การพัฒนาการตรวจหาเชื้อไข้หวัดนกสายพันธุ์ H5N1 ที่ดื้อต่อยา Oseltamivir และอณูชีววิทยาทางพันธุศาสตร์ของเชื้อไข้หวัดใหญ่ชนิด A ที่มีในประเทศไทย

นางสาว สลิล ชุตินิมิตกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

OSELTAMIVIR-RESISTANCE DETECTION OF AVIAN INFLUENZA H5N1 AND MOLECULAR GENETICS OF INFLUENZA A VIRUS IN THAILAND

Ms. Salin Chutinimitkul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2008

Thesis Title	OSELTAMIVIR-RESISTANCE DETECTION OF AVIAN INFLUENZA H5N1 AND MOLECULAR GENETICS OF INFLUENZA A VIRUS IN THAILAND
By	Ms.Salin Chutinimitkul
Field of study	Biomedical Sciences
Thesis Principal Advisor	Professor Yong Poovorawan, MD
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สลิล ชุตินิมิตกุล : การพัฒนาการตรวจหาเชื้อไข้หวัดนกสายพันธุ์ H5N1 ที่ดื้อต่อยา Oseltamivir และอณูชีววิทยาทางพันธุศาสตร์ของเชื้อไข้หวัดใหญ่ชนิด A ที่มีในประเทศไทย (Oseltamivir-resistance detection of avian influenza H5N1 and molecular genetics of influenza A virus in Thailand) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.ยง ภู่วรวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สัตวแพทย์หญิง ดร.สุดารัตน์ ดำรง วัฒนโภคิน, 104 หน้า.

โรคติดเชื้อในระบบทางเดินหายใจสามารถเกิดได้จากหลายสาเหตุ สาเหตุสำคัญหนึ่งคือการติดเชื้อไวรัส เชื้อไวรัสไข้หวัดใหญ่สามารถแพร่กระจายติดเชื้อในระบบทางเดินหายใจได้ในทุกช่วงอายุ ซึ่งทำให้มีอาการไข้สูงรวมทั้งสามารถทำให้ทารก เด็ก และผู้สูงอายุที่ติดเชื้อเสียชีวิตได้จากอาการปอดบวม ในวิทยานิพนธ์เล่มนี้ได้กล่าวถึงสายพันธุ์ของเชื้อไวรัสไข้หวัดใหญ่ที่ตรวจพบในคน สัตว์ปีก และสุกร ซึ่งได้ทำการศึกษาเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H1N1 H1N2 H3N2 รวมถึง H5N1 ซึ่งเริ่มพบการระบาดในสัตว์ปีกในประเทศไทยตั้งแต่ปี 2547 และข้ามมาติดเชื้อในคน เนื้อหาการวิจัยประกอบด้วย ส่วนที่หนึ่งเป็นการตรวจพิสูจน์น้ำเลือดของผู้ป่วยที่ติดเชื้อไข้หวัดใหญ่สายพันธุ์ H5N1 เพื่อแสดงให้เห็นถึงการมีอยู่ของไวรัสที่สามารถเพิ่มจำนวนได้ในเซลล์เพาะเลี้ยง ส่วนที่ 2 ได้ออกแบบการทดลองเพื่อตรวจหาเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H5N1 ที่ดื้อต่อยา Oseltamivir ซึ่งเป็นยาที่มีประสิทธิภาพในการรักษาผู้ป่วยที่ติดเชื้อไข้หวัดใหญ่สายพันธุ์ H5N1 โดยใช้เทคนิค Real-time PCR จากการออกแบบ TaqMan probe ให้จำเพาะต่อเชื้อไวรัสที่ดื้อยาและไม่ดื้อยาซึ่งสามารถตรวจแยกเชื้อไวรัสได้ถึงแม้ว่าเชื้อที่ดื้อต่อยาผสมกับเชื้อที่ไม่ดื้อยา ส่วนที่ 3 วิเคราะห์จีโนไทป์ของไวรัสไข้หวัดใหญ่สายพันธุ์ H5N1 ที่ระบาดในประเทศไทย เนื่องจากเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H5N1 ที่ระบาดในแต่ละพื้นที่มีความแตกต่างในด้านของรหัสพันธุกรรมในส่วนของยีนที่สำคัญ ยกตัวอย่างเช่น ความแตกต่างในด้านการทนต่อยาต้านไวรัส พบว่าเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H5N1 ที่ระบาดในประเทศไทยมีเชื้อไวรัส 2 สายพันธุ์ โดยสายพันธุ์แรกคล้ายกับเชื้อไวรัสจากประเทศเวียดนามซึ่งเป็นจีโนไทป์ Z และอีกสายพันธุ์คล้ายกับเชื้อไวรัสจากตอนใต้ของประเทศจีนซึ่งเป็นจีโนไทป์ V ส่วนที่ 4 ศึกษาการเปลี่ยนแปลงของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H1N1 และ H3N2 ที่พบในทารกและเด็กที่เข้ารับการรักษาในโรงพยาบาลจุฬาลงกรณ์ในช่วงปี 2549 – 2550 โดยศึกษาถึงความคล้ายคลึงของเชื้อที่ตรวจพบกับเชื้อสายพันธุ์ที่นำมาทำวัคซีนที่ใช้กันทั่วไป พบว่าเชื้อยังมีความเหมือนกับเชื้อสายพันธุ์ที่ใช้ทำวัคซีนหมายถึงวัคซีนที่ใช้นั้นยังสามารถป้องกันการติดเชื้อไข้หวัดใหญ่สายพันธุ์ที่มีอยู่ได้ และสุดท้ายเพื่อศึกษาถึงเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ที่พบในสุกร ได้ทำการศึกษารหัสพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่ในสุกรพบว่าเชื้อไวรัสไข้หวัดใหญ่ในสุกรที่พบนั้นมี 3 สายพันธุ์คือ H1N1 H1N2 และ H3N2 และเมื่อเปรียบเทียบกับเชื้อไวรัสที่พบในฐานข้อมูลพบว่าเชื้อไวรัสไข้หวัดใหญ่ในสุกรมีความคล้ายกับเชื้อไวรัสในหลายพื้นที่ทั้งทวีปยุโรป อเมริกา

สาขาวิชา ชีววิทยาศาสตร์ ลายมือชื่อนิสิต.....สลิล ชุตินิมิตกุล.....

ปีการศึกษา 2551 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

468 96991 20 : MAJOR BIOMEDICAL SCIENCES

KEY WORD: INFLUENZA A VIRUS/ AVIAN INFLUENZA/ H5N1/ OSELTAMIVIR RESISTANCE

SALIN CHUTINIMITKUL : OSELTAMIVIR-RESISTANCE DETECTION OF AVIAN INFLUENZA H5N1 AND MOLECULAR GENETICS OF INFLUENZA A VIRUS IN THAILAND. THESIS PRINCIPAL ADVISOR : PROF. YONG POOVORAWAN, MD., THESIS COADVISOR : SUDARAT DAMRONGWATANAPOKIN, DVM, Ph.D., 104 pp.

Severe respiratory illness can be caused by several factors. The important factor is viral infection especially influenza A virus infection. Influenza A virus can cause severe respiratory illness in every age which makes patient has high fever, cough, sneeze including pneumonia particularly in children or elderly people. In this thesis, the molecular genetics of influenza A virus in Thailand were studied. There were influenza A virus subtype H1N1, H1N2, H3N2 and also H5N1 which spread among avian species in Thailand since 2004 and cross species to infect human. The studies consisted of; firstly, the H5N1 influenza virus from human plasma was isolated and proved that the virus can replicate in the cell culture experiment. Secondly, Oseltamivir resistant detection of H5N1 influenza virus was designed using 2 specific TaqMan probes. This method can detect the mixture of both wild type and Oseltamivir resistant virus which could be the advantage for monitoring the change of virus in patient during Oseltamivir treatment. Thirdly, the 2 distinct genotypes, genotype Z and V of H5N1 influenza virus spreading in Thailand were identified and characterized which showed the difference in the sensitivity of Amantadine drug. Fourthly, influenza A virus, H1N1 and H3N2, in infants and children who admitted to Chulalongkorn Memorial hospital since 2006 -2007 were detected. The sequences of HA and NA gene were characterized for comparing the receptor binding and antigenic site of each subtype. In addition, they were compared with H1N1 and H3N2 vaccine strain from Northern Hemisphere 2007-2008, the finding showed the isolated subtypes were not different from vaccine strain. As a final, subtypes of swine influenza virus were isolated. Three subtypes, H1N1, H1N2 and H3N2, are described. Phylogenetic analysis of the SIV hemagglutinin and neuraminidase genes shows individual clusters with swine, human or avian influenza virus at various global locations.

Field of study Biomedical Science Student's signature.....

Academic year 2008 Principal Advisor's signature.....

Co-advisor's signature.....

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LIST OF ABBREVIATIONS

BLAST	=	Basic Local Alignment Search Tool
bp	=	Base pair
CBC	=	Complete blood count
cDNA	=	Complementary deoxyribonucleic acid
CNS	=	Central nervous system
EDTA	=	Ethylenediamine tetraacetic acid
Gal	=	Galactose
HA	=	Hemagglutinin
HMPV	=	Human metapneumovirus
HPAI	=	Highly Pathogenic Avian Influenza
IBDV	=	Infectious bursal disease virus
IBV	=	Infectious bronchitis virus
K	=	Kilo
LPAI	=	Low Pathogenic Avian Influenza
M	=	Matrix protein
MDCK	=	Madin–Darby Canine Kidney
mg	=	milligram
MGB	=	Minor groove binding
ml	=	millilitre
mM	=	millimolar
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
NA	=	Neuraminidase
NeuAc	=	N-acetylneuraminic acid
NeuGc	=	N-glycolylneuraminic acid
NAI	=	Neuraminidase inhibitor
NDV	=	Newcastle disease virus

NJ	=	Neighbour-joining
nm	=	nanometre
NP	=	Nucleoprotein
NP suction	=	Nasopharyngeal suction
NS	=	Non-structural protein
OIE	=	Office International des Epizooties
PA	=	Polymerase Acid protein
PB1	=	Polymerase Basic protein 1
PB2	=	Polymerase Basic protein 2
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
RNP	=	Ribonucleoprotein
RSV	=	Respiratory syncytial virus
RT-PCR	=	Reverse-transcription polymerase chain reaction
SA	=	Sialic acid
SIV	=	Swine influenza virus
U	=	Unit
UV	=	Ultra violet
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

The common cold and influenza (flu) are the most common syndromes of infection in human beings. Influenza is an infectious disease of birds and mammals caused by an RNA virus of the family *Orthomyxoviridae*. In humans, common symptoms of influenza infection are fever, sore throat, muscle pains, severe headache, coughing, and weakness and fatigue. In more serious cases, influenza causes pneumonia, which can be fatal, particularly in young children and the elderly. 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 occur through contact with these bodily 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 indefinitely at very low temperatures; however, most influenza strains can be inactivated easily by disinfectants and detergents (Eccles, 2005). Flu spreads throughout the world in seasonal epidemics, killing millions of people in pandemic years and hundreds of thousands in non-pandemic years. Three worldwide (pandemic) outbreaks of influenza occurred in the 20th century: in 1918, 1957, and 1968 and killed tens of millions of people, with each of these pandemics being caused by the appearance of a new strain of the virus in humans. Recurrently, these new strains result from the spread of an existing flu virus to humans from other animal species. Since it first killed humans in Asia in the 1990s, a deadly avian strain of H5N1 has posed the greatest treat for a new influenza pandemic; however, this virus has not mutated to spread easily between people (Kilbourne, 2006). Since 2004, when the avian influenza A subtype H5N1 widespread in Thailand, many Thai researchers enthusiastically explored the basic data of this virus. Together with this thesis, the aim of each experiment is to provide advance research in developing laboratory diagnostic techniques both in

human and animal and study the genetic characterization in many subtypes of influenza A virus in several species of Thailand. The detail of this thesis can be divided into 5 parts that is 1) H5N1 influenza A virus and infected human plasma; 2) H5N1 Oseltamivir-resistance detection by real-time PCR using two high sensitivity labeled TaqMan probes; 3) New strain of influenza A virus (H5N1), Thailand; 4) Molecular characterization and phylogenetic analysis of H1N1 and H3N2 human influenza A viruses among infants and children in Thailand and 5) Genetic characterization of H1N1 H1N2 and H3N2 swine influenza virus.

Hypothesis

1. Infectious H5N1 influenza A virus can be isolated in human fatal case.
2. Rapid detection of wild type and Oseltamivir resistance of H5N1 influenza virus designed by real-time PCR using a two labeled probe set provide high accuracy and sensitivity in several sources of specimens.
3. The strain of H5N1 influenza virus in Northeastern Thailand is different from other parts of Thailand.
4. The current vaccine strain for the Northern Hemisphere 2007-2008 is suitable for influenza A virus in the year of 2007 in Thailand.
5. Swine influenza virus share receptor properties of both avian and human influenza virus

Objectives

1. To prove that H5N1 influenza A virus can be isolated from plasma of H5N1 infected human.
2. To remind the necessity to carefully handle and transport serum or plasma specimens from patients suspected of H5N1 infection.
3. To develop rapid method for differentiation between influenza A virus H5N1 Oseltamivir-resistant strain and wild type at H274Y of neuraminidase gene and expand the detection in several sources of specimens

4. To survey H5N1 influenza virus strains in Thailand for evaluate the potential H5N1 influenza vaccine strain.
5. To investigate subtype of human influenza virus during the year of 2006 to 2007 and evaluate the difference in both hemagglutinin and neuraminidase gene with past and current influenza vaccine strain.
6. To investigate subtype of swine influenza virus and deeply examine in hemagglutinin and neuraminidase gene for studying the relatedness of swine influenza virus to avian and human influenza virus.

Conceptual Framework

Part 1 H5N1 Influenza A virus and Infected Human Plasma

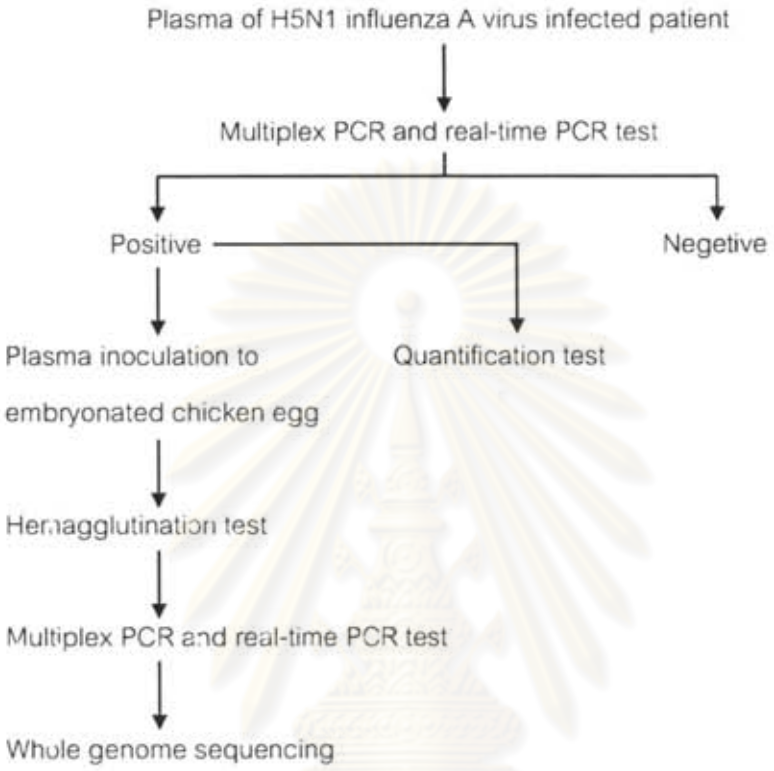
Part 2 H5N1 Oseltamivir-resistance detection by real-time PCR using two high sensitivity labeled TaqMan probes

Part 3 New Strain of Influenza A Virus (H5N1), Thailand

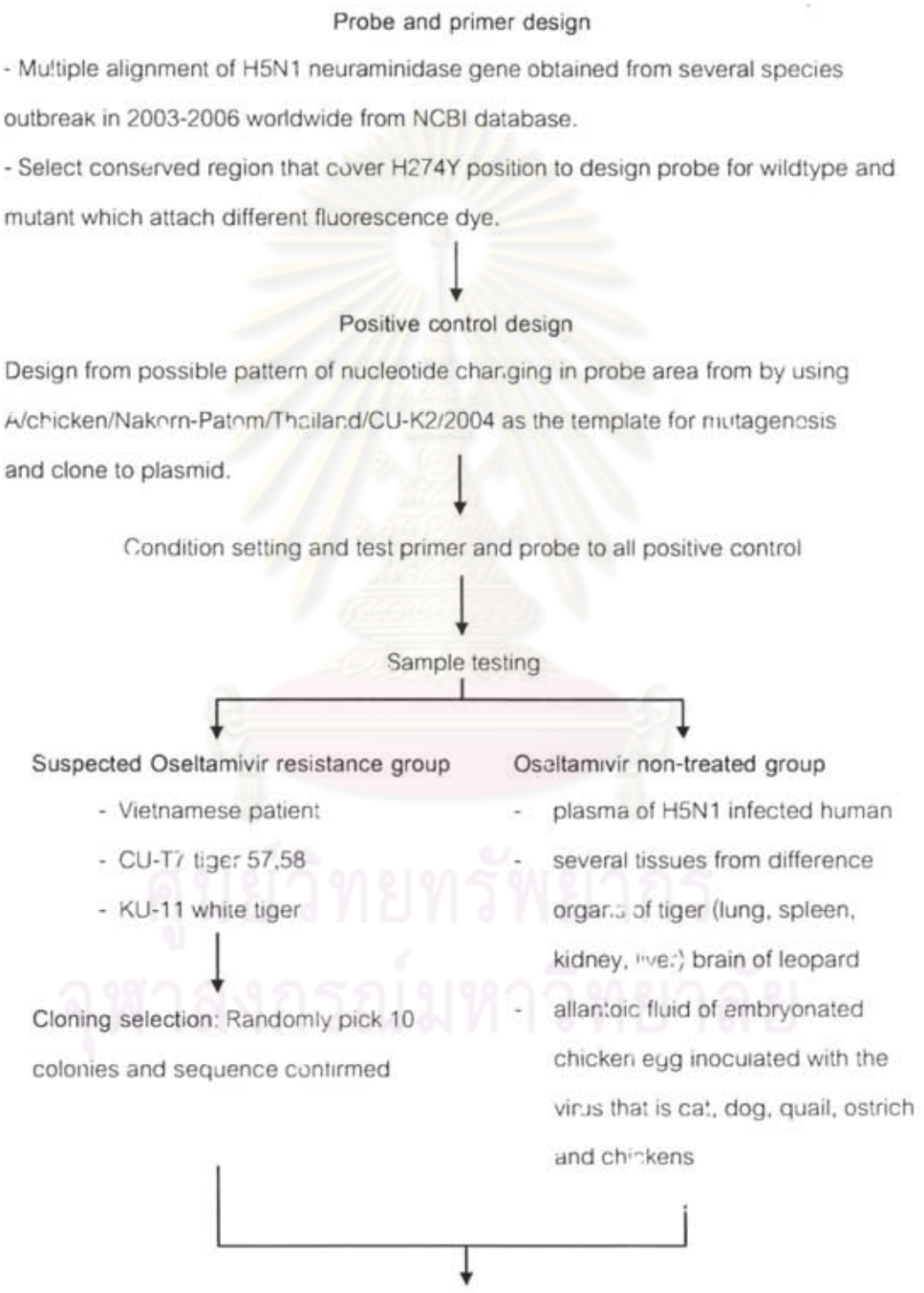
Part 4 Molecular characterization and phylogenetic analysis of H1N1 and H3N2 human influenza A viruses among infants and children in Thailan

Part 5 Genetic characterization of H1N1 H1N2 and H3N2 swine influenza virus

Part 1: H5N1 Influenza A virus and Infected Human Plasma



Part 2: H5N1 Oseltamivir-resistance detection by real-time PCR using two high sensitivity labeled TaqMan probes



Specificity test (cross reactivity study)

- Different NA subtypes (N2-N9) of influenza
- Newcastle disease virus (NDV)
- Respiratory syncytial virus (RSV) subgroups A and B
- Infectious bursal disease virus (IBDV)
- Infectious bronchitis virus (IBV)
- Human Metapneumovirus (HMPV)
- Coronavirus OC43 and 229E

↓

Sensitivity test

Mixing different ratios of wild type and mutant plasmid

and diluting over a range of concentrations

Part 3: New Strain of Influenza A Virus (H5N1), Thailand

RNA extracted from H5N1 infected chickens (n=20)

↓

HA and NA whole gene sequencing (n=20)

↓

Select the different for whole genome sequencing and data analysis

Part 4: Molecular characterization and phylogenetic analysis of H1N1 and H3N2 human influenza A viruses among infants and children in Thailand

302 NP suction specimens were collected during Feb 2006 to 2007

↓

RNA extraction and screening for influenza A virus positive

↓

HA and NA whole gene sequencing and data analysis

Part 5: Genetic characterization of H1N1 H1N2 and H3N2 swine influenza virus

RNA extracted from 12 positive swine influenza virus

↓

HA and NA whole gene sequencing and data analysis

Key Words

Influenza A virus, H1N1, H1N2, H3N2, H5N1, phylogenetic analysis, molecular characterization, Oseltamivir resistance, Real-time PCR

Expected Benefits

1. Distribute knowledge/information that infectious influenza A virus H5N1 was isolated from human blood and propose the necessity to carefully handle and transport infectious serum or plasma specimens from patients suspected H5N1 AI infection for hospital staff and care-takers.
2. Develop a new testing method for rapid and accurate detection of bird flu resistance to the anti-viral drug (oseltamivir) for clinical laboratory.
3. Identify H5N1 influenza virus strain in Thailand for evaluating the potential H5N1 influenza vaccine strain.
4. Reveal the subtype of human influenza virus isolated from infants and children in Thailand during 2006 to 2007; assess the suitable influenza vaccine strain in Thailand and genectic variation of human influenza virus.
5. Reveal the subtype of swine influenza virus in 2005 and determine the relatedness of swine influenza virus to avian and human influenza viruses.

