the human trachea. In contrast avian influenza viruses preferentially recognize *N*-acetylsialic acid linked to galactose by the α2,3 linkage (NeuAcα2,3Gal) that is matched by NeuAcα2,3Gal on epithelial cells in the intestinal tract of waterfowl (Rogers and Paulson, 1983). The predominant sialic acid-galactose linkages of sialyloigosaccharides in epithelial cells at viral replication sites differ by the host animal species. Interestingly, the epithelial cells in the pig trachea contain both NeuAcα2,3Gal and NeuAcα2,6Gal linkages (Ito et al., 1998), explaning why this animal is highly susceptible to both human and avian influenza viruses. The host receptor-binding specificity suggests that influenza viruses need to acquire the ability for interspecies transmission. Indeed, the earliest isolates of the 1918, 1957 and 1968 virus possessed HA recognized to human receptor, even though that is avian origin. The infection of HPAI avian H5N1 viruses seem to be surprising, the isolated from infected individuals in Hong Kong in 1997 preferentially recognized NeuAcα2,3Gal (Matrosovich et al., 1999). However, studies showed avian-type receptors on human epithelial cells that line the respiratory bronchiole and the alveolar walls, but human-type receptors on human epithelial cells in nasal mucosa, paranasal sinuses, pharynx, trachea and bronchi (Shinya et al., 2006; van Riel et al., 2006). Another study, however, showed the infection of human upper respiratory organs with an H5N1 avian virus

(Nicholls et al., 2007). Still, the finding of avian-type receptors in human lungs explains the severe pneumonia seen in humans with highly pathogenic avian H5N1 viruses.

Such differences in specificity are determined by the structure of the HA receptor-binding site. The differences in receptor-binding specificity of human and avian viruses are determined by the amino acid residues in the HA receptor-binding pocket. Gln at position 226 (Gln-226) and Gly at position 228 (Gly-228) of H2 and H3 HAs confer binding to avian-type receptors, whereas Leu (Leu-226) and Ser (Ser-228) at these positions determine binding to human-type receptors. For H1 HAs, amino acids at position 190 and 225 (H3 numbering) determine receptor-binding specificity. Asp at position 190 (Asp-190) and Asp at position 225 (Asp-225) in human H1 confer binding to human-type receptors (found in human H1 HAs), whereas Glu (Glu-190) and Gly (Gly-225) confer binding to avian-type receptors (found in avian H1 HAs) (Stevens et al., 2004). For H5N1 viruses, amino acid changes at positions 133, 138, 186, 192 and 227 (H3 numbering) have been identified in human isolates and confer human-type receptor recognition (Gambaryan et al., 2006; Yamada et al., 2006; Auewarakul et al., 2007). The experimental changes at positions 226 and 228 (Gln226Leu and Gly228Ser), not included position 190 (Glu190Asp), resulted in the recognition of human-type receptors as well as avian-type receptors (Stevens et al., 2006), however, the respective amino acid changes at positions 226 and 228 have not been detected in human H5N1 virus isolates.

The essential structure of HA for viral infectivity is HA cleavage site, because exposure of the amino terminus of HA2, fusion peptide, mediates fusion between the viral envelope and the endosomal membrane, an essential step for vRNP release to the cytoplasm. The amino acid sequence at the cleavage site determines the cleavability of HA. Low pathogenic avian viruses (LPAI) and non-avian influenza viruses (with the exception of H7N7 equine influenza viruses) possess a single Arg residue at the cleavage site that is cleaved by proteases in the respiratory and/or intestinal organs, and hence restricts viral replication locally. There are usually cleaved in only a limited number of organs, resulting in mild or asymptomatic infection. In contrast, highly pathogenic H5 and H7 viruses possess several basic amino acids at the HA cleavage

site (Kawaoka & Webster, 1988). This motif is cleaved by ubiquitous proteases, such as furin and PC6 (also as known as PCSK5), which are present in a broad range of different host cells, supporting lethal systemic infection in poultry (Stieneke-Grober et al., 1992; Horimoto et al., 1994). A carbohydrate side chain near the cleavage site can affect HA cleavability by interfering with the accessibility of the host proteases to the cleavage site (Kawaoka et al., 1984; Kawaoka and Webster, 1988). Therefore, HA cleavability is considered the main determinant of the tissue tropism of avian influenza viruses (Horimoto and Kawaoka, 1994), and differences in the tissue distribution of proteases and HA susceptibility to these enzymes can determine the outcome of virus infection. For several outbreaks in poultry, increased pathogenicity of avian influenza viruses has been linked to the acquisition of multibasic HA cleavage sites, a finding that underscores the significance of the HA cleavage motif for virulence.

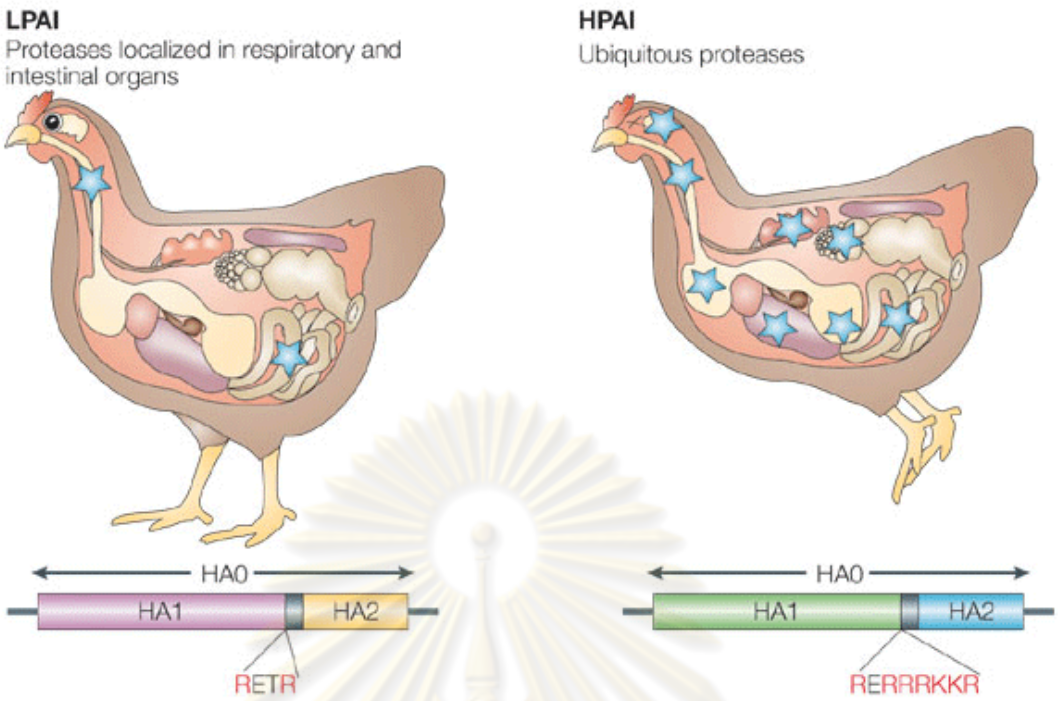


Figure 6 Post-translational proteolytic cleavage of the HA precursor molecule (HA0) into HA1 and HA2 subunits by host proteases. Therefore, proteolytic activation of the HA molecule is essential for viral infectivity. The HAs of low-pathogenicity avian influenza (LPAI) viruses do not contain a series of basic amino acid (RETR) at the protease cleavage site and are cleaved by proteases that are localized in respiratory and intestinal organs, resulting in mild localized infections. By contrast, the HAs of high-pathogenicity avian influenza (HPAI) viruses possess multiple basic amino acids at the cleavage site (RERRRKKR), which are cleaved by ubiquitous proteases in a wide range of organs, resulting in lethal systemic infection (Horimoto and Kawaoka, 2005).

The viral replication complex has been recognized as an important contributor to viral pathogenicity, probably by affecting viral growth. The amino acid at position 627 of the PB2 protein was first described as a host range determinant, on the basis of cell culture studies (Subbarao et al., 1993). The respective amino acid change was shown to determine the pathogenicity of H5N1 influenza viruses in mice (Hatta et al., 2001). Viruses with lysine at this position were pathogenic in mice, whereas those with glutamic acid were non-pathogenic in these animals. Notably, almost all human influenza viruses possess lysine at this position, whereas most avian viruses (with the exception of the

'Qinghai Lake' lineage of H5N1 viruses and their descendants) possess glutamic acid at PB2-627. Lysine at position 627 of PB2 is now recognized as a determinant of viral pathogenicity in several mammalian species. Several studies have addressed the mechanism by which PB2- Lys 627 affects virulence. The amino acid change does not affect tissue tropism in mice but viral replicative ability. The amino acid at position 701 of PB2 has also emerged as a determinant of virulence (Li et al. 2005; Gabriel et al.,2007), a role probably related to its facilitation of binding of PB2 to importin α (a cellular nuclear import factor) in mammalian cells (Gabriel et al., 2008). In addition to PB2, other components of the replication complex may also contribute to viral pathogenicity (Salomon et al., 2006). A recent study also suggested that the replication complex, particularly the PB1 protein, contributes to the virulence of the 1918 pandemic virus in ferrets (Watanabe et al., 2009).

The NS1 protein is an interferon antagonist (Garcia-Sastre, 2001; Garcia-Sastre, A. et al. , 1998) that blocks the activation of transcription factors and IFN-β-stimulated gene products, and binds to double-stranded RNA (dsRNA) to prevent the dsRNA-dependent activation of 29-59 oligo(A) synthetase, and the subsequent activation of RNase L, an important player in the innate immune response. Recently obtained structural data are expected to help in the identification of domains that are critical for the biological functions of NS1. The NS1 proteins of H5N1 viruses confer resistance to the antiviral effects of interferon and are associated with high levels of pro-inflammatory cytokines (Peiris, J. S. et al., 2004; To, et al., 2001; Cheung, et al., 2002; Seo et al., 2002; Guan et al., 2004). The resulting cytokine imbalance probably contributes to the high mortality of H5N1 virus infections in humans. Several amino acids in NS1 have now been shown to affect virulence (Seo et al., 2002; Jiao et al., 2008; Li et al., 2006).

The PB1 gene of most avian and human influenza A viruses encodes a second protein, PB1-F2, that is expressed from the +1 reading frame (Chen et al., 2001). The length of PB1-F2 of swine influenza viruses differs depending on their origin, classical swine viruses possess truncated PB1-F2 proteins of 8–11 amino acids, whereas Eurasian avian-like swine viruses possess full-length PB1-F2 proteins (87–89 amino acids). PB1-F2 induces apoptosis, probably by interaction with two mitochondrial

proteins (Chen et al., 2001; Zamarin et al., 2005), enhances inflammation in mice, and increases the frequency and severity of secondary bacterial infections (McAuley et al., 2007). It may also affect virulence by interacting with the PB1 protein to retain it in the nucleus for efficient viral replication (Mazur et al., 2008). A recent study demonstrated that the amino acid at position 66 of PB1-F2 affects the pathogenicity of an H5N1 virus in mice (Conenello et al., 2007).

Human influenza and vaccination

Shortly after the first human influenza virus isolation in 1933 (Smith et al. 1993), recognition of the changeability in antigenic characteristics led to the WHO establishment of a global Influenza Surveillance Network for monitoring changes in the viruses causing outbreaks of influenza throughout the year in different parts of the world. The principal objectives are: (i) the early detection and characterization of novel subtypes of human influenza A with the potential for causing pandemics and (ii) the identification of new antigenic variants among currently circulating influenza A and B viruses in order to ensure that influenza vaccines contain components that reflect the immunological characteristics of the prevalent viruses. The WHO has published formal recommendations for the compositions of influenza vaccines based on cumulative data since 1973. More recently, since 1998, two recommendations per year in February and September relate to vaccines for use during the following winters in the northern and southern hemisphere respectively. The chosen strains are the influenza A subtype H1N1, H3N2, and influenza B strains thought most likely to cause significant human suffering in the coming season. The timing and speed as well as the nature of antigenic changes are unpredictable. Changes in the prevalence of H3N2 variants generally occur rapidly, for example A/Sydney/5/97(H3N2)-like viruses spread to all parts of the world within 6 months of their initial detection and rapidly replaced the previously prevalent A/Wuhan/359/95-like viruses. Co-circulation of antigenically distinguishable variants of influenza A subtype H1N1 and influenza B viruses reflects the slower emergence of novel antigenic strains.

The emerging of HPAI H5N1

The emergence of H5N1 viruses in Asia since 1996 was a key determinant of the 2004 epidemic. In 1996, highly pathogenic H5N1 virus was isolated from geese during an outbreak in Guangdong Province in southern China (influenza A/Goose/Guangdong/1/96 (Gs/Gd/96)) (Xu et al., 1999). The hemagglutinin (HA) gene of all subsequent isolates of H5N1 virus in Asia is related to this or similar viruses, although considerable genetic variation within this viral lineage has occurred as the viruses evolved (Guan et al., 2002; Li et al., 2004). Similarly, the NA genes for all but the 1997 Hong Kong H5N1 viruses are also related to that of the Gs/GD/96-like lineage. The 1997 outbreak of H5N1 HPAI in Hong Kong was the first occasion that cases of serious disease associated with an AI virus occurred in both terrestrial poultry and man (Claas et al., 1998). It was caused by a viral reassortment. A Gs/GD/96-like virus is considered to be the donor of the HA gene (Sims et al., 2005) of the 1997 viruses. The other seven genes were probably derived from other avian influenza viruses circulating at the time, with the most likely candidates being viruses isolated from quail (H9N2) and teal (H6N1) (Guan et al., 1999; Chin, et al., 2002).

By 1999 it was apparent that goose viruses similar to Gs/GD/96 continued to circulate in China (Cauthen et al., 2000) as a number of viruses were isolated from geese imported for slaughter in Hong Kong that originated in mainland China. Another H5N1 virus with a novel nonstructural protein gene, presumably derived from another influenza virus from an aquatic avian species, was also identified in Guangxi province (Chen et al., 2004) in samples collected in 1999 from a duck. Over the next 3 years, multiple genotypes of H5N1 virus were detected in ducks and geese from southern China, including several with genetic constellations similar to viruses found in terrestrial poultry in 2001 (Guan et al., 2002). Two H5N1 HPAI viruses were also detected in geese in markets in one small surveillance study in live poultry markets in Vietnam (Nguyen et al., 2005). Genetic characterization of these viruses is continuing but the HA of these viruses differs considerably from that found in viruses in Vietnam in 2004. Based on the HA gene it appears to be similar to other viruses found in domestic waterfowl elsewhere in Asia in 2001–2002. The genotype of one of the viruses isolated from a duck in Guangxi in 2001 (A/Duck/Guangxi/50/2001) (Nguyen et al., 2005) was very similar

genetically (>99% homology for all genes) to that of viruses that subsequently emerged as the dominant genotype in the region—the so-called “Z” genotype (Sims et al., 2005). Samples collected from live bird markets in southern China from 2002 to 2004 confirmed that “Z” genotype viruses persisted in domestic waterfowl throughout this period. The genotype isolated most frequently was the so-called “Z” genotype. This continued to be the dominant (but not the only) genotype through 2003 and 2004.

From late 2003 to early 2004, the virus reached endemic levels among poultry in several Southeast Asian countries and spread to Europe and Africa during 2005, with H5N1 virus infected birds discovered in more than 50 countries. The 2004-2007 outbreaks in various countries have highlighted the highly pathogenic avian influenza (HPAI) subtype H5N1 virus as the cause of a major epidemic, with potentially vast repercussions on economics, public health and society at large. Not only has this AI virus infected poultry but it has also proven highly pathogenic and fatal to mammalian species including humans and other mammals (Keawcharoen et al., 2004; Songsermn et al., 2006a; Songserm et al., 2006b; Chotpitayasunondh et al., 2005). Many countries had been affected by the spread of influenza H5N1 virus infections in poultry, and transmission to humans engenders a high mortality rate with nearly 60% of cases diagnosed as H5N1 infection having proved fatal. Because different names have been used to describe emerging lineages of the H5N1 virus, WHO/OIE/FAO H5N1 Evolution Working Group describes a unified nomenclature system to facilitate discussion and comparison of subtype H5N1 lineages (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). Base on HA gene phylogenetic analysis, they have identified 10 unique first-order numbered clades of the HPAI viruses (H5N1) in the Gs/GD-like lineage (clades 0–9).

The emerging of HPAI H5N1 in Thailand

By 2008, the outbreak of HPAI H5N1 viruses in Thailand occurred in 8 waves with 17 out of 25 infected human succumbing to the disease. The first wave between January and May 2004, the viruses isolated from poultry, wild and domestic bird populations, and domestic cat infected by eating a pigeon carcass (Songserm et al., 2006a), a tiger and leopard from Suphanburi province in Thailand (Keawcharoen et al.,

2004). The second wave of the outbreaks occurred between July 2004 and April 2005 during which time avian influenza H5N1 virus was most widespread among poultry in Thailand. Not only is avian influenza H5N1 virus known to cross the species barrier and infect humans and felines; in this period, fatal H5N1 infection in a domestic dog following ingestion of an H5N1-infected duck was reported from Suphanburi province (Songserm et al., 2006b). The studies performed on felines and canines demonstrate that H5N1 virus infection causes systemic disease and can spread within and between mammalian hosts. Although no direct transmission of H5N1 from cats to humans has been reported, the possibility of humans acquiring H5N1 infection from direct contact with infected cats and dogs warrants concern and highlights the necessity for monitoring domestic animals during H5N1 outbreaks. The third wave of the outbreaks occurred between July and November 2005. The entire genome was sequenced and compared with chickens and quail from this particular outbreak. Sequence analysis of eight gene segments revealed that the 2005 H5N1 viruses isolated in October 2005 were closely related to those recovered from chicken, tiger(s) and human(s) between January and July 2004 (Amonsin et al., 2006). The fourth wave began on July 23, 2006 and had spread by July 29, 2006. These outbreaks affected chickens and encompassed 2 distinct areas: Phichit and Nakhon Phanom Province. The sequences of all 8 gene segments were obtained from both provinces. The samples from Phichit closely resembled H5N1 strains that had circulated in Thailand during 2004 and 2005, but the samples from Nakhon Phanom were newly emerged in Thailand and more closely related to H5N1 strains, that had been circulating since 2005 in south-east China and Laos. Phylogenetic analysis also showed that the viruses isolated from Phichit belonged to genotype Z, whereas virus isolated from Nakhon Phanom belonged to genotype V (Chutinimitkul et al., 2007). According to WHO/OIE/FAO H5N1 Evolution Working Group reports, the virus isolated from Nakhon Phanom belonged to clade 2 subclade 3. clades. In Thailand, H5N1 influenza virus isolated during the four episodes of the outbreak can be separated into clade 1 and clade 2 subclade 3. The fifth wave occurred between January and March 2007 affecting four provinces: Ang Thong, Phitsanulok, Nong Khai and Mukdahan. Isolates from this episode can be divided into clad 1 which founded in Ang Tong, Phisanulok and Mukdahan provinces and clade 2

subclade 3 were founded in Nong Khai province (Amonsin et al., 2008). In January 2008, the sixth wave occurred in two provinces, Phichit and Nakhonsawan no human cases were reported (Chaichoune et al., 2009). In November 2008, the seventh wave occurred in the neighboring provinces.

H5N1 in Tiger

At first time, in early 2004, H5N1 viruses caused major devastation in Suphanburi zoo. Two tigers (*Panthera tigris*) and two leopards (*P. pardus*) showed clinical signs, including high fever and respiratory distress, and they died unexpectedly. The animals had been fed fresh chicken carcasses from a local slaughterhouse (Keawcharoen et al., 2004). At that time many chickens around Suphanburi were dying with respiratory and neurologic symptoms of what was retrospectively identified as H5N1 virus infection. All four animals were severe pulmonary consolidation and multifocal hemorrhage in several organs, including lung, heart, thymus, stomach, intestine, liver, and lymph nodes. One tiger and one leopard had evidence of encephalitis, characterized by multifocal infiltration by neutrophils and macrophages. The genetically of viruses are similar to the viruses isolated from poultry during the same period. The second outbreak, in mid-October, was reported in tiger zoo, Sriracha, Chonburi where 147 out of 441 tigers either died or had to be culled. The animals had been fed raw chicken carcasses that were possibly contaminated with the HPAI H5N1 virus. Microscopic findings showed moderate congestion of the brain with mild nonsuppurative meningoencephalitis, severe diffuse lung hemorrhage and edema, and moderate multifocal necrotizing hepatitis. Approximately 12 days after stopping the feeding of the tigers with raw chicken carcasses, these symptoms were still present. Moreover, except for the tigers no other avian or mammalian species kept in the zoo had been infected during this outbreak. Hence, after cessation of feeding raw chicken carcasses, the tigers were probably infected by horizontal transmission (Thanawongnuwech et al., 2005). Administration of oseltamivir therapy might suppress H5N1 virus and prolong its incubation period but this is unlikely.

CHAPTER III

TYPING (A/B) AND SUBTYPING (H1/H3/H5) OF INFLUENZA A VIRUSES BY MULTIPLEX REAL-TIME RT-PCR ASSAYS

To date, 16 HA and 9 NA subtypes of influenza A viruses have been detected in wild birds and poultry throughout the world (Fouchier et al., 2005a). As viruses of all subtypes have been isolated from aquatic birds, they are considered a reservoir of influenza A viruses for other animal species. The number of viral subtypes found in mammals is limited; the most common subtypes of influenza A virus causing disease in humans have beenH1N1, H2N2, and H3N2 (Harper et al., 2004). However, various additional subtypes of influenza A virus including H5N1, H7N7, H7N3 and H9N2 have been reported in humans over the past few years (Fouchier et al., 2005b). One of the mechanisms resulting in the emergence of a pandemic influenza strain is genesis of a virus combining the highly pathogenic nature of avian strains with the highly transmissible nature of human strains by genetic reassortment subsequent to co-infection of a human host with an avian strain and a human strain. Therefore, rapid detection and identification of these subtypes is particularly important for early diagnosis, leads to suitable treatment and prevention of widespread transmission.

This part was advanced further and provides an even more rapid, specific and sensitive assay based on a single-step multiplex real-time RT-PCR using primers and various LNA-mediated TaqMan probes in two separate assays. The first assay consisted of primers and probes corresponding to the matrix gene (M1) of influenza A virus, matrix gene (M1) of influenza B virus and GAPDH gene of host cells for typing of influenza virus and serving as an internal control, respectively. The other assay employed primers and probes specific to the hemagglutininH1, H3 and H5 subtypes in order to identify the three most prominent subtypes of influenza A virus capable of infecting humans.

1. Objective

To develop the early and rapid assay for detection Influenza A/B and subtype H1, H3 and H5.

2. Materials and methods

2.1 Sources of clinical specimens

Fifty-two influenza virus specimens were used to validate the multiplex real-time RT-PCR assays. This part was performed on influenza A virus human subtypes H1N1 and H3N2 isolated from humans nasopharyngeal suctions in Thailand during 2006 to early 2008 (N= 10 and 12, respectively) and subtype H5N1 (N= 19) in Thailand between 2005 and 2008. Eleven influenza B virus consist of, one from human nasopharyngeal suctions in Thailand, and 10 cDNA from isolates were provided by the Minnesota Department of Health were performed.

The RNA extracted from allantoic fluid of the H5N1 influenza inoculated eggs were provided by: the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; the Department of Livestock Development, Bangkok, Thailand; the Faculty of Veterinary Science, Kasetsart University, Kampaengsaen Campus, Nakorn Pathom, Thailand and the Department of Pediatrics, Faculty of Medicine, Srinakharinwirot University, Nakhon Nayok, Thailand.

2.2 Primers and LNA-mediated TaqMan probe design

In this part, a specific and sensitive one-step multiplex real-time RT-PCR was developed by using primers and a number of specific LNA-mediated TaqMan probes in two separate assays. The first assay consisted of primers and probes specific to the matrix (M1) gene of influenza A virus, matrix (M1) gene of influenza B virus for typing of influenza virus as well as GAPDH gene of host cells for verification as an internal control to assure correct collection of the specimens.

A one-step multiplex real-time RT-PCR using LNA-mediated TaqMan probes was developed. The locked nucleic acids (LNAs) are nucleotide analogues that are locked

conformationally in a C3'-endo/N-type sugar conformation by O2' to C4' methylene linkage (Koshkin et al., 1998; Ichikawa et al., 1999; Wang et al., 1999) that leads to reduced conformational flexibility (Braasch and Corey, 2001). LNA increases the thermal stability of oligonucleotides (about 3–8 ˚C per modified base in the probe) (Letertre et al., 2003; Kennedy et al., 2005). This can be used to increase the sensitivity and specificity of TaqMan probes.

Primers and probes for discrimination between the influenza A and influenza B virus were chosen from conserved nucleotide sequences specific for the matrix gene (M1) of influenza A virus for influenza A detection (Payungporn et al., 2008) and specific for the matrix gene (M1) of influenza B virus for influenza B detection. The primers and probe for GAPDH gene detection of the host cell were chosen from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) nucleotide sequence. For the assay aimed at differentiating between influenza A virus subtypes H1, H3 and H5, the specific regions of subtype H1, H3 and H5 hemagglutinin (HA) were chosen for designation of primers and probes to distinguish between subtypes H1, H3 and H5 respectively. Primers and probes were analyzed using the primer design software (OLIGOS, Version 9.1 by Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) and Exiqon Tm prediction 1.1 (http://lnatm.com/) to ensure that they could be combined in a multiplex format and subjected to identical PCR conditions. The probes label with three different fluorescent reporter dyes (FAM, HEX and Cy5 with emission wavelengths at 518, 556 and 667 nm, respectively) were applied for simultaneous detection in multiplex format of the sequences corresponding to the target genes. The primers and probes used in this part showed in Table 1 and 2.

Table 1 Multiplex PCR primers and LNA mediated TaqMan probes for typing of influenza virus.

Target gene	Primers/Probes	Sequence (5'-3')	Position	Strand
GAPDH	GAPDH-F85	GTGAAGGTCGGAGTCAACGG	85-104	Sense
	GAPDH-P121	HEX-CGCCTGGTCA**C**CAGGGCTGC-	121-140	Sense
	GAPDH-R191	TCAATGAAGGGGTCATTGATGG	191-169	Antisense
M Gene Flu A	FluA-M-F151	CATGGARTGGCTAAAGACAAGACC	151-175	Sense
	FluA-M-P218	FAM-ACGC**T**CACCG**T**GCCC**A**GT-BHQ1	218-235	Sense
	FluA-M-R276	AGGGCATTTTGGACAAAKCGTCTA	276-252	Antisense
M Gene Flu B	FluB-MF439	CTCTGTGCTTTRTGCGARAAAC	439-460	Sense
	FluB-P135	Cy5-TCAG**C**A**A**T**GA**A**C**ACAGCAA-BHQ3	541-559	Sense
	FluB-MR	CCTTCYCCATTCTTTTGACTTGC	671-649	Antisense

Bold and underlined nucleotides represent LNA residues

Table 2 Multiplex PCR primers and LNA mediated TaqMan probes for differentiating between influenza A virus subtypes H1, H3 and H5.

Target gene	Primers/Probes	Sequence (5'-3')	Position	Strand
H1	H1_F	ACTACTGGACTCTGCTKGAA	750-769	Sense
	H1_P	FAM-TTGAGGCAAATGGAAATCTAATAGC-TAMRA	789-813	Sense
	H1_R	AAGCCTCTACTCAGTGCGAA	846-827	Antisense
H3	H3_F	TGCTACTGAGCTGGTTCAGAGT	139-160	Sense
	H3_P	HEX-AGAT**G**C**T**C**T**A**T**T**G**G**G**AGACC-BHQ1	226-245	Sense
	H3_R	AGGGTAACAGTTGCTGTRGGC	322-302	Antisense
H5	H5-F1512	TGGAAAGTGTAARAAACGGAACGT	1512-1536	Sense
	H5-P	Cy5-A**C**TC**C**A**C**TTA**T**T**C**CT**C**T**C**T-BHQ3	1593-1574	Antisense
	H5-R1660	TGCTAGGGAACTCGCMACTG	1660-1640	Antisense

Bold and underlined nucleotides represent LNA residues

2.3 Multiplex real-time RT-PCR condition

A single step multiplex real-time RT-PCR was carried out using the SuperScript III Platinum One-Step RT-PCR system (Invitrogen, California, USA). Three sets of primers and LNA mediated TaqMan probes were used in the multiplex format (Table 1 and 2), each primer and probe at a final concentration of 0.25 μM and 0.125 μM, respectively. The reaction comprised a combination of 1.0 μl RNA sample with a reaction mixture containing 5 μl of 2× Reaction Mix, 0.2 μl of SuperScript III RT Platinum® Taq Mix, additional 0.1 mM of MgCl2 and RNase-free water in a final volume of 10 μl. One-step multiplex real-time RT-PCR was performed on Rotor-Gene 3000 (Corbett Research, New South Wales, Australia). Cycling conditions included a reverse transcription step at 50 °C for 30 min. After an initial denaturation step at 95 °C for 10 min to activate the Platinum® Taq DNA polymerase, amplification was performed during 40 cycles including denaturation (95 °C for 15 s), annealing and extension (60 °C for 30 s, respectively) Multiple fluorescent signals were obtained once per cycle upon completion of the extension step with detectors corresponding to FAM (518 nm), HEX (556 nm) and Cy5 (667 nm). Data acquisition and analysis of the real-time PCR assay were performed using the Rotor-Gene data analysis software, Version 6.0 (Corbett research supporting program).

2.4 Positive controls

To develop and optimize the assays for typing (A/B) and subtyping (H1/H3 and H5) of influenza viruses by multiplex real-time RT-PCR assay, plasmid DNAs were constructed by insertion of the matrix gene of influenza A virus (nt 1-1027; A/chicken/Nakorn-Patom/Thailand/CU-K2/2004), matrix gene of influenza B virus (nt 1-682; B/Malaysia/2506/2004-like vaccine strain), GAPDH gene (nt 85-191), H1 gene (nt 266-1672; A/Thailand/CU51/2006(H1N1)), H3 gene (nt 10-1771; A/Thailand/CU46/2006(H3N2)) and H5 gene (nt 880-1694; A/chicken/Nakorn-Patom/Thailand/CU-K2/2004(H5N1)) into the pGEM-T Easy Vector (Promega, Madison, WI) by TA-cloning strategy.

2.5 Specificity test

The specificity of the multiplex real-time RT-PCR assay was evaluated by cross-reaction tests with RNA extracted from other subtypes of influenza A viruses including: A/Singapore/1/57 (H2N2), A/duck/Czeck/56 (H4N6), A/turkey/Massachusetts/3740/65 (H6N2), A/seal/Massachusetts/1/80 (H7N7), A/turkey/Ontario/67 (H8N4), A/turkey/Wisconsin/66 (H9N2), A/chicken/Germany/N/49 (H10N7), A/duck/England/1/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/gull/Maryland/704/77 (H13N6), A/mall/Astrakhan/263/82 (H14N5) and A/duck/Australia/341/83 (H15N8). The other subtypes of influenza A virus specimens were provided by the Department of Livestock Development, Bangkok, Thailand. The assays were also evaluated against other respiratory viruses including human bocavirus (HBoV) (N=5), adenovirus (N=2), parainfluenza (PIVs) (N=3), respiratory syncytial virus (RSV) subgroups A & B (N=5), metapneumovirus (MPV) (N=7) and the non-specific pathogen specimens (N=12), which were negative for influenza A and B virus.

2.6 RNA standard for sensitivity test

To generate an RNA standard for the sensitivity test, in vitro transcription was performed by using the RiboMAXTM Large Scale RNA Production System-T7 (Promega, Madison, WI) following the manufacturer's recommendations. The resulting RNA was extracted with phenol/chloroform followed by ethanol precipitation. The concentration of the in vitro transcribed RNAs was calculated by measuring absorbance at 260 nm. The copy number was calculated by the formula: Transcript copy (number/µl) = [RNA concentration (g/µl)×6.02×1023]/[length of in vitro transcribed RNA (bp)×340]. RNAs were then diluted serially 10-fold, ranging from 10^8 to 10 copies/µl and used for the sensitivity test.

2.7 Direct sequencing

Full length of HA and NA genes were amplified by using primers described previously (Hoffmann et al., 2001). For automated DNA sequencing, the PCR products

of interest were purified from the agarose gel using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg, Germany). The sequencing reaction was performed using BigDye Terminator, Version 3.1 (Applied Biosystems) according to the manufacturer's specifications. The sequencing products were subjected to a PerkinElmer 310 Sequencer (PerkinElmer). The results were analyzed and influenza A virus subtypes were determined by BLAST analysis.

3. Results

3.1 Interpretation of typing (A/B) and subtyping (H1/H3/H5) of influenza viruses by single-step multiplex real-time RT-PCR assays

In the assay aimed at typing of influenza A and influenza B virus, the results were obtained by using multiple LNA-mediated TaqMan probes label with the FAM, Cy5 and HEX fluorescent signal corresponding to the matrix gene of influenza A virus, matrix gene of influenza B virus and GAPDH gene, respectively. The fluorescent signals obtained from the typing assay can be interpreted as shown in Figure 7. The results showed a clear signal obtained from each detection channel without any unexpected noise. Influenza A virus yielded an amplification signal in the FAM channel, influenza B virus produced a fluorescent signal in the Cy5 channel and the GAPDH gene of the host cell yielded a signal only detected in the HEX channel.

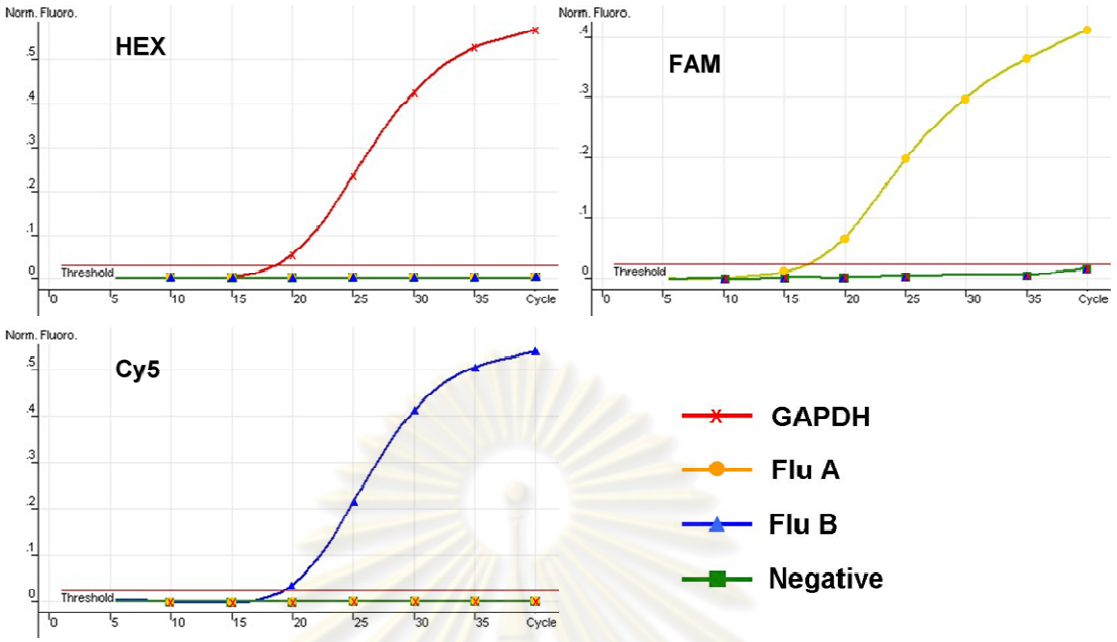


Figure 7 Interpretation of influenza virus type detection by single-step multiplex real-time RT-PCR. The influenza virus type detection (A) shows fluorescent signals of HEX, FAM and Cy5 corresponded to the GAPDH, M gene of influenza A virus and M gene of influenza B virus, respectively.

In the subtyping (H1/H3/H5) assay, the presence of H1, H3 and H5 subtypes was detected by using multiple LNA-mediated TaqMan probes labeled with the FAM, HEX and Cy5 fluorescent dyes, respectively (Figure 8). The results revealed a clear signal obtained from each detection channel without any unpredicted noise. Samples yielding a positive FAM signal have been interpreted as H1 subtype, samples yielding a positive HEX signal as H3 subtype and samples yielding a positive Cy5 signal as H5 subtype.

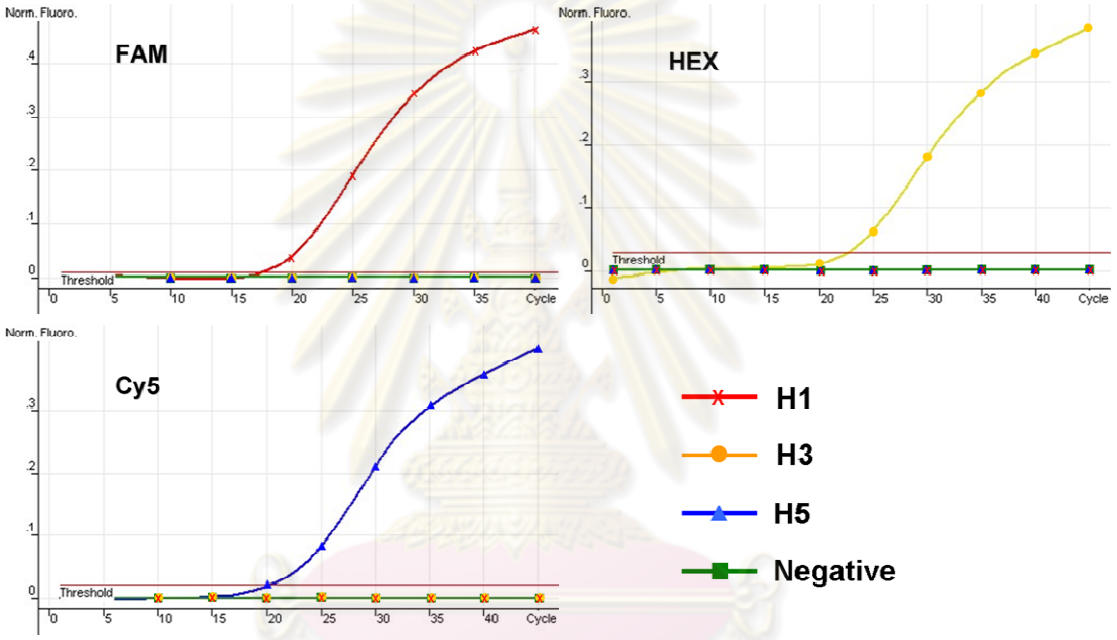


Figure 8 Interpretation of influenza virus subtype detection by single-step multiplex real-time RT-PCR. The subtype of influenza virus detection shows fluorescent signals of FAM, HEX and Cy5 corresponded to H1, H3 and H5, respectively.

3.2 Specificity test

The specificity of both the typing and subtyping multiplex realtime RT-PCR assays was evaluated by cross-reaction tests against other respiratory viruses including HBoV, adenovirus, PIVs, RSV subgroups A and B, MPV and the unknown specimens, which were negative for influenza A and B viruses. The results did not reveal any cross-amplification signal when testing against other respiratory viruses.

Other subtypes of influenza A virus including H2N2, H3N8, H4N6, H6N2, H7N7, H8N4, H9N2, H10N7, H11N6, H12N5, H13N6, H14N5 and H15N8 were also included in order to validate the assays in terms of specificity. The results revealed that the typing assay (A/B) produced exclusively the FAM amplification signal against all subtypes of influenza A virus tested, indicating the broad detection range of the primers and probe for the matrix gene of influenza A virus in the typing assay which can therefore be used for broad detection against all subtypes of influenza A virus. The subtyping (H1/H3/H5) assay did not produce any amplification signal against other subtypes of influenza A virus (H2, H4 and H6-H15), demonstrating the high specificity of the primers and probes used (Table 3).

Table 3 The results of specificity test with other subtype of influenza A virus.

Strain name	Results		Subtype
	Typing	Subtyping	
A/Singapore/1/57 (H2N2)	A	-	H2
A/duck/Czeck/56 (H4N6)	A	-	H4
A/turkey/Massachusetts/3740/65 (H6N2)	A	-	H6
A/seal/Massachusetts/1/80 (H7N7)	A	-	H7
A/turkey/Ontario/67 (H8N4)	A	-	H8
A/turkey/Wisconsin/66 (H9N2)	A	-	H9
A/chicken/Germany/N/49 (H10N7)	A	-	H10
A/duck/England/1/56 (H11N6)	A	-	H11
A/duck/Alberta/60/76 (H12N5)	A	-	H12
A/gull/Maryland/704/77 (H13N6)	A	-	H13
A/mall/Astrakhan/263/82 (H14N5)	A	-	H14
A/duck/Australia/341/83 (H15N8)	A	-	H15

3.3 Sensitivity test

In order to determine the sensitivity of the multiplex real-time RT-PCR assays, 10-fold serial dilutions of the standard in vitro transcribed RNAs (ranging from10^8 to 10 copies/µl) of matrix influenza A virus, matrix influenza B virus, GAPDH, H1,H3 and H5 genes were prepared and 1µl of each serially diluted RNA sample was used as a template in the real-time RT-PCR assay. As expected, the threshold cycle (Ct) increased in direct proportion to the dilution of the RNA standards. In the typing assay, the fluorescent signal can be detected at RNA standard concentrations of GAPDH, matrix influenza A virus and matrix influenza B virus as low as 10, 10 and 10^3 copies/µl, respectively whereas the fluorescent signal of the subtyping assay can be detected at standard concentrations as low as 10^2, 10^3 and 10 copies/µl for H1, H3 and H5 RNA, respectively

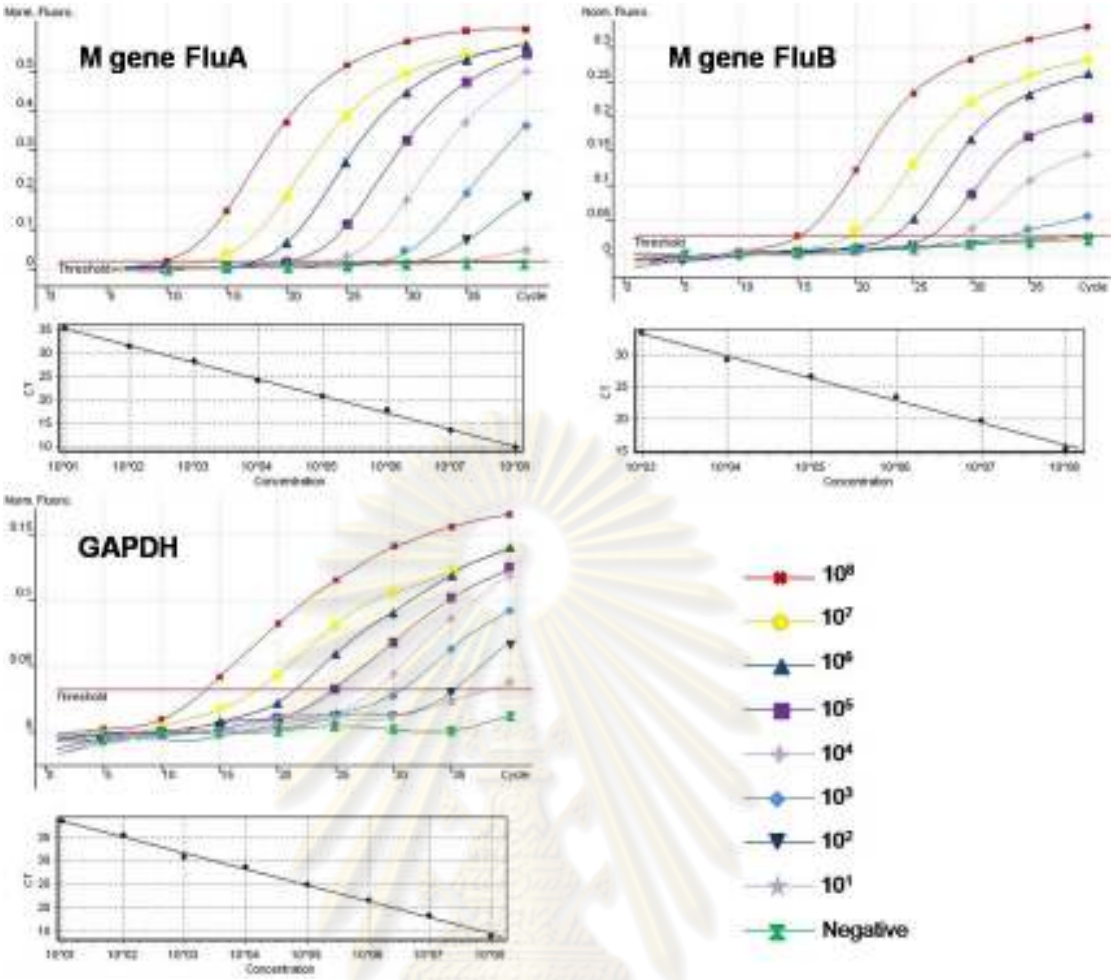


Figure 9 The sensitivity test for typing (A/B) of influenza viruses. The fluorescent signal can be detected at RNA standard concentrations of matrix gene of influenza A virus, matrix gene of influenza B virus and GAPDH as low as 10, 10^3 and 10 copies/µl, respectively.

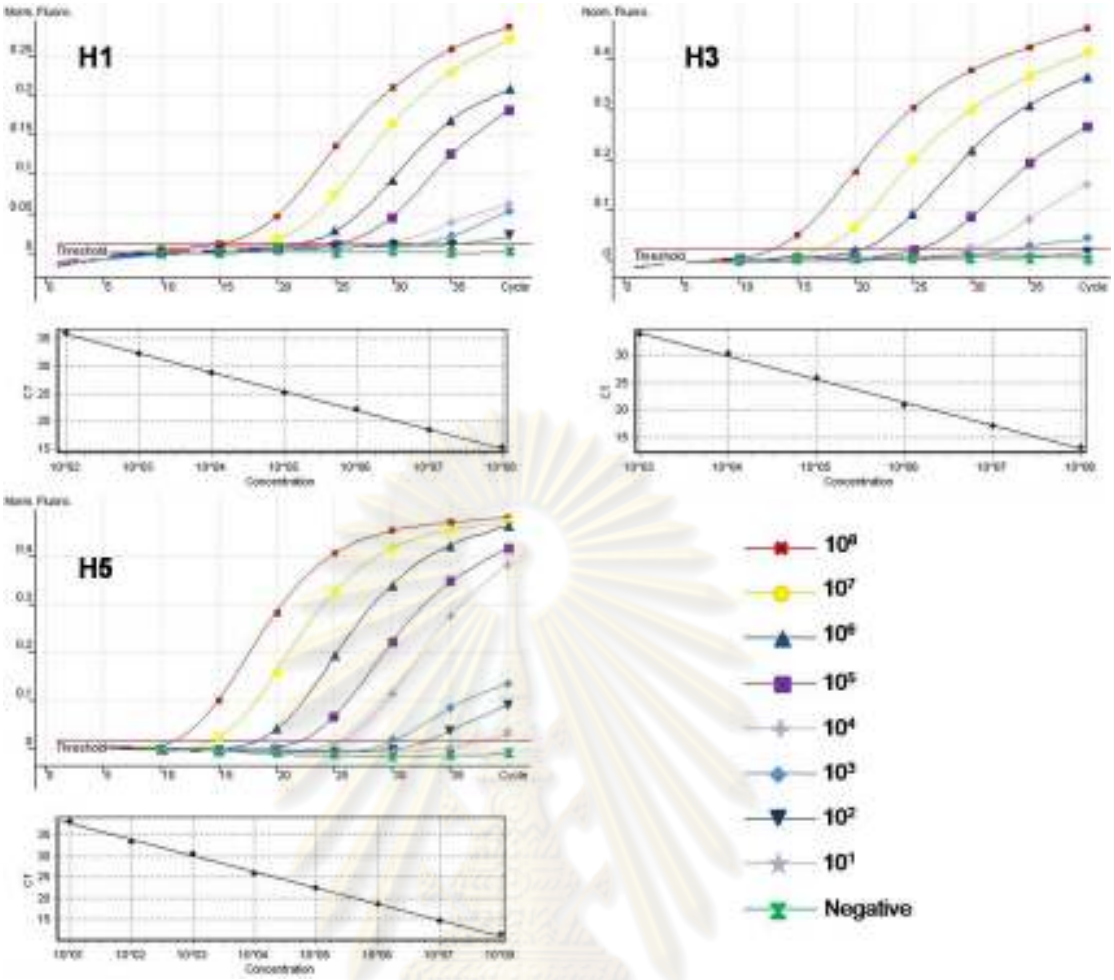


Figure 10 The sensitivity test for subtyping (H1, H3 and H5) of influenza A viruses. The fluorescent signal can be detected at RNA standard concentrations of hemagglutinin gene of influenza A virus subtype H1, H3 and H5 as low as 10^2, 10^3 and 10 copies/µl, respectively.

3.4 Evaluation of the assays using clinical specimens

In this part, 52 specimens containing influenza viruses were used to evaluate the efficiency of the multiplex real-time RT-PCR assays. The results obtained from the typing assay (A/B) of 52 clinical specimens showed 41 specimens infected with influenza A virus and 11 specimens with influenza B virus. The 41 influenza A virus positive specimens were subjected to further subtyping for H1, H3 and H5, respectively. The results demonstrated subtype H1 in 10 samples, subtype H3 in 12, and subtype H5 in 19 samples, respectively. None of the samples tested produced any significant false positive or non-specific signal. Nucleotide sequencing was performed in all positive

specimens for confirmation of type and subtype identification. Typing and subtyping results obtained from the multiplex real-time RT-PCR were identical with the results obtained from nucleotide sequencing and BLAST analysis (Table 4), indicating that typing and subtyping of influenza viruses had been accurately performed by the multiplex real-time RT-PCR assay.

Table 4 Results of typing and subtyping of influenza viruses obtained for evaluate of the assays against clinical specimens.

Strain name	Real-time PCR result		Direct sequencing
	Typing	Subtyping	
A/Thailand/CU41/2006(H1N1)	A	H1	H1
A/Thailand/CU53/2006(H1N1)	A	H1	H1
A/Thailand/CU67/2006(H1N1)	A	H1	H1
A/Thailand/CU88/2006(H1N1)	A	H1	H1
A/Thailand/CU51/2006(H1N1)	A	H1	H1
A/Thailand/CU57/2006(H1N1)	A	H1	H1
A/Thailand/CU44/2006(H1N1)	A	H1	H1
A/Thailand/CU68/2006(H1N1)	A	H1	H1
A/Thailand/CU75/2006(H1N1)	A	H1	H1
A/Thailand/CU32/2006(H1N1)	A	H1	H1
A/Thailand/CU23/2006(H3N2)	A	H3	H3
A/Thailand/CU46/2006(H3N2)	A	H3	H3
A/Thailand/CU272/2007(H3N2)	A	H3	H3
A/Thailand/CU280/2007(H3N2)	A	H3	H3
A/Thailand/CU228/2006(H3N2)	A	H3	H3
A/Thailand/CU282/2007(H3N2)	A	H3	H3
A/Thailand/CU259/2006(H3N2)	A	H3	H3
A/Thailand/CU260/2006(H3N2)	A	H3	H3
A/Bangkok/CU231/2006(H3N2)	A	H3	H3
A/Bangkok/CU-01/2008(H3N2)	A	H3	H3
A/Bangkok/CU-02/2008(H3N2)	A	H3	H3
A/Bangkok/CU-03/2008(H3N2)	A	H3	H3
A/leopard/Thailand/Leo-1/2004(H5N1)	A	H5	H5
A/tiger/Suphanburi/Thailand/Ti-1/04(H5N1)	A	H5	H5
A/clouded leopard/Thailand/KU-11/2004(H5N1)	A	H5	H5
A/dog/Thailand-Suphanburi/KU-08/04(H5N1)	A	H5	H5

Table 4 (*continue*) Results of typing and subtyping of influenza viruses obtained for evaluate of the assays against clinical specimens.

Strain name	Real-time PCR result		Direct sequencing
	Typing	Subtyping	
A/Chicken/Thailand/PC-168/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/PC-170/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/NP-172/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/167/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/169/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/173/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/174/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/1947/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/3277/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/3348/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/3886/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/4802/2006(H5N1)	A	H5	H5
A/Chicken/Thailand/5295/2006(H5N1)	A	H5	H5
A/chicken/Thailand/NS-339/2008(H5N1)	A	H5	H5
A/chicken/Thailand/PC-340/2008(H5N1)	A	H5	H5
B/Thailand/CU243/2006	B	-	B
B/Shanghai/MDH1/2007	B	-	B
B/Shanghai/MDH2/2007	B	-	B
B/Shanghai/MDH3/2007	B	-	B
B/Malaysia /MDH4/2007	B	-	B
B/Shanghai/MDH5/2007	B	-	B
B/Shanghai/MDH6/2007	B	-	B
B/Shanghai/MDH7/2007	B	-	B
B/Shanghai/MDH8/2007	B	-	B
B/Shanghai/MDH9/2007	B	-	B
B/Shanghai/MDH10/2007	B	-	B

4. Discussion

In this part, two methods based on single-step multiplex real-time RT-PCR assays were developed and validated in terms of specificity, sensitivity and efficiency. The first assay consisted of primers and probes specific to the matrix gene of influenza A virus, matrix gene of influenza B virus and GAPDH gene of host cells for typing (A/B) of influenza viruses and verification by an internal control, respectively. The other assay employed primers and probes specific to the hemagglutinin gene of H1, H3 and H5 subtypes in order to identify the three predominant subtypes of influenza A viruses capable of infecting humans. However, the hemagglutinin gene of H16 was not tested.

The single-step multiplex real-time RT-PCR assays were developed to meet the following objectives: to detect the presence of RNA from influenza B virus or any subtypes of influenza A viruses; by including an internal control gene (GAPDH), to provide evidence that failure to identify viral sequences was not attributable to degradation of the target RNA or the presence of enzyme inhibitors or detection quenchers; and to identify positively the three most common subtypes (H1/H3/H5) of influenza A virus capable of infecting humans. Each method represents a single-step process which less time consuming than the two-step process and thus, minimizes the risk of cross-contamination.

The single-step multiplex real-time RT-PCR assay for typing of influenza viruses relies on three molecular probes which target GAPDH from the host cell as well as the matrix gene from the influenza A and influenza B virus genomes, respectively. Amplification of the host GADPH gene serves to establish specimen quality and integrity and assay functionality. Clinical necropsy or laboratory samples containing influenza A virus are expected to yield two amplification signals, FAM for the influenza A matrix gene and HEX for the GAPDH gene. Specimens yielding the two amplification signals Cy5, for the influenza B matrix gene and HEX for the GAPDH gene would be indicative of an influenza B virus infection. Properly collected and handled specimens lacking influenza A and influenza B viruses yield the GAPDH amplification signal (HEX) only. Finally, specimens failing to yield amplification signals with the three probes are indicative of defective specimen collection or handling, degradation, faulty RNA extraction or the presence of inhibitors of the polymerases or fluorochromes used in real-time PCR.

The probes used in this part were LNA-mediated TaqMan probes to increase thermal stability (Kennedy et al., 2005). Therefore the LNA-mediated TaqMan probes are shorter than the conventional TaqMan probes at the same melting temperatures. For influenza A virus detection, a short LNA-TaqMan probe is more suitable than the conventional TaqMan probe as it is difficult to find a long conserved region (25–30 nt) when aligning different strains of influenza A virus for designing a TaqMan probe providing perfect match with all subtypes of influenza A viruses. A TaqMan MGB may serve as an alternative choice because the probe is coupled with a minor groove binder

(MGB), which increases the Tm of the probe. This part was performed on Rotor-Gene 3000. Primers and probes in this part can be applied into other platforms by using compatible fluorescents/quenchers with other platforms.

The multiplex real-time RT-PCR assays described above provide adequate sensitivity values since the detection limits have been comparable with previous studies based on real-time PCR for influenza virus detection which had yielded sensitivity ranges from 10 to 10^3 copies (Spackman et al., 2002; Lee and Suarez, 2004; Payungporn et al., 2006).