CHAPTER II

REVIEW AND RELATED LITERATURES

Influenza is a highly infectious disease that affects the respiratory (breathing) tract. It is also known as the flu or grippe. Influenza viruses are classified in family *Orthomyxoviridae* of which there are three types A, B and C. Type A virus can infect many different kinds of animals, including humans, pigs, horses, and birds. Type B and C infect only humans. Influenza A is responsible for most cases of the disease in humans. Types B and C are less common and produce a milder form of infection. Nevertheless, the subject of this thesis was specific to influenza A virus. Naturally, influenza A viruses infect a variety of mammalian and avian species and are associated with the major human pandemics. Influenza A viruses are categorised by their two surface antigens HA, of which there are 16 types (H1–H16), and NA, of which there are 9 (N1–N9) (Fouchier et al., 2005). The natural hosts, waterfowl, shorebirds and gulls, harbour a large reservoir of influenza viruses, representative of all HA and NA types from which mammalian influenza viruses are directly or indirectly derived.

Influenza A virus Morphology and genome

The virions of influenza A virus consist of an envelope, a matrix protein, a nucleoprotein complex, a nucleocapsid, and a polymerase complex. Virus capsid is enveloped. Virions are spherical to pleomorphic; filamentous forms occur (sometimes). Virions measure 80-120 nm in diameter and 200-300(-3000) nm in length. Surface projections are densely dispersed, about 500 clustered, distinctive spikes that cover the surface evenly and comprise major glycoprotein hemagglutinin and neuraminidase that are interspersed in a ratio of HA to NA about 4-5 to 1. Surface projections are composed of different types of proteins. Surface projections are 10-14 nm long; 4-6 nm in diameter. Capsid/nucleocapsid is elongated with helical symmetry. The nucleocapsid is helical; and segments have different size classes with clear predominate lengths with a length of 50-130 nm (in different size classes). The nucleocapsids are segmented with loops at one end (ICTVdB Management, 2006).

The genome is segmented and consists of eight segments of linear negative-sense, single-stranded RNA. The complete genome is approximately 13580 nucleotides long. The genome has terminally redundant sequences and repeated at both ends. Nucleotide sequences at the 3'-terminus are identical. The 5'-terminal sequence has conserved regions and repeats complementary to the 3'-terminus (5'-AGUAGAAACAAGG..., terminal repeats at the 5'-end are 13 nucleotides long. The 3'-terminus has conserved nucleotide sequences; of 12 nucleotides in length; in viruses of same species; sequence has conserved regions (3'-UCG(U/C)UUUCGUCC..., in all RNA species. The multipartite genome is encapsidated, each segment in a separate nucleocapsid, and the nucleocapsids are surrounded by one envelope. Each virion contains defective interfering copies (may be present) (Hoffmann et al., 2001). The eight influenza A viral RNA segments encode 10 recognized gene products. These are PB1, PB2, and PA polymerases, HA, NP, NA, M1 and M2 proteins, and NS1 and NS2 proteins (Table 1) (Webster et al., 1992; Baigent and McCauley, 2003).

Emergence of influenza

The epidemiological behaviour of influenza in people is related to the two types of antigenic variation of its envelope glycoproteins - antigenic drift and antigenic shift. During antigenic drift, new strains of virus evolve by accumulation of point mutations in the surface glycoproteins. The new strains are antigenic variants but are related to those circulating during preceding epidemics. This feature enables the virus to evade immune recognition, leading to repeated outbreaks during interpandemic years. Antigenic shift occurs with the emergence of a new potentially pandemic, influenza A virus that possesses a novel hemagglutinin alone or with a novel neuraminidase. The new virus is antigenically distinct from earlier human viruses and could not have arisen from them by mutation. Three such major global pandemics caused by novel antigen variants of influenza viruses have affected the human population, the "Spanish flu" in 1918 (H1N1 subtype), the "Asian flu" in 1957 (H2N2 subtype), and the "Hong Kong flu" in 1968 (H3N2 subtype) resulting in millions of deaths (Stephenson and Zambon, 2002).

Table 1 Influenza virus genes and proteins

Seg	Protein	Localisation & features	Function
1	PB2, 96K	Virion interior, infected cell nuclei	Viral replication and transcription, in a complex of these three proteins. Replicate the viral RNA in a complex with NP. PB2 may be host specific in replication.
2	PB1, 87K	Virion interior, infected cell nuclei	
3	PA, 85.5K	Virion interior, infected cell nuclei	
4	Haemagglutinin (HA), 220K homotrimer	Virion envelope, infected cell surface Globular head bears antigenic sites & receptor binding site; stem; transmembrane span; cytoplasmic tail	Virus binding to sialic acid-containing receptors on host cell; penetration of virus genome into host cell cytoplasm by fusion of virus & host cell membranes; major antigenic determinant
5	Nucleoprotein (NP), 55K	Virion interior, associated with Polymerase complex & viral RNA	Role in controlling the replication of the viral RNA, in a complex with PB2, PB1, and PA
6	Neuraminidase (NA), 240K homotetramer	Virion envelope, infected cell surface Cytoplasmic tail; transmembrane span; extracellular stalk; globular head bears antigenic sites & enzyme active site	Sialidase enzyme catalysing cleavage of terminal sialic acid residues from glycoconjugates thereby: digesting mucin to enable virus to reach target epithelium & facilitating release of infectious progeny virus; antigenic molecule
7	Matrix protein (M1), 28K	Beneath lipid bilayer of virion envelope; associates with vRNPs in mature virion to form nucleocapsid	Controls the transport of viral ribonucleoprotein complexes into and out of the nucleus. Involved in the budding of the RNPs at the plasma membrane during virion formation
	M2, 15K homotetramer	Virion envelope, infected cell surface (abundant) Extracellular region; transmembrane span; cytoplasmic tail; tetramers form cation-selective channel	Forms ion channel in virion to allow H+ ions to enter the virion and allow M/NP to dissociate, enabling the RNP to travel to the nucleus; modulates the pH of the Golgi to allow acid-sensitive HA molecules to pass to the cell surface intact.
8	Non-structural protein 1 (NS1), 25K dimer	Infected cell nuclei	Binds and sequesters RNA, prevents activation of PKA, and prevents cellular apoptosis.
	Non-structural protein 2 (NS2), 14K	Associated with core components of virion; cytoplasm of infected cells	Involved in the nuclear export of viral RNPs.

The origins of the human influenza virus genomic segments during the emergence of the pandemic strains in 1918, 1957, and 1968, and the further reassortment of the viral segments between the H3N2 and the H2N2 viruses that occurred after the emergence of the H3N2 virus was shown in Figure 1. A much more complex, multi-factorial genetic mechanism allows for the formation of viral bridges from avian onto mammalian species at large, usually bringing about notable, and at times profound, clinical manifestations in the infected mammalian host. Those bridges readily form towards pigs; they are long established, though appreciably narrower, towards horses: they occasionally form towards seals; and they have but once formed towards minks, cats, bats, deers, squirrels and whales. In all those cases, apparently, the mammalian virus strains thus given birth to became intra-species contagious. Infectivity towards man has formed, for now, with regard to the avian-originated antigenic subtypes H1N1, H2N2, H3N2, H5N1, H7N2, H7N3, H7N7 and H9N2. H1N2 (A/Wisconsin/2001) and H3N1 (A/Memphis/1971) were also isolated from humans, independently, as well as H10N7 (A/Egypt/2004). All that can be observed, then, is that any antigenic subtype already showing infectivity towards humans, tentatively has somewhat higher chance to be the progenitor of the next pandemic virus, as compared to other subtypes (Shoham, 2006).

Molecular controls of host range and interhost transmission

For a virus to become a successful pathogen of a new host it must gain the ability to bind, enter, and infect the cell, overcome or avoid the cellular antiviral and host innate immune responses, complete its replication cycle, and spread among the appropriate tissues of the hosts. It must also spread within the host and be released or transmitted to new host animals. Most influenza viruses are able to infect a variety of hosts including embryonated eggs or cultured cells, and there are many examples of avian or porcine influenza viruses causing dead-end infection in humans without spreading efficiently to other individuals (Parrish and Kawaoka, 2005; Lipatov et al., 2004; Webster et al., 1997; Baigent and McCauley 2003). This suggests that host animal infection by those viruses is inefficient but that once infection occurrs the virus can

replicate in the new host and spread within tissues; however, barriers prevent host to host spread. In experimental infections avian viruses do not efficiently replicate in primates, and human viruses do not replicate well in ducks (Hatta et al., 2002). Those differences include cell surface receptors, intracellular environment, body temperature, and innate and adaptive antiviral immune responses. Sialic acid (SA) types and their linkages within the oligosaccharides on target cell glycoproteins or glycolipids differ between cells and tissues of humans and birds. The viruses in humans cause mainly respiratory infections and in aquatic birds the viruses primarily infect the intestinal tract. As SAs are a critical component of the receptors for host cell binding and infection, the host jumping viruses must therefore adapt from the avian to the human forms of SA, and the viruses from different hosts show specificity for the SA of the host tissues in which they replicate (Gkehei and Wiley, 2000; Suzuki et al., 2000). That adaptation involves the complementary activities of the HA and NA. Influenza viruses recognize two species of SA (N-acetylneuraminic acid, NeuAc, and N-glycolylneuraminic acid, NeuGc) which are attached to galactose in SAα-2,3Gal or SAα-2,6Gal linkages. The host animal might exert selective pressures on receptor specificity of the virus, since the abundance of receptor types on cells at the sites of virus replication varies. Human tracheal epithelium has predominantly NeuAcα-2,6Gal; equine tissues possess both NeuAc and NeuGc, the major SA-Gal moiety in horse trachea being NeuGcα-2,3Gal; duck intestine contains mainly NeuAcα-2,3Gal (also NeuGcα-2,3Gal). This may explain why horses are susceptible to direct transmission of avian viruses though apparently not to human viruses. Swine tissues possess both NeuAc and NeuGc, the trachea having both SAα-2,3Gal and SAα-2,6Gal. This confers susceptibility to avian and human viruses, both experimentally and in the field (Ito et al., 1998). Changes in the HA allow the binding to sialyoligosaccharides possessing NeuAc linked to galactose by α2,6 linkages (NeuAcα2,6Gal) found in humans rather than by the NeuAcα2,3Gal linkage found in birds (Ito and Kawaoka, 2000). The receptor-binding site lies in a depression near the tip of the HA molecule, and changes in that site control the specificity of binding. The binding to the NeuAcα2,6Gal linkage found in human SAs is determined by combinations of residues Leu-226 is associated with Ser-228 and in equine and avian

viruses Gln226 is associated with Gly228 allowing the proper orientation of the SA in the receptor-binding site in the H2 and H3 subtypes but not in H1 (Vines et al., 1998). In H1 viruses of swine and humans, mutation of Glu190 to Asp and Gly225 to Glu is associated with acquisition of SAα-2,6Gal specificity during adaptation of avian viruses to these hosts (Matrosovich et al., 2000). In order to establish why H5N1 avian influenza virus is so lethal to humans and why the virus is not easily expelled by coughing and sneezing, scientists employed marker molecules, lectins specific for SAα-2,3Gal and SAα-2,6 Gal to indicate the cell type in human respiratory tissues. They found that SAα-2,6 Gal is dominant on epithelial cells in the nasal mucosa, with SAα-2,3 Gal being occasionally detected on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and the alveolus at the human lower respiratory tract (Shinya et al., 2006). At this site, H5N1 virus attached predominantly to type II pneumocytes and alveolar macrophages, which may contribute to the severity of the pulmonary lesion since type II pneumocytes are metabolically active and are the most numerous cell type lining the alveoli which can impair their functions, including re-epithelialization after alveolar damage, ion transport, and surfactant production, and thus may inhibit tissue repair.

Among experimental animals, the lower respiratory tract of cats and ferrets resembles human tissues more closely than that of mice and macaques. Due to the similarity in viral attachment, these two species are the most suitable animal models for H5N1 viral pneumonia in humans (van Riel et al., 2006). Influenza species barriers illustrate the interaction of factors that limit transmission and subsequent establishment of an infection in a novel host species.

For a virulent virus, the receptor-binding properties of HA should be functionally compatible with the cleavage specificity of NA and the stalk length of NA since release of virus from the cell surface requires cleavage of the receptor by NA. Incompatibility between HA and NA can restrict the virulence of reassortant viruses, while some combinations of HA and NA are associated with infection of certain host species. NAs can cleave SAα-2,3 and/or SAα-2,6 linkages. NA cleavage specificity should also match the predominant linkage type in the host species. During the evolution of N2

avian influenza viruses in man, α-2,3 cleavage activity of NA has been maintained and α-2,6 activity increased, possibly conferring a selective advantage to human viruses where the HA has α-2,6 receptor specificity (Baum and Paulson, 1991). Evolution of these viruses also correlates with decreased enzymatic activity of NA due to amino acid substitutions in and near the active site. Since affinity of human virus HAs for their respective receptors is less than that of avian virus HAs, it is likely that reduction in specific activity of NA is required to maintain an optimal balance between HA and NA activity during evolution of these viruses in man (Kobasa et al., 2001). The NA stalk, which holds the active site above the virion envelope, varies in sequence and length. A short-stalked NA is inefficient in disaggregating progeny virus because the active site cannot access its substrate efficiently. A shortened NA stalk reduces ability of virus to elute from erythrocytes, can decrease virus growth in MDCK cells and eggs and can decrease virulence in mice. However, naturally occurring avian viruses having short NA stalks are fully virulent in poultry, showing a long stalk is not essential for virulence in chickens (Castrucci and Kawaoka, 1993).

Zoonotic potential and role of pigs in the influenza viruses' transmission

Influenza as a disease of pigs was first recognized during the Spanish influenza pandemic of 1918–1919 in the United States of America. Influenza virus was first isolated from pigs in 1930 with the virus isolated from humans several years later (Myers et al., 2007). The first isolation of a swine influenza virus from a human occurred in 1974 (Ito et al., 1998), confirming speculation that swine origin influenza viruses could infect humans. Pigs are thought to have an important role in interspecies transmission of influenza, because they have receptors to both avian and human influenza virus strains. Consequently, they have been considered a possible "mixing vessel" in which genetic material can be exchanged, with the potential to result in novel progeny viruses to which humans are immunologically naïve and highly susceptible (Webster et al., 1992).

Influenza viruses of three different subtypes, H1N1, H3N2 and H1N2, are circulating in swine worldwide. Unlike for human influenza viruses, the origin and nature of swine influenza viruses (SIV) differ on different continents. The predominant H1N1 SIV

in Europe is entirely of avian origin and they were introduced from wild ducks into the pig population in 1979. Two types of H1N1 SIV are circulating in the USA: the so-called "classical" H1N1 viruses that have been present since the early 20th century and novel reassortants with the surface glycoproteins of the classical virus and internal proteins of more recently emerged H3N2 or H1N2 SIV.

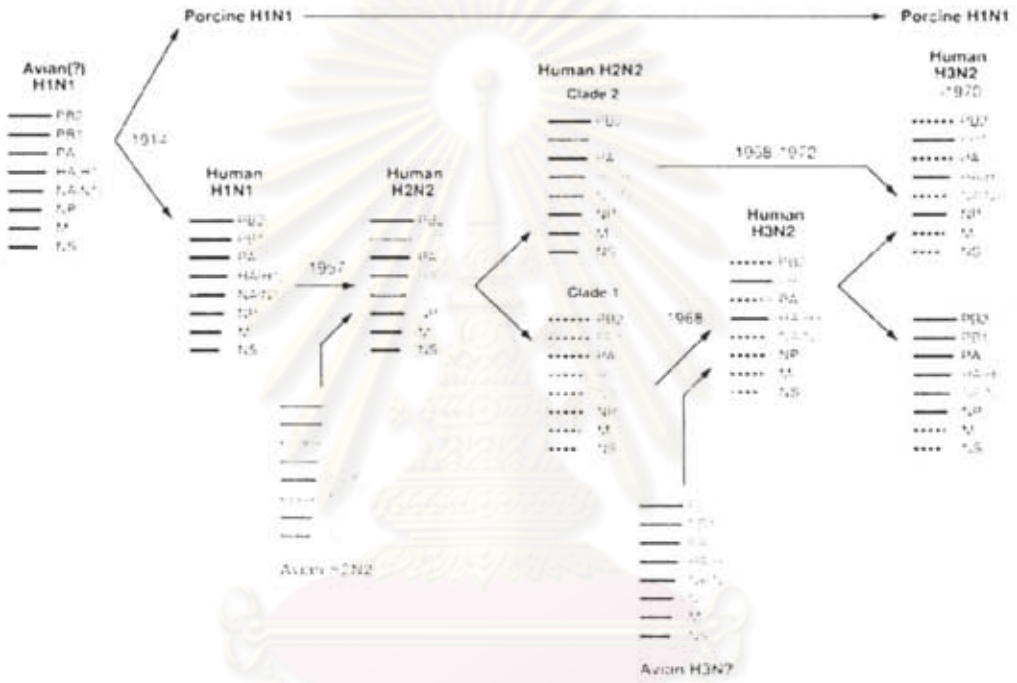


Figure 1 The sources of genomic segments acquired from different viruses are shown in different colors. The H1N1-derived gene segments are black, those from avian-like viruses encoding the HA, NA, or PB1 proteins during the derivation of the H2N2 virus strain in 1957 are red, and those acquired from a second avian ancestor during the emergence of the H3N2 virus are blue. The H2N2 viruses had separated into two clades before 1968 (solid and hatched lines), and the derivation of the original H3N2 from clade 1 parents is shown, along with the secondary reassortment of that H3N2 virus with clade 2 human H2N2 viruses to give the later strains of the H3N2 virus. The origin and continued circulation of the porcine H1N1 virus, which also emerged around 1918 from a source closely related to the human H1N1 virus, is also shown (Parrish and Kawaoka, 2005).

Viruses of both other subtypes also have a different origin in Europe and in the USA and were introduced in the swine population at different times (van Reeth, 2007). (Table 2).

Table 2 Influenza A virus subtypes infecting pigs endemically in Europe and North America and their antigenic/genetic characteristics (Van Reeth, 2007).

Continent	Subtype	Year of introduction	Antigenic/genetic characteristics
Europe	H1N1	1979	Wholly avian virus
	H3N2	1984[a]	Reassortant
			human H3N2 (Hong Kong /68-like HA and NA) X swine H1N1
	H1N2	1994	Reassortant
			human H1N1 (England/8(like HA) X swine H3N2 (NA) X swine H1N1
North America	H1N1	1918	"Classical" SIV
	H1N1	1998	Reassortant
			classical SIV (HA, NA) X swine H3N2 OR swine H1N2
	H3N2	1998	Reassortant
			human H3N2 (HA, NA) X classical SIV
	H3N2	1998	"Triple" reassortant
			human H3N2 (HA, NA) X classical SIV X avian
	H1N2	1998	Reassortant
			classical SIV (HA) X triple reassortant H3N2 (NA

Progenitor virus lineages of the reassortant SIV are shown in Italics.

[a]A wholly human Hong Kong/68-like influenza virus was already transmitted to pigs in the early 1970s, but genetic reassortment with the "avian-like" H1N1 SIV occurred in the mid 1980s.

SIV have been occasionally isolated from the respiratory secretions or lungs of humans in Europe, Asia and the USA. Most SIV infections in people are not clinically distinguishable from human influenza virus infections, but fatal cases have been seen in humans infected with classical H1N1 SIV. During the so-called "New Jersey" incident in the USA in 1976, an approximate 500 humans became infected with an H1N1 virus identical to viruses isolated from pigs (van Reeth, 2007).

For a long time, it was thought that transmission of avian influenza viruses to humans does not occur directly but via the pig as an intermediate host. Pigs are clearly susceptible to infection with both LPAI and HPAI viruses. Though most of these viruses

have an H1 or H3 HA, HA subtypes that are usually restricted to birds can also cross the species barrier to pigs. Serological investigations in Asia, for example, have shown evidence for infections of pigs with avian H4, H5 and H9 viruses (Ninomiya et al., 2002).

Importantly, however, only the H1N1 virus that crossed from wild birds to swine in Europe in 1979 has become established in pigs, whereas the other viruses have disappeared. In an experimental study, most LPAI viruses are able to infect pigs after experimental intranasal inoculation of a high virus dose, but there are strong indications that avian influenza viruses replicate much less efficiently than the typical SIV. Therefore, that the circulation of entirely AI viruses in pigs in nature is a relatively rare event. Still, there is circumstantial evidence that the genes of avian viruses may persist after reassortment with one or more influenza viruses endemic in pigs. As an example, H3N2 and H1N2 influenza viruses carrying mixtures of avian, swine and human influenza virus genes have become enzootic in Europe and North America.

There are many factors that limit the transmission of influenza viruses from one species to another. A first possible barrier is a lack of suitable receptors on the host cell, so that the HA of a virus from another species cannot attach (Kuiken et al., 2006). Even if an influenza virus succeeds to enter the cell of a new host, it must successfully co-opt host cell processes to replicate there. The polymerases of the virus, which are responsible for the replication and transcription of viral RNA, play a key role at this stage (Salomon et al., 2006; Gabriel et al., 2005). Finally, the influenza virus must escape from the cell it has infected. During this step, the viral HA tends to re-bind to receptors on the cell surface and the NA helps to break this binding. Like the HA, the NA also has a preference for one of both types of sialic acid linkages and thus for humans or birds. It is reassuring that a large number of viral mutations, or genetic reassortments, are obviously needed for this adaptation and that most of such genetic changes will be deleterious for the virus. The exact nature of these changes remains unknown and the genetic basis of influenza virus transmissibility appears to be highly complex (van Reeth, 2007).

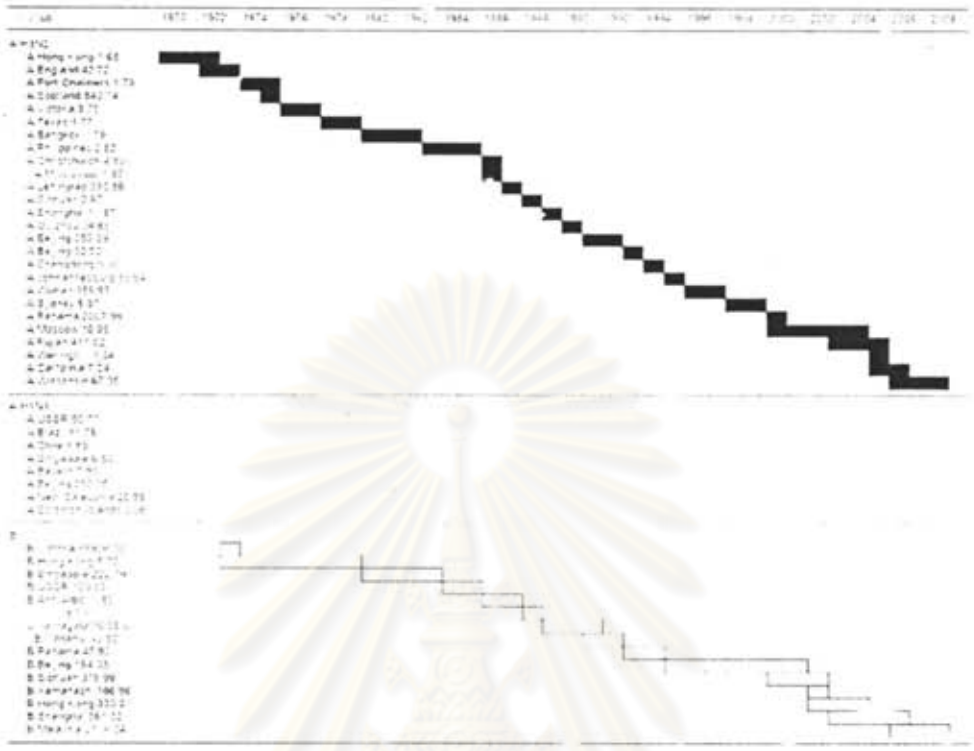


Figure 2 Changes in the influenza vaccine compositions recommended by the WHO, 1973-2008. The viruses listed are the prototypes recommended for inclusion in the bivalent or trivalent (1977 onwards) vaccine (Adapted from Hay et al., 2001).

Vaccination and influenza virus in Thailand

The outstanding feature of influenza viruses is their capacity for evading host immunity and causing recurrent annual epidemics of disease and major worldwide pandemics due to the introduction of antigenically novel viruses into an immunologically naïve human population. After the isolation of the first human influenza virus in 1993, WHO has established a global Influenza Surveillance Network for monitoring changes in the viruses causing outbreaks of influenza throughout the year in different parts of the world and published formal recommendations for the compositions of influenza vaccines based on cumulative data since 1973. The epidemiology of human influenza reflects the particular characteristics of the virus genome (segmented single-stranded RNA) and the diversity and host range of the viruses. Two types, namely influenza A and influenza B

viruses are responsible for recurrent annual epidemics. They are antigenically distinct and do not exhibit cross-immunity, nor do they undergo intertypic genetic reassortment (recombination). Although influenza B viruses have been responsible for severe epidemics, the impact of influenza A viruses is greater in terms of annual epidemics as well as the infrequent more devastating pandemics. The latter characteristics and the evolution of influenza A viruses is a consequence of their greater genetic diversity and, in particular, their unique host range. Whereas influenza B viruses almost exclusively infect humans, influenza A viruses are essentially avian viruses that periodically transmit to other species, including mammals. Furthermore, influenza A viruses comprise a large variety of antigenically distinct subtypes, with different combinations of 16HA and 9NA subtypes, that replicate asymptomatically in the intestine of aquatic birds, particularly ducks and constitute a large reservoir of potential pandemic viruses. The extent of variation in the antigenic properties of the viruses is reflected in the number of changes recommended in vaccine composition (Figure 2). The greater antigenic variability of AH3N2 viruses has required 26 changes in the vaccine component over 38 years. In contrast, the 14 changes to the influenza B component and eight changes to the AH1N1 component made during this period reflect the slower rates of antigenic change of these viruses (Webster et al., 1992; Hay et al. 2001)

The requirements for annual vaccination and the high cost of the influenza vaccine when compared to other vaccines recommended by WHO are likely to be significant obstacles to vaccine introduction in less wealthy countries. In Thailand, influenza vaccine is not widely used and is mostly limited to persons who can afford to pay private health care providers for the immunization. Three companies currently distribute inactivated influenza vaccine in Thailand at an average retail cost of approximately 300 Thai baht (US$ 7) per dose. The virological surveillance is conducted at the Thailand National Institute of Health (NIH) by conducting laboratory surveillance, subtyping viruses responsible for disease outbreaks, and contributing strain surveillance data which identified influenza virus throughout the year in Thailand with a peak in the proportion of positive isolates typically occurring between June and October (Figure 3A). Sharp peaks in reported influenza cases are observed during the months of June

through September with smaller increases in reported cases sometimes being seen in January and February (Figure 3B). The age distribution of reported cases of influenza infection in Thailand during 1999–2002 indicates that influenza may be more likely to be diagnosed in older age groups (Figure 3C) (Simmerman et al., 2004).

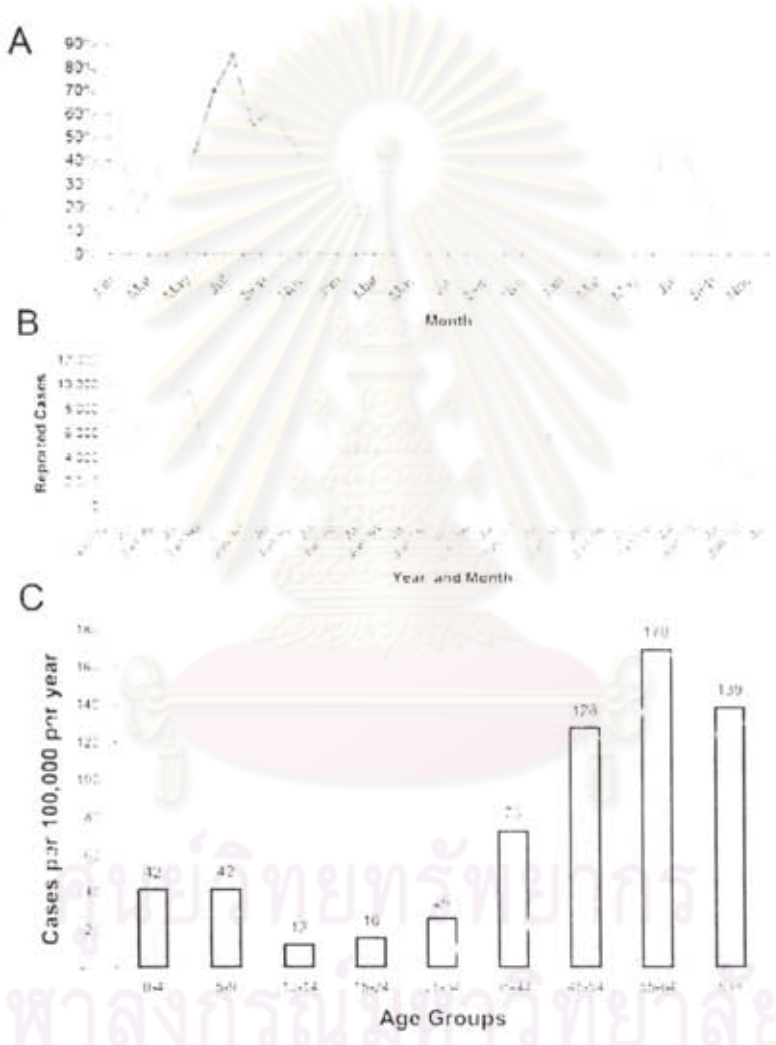


Figure 3 The virological surveillance at the Thailand National Institute of Health (NIH) (A) Proportion of specimens positive for influenza virus by month in Thailand: January 2001–September 2003. Source: Thailand National Institute of Health. (B) Seasonal distribution of reported influenza 1988–2002. Source: Bureau of Epidemiology, Thailand Ministry of Public Health. (C) Age Distribution of reported influenza in Thailand 1999–2002. Source: Bureau of Epidemiology, Thailand Ministry of Public Health (Simmerman et al., 2004).

The beginning of H5N1 in Thailand

When an epidemic of disease happened, the first important thing is the coordination of high potential organization to study the etiology of that disease for prompt treatment, effective management and successfully defend for recurrent of disease. The avian influenza A subtype H5N1 widespread in Thailand was the imperative samples to draw attention to research collaboration. The avian influenza A subtype H5N1 has been reported to have emerged in Hong Kong in 1997. Since 2003 to early 2004, the virus has reached endemic levels among poultry in several south-east Asian countries and during 2005 in Europe and Africa with H5N1 virus infected birds in more than 50 countries. The 2004-2007 outbreaks in various countries and highlights 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 has also proven highly pathogenic and fatal to mammalian species including humans and other mammals. By July, 2007 many countries had been affected by the spread of influenza H5N1 virus infections in poultry and the mortality rate of highly pathogenic H5N1 avian flu in a human is high nearly 60% of cases classified as H5N1 resulted in death.

The 2004-2007 outbreaks of highly pathogenic avian influenza (HPAI) subtype H5N1 virus in Thailand can cause 5 periods of the outbreaks which 17 from 25 infected human deaths. In the first period during January 2004 to May 2004, the genome sequence analysis of H5N1 avian Influenza A Virus isolated from the outbreak among poultry, wild and domestic bird populations, a domestic cat infected by eating a pigeon carcass, a tiger and leopard from Suphanburi province in Thailand was promptly reported (Songserm et al., 2006a; Songserm et al., 2006b; Keawcharoen et al., 2004). After the molecular characterization, HA gene revealed a common characteristic of a highly pathogenic AI (HPAI), a 20-codon deletion in the neuraminidase gene, a 5-codon deletion in the NS gene and polymorphisms of the M2, amantadine resistance, and a single amino acid substitution at the position 627 in the polymerase basic protein 2 (PB2). Moreover, the HA and NA genes of the Thai avian influenza virus displayed high similarity to those of the AI viruses isolated from human cases during the same epidemic

(Viseshakul et al., 2004; Amonsin et al., 2006a). Then, the rapid single-step multiplex RT-PCR based on conventional PCR and real-time PCR for influenza A virus subtype H5N1 detection was developed for screening the massive samples (Payungporn et al., 2004; Payungporn et al., 2006a). Based on virulence, the H5 influenza virus subtype can be further differentiated into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Payungporn et al., 2006b). The HPAI virus causes systemic lethal infection, which can kill numerous birds rapidly, whereas it is uncommon for the LPAI virus to generate outbreaks of severe disease. Hence, morbidity and mortality rates of LPAI virus infections are lower than those of HPAI viruses. Due to different post-translational proteolytic cleavage of the HA precursor molecule (HA0) into HA1 and HA2 subunits, LPAI do not contain a series of basic amino acid at the protease cleavage site but are cleaved by proteases localized in respiratory and intestinal organs, resulting in mild localized infections. In contrast, the HA of HPAI virus harbors multiple basic amino acids at the cleavage site, for example RERRRKKR, which are cleaved by ubiquitous proteases in a wide range of organs, resulting in lethal systemic infection. The way to discriminate HPAI and LPAI is sequencing, however, using of one-step real-time RT-PCR with melting curve analysis was attractive for large-scale screening of suspected subtype H5 influenza A virus during outbreaks to identify candidate LPAI that could be used as vaccine strains.

The second period of the outbreaks occurred during July 2004 to April 2005 which was the most widely spreading period of avian influenza H5N1 virus in poultry of Thailand. The vast devastated by H5N1 influenza virus happened on October 2004 in tiger zoo in Sriracha, Chonburi, Thailand which cause a total of 147 from 441 tigers died or were euthanized. 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. The researcher also found that after stop feeding the tigers with raw chicken carcasses in ≈12 days, and no other avian or mammal species kept in the zoo but tigers had been infected during this outbreak. It can be pointed toward tigers were probably infected by

horizontal transmission since the animals had not been fed raw chicken carcasses (Amonsin et al., 2006a; Thanawongnuwech et al., 2005). Administration of oseltamivir therapy could suppress and prolong the incubation period of the H5N1 virus infection, but it is unlikely. Not only avian influenza H5N1 virus is known to cross the species barrier and infect humans and felines, in this period, fatal H5N1 infection in a dog following ingestion of an H5N1-infected duck was reported from Suphanburi province. H5N1 influenza virus can be isolated from lung, liver, kidney, and urine of dog's specimens and this study is the first report of H5N1-related systemic disease in a domestic dog (Songserm et al., 2006b). The studies in felines and canine 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 need for monitoring domestic animals during H5N1 outbreaks

The third period of the outbreaks carried on July 2005 to November 2005. The H5N1 viruses in this period were characterized from one human case and three poultry cases. A plasma sample from an H5N1 infected patient which stored at –20°C for 12 days and then stored at –70°C was processed for virus isolation by embryonated egg injection within 48 hours, and the allantoic fluid was shown to contain 2,048 hemagglutinin (HA) units. Whole genome sequencing was performed and compared with chickens and quail from this period outbreak. The 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) in January and July 2004. Furthermore, the genetic changes of the AI isolates at the HA cleavage site have been observed (the details in this thesis) (Chutinimitkul et al., 2006). The broadly use Tamiflu (Oseltamivir) for treat H5N1 infected patients cause the prevalence of oseltamivir-resistant H5N1 viruses among patients treated with this drug is being stockpiled in many countries potentially affected by the influenza A virus subtype H5N1 epidemic. For identifying this change in Oseltamivir-treated patients, a method based on real-time PCR using two labeled TaqMan probes or conventional PCR technique was

developed for detection the substitution of amino acid H274Y in Neuraminidase gene of H5N1 influenza A virus in many species and various sources of specimens with high sensitivity and specificity (the details in this thesis) (Chutinimitkul et al., 2007b).