In conclusion, both typing and subtyping assays based on the single-stepmultiplex real-time RT-PCR assays described above provide a rapid, specific, sensitive and cost-effective approach for typing (A/B) and subtyping (H1/H3/H5) of influenza viruses thus rendering it feasible and attractive for large-scale screening, particularly at times of influenza A virus outbreaks. Rapid laboratory diagnosis of influenza virus infections at an early stage of the disease can yield information relevant to patient management and help facilitate biosecurity protocols.

CHAPTER IV

MOLECULAR EVOLUTION OF HUMAN INFLUENZA (H1N1 AND H3N2) IN THAILAND, 2006-2008

The WHO has published recommendations on the composition of influenza vaccine for the northern and southern hemispheres. For the northern hemisphere, the WHO has issued the recommendation for strains to be included in the trivalent vaccine for the next season based on epidemiological data and genetic analyses of circulating strains.

The ability to predict emergence of circulating influenza strains for subsequent annual vaccine development has become vital. Comparisons between antigenic differences and phylogenetic analyses are essential to further the understanding of multiple lineages of influenza virus variants. Therefore, this part has been aimed at elucidating the complete genome evolution of influenza A H1N1 and H3N2 isolated from Thailand over a time period of three years, from early 2006 to the end of 2008. Genome evolution may have implications on predicting the strains emerging during the following season in order to match vaccine composition.

1. Objective

To investigate and study the molecular evolution of human influenza A virus subtype H1N1 and H3N2 by using the whole genome sequences analysis in Thailand.

2. Materials & Methods

2.1 Clinical samples

Nasopharyngeal suction specimens were collected from 383 patients diagnosed with respiratory illness in Thailand from 2006-2008. All clinical samples were provided by the Department of Pediatrics, King Chulalongkorn Memorial Hospital. All clinical

samples were screened for influenza A virus subtypes H1 and H3 by using one-step multiplex real-time RT-PCR as previously described in CHAPTER III.

2.2 RNA extraction and whole genome sequencing

Viral RNA was extracted from 200-µl nasopharyngeal suction samples by Real Genomics Viral Nucleic Acid Kit (RBC Bioscience, Taiwan). cDNAs were synthesized at 37°C for 3 hours using the M-MLV reverse-transcription system (Promega, Madison, WI) and 1 µM universal primer as described by Hoffmann and co-worker (Hoffmann et al., 2001). The whole genome sequences were amplified using the primer sets for human H1N1 (Table 5) and H3N2 (Table 6) influenza A virus. Briefly, 1 µl of cDNA template was added to the reaction mixture containing 10 µl of 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), 0.5 µM forward and reverse primers and nuclease free water to a final volume of 25 µl. The amplification reaction was performed in a thermal cycler (Eppendorf, Germany) under the following conditions: Denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec (for PB2, PB1, PA genes) and 55°C for 30 sec (for HA, NP, NA, MP, NS genes) followed by extension at 72°C for 90 sec, and concluded by a final extension at 72°C for 7 min. After electrophoresis in a 2% agarose gel stained with ethidium bromide on preparation, the expected PCR product was visualized on a UV trans-illuminator. PCR products were purified from the 2% agarose gel using the Perfectprep Gel Cleanup kit (Eppendorf, GmbH, Germany). DNA sequencing was performed using the Gene Amp PCR system 9600 (Perkin-Elmer, Boston, MC).

Table 5 Primer sets for H1N1 whole genome amplification

Target Gene	Primer	Sequence (5'-3')	Position	Strand
PB2	PB2-F5'	AGCAAAAGCAGGTCAATTATATTC	1-23	Sense
	PB2_H1_F529	TTCCCTAATGAAGTGGGAGC	529-5148	Sense
	PB2_H1_R626	GGGGAAATTTTGCAATCCTG	626-607	Antisense
	PB2_H1_F1099	GAGGGATATGAAGAGTTCAC	1099-1118	Sense
	PB2_H1_R1217	GCTTCGACTATTGACTGTTC	1217-1198	Antisense
	PB2_H1_F1721	CTCAGAACCCTACAATGCTAT	1721-1741	Sense
	PB2_H1_R1850	CCAAGCACATCCCTCATTTG	1850-1831	Antisense
	PB2-R3'	AGTAGAAACAAGGTCGTTTTTAAA	2341-2317	Antisense
PB1	PB1-F5'	AGCAAAAGCAGGCAAACCATTTG	1-23	Sense
	PB1_H1_F506	GGAGGCTAATAGACTTCCTTA	506-526	Sense
	PB1_H1_R701	GTGTTCAGGGTTAATGCCCT	701-682	Antisense
	PB1_H1_F1232	TGAGTCCTGGAATGATGATG	1232-1251	Sense
	PB1_H1_R1319	GTGTATCTCTTTTGCCCAAGA	1319-1299	Antisense
	PB1_H3_F1705	TGCCACAGAGGTGACACAC	1705-1723	Sense
	PB1_H1_R1863	CCATTTCAAGCAGACTTCAG	1863-1844	Antisense
	PB1-R3'	AGTAGAAACAAGGCATTTTTTCA	2341-2319	Antisense
PA	PA-F5'	AGCAAAAGCAGGTACTGATCCG	1-22	Sense
	PA_H1_F481	GAAGAAATGGCCACAAAGGC	481-500	Sense
	PA_H1_R613	CTCTTTCGGACTGACGAAAG	613-594	Antisense
	PA_H1_F1246	CAGAATGAGTTCAACAAGGC	1246-1265	Sense
	PA_H1_R1383	GCAATGGGACACCTCAGCT	1383-1365	Antisense
	PA_H1_R1921	TGGAACCTTCTTCCACTCCT	1921-1902	Antisense
	PA-R3'	AGTAGAAACAAGGTACTTTTTTGG	2234-2211	Antisense

Table 5 ***(continue)*** Primer sets for H1N1 whole genome amplification

Target Gene	Primer	Sequence (5'-3')	Position	Strand
HA	HA-F5'	AGCAAAAGCAGGGGTCTGATCTG	1-23	Sense
	H1 F266	CTTAGGAAACCCAGAATGCG	266–285	Sense
	H1 R627	ACGGGTGATGAACACCCCA	627–609	Antisense
	H1 F1062 5_	GGTTTGTTTGGAGCCATTGC	1062–1081	Sense
	H1 R1525 5_	CCATTTTTCACACTTTCCATGC	1525–1504	Antisense
	HA-R3'	AGTAGAAACAAGGGTGTTTTTAACTAC	1778-1754	Antisense
NP	NP-F5'	AGCAAAAGCAGGGTAGATAATC	1-22	Sense
	NP_H1_F542	TGATGCAGGGTTCAACTCTC	542-561	Sense
	NP_H1_R649	CTCCAGAAGTTTCGGTCATT	649-668	Antisense
	NP_H1_F1018	AAGAGTCAGTTGGTGTGGAT	1018-1037	Sense
	NP_H3_R1205	GCCCAGTATCTGCTTCTCA	1205-1187	Antisense
	NP-R3'	AGTAGAAACAAGGGTATTTTTCT	1564-1543	Antisense
NA	NA-F5'	AGCAAAAGCAGGAGTTTAAAATG	1-23	Sense
	N1 F479	CCCYTATAGGGCYTTAATGAG	479–499	Sense
	N1 R620	ATTRTCTGGACCAGAAATTCC	620–600	Antisense
	N1 F653	AATAACTGAAACCATAAAAAGTTG	653–676	Sense
	N1 R1159	CATCCATTAGGATCCCAAATCA	1159–1138	Antisense
	NA-R3'	AGTAGAAACAAGGAGTTTTTTGAAC	1458-1434	Antisense
M	M-F5'	AGCAAAAGCAGGTAGATGTTG	1-21	Sense
	M-R3'	AGTAGAAACAAGGTAGTTTTTTAC	1022-1004	Antisense
NS	NS-F5'	AGCAAAAGCAGGGTGACAAAAAC	1-23	Sense
	NS-R3'	AGTAGAAACAAGGGTGTTTTTTAT	890-867	Antisense

Table 6 Primer sets for H3N2 whole genome amplification

Target Gene	Primer	Sequence (5'-3')	Position	Strand
PB2	PB2-F5'	AGCAAAAGCAGGTCAATTATATTC	1-23	Sense
	PB2_H3_F493	GCCAAGGAGGCACAAGATG	493-511	Sense
	PB2_H3_R642	GTATGCAACCATCAAGGGAG	642-623	Antisense
	PB2_H3_F1059	GCTTACAGGCAATCTCCAAAC	1059-1079	Sense
	PB2_H3_R118	CACTCACTATGAGCTGAACC	1189-1170	Antisense
	PB2_H3_F1637	GGGAGATTAACGGTCCTGA	1637-1655	Sense
	PB2_H3_R185	CCCAAGTACATCTCTCATTTG	1851-1831	Antisense
	PB2-R3'	AGTAGAAACAAGGTCGTTTTTAAA	2341-2317	Antisense
PB1	PB1-F5'	AGCAAAAGCAGGCAAACCATTTG	1-23	Sense
	PB1_H3_F491	CAGCTAATGAATCAGGAAGGC	491-511	Sense
	PB1_H3_R762	CATCCCGGGTGTTGCAATAG	762-743	Antisense
	PB1_H3_F1116	CCGAACACAAATACCCGCAG	1116-1135	Sense
	PB1_H3_R122	GTGCCATCTATTAGAAGAGG	1225-1204	Antisense
	PB1_H3_F1704	GTGCCATAGAGGAGACACAC	1704-1723	Sense
	PB1_H3_R186	GCTCCCACTTTAAGCAGACT	1867-1848	Antisense
	PB1-R3'	AGTAGAAACAAGGCATTTTTTCA	2341-2319	Antisense
PA	PA-F5'	AGCAAAAGCAGGTACTGATCCG	1-22	Sense
	PA_H3_F472	TTCACTGGGGAGGAAATGGCC	472-492	Sense
	PA_H3_R681	GAGACTTTGGTCGGCAAGCC	681-662	Antisense
	PA_H3_F1129	GCTCTTGGTGAAAACATGGC	1129-1148	Sense
	PA_H3_R1247	CTGTATCCAGCTTGAAAGTGA	1247-1228	Antisense
	PA_H3_F1585	GTGAGCATGGAGTTTTCTCTC	1585-1605	Sense
	PA_H3_R1778	AGGCAACGTCTCATCTCCAT	1778-1759	Antisense
	PA-R3'	AGTAGAAACAAGGTACTTTTTTGG	2234-2211	Antisense

Table 6 *(continue)* Primer sets for H3N2 whole genome amplification

Target Gene	Primer	Sequence (5'-3')	Position	Strand
HA	HA-F5'	AGCAAAAGCAGGGGTCTGATCTG	1-23	Sense
	H3 F598	TTGACAAATTGTACATTTGGGG	598–619	Sense
	H3 R797	TCCCGGATTTACTATTGTCCA	797-777	Antisense
	H3 F1013	CACTCTGAAATTGGCAACAGG	1013–1033	Sense
	H3 R1184	GCTTTTGAGATCTGCTGCTTG	1184–1164	Antisense
	HA-R3'	AGTAGAAACAAGGGTGTTTTTAACTAC	1778-1754	Antisense
NP	NP-F5'	AGCAAAAGCAGGGTAGATAATC	1-22	Sense
	NP_H3_F522	GGATCCCAGAATGTGCTCTC	522-541	Sense
	NP_H3_R680	CCATTCTCACCTCTCCAGAA	680-661	Antisense
	NP_H3_F1023	TCAGCTGGTGTGGATGGCA	1023-1041	Sense
	NP_H3_R1221	ACTCCTGGTCCTTATGGCCC	1221-1202	Antisense
	NP-R3'	AGTAGAAACAAGGGTATTTTTCT	1564-1543	Antisense
NA	NA-F5'	AGCAAAAGCAGGAGTTTAAAATG	1-23	Sense
	N2 F557	AGCTCAAGTTGTCACGATGG	557–576	Sense
	N2 R564	CTTGAGCTGGACCATGCTAT	564–545	Antisense
	N2 F766	AGCTGATACTAAAATACTATTCAT	766–789	Sense
	N2 R1120	GCTGATCGTTCTTCCCATCC	1120–1101	Antisense
	NA-R3'	AGTAGAAACAAGGAGTTTTTTGAAC	1458-1434	Antisense
M	M-F5'	AGCAAAAGCAGGTAGATGTTG	1-21	Sense
	M-R3'	AGTAGAAACAAGGTAGTTTTTTAC	1022-1004	Antisense
NS	NS-F5'	AGCAAAAGCAGGGTGACAAAAAC	1-23	Sense
	NS-R3'	AGTAGAAACAAGGGTGTTTTTTAT	890-867	Antisense

2.3 Phylogenetic analysis

Nucleotide sequences were aligned with ClustalX v1.83 (Thompson et al., 1997). Phylogenetic trees were constructed using the Neighbor-joining (NJ) approach implemented in MEGA 4 (Tamura et al., 2007). Bootstrapping support for tree topologies was accomplished using NJ methods implemented in MEGA with 1,000 replicates. Genetic distances based on NJ phylogenetic trees were calculated applying Kimura's two-parameter method using MEGA 4.

2.4 Prediction of N-Glycosylation

Potential N-glycosylation sites (amino acids Asn-X-Ser/Thr, where X is not Pro) were predicted using nine artificial neural networks with the NetNGlyc server 1.0 (Gupta et al., 2004). A threshold value of >0.5 average potential score was set to predict glycosylated sites. The N-Gly-cosite prediction tool at Los Alamos (Zhang et al., 2004) was used to visualize the fraction of isolates possessing certain glycosylated sites along the sequence alignment.

2.5 Measurement of Selection Pressures

To determine the overall selection pressures for each gene, the mean numbers of dN/dS ratio, in which dN is the non-synonymous substitution rate (non-synonymous changes per non-synonymous site) and dS is the synonymous substitution rate (synonymous changes per synonymous site) were estimated. The ratio between dN and dS substitutions and the individual site specific selection pressure was measured by using the likelihood based single likelihood ancestor counting (SLAC), two rate fixed effects likelihood (FEL) and random effects likelihood (REL) methods contained in the HYPHY package (Kosakovsky Pond et al., 2005) and accessed the dataset on the website of Datamonkey interface (http://www.datamonkey.org). In all cases, dN/dS estimates were based on neighbor-joining trees under the GTR substitution model. The accepted significance level for a positively selected site was set at <0.1 (two-tailed binominal distribution) for SLAC and FEL analyses and >50 bayes factor for REL.

2.6 Calculation of antigenic distance

The specific measure of antigenic distance between two strains of influenza virus was calculated as $P_{epitope}$ values employing the method suggested by Muñoz et al. (Muñoz et al., 2005). The $P_{epitope}$ value was calculated as the number of mutations at an antibody's antigenic site divided by the number of amino acids defining the site. It is assumed that the antigenic epitope with the greatest percentage of mutations is dominant as this epitope is under the greatest selective pressure from the immune system. The $P_{epitope}$ distance is defined as the fractional change between the dominant antigenic epitopes of one strain compared to another. The $P_{epitope}$ Calculator (Gupta et al., 2004) was applied for H3 sequences.

3. Results

3.1 Prevalence of Influenza A virus in Thailand from 2006 to 2008

The relative prevalence of influenza virus from 2006 to 2008 has been subjected to variation. In the course of this period, 383 patients were diagnosed with respiratory illness. Forty one samples were positive for the matrix gene of influenza A virus. Distinction between subtypes H1N1 and H3N2 differentiated 30 H3N2 samples from 11 H1N1 samples. Between April and August 2006, a higher prevalence of H1N1 viruses co-circulating with H3N2 was observed. Influenza A virus subtype H3N2 was the dominant strain in Thailand from 2006 to 2008. Of the influenza A virus positive samples, 13 H3N2 and 5 H1N1 samples were selected representing the respective time of collection. The details are shown in Table 7.

Table 7 The details of the human influenza A virus subtype H1N1 and H3N2 specimens

Strain	Subtype	Date	Sex	Age	Accession number
A/Thailand/CU23/2006	H3N2	22-03-2006	Female	1 year	EU021266, EU021267, FJ912901-FJ912906
A/Thailand/CU32/2006	H1N1	13-04-2006	Female	3 years	EU021264, EU021265, FJ912907-FJ912912
A/Thailand/CU44/2006	H1N1	13-05-2006	Female	11 years	EU021258, EU021259, FJ912912-FJ912918
A/Thailand/CU46/2006	H3N2	18-05-2006	Male	11 months	EU021268, EU021269, FJ912919-FJ912924
A/Thailand/CU51/2006	H1N1	30-05-2006	Male	2 years	EU021254, EU021255, FJ912925-FJ912930
A/Thailand/CU68/2006	H1N1	26-06-2006	Female	8 months	EU021260, EU021261, FJ912931-FJ912936
A/Thailand/CU88/2006	H1N1	25-07-2006	Female	5 months	EU021252, EU021253, FJ912937-FJ912942
A/Thailand/CU228/2006	H3N2	11-10-2006	Male	2 years	EU021274, EU021275, FJ912943, FJ912948
A/Thailand/CU231/2006	H3N2	14-11-2006	Male	1 year	EU021282, EU021283, FJ912949-FJ912954
A/Thailand/CU260/2006	H3N2	27-12-2006	Female	1 year	EU021280, EU021281, FJ912955-FJ912960
A/Thailand/CU280/2007	H3N2	26-01-2007	Female	2 years	EU021272, EU021273, FJ912961-FJ912966
A/Thailand/CU282/2007	H3N2	02-02-2007	Male	3 years	EU021276, EU021277, FJ912967-FJ912972
A/Thailand/CU1101/2008	H3N2	25-02-2008	Female	3 years	EU625363, EU625366, FJ912997-FJ913002
A/Thailand/CU1102/2008	H3N2	20-03-2008	Male	3 years	EU625364, EU625367, FJ913003-FJ913008
A/Thailand/CU1103/2008	H3N2	24-03-2008	Male	10 years	EU625365, EU625368, FJ913009-FJ913014
A/Thailand/CU356/2008	H3N2	25-03-2008	Male	11 months	FJ912973-FJ912980
A/Thailand/CU370/2008	H3N2	16-06-2008	Male	5 months	FJ912981-FJ912988
A/Thailand/CU379/2008	H3N2	04-07-2008	Female	9 months	FJ912989-FJ912996

3.2 Phylogenetic Analysis

Based on phylogenetic analysis of the nucleotide sequence, 13 H3N2 isolates were compared with vaccine strains (Figures 11-13). The results showed H3 and N2 nucleotide sequences formed seasonal phylogenetic clusters representative for the 2005-2006, 2006-2007 and 2007-2008 seasons. The H3 nucleotide sequences of the 2005-2006 strains (A/Thailand/CU23/2006 and A/Thailand/CU46/2006) were closely related to the A/Wisconsin/67/2005 like lineage (vaccine strain for 2006-2007 Northern Hemisphere, 2007 Southern Hemisphere, 2007-2008 Northern Hemisphere). The average percentage of nucleotide homologies of HA to this vaccine strain is 99.49% and 98.53% based on amino acids. The 2006-2007 strains (A/Thailand/CU228/2006, A/Thailand/CU231/2006, A/Thailand/CU260/2006, A/Thailand/CU280/2006, A/Thailand/CU282/2006) were different from the 2005-2006 strains. The average percentage of nucleotide homologies among the 2006-2007 and 2005-2006 strains amounts to 99.029% (98.88% based on amino acids) and the homologies of the 2006-2007 strain to the A/Wisconsin67/2005 like lineage has decreased to 98.985% (97.64% based on amino acids). During the following season, 2007-2008 (A/Thailand/CU1101/2008, A/Thailand/CU1102/2008, A/Thailand/CU1003/2008, A/Thailand/CU356/2008, A/Thailand/CU370/2008 and A/Thailand/CU379/2008), the H3 nucleotide sequence had drifted towards a more A/Brisbane/10/2007 like lineage (vaccine strain for 2008 Southern Hemisphere and 2008-2009 Northern Hemisphere). The H3 nucleotide homologies to this vaccine strain are 99.392% and 99.24% based on amino acids. The average percentage of nucleotide and amino acid homologies between H1 isolates and vaccine strains are shown in Table 8.

Table 8 The average percentage of nucleotide and amino acid of H3 and vaccine strains.

	Nucleotide		Amino acid	
	A/Wisconsin/67/2005	A/Brisbane/10/2007	A/Wisconsin/67/2005	A/Brisbane/10/2007
2005-2006	99.38	99.203	98.256	97.674
2006-2007	98.99	99.026	97.326	97.442
2007-2008	98.686	99.395	96.609	98.353

The N2 nucleotide sequences of the 2005-2006 strain were closely related to the A/Wisconsin/67/2005 like lineage (99.52% for nucleotides and 99.44% for amino acids). The isolates from the 2006-2007 season were slightly different from the 2005-2006 strain (99.02% for nucleotides and 98.77% for amino acids) and from the A/Wisconsin/67/2005 like lineage (98.85% for nucleotides and 98.65% for amino acids). During the 2007-2008 season, the N2 nucleotide sequences had drifted towards the A/Brisbane/10/2007 like lineage (99.59% for nucleotides and 99.40% for amino acids). Few isolates from the 2006-2007 strain (A/Thailand/CU231/2006) are closely related to the 2007-2008 strain (99.06% for nucleotides and 99.41% for amino acids). The H3N2 internal genes of the 2007-2008 strain are closely related to the A/Brisbane/10/2007 like lineage (Figure 12-13).

The 5 H1N1 isolates from the 2006 season were compared with the vaccine strains. The H1 and N1 nucleotide sequences from the 2006 season were differentiated into 2 lineages (Figures 12-13). Two isolates (A/Thailand/CU88/2006 and A/Thailand/CU51/2006) were closely related to the A/Solomon Island/3/2006 like lineage (vaccine strain for 2007-2008 Northern Hemisphere) (Figure 13). The average per cent nucleotide and amino acid homologies of HA to this vaccine strain are 98.86% and 98.08%, respectively and homologies of NA to this vaccine amount to 99.16% and 99.01%, respectively. Two isolates (A/Thailand/CU44/2006 and A/Thailand/CU68/2006) were distinct from the A/Solomon Island/3/2006 like lineage and closely related to the

A/Brisbane/59/2007 like lineage (vaccine strain for 2008-2009 Northern Hemisphere). Percent homologies to the A/Brisbane/69/2007 like lineage were determined at 98.66% for the H1 nucleotide sequence, 98.46% for the H1 amino acid sequence, 97.76% for the N1 nucleotide sequence and 97.60% for the N1 amino acid sequence. One isolate (A/Thailand/CU32/2006) was closely related to the A/New Caledonia/20/1999 like lineage (vaccine for 2006-2007 Northern Hemisphere). The phylogenetic trees of the internal genes showed the same topology (Figures 12-13). The average percentage of nucleotide and amino acid homologies between H1 isolates and vaccine strains are shown in Table 9.

Table 9 The average percentage of nucleotide and amino acid of H1 and vaccine strains. A/Solomon Islands/3/2006 like lineage (S-like), A/Brisbane-like/59/2007 like lineage (B-like), A/New Caledonia/20/1999 like lineage (NC-like)

	Nucleotide			Amino acid		
	A/NewCaledonia/20/ 1999	A/SolomonIsland/3/ 2006	A/Brisbane/69/ 2007	A/NewCaledonia/20/ 1999	A/SolomonIsland/3/ 2006	A/Brisbane/69/ 2007
NC-like	97.865	96.413	96.243	97.949	96.667	97.179
S-like	97.267	98.89	97.737	96.282	98.077	97.308
B-like	97.523	98.463	98.463	97.436	98.462	98.462

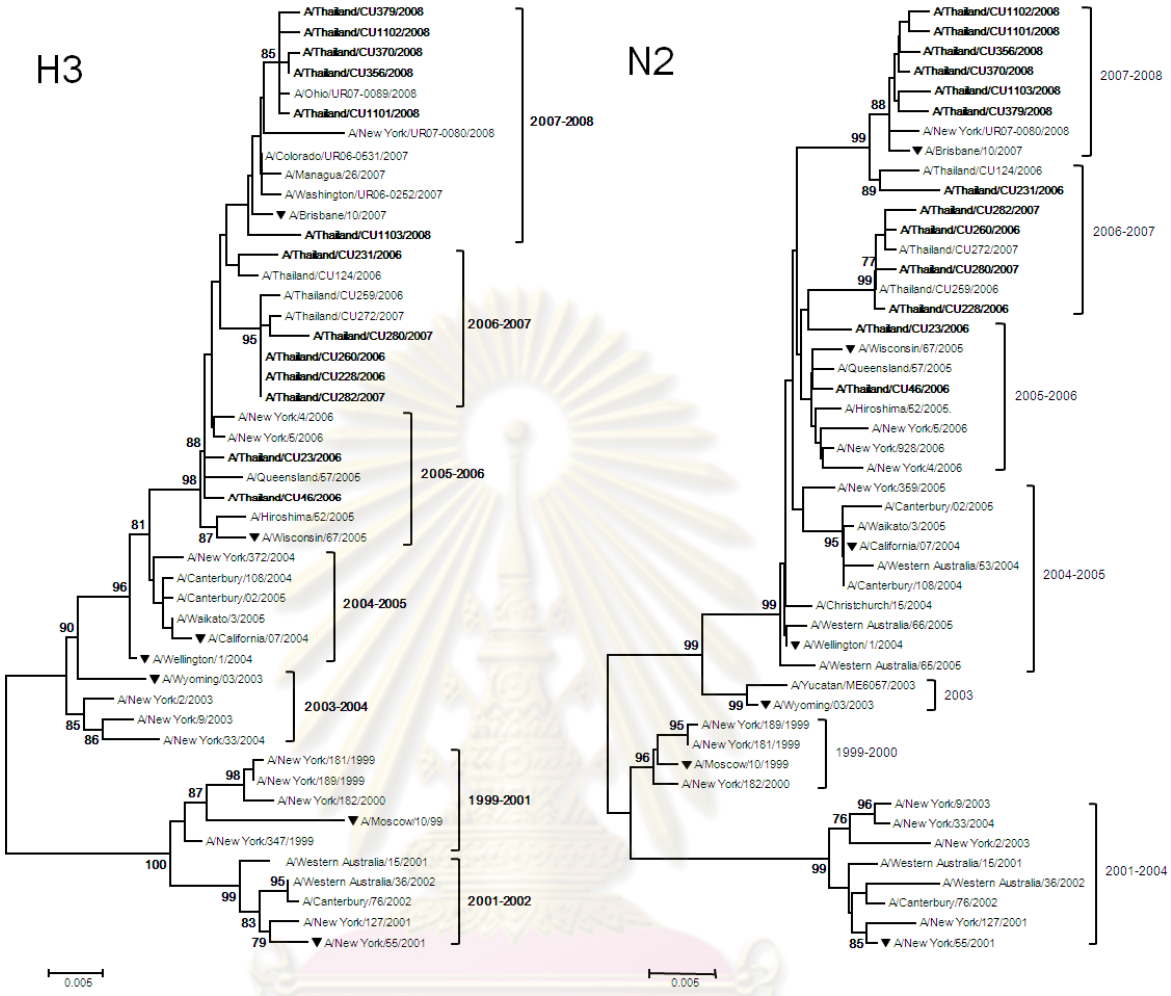


Figure 11 Phylogenetic tree of H3 and N2 genes. The trees were constructed by the Neighbor-Joining method with bootstrap support by 1,000 replicates. (Bold font represents isolates from this project and ▼ represents vaccine strain)