The fourth wave began on July 23, 2006 and spreding shortly until July 29, 2006. These outbreaks involved chickens and encompassed 2 distinct areas: Phichit and Nakhon Phanom Province. All 8 gene segments of both provinces were sequenced. Whole genome analysis showed that all 3 samples had undergone minor mutations that are typical of circulating influenza A viruses. All at once, this outbreak was associated with 2 strains of the virus. The samples from Phichit closely resembled H5N1 strains that had circulated in Thailand during 2004 and 2005, although samples from Nakhon Phanom was newly observed in Thailand and more closely related to H5N1 strains that had been circulating since 2005 in southeast People's Republic of China and Lao's PDR. The whole genome phylogenetic analysis also showed that the viruses isolated from Phichit belonged to genotype Z, whereas virus isolated from Nakhon Phanom belonged to genotype V. According to previous World Health Organization reports, the HA sequences of most influenza (H5N1) viruses that circulated in avian species during the past 3 years are separated into 2 distinct phylogenetic clades. In Thailand, from 4 periods of the outbreaks, H5N1 influenza virus can be separated into Clade 1 and Clade 2 subclade 3 (the details in this thesis) (Chutinimitkul et al., 2007a).

The last outbreaks occurred during January 2007 to March 2007 which effected to 4 provinces; Ang Thong, Phitsanulok, Nong Khai and Mukdahan which can be divided viruses in to 2 genotype genotype V and Z, Clade 1 and Clade 2 subclade 3, as in the fourth outbreaks.

The H5N1 influenza virus epidemics enlighten us the important lesson. There are some evidences which show that the virus was probably transmitted from humans to humans in Thailand, Vietnam and also Indonesia as well as evidence of tiger to tiger transmission. Symptomatic of H5N1 avian influenza infection in human and mammals had high mortality rate. The virus has high invasive properties, not only pulmonary tract involvement, but also pathogenicity occurring in the extra respiratory system such as CNS (encephalitis), kidney (renal failure). H5N1 avian influenza virus could morph into a

pandemic in two ways: the mutation of the virus in order to transmit from human to human or gene exchange with common human influenza strain. The pandemic will occur at what time the adapted virus suitable for the most of people who have no immune protection. We believe that the outbreak of H5N1 is unlikely to be the last in the near future. We have to prepare and take preventive measures for a pandemic by extensive influenza surveillance, vaccine development and production, antiviral therapy and influenza related research.

The treatment of influenza with antiviral drugs

Influenza is responsible for more morbidity and mortality each year than all other respiratory diseases combined, and it results in tremendous economic costs both from admissions to hospital and loss of productivity. Preventive strategies are the key to reducing the impact of influenza on our communities, and effective vaccination strategies have been in place for half a century and continue to be improved upon. However, for the individual physician faced with a severely ill patient during an influenza epidemic, effective treatment is required. This need has led to the development of antiviral agents that halt or impede the ability of the virus to infect respiratory epithelial cells. Two main classes of drug interfere with influenza virus infection; the first drug to be developed was amantadine, which belongs to the ion channel blocker group of anti-influenza drugs. It has been used to treat and prevent influenza A since the mid-to-late 1960s. In the United States, rimantadine, an agent with fewer side effects than amantadine is more commonly used. The second class of agents consists of the viral neuraminidase inhibitors (NAIs), zanamivir (Relenza) and oseltamivir (Tamiflu). There are 2 major proteins on the surface of the influenza virus, the hemagglutinin (HA) and the neuraminidase (NA). The hemagglutinin mediates attachment of the virus particles to the respiratory epithelial cells via specific receptors. Once the virus has bound to its host cell, it is transported into the cytoplasm in an endosome. The acid pH in the endosome activates or opens an ion channel called the M2 protein, permitting hydrogen ions to enter the virion. The resulting acidification of the virus is necessary for viral uncoating, another essential step in viral replication (Figure 4) (Stiver, 2003).

Amantadine, the M2 inhibitors act by blocking the influx of hydrogen ions through the M2-proton channel of influenza A and inhibiting the uncoating and release of free viral ribonucleoproteins into the cell cytoplasm. In vitro and animal models have demonstrated a benefit from M2 and NAI therapy in combination. Amantadine is only effective against influenza A, and not influenza B, because influenza B does not have an M2 protein, but a substitute protein called NB that is not affected by amantadine (Hayden, 2006). There are several concerns with the use of M2 inhibitors (amantadine and rimantadine), for seasonal influenza. These concerns include central nervous system (dizziness, nervousness, and insomnia) and gastrointestinal toxicities, as well as antiviral resistance (Peters et al., 2001). However, amantadine might be considered as part of combination antiviral therapy by clinicians treating severely ill patients in a pandemic. Current WHO treatment guidelines for pandemic influenza recommend the use of an M2 inhibitor along with an NAI only if administered in the context of prospective data collection and if local surveillance data show that the H5N1 variant of concern is known or likely to be susceptible to this drug (WHO, 2006b). The dosing schedules for amantadine and rimantadine are shown in Table 3.

Neuraminidase is a viral enzyme that cleaves the neuraminic acid component of sialic acid in the respiratory epithelial cell hemagglutinin receptors. After replication, in order to exit the cell and infect other cells, influenza virus particles bud off the host cell membrane. The viral neuraminidase is required to release the budding virus particles by digesting the hemagglutinin receptors holding the viruses to the cell. The virus particles thus released still have hemagglutinin receptors from the cell membrane coating them, and the hemagglutinins of other newly released viruses bind to these causing clumping. The neuraminidase cleaves these residues, allowing the viruses to disperse, enhancing their ability to infect other cells. The third function of the neuraminidase is to digest neuraminic acid in respiratory mucus, perhaps facilitating viral spread. The NAI drugs, zanamivir and oseltamivir, bind to the active site on the viral neuraminidase, blocking its activity. Thus, virus particles cannot exit the cells as easily, and they tend to clump and not disperse. This impedes their ability to infect more cells and attenuates the patient's infection. However, there is still subclinical or mild infection that actively immunizes the

patient against that strain. The NAIs are active against both influenza A and B (Stiver, 2003). Oseltamivir is an oral preparation (either a capsule or liquid suspension) and after absorption is widely distributed throughout the body. Zanamivir is delivered by inhalation or, rarely, intravenous infusion. When administered through a diskhaler, zanamivir is concentrated in the respiratory tract and is effective within 10 seconds. Because the replication of influenza virus is at a peak from 24 to 72 hours after illness onset, the NAIs must be administered as early as possible after symptoms appear. The dosing schedules for oseltamivir and zanamivir are shown in Table 3.

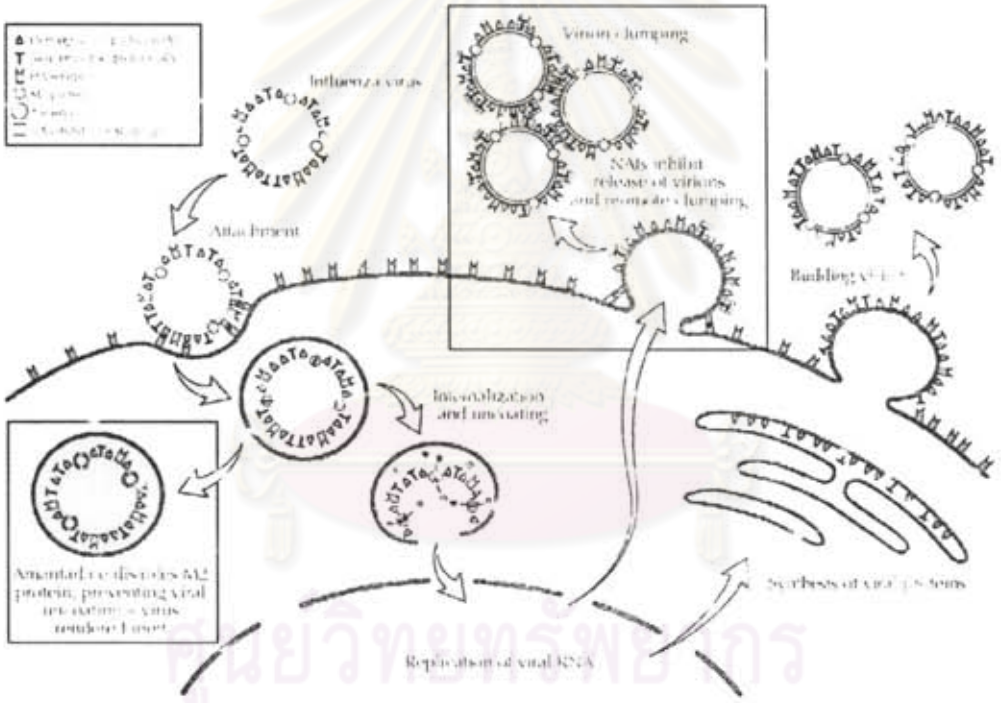


Figure 4 Schematic representations of influenza virus attachment, internalization, replication and exit from the host respiratory cell and steps inhibited by antiviral drugs. Amantadine blocks viral internalization and uncoating. Neuraminidase inhibitors prevent the neuraminadase from releasing budding viruses and dispersing virions. Photo: Myra Rudakewich (Stiver, 2003).

Table 3 Dosing schedule of antivirals for the treatment and prevention of influenza, according to patient's age and coexisting illness (Harrod et al., 2006; Montalto et al., 2000).

Drug	Recommended dose according to age			
	1–6 years	7–12 years	13–64 years	≥ 65 years
Treatment				
Zanamivir	NA	10 mg (2 inhalations) twice daily for 5 days for children > 5 years	10 mg (2 inhalations) twice daily for 5 days	10 mg (2 inhalations) twice daily for 5 days
Oseltamivir	2 mg/kg up to 75 mg twice daily for 5 days	2 mg/kg up to 75 mg twice daily for 5 days	75 mg twice daily for 5 days	75 mg twice daily for 5 days
Amantadine	5 mg per kg per day, up to 150 mg given in two divided doses	100 mg twice daily	100 mg twice daily	<100 mg twice daily
Rimantadine	NA	NA	100 mg twice daily	100 or 200 mg per day
Prophylaxis				
Zanamivir	NA	NA	10 mg (2 inhalations) once daily for 10–28 days	NA
Oseltamivir	NA	NA	75 mg once daily for > 7 days (up to 6 weeks)	75 mg once daily for > 7 days (up to 6 weeks)
Amantadine	NA	100 mg once daily for children aged 5–9 years	100 mg twice daily for people > 9 years	100 mg once daily
Rimantadine	5 mg per kg per day, up to 150 mg given in twodivided doses	100 mg twice daily	100 mg twice daily	100 mg or 200 mg twice daily

NA = not applicable

Emergence of antiviral resistant strains

Up to approximately one-third of patients may shed resistant viruses when amantadine or rimantadine is used for therapy. *In virtro* sensitive viruses became resistant after three or five passages in the presence of 2 µg/ml amantadine. Naturally occurring influenza A viruses can be viewed as mixtures of sensitive and resistant strains with a ratio of 10000: 1, the latter would be selected within 2–3 days of starting amantadine therapy (Hayden et al., 1991). Persons who have influenza A infection and who are treated with amantadine can shed sensitive viruses early in the course of treatment, and later shed drug-resistant viruses, especially after 5–7 days of therapy. Such persons can benefit from therapy even when resistant viruses emerge. However, amantadine-resistant viruses are not more virulent or transmissible than -sensitive viruses. The molecular changes associated with resistance have been identified as single-nucleotide changes leading to corresponding amino-acid substitutions of one of four critical sites, amino acids 26, 27, 30, and 31, in the transmembrane region of the M2 protein (Holsinger et al., 1994, Suzuki et al., 2003).

Zanamivir and oseltamivir, the currently marketed influenza virus neuraminidase inhibitors (NAIs), are prescribed for the treatment and prophylaxis of influenza and are being stockpiled for pandemic influenza. Oseltamivir resistance has been reported in up to 2% of patients in clinical trials of oseltamivir and in up to 18% of treated children. There are also reports in at least three patients treated with oseltamivir for influenza A (H5N1) infections. At this stage, there are no reports of resistance occurring to zanamivir in immunocompetent patients. Zanamivir and oseltamivir bind differently at the neuraminidase catalytic site and this contributes to different drug resistance profiles (Reece, 2007). Oseltamivir resistance owing to neuraminidase mutations have been rising both in challenge studies and in patients with naturally acquired infections. The rates of resistance are estimated to be around 1% in the adult population and 5% in pediatric patients (Whitley et al., 2001; Jackson et al., 2000). In 2004, influenza A viruses (H3N2) were collected from 50 children before and during treatment with Oseltamivir. Eighteen percent of the children (N=9/50) had neuraminidase mutations at Arg292Lys (N=6/9) or Glu119Val (N=2/9) or Asn294Ser (N=1/9) (Kiso et al., 2004). Data from

volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) virus and treated with Oseltamivir show a substitution H274Y in the neuraminidase active site (Gubareva et al., 2001). This mutation following Oseltamivir phosphate treatment leave virus severely compromised both *in vitro* and *in vivo* (Ives et al., 2002) and confers about 400- to 600- fold resistance (Wetherall et al., 2003). The mutation at position 274 can influence the sensitivities of influenza N1 NA but not of N2 NA to Oseltamivir carboxylate by rearrangement the shape of active site to create a pocket for Oseltamivir (Moscona, 2005b; Gubareva, 2004; Wang et al., 2002).

CHAPTER III

H5N1 INFLUENZA A VIRUS AND INFECTED HUMAN PLASMA

(Published in Emerg Infect Dis. 2006. 12: 1041-1043)

Since January 2004, a total of 22 persons have been confirmed infected with avian influenza A virus (H5N1) in Thailand; 14 of these patients died. Three waves of outbreaks occurred during the past 2 years. The last patient of the third wave was a 5-year-old boy whose symptoms developed on November 28, 2005; he was hospitalized on December 5 and died 2 days later. The child resided in the Ongkharak District, Nakhon Nayok Province, 70 km northeast of Bangkok. Villagers informed the Department of Livestock after the patient's illness was diagnosed. Five dead chickens had been reported in this area from November 28 to December 1, 2005. Samples from these chickens could not be obtained, thus, no H5N1 testing was performed. The boy had fever, headache, and productive cough for 7 days before he was admitted to the Her Royal Highness Princess Maha Chakri Sirindhorn Medical Center. Clinical examination and chest radiograph showed evidence of lobar pneumonia. He was treated with antimicrobial drugs (midecamycin and penicillin G) and supportive care, including oxygen therapy. On December 7, the patient's condition worsened, and severe pneumonia with adult respiratory distress syndrome developed. Laboratory tests showed leukopenia (2,300 cells/mm^3), acidosis, and low blood oxygen saturation by cutaneous pulse oximetry (81.6%). Oseltamivir was administered after his parents informed hospital staff about the boy's contact with the dead chicken. However, the boy died the same day; no autopsy was performed. On December 9, the cause of death was declared by the Ministry of Public Health to be H5N1 influenza virus.

A blood sample was collected from the patient on December 7; anticoagulation was accomplished with ethylenediaminetetraacetic acid (EDTA) for repeated biochemistry analysis and complete blood count. The plasma from the EDTA blood sample was separated 2 days later and stored at –20°C for 12 days. The sample was subsequently given to the Center of Excellence in Viral Hepatitis, Faculty of Medicine, Chulalongkorn University, for molecular diagnosis and then stored at –70°C, where

specific precautions implemented for handling highly infectious disease specimens such as H5N1 influenza virus were observed. Plasma was examined by multiplex reverse transcription–polymerase chain reaction (RT-PCR) (Payungporn et al., 2004) and multiplex realtime RT-PCR (Payungporn et al., 2005), both of which showed positive results for H5N1 virus. The virus titer obtained from the plasma was 3.08×10^3 copies/mL. The plasma specimen was processed for virus isolation by embryonated egg injection, according to the standard protocol as described previously (Harmon, 1999). Briefly, 100 µL 1:2 diluted plasma was injected into the allantoic cavity of a 9-day-old embryonated egg and incubated at 37°C. The infected embryo died within 48 hours, and the allantoic fluid was shown to contain 2,048 hemagglutinin (HA) units; also, subtype H5N1 was confirmed (Payungporn et al., 2004; Payungporn et al., 2006a). Whole genome sequencing was performed and submitted to the GenBank database under the strain A/Thailand/NK165/05 accession no. DQ372591-8. The phylogenetic trees of the HA and neuraminidase (NA) genes were constructed by using MEGA 3 (Kumar et al., 2004) for comparison with H5N1 viruses isolated from humans, tigers, and chickens from previous outbreaks in 2004 and 2005 (Figure 5). The sequence analyses of the viruses showed that the HA cleavage site contained SPQRERRKKR, which differed from the 2004 H5N1 virus by an arginine-to-lysine substitution at position 341.

That finding had also been observed in wild bird species during earlier outbreaks in Thailand in 2004 (Keawcharoen et al., 2005). Similar to the 2004–2005 H5N1 isolates from Thailand, a 20–amino acid deletion at the NA stalk region was observed. Moreover, the amino acid residues (E119, H274, R292, and N294) of the NA active site were conserved, which suggests that the virus was sensitive to oseltamivir. In addition, a single amino acid substitution from glutamic acid to lysine at position 627 of PB2 showed increased virus replication efficiency in mammals (Shinya et al., 2004). Observing live influenza virus in human serum or plasma is unusual. However, in 1963, low quantities of virus were isolated from blood of a patient on day 4 of illness (Naficy, 1963), and in 1970, the virus was cultivated from blood specimens from 2 patients (Lehmann and Gust, 1971). Recently, a fatal case of avian influenza A (H5N1) in a Vietnamese child was reported. The diagnosis was determined by isolating the virus

from cerebrospinal fluid, fecal, throat, and serum specimens (de Jong et al., 2005); viral RNA was found in 6 of 7 serum specimens 4–9 days after the onset of illness (WHO 2005). In this case, the H5N1 virus could be isolated from plasma on day 10 after symptoms developed. This case showed the virus in the patient's blood, which raises concern about transmission among humans. Because probable H5N1 avian influenza transmission among humans has been reported (Ungchusak et al., 2005), this case should be a reminder of the necessity to carefully handle and transport serum or plasma samples suspected to be infected with H5N1 avian influenza. Because viable virus has been detected in blood samples, handling, transportation, and testing of blood samples should be performed in a biosafety (category III) containment laboratory to prevent the spread of the virus to healthcare and laboratory workers.

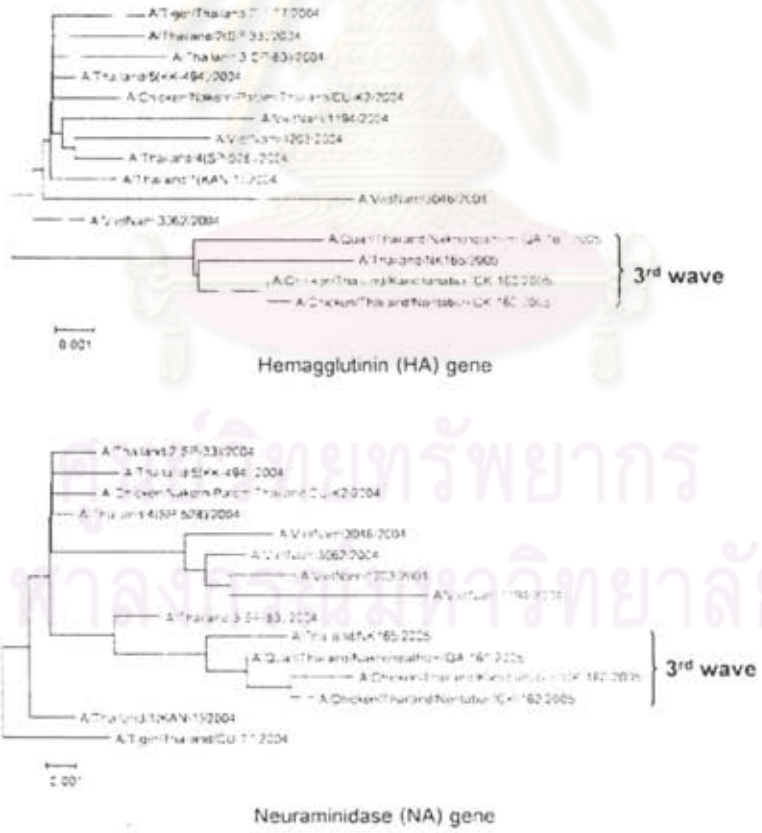


Figure 5 Phylogenetic analysis of the hemagglutinin and neuraminidase genes of H5N1 from study patient compared with sequences from previous outbreaks (2004–2005).

CHAPTER IV

H5N1 OSELTAMIVIR-RESISTANCE DETECTION BY REAL-TIME PCR USING TWO HIGH SENSITIVITY LABELED TAQMAN PROBES

(Published in J Virol Methods. 2007. 139: 44-49)

A single amino acid substitution, from histidine to tyrosine at position 274 of the neuraminidase gene has converted Oseltamivir sensitive H5N1 influenza A virus into a resistant strain. Currently, Oseltamivir is being stockpiled in many countries potentially affected by the influenza A virus subtype H5N1 epidemic. To identify this change in Oseltamivir-treated patients, a method based on real-time PCR using two labeled TaqMan probes was developed for its rapid detection. In order to validate the method, Oseltamivir specimen from treated (Oseltamivir-resistant strain from a Vietnamese patient, two Oseltamivir-treated tigers) and untreated subjects have been used for this study. The results thus obtained as well as those derived from clone selection and sequencing showed that TaqMan probes could clearly discriminate wild type H274 from the mutant 274Y variant. The sensitivity of this assay was as low as 10 copies/μl and allowed the detection of the mutation in a mixture of wild type and mutant. Overall, the assay based on real-time PCR with two labeled TaqMan probes described here should be useful for detecting Oseltamivir-resistant H274Y H5N1 influenza A virus in many species and various sources of specimens with high sensitivity and specificity. Such studies can address potential differences in the diagnostic outcomes between patients who develop detectable Oseltamivir resistance and those who retain only the wild type strain of H5N1.

1. Introduction

Influenza virus is a RNA virus from the Orthomyxoviridae family. Annually, influenza viruses may develop symptomatic influenza in 20% of children and5%of adultsworldwide (Turner et al., 2003). From the three types A–C only A and B cause widespread outbreaks. Further subtyping of influenza A virus is based on the antigenic differences between two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Nowadays, 16 HA (H1–H16) and 9 NA (N1–N9) subtypes have been described

(Fouchier et al., 2005; Nicholson et al., 2003). Since 2004, the influenza A virus subtype H5N1 has been the cause of severe disease in various poultry and mammals. The clinical spectrum of avian influenza (H5N1) in humans comprises initial symptoms of high fever (exceeding 38 °C), lower respiratory tract symptoms, clinically significant lymphopenia, abnormalities on chest radiography, and in some cases diarrhea, vomiting (Tran et al., 2004) and encephalitis. The overall fatality rate among hospitalized patients with avian influenza A (H5N1) infection has amounted to 57% (WHO, 2006a). Two groups of antiviral agents are currently available for the treatment of influenza infection. The Adamantanes (Amantadine and Rimantadine) block the function of the M2 protein; however, drug resistance in patients has increased to 30% (Hayden and Hay, 1992). The more recently developed class of viral neuraminidase inhibitors includes Zanamivir (Relenza) and Oseltamivir (Tamiflu). An important antiviral medication used against all strains of influenza A virus is Oseltamivir, which is the first orally active neuraminidase inhibitor in the form of a capsule or powder for liquid suspension (Kim et al., 1997). The neuraminidase inhibitor (NAI) Oseltamivir imitates natural neuraminidase substrate molecules and binds to the active site of the enzyme in addition to interfering with the release of progeny influenza virus from infected host cells (Moscona, 2005a). Therefore, neuraminidase (NA) cannot cleave a terminal N-acetylneuraminic acid residue from an oligosaccharide chain and thus, the virions self-aggregate and bind to the surface of infected cells. Oseltamivir resistance due to neuraminidase mutations have arisen both in challenge studies and in patients with naturally acquired infections. Rates of resistance are estimated at around 1% in the adult population and 5% in pediatric patients (Jackson et al., 2000; Whitley et al., 2001). In 2004, Kiso et al. (2004) analyzed influenza A viruses (H3N2) collected from 50 children before and during treatment with Oseltamivir. Eighteen percent of the children (N= 9/50) had neuraminidase mutations at Arg292Lys (N= 6/9) or Glu119Val (N= 2/9) or Asn294Ser (N= 1/9). Volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) virus and treated with oseltamivir have shown an H274Y substitution at the neuraminidase active site (Gubareva et al., 2001). This mutation in response to oseltamivir phosphate treatment leaves the virus severely compromised both in vitro and in vivo (Ives et al., 2002) and

confers about 400–600-fold resistance (Wetherall et al., 2003). The mutation at position 274 can influence the sensitivity of influenza N1 NA yet not of N2 NA to Oseltamivir carboxylate by rearranging the shape of the active site to create a pocket for Oseltamivir (Gubareva, 2004; Moscona, 2005b; Wang et al., 2002). Safeguarding against a potential influenza A virus subtype H5N1 epidemic, many countries now stockpile Oseltamivir. It has recently been reported that Oseltamivir-resistant influenza A (H5N1) viruses with the H274Y mutation have been isolated from three patients. H5N1 viruses with pronounced Oseltamivir resistance were isolated from two of eight Vietnamese patients during Oseltamivir treatment. Both patients died in January 2005 and another resistant case died in February 2005 (Beigel et al., 2005; de Jong et al., 2005; Le et al., 2005). As an increase in Oseltamivir-resistant viruses seems likely, a method aimed at rapidly identifying resistant H5N1 strains applying real-time PCR using TaqMan probes was designed. The assay enables to identify the H274Y mutation from samples originated directly from infected tissue and plasma.

2. Materials and methods

2.1. Sources of clinical specimens

Oseltamivir-treated and non-treated specimens of several species infected with avian influenza A subtype H5N1, previously detected using the method described by Payungporn et al. (2005), were used. The Oseltamivir-treated specimens were: H5N1 Oseltamivir-resistant strain in a Vietnamese patient (N= 1), Oseltamivir-treated tiger CU-T7; *Panthera tigris tigris* (N= 1) white tiger KU-11; *P. tigris tigris* (N= 1). The Oseltamivir untreated specimens were: plasma of H5N1 infected human (N=1) (Chutinimitkul et al., 2006), several tissues from different organs of tiger, lung (N= 1), spleen (N= 1), kidney (N= 1), liver (N= 1), brain of leopard (*Panthera pardus*) (N= 1), allantoic fluid of embryonated chicken eggs inoculated with the virus according to the method described by the Office International des Epizooties (OIE) originating from a cat; *Felis catus* (N= 1), a dog; *Canis familiaris* (N= 1), a quail (N= 1), an ostrich (N= 1) and chicken (N= 6). These specimens 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; (4) Department of Pediatrics, Faculty of Medicine, Srinakharinwirot University, Nakhon Nayok, Thailand; (5) National Institute of Hygiene and Epidemiology, Hanoi, Vietnam.

2.2. Primer and TaqMan probe design

The nucleotide sequences (N= 246) of the neuraminidase gene of influenza A virus (H5N1) were taken from the Genbank database going back as far as 2003–2006 and hence, comprising entries isolated from various species, such as avian, cats, dog, tigers, swine and humans, including DQ250165, the sequence of one Vietnamese Oseltamivir-resistant patient (A/Vietnam/CL2009/2005(H5N1)). The alignments were performed using CLUSTAL X (Version 1.81 from ftp://ftpigbmc.u-strasbg.fr/pub/ClustalX) and BioEdit sequence alignment Software Version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Assay target regions were first identified by visual inspection of the sequence alignment. Primers were chosen from constant regions of all sequences specific for the neuraminidase gene N1 of influenza A virus most closely related to the probes. MGB TaqMan probes were chosen from the region covering the drug resistant area (H274Y) and designed to be specific for both wild type and mutant. Both primers and probes were analyzed using the primer design software (OLIGOS Version 9.1 by Rusian Kalendar, Institute of Biotechnology, University of Helsinki, Finland) and Primer Express Software Version 2.0 (Applied Biosystems, CA). The wild type (H) and mutant (Y) MGB Taq-Man probes were labeled with FAM and VIC with emission wavelengths at 530 and 560 nm, respectively. The primers and probes used in this study are shown in Table 4.

2.3. Oligonucleotides designed for H274Y mutagenesis

An RNA sample extracted from embryonated chicken eggs and previously identified as influenza A virus subtype H5N1 (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004(H5N1)) was applied to design a series of mutagenesis. The oligonucleotides depicted in Table 1 were used to generate the series of mutagenesis at

amino acid position 274 of the neuraminidase N1 gene which served as a control for all possible patterns of nucleotide change in the area of probe binding. The primers N1MuF and N1MuR served as the outer primers of each mutagenesis group, which paired with

Table 4 H274Y detection primers and TaqMan MGB probes and series of mutagenesis primers at amino acid position 274

Primer/Probe	Sequence (5'-3')	Position*	Strand
Primer_F	5'-ATACTGAGAACTCAAGAGTC-3'	583-602	Sense
Primer_R	5'-TTATCCCTGCACACACATG-3'	800-702	Antisense
Probe_H274	5'-6FAM-TCCTCATAGTGRTAATT-MGBNFQ-3'	749-733	Antisense
Probe_274Y	5'-VIC-TCCTCATAGTARTAATT-MGBNFQ-3'	749-733	Antisense
N1MuF	5'-GGGGCTGTGGCTGTATTG-3'	517-534	Sense
N1MuR	5'-GGGGCGTGGATTGTCTCC-3'	900-883	Antisense
N1Mu1F	5'-ATTATCACTATGAGGAATGCTC-3'	734-755	Sense
N1Mu1R	5'-GAGCATTCCTCATAGTGATAAT-3'	755-734	Antisense
N1Mu2F	5'- ATTATTACTATGAGGAATGCTC-3'	734-755	Sense
N1Mu2R	5'-GAGCATTCCTCATAGTAATAAT-3'	755-734	Antisense
N1Mu3F	5'-ATTACCACTATGAGGAATGCTC-3'	734-755	Sense
N1Mu3R	5'-GAGCATTCCTCATAGTGGTAAT-3'	755-734	Antisense
N1Mu4F	5'-ATTACTACTATGAGGAATGCTC-3'	734-755	Sense
N1Mu4R	5'-GAGCATTCCTCATAGTAGTAAT 3'	755-734	Antisense

[a] The neuraminidase gene of A/chicken/Nakorn-Patom/Thailand/CU-K2/2004 GenBank accession number AY590567 served as reference.

the mutagenesis primers. Mutagenesis primer group 1 for H274 is N1Mu1F and N1Mu1R. Mutagenesis primer group 2 for 274Y is N1Mu2F and N1Mu2R. Mutagenesis primer groups 3 and 4 were designed for possible varied strains occasionally found. The mutagenesis products of groups 1 and 2 were used to construct mutagenesis groups 3 and 4. Mutagenesis group 3 for H274 is N1Mu3F and N1Mu3R. Mutagenesis group 4 for 274Y is N1Mu4F and N1Mu4R. The primary mutagenesis PCR reaction mixture comprised 0.5 µl of cDNA CU-K2, 0.5 µM forward primer (outer or mutagenesis primer), 0.5 µM reverse primer (outer or mutagenesis primer), 10 µl of 2.5x MasterMix (Eppendorf, Hamburg, Germany) and nuclease-free water to a final volume of 25 µl. The secondary mutagenesis PCR reaction mixture comprised 0.5 µl PCR product representative for each mutagenesis group, 0.5 µM N1MuF, 0.5 µM N1MuR, 10 µl 2.5x MasterMix (Eppendorf) and nuclease-free water to a final volume of 25 µl. Both amplification reactions were performed in a Mastercycler personal (Eppendorf) under the following conditions: predenaturation at 94 °C for 2 min followed by 40 amplification cycles consisting of 30 s denaturation at 94 °C, 30 s annealing at 52 °C and 1 min extension at 72 °C and concluded by a final 7 min extension at 72 °C. Four groups of mutagenesis PCR products were separated by 2% agarose gel electrophoresis and purified using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg, Germany). These purified products were inserted into the pGEM-T Easy Vector System (Promega, Madison, WI) and plasmids were purified by using the High Pure Plasmid Isolation Kit (Roche, GmbH, Germany) according to the manufacturer's specifications. The series of H274Y mutations were sequenced and used as controls.

2.4. RNA extraction and reverse transcription

Viral RNAwas extracted from 140 µl samples of the allantoic fluid of inoculated embryonated eggs, plasma and the supernatant resulting from tissue extraction using the QIAmp viral RNA mini kit (Qiagen, GmbH, Germany) according to the manufacturer's specifications. Reverse transcription was performed on 12 µl of each RNA sample at 37 °C for 1 h using 200 units of M-MLV reverse-trancriptase (Promega), 5 µl of 5x M-MLV reaction buffer (Promega), 5 µl of 10mM dNTP (Promega), 25 units of

rRNasin® Ribonuclease Inhibitor (Promega), 1 µM universal primer as described by Hoffmann et al. (2001). Twelve microliters of RNA from the RNA extraction kit were heated to 70 °C for 5 min and cooled on ice before adding nuclease-free water to a final volume of 25 µl.

2.5. Real-time PCR conditions

Real-time PCR was performed using the Biotools QuantiMix EASY PROBES KIT (Biotools, Madrid, Spain). Both probes and primer pairs depicted in Table 1 were used in multiple formats, each primer and probe at a final concentration of 0.5 µM and 0.20 µM, respectively. A combination of 0.5 µl cDNA from embryonated eggs or 2 µl cDNA from tissue, serum or plasma with a reaction mixture containing 10 µl of QUANTIPROBES, 4.0mM $MgCl_2$ and nuclease-free water was adjusted to a final volume of 20 µl. Real-time PCR amplification was carried out in a Rotor-Gene 3000 Instrument (Corbett Research, Sydney, Australia). The amplification reaction consisted of a preincubation step at 95 °C for 10 min to activate the HotStarTaq DNA polymerase. This was followed by 40 cycles of amplification including denaturation at 95 °C for 10 s, annealing at 55 °C for 15 s and extension at 72 °C for 20 s. Two fluorescent signals were obtained once per cycle at the end of the extension step with detectors corresponding to the FAM (530 nm) and VIC (560 nm) channels, respectively. 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).

2.6. Selection of drug resistant clones

cDNAs were amplified by PCR in a reaction mixture containing 10 µl of 2.5x MasterMix (Eppendorf) 0.5 µM primer F: 5'-ATACTGAGAACTCAAGAGTC-3', 0.5 µM primer R: 5'-TTATCCCTGCACACACATG-3' and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a Mastercycler personal (Eppendorf) under the following conditions: predenaturation at 94 °C for 2 min followed by 40 amplification cycles comprising denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s and concluded by a final extension at 72 °C for 7

min. The PCR products were separated by 2% agarose gel electrophoresis and purified using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg). The purified products were inserted into pGEM-T Easy Vector System (Promega), 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) 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).

2.7. Specificity and sensitivity test

The specificity of the dual probe real-time PCRwas evaluated by cross-reaction tests carried out between RNA extracts from isolates or clinical specimens expressing the entire spectrum of NA subtypes (N2-N9) of WHO reference strain influenza and other viral pathogens, such as Newcastle disease virus (NDV), respiratory syncytial virus (RSV) subgroups A and B, human metapneumovirus (HMPV), coronavirus OC43, coronavirus 229E, infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV). The sensitivity was established with plasmids containing a copy of the wild type H274 and mutant 274Y serving as reference. The wild type H274 and mutant 274Y plasmids were used to determine the capacity of the real-time PCR assay to detect wild type and Oseltamivir-resistant codon 274 mutants in a single sample. DNA concentration was determined by measuring absorbance at 260 and 280 nm. The two control plasmids were diluted to 10^4, 10^3, 10^2 and 10 copies/µl and each resulting concentration was mixed at 100:0, 75:25, 50:50, 25:75 and 0:100 wild type-to-variant ratios. Real-time PCR analysis of potential codon 274 variants was performed on each ratio at each concentration under the conditions described above.

3. Results

3.1. Detection of oseltamivir resistance by real-time PCR using two labeled TaqMan probes

The result was obtained by using two TaqMan probes labeled with the FAM and VIC fluorescent signal for wild type and mutant detection, respectively. The fluorescent signal resulting from real-time PCR can be interpreted as shown in Figure 6. A sample containing only the wild type strain will emit the fluorescent signal exclusively via the FAM channel (530 nm) whereas a sample containing the Oseltamivir-resistant variant with a nucleotide alteration at position 274 of the neuraminidase gene will emit the fluorescent signal via the VIC channel (560 nm). In order to develop and optimize the assay these probes were tested on four sets of mutagenesis and obtained clearly discernible results irrespective of the concentrations of wild type and mutant plasmids or the ratio in which they had been mixed. The clinical samples tested in this assay were isolated from humans, tigers, leopard, cat, dog and various avian species previously infected with H5N1. Two human specimens in this experiment were investigated. The first one isolated from human plasma and collected in Nakhon nayok province showed a positive result for the wild type only. In contrast, the second one obtained from a Vietnamese patient previously reported by Le et al. (2005) emitted signals specific for both wild type and mutant. The remaining specimens originating from tiger lung, spleen, kidney and liver, leopard brain and the allantoic fluid of various avian species, cat and dog displayed positive results specific for the wild type only.