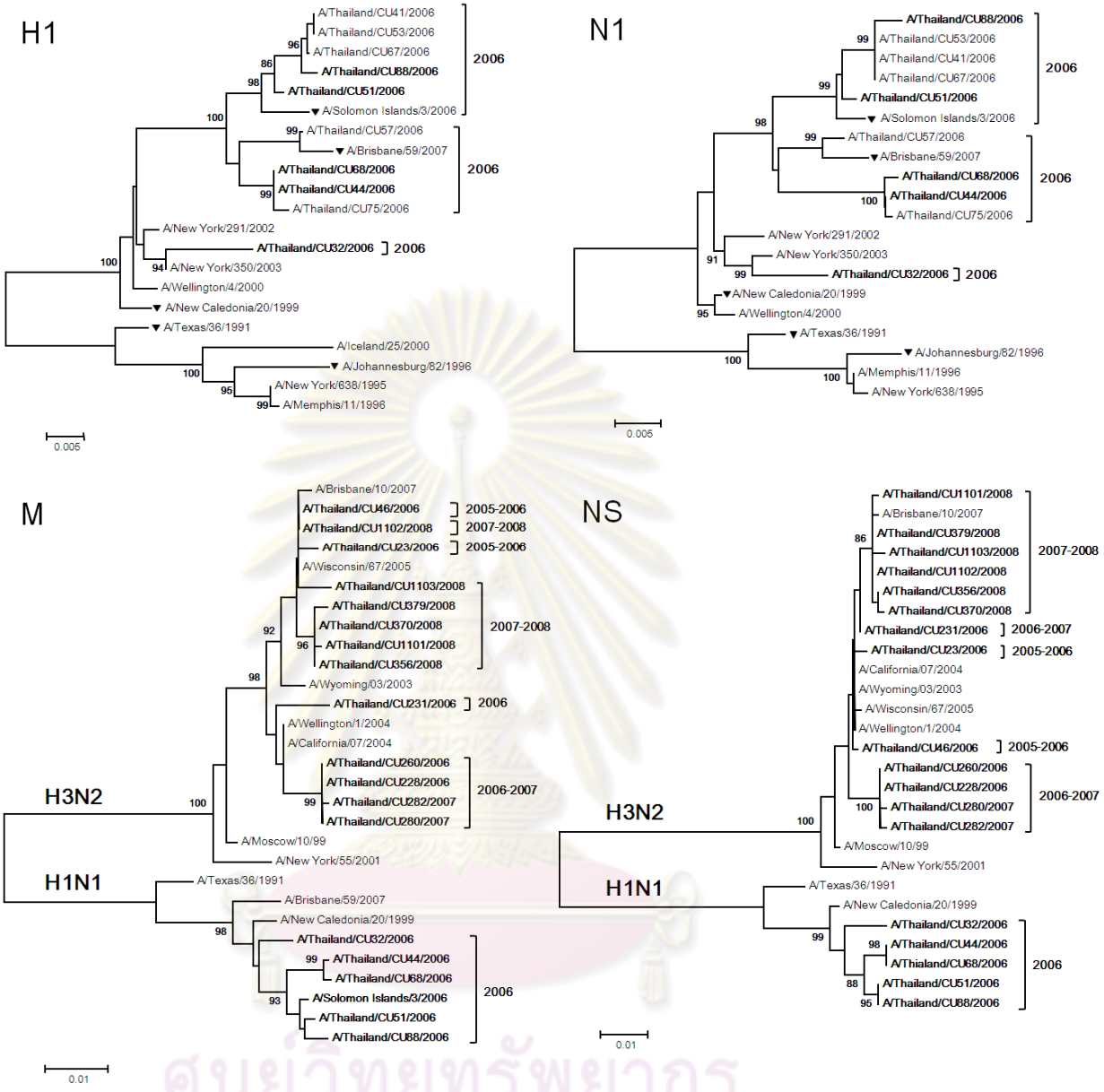


Figure 12 Phylogenetic tree of internal genes of H1N1 and H3N2 and H1 and N1. The trees were constructed by the Neighbor-Joining method with bootstrap support by 1,000 replicates. (Bold font represents isolates from this project and ▼ represents vaccine strain)

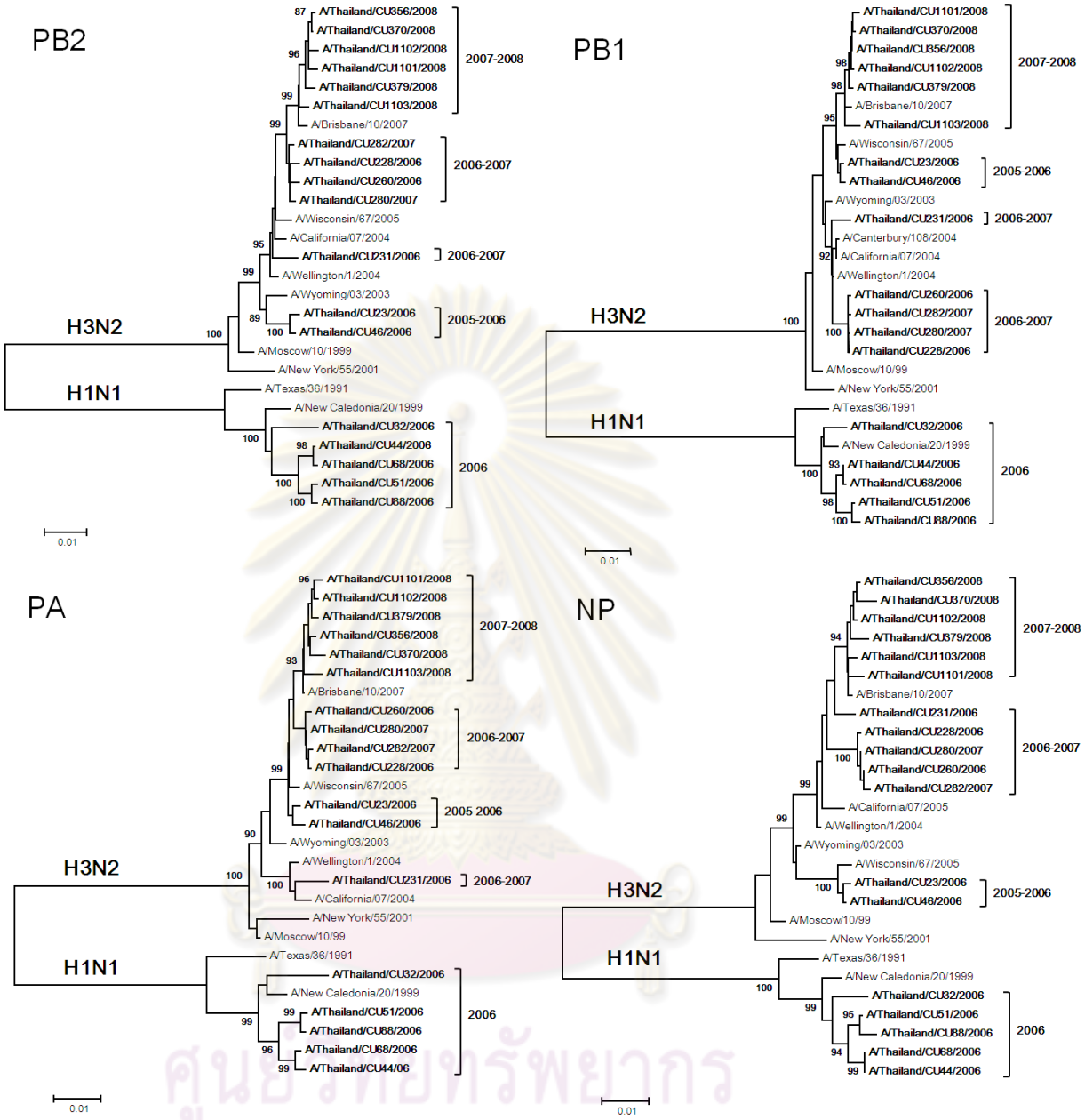


Figure 13 Phylogenetic tree of internal genes of H1N1 and H3N2. The trees were constructed by the Neighbor-Joining method with bootstrap support by 1,000 replicates. (Bold font represents isolates from this project)

3.3 Variations in hemagglutinin and neuraminidase

The amino acid positions in the 2005-2006 H3 strains that have undergone mutations in the 2006-2007 strain's HA are I6N, I140K, R142G, L157S, K173E, D375N and K450R (numbering according to H3 structure) (Table 10). In the 2007-2008 strain, positions 50 and 361 had changed to Glu and Arg, respectively. Positions 6 and 157 have remained Asn and Ser. After the 2006-2007 season, positions 140, 375 and 450 have been stable. Position 173 had the highest variability represented by three different amino acids. The altered amino acids were detected at antigenic sites A, B and E: K/I-140 and R/G-142 at site A, L/S-157 at site B, and E/K-173 at site E. The 2005-2006 strain had undergone 2 substitutions at the antigenic site A in HA in comparison with the 2006-2007 strain and the antigenic site preferred for mutation was antigenic site A ($P_{epitope}$ = 0.052). The 2007-2008 strain showed changes at 7 positions compared to the 2006-2007 strain and the antigenic site preferred for mutation was site A ($P_{epitope}$ = 0.105).

The N2 from the 2006-2007 strains has remained identical to the 2005-2006 strains due to S43N, D93N and R315S. The changes observed at site 93 in NA from the 2006-2007 and 2007-2008 strains are located in the HLA-A*0201 NA90-99 (PQCNITGFAP) CTL epitope (Gianfrani et al., 2000). 2006-2007 strains has become the 2007-2008 strain due to amino acid alterations to R150H, I194V, V215I, S315R, L372S, K366N in NA.

Table 10 Amino acid alterations in H3 and N2 between seasons. Amino acids in brackets indicate the minor amino acid substitutions within a season.

HA				NA			
Amino acid	2005-2006	2006-2007	2007-2008	Amino acid	2005-2006	2006-2007	2007-2008
6	N	I	N	43	N	S(N)	N
50	G	G	E	93	N	D	D
140	K	I	I	150	H	H(R)	R
142	R	G	R(K)	194	V	V(I)	I
157	L	S	L	215	I	I	V
173	K	E	Q	310	Y	Y(H)	Y
361	I	I	R	315	S	R(S)	S
375	N	D	D	372	S	S(L)	L
450	R	K	K	366	N	N	K

In the H1 HA, the amino acids of isolates closely related to the A/Solomon Island/3/2006 like lineage were different from isolates closely related to the A/Brisbane/59/2007 like lineage (Table 11). Four positions in the A/Solomon Island/3/2006 like lineage were different from the A/Brisbane/59/2007 like lineage (73, 77, 88 132). Positions 73 and 77 were part of the antigenic Sa site. As for the N1 NA, five positions were different between the two lineages (23, 64, 77, 173 and 266).

Table 11 Amino acid comparison of H1N1 isolated from Thailand to A/Solomon Islands/3/2006 like lineage (S-like), A/Brisbane-like/59/2007 like lineage (B-like) and A/New Caledonia/20/1999 like lineage (NC-like).

H1				N1			
Amino acid	S-like	B-like	NC-like	Amino acid	S-like	B-like	NC-like
73	S	L	L	23	I	M	M
77	R	K	K	64	H	N	N
86	K	K	T	77	E	G	G
88	S	N	N	173	R	K	K
98	Y	Y	H	234	M	M	V
132	A	V	V	266	T	S	S
149	K	K	R				
212	K	K	R				
270	N	N	T				
419	I	I	L				

Genetic indication of neuraminidase drug resistance at positions 119, 152, 274, 292, 294 was not found in either subtype.

3.4 Variations in the internal genes

The PA protein harbors the amino acids essential for the HLA epitope. The sequence HLA-A*0201 PA225-233 (CLENFRAYV) (Gianfrani et al., 2000) was found in all H3N2 isolates. In H1N1, the substitution I226L was found in this epitope. The substitution V602I located in HLA-B*8 PA601-609 (SVKEKDMTK) (Wang et al., 2007) was found in one H3N2 isolate (A/Thailand/CU-231/2006).

The H3N2 isolates from the 2005-2006 season (A/Thailand/CU23/2006, A/Thailand/CU46/2006) displayed the original HLA-A*01 NP44-52 (CTELKLSDY) (DiBrino et al., 1993) in the NP protein, while the substitution Y52H had occurred in isolates since the 2006-2007 season (A/Thailand/CU228/2006, A/Thailand/CU231/2006, A/Thailand/CU260/2006) until the 2007-2008 season. The H1N1 isolates had undergone a S50N replacement in this epitope.

The H3N2 isolates from 2006 to 2008 exhibited the original CTL epitopes HLA-A*6801 NP 91-99 (KTGGPIYRR) (Guo et al., 1992), HLA-B*1508 NP103-111 (KWMRELVLY) and HLA-B*4002 NP251-259 (AEIEDLIFL) (Berkhoff et al., 2007). The H1N1 isolates have experienced K98R, V105M and T257I substitutions in these epitopes, respectively. H1N1 displayed the original CTL epitope HLA-B*1402 NP146-154 (TTYQRTRAL) (DiBrino et al., 1994), while H3N2 has undergone a T146A replacement. H1N1 showed L191M and V197I substitutions in the CTL epitope HLA-A*1101 NP188-198 (TMVMELIRMVK) (Gianfrani et al., 2000) and two isolates of H3N2 from 2006 (A/Thailand/CU231/2006) and 2008 (A/Thailand/CU379/2008) exhibited a V197I replacement in this epitope. The H3N2 isolates from the 2006-2007 season to the 2007-2008 season had undergone alterations to the original CTL epitope HLA-DQA1*0501/HLA-DQB1*0201 NP365-379 (IASNENMDNMGSSTL) (Vartdal et al., 1996) while the 2005-2006 season viruses (A/Thailand/CU23/2006, A/Thailand/CU46/2006) displayed the substitution S377G. The H1N1 viruses had three amino acid differences in this epitope (N373A, M374I and G375V). The H3N2 isolates had the G384Y substitution in the CTL epitope HLA*B27 NP383-391 (SPYWAIRTR) (Wang et al., 2007) while H1N1 showed the R384Y replacement in this epitope. The H3N2 isolates had experienced the substitution V425I in the CTL epitope HLA-B*0702/HLA-B*3501 NP418-426

(LPFEKSTVM) (Rohrlich et al., 2003). E421D and S423T were found at this epitope in H1N1 viruses.

The H1N1 sequences showed the original CTL epitope HLA-B*39 M1173-181 (IRHENRMVL) (Wang et al., 2007) in the M1 protein, while the substitution R174K was found in four H3N2 isolates, two from the 2005-2006 season (A/Thailand/CU23/2006, A/Thailand/CU46/2006) and two from the 2007-2008 season (A/Thailand/CU1102/2008, A/Thailand/CU1103/2008). The S31N substitution in the M2 protein indicates resistance to amantadine, the influenza matrix ion channel inhibitory drug. Five H3N2 isolates from the 2006-2007 season (A/Thailand/CU228/2006, A/Thailand/CU231/2006, A/Thailand/CU260/2006, A/Thailand/CU280/2007 and A/Thailand/CU282/2007) displayed Asn at this position indicating amantadine sensitivity. But in the 2007-2008 H3N2 strain, this position had changed to S31N implying drug resistance. The H1N1 sequences showed Asn at this position.

The CTL epitope HLA-DR*03 NS134-42 (DRLRRDQKS) (Jameson et al., 1999) had been identified in NS1 in H1N1. The H3N2 viruses display the substitution K41R and 4 isolates from the 2006-2007 season (A/Thailand/CU228/2006, A/Thailand/CU260/2006, A/Thailand/CU280/2007 and A/Thailand/CU282/2007) have undergone the substitution L36V in this epitope. In H3N2 viruses, the HLA-A*0201 NS1122-130 (AIMDKNIIL) epitope (DiBrino et al., 1995) displays amino acids D125E and I129M.

3.5 Glycosylation patterns

Ten potential N-glycosylation sites were predicted in H3 HA1 (8, 22, 38, 63, 126, 133, 165,246 and 285) and one (483) in HA2 (Table 12). These sites were found to be conserved among all isolates obtained during the 3 seasons of this study. In comparison with the 2005-2006 strain, the majority of 2006-2007 viruses had lost the predicted glycosylation at position 144; one isolate showed glycosylation at this position (A/Thailand/CU231/2006). The predicted score at position 144 has been below the set threshold value of 0.5 and was therefore not included in the count. All isolates from the 2007-2008 season had lost this position. Three positions are located at the antigenic site A (126, 133 and 144).

Six potential N-glycosylation sites were predicted for N2 NA from the 2005-2006 season, (61, 70, 86, 93, 146 and 234). The virus from the 2006-2007 and 2007-2008 seasons had lost the potential site at position 93.

For H1 HA, seven potential N-linked glycosylation sites were predicted in HA1 (15, 27, 58, 91, 129, 163 and 290) and two in HA2 (484 and 543). Positions 129 and 163 are located at the antigenic site Sa. Eight predicted potential N-linked glycosylation sites were detected in the N1 NA (44, 58, 63, 70, 88, 146, 235 and 434).

Table 12 N-Glycosylation sites predicted in the HA and NA with a threshold value of >0.5 average potential score. : Bold fond represents an antigenic site. Position in brackets indicates that some strains display N-glycosylation at this position.

	Season	Amino acid position
H3	2005-2006	8, 22, 38, 63, **126**, **133**, **144**, 165, 246, 285, 483
	2006-2007	8, 22, 38, 63, **126**, **133**, **(144)**, 165, 246, 285, 483
	2007-2008	8, 22, 38, 63, **126**, **133**, 165, 246, 285, 483
N2	2005-2006	61, 70, 86, 93, 146, 234
	2006-2007	61, 70, 86, 146, 234
	2007-2008	61, 70, 86, 146, 234
H1	2006	15, 27, 58, 91, **129**, **163**, 290, 484, 543
N1	2006	44, 58, 63,70, 88, 146, 235, 434

3.6 Selection pressure

Based on the ratio of non-synonymous versus synonymous substitutions no single influenza A gene was directly influenced by positive selection (dN/dS<1) (Table 13). The HA1 region of both subtypes has rather been subjected to evolutionary pressure. The M2 protein displays a slightly higher dN/dS ratio than the other internal genes. FEL and SLAC maximum-likelihood methods were applied to estimate positively selected individual sites. Positively selected site could not be determined by either method. The REL analysis retrieved four sites in the M1 gene at positions 162, 174, 211 and 219. Neither method could predict any positively selected site for NA and the internal genes. Similarly, positively selected sites could not be determined for H1 and N1 by any of the three methods.

Table 13 Non-synonymous/synonymous substitution ratio for H3N2 isolates. dN/dS calculated by SLAC likelihood analysis.

Gene	dN/dS	
	H3N2	H1N1
PB2	0.098	0.055
PB1	0.061	0.049
PA	0.084	0.040
HA	0.229	0.176
HA1	0.346	0.252
HA2	0.134	0.051
NP	0.059	0.027
NA	0.263	0.288
M1	0.105	0.093
M2	0.680	0.286
NS1	0.233	0.269
NS2	0.079	0.065

4. Discussion

Of the 383 patient samples tested for influenza A virus from early 2006 to 2008, 41 (10.7%) were found positive, with 30 samples harboring H3N2 virus and 11 H1N1. During the seasonal outbreaks recorded for this period, H3N2 viruses were the dominant influenza A subtype in Thailand. Higher prevalence of H1N1 viruses co-circulating with H3N2 was observed between April and August 2006.

Each year, WHO recommends the most suitable composition of influenza vaccine strains for the Northern and Southern Hemispheres, respectively. The phylogenetic trees of HA and NA showed seasonal clusters. Genetic comparison between the HA and NA sequences determined in this project and vaccine strains showed seasonal clusters are closely related to the vaccine strains recommended. H3N2 isolates from the 2005-2006 season are closely related to the vaccine strains recommended for 2006-2007 Northern Hemisphere, 2007 Southern Hemisphere and 2007-2008 Northern Hemisphere (A/Wisconsin/67/2005 like lineage). During the 2007-2008 season, viruses had drifted towards the sequence for a vaccine strain for 2008 Southern Hemisphere and 2008-2009 Northern Hemisphere (A/Brisbane/10/2007 like lineage). Most H1N1 isolates from 2006 could be differentiated into two lineages, with one closely related to the vaccine strain for 2007-2008 Northern Hemisphere (A/Solomon Island/3/2006 like lineage) and the second to the vaccine strain for 2008-2009 Northern Hemisphere (A/Brisbane/59/2007 like lineage). Few H1N1 isolates were closely related to the vaccine strain for 2006-2007 Northern Hemisphere (A/New Caledonia/20/1999 like lineage).

Sequence analysis of HA showed high variation in HA1, which might be due to its receptor-binding properties and to it being targeted by neutralizing antibodies since it represents the membrane fusion glycoprotein of influenza virus. The residues within the receptor-binding site are relatively conserved but the residue mainly responsible for NeuAcα2,6Gal linkage specific for the H3 subtype was Ile226 instead of Leu226 [19]. In H3, amino acid substitutions were detected at three antigenic sites, A, B and E. The antigenic site preferred for mutation was antigenic site A. Positions in the H1N1 isolates differentiating them from the A/Solomon Island/3/2006 like lineage and

A/Brisbane/59/2007 like lineage were included in the antigenic Sa site. T-cell epitopes in proteins of influenza A virus are more conserved than antibody epitopes. The reason for this higher degree of conservation is that 80% of the antibody epitopes are located in the variable glycoproteins HA and NA, while only 40% of the T-cell epitopes are found in these proteins (Bui et al., 2007). Most substitutions in regions involved in protective T-cell response were detected in the NP protein, as most T-cell epitopes are defined for the NP protein and this protein constitutes the main target for the host's cytotoxic immune response (Townsend et al., 1984, Winter et al., 1981).

Oligosaccharides in the surface proteins HA and NA might more readily facilitate viral escape from the immune system than single amino acid changes at the antigenic sites. Oligosaccharides may trigger conformational changes in the molecule and mask antigenic sites, which in turn would prevent binding of host antibodies. The number of N-linked glycosylation sites in the H3 protein has decreased during three years. The predicted N-linked glycosylation at position 144 of the HA antigenic site A has not been observed since the 2006-2007 season. This position may not play any major role in escape from the immune system. Six potential N-glycosylation sites have been conserved in the N2 protein. The majority of isolates since the 2006-2007 season have lost the site at position 93 which is located in a CTL epitope (HLA-A*0201) region of NA (Gianfrani et al., 2000). Thus, NA may now be more easily recognized by the cellular immune response.

During the 2008-2009 season, the prevalence of the oseltamivir resistance was very high among the over 30 countries (WHO, 2009). However, the indicated nuraminidase drug resistance was not found in both subtypes during 2006-2008 in Thailand. The amantadine drug resistance H3N2 viruses were increased in 2007-2008 season in Thailand, while H1N1 in 2006 showed sensitive to amantadine.

Selection at the protein level can be measured by dN/dS. If amino acid changes provide better fitness or have a positive selection advantage, the mutations will be fixed at a higher rate than synonymous mutations. This will result in a dN/dS ratio >1. The expected higher dN/dS ratio was observed for the surface glycoproteins, although none were directly subjected to positive selection. In this study, the genes encoding the HA1

and M2 proteins displayed a slightly higher global dN/dS ratio than the other genes. This ratio was higher for the surface protein genes than for the internal genes. Genes coding for surface proteins are subject to strong selection pressure by neutralizing antibodies of host immune systems (Gammelin et al., 1990, Gorman et al., 1991). The M2 protein is a membrane ion channel protein on the surface of the viral molecule and thus, a higher dN/dS ratio for this protein compared to the internal proteins can be expected. The ratio of M2 might be biased because the M2 protein is overlapped by M1 and the dS is suppressed for overlapping regions giving a higher dN/dS ratio. In general, when multiple genes overlap in different reading frames, nucleotide substitution is expected to be suppressed for each gene due to the superimposition of functional constraints operating on the overlapping genes. The mutation rates for these regions are similar and the synonymous mutations are close to selectively neutral (Suzuki 2006). Positively selected sites could not be detected employing SLAC and FEL methods, while REL analysis identified four sites under positive selection pressure in M1. The results may vary depending on the dataset applied and the method used for a site to be classified as positively selected.

Complete genome analysis of human influenza A viruses was necessary to obtain a comprehensive picture of the evolution of viruses. In the course of this project, our group has detected variations due to amino acid substitutions in surface proteins and internal proteins including the potential N-linked glycosylation of HA and NA. Nucleotide sequence comparisons between Thailand isolates and vaccine strains have shown that seasonal influenza strains closely resemble the vaccine strains for each season.

CHAPTER V

MOLECULAR EVOLUTION OF H5N1 IN THAILAND BETWEEN 2004 AND 2008

HPAI H5N1 viruses have seriously affected the Asian poultry industry since their occurrence in 2004. Thailand has been one of those countries exposed to HPAI H5N1 outbreaks.

Seven waves of H5N1 outbreaks in Thailand were detected in avian species since early 2004 (Amonsin et al., 2006; Tiensin et al., 2007). Between January and March 2004, the first wave of outbreaks had lead to 12 cases of human infection with 8 fatalities. The second wave occurred in July, 2004, and had run its course by the end of the year leaving in its wake five human cases with four fatalities (Tiensin et al., 2005). Between October and December 2005, the third wave of outbreaks was reported resulting in five human cases with two fatalities. No human cases were reported during the fourth (January-March 2006) and fifth waves (November-March 2007). In January 2008, the sixth wave occurred in 2 provinces, located in the lower northern part of Thailand and as with the previous two outbreaks, no human cases were reported (Amonsin et al., 2008; Chaichoune et al., 2009). In late 2008, the seventh wave occurred in the nearby provinces.

During the four years with seven waves of outbreaks, numerous nucleotide sequence data of HPAI H5N1 viruses have been obtained in Thailand. The aim of this part was to compare the molecular evolution of HPAI H5N1 in Thailand between 2004 and 2008. In order to understand the relationship between geographic distribution and clade, the geographic location of each clade was identified.

This part was designed to compare the molecular evolution of HPAI H5N1 in Thailand between 2004 and 2008.

1. Objective

To investigate and study the molecular evolution of H5N1 influenza A virus by using the whole genome sequences in Thailand.

2. Materials and methods

2.1 Viruses and Datasets.

From 2006 through 2008, the faculty of veterinary science at Chulalongkorn University (CU) in collaboration with the faculty of veterinary science at Kasetsart University and the Department of Livestock Development (DLD) of Thailand has conducted surveillance to detect H5N1 infections in poultry. Collection sites included poultry farms, backyard flocks, and live bird markets in the northern, north-eastern and central region of Thailand. Eight H5N1 viruses were performed sequencing from different time waves, 1 isolate from 2006-2007 during the fifth wave, 4 isolates from January 2008 during the sixth wave and 3 isolates from November 2008 during the seventh wave (Table 14). 97 publicly accessible isolates collected between the first and sixth wave in Thailand from 2004 to 2008 and obtainable at the Influenza Database, National Center for Biotechnology Information (NCBI) were also included analysis. Altogether 105 H5N1 isolates obtained from different regions of Thailand between 2004 and 2008 were subjected to analysis (Table 14).

Table 14 The H5N1 viruses analyzed in this study.