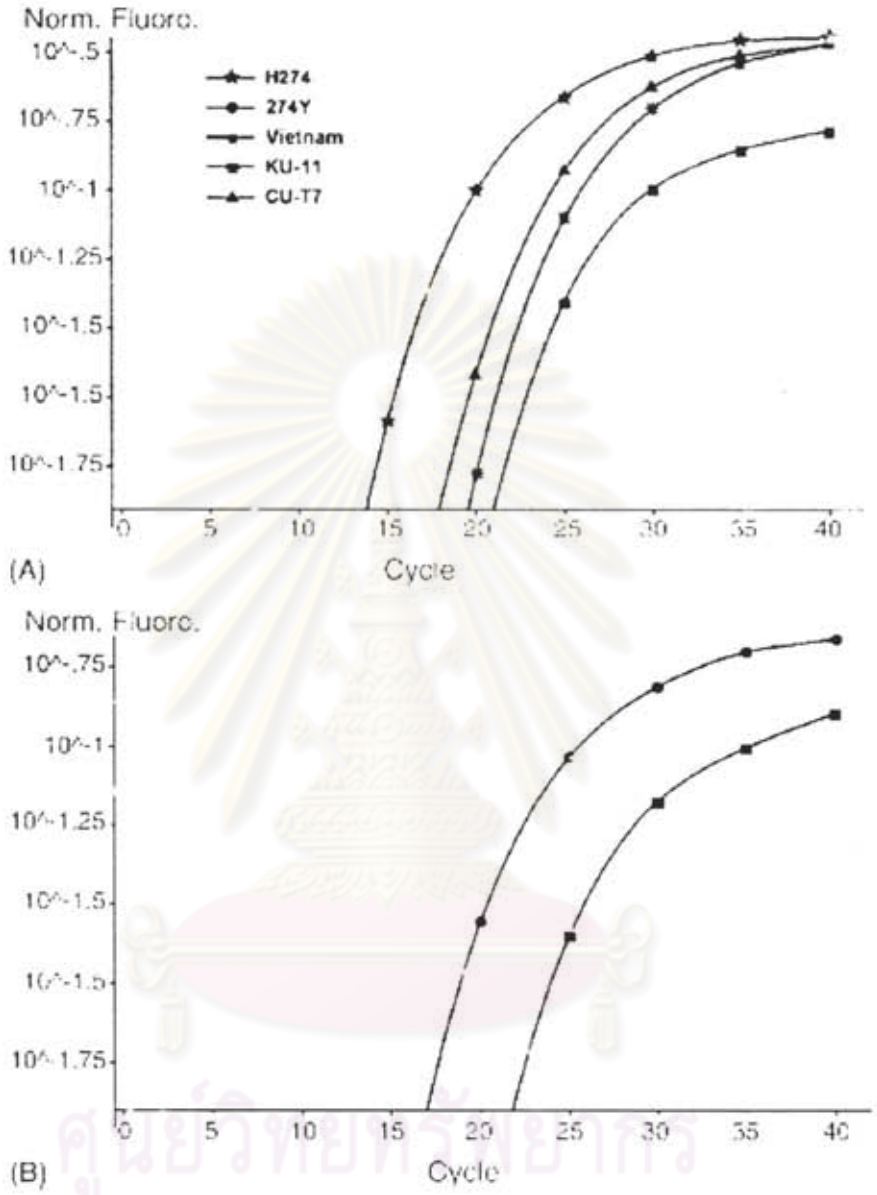


Figure 6 Two fluorescent signals for wild type H274 and mutant 274Y detection emitted by TaqMan probes labeled with FAM (A) and VIC (B), respectively. H274: positive control plasmid, 274Y: positive control plasmid; Vietnam: Oseltamivir-treated Vietnamese patient (Le et al., 2005) showing both fluorescence signals indicative of a combination between wild type and resistant strain, KU-11: Oseltamivir treated white tiger, CU-T7: Oseltamivir-treated tiger showing only the wild type signal.

3.2. Selection of drug resistant clones

Three specimens, treated with Oseltamivir, were chosen to be cloned into plasmids for confirmation. The first specimen was from a tiger (A/Tiger/Thailand/CU-T7/04) isolated from zoo tigers that had perished during the mid-October 2004 H5N1 influenza outbreak. CU-T7 was isolated from a nasal swab of a tiger that eventually perished but had been treated with Oseltamivir at 75 mg/60 kg twice daily for 4 days prior to specimen collection (Amonsin et al., 2006; Thanawongnuwech et al., 2005) and inoculated into SAN-fowl eggs according to the method described by the Office International des Epizooties (OIE). Ten clones of CU-T7 were randomly selected and sequenced. All the clones were sensitive to Oseltamivir. The second specimen was white tiger (A/Tiger/Thailand/KU-11/04) was isolated from a sick white tiger found positive for H5N1 by nasal swab and subsequently treated with Oseltamivir at 75 mg/60 kg twice daily for 4 days. This tiger survived and a rectal swab was taken. This specimen was inoculated into SAN fowl eggs. Ten clones of KU-11 were randomly selected and sequenced. All the clones were sensitive to Oseltamivir. The third specimen was cDNA from a Vietnamese patient (Le et al., 2005). Ten clones of this strain were randomly selected and sequenced. Nine of the 10 clones were resistant to Oseltamivir and only 1 clone was sensitive.

3.3. Specificity and sensitivity test

The specificity of the assay by cross-contamination tests were evaluated and found no cross-reactivity to total human DNA, any of the different NA subtypes of influenza A virus, Newcastle disease virus (NDV), respiratory syncytial virus (RSV) subgroups A and B, human metapneumovirus (HMPV), coronavirus OC43, coronavirus 229E, infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV). Likewise, any significant false positive or non-specific signal in any of the samples tested was not observed. Overall, the results obtained on Oseltamivir resistance with the two labeled probes indicate a high specificity of both primers and probes used for amplification. As for the sensitivity of real-time PCR, the threshold concentration for detecting both wild type (H274) and mutant (274Y) was 10 copies/μl. Furthermore, in

order to establish the limits of real-time PCR to detect both wild type and mutant in the same sample, wild type and mutant plasmids were mixed in various ratios and diluted them over a range of concentrations 100:0, 75:25, 50:50, 25:75 and 0:100 ratios of wild type and mutant. The result showed the high sensitivity of the detection that can be detected although the reaction had 7.5:2.5 plasmid copies/µl of wild type and mutant.

4. Discussion

At present, there is a substantial risk of a global influenza A virus subtype H5N1 epidemic not only affecting poultry but also mammalian species including humans. A medication capable of preventing the spread to humans is the neuraminidase inhibitor Oseltamivir. Yet, influenza A virus subtype H5N1 has already developed drug resistance by mutations in the neuraminidase gene leading to amino acid substitutions predominantly at positions 119, 152 274 and 292 (N2 numbering system) of the enzyme's active site (Gubareva et al., 2000). The amino acid substitution at position 274 identified in mutants selected in the presence of NA inhibitors both in vivo and in vitro has exclusively been found in N1 (Gubareva et al., 2002). In 2005, H5N1 virus resistant to oseltamivir due to an amino acid change from Histidine (H) to Tyrosine (Y) based on a single nucleotide alteration at position 274 has been isolated from three Vietnamese patients one of whom, a 14 years old, recovered (Le et al., 2005) while the remaining two, a 13 and 18 years old, succumbed to the infection (de Jong et al., 2005). Hence, patients found positive for H5N1 infection ought to be monitored for the nucleotide change at position 274 causing resistance to Oseltamivir before the onset of treatment.With the mutation detected in time, alternative treatment applying, for example Zanamivir might save the patient's life.

In this experiment, both probes and primers were specifically designed to detect the nucleotide change causing Oseltamivir resistance. The amino acid substitution of Histidine (H) with Tyrosine (Y) is the consequence of a single nucleotide in the first codon of this amino acid change from C to T. The alignment of theH5N1 neuraminidase gene sequence with more than 200 sequences stored in the Genbank database showed a nucleotide change in the specific probe area yet at a position not triggering the critical

amino acid alteration. Hence, the probewas designed to allow for this inconsequential mutation by using a degenerate nucleotide at that position thus closely mimicking the natural situation. Moreover, since the conserved area of the gene restricted the probe's length to 17 nucleotides TaqMan MGB was chosen. The probe was coupled with a minor groove binder enhancing its Tm and had a non-fluorescent quencher attached to the 3' end, which does not interfere with fluorescent signal detection. After having tested the probes with the mutagenesis control, the result showed high sensitivity and correct distinction between wild type and mutant upon mixing different ratios of wild type and mutant plasmid at low concentrations.

In conclusion, real-time PCR using two labeled TaqMan probes provides a highly specific and sensitive method to detect the amino acid alteration at position 274 of the influenza A subtype H5N1 neuraminidase gene causing oseltamivir resistance. Studies as the one described here could address the potential differences in diagnostic outcomes between patients who develop detectable Oseltamivir resistance and patients who retain only the wild type strain of H5N1. However, the other point mutations of Oseltamivir resistance in H5N1 infected mammalian species need for the further investigation.

CHAPTER V

NEW STRAIN OF INFLUENZA A VIRUS (H5N1), THAILAND

(Published in Emerg Infect Dis. 2007. 13:506-507)

During 2004–2005, 3 major waves of avian influenza outbreaks occurred in Thailand (Amonsin et al., 2006a). The first wave was reported in early January 2004, the second in July 2004, and the third in October–December 2005. In total, 22 persons were infected and 14 died. Recently, a fourth wave began on July 23, 2006. The Thai Ministry of Public Health reported that avian influenza A (H5N1) virus killed 2 infected persons. The first patient, a 17-year-old man in Phichit Province, began to experience symptoms on July 15, 2006, and died on July 24, 2006 (Hopp, 2006). The second patient, a 27-year-old man in Uthai Thani Province, began to experience symptoms on July 24, 2006, and died on August 3, 2006 (Gale, 2006). The fourth wave of these outbreaks involved chickens and encompassed 2 distinct areas: Phichit Province identified on July 23, 2006 (Dudley, 2006), and Nakhon Phanom Province, identified on July 28, 2006 (Marshall, 2006). We sequenced all 8 gene segments of the 2 viruses isolated from Phichit and 1 virus isolated from Nakhon Phanom and then submitted to GenBank as follows: A/chicken/Thailand/PC-168/2005 (DQ999879–86) and A/chicken/Thailand/PC-170/2006 (DQ999887–94) from Phichit and A/chicken/Thailand/NP-172/2006 (DQ999871–8) from Nakhon Phanom. Whole genome analysis showed that all 3 samples had undergone minor mutations that are typical of circulating influenza A viruses. Unexpectedly, this outbreak was associated with 2 strains of the virus. The 2 samples from Phichit closely resembled H5N1 strains that had circulated in Thailand during 2004 and 2005. The sample from Nakhon Phanom was newly observed in Thailand and more closely related to H5N1 strains that had been circulating since 2005 in southeast People's Republic of China. The whole genome phylogenetic analysis also showed that the viruses isolated from Phichit belonged to genotype Z, whereas virus isolated from Nakhon Phanom belonged to genotype V, which differs from genotype Z in the PA gene (Mase et al., 2005) (Figure 7). The phylogenetic tree of the hemagglutinin (HA) gene (Figure 8) showed that the Phichit samples were similar to the cluster of samples isolated during

2004 and 2005 in Thailand and Vietnam. In contrast, the Nakhon Phanom sample was clustered into the same group with viruses isolated from southeast People's Republic of China, including Zhejiang, Shantou, Hunan, Fujian, Guangxi, and Lao People's Democratic Republic (Boltz et al., 2006) with the differences in the cleavage site, SPLRERRRK-R/G (underline and dash indicate differences), which had never been found in Thailand. The N-link glycosylation sites (positions 154–156) of the Pichit isolates were NST residues, whereas in the Nakhon Phanom isolate, NNT residues were observed. However, the receptor binding site of HA (positions 222 and 224) was unchanged. In the neuraminidase (NA) gene, the new isolates contain 20 amino acid deletions within the stalk region, the same as previously described (Amonsin et al., 2006a). The ESEV residues in the C-terminal and Asp92 of NS1 were observed in the 2006 isolates and in viruses that have been isolated from Thailand, Vietnam, and People's Republic of China. This finding indicates that the new isolates were highly virulent but sensitive to treatment with interferon and tumor necrosis factor-alpha (Krug, 2006). The 2006 isolates contain Glu627 of PB2, identical to the previous isolates from Thailand and Indonesia, which may indicate that the new isolates had less efficient replication capability in mammalian hosts (Shinya et al., 2004). Drug resistance or sensitivity is based on sequences of M2 and NA. Substitution within residues including L26I, V27A/I, A30S, and S31N of the M2 ion channel protein was used to predict amantadine-resistant mutants, and H274Y of the NA was used to predict for oseltamivir resistance (Scholtissek et al., 1998). The virus observed in 2006 isolates from Phichit was resistant to amantadine but sensitive to oseltamivir, whereas the isolate from Nakhon Phanom was sensitive to amantadine and oseltamivir, which implies that infected patients received different antiviral drugs. According to previous World Health Organization reports, the HA sequences of most influenza (H5N1) viruses that circulated in avian species during the past 3 years are separated into 2 distinct phylogenetic clades. Clade 1 viruses that circulated in Cambodia, Thailand, and Vietnam were responsible for human infections in those countries during 2004 and 2005. Clade 2 viruses that circulated in birds in People's Republic of China and Indonesia during 2003–2004 and 2005–2006 spread westward to the Middle East, Europe, and Africa.

This latter genetic group of viruses has been principally responsible for human infections during late 2005 and 2006 (WHO, 2005). The latest wave of the outbreaks in Thailand was caused by viruses closely related to those that caused outbreaks in Thailand in 2004–2005 and to viruses recently circulating in southeast People's Republic of China and other Southeast Asian countries. This finding raises concern for development of new candidate influenza (H5N1) vaccine strains. Geographic spreading, epidemiology, and genetic properties of recently circulating influenza (H5N1) viruses should be considered when developing candidate H5N1 strains of influenza vaccine.

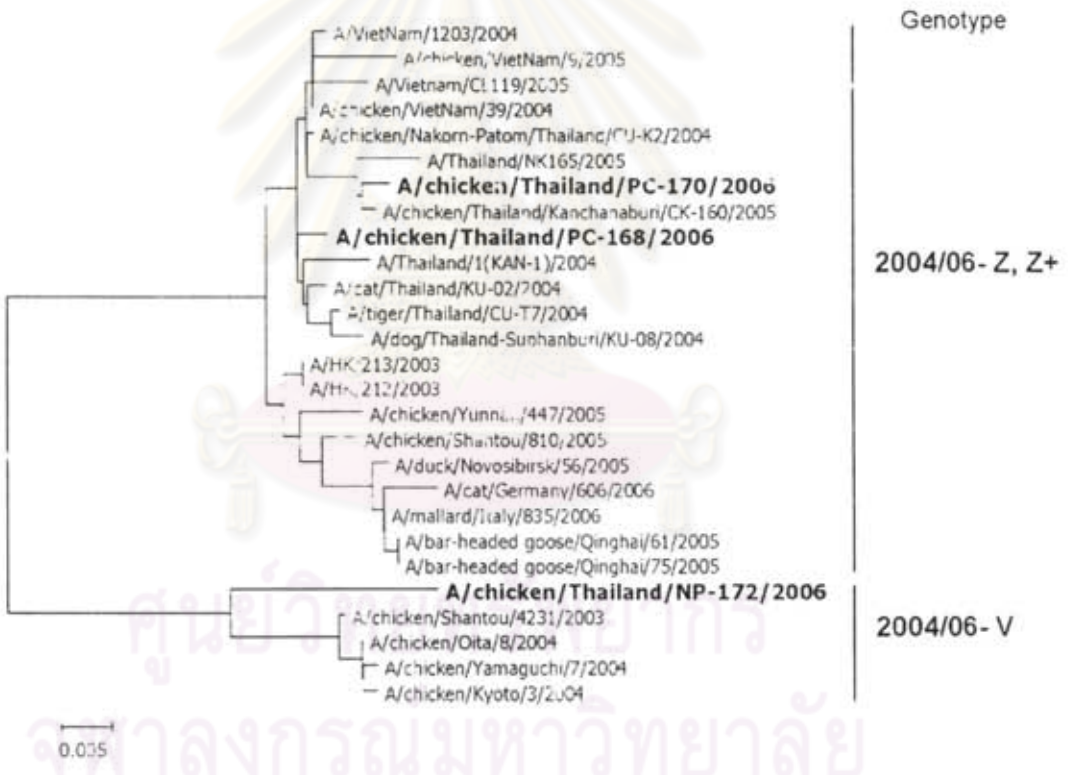


Figure 7 Phylogenetic relationships of the polymerase acid protein (PA) gene comparing genotype Z, Z+, and V.

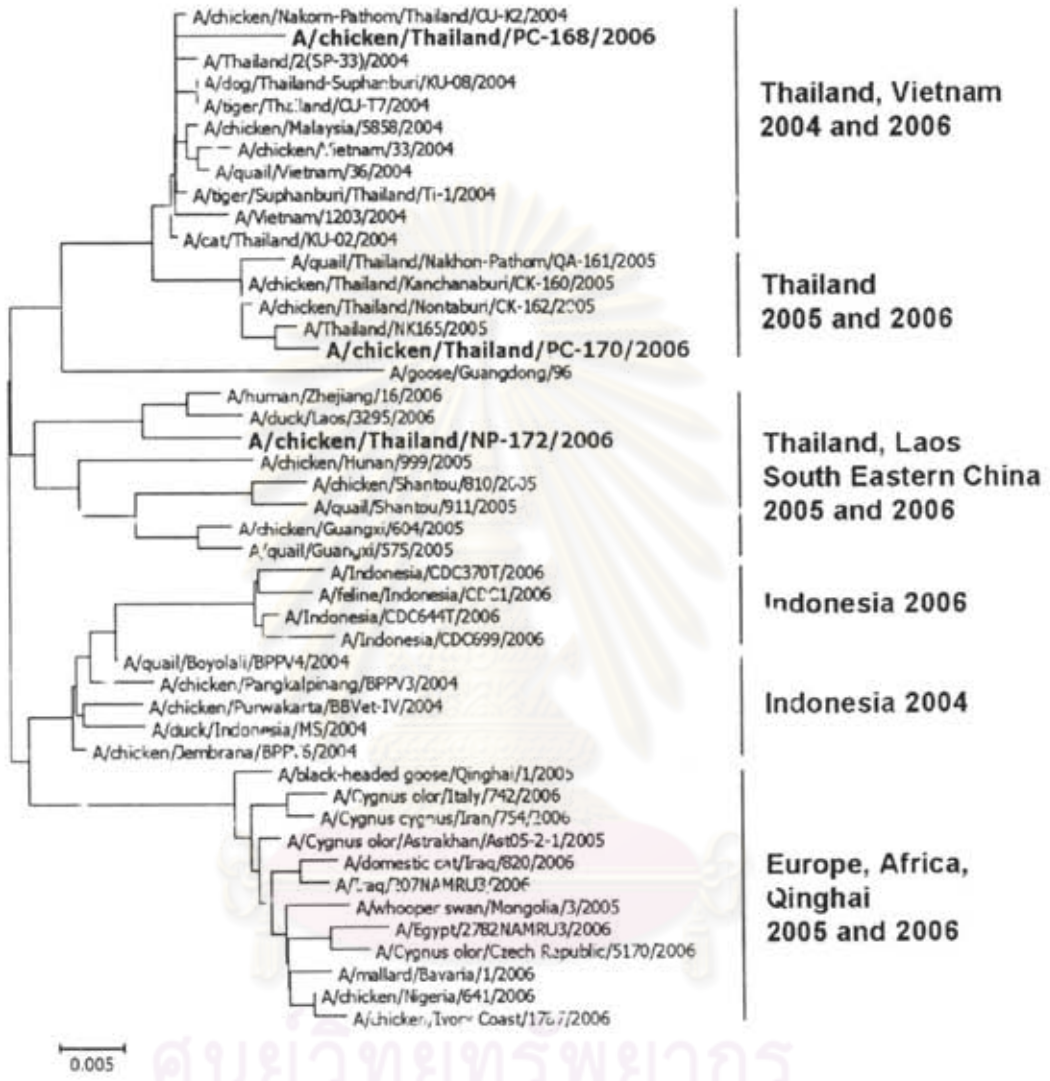


Figure 8 Hemagglutinin (HA) gene of influenza A (H5N1) viruses in Thailand 2006 compared with several other strains worldwide.

CHAPTER VI

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF H1N1 AND H3N2 HUMAN INFLUENZA A VIRUSES AMONG INFANTS AND CHILDREN IN THAILAND

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The annual influenza outbreaks can cause a high mortality rate among infants and children. In the tropics, influenza shows no clear dependence on seasons. In the present study, we performed molecular and phylogenetic analysis of H1N1 and H3N2 influenza virus isolated from infants and children diagnosed with respiratory tract illness between February 2006 and February 2007. A total of 33 samples (10.92%) were found positive for human influenza virus infection. Characterization of the hemagglutinin gene revealed conserved sequences at the receptor-binding site as well as variations due to amino acid substitutions at the antigenic site, potentially resulting in an N-linked glycosylation site. As for the neuraminidase gene, amino acid substitutions were found in N1 and N2 but not directly at the catalytic or framework sites of this enzyme. Based on the phylogenetic tree, the hemagglutinin 1 (HA1) region and the neuraminidase (NA) gene of both H1N1 and H3N2 isolated subtypes clustered with the current vaccine strain for the Northern Hemisphere 2007-2008. This finding contributes to understanding the evolution of influenza A viruses in humans and is useful for surveillance and vaccine strain selection.

1. Introduction

Influenza A viruses are members of the *Orthomyxoviridae* family which can be divided into subtypes based on the antigenic properties of the surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA) (Webster et al., 1992). All influenza A virus subtypes have been discovered in avian species, but only a few subtypes have been found in humans. Every year, in excess of 200,000 people worldwide succumb to severe respiratory illness caused by influenza A virus. Three such major global pandemics caused by novel antigen variants of influenza viruses have affected the human population, the "Spanish flu" in 1918 (H1N1 subtype), the "Asian flu" in 1957 (H2N2

subtype), and the "Hong Kong flu" in 1968 (H3N2 subtype) resulting in millions of deaths (Stephenson and Zambon, 2002). The recent circulation of highly pathogenic avian H5N1 viruses since 2003 has brought about more than 300 infected cases and nearly 200 human deaths (WHO, 2007b) which has raised concern as to the emergence of a new pandemic. However, only cyclical alterations of the H1N1 and H3N2 viruses are predominant strains in humans. During 2006–2007, influenza A virus subtypes H1N1 and H3N2 circulated in many parts of the world. The influenza A surface glycoprotein hemagglutinin (HA) is under selective pressure to undergo changes in order to evade the host's immune system (Holmes et al., 2005). Each year, WHO publish recommendations on the composition of influenza vaccine for the Northern and Southern Hemispheres. In the Northern Hemisphere, many isolates of H1N1 influenza viruses were antigenically similar to the current reference virus, A/New Caledonia/20/1999, but an increasing proportion of recent viruses were more closely related to A/Solomon Islands/3/2006. Likewise, H3N2 influenza viruses were antigenically similar to the current reference virus, A/Wisconsin/67/2005, but showed an increasing proportion of antigenic differences (WHO, 2007a). Predicting variations of circulating influenza strains for subsequent annual vaccine development has become vital. Furthermore, comparisons between antigenic differences and phylogenetic analyses are necessary to further the understanding of multiple lineages of influenza virus variants. Therefore, between February 2006 and February 2007, we collected 302 clinical samples of nasopharyngeal suction from patients diagnosed with respiratory illness in the Pediatrics ward at King Chulalongkorn Memorial Hospital, Thailand for influenza A virus detection. On all positive samples, we performed sequence analysis to study the antigenic differences and compare them with the vaccine strain.

2. Materials and methods

2.1. Clinical samples

The study protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok. The parents of all participating children were informed about the study objective and their written consent was obtained.

Nasopharyngeal suction specimens were collected from 302 infants or children (age range: 5 days to 14 years) suffering from respiratory illness from 14 February 2006 to 28 February 2007. All clinical samples were provided by the Department of Pediatrics, King Chulalongkorn Memorial Hospital. NP suction samples were collected in transport medium consisting of phosphate buffered saline with antibiotics (250 U/mL of Penicillin G sodium and 250 µg/mL of Streptomycin sulfate) and stored at -70°C until tested.

2.2. RNA extraction and cDNA synthesis

RNA was extracted from 150 µL of each NP suction sample using TRI REAGENT® LS (Molecular Research Center, Inc., Cincinnati, OH) and dissolved in 12 µL of DEPC-treated water. cDNAs were synthesized at 37°C for 2 h using the M-MLV reverse-transcription system (Promega, Madison, WI) consisting of 200U of M-MLV reverse transcriptase, 5 µL of 5x M-MLV reaction buffer, 5 µL of 10mM dNTP, 25U of RNasin® ribonuclease inhibitor, 0.5 µg/µL of random primer, 12µL of RNA heated to 70°C for 5 min, then cooled on ice and nuclease free water to a final volume of 25 µL. These samples were used for additional respiratory screening described by Chieochansin et al. (2007).

2.3. Influenza A virus detection

Influenza A virus detection was performed by conventional PCR using 1 µL of cDNA, 0.5 µM of FluA_M_F: 5'-RGGCCCCCTCAAAGCCGA-3' (nt 76–93), 0.5 µM of FluA_M_R: 5'-ACTGGGCACGGTGAGYGT-3' (nt 235–218) (the matrix gene from GenBank, accession number NC 0020166, served as the reference), 10 µL of 2.5x Eppendorf MasterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µL. The house keeping gene glyceraldehyde-3-phosphate dehydrogenase; GAPDH, of each sample was amplified in one additional reaction mixture of identical volume with primers GAPDH_F: 5'-GTGAAGGTCGGAGTCAACGG-3' (nt 112–131) and GAPDH_R: 5'-GTTGTCATGGATGACCTTGGC-3' (nt 603–583) (the GAPDH gene from GenBank, accession number NM 002046, served as the reference) at a 0.5 µM final concentration, each. The amplification reaction was performed in a

thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94°C for 3 min, followed by 40 amplification cycles consisting of 94°C for 30 s (denaturation), 55°C for 30 s (primer annealing), and 72°C for 1 min (extension), and concluded by a final extension step at 72°C for 7 min. After 2% agarose gel electrophoresis, gel were stained with ethidium bromide and visualized on a UV transilluminator. The expected products of influenza A virus and the house keeping gene was 160 bp and 492 bp, respectively.

2.4. Full-length PCR amplification of HA and NA

Representative for each month of collection, we selected 20 matrix gene positive samples of sufficient volume for full length hemagglutinin (HA) and neuraminidase (NA) gene PCR amplification. We re-extracted RNA and performed reverse transcription using 1 µM of universal primer (Uni12 primer 5'-AGCAAAAGCAGG-3') as described by Hoffmann et al. (2001) under identical conditions as described above. Subsequently, we amplified the full length HA and NA genes using forward and reverse primers published by Hoffmann et al. (2001) with modifications. The HA gene was amplified using 0.5 µM of HAF5': 5'-CAGGGAGC AAAAGCAGGCG-3' and 0.5 µM of HAR3': 5'-CCAGTAGAAACAAGGGTGTTTT-3' or NA gene was amplified by using 0.5 µM of NAF5': 5'-CAGGGAGCAAAAGCAGGAGT-3' and 0.5 µM of NAR3':5'-CCAGTAGAAACAAGGAGTTTTTT-3' with 10 µL of 2.5x Eppendorf MasterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µL. The amplification reaction was performed in a thermocycler (Eppendorf, Germany) under the following conditions: denaturation at 94°C for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 2 min, and concluded by a final extension step at 72°C for 7 min.

2.5. HA and NA sequencing

The resulting amplicons were analyzed by 2% agarose gel electrophoresis and purified with the Perfectprep Gel Cleanup Kit (Eppendorf, Hamburg,

Germany).DNA sequencing was carried out using the Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction kit (ABI, Foster City, CA) together with the inner primer of each subtype shown in Table 5. Subsequently, any unincorporated labeled ddNTPs

Table 5 Inner sequencing primer for each subtype of human influenza A virus

Subtype	Primer	Sequence 5'-3'	Tm	Position
H1 [a]	H1_F266:	5'-CTTAGGAAACCCAGAATGCG-3'	60	266-285
	H1_R394:	5'-TGCTCCCTCAGTTCCTCATA-3'	60	394-375
	H1_R627:	5'-ACGGGTGATGAACACCCCA-3'	60	627-609
	H1_F766:	5'-ACTACTGGACTCTGCTGGAA-3'	60	766-785
	H1_F1062:	5'-GGTTTGTTTGGAGCCATTGC-3'	60	1062-1081
	H1_R1525:	5'-CCATTTTTCACACTTTCCATGC-3'	62	1525-1504
H3 [b]	H3_F360:	5'-AGCAACTGTTACCCTTATGATG-3'	62	360-381
	H3_R431:	5'-CACTGTGCCGGATGAGGC-3'	60	431-414
	H3_F598:	5'-TTGACAAATTGTACATTTGGGG-3'	60	598-619
	H3_R797:	5'-TCCCGGATTTACTATTGTCCA 3'	60	797-777
	H3_F1013:	5'-CACTCTGAAATTGGCAACAGG-3'	62	1013-1033
	H3_R1184:	5'-GCTTTTGAGATCTGCTGCTTG-3'	62	1184-1164
N1 [c]	N1_R330:	5'-TGTCTTTTGTGTATATAGCCCA-3'	60	330-309
	N1_F479:	5'-CCCYTATAGGGCYTTAATGAG-3'	62	479-499
	N1_R620:	5'-ATTRTCTGGACCAGAAATTCC-3'	59	620-600
	N1_F653:	5'-AATAACTGAAACCATAAAAAGTTG-3'	60	653-675
	N1_F849:	5'-GAGGAATGTTCCTGTTACCC-3'	60	849-868
	N1_R1159:	5'-CATCCATTAGGATCCCAAATCA-3'	62	1159-1138
N2 [d]	N2_F367:	5'-GACAAGACTACCTTATGTGTC-3'	60	367-387
	N2_F557:	5'-AGCTCAAGTTCTCACGATGG-3'	60	557-576
	N2_R564:	5'-CTTGAGCTGGACCATGCTAT-3'	60	564-545
	N2_F766:	5'-AGCTGATACTAAAATACTATTCAT-3'	60	766-789
	N2_R1120:	5'-GCTGATCGTTCTTCCCATCC-3'	62	1120-1101
	N2_R1298:	5'-TCAACTCCACATAAAAGCACC-3'	60	1298-1278

[a] Reference position of the H1 gene from A/New York/399/2003(H1N1) Accession number CY002808

[b] Reference position of the H3 gene from A/Taiwan/30005/2004(H3N2) Accession number DQ249261

[c] Reference position of the N1 gene from A/Taiwan/30017/2002(H1N1) Accession number DQ249258

[d] Reference position of the N2 gene from A/New York/396/2005(H3N2) Accession number CY002074

were removed by ethanol precipitation. The reactions were resolved on an ABI-Prism 310 Genetic Analyzer (PerkinElmer, Norwalk, CT), sequences were edited by Chromas Lite version 2.01 (Technelysium Pty Ltd., Australia) and the Bioedit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999). Finally, sequences were aligned using the SeqMan program (DNASTAR, Madison, WI). The nucleotide sequences of all samples were submitted to GenBank under the accession numbers shown in Table 6.

2.6. Phylogenetic analysis

Phylogenetic trees were constructed based on the continuous nucleotide sequences aligned with ClustalX (Thompson et al., 1997). Genetic distances were calculated applying Kimura's two-parameter method using MEGA3.1 (Kumar et al., 2004), and used to construct neighbour-joining (NJ) trees. Confidence values for the tree topologies were evaluated by bootstrap analysis of 1000 pseudo-replicate datasets.

3. Results

Three hundred and two nasopharyngeal (NP) suction specimens from infants or children aged between 5 days and 14 years diagnosed with respiratory illness between 14 February, 2006 and 28 February, 2007 were tested by PCR for the matrix genes of influenza A virus with the house keeping gene serving as internal control. Since February, the number of respiratory illness samples had been slightly increasing. It peaked in August and was again on the decrease by September. Every sample displayed the house keeping gene but only 33 samples proved positive for the matrix gene of influenza A virus. The average age of patients testing positive was 2 years 11 months, with a minimum age of 4 months and a maximum age of 13 years. Of the 33 matrix gene positive samples, we selected 20 representing each month of collection and of sufficient volume for further testing. Each patient's details are shown in Table 6. Sequencing revealed 10 samples as influenza A/H1N1 primarily identified from March to July 2006 and 10 samples as influenza A/H3N2 tentatively identified from March to May 2006 whereas after August 2006, this subtype was predominantly identified as shown in Figure 9.

Table 6 Specimen details and sequence accession numbers

Patient	Strain	Subtype	Date* (dd/mm/yy)	Sex	Age	Gene	Accession Number
1	A/Thailand/CU23/2006	H3N2	22-03-06	Female	1 Y	HA	EU021266
						NA	EU021267
2	A/Thailand/CU32/2006	H1N1	13-04-06	Female	3 Y	HA	EU021264
						NA	EU021265
3	A/Thailand/CU41/2006	H1N1	18-05-06	Male	4 Y	HA	EU021246
						NA	EU021247
4	A/Thailand/CU44/2006	H1N1	13-05-06	Female	11 Y	HA	EU021258
						NA	EU021259
5	A/Thailand/CU46/2006	H3N2	18-05-06	Male	11 M	HA	EU021268
						NA	EU021269
6	A/Thailand/CU51/2006	H1N1	30-05-06	Male	2 Y	HA	EU021254
						NA	EU021255
7	A/Thailand/CU53/2006	H1N1	06-05-06	Female	3 Y	HA	EU021248
						NA	EU021249
8	A/Thailand/CU57/2006	H1N1	10-06-06	Male	1 Y	HA	EU021256
						NA	EU021257
9	A/Thailand/CU67/2006	H1N1	23-06-06	Male	2 Y	HA	EU021250
						NA	EU021251
10	A/Thailand/CU68/2006	H1N1	26-06-06	Female	8 M	HA	EU021260
						NA	EU021261
11	A/Thailand/CU75/2006	H1N1	06-07-06	Female	6 Y	HA	EU021262
						NA	EU021263
12	A/Thailand/CU88/2006	H1N1	25-07-06	Female	5 M	HA	EU021252
						NA	EU021253
13	A/Thailand/CU124/2006	H3N2	18-08-06	Female	2 Y	HA	EU021284
						NA	EU021285
14	A/Thailand/CU228/2006	H3N2	11-10-06	Male	2 Y	HA	EU021274
						NA	EU021275
15	A/Thailand/CU231/2006	H3N2	14-11-06	Male	1 Y	HA	EU021282
						NA	EU021283
16	A/Thailand/CU259/2006	H3N2	27-12-06	Male	1 Y	HA	EU021278
						NA	EU021279
17	A/Thailand/CU260/2006	H3N2	27-12-06	Female	1Y	HA	EU021280
						NA	EU021281
18	A/Thailand/CU272/2006	H3N2	11-01-07	Male	11 M	HA	EU021270
						NA	EU021271
19	A/Thailand/CU280/2006	H3N2	26-01-07	Female	2 Y	HA	EU021272
						NA	EU021273
20	A/Thailand/CU282/2006	H3N2	02-02-07	Male	3 Y	HA	EU021276
						NA	EU021277

* Date of specimen collection; Y, Year; M, Month; HA, Hemagglutinin gene; NA, Neuraminidase gene

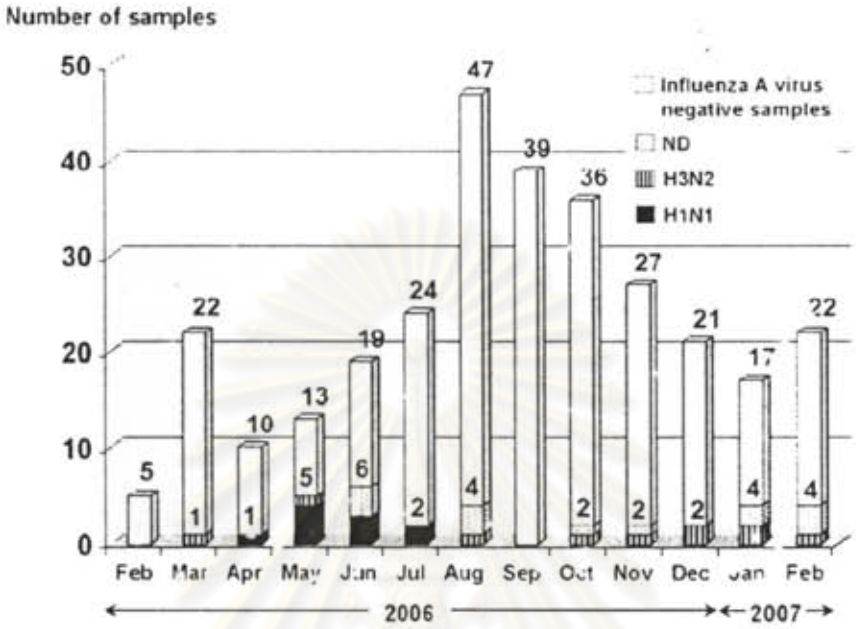

Figure 9 Number of clinical samples obtained each month between February 2006 and February 2007 from 302 infants and children with respiratory tract illness showing a slight increase from February onwards, peaking in August and decreasing by September. Thirty-three samples were positive for influenza A virus, of which 10 harbored subtype A/H1N1 and another 10 subtype A/H3N2. ND, no subtype detected.

The nucleotide and deduced amino acid sequences of hemagglutinin 1 (HA1) from 20 isolated samples were compared with current vaccine strains. For H1N1 HA1, A/Solomon Islands/3/2006 and A/New Caledonia/20/1999 were used as vaccine strains in the Northern Hemisphere 2007–2008 and Southern Hemisphere 2007, respectively. Ten H1N1 isolates from Thailand indicated a higher average of per cent similarity to A/Solomon Islands/3/2006 (98.30% based on nucleotides and 98.10% based on amino acids) than A/New Caledonia/20/1999 highest average per cent similarity to this vaccine strain with 99.09% based on nucleotides and 98.04% based on amino acids. The average per cent nucleotide and amino acid similarities of the previous H3N2 HA1 vaccine strain to A/California/7/2004 were 98.59% and 98.04% to A/Wellington/1/2004

98.49% and 97.14% and to A/Fujian/411/2002 98.09% and 96.04%, respectively. Genetic relationships of the HA1 region and NA gene of the H1N1 (Figure 10) and H3N2 (Figure 11) isolates with vaccine strains and other influenza viruses were constructed by Neighbor-Joining analysis with 1000 bootstrapped replicates. Both showed continuous evolution and the isolates were relevant to the recent vaccine strain.

HA constitutes the receptor-binding and membrane fusion glycoprotein of influenza virus. The alignment of the terminal sialic acid (SA) residues of glycoproteins and glycolipids representing the cellular receptors for influenza virus, the targets for neutralizing antibodies, and N-linked glycosylation sites are shown in Figures 12 and 13. Five conserved amino acid residues in both H1 and H3 influenza A virus, Tyr(Y)-98, Ser(S)-136, Trp(W)-153, His(H)-183 and Tyr(Y)-195 (numbering according to H3 structure) at the HA receptor-binding site have been described by Skehel and Wiley (2000). These five amino acid residues within the receptor-binding site of both H1N1 and H3N2 isolates were relatively conserved. The amino acids at the terminal sialic acid (SA) of all H1 isolates were Asp(D)-190 and Asp(D)-225 which have been previously reported (Stevens et al., 2006; Matrosovich et al., 1997) as the SA binding specific to the NeuAcα2,6Gal amino acid linkage, except for A/Thailand/CU75/06 with Ala(A)-190. Residues mainly responsible for NeuAcα2,6Gal linkage of H3 are Leu(L)-226 and Ser(S)-228 (Vines et al., 1998); however, the amino acids at the terminal SA of all H3 isolates were Ile(I)-226 and Ser(S)-228, similar to the previous report by Lindstrom et al. (1996). The patterns of the antigenic site in theHAgene can be observed by amino acid alignment. The antigenic sites of H1N1 and H3N2 isolates were related to the sites already defined in a previous study of HA1. As for H1N1, antibodies are directed to each of the two strain-specific (Sa and Sb) and common antigenic sites (Ca and Cb) of the virus hemagglutinin (Caton et al., 1982).We detected four altered amino acids at the H1 HA1 antigenic sites; amino acids Ser(S)/Leu(L)-73 and Arg(R)/Lys(K)-77 at the Cb site and amino acids Arg(R)/Lys(K)-192 andLys(K)/Thr(T)-197 at the Sb site (Figure 12). As for H3N2, the antigenic sites A–E have been described by Wiley et al. (1981). We found three altered (95.78% based on nucleotides and 94.68% based on amino acids).