					Accession number							
wave	Year	Location	Region	Strain	PB2	PB1	PA	HA	NP	NA	M	NS
1	2004	Kanchanaburi	West	A/Thailand/1(KAN-1)/2004	AY626149	AY626148	AY626147	AY555150	AY626145	AY555151	AY626144	AY626146
1	2004	Nakorn-Patom	Central	A/chicken/Nakorn-Patom/Thailand/CU-K2/2004	AY590581	AY590582	AY551934	AY590568	AY590579	AY590567	AY590578	AY590580
1	2004	Suphanburi	Central	A/leopard/Thailand/Leo-1/2004	AY646182	AY646181	AY646179	AY646175	AY646177	AY646176	AY646180	AY646178
1	2004	Suphanburi	Central	A/tiger/Suphanburi/Thailand/Ti-1/2004	AY646174	AY646173	AY646171	AY646167	AY646169	AY646168	AY646172	AY646170
1	2004	Suphanburi	Central	A/chicken/Suphanburi/Thailand/CU-1/2004				DQ083550		DQ083586	DQ083660	DQ083622
1	2004	Chachoengsao	East	A/chciken/Thailand/CU-10/2004				DQ083558		DQ083594	DQ083668	DQ083630
1	2004	Chachoengsao	East	A/chicken/Chachoengsao/Thailand/CU-11/2004				DQ083559		DQ083595	DQ083669	DQ083631
1	2004	Nakhonsawan	Central	A/chicken/Nakhon Sawan/Thailand/CU-12/2004				DQ083560		DQ083596	DQ083670	DQ083632
1	2004	Nakhonsawan	Central	A/chicken/Nakhon Sawan/Thailand/CU-13/2004				DQ083561		DQ083597	DQ083671	DQ083633
1	2004	Nakhon Pathom	Central	A/chicken/Nakhon Pathom/Thailand/CU-14/2004				DQ083562		DQ083598	DQ083672	DQ083634
1	2004	Bangkok	Central	A/crow/Bangkok/Thailand/CU-15/2004				DQ083563		DQ083599	DQ083673	DQ083635
1	2004	Bangkok	Central	A/white peafowl/Bangkok/Thailand/CU-16/2004				DQ083564		DQ083600	DQ083674	DQ083636
1	2004	Saraburi	Central	A/chicken/Saraburi/Thailand/CU-17/2004				DQ083565		DQ083601	DQ083675	DQ083637
1	2004	Bangkok	Central	A/Kalji Pheasant/Bangkok/Thailand/CU-18/2004				DQ083566		DQ083602	DQ083676	DQ083638
1	2004	Samut Prakan	Central	A/Ostrich/Samut Prakan/Thailand/CU-19/2004				DQ083567		DQ083603	DQ083677	DQ083639
1	2004	Chonburi	East	A/duck/Thailand/CU-2/2004				AY779048		AY779049	DQ083696	DQ083658
1	2004	Bangkok	Central	A/chicken/Bangkok/Thailand/CU-20/2004				DQ083568		DQ083604	DQ083678	DQ083640
1	2004	Bangkok	Central	A/chicken/Thailand/CU-21/2004				AY779050		AY779051	DQ083697	DQ083659
1	2004	Bangkok	Central	A/chicken/Bangkok/Thailand/CU-3/2004				DQ083551		DQ083587	DQ083661	DQ083623
1	2004	Bangkok	Central	A/crow/Bangkok/Thailand/CU-4/2004				DQ083552		DQ083588	DQ083662	DQ083624
1	2004	Chonburi	East	A/duck/Chonburi/Thailand/CU-5/2004				DQ083553		DQ083589	DQ083663	DQ083625
1	2004	Bangkok	Central	A/chicken/Bangkok/Thailand/CU-6/2004				DQ083554		DQ083590	DQ083664	DQ083626
1	2004	Chonburi	East	A/chicken/Chonburi/Thailand/CU-7/2004				DQ083555		DQ083591	DQ083665	DQ083627
1	2004	Prachinburi	East	A/chicken/Prachinburi/Thailand/CU-8/2004				DQ083556		DQ083592	DQ083666	DQ083628
1	2004	Suphanburi	Central	A/chicken/Suphanburi/Thailand/CU-9/2004				DQ083557		DQ083593	DQ083667	DQ083629
1	2004	Khon Kaen	NorthEast	A/Thailand/5(KK-494)/2004	AY627892	AY627891	AY627890	AY627885	AY627889	AY627886	AY627887	AY627888

Table 14 (*continue*) The H5N1 viruses analyzed in this part.

					Accession number							
wave	Year	Location	Region	Strain	PB2	PB1	PA	HA	NP	NA	M	NS
1	2004	Suphanburi	Central	A/Thailand/2(SP-33)/2004	AY627898	AY627897	AY627896	AY555153	AY627895	AY555152	AY627893	AY627894
1	2004	Kalasin	NorthEast	A/chicken/Kalasin/NIAH316/2004	AB450502	AB450518	AB450534	AB450550	AB450580	AB450596	AB450626	AB450642
1	2004	KohnKaen	NorthEast	A/chicken/Kohn Kaen/NIAH330/2004	AB450503	AB450519	AB450535	AB450552	AB450581	AB450598	AB450627	AB450643
2	2004	Ayutthaya	Central	A/chicken/Ayutthaya/Thailand/CU-23/2004	AY770993	AY770994	AY770995	AY770991	AY770996	AY770992	AY770998	AY770997
2	2004	Chonburi	East	A/tiger/Thailand/CU-T3/2004	AY907672	AY972550	AY972549	AY842935	AY972548	AY842936	AY972547	AY907673
2	2004	Chonburi	East	A/tiger/Thailand/CU-T7/2004	AY907671	AY972554	AY972553	AY866475	AY972552	AY866476	AY972551	AY907674
2	2004	Samut Prakan	Central	A/pigeon/Samut Prakan/Thailand/CU-202/2004				DQ083583		DQ083619	DQ083693	DQ083655
2	2004	Phang-Nga	South	A/sparrow/Phang-Nga/Thailand/CU-203/2004				DQ083584		DQ083620	DQ083694	DQ083656
2	2004	Ranong	South	A/Mynas/Ranong/Thailand/CU-209/2004				DQ083585		DQ083621	DQ083695	DQ083657
2	2004	Ayutthaya	Central	A/chicken/Ayutthaya/Thailand/CU-24/2004				DQ083569		DQ083605	DQ083679	DQ083641
2	2004	Chonburi	East	A/tiger/Thailand/CU-T4/2004				AY972539		AY972543		
2	2004	Chonburi	East	A/tiger/Thailand/CU-T5/2004				AY972540		AY972544		
2	2004	Chonburi	East	A/tiger/Thailand/CU-T6/2004				AY972541		AY972545		
2	2004	Chonburi	East	A/tiger/Thailand/CU-T8/2004				AY972542		AY972546		
1	2004	Suphanburi	Central	A/cat/Thailand/KU-02/2004	DQ236079	DQ236080	DQ236081	DQ236077	DQ236082	DQ236078	DQ236084	DQ236083
2	2004	Suphanburi	Central	A/dog/Thailand-Suphanburi/KU-08/2004	DQ530170	DQ530171	DQ530172	DQ530173	DQ530174	DQ530175	DQ530176	DQ530177
2	2004	Bangkok	Central	A/crow/Bangkok/Thailand/CU-25/2004				DQ083570		DQ083606	DQ083680	DQ083642
2	2004	Bangkok	Central	A/rollers/Bangkok/Thailand/CU-26/2004				DQ083571		DQ083607	DQ083681	DQ083643
2	2004	Saraburi	Central	A/chicken/Saraburi/Thailand/CU-27/2004				DQ083572		DQ083608	DQ083682	DQ083644
2	2004	Samut Prakan	Central	A/ostrich/Samut Prakan/Thailand/CU-31/2004				DQ083574		DQ083610	DQ083684	DQ083646
2	2004	Bangkok	Central	A/crow/Bangkok/Thailand/CU-35/2004				DQ083575		DQ083611	DQ083685	DQ083647
2	2004	Lopburi	Central	A/chicken/Lopburi/Thailand/CU-38/2004				DQ083576		DQ083612	DQ083686	DQ083648
2	2004	Nakhonsawan	Central	A/chicken/Nakhon Sawan/Thailand/CU-39/2004				DQ083577		DQ083613	DQ083687	DQ083649
2	2004	Ratchaburi	West	A/chicken/Ratchaburi/Thailand/CU-68/2004				DQ083578		DQ083614	DQ083688	DQ083650
2	2004	Nakhon Pathom	Central	A/duck/Nakhon Pathom/Thailand/CU-71/2004				DQ083579		DQ083615	DQ083689	DQ083651
2	2004	Chonburi	East	A/chicken/Chonburi/Thailand/CU-73/2004				DQ083580		DQ083616	DQ083690	DQ083652

Table 14 (*continue*) The H5N1 viruses analyzed in this part.

					Accession number							
wave	Year	Location	Region	Strain	PB2	PB1	PA	HA	NP	NA	M	NS
2	2004	Saraburi	Central	A/duck/Saraburi/Thailand/CU-74/2004				DQ083581		DQ083617	DQ083691	DQ083653
2	2004	Bangkok	Central	A/white peafowl/Bangkok/Thailand/CU-29/2004				DQ083573		DQ083609	DQ083683	DQ083645
2	2004			A/clouded leopard/Thailand/KU-11/2004				EU221247		EU221248		
2	2004	Prachinburi	East	A/chicken/Prachinburi/Thailand/CU-104/2004				DQ083582		DQ083618	DQ083692	DQ083654
2	2004	Phathum Thani	Central	A/quail/Phathumthani/NIAH2711/2004	AB450504	AB450520	AB450536	AB450554	AB450582	AB450600	AB450628	AB450644
2	2004	Nara Thiwat	South	A/chicken/NaraThiwat/NIAH1703/2004	AB450505	AB450521	AB450537	AB450556	AB450583	AB450602	AB450629	AB450645
2	2004	Suphanburi	Central	A/chicken/Suphanburi/NIAH7540/2004	AB450506	AB450522	AB450538	AB450561	AB450584	AB450607	AB450630	AB450646
2	2004	Angthong	Central	A/duck/Angthong/NIAH8246/2004	AB450507	AB450523	AB450539	AB450563	AB450585	AB450609	AB450631	AB450647
3	2005	Kanchanaburi	West	A/chicken/Thailand/Kanchanaburi/CK-160/2005	DQ334757	DQ334758	DQ334759	DQ334760	DQ334761	DQ334762	DQ334763	DQ334764
3	2005	Nontaburi	Central	A/chicken/Thailand/Nontaburi/CK-162/2005	DQ334773	DQ334774	DQ334775	DQ334776	DQ334777	DQ334778	DQ334779	DQ334780
3	2005	Nakhonnayok	Central	A/Thailand/NK165/2005	DQ372598	DQ372597	DQ372596	DQ372591	DQ372594	DQ372593	DQ372592	DQ372595
3	2005	Nakhon Pathom	Central	A/quail/Thailand/Nakhon Pathom/QA-161/2005	DQ334765	DQ334766	DQ334767	DQ334768	DQ334769	DQ334770	DQ334771	DQ334772
3	2005	Kanchanaburi	West	A/pigeon/Thailand/VSMU-11-KRI/2005	AB450513	AB450529	AB450545	AB450573	AB450591	AB450619	AB450637	AB450653
3	2005	Kanchanaburi	West	A/pigeon/Thailand/VSMU-13-KRI/2005	AB450514	AB450530	AB450546	AB450574	AB450592	AB450620	AB450638	AB450654
3	2005	Kanchanaburi	West	A/tree sparrow/Thailand/VSMU-14-KRI/2005	AB450515	AB450531	AB450547	AB450576	AB450593	AB450622	AB450639	AB450655
3	2005	Angthong	Central	A/open-bill stork/Thailand/VSMU-15-ATG/2005	AB450516	AB450532	AB450548	AB450577	AB450594	AB450623	AB450640	AB450656
3	2005	Ratchaburi	West	A/tree sparrow/Thailand/VSMU-16-RBR/2005	AB450517	AB450533	AB450549	AB450578	AB450595	AB450624	AB450641	AB450657
3	2005	Suphanburi	Central	A/chicken/Suphanburi/NIAH108192/2005	AB450508	AB450524	AB450540	AB450565	AB450586	AB450611	AB450632	AB450648
3	2005	NakhonPathom	Central	A/quail/Nakhon Pathom/NIAH7562/2005	AB450509	AB450525	AB450541	AB450566	AB450587	AB450612	AB450633	AB450649
4	2006	Nakhonphanom	NorthEast	A/chicken/Thailand/NP-172/2006	DQ999878	DQ999875	DQ999876	DQ999872	DQ999877	DQ999873	DQ999874	DQ999871
4	2006	Phichit	Central	A/chicken/Thailand/PC-168/2006	DQ999884	DQ999886	DQ999885	DQ999880	DQ999883	DQ999881	DQ999882	DQ999879
4	2006	Phichit	Central	A/chicken/Thailand/PC-170/2006	DQ999893	DQ999892	DQ999894	DQ999887	DQ999891	DQ999888	DQ999889	DQ999890
4	2006	Phichit	Central	A/chicken/Phichit/NIAH606988/2006	AB450510	AB450526	AB450542	AB450568	AB450588	AB450614	AB450634	AB450650
5	2006		Central	A/quail/Thailand/CU-330/2006	EU616858	EU616857	EU616856	EU616851	EU616855	EU616852	EU616853	EU616854
5	2006		Central	A/quail/Thailand/CU-331/2006	EU616866	EU616865	EU616864	EU616859	EU616863	EU616860	EU616861	EU616862
5	2006		Central	A/quail/Thailand/CU-332/2006	EU616874	EU616873	EU616872	EU616867	EU616871	EU616868	EU616869	EU616870

Table 14 (*continue*) The H5N1 viruses analyzed in this part.

					Accession number							
wave	Year	Location	Region	Strain	PB2	PB1	PA	HA	NP	NA	M	NS
5	2006		Central	A/quail/Thailand/CU-333/2006	EU616882	EU616881	EU616880	EU616875	EU616879	EU616876	EU616877	EU616878
5	2006		Central	A/watercock/Thailand/CU-334/2006	EU616890	EU616889	EU616888	EU616883	EU616887	EU616884	EU616885	EU616886
5	2006		Central	A/moorhen/Thailand/CU-317/06				EU616825		EU616826		
5	2006		Central	A/moorhen/Thailand/CU-318/06				EU616827		EU616828		
5	2006		Central	A/watercock/Thailand/CU-319/2006				EU616829		EU616830		
5	2006		Central	A/quail/Thailand/CU-320/2006				EU616831		EU616832		
5	2006		Central	A/chicken/Thailand/CU-321/06				EU616833		EU616834		
5	2006	Nakhonphanom	NorthEast	A/chicken/Nakhonphanom/NIAH113718/2006				EF419242				
5	2007		Central	A/duck/Thailand/CU-328/2007	EU616842	EU616841	EU616840	EU616835	EU616839	EU616836	EU616837	EU616838
5	2007		Central	A/duck/Thailand/CU-329/2007	EU616850	EU616849	EU616848	EU616843	EU616847	EU616844	EU616845	EU616846
5	2007	Nongkhai	NorthEast	A/duck/Nong-Khai/Thailand/KU-56/2007	EU221256	EU221255	EU221254	EU221249	EU221252	EU221251	EU221250	EU221253
5	2007	Phitsanulok	Central	A/chicken/Thailand/ICRC-195/2007	EU669192	EU669191	EU669190	EU497920	EU669189	EU669188	EU669193	EU669194
5	2007	Sukhothai	North	A/chicken/Thailand/ICRC-213/2007	EU875391	EU875392	EU875393	EU497921	EU875394	EU875395	EU875396	EU875397
5	2007	Sukhothai	North	A/chicken/Thailand/ICRC-V143/2007	EU233413	EU233414	EU233415	EU233416	EU233417	EU233418	EU233419	EU233420
5	2007	Nongkhai	NorthEast	A/chicken/Nongkhai/NIAH400802/2007				EF419243				
6	2008	Nakhonsawan	Central	A/chicken/Thailand/NS-339/2008	EU620652	EU620653	EU620654	EU620655	EU620656	EU620657	EU620658	EU620659
6	2008	Nakhonsawan	Central	A/chicken/Thailand/NS-341/2008	EU850413	EU850414	EU850415	EU850416	EU850417	EU850418	EU850419	EU850420
6	2008	Nakhonsawan	Central	A/chicken/Thailand/NS-342/2008	EU850421	EU850422	EU850423	EU850424	EU850425	EU850426	EU850427	EU850428
6	2008	Phichit	Central	A/chicken/Thailand/PC-340/2008	EU620660	EU620661	EU620662	EU620663	EU620664	EU620665	EU620666	EU620667
6	2008	Phitsanulok	Central	A/chicken/Thailand/ICRC-618/2008	EU875390	EU875389	EU547800	EU547798	EU547799	EU875388	EU669187	EU547801
6	2008	Nakhonsawan	Central	A/chicken/Thailand/ICRC-V586/2008	EU669195	EU669196	EU669197	EU497919	EU669198	EU669199	EU669201	EU669200
6	2008	Phitsanulok	Central	A/duck/Thailand/ICRC-V629/2008	EU676312	EU676313	EU676306	EU676307	EU676308	EU676311	EU676309	EU676310
6	2008	Suphanburi	Central	A/Kalij pheasant/Thailand/vsmu-1/2008	EU676314	EU676315	EU676316	EU676317	EU676318	EU676319	EU676320	EU676321
7	2008	Sukhothai	North	A/chicken/Thailand/ST-351/2008	FJ868019	FJ868020	FJ8680121	FJ868014	FJ868015	FJ868016	FJ868017	FJ868018
7	2008	Uthaithani	North	A/chicken/Uthaithani/NIAH115067/2008	FJ868030	FJ868031	FJ868032	FJ868033	FJ868034	FJ868035	FJ868036	FJ868037
7	2008	Sukhothai	North	A/chicken/Sukhothai/NIAH114843/2008	FJ868022	FJ868023	FJ868024	FJ868025	FJ868026	FJ868027	FJ868028	FJ868029

2.2 Whole genome sequencing

The RNAs of H5N1 subtype was extracted from allantoic fluid of the avian influenza inoculated eggs. The RNA extracted from allantoic fluid of the avian influenza inoculated eggs were provided by: the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; the Department of Livestock Development, Bangkok, Thailand; the Faculty of Veterinary Science, Kasetsart University, Kampaengsaen Campus, Nakorn Pathom, Thailand. RNA extracted were performed reverse transcription using 1 μM universal primer (Uni12 primer 5'-AGCAAAAGCAGG-3') as described by Hoffmann et al (Hoffmann et al., 2001) under identical conditions as described above. The whole genome sequences were amplified by using the primers set for H5N1 influenza A virus (Table 15). Briefly, 1 μl of cDNA template will be added to the reaction mixture containing 10 μl of 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), 0.5 μM forward and reverse primers and nuclease free water to a final volume of 25 μl. The amplification reaction will be performed in a thermal cycler (Eppendorf, Germany) under the following conditions: Denaturation at 94 ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec (for PB2, PB1, PA gene) and 55°C for 30 sec (for HA, NP, NA, MP, NS gene) then extension at 72°C for 90 sec, and concluded by a final extension at 72°C for 7 min. After electrophoresis in a 2% agarose gel stained with ethidium bromide on preparation, the expected PCR product will be visualized on a UV trans-illuminator. PCR products will be purified from the 2% agarose gel using the Perfectprep Gel Cleanup kit (Eppendorf, GmbH, Germany). DNA sequencing will be performed using the Gene Amp PCR system 9600 (Perkin-Elmer, Boston, MC).

Table 15 Primer sets for H5N1 whole genome amplification

Target Gene	Primer	Sequence (5'-3')	Position	Strand
PB2	PB2-F5'	AGCAAAAGCAGGTCAATTATATTC	1-23	Sense
	PB2-R1+	TTTGGAGGTTGCCTGTAAGC	1078-1059	Antisense
	PB2-F2	AGCACAAGATGTCATCATGGA	501-521	Sense
	PB2-F3	GTAGATATATGCAAGGCAGCA	949-969	Sense
	PB2-R3	CGACCACTCTTTCAGTGCTAG	1493-1513	Antisense
	PB2-F4	GTGCTGTTTCAAAACTGGGGA	1357-1377	Sense
	PB2-R5	GCTGTCTGGCTGTCAGTAAGT	2276-2256	Antisense
	PB2-R3'	AGTAGAAACAAGGTCGTTTTTAAA	2341-2317	Antisense
PB1	PB1-F5'	AGCAAAAGCAGGCAAACCATTTG	1-23	Sense
	PB1-R2	AGCTCTGTATCTTGTGAGTTA	917-897	Antisense
	PB1-F3	GCATCTGTGAAAAACTTGAGC	806-826	Sense
	PB1-R4	ATCACTGTAACTCCAATGCTC	1613-1593	Antisense
	PB1-F3+	TCCTCTGATGATTTCGCTCTC	1351-1371	Sense
	PB1-R3'	AGTAGAAACAAGGCATTTTTTCA	2341-2319	Antisense
PA	PA-F5'	AGCAAAAGCAGGTACTGATCCG	1-22	Sense
	PA-R1	GTCTCTTCGCCTCTCTCGG	624-606	Antisense
	PA-F2	GATGAAGAGAGCAGGGCAAG	515-534	Sense
	PA-F3	TGGAAGCAGGTGCTGGCAG	1037-1055	Sense
	PA-R3	CTCATTTCCATGCCCCATTTC	1770-1750	Antisense
	PA-F4	AACTTTGTGAGTATGGAATTCTC	1580-1602	Sense
	PA-R3'	AGTAGAAACAAGGTACTTTTTTGG	2234-2211	Antisense

Table 15 (*continue*) Primer sets for H5N1 whole genome amplification

Target Gene	Primer	Sequence (5'-3')	Position	Strand
HA	HA-F5'	AGCAAAAGCAGGGGTCTGATCTG	1-23	Sense
	H5-R1	GCTCCTCTTTATTGTTGGGTATG	565-543	Sense
	H5-F2	TGAGAAAATTCAGATCATCCCC	409-430	Antisense
	H5-R2	CAACGGCCTCAAACTGAGTGT	1265-	Sense
	H5-F3	ACTCCAATGGGGGCGATAAAC	914-934	Antisense
	HA-R3'	AGTAGAAACAAGGGTGTTTTTAACTAC	1778-	Antisense
NP	NP-F5'	AGCAAAAGCAGGGTAGATAATC	1-22	Sense
	NP-R1	CCATCGTCCCGACTCCCTTTA	613-593	Antisense
	NP-F2	TGATGCCACATACCAGAGAAC	477-497	Sense
	NP-R3'	AGTAGAAACAAGGGTATTTTTCT	1564-	Antisense
NA	NA-F5'	AGCAAAAGCAGGAGTTTAAAATG	1-23	Sense
	N1-R1	TGATAGTGTCTGTTATTATGCC	669-648	Antisense
	N1-F2	GTTTGAGTCTGTTGCTTGGTC	539-559	Sense
	NA-R3'	AGTAGAAACAAGGAGTTTTTTGAAC	1458-	Antisense
M	M-F5'	AGCAAAAGCAGGTAGATGTTG	1-21	Sense
	M-R3'	AGTAGAAACAAGGTAGTTTTTTAC	1022-	Antisense
NS	NS-F5'	AGCAAAAGCAGGGTGACAAAAAC	1-23	Sense
	NS-R3'	AGTAGAAACAAGGGTGTTTTTTAT	890-867	Antisense

2.3 Genetic comparison and Phylogenetic analysis

The H5N1 sequences (n=104) were aligned using Clustal X version 1.8.3 (Thompson et al., 1997). Columns with gaps were removed from the alignments. The nucleotide regions used for analysis were PB2 (nt 73-2220), PB1 (nt 49-1971), PA (nt 28-2117), HA (nt 48-1626), NP (nt 37-1434), NA (nt 25-1434), M (nt 25-1296) and NS (nt 25-789). Phylogenetic trees of alignment were constructed using the Neighbor-joining approach implemented in MEGA 4 (Tamura et al., 2007). Bootstrapping support for tree topologies was performed using NJ methods implemented in MEGA with 1,000 replicates. Bayesian tree was estimated by MrBayes version 3.1.2 (Huelsenbeck et al., 2001) with 1 million generations, sampling every 100 generations, using the default heating parameter. The consensus trees were calculated using the 'Allcompat' option for the final 10,001 trees from each run. The posterior probability for each split was generated when Bayesian trees were estimated by using the MrBayes 'sumt' command option with a 25% burnin. These posterior probabilities were used as an alternative measure of clade assignment support.

2.3 Substitution Rate

To estimate the rates of nucleotide substitution among H5N1 clade 1 viruses, the Bayesian Markov chain Monte Carlo (BMCMC) method as implemented in the BEAST v1.4.7 was applied (Drummond & Rambaut, 2007). Each gene was analyzed with the codon based SRD06 nucleotide substitution model (Shapiro et al., 2006). Three clock models including strict clock, uncorrelated lognormal relaxed (UCLD) clock and uncorrelated exponential relaxed (UCED) clock (Drummond et al., 2006) were attempted independently, and the most appropriate clock model was selected by comparing their posterior contribution calculated using the Bayes factor (Suchard et al., 2001). The BMCMC analyses contained $1x10^8$ states, with samples taken at 1000-state intervals, and the first 10% of each chain were discarded as burn-in. Convergences and effective sample sizes of the estimates were checked using Tracer v1.4 (Rambaut & Drummond, 2007).

2. 4 Geographic mapping

In order to visualize the geographic location of H5N1 isolates in this study, the viruses and their clade identity were plotted on the map of Thailand. To examine the geographic spread of viral variation, the location of variation based on phylogenetic tree analysis was projected onto the map in relation to the period of outbreaks.

3. Results

3.1 H5N1 dataset

In this study, the eight whole genome of H5N1 were performed and 97 publicly accessible isolates collected between the first and sixth wave in Thailand from 2004 to 2008 and obtainable at the Influenza Database, National Center for Biotechnology Information (NCBI) were also included analysis. Altogether 105 isolates (Table 14) from the first to the last wave from all regions of the country consist of: 29 isolates from first wave in 2004, 30 isolates from second wave in 2004, 12 isolates from third wave in 2005, 4 isolates from fourth wave in 2006, 11 isolates from fifth wave in 2006, 7 isolates from fifth wave in 2007, 8 isolates from sixth wave in 2008 and 3 isolates from seventh wave in 2008. The locations of these isolates were plotted on the map of the Thailand (Figure 14).

Figure 14 Location of H5N1 isolates in this study.

3.2 Genetic comparison and Phylogenetic analysis

The phylogenetic trees of the H5N1 viruses isolated during outbreaks in Thailand were constructed from 8 genes, using a NJ approach with bootstrapping supported by NJ and BMCMC methods (Figure 15-22). The 105 H5N1 isolates obtained from the first to seventh wave between 2004 and 2008 were analyzed. The phylogenetic tree confirmed the two distinct clades among the Thai isolates. Most isolates in this analysis belong to a distinct lineage designated clade 1 (Thai-Vietnam strains). The few outbreaks occurring in the north-east of Thailand were caused by clade 2.3.4 (Fujian-like strains) (Chutinimitkul et al., 2007). The 4 H5N1 isolates belonging to this distinct lineage, designated clade 2.3.4 are Ck/NIAH113718/06, A/Ck/Thailand/NP-172/06, Ck/NIAH400802/07 and Dk/Thailand/KU-56/07.

Genetic relationships showed that clade 1 can be divided into 3 distinct lineages (CUK2-like, PC168-like and PC170-like) with bootstrap support and posterior probabilities support over 90 applying NJ and BMCMC methods. A similar finding has been reported by Uchida and coworkers (Uchida et al., 2008) and designated 1.p1 and 1.p2. Since early 2004, the CUK2-like lineage has circulated in Thailand and spread throughout the country. The genetic homologies of CUK2-like isolates within the lineage are ranging from 99.5-99.7%.

The PC168-like and PC170-like lineage viruses were distinct from CUK2-like virus. Based on phylogenetic analysis, they clustered into two sub-branches by bootstrap and posterior probabilities values support over 90. During the third wave in 2005, PC170-like lineage viruses were detected. This lineage was consistently detected until the year 2006. During the course of the wave in 2006, PC168-like viruses had been detected. Both lineages persisted until the year 2007 (the fifth wave). The genetic distances of PC168-like and PC170-like viruses compared to the CUK2-like lineage are ranging from 98.4-99.4% and 98.7-99.5%, respectively. Upon phylogenetic analysis, they can be divided into two branches with reference to all genes except for the M gene. Based on M gene analysis, CUK2-like virus can be distinguished from PC168-like isolates. Yet, this does not apply to the P170-like lineage since its M gene belongs to the CUK2-like lineage (Figure 21).

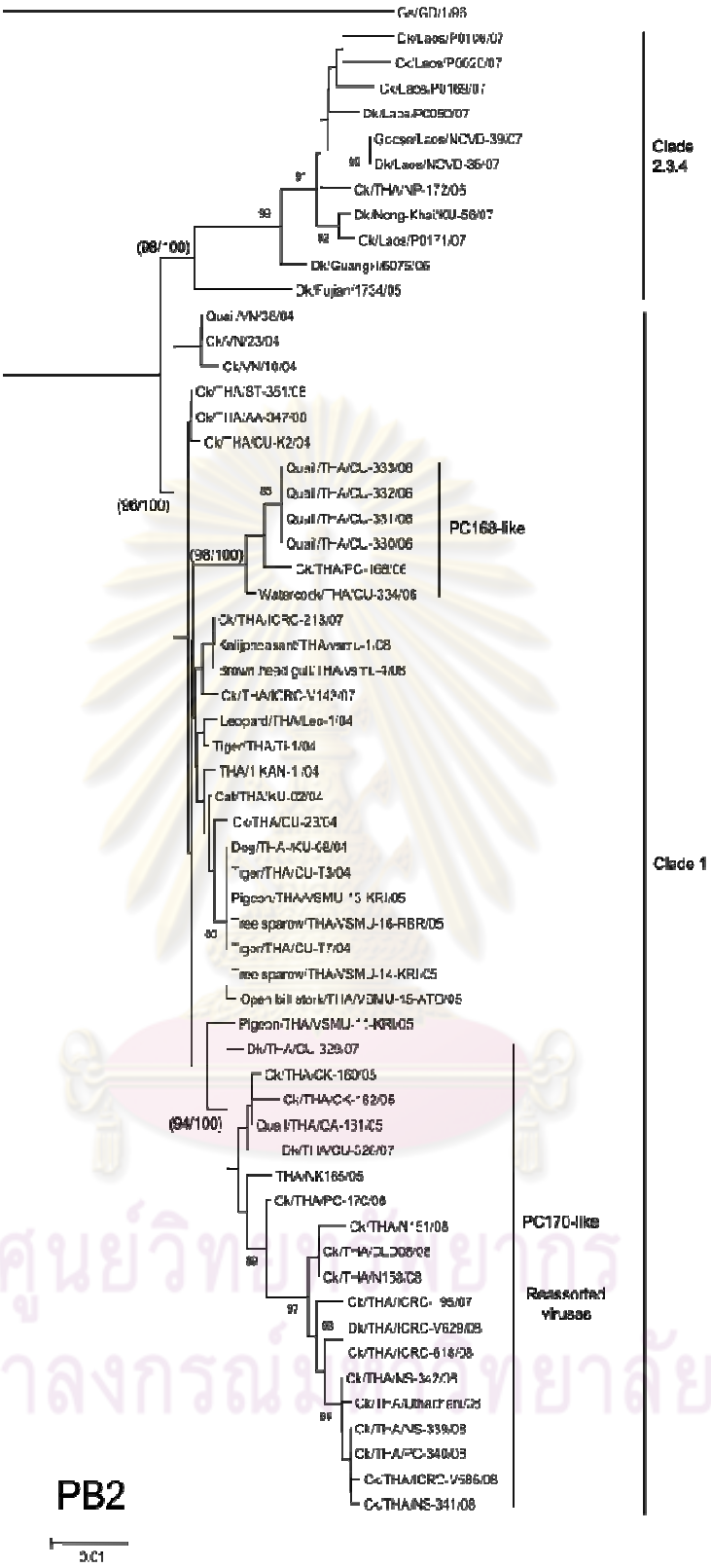


Figure 15 Phylogenetic tree of the PB2 gene of the sequences in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

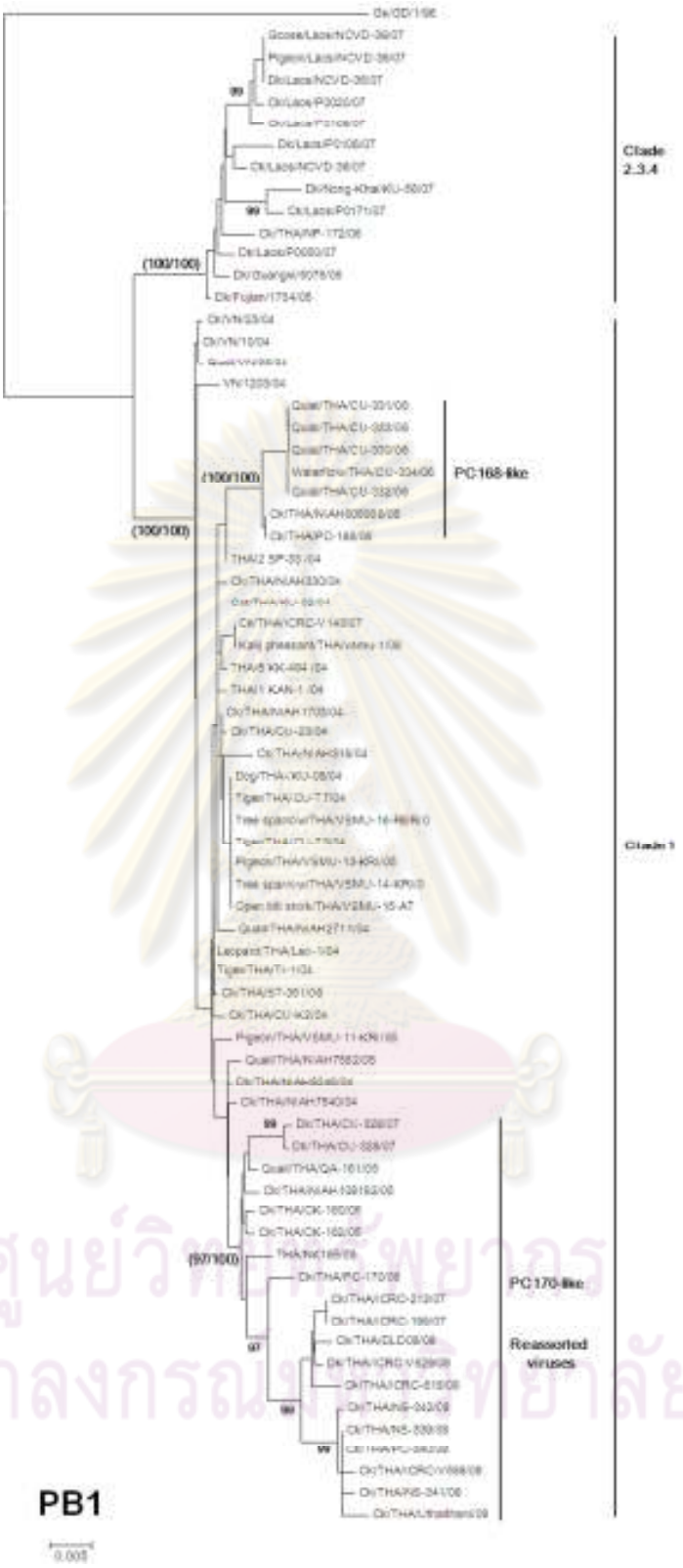


Figure 16 Phylogenetic tree of the PB1 gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

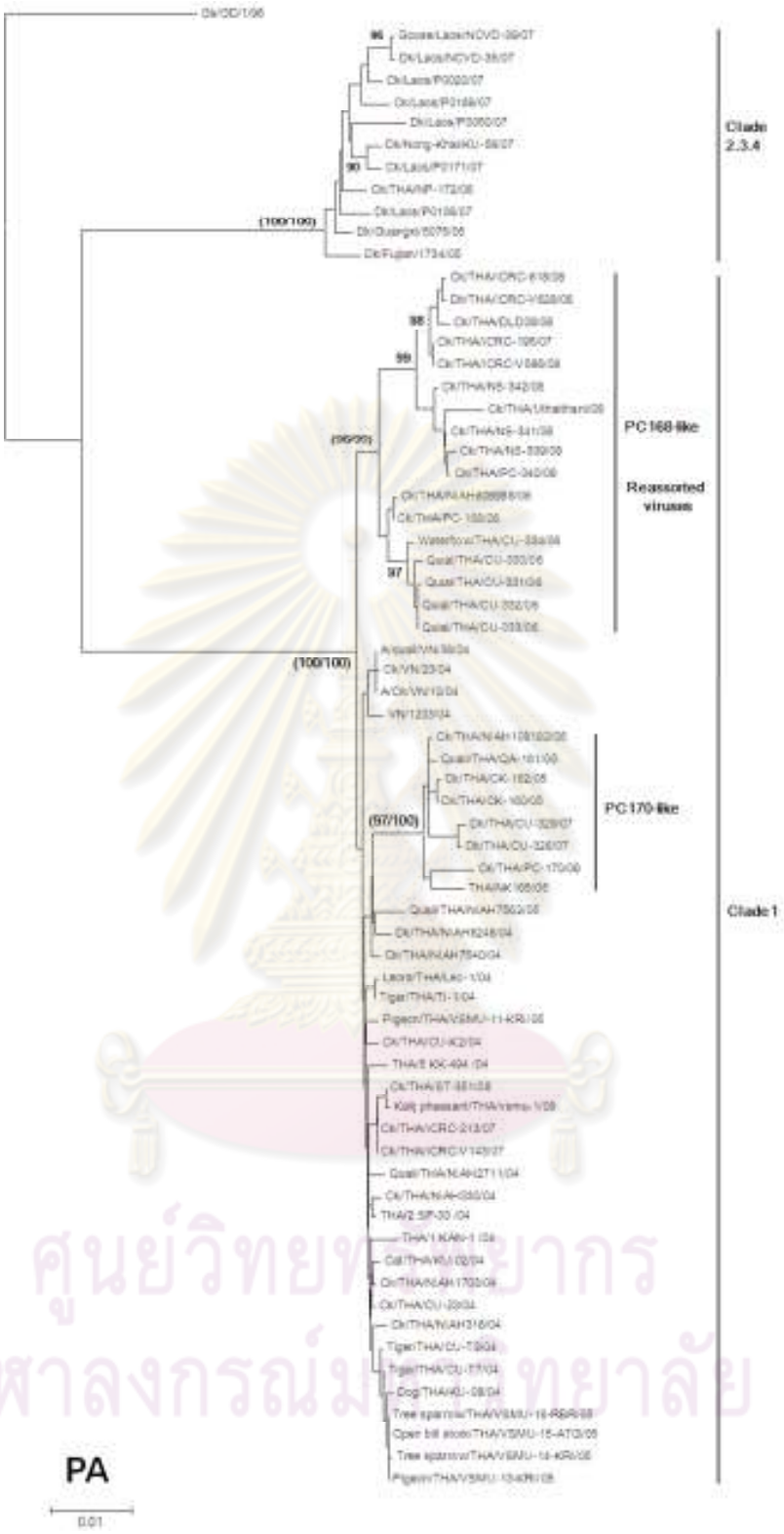


Figure 17 Phylogenetic tree of the PA gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

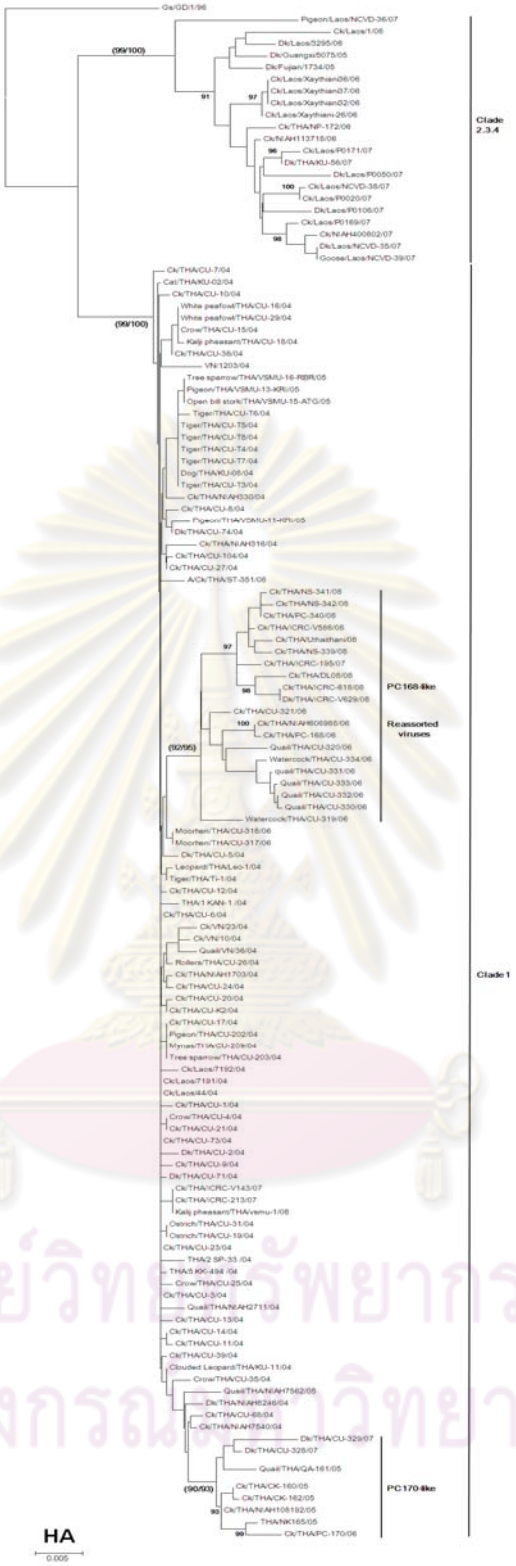


Figure 18 Phylogenetic tree of the HA gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

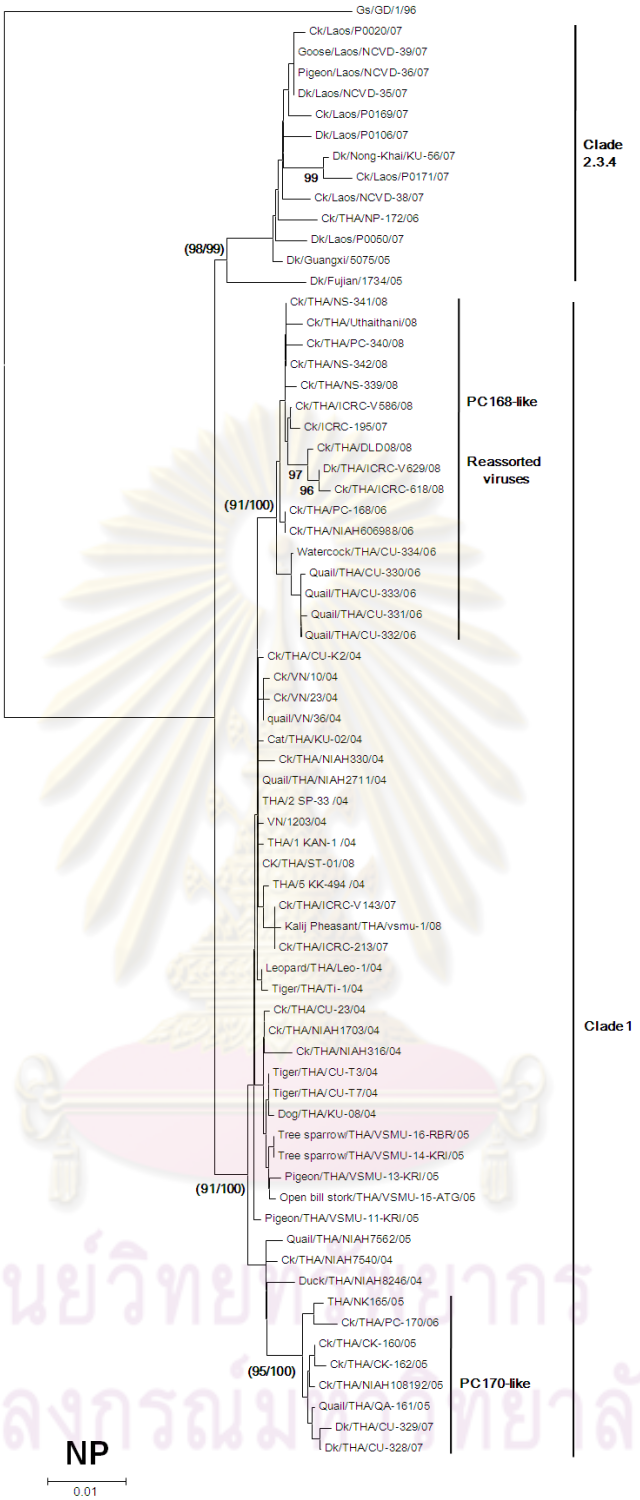


Figure 19 Phylogenetic tree of the NP gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

Figure 20 Phylogenetic tree of the NA gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

Figure 21 Phylogenetic tree of the M gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

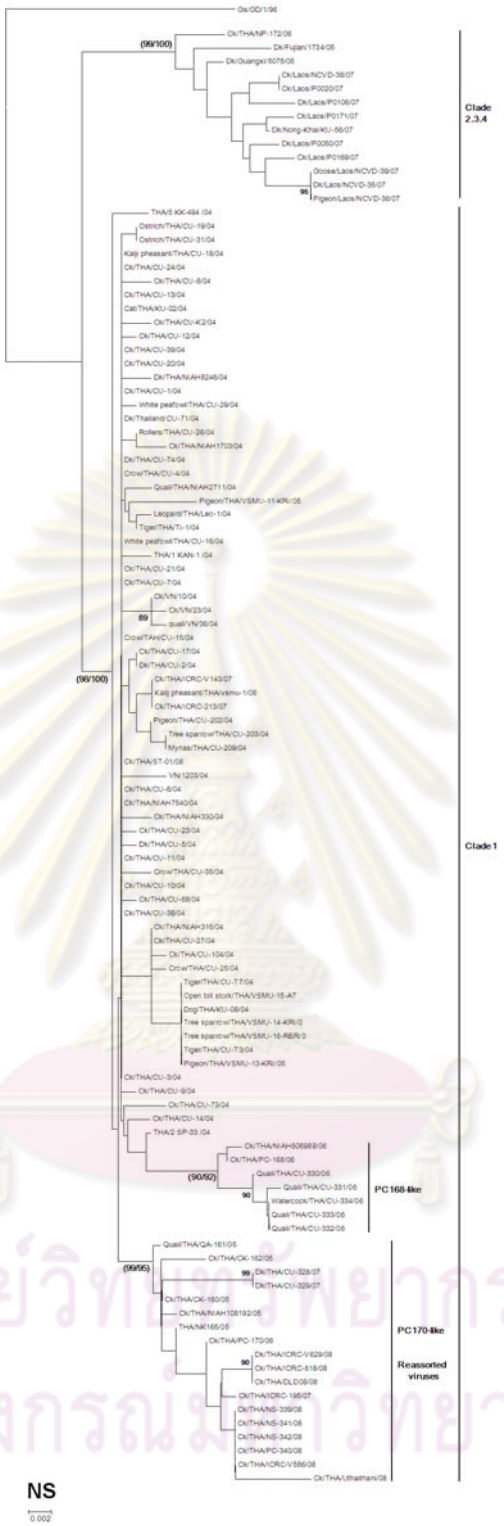


Figure 22 Phylogenetic tree of the NS gene of the sequence in this study. The phylogenetic tree was constructed by using a NJ method. Topological support summarized from bootstrap replication and posterior probability from BMCMC analyses is also shown (NJ/BMCMC).

Evidently, the 2008 strains had been re-assorted. The re-assorted gene could be detected in eight strains (Ck/Thailand/NS-339/08, Ck/Thailand/NS-341/08, Ck/Thailand/NS-342/08, Ck/Thailand/PC-340/08, Ck/Thailand/NIAH115067/08 and Ck/Thailand/NIAH114843/08). Re-assorted viruses were derived from 2 lineages (PC168-like and PC170-like viruses) with 4 segments (PA, HA, NP and M) (Fig 5, 6, 7 and 9) from the PC168-like and 4 segments (PB2, PB1, NA and NS) (Fig 3, 4, 8 and 10) from the PC170-like lineage. This evident is confirmed by similarity plot and bootscan analysis (Figure 23) In addition, CUK2-like viruses (non-reassorted) have still been circulating in 2008 (Pheasant/Thailand/VSMU-1-SPB/08, Ck/Thailand/ST-351/08). This analysis indicated that only 2 variants persisted, the CUK2-like viruses which were still in circulation and the re-assorted strains, while neither the PC168-like nor the PC170-like lineage was detected.

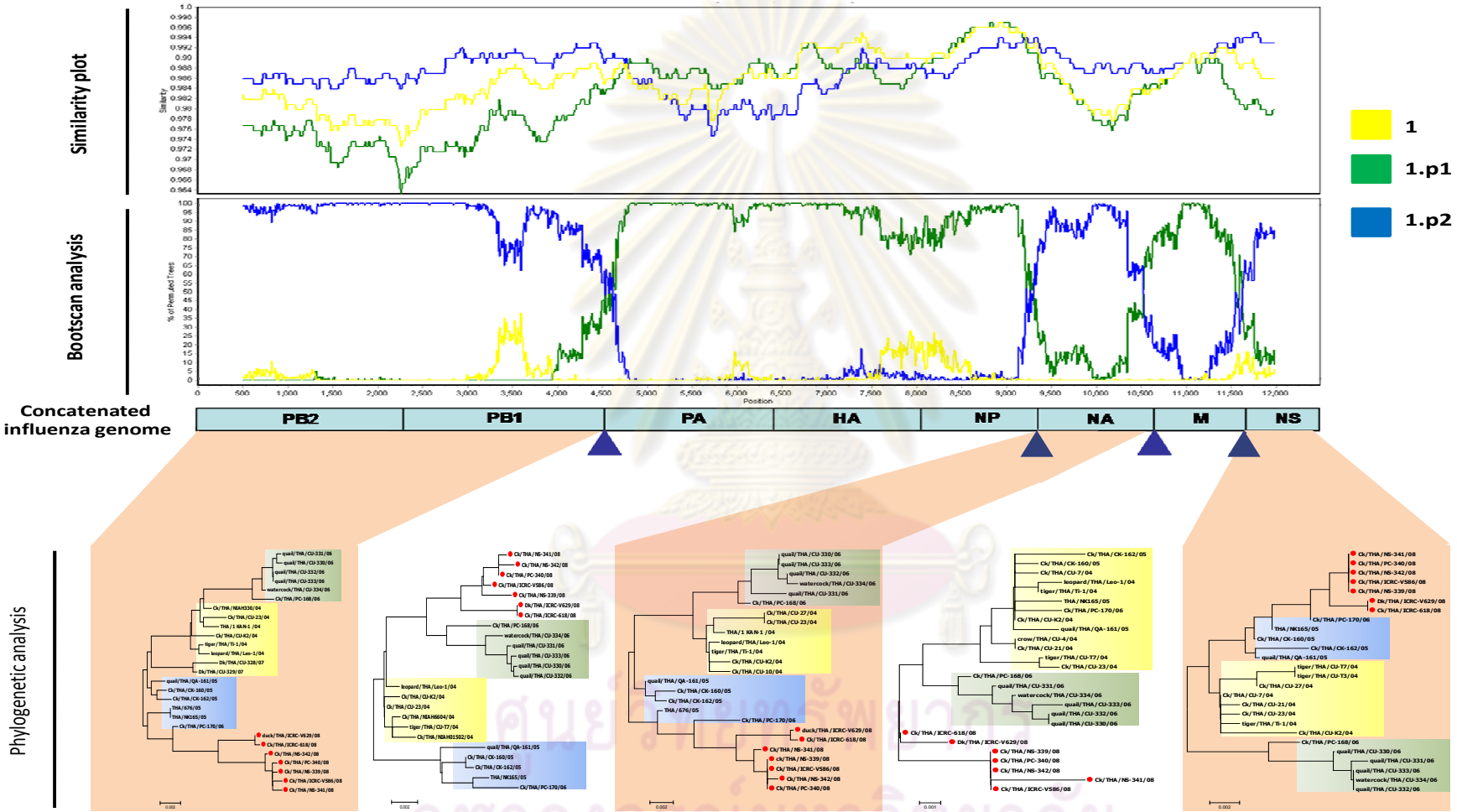


Figure 23 Similarity plot and bootscan analysis. These results support the evident of re-assorted viruses with phylogenetic analysis.

Genetic characterization of clade 1 viruses did not reveal any mutations at key determinant residues. The receptor-binding preference site at the hemagglutinin (HA) residue (positions 222, 224) also contained glutamine and glycine, respectively. The viruses harbor multiple insertions of basic amino acids at the HA cleavage site. Twenty amino acid deletions at the NA stalk region (positions 49–68) were discovered in all 2004–2008 isolates. Five amino acid deletions at positions 79–83 of the NS gene were also observed in all virus samples. The oseltamivir resistance residue in the NA gene at position 274 was not discernible. Alterations of the amino acid at position 31 (serine (S)) of the M2 protein can result in amantadine resistance.

3.3 Rate of nucleotide substitution

Analysis of the clade 1 viruses in Thailand showed that the mean substitution rates for the HA, NA and NS genes were 2.46 $x10^{-3}$, 2.47 $x10^{-3}$ and 2.56 $x10^{-3}$ substitutions per site, per year (subst/site/year), respectively, which are higher than the substitution rates established for the other gene segment (1.39-2.15 x 10^{-3} subst/site/year) (Table 16). However, this analysis did not display any significant difference in substitution rates among genes.

Table 16 Nucleotide substitution rates among clade 1 H5N1 viruses in Thailand from 2004 to 2008.

Gene	Mean substitution rate ($x10^{-3}$)	Substitution rate HPD ($x10^{-3}$)
PB2	1.70	1.31-2.08
PB1	2.15	1.71-2.62
PA	1.71	1.24-2.20
HA	2.46	1.91-3.06
NP	1.39	0.92-1.87
NA	2.47	1.93-3.06
M	1.53	0.96-2.10
NS	2.56	1.78-3.34

3.4 Geographical expansion of genetic variation

The geographical location of each H5N1 group was plotted onto a map of Thailand in order to trace both time and location of its respective emergence and subsequent dissemination throughout the country (Figure 24). During 2006-2007, clade 2.3.4 (Fujian-like) strains were detected only in 2 provinces in the northeast of Thailand (Nong-Khai, and Nakhon Panom). In contrast, the clade 1 strains spread throughout Thailand. During the early outbreak in 2004, the CUK2-like lineage spread throughout the center, south, north and east of Thailand (Tiensin et al., 2007). In 2005, the CUK2-like lineage persisted in the central region and the PC170-like virus was also detected in the central region (Ck/Thailand/CK-161/05, Ck/Thailand/CK-162/05, Thailand/NK-165/05 and Quail/Thailand/QA-161/05). In 2006-2007, the PC168-like lineage was detected in some provinces of central and lower northern Thailand (Moorhen/Thailand/CU-317/06, Moorhen/Thailand/CU-318/06, Watercock/Thailand/CU-319/06, Quail/Thailand/CU-320/06, Quail/Thailand/CU-330/06, Quail/Thailand/CU-331/06, Quail/Thailand/CU-332/06, Quail/Thailand/CU-333/06, Watercock/Thailand/CU-334/06 and Ck/Thailand/PC-168/06,). PC170-like viruses persisted in central Thailand (Dk/Thailand/CU-328/07, Dk/Thailand/CU-329/07) and in lower northern Thailand (Ck/Thailand/PC-170/06). In Phichit province, located in the lower part of the north, 2 lineages were detected (Ck/Thailand/PC-168/06 and Ck/Thailand/PC-170/06).

In 2008, the re-assorted viruses were found in the lower northern part of Thailand (Ck/Thailand/NS-339/08, Ck/Thailand/NS-341/08, Ck/Thailand/NS-342/08 and Ck/Thailand/PC-340/08). During the seventh wave of the outbreaks, the re-assorted viruses spread throughout the lower northern and the upper central part of Thailand. (Ck/Thailand/NIAH115067/08, Ck/Thailand/NIAH114843/08). CUK2-like viruses were still detected in the center of Thailand (Pheasant/Thailand/VSMU-1-SPB/08) and the lower part of the north (Ck/Thailand/ST-351/08). Interestingly, neither the PC168-like nor the PC170-like lineage was detected in the course of this year.

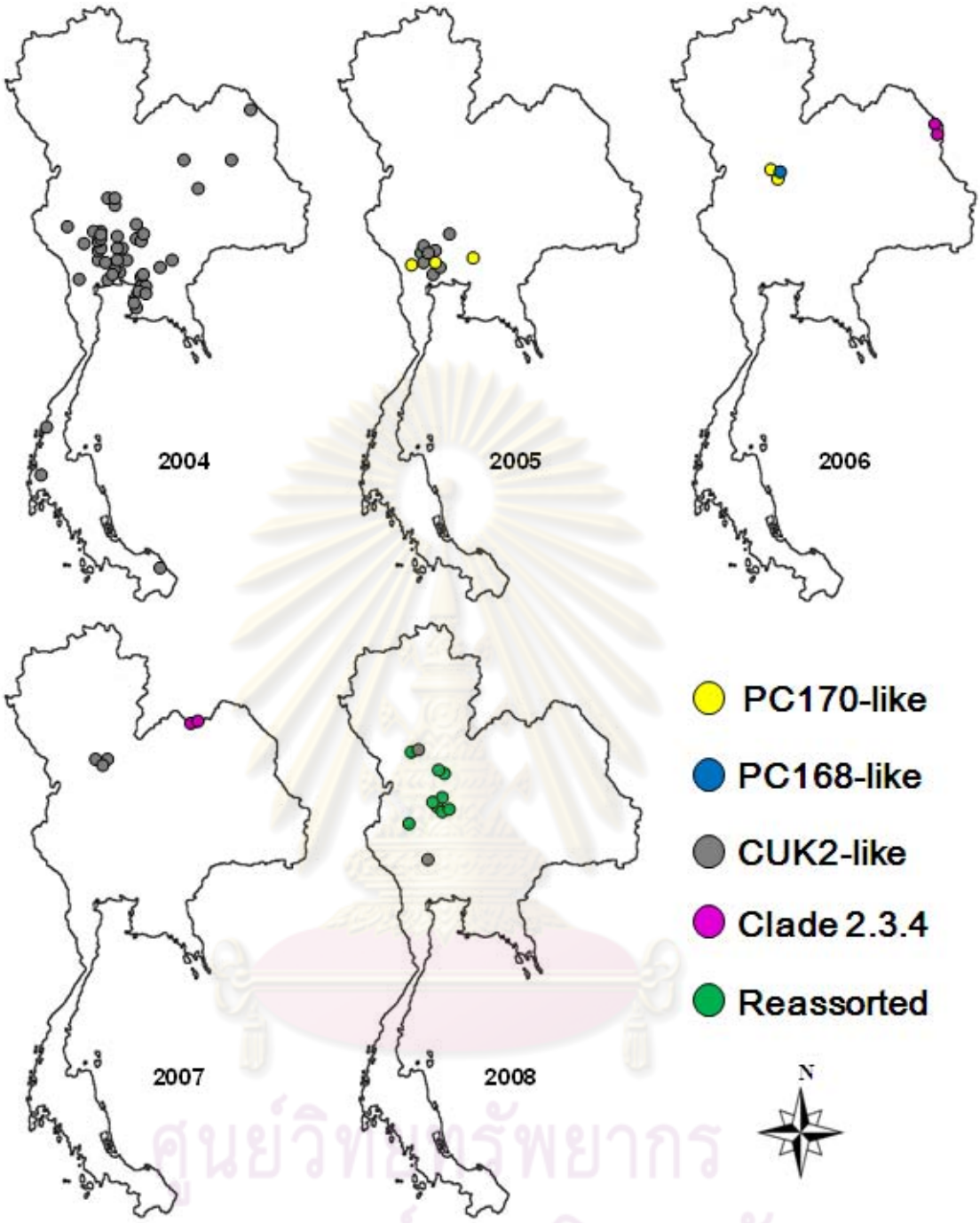


Figure 24 Geographic distribution of the genetic variation of the H5N1 isolates in Thailand from 2004 to 2008.

4. Discussion

Thailand has been one of the countries exposed to HPAI H5N1 outbreaks. In early 2004, the first outbreak of H5N1 has led to dramatic economic losses to the nation's poultry industry. The virus emerged in early 2004 and subsequently spread through many parts of the country. H5N1 viruses were also detected in humans and other mammalian species. H5N1 infections were mainly detected in backyard chickens and domestic ducks. During the first and second outbreaks, the virus crossed the species barrier and infected a domestic cat, a dog, captive tigers and leopards. During the following outbreaks, the number of dead animals decreased with time. Frequent disinfection of farms, monitoring transport and control on import of fowl and their eggs across borders, and raising public awareness of the disease nationwide has led to a decrease in the number of viruses isolated during the surveillance. However, sporadic outbreaks were still reported from 2006 to late 2008 (Amonsin et al., 2006; Buranathai et al., 2007; OIE, 2008; Chutinimitkul et al., 2007). The surveillance program in 2006 and 2007 which was conducted in live birds and food markets in 10 provinces of the center of Thailand indicated a risk for H5N1 contamination in bird meats, especially quail (Amonsin et al., 2008) suggesting that the virus persisted.

Genetic comparison showed the emergence of clade 2.3.4 viruses (Fujian-like strains). These viruses were limited to the north-east of the country in 2006-2007. Based on genetic characterization of samples obtained from the neighboring country, Laos, the clade 1 virus had been identified in 2004. Clade 2.3.4 virus was also observed in 2006-2007. Phylogenetic analysis demonstrated that viruses from both Thailand and Laos originated from the same source. The locations where virus was detected, Nakhon Panom and Nongkhai are located in the valley of the Mekong River, which also forms the border with Laos (Puthavathana et al., 2009).

Most Thai isolates belong to clade 1 and are still evolving. Upon genetic characterization, clade 1 viruses, the native strains in Thailand, were divided into 3 distinct lineages. CUK2-like virus was detected from the first to the last outbreaks. Analysis of recently isolated virus showed that the genetic distance was slightly different from virus of the early outbreak and that CUK2-like viruses comprise the native strain.

Between 2005 and 2007, PC168-like and PC170-like viruses were detected at various locations around the central and lower northern part of Thailand. Viruses obtained from Phichit province displayed 2 divergent lineages. The isolation of genetically divergent strains within the same province may suggest that viruses that evolved in separate environments may have converged in Phichit province due to as yet unidentified causes. Phylogenetic analysis has shown that these lineages share the same origin as the CUK2-like viruses. Genetic comparison has further demonstrated genetic drift from CUK2-like viruses. The distance was insufficient to distinguish a new clade (98.4-99.4% and 98.7-99.5%) (WHO, 2007). However, hemagglutination inhibiton test with a panel of monoclonal antibodies demonstrated a possible antigenic drift between 2 lineages as reported by a previous study (Uchida et al., 2008).

Numerous viruses re-assorted from 2 lineages, PC168-like and PC170-like viruses, were initially isolated in the lower northern provinces of Thailand and subsequently spread to the upper central part of the country. Re-assorted viruses were found in four neighboring provinces, Phichit, Sukhothai, Nakhonsawan and Phitsanulok. Although re-assorted viruses caused the outbreak in 2008, CUK2-like viruses were still detected around the lower northern and the upper central provinces of Thailand. Furthermore, upon emergence of re-assorted viruses, the PC168-like and PC170-like lineages could not be detected suggesting that the remaining strains still circulating in Thailand were CUK2-like and re-assorted viruses.

The rate of nucleotide substitution arrived at in this project was lower than those previously described for other countries (Vijaykrishna et al., 2008; Chen et al., 2006; Ducatez et al., 2007). The substitution rates of the viruses in Africa and China were high, reaching on average 2.6x10-3 and 2.8x10-3, respectively. In China, a combination of strong ecological and evolutionary factors led to a rapid increase in diversity, the spread of the virus through large, immunologically naïve poultry populations consisting of diverse species coupled with relatively high rates of nucleotide substitution (Vijaykrishna et al., 2008). But in Thailand, outbreaks were limited to the same area which might explain the lower nucleotide substitution rate.

The substitution rates of the surface genes (HA and NA) were higher than of the internal gene. Genes that code for surface protein are subject to strong selection pressure by neutralizing antibodies of host immune systems. On the other hand, genes coding for internal protein may not be subjected to strong host immune selection pressure but are thought to undergo significant host-specific adaptive evolution (Gammelin et al., 1990; Gorman et al., 1991).

Analysis of the molecular evolution of HPAI H5N1 isolated in Thailand during 2004-2008 has demonstrated that on an evolutionary scale, the virus in Thailand can be divided into 2 branches with re-assorted strains having emerged in the lower northern and upper central provinces of Thailand. Although more recent results could pinpoint the locations of re-assortment for some parts of the country, surveillance of poultry should continue throughout the country. Prevention and control initiatives should be applied country-wide to guard against the emergence of new variant HPAI H5N1 strains, which would expand the H5N1 viral gene pool. Surveillance programs conducted in poultry would help decrease the genetic reservoir and the number of variant viruses in particular, thus reducing the threat to public health.

CHAPTER VI

QUASISPECIES OF H5N1 INFLUENZA A VIRUS IN MAMMALS

Influenza viruses have a high error rate during the transcription of their genomes because of the low fidelity of RNA polymerase (Stech et al., 1999). The high error rate produce quasispecies, a phenomenon where many different viral genotypes co-circulate in the host, each virus subtype potentially associated with varying levels of fitness for that host (Domingo et al., 1985). As defined by Domingo et al, ‘‘viral quasispecies are closely related (but nonidentical) mutants and recombinant viral genomes subjected to continuous genetic variation, competition, and selection’’ (Domingo et al., 1998). This high error rate in replication operates as a double-edged sword - improving the ability of the virus to rapidly adapt to a new host via genetic changes that aid in replication and transmission efficiency while leading to the production of defective subtypes that have reduced fitness for the current host. Some or most of these quasispecies or mixed subtypes may be missed during viral culture because a ‘‘host’’ (chicken embryo or cell culture) adaptation pressure (Wang et al., 2008). This study used the second generation sequencing approach to sequence the entire genome of H5N1 influenza A viruses isolated from animals, Tigers and Leopard, to detect quasispecies.

454 sequencing, second generation sequencing

Since the early 1990s, DNA sequence production has almost exclusively been carried out with capillary-based, semi-automated implementations of the Sanger biochemistry (Figure 25). Alternative strategies for DNA sequencing, the second generation sequencing, the cyclic-array sequencing have recently been realized in a commercial product (e.g., 454 sequencing (used in the 454 Genome Sequencers, Roche Applied Science; Basel), Solexa technology (used in the Illumina (San Diego) Genome Analyzer), the SOLiD platform (Applied Biosystems; Foster City, CA, USA), the Polonator (Dover/Harvard) and the HeliScope Single Molecule Sequencer technology

(Helicos; Cambridge, MA, USA)). The concept of cyclic-array sequencing can be summarized as the sequencing of a dense array of DNA features by iterative cycles of enzymatic manipulation and imaging-based data collection (Mitra et al., 1999; Mitra et al., 2003). Although these platforms are quite diverse in sequencing biochemistry as well as in how the array is generated, their work flows are conceptually similar (Figure 25)

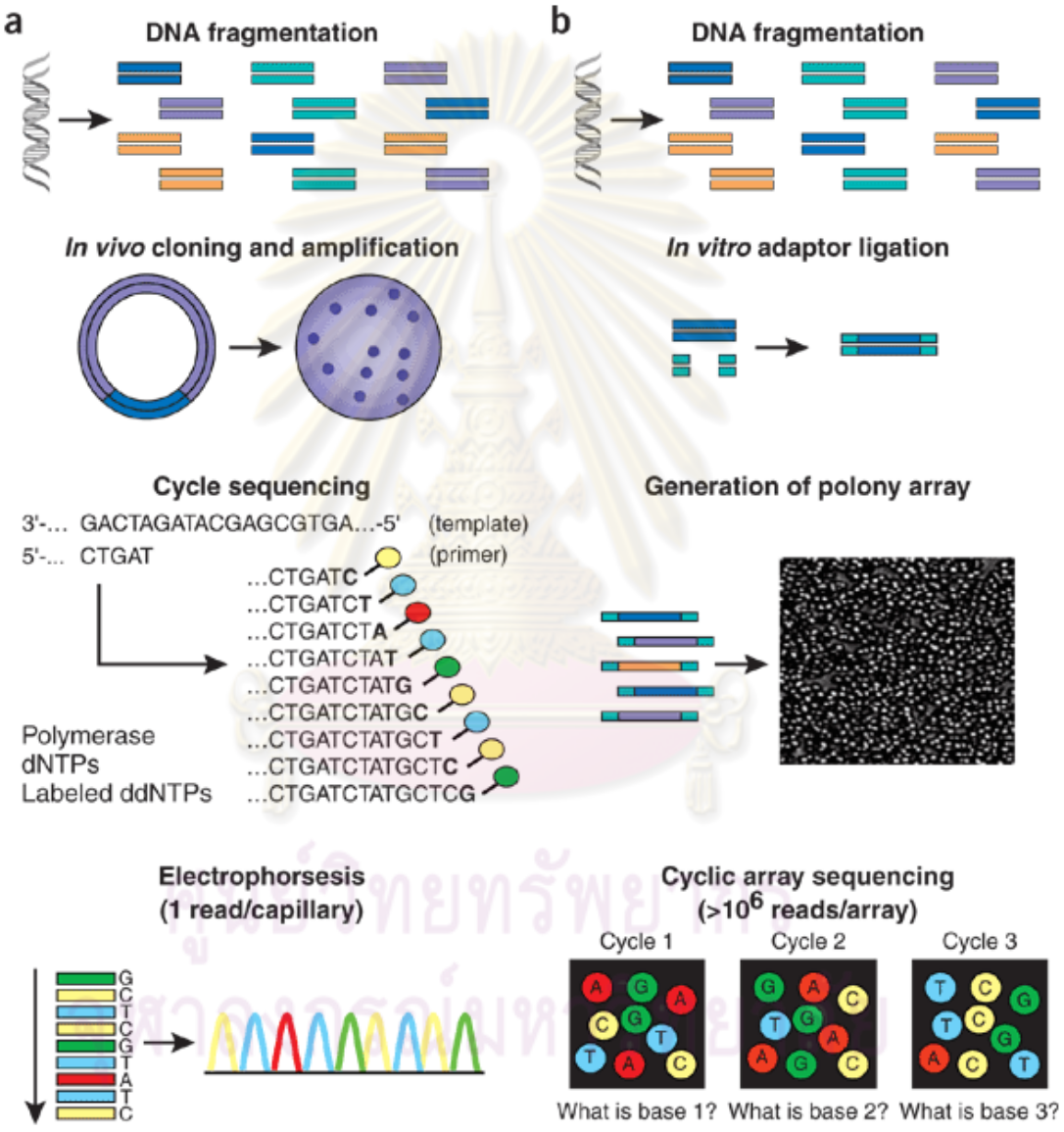


Figure 25 Work flow of conventional versus second-generation sequencing. (a) With high-throughput shotgun Sanger conventional sequencing. (b) In shotgun sequencing with cyclic-array methods. (Shendure and Ji, 2008)

The 454 system was the first next-generation sequencing platform available as a commercial product (Margulies et al., 2005). At the first step, Library preparation is accomplished by random fragmentation of DNA, followed by in vitro ligation of common adaptor sequences. The short adaptors (A and B) are added to each fragment. The adaptors are used for purification, amplification, and sequencing steps (Figure 26). Single-stranded fragments (sstDNA) with A and B adaptors compose the sample library used for subsequent workflow steps.

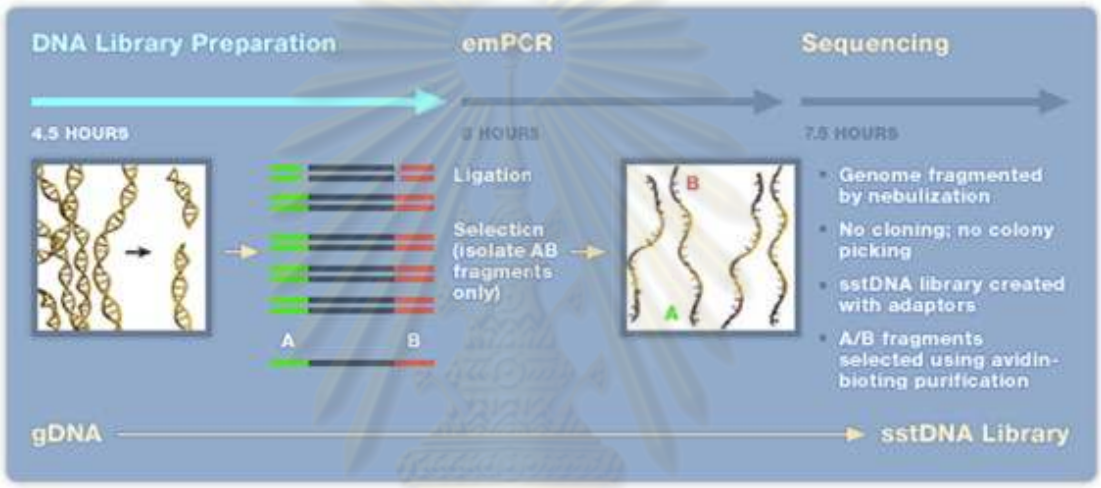


Figure 26 Generation of single-stranded fragments (sstDNA) with A and B adaptors compose the sample library used for subsequent workflow steps (Droege and Hill, 2008).

During the following emulsion PCR procedure (Figure 27) the sstDNA library is first mixed with an excess of sepharose beads carrying oligonucleotides that complementary to sstDNA. As a result most of these beads carry a unique single-stranded DNA library fragment. The beadbound library is then emulsified with amplification reagents in a water-in-oil mixture. Each bead is now captured within its own microreactor where clonal amplification of the single-stranded DNA fragments occurs. This results in bead-immobilized, clonally amplified DNA fragments.

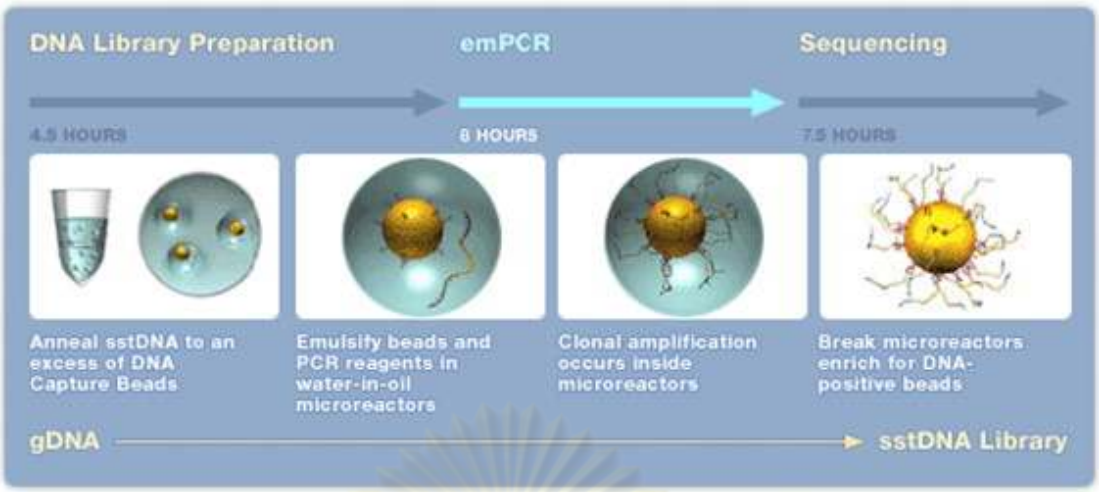


Figure 27 Clonal amplification of single sstDNA on beads during the so-called emulsion PCR (Droege and Hill, 2008).

The sstDNAlibrary beads are added to the DNA Bead Incubation Mix (containing DNA polymerase) and are layered with Enzyme Beads (containing sulfurylase and luciferase) onto the 454 PicoTiterPlate™ device (Figure 28). Only one library bead fits into one well. The bead-deposition process maximizes the number of wells that contain a single-amplified library bead (avoiding more than one sstDNA library bead per well).

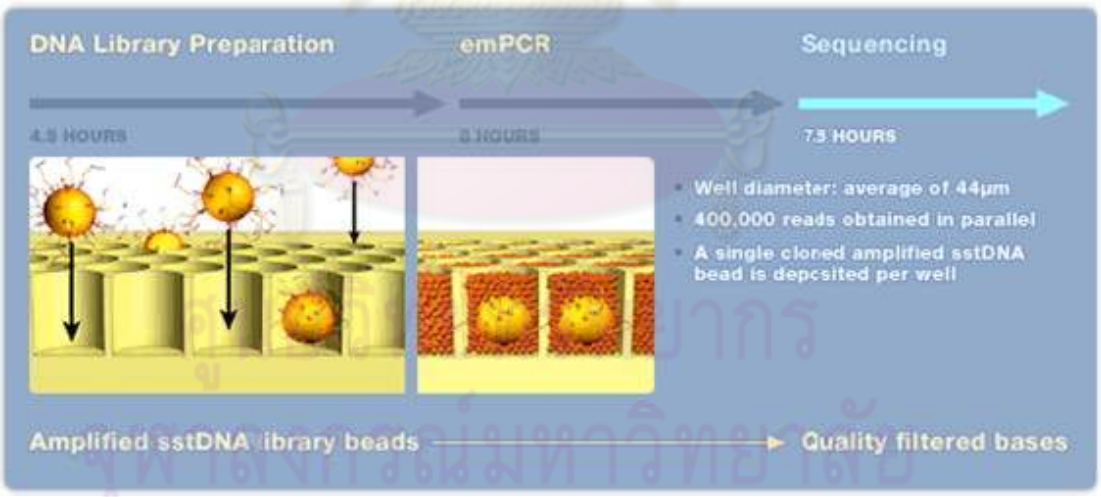


Figure 28 The sstDNA library beads are added to the DNA Bead Incubation Mix (containing DNA polymerase) and are layered with Enzyme Beads (containing sulfurylase and luciferase) onto the 454 PicoTiterPlate™ device (Droege and Hill, 2008).

The loaded PicoTiterPlate device is placed into the Genome Sequencer FLX™ Instrument for sequencing. Sequencing is performed by the pyrosequencing method (Ronaghi et al., 1996). The amplicon-bearing beads are preincubated with sequencing reagents (containing buffers and nucleotides) and then deposited on to a microfabricated array of picoliterscale wells (with dimensions such that only one bead will fit per well). Nucleotides are flowed sequentially in a fixed order across the PicoTiterPlate device during a sequencing run. During the nucleotide flow, hundreds thousands of beads carrying millions of copies of a unique single-stranded DNA molecule are sequenced in parallel. If the nucleotide complementary to the template strand is flowed into a well, the polymerase extends the existing DNA strand by adding nucleotide(s). Addition of one (or more) nucleotide(s) results in a reaction that generates a light signal that is recorded by the CCD camera in the instrument (Figure 29).

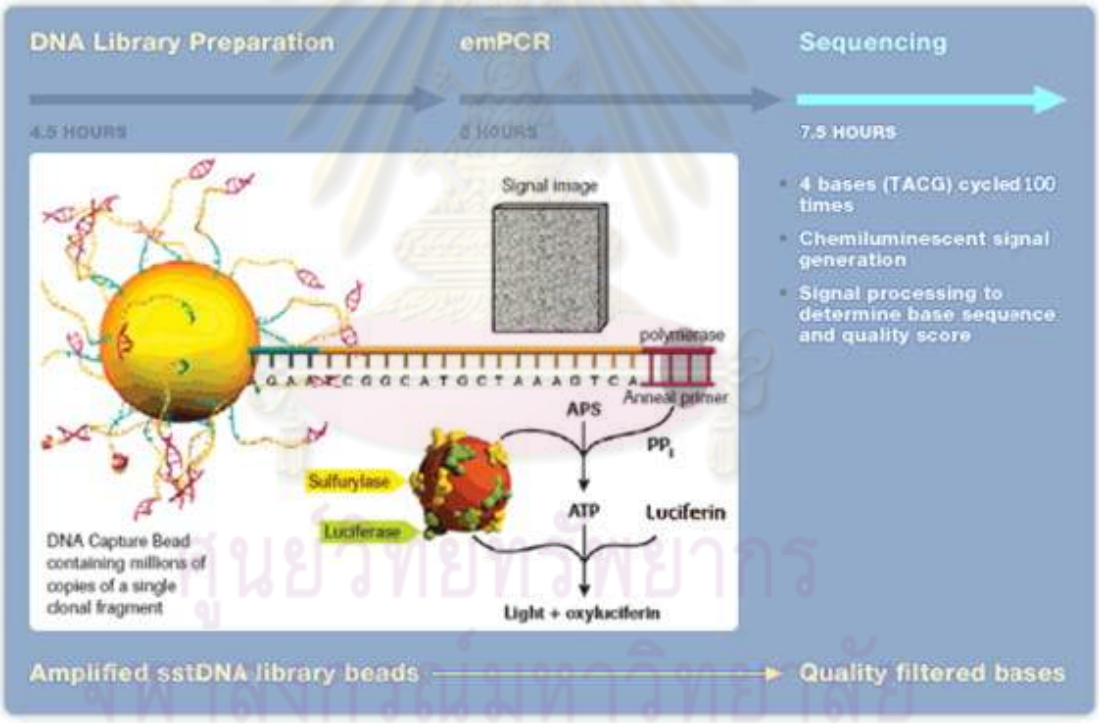


Figure 29 The scheme of 454 pyrosequencing (advanced pyrosequencing technology). (Droege and Hill, 2008)

To investigate the viral quasispecies of H5N1 influenza virus in mammal, in this study was separated into 2 parts. First, the quasispecies in the viral PB2 protein at position 627 was determined by using direct conventional clonal sequencing. Many kind tissues of tigers and leopard were determined. Second, the high resolution sequencing

of 454 sequencing determined the quasispecies in the respiratory tract tissue from tigers and leopard. Moreover, the comparison of viruses from direct tissue and virus from tissue culture was determined by using high resolution sequencing.

1. Objective

To investigate the viral quasispecies of H5N1 influenza virus in mammal.

2. Clonal sequencing of PB2 gene in tissue tropism

Subbarao and team have established a role for the viral protein PB2 in determining influenza virus host range in tissue culture (Subbarao et al., 1993). PB2 is a component of RNA polymerase, along with two other viral proteins, PB1 and PA. Within PB2, residue 627 normally show glutamic acid (E) in avian isolates but lysine (K) in viruses from humans. Altering residue 627 alone changed the host range of an avian PB2 single-gene reassortant so that it replicated in mammalian cells (Subbarao et al., 1993). Hatta et al. selected a pair of model of H5N1, the highly pathogenic H5N1 (A/Hong Kong/483/97) and the nonlethal H5N1 (A/Hong Kong/486/97) to explore the molecular basis of virulence of these viruses for mice. By using reverse genetics, they discovered that a mutation at position 627 in the PB2 protein influenced the outcome of infection in mice (Hatta et al. 2001). The virulent high pathogenic and nonlethal viruses a different in ability to invade systemic organs, suggesting that the PB2 protein could be a determinant of cell tropism. In this study, the qusispecies at position 627 in the viral protein PB2 was determined in mammals by using clonal sequencing

2.1 Material and method

2.1.1 Virus from tissue

Fifteen virus isolates were isolated from direct tissue of three tigers and one leopard. These viruses were isolated and provided by: (i) the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; (ii) the Department of Livestock Development, Bangkok, Thailand; (iii) Faculty of Veterinary Science, Kasetsart University, Kampaengsaen Campus, Nakorn Pathom, Thailand; These viruses were indicated in Table 17.

Table17 Source of virus sample for clonal sequencing.

Strain	Virus Isolated from
A/leopard/Suphanburi/Thailand/Leo-1/2004	Lung
	Brain
A/tiger/Suphanburi/Thailand/Ti-1/2004	Lung
	Brain
	Liver
A/tiger/Chonburi/Thailand/C2/2004	Kidney
	Lung
	Heart
	Spleen
	Pancreas
A/tiger/Chonburi/Thailand/C3/2004	Kidney
	Liver
	Spleen
	Pancreas
	Trachea

2.1.2 RNA Extraction, RT-PCR and Clonal sequencing

Viral RNA was extracted from 140 µl of tissue homogenized samples using QIAmp viral RNA mini kit (Qiagen, GmbH, Germany)) as per the manufacturer's instructions. RNA extraction was performed in biosafety level 3. RNA was amplified by single step RT-PCR by using the SuperScript III Platinum One-Step RT-PCR system (Invitrogen, California, USA). The reaction comprised a combination of 1.0 µl RNA sample with a reaction mixture containing 10 µl of 2× Reaction Mix, 0.5 µl of SuperScript III RT Platinum® Taq Mix (Invitrogen, California, USA), additional 0.1 mM of $MgCl_2$, 0.5M

primer PB2F: 5'-GTAGCAATGGTGTTCTCACAG-3' (nt 1198-1218), 0.5M primer PB2R: 5'-ACCTGCGTCCTTTCCAAGAA-3' (nt 2019-2000) and RNase-free water to a final volume of 25 µl. The amplification reaction was performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) under the following conditions: reverse transcription at 42°C for 30 min, predenaturation at 94 °C for 2 min followed by 40 amplification cycles comprising denaturation at 94°C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min and concluded by a final extension at 72 °C for 10 min. The PCR products were separated by 2% agarose gel electrophoresis and purified using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg, Germany). The purified products were inserted into pGEM-T Easy Vector System (Promega, Madison, WI), according to the manufacturer's protocol. Ten clones were randomly selected from the resultant white colonies and plasmids were purified using the High Pure Plasmid Isolation Kit (Roche, GmbH, Germany) according to the manufacturer's specifications. For automated DNA sequencing, all plasmids were amplified using the Gene Amp PCR System 9600 (Perkin-Elmer, MA). The sequenced products were subjected to a Perkin-Elmer 310 Sequence Analyzer (Perkin-Elmer).

2.2 Clonal sequencing of PB2 gene in tissue tropism result

The amino acid at position 627 in PB2 viral protein sequencing from directly RT-PCR product were detected for the major sequence which before clonal sequencing as follow table 18.

Table 18 The result of amino acid at position 627 in PB2 protein from direct RT-PCR sequencing

Strain	Virus Isolated from	Amino acid at position 627 in PB2 protein
A/leopard/Suphanburi/Thailand/Leo-1/2004	Lung	K
	Brain	K
A/tiger/Suphanburi/Thailand/Ti-1/2004	Lung	E
	Brain	E
	Liver	E
A/tiger/Chonburi/Thailand/C2/2004	Kidney	K
	Lung	K
	Heart	K
	Spleen	K
	Pancreas	K
A/tiger/Chonburi/Thailand/C3/2004	Kidney	K
	Liver	K
	Spleen	K
	Pancreas	K
	Trachea	K

Ten clones were randomly selected in each samples were then determined the sequence at position 627 in viral PB2 protein. The results of amino acid sequencing at position 627 in viral PB2 protein shown in Table19.

Table 19 The ratio of amino acid at position 627 in PB2 protein from clonal sequencing

Strain	Tissue organ	Number of colonies which have amino acid at position 627 in PB2 protein are:	
		glutamicacid (E)	lysine (K)
A/leopard/Suphanburi/Thailand/Leo-1/2004	Lung	0	10
	Brain	0	10
A/tiger/Suphanburi/Thailand/Ti-1/2004	Lung	10	0
	Brain	10	0
	Liver	10	0
A/tiger/Chonburi/Thailand/C2/2004	Kidney	0	10
	Lung	0	10
	Heart	0	10
	Spleen	0	10
	Pancreas	0	10
A/tiger/Chonburi/Thailand/C3/2004	Kidney	0	10
	Liver	0	10
	Spleen	0	10
	Pancreas	0	10
	Trachea*	8	12

* 20 colonies of Trachea A/tiger/Chonburi/Thailand/C3/2004 were picked up for clonal sequencing.

2.3 Discussion

The major sequencing from direct clonal RT-PCR showed that the isolate of A/tiger/Suphanburi/Thailand/Ti-1/2004 is the only one strain which resulted glutamicacid (E) at position 627 in PB2 viral proteins and the others showed lysine (K). The mixing strain between glutamicacid (E) and lysine (K) was shown in only trachea of A/tiger/Chonburi/Thailand/C3/2004 which was detected by clonal sequencing. Twenty colonies were picked up for DNA sequencing. The variation ratio of this virus population from this tissue is 8:12 for glutamic acid (E): lysine (K). These results showed the quasispecies in viral proteins. The quasispecies of the other positions in genomic are needed to use the high resolution sequencing method for the quasispecies detection the in viral genome.

3. High resolution genome sequencing of H5N1

The quasispecies and mixed infections of influenza A viruses had been detected by de novo sequencing (Ramakrishnan et al., 2009). The mixed infections and quasispecies were identified in low embryonated chicken egg passage of influenza A and cloacal swabs. An avian isolate and the second cloacal swab showed evidence of mixed infections by two and three HA subtypes. Multiple sequence differences were identified between cloacal swab and the virus recovered using embryonated chicken egg passage. Previously, genomic sequencing of tiger isolates were determined by using embryonated chicken egg passage isolates as templates but in this study we use the RNA from direct tissue. To compare the quasispecies of influenza A virus between directly tissue organism and embryonated chicken egg passage isolate, the high resolution sequencing were performed. Furthermore, the quasispecies in tissue organism were determined by using this technique.

3.1 Material and Method

3.1.1 Sample and virus

Five virus isolates form direct tissue of four tigers and one leopard and the four parallel isolated from embryonated chicken eggs passage of three tigers and a leopard were included for the quasispecies comparing between before (direct tissue isolates) and after (embryonated chicken egg isolates) cultural processing. These

viruses were isolated and provided by: (1) the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; (2) the Department of Livestock Development, Bangkok, Thailand; (3) Faculty of Veterinary Science, Kasetsart University, Kampaengsaen Campus, Nakorn Pathom, Thailand; These viruses were indicated in table 20.

Table 20 Viruses samples list for high resolution sequencing

No.	Virus Isolated from	Strain
1	Direct tissue (Lung)	A/leopard/Suphanburi/Thailand/Leo-1/2004
2	Embryonated chicken egg passage	A/leopard/Suphanburi/Thailand/Leo-1/2004
3	Direct tissue (Lung)	A/tiger/Suphanburi/Thailand/Ti-1/2004
4	Embryonated chicken egg passage	A/tiger/Suphanburi/Thailand/Ti-1/2004
5	Direct tissue (Lung)	A/tiger/Chonburi/Thailand/C2/2004
6	Direct tissue (Treachea)	A/tiger/Chonburi/Thailand/C3/2004
7	Embryonated chicken egg passage	A/tiger/Chonburi/Thailand/C3/2004
8	Direct tissue (Lung)	A/tiger/Thailand/CU-T3/2004
9	Embryonated chicken egg passage	A/tiger/Thailand/CU-T3/2004

3.1.2 RNA Extraction and Enrichment of influenza RNA segments

RNA was extracted from tissue organ and allantoic fluid by using QIAmp viral RNA mini kit (Qiagen, GmbH, Germany) as per the manufacturer's instructions. To reduce the contaminating host nucleic acids commonly observed in viral RNA preparations, RNA of influenza A virus molecules were captured and enriched through the hybridization of a biotin-labeled oligonucleotide directed to the conserved 5'-end of all eight segments of influenza A virus genome. Total RNA (50µL; ~50 ng/µl) was incubated in the presence of 200 µl of 6X SSPE buffer containing 0.1 units/µl of

SUPERase-In (Ambion,Texas, USA) and 0.5 μM of the 5'-Capture Oligo (5'-CCTTGTTTCTACT-biotin-3') at 70°C for 5 minutes followed by 15 minutes at 39°C. Equal volume (240 μl) of 2X binding and washing buffer containing 0.5 mg of washed Dynabeads MyOne Streptavidin C1 (Invitrogen, California, USA) was added to the above RNA samples and mixed thoroughly with a pipette. Fifty micro liters (a total of 0.5 mg) of Dynabeads MyOne Streptavidin C1 beads were washed with 1X binding and washing buffer as per the manufacturer's instructions and resuspended in 240 μl of 2X binding and washing buffer (10 mM Tris-HCL pH 7.5, 1 mM EDTA, 2 M NaCl, 0.1% Tween 20). The solution was incubated at room temperature for 30 min. with gentle shaking in the orbital shaker and then placed on a magnetic stand for 3 min. The supernatant was removed by aspiration with a pipette and the coated beads were washed four times with 1X binding and washing buffer. The captured RNA was eluted from the beads by incubating at 65°C for 5 min with 40 μl of 10 mM EDTA, pH 8.2, in 99% formamide. The tube was placed on the magnetic stand for 3 min. and the supernatant, containing enriched RNA, was aspirated with a pipette.

3.1.3 Fragmentation RNA

The enriched viral RNA was fragmented into a size range compatible with sequencing on the Genome Sequencer FLX. Five micro liters of 5X RNA Fragmentation Buffer (200 mM Tris-acetate, pH 8.1, 500 mM Potassium acetate, 150 mM Magnesium acetate) was added to 20 μl of enriched viral RNA. The samples were mixed thoroughly by pipetting, incubated for 2 min at 82°C, and then immediately transferred to ice to stop the fragmentation reaction. The reaction volume was increased to 50 μl by adding RNase free water, purified with RNAClean (Agencourt, Bourgogne, France) as per the manufacturer's instructions and eluted with 20 μl of RNase free water.

3.1.4 cDNA synthesis

The fragment of RNA sample was reverse transcribed in the final volume of 20 μL by using random hexamer (5'-phosphate-NNNNNNN-3') and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, California) as per the manufacturer's instructions. Each reaction consisted of 7 μl of fragmented RNA and 2 μl of 500 μM primers. After reverse transcription, the RNA was removed by hydrolysis by adding 20 μl

of Denaturation Solution (0.5 M NaOH, 0.25 M EDTA) and incubating at 65°C for 20 minutes. The mixture was then neutralized by adding 20 µl of 0.5 M HCl in 0.5 M Tris-HCl, pH 8.0. The resultant Single-stranded cDNA (sscDNA) was recovered with RNAClean (Agencourt, Bourgogne, France) as per the manufacturer's instructions and eluted from the beads with 20 µL of RNase free water.

3.1.5 Adaptor preparing

cDNA Adapter Set A;

The reaction comprised a combination mixture of 50 µM cDNA Adapter Oligo A, 60 µM of cDNA Adapter Oligo A-prime, 10 mM 1 M Tris-Cl pH 7.5 and DNase/RNase-free water in a final volume of 100 µl. The reaction was performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) under the following conditions of 80°C for 5 min, 65°C for 7 min, 60°C for 7 min, 55°C for 7 min, 50°C for 7 min, 45°C for 7 min, 40°C for 7 min, 35°C for 7 min, 30°C for 7 min and the final of 25°C for 7 min.

cDNA Adapter Set B;

The reaction comprised a combination of 240 µM of cDNA Adapter Oligo B, 200 µM of cDNA Adapter Oligo B-prime, 10 mM of 1 M Tris-Cl pH 7.5 and DNase/RNase-free water in a final volume of 100 µl. The reaction preparation was performed in a Mastercycler personal (Eppendorf) under the following conditions: 80°C for 5 min, 65°C for 7 min, 60°C for 7 min, 55°C for 7 min, 50°C for 7 min, 45°C for 7 min, 40°C for 7 min, 35°C for 7 min, 30°C for 7 min and the final of 25°C for 7 min.

3.1.6 Adaptor ligation

For clonal amplification and sequencing on the Genome Sequencer FLX the sscDNA requires the addition of adaptors to each terminus. The adaptors have been designed to enforce directional ligation to the sscDNA, such that one will be uniquely ligated to the 5'-end (sscDNA Adaptor A) and the other to the 3'-end (sscDNA Adaptor B) of the sscDNA. Each adaptor is comprised of two complimentary oligonucleotides that are annealed together as described below. The 3'-end adaptor consists of "sscDNA Oligo B" (5'-biotin- GCCTTGCCAGCCCGCTCAGNNNNNN-phosphate- 3') and "sscDNA Oligo B-prime" (5'-phosphate- CTGAGCGGGCTGGCAAGG-dideoxyC-3'). After

annealing there will be resulted the “sscDNA Adaptor B” with 3’-random overhang with six nucleotides. In the same way, the 5'-end adaptor consists of “sscDNA Oligo A-prime” (5’-NNN NNN CTG ATG GCG CGA GGG AGG dideoxyC-3”) and “sscDNA Oligo A” (5'-GCCTCCCTCGCGCCATCAG-3') which form the “sscDNA Adaptor A” with a six nucleotide 5'-end overhang.

The adapter ligation reaction was carried out using T4 DNA ligase (New England Biolabs, Massachusetts) in a total volume of 30 µl, containing 3 µl of 10X ligase buffer, 1µl of (1.67 mM final conc.) adapter A, 1 µl of (6.67 mMfinal conc.) adapter B, 5 µL (2000 cohesive end units) of T4 DNA ligase, 15 µl of sscDNA and 5 µl of water. The reaction mixture was incubated at room temperature for 2 hours; the ligated sscDNA was recovered with Dynabeads MyOne Streptavidin C1 (20 µl beads per sample) and eluted by incubating at 65°C for 5 min with 40 µl of 10 mM EDTA, pH 8.2, in 99% formamide. The final sscDNA was purified with two rounds of RNAClean (Agencourt, Bourgogne, France) and eluted in 20 µL of nuclease free water.

3.1.7 Double stranded library preparation

The final adapted sscDNA was amplified using Advantage2 PCR Kit (Clontech) in a total volume of 50 µl containing 5 µl of 10X Advantage 2 buffer, 2 µl of 50X dNTP mix (10 mM each), 10 µl (10 µM) Primer A (59-GCC TCC CTCGCG CCA-39), 10 µl (10 µM) Primer B (59-GCC TTG CCA GCC CGC-39), 1 µl of 50X Advantage polymerase mix, 10 µl of sscDNA, and 12 µl of nuclease free water. The PCR condition used was: 96°C for 4 min; 30 cycles of 94°C for 30 s and 64°C for 30 s; 68°C for 3 min; hold at 14°C. The PCR product was purified with two rounds of AMPure (Agencourt, Bourgogne, France) followed the manufacturer’s instructions. The double stranded DNA library was eluted with 20 µl of water.

3.1.8 Quantify and sequencing

The double stranded DNA library was quantified with the Quant-iT Picogreen dsDNA Assay Kit (Invitrogen , California, USA). Emulsion PCR amplification was carried out using either primer A or primer B or both for bidirectional sequencing. The sequencing reactions were carried out in small regions of the PicoTiterPlate (1–9 regions/sample) on the Genome Sequencer FLX (GS FLX) platform.

3.1.9 Sequencing Data analysis

Data analyses were performed on the Linux servers work station at the Minnesota Supercomputing Institute. All the sequencing reads were blasted against influenza genome in NCBI blast version.2.2.16. The 'non influenza' sequences were filtered out by perl script and only influenza reads were assembled in GS De novo Assembler Version 2.0.00.20 and mapped in GS Reference Mapper Version 2.0.00.20. The influenza contigs obtained using the above software were reassembled in Sequencher Version 4.8 (Genecodes).

3.2 Result of high resolution genome sequencing of H5N1

Preparing process was shown fragmented DNA on gel electrophoresis. Quantify double stranded library DNA shown the 1 molecule of DNA fragment per bead. All of the samples were loaded into 9 region of PicoTiterPlate. The total of nucleotide sequencing from Genome Sequencer FLX should be around 10 million bp. The result was underestimating from expected results. The total bases shown lower than usually run (Table 21). Totally bases from this run were just 691,299 bp that was underestimating from usually.

The control sequencing also showed the lower quality and quantity sequences (Table 22). The totally control sequencing was 32,428 bp. This run has shown the underestimate quality and quantity performance (normally should be approximately 700,000-1,000,000 bp).

Table 21 The sample sequence statistics from Genome Sequencer FLX. In this study, samples were loaded in region 1 to 9.

SAMPLE SEQUENCE STATISTICS

Regions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Tot/Ave
Total Bases	636	5380	3841	715	3203	3467	4357	13951	135549	18144	124040	124121	31842	160026	50237	11790	691299
Raw Wells	37936	42173	39328	41290	38993	37380	39367	38201	38749	39107	40082	30046	36192	34001	38197	38726	609768
Keypass Wells	23346	23574	16341	25555	21657	22993	25109	22824	21328	21905	24092	10734	19241	23300	27244	33761	363004
Passed Filter Wells	4	70	51	16	43	42	64	139	1316	134	696	590	191	804	331	95	4586
Num Dot Failed	17376	19758	14672	20179	19052	21394	21532	20008	15531	19559	20771	8862	17751	16462	21368	27878	302153
Num Mixed Failed	5426	2631	1048	4591	1599	933	2378	1422	2258	1100	983	600	629	3787	2992	3494	35871
Short Quality	508	1021	484	708	816	373	1054	1231	2147	1055	1586	576	633	2227	2521	2258	19198
Short Primer	32	94	86	61	147	251	81	24	76	57	56	106	37	20	32	36	1196
% Dot + Mixed	97.67	94.97	96.20	96.93	95.35	97.10	95.22	93.89	83.41	94.31	90.30	88.15	95.53	86.91	89.41	92.92	93.12
% Short Quality Primer	2.31	4.73	3.49	3.01	4.45	2.71	4.52	5.50	10.42	5.08	6.82	6.35	3.48	9.64	9.37	6.79	5.62
% Passed Filter	0.02	0.30	0.31	0.06	0.20	0.18	0.25	0.61	6.17	0.61	2.89	5.50	0.99	3.45	1.21	0.28	1.26

Sequence Results	1.TCA	2.TCA	3.TCA	4.TCA	5.TCA	6.TCA	7.TCA	8.TCA	9.TCA	10.TCA	11.TCA	12.TCA	13.TCA	14.TCA	15.TCA	16.TCA	Tot/Ave
Sequence numbers	4	65	50	8	40	40	59	138	1311	134	692	589	189	798	325	95	4537
Average Seq Length	159.0	82.8	76.8	89.4	80.1	86.7	73.8	101.1	103.4	135.4	179.2	210.7	168.5	200.5	154.6	124.1	-
Ave Seq Len Std Dev	41.4	60.3	52.7	62.9	63.0	69.8	54.8	67.1	65.9	83.4	78.7	71.1	79.9	72.1	82.2	71.2	-
Average Quality Score	13.6	22.5	21.2	25.7	21.3	26.8	21.1	26.1	30.9	29.5	31.0	31.5	28.3	29.6	23.2	25.5	-
Ave Qual Score Std Dev	4.8	7.7	8.1	8.1	8.4	8.4	8.0	8.3	7.1	7.7	7.2	7.5	7.8	8.1	8.4	7.9	-

Table 22 The control sequence statistics from Genome Sequencer FLX.

CONTROL SEQUENCE STATISTICS

Regions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Tot/Ave
Total Bases	4427					1310	176	337	871	159	419	14373	2695	7204		457	32428
Raw Wells	37936	42173	39328	41290	38993	37380	39367	38201	38749	39107	40082	30046	36192	34001	38197	38726	1219536
Keypass Wells	96	40	64	2	94	196	176	118	246	71	176	345	125	318	27	36	2130
Passed Filter Wells	27	0	0	0	0	13	4	5	5	1	3	78	13	40	0	2	191
Num Dot Failed	9	2	6	0	4	9	10	6	47	12	44	4	2	33	0	1	189
Num Mixed Failed	5	3	8	1	9	19	24	16	26	8	21	31	14	48	7	5	245
Short Quality	55	35	50	1	81	155	138	91	168	50	108	232	96	197	20	28	1505
Short Primer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
% Dot + Mixed	14.58	12.50	21.88	50.00	13.83	14.29	19.32	18.64	29.67	28.17	36.93	10.14	12.80	25.47	25.93	16.67	20.38
% Short Quality Primer	57.29	87.50	78.12	50.00	86.17	79.08	78.41	77.12	68.29	70.42	61.36	67.25	76.80	61.95	74.07	77.78	70.66
% Passed Filter	28.12	0.00	0.00	0.00	0.00	6.63	2.27	4.24	2.03	1.41	1.70	22.61	10.40	12.58	0.00	5.56	8.97

Sequence Results	1.ATG	2.ATG	3.ATG	4.ATG	5.ATG	6.ATG	7.ATG	8.ATG	9.ATG	10.ATG	11.ATG	12.ATG	13.ATG	14.ATG	15.ATG	16.ATG	Tot/Ave
Sequence numbers	23					10	3	3	5	1	3	74	12	36		2	172
Average Seq Length	192.5	0.0	0.0	0.0	0.0	131.0	58.7	112.3	174.2	159.0	139.7	194.2	224.6	200.1	0.0	228.5	-
Ave Seq Len Std Dev	66.8	0.0	0.0	0.0	0.0	65.2	7.3	64.7	63.3	0.0	60.5	60.2	35.4	44.7	0.0	9.5	-
Average Quality Score	25.9	0.0	0.0	0.0	0.0	26.4	18.2	27.8	33.3	25.6	24.0	29.9	24.3	29.5	0.0	22.4	-
Ave Qual Score Std Dev	8.3	0.0	0.0	0.0	0.0	7.9	4.2	8.8	5.2	5.8	8.1	6.7	7.5	6.5	0.0	7.3	-

3.3 Discussion

The result from Genome Sequencing FLX should be around 10 million bp. The total samples sequencing in this study showed only 691,299 bp. The numbers of nucleotides sequencing results were less than actual value approximately by 10 times. Not only samples sequencing results, but also control sequencing results underestimated actual value. The result from this run showed low quality and quantity performance. Machine running failure, results might be low quality and quantity. This machine was out of control so this experiment needs the further repeat.

4. Discussion

The high error rate during the transcription process of genomes of influenza A viruses may lead to produce the quasispecies. Hatta et al. had showed the mouse experiments with single-gene reassortant viruses (Hatta et al., 2001), the PB2 viral protein which is the viral RNA-dependent RNA polymerase subunit was responsible for the differenciation of virulence between the two Hong Kong H5N1 viruses. This finding was subsequently narrowed to amino acid residue 627 of the PB2 protein, which reportedly contributes to the host range of influenza A viruses (Subbarao et al., 1993). The virulent reassortant viruses and non-virulent reassortant viruses also differed in their ability to invade systemic organs, suggesting that the PB2 protein could be a determinant of cell tropism. In this study, the viral PB2 gene at position 627 was determined the quasispecies by using conventional clonal sequencing technique. Twenty colonies were picked up from trachea organ of A/tiger/Chonburi/Thailand/C3/2004 and performed the nucleotide sequencing. The quasispecies were detected 8:12 of glutamic acid (E): lysine (K). The evidence of quasispecies might be detected only in respiratory organ but not in trachea organ from the other tigers.

The quasispecies of H5N1 in sequences covering the receptor-binding domain of the hemagglutinin gene have been reported from fatal human cases (Kongchanagul et al., 2008). The highly variation in HA gene may lead the cause of viral adaptation in human host. The high resolution sequencing is the suitable technique for quasispecies

detection of viral whole genome. This technique is the large-scale capable for 400-600 million base pairs sequencing per run with 400-500 base pair read lengths. The DNA library preparation of genomics samples eliminates the laborious tasks of cloning and colony picking. The respiratory organ from tiger were detected the viral genome quasispecies by using the high resolution sequencing. Furthermore, viral genome quasispecies from direct tissue organ were compared with viral genome quasispecies from embryonated chicken egg. The mixed infection and quasispecies of influenza A virus have been reported in low embryonated chicken egg passage and direct clinical specimens (Ramakrishnan et al., 2009).

Unfortunately, the results from high resolution sequencing were failed. The controls running showed the low quality and quantity of results as well as samples running. The experiment needs to be repeated. But, the limitation of funding time is the problem for performing the repeat running. The Genome Sequence FLX running cost is very expensive. The cost of PicotiterPlate was up to 10,000 US dollar. This facility machine was provided and controlled by BioMedical Genomic Center (BMGC), University of Minnesota, USA. BMGC considered this problem and tried to find the way to solve this problem. This problem was already reported to Roche Applied Science (Indianapolis, USA), technical supported company.

The expected result in high resolution sequencing might be showed the quasispecies in other genes. HA gene has been reported the quasispecies for adaptation of the receptor-binding domain of H5N1 virus in infected human tissues (Kongchanagul et al., 2008). And, the viral tissue tropism were detected differences HA quasispecies in differences organ tropism. However, the conventional clonal sequencing showed the quasispecies in viral PB2 protein in trachea. Not only surface protein but might be also internal viral proteins were detected the quasispecies. The position of viral adaptation to mammal host is not clear. This study approaches the preliminary results of viral quasispecies for host adaptation, which need the further study for determining the viral quasispecies in other viral genome position.

CHAPTER VII

SUMMARIZING DISCUSSION

These studies mainly focused on molecular analysis of influenza A virus strains, not including pandemic H1N1 2009. Unfortunately, these studies were limited by the availability of samples and sample processing techniques. These molecular analysis of genetic materials may not answer all research questions, however, they can be used for the basic analyses and monitoring changes of the virus that may result in epidemic or even a pandemic.

In chapter III, usually, H1N1 and H3N2 are the most common subtypes of influenza A virus found to infect humans. However, during the past few years, influenza A virus subtype H5N1 has been reported to infect humans in many countries. Therefore, rapid detection and identification of these subtypes are particularly important in early diagnosis, suitable treatment and prevention of virus transmission. The two methods based on single-step multiplex real-time RT-PCR assays were developed and validated in terms of specificity, sensitivity and efficiency. The first assay consisted of primers and probes specific to the matrix gene of influenza A virus, matrix gene of influenza B virus and GAPDH gene of host cells for typing (A/B) of influenza viruses and verification by an internal control, respectively. The other assay employed primers and probes specific to the hemagglutinin gene of H1, H3 and H5 subtypes in order to identify the three subtypes of influenza A viruses capable of infecting humans.

In chapter IV, the complete genome analysis of human influenza A viruses subtype H1N1 and H3N2 which isolated between 2006 and 2008 obtained a comprehensive picture of the evolution of viruses in Thailand. Phylogenetic analysis of viral surface genes formed seasonal phylogenetic clusters. The seasonal clusters were

closely related to the WHO recommended vaccine strains in each season. The surface glycoprotein's showed the higher selection pressure than the other internal gene.

In chapter V, we described the evolution of HPAI H5N1 isolated in different regions in Thailand since early outbreak until seventh outbreak in 2008. H5N1 in clade 1, hemagglutinin were first observed in early 2004 and persisted until 2008. Viruses with clade 2.3.4 HA were first observed in the northeastern region of Thailand between 2006 and 2007. Phylogenetic analysis among Thai isolates indicated that clade 1 viruses in Thailand consist of three distinct lineages: CUK2-like, PC168-like, and PC170-like. The CUK2-like virus represents the predominant lineage and has been circulating throughout the 4-year outbreaks. Analysis of recently isolated viruses has shown that the genetic distance was slightly different from viruses of the early outbreak and that CUK2-like viruses comprise the native strain. Between 2005 and 2007, PC168-like and PC170-like viruses were first observed in several areas around central and lower northern Thailand. In 2008, viruses reassorted from these two lineages, The PC168-like and PC170-like viruses were initially isolated in the lower northern provinces of Thailand and subsequently spread to the upper central part of Thailand. On the other hand, CUK2-like viruses were still can detected around the lower northern and the upper central part of Thailand. Furthermore, upon emergence of the reassorted viruses, the PC168-like and PC170-like lineages could not be detected, suggesting that the only predominant strains still circulating in Thailand were CUK2-like and reassorted viruses. The substitution rate among clade 1 viruses in Thailand was lower. The virus being limited to the same area might explain the lower nucleotide substitution rate.

In chapter VI, H5N1 quasispecies were illustrated in Tiger. This study was tried to find H5N1 viral quasispecies in mammal by using two methods. Conventional clonal sequencing showed the viral quasispecies at PB2 gene position 627 in trachea of tiger. Further study by high resolution sequencing is recommended for viral genome quasispecies detection.

FUTURE PERSPECTIVES

The data presented in these studies can be used as the basis for future research. The single-step multiplex real-time RT-PCR could be applied for prevention and identification the prevalence of influenza A viruses subtypes. The single-step multiplex real-time RT-PCR could be developed for other influenza A viruses subtype and including H1N1 2009 pandemic influenza. The studies of molecular evolution of influenza A viruses in Thailand could be used for predict the outbreak strains and applied for surveillance programs. Although the current vaccine strains of human influenza A viruses subtype H1N1 and H3N2 are efficient in controlling the circulating outbreak subtypes, continuous surveillance is necessary to provide unambiguous information on emergent viruses. These findings contribute the understanding of evolution in influenza A viruses in humans and are useful for the routine surveillance and vaccine strain selection. The evolution of H5N1 study has demonstrated that nationwide attempts to monitor the virus may help curb access and propagation of new HPAI viral genes. The study of H5N1 influenza A virus surveillance in Thailand should be continued since this virus is highly pathogenic. Monitoring the variations can predict the fundamental characteristic of H5N1 influenza virus and may be important for vaccine development.

The conventional clonal sequencing showed the quasispecies in viral PB2 gene at position 627. The quasispecies might be found in other genome position. So, high resolution sequencing is recommended for further study.

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