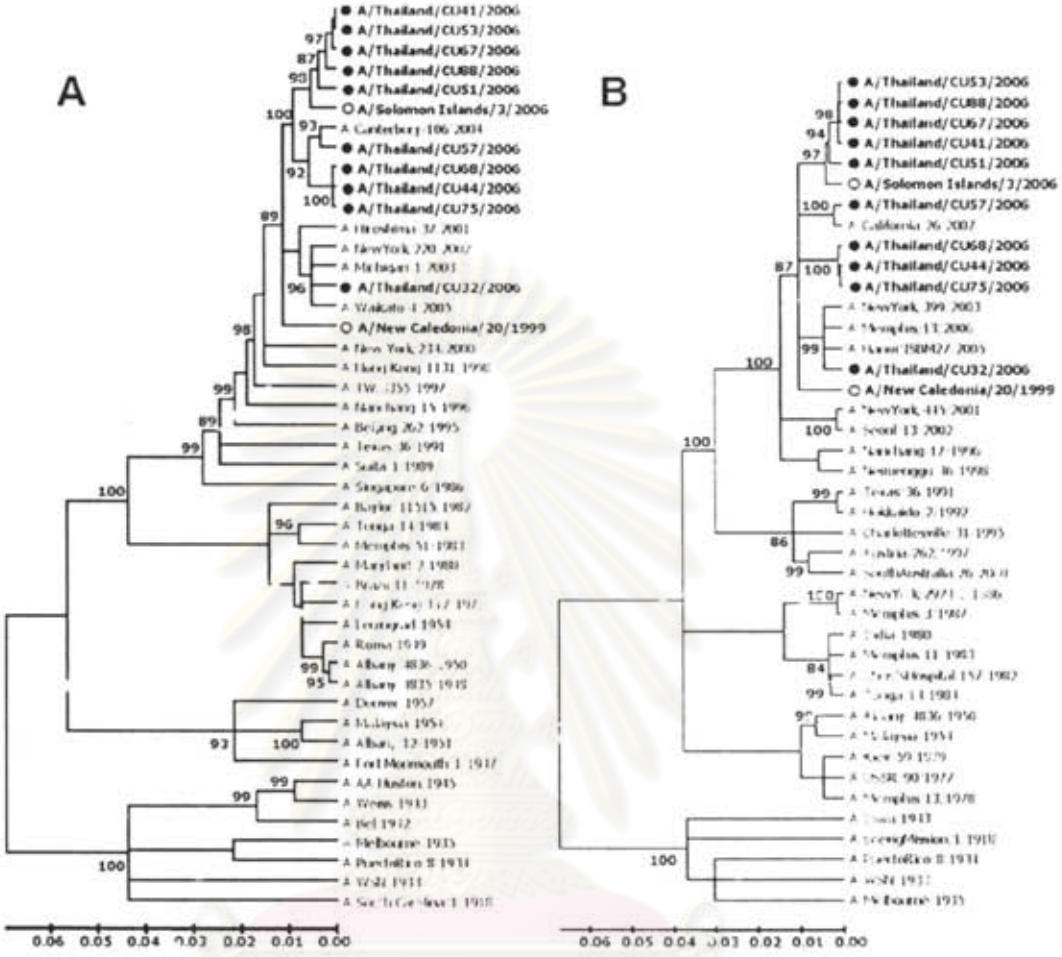

Figure 10 Phylogram of the hemagglutinin (HA) 1 region of the HA gene (A) and neuraminidase (NA) gene (B) of H1N1 isolates with the H1N1 vaccine strain and other H1N1 influenza viruses since 1918. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node (●; H1N1 isolates, ○; vaccine strain for subtype H1N1).

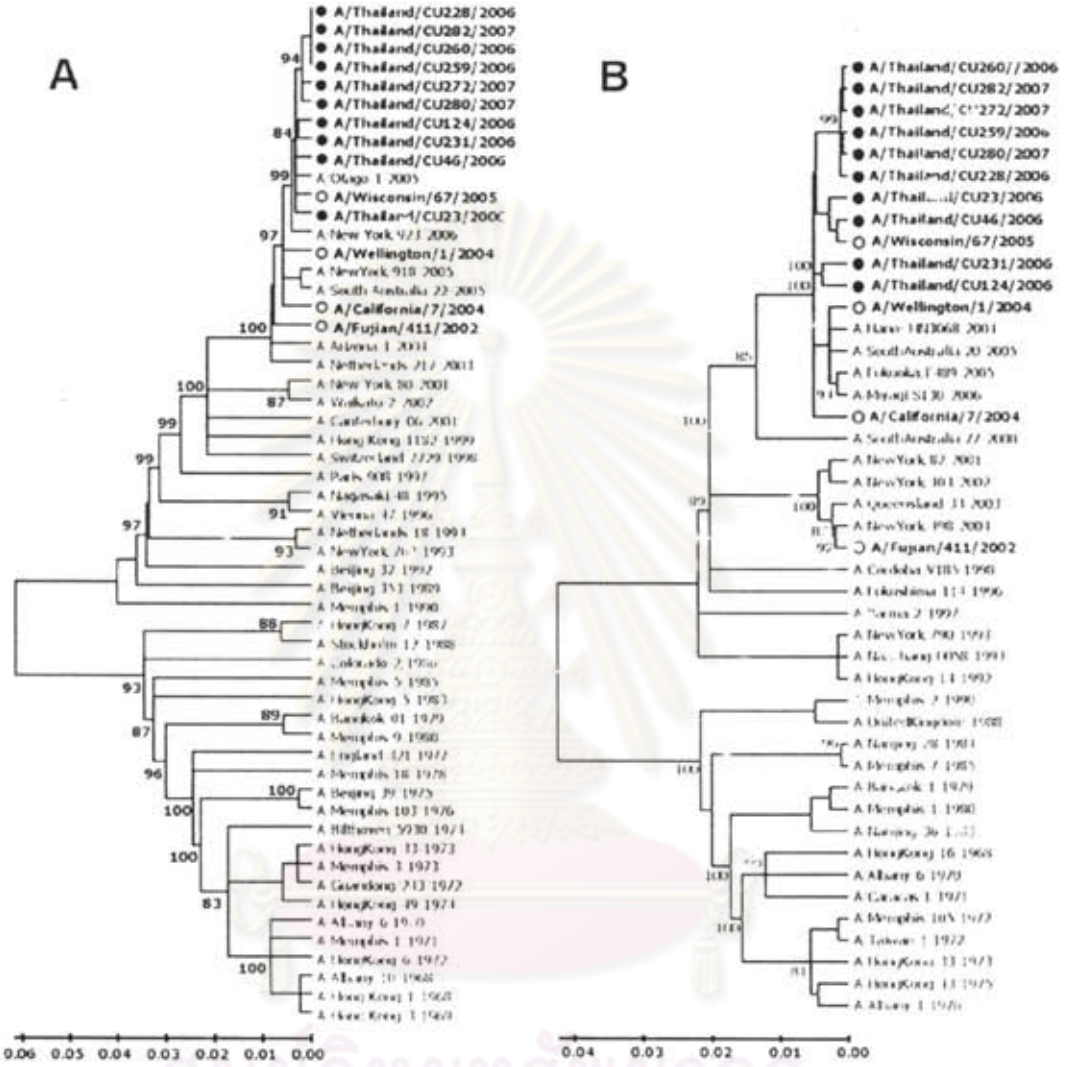

Figure 11 Phylograms of the hemagglutinin (HA) 1 region of the HA gene (A) and neuraminidase (NA) gene (B) of H3N2 isolates with H3N2 vaccine strains and other H3N2 influenza viruses since 1968. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node (●; H3N2 isolates, ○; vaccine strain for subtype H3N2)

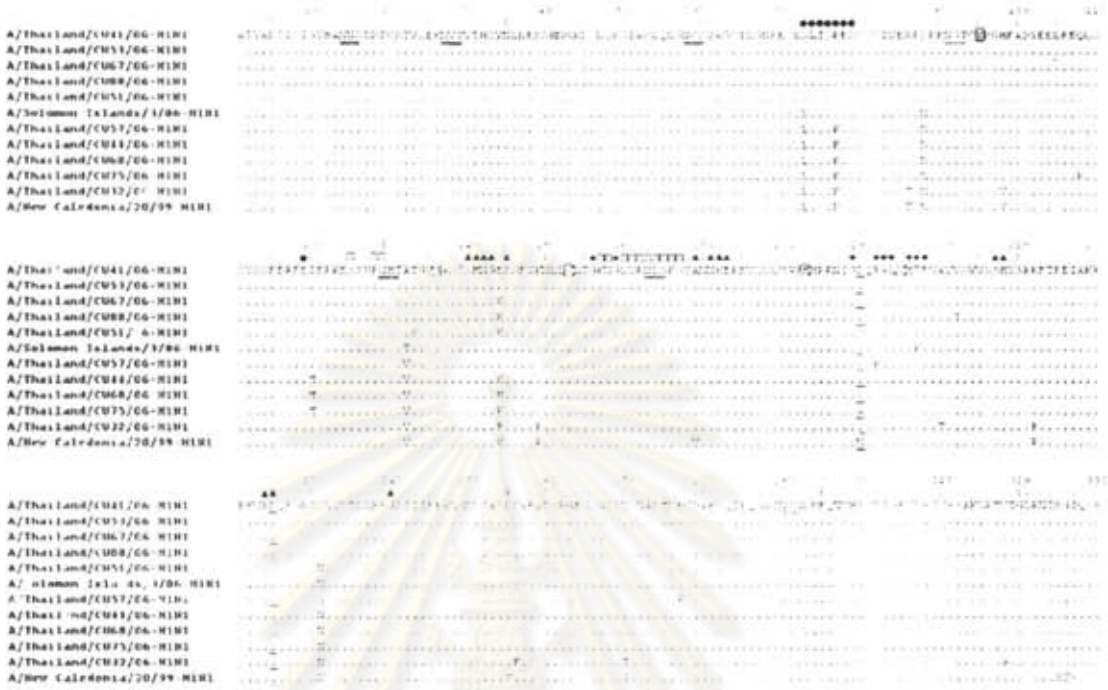

Figure 12 Amino acid comparison between HAI domains of H1N1 isolates and vaccine strains A/Solomon Islands/3/06 and A/New Caledonia/20/99. Dots represent amino acids similar to the consensus. The conserved amino acid residues at the receptor-binding site are shown as small rectangles. Alternative amino acids for sialic acid linkages of HA are underlined. The amino acid residues mapped at previously defined antigenic sites are shown as follows: site Sa (□), site Sb (◆), site Ca (▲), and site Cb (●). All potential N-linked glycosylation sequons (NXS/T) are double-underlined.

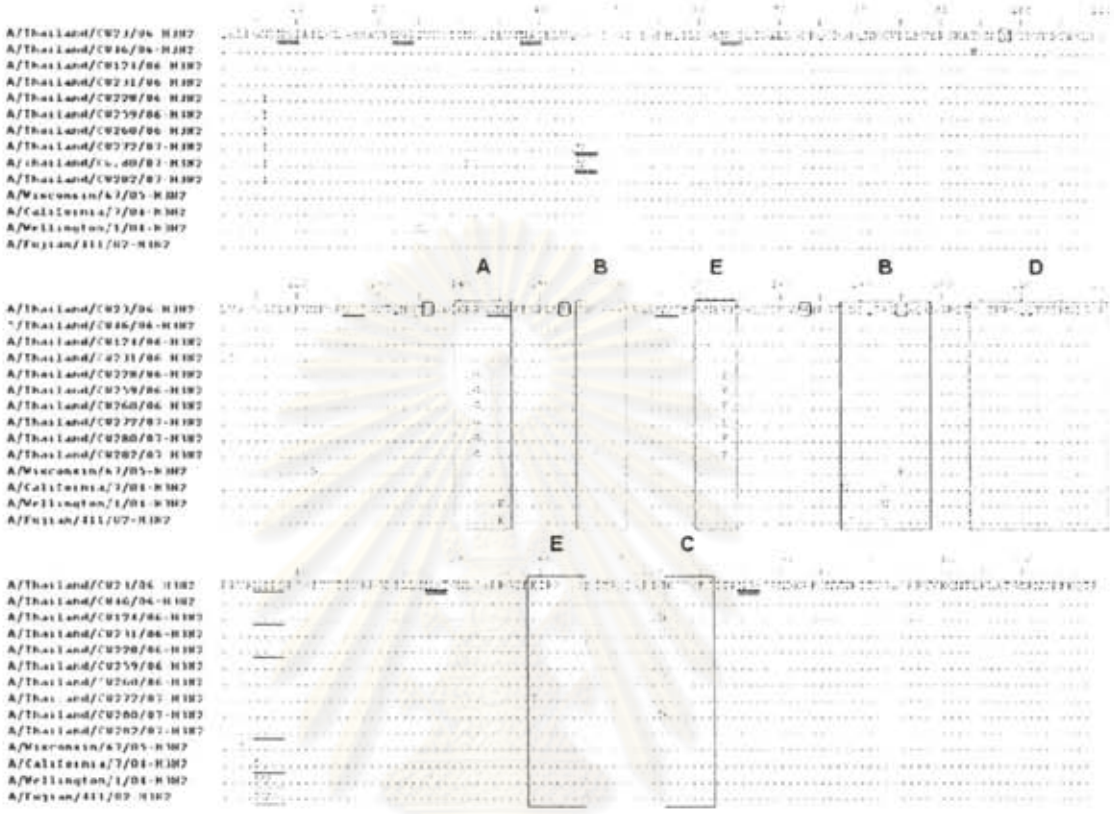

Figure 13 Amino acid comparison between HAI domains of H3N2 isolates and vaccine strains A/Wisconsin/67/05, A/California/7/04, A/Wellington/1/04 and A/Fujian/411/02. Dots represent amino acids similar to the consensus. The conserved amino acid residues at the receptor-binding site are shown as small rectangles. Alternative amino acids for sialic acid linkages of HA are underlined. The amino acid residues mapped at previously defined antigenic sites A-E are shown as large rectangles. All potential N-linked glycosylation sequons (NXS/T) are double-underlined.

For H3N2 HA1, A/Wisconsin/67/05 was used as the vaccine strain both in the Northern Hemisphere 2007–2008 and the Southern Hemisphere 2007. Ten H3N2 isolates indicated the amino acids at the H3 HA1 antigenic sites; Gly(G)/Arg(R)-142 at site A, Leu(L)/Ser(S)-157 at site B, and Glu(E)/Lys(K)-173 at site E (Figure 13). N-linked glycosylation was commonly found in HA of influenza A virus with (Asn-X-Ser/Thr) as the specific polypeptide for glycosylation where X can be any amino acid except for aspartic acid or proline. This sequence is known as a glycosylation sequon (Helenius and Aebi, 2004). There were 7 and 12 potential glycosylation sites on H1 and H3 HA, respectively (Figures 12 and 13). The HA2 sequences of both H1N1 and H3N2 isolates were largely conserved (data not shown).

We compared the nucleotide and deduced amino acid sequences of the NA from 20 isolated samples with the vaccine strains. As for H1N1 NA, 10 isolates from Thailand indicated a higher average of per cent similarity to A/Solomon Islands/3/2006 (98.34% based on nucleotides and 98.21% based on amino acids) than to A/New Caledonia/20/1999 (97.70% based on nucleotides and 97.73% based on amino acids). As for H3N2 NA, 10 isolates showed a higher average of per cent nucleotide and amino acid similarity to the vaccine strains A/Wisconsin/67/2005 (98.99%, 98.71%), A/California/7/2004 (98.85%, 99.09%), and A/Wellington/1/2004 (99.08%, 98.52%) than to A/Fujian/411/2002 (95.54%, 95.80%). The analysis of NA of H1N1 and H3N2 isolates showed conserved residues in all NA subtypes including the catalytic sites (R118, D151, R152, R224, E276, R292, R371, and Y406) (N2 numbering) and framework sites supporting the catalytic residues (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, and E425) (Colman et al., 1993; Colman, 1994). However, we detected amino acid substitutions in the neuraminidase gene of influenza virus subtype H1N1 and H3N2 isolates not related to catalytic or framework sites as shown in Table 7.

Table 7 Amino acid substitutions in the neuraminidase gene of influenza virus subtypes H1N1 and H3N2 isolated in Thailand compared with vaccine strains.

Virus strain	Amino acid position of NA gene														
H1N1	23	41	59	64	77	94	173	214	234	266	267	273	359	382	452
(N2 numbering)	(23)	(41)	(56)	(61)	(74)	(94)	(172)	(213)	(233)	(265)	(266)	(272)	(362)	(385)	(452)
A/Thailand/CU53/06	I	E	S	N	E	I	R	E	M	T	M	N	M	N	G
A/Thailand/CU88/06	I	E	S	N	E	I	R	E	M	T	M	N	M	N	G
A/Thailand/CU67/06	I	E	S	N	E	I	R	E	M	T	M	N	M	N	G
A/Thailand/CU41/06	I	E	S	N	E	I	R	E	M	T	M	N	M	N	G
A/Thailand/CU51/06	I	G	S	N	E	I	R	E	M	T	I	N	I	N	G
A/Solomon Islands/3/06	I	G	S	N	E	I	R	E	M	T	I	N	I	N	G
A/Thailand/CU44/06	M	G	N	H	G	V	K	E	M	S	I	S	I	N	N
A/Thailand/CU75/06	M	G	N	H	G	V	K	E	M	S	I	S	I	N	N
A/Thailand/CU68/06	M	G	N	H	G	V	K	G	M	S	I	S	L	N	N
A/Thailand/CU57/06	M	G	S	H	E	I	K	G	M	S	I	N	I	N	D
A/Thailand/CU32/06	M	G	S	H	G	I	K	E	V	S	R	N	I	D	D
A/New Caledonia/20/99	M	G	S	H	G	I	K	E	V	S	I	N	I	D	D

H3N2	43	93	150	194	310	295	370	372
A/Thailand/CU272/07	S	D	H	V	Y	R	S	S
A/Thailand/CU260/06	S	D	H	V	Y	R	S	S
A/Thailand/CU282/07	S	D	H	V	Y	R	S	S
A/Thailand/CU228/06	S	D	H	V	Y	R	S	S
A/Thailand/CU259/06	S	D	H	V	Y	R	S	S
A/Thailand/CU280/07	S	D	H	V	Y	R	S	S
A/Thailand/CU23/06	N	N	H	V	Y	S	S	S
A/Thailand/CU231/06	N	D	R	I	H	S	S	L
A/Thailand/CU124/06	N	D	R	I	H	S	S	L
A/Thailand/CU46/06	N	N	H	V	Y	S	L	S
A/Wisconsin/67/05	N	N	H	V	Y	S	L	S
A/California/7/04	N	D	H	V	Y	S	L	S
A/Wellington/1/04	N	D	H	V	Y	S	L	S
A/Fujian/411/02	N	N	H	V	Y	S	L	S

4. Discussion

From February 2006 to February 2007, we colleted nasopharyngeal suction samples by inserting a nasal catheter into the posterior nasopharynx of pediatric patients diagnosed with respiratory tract illness, and placed them in virus transport medium. We extracted viral RNA and dissolved it in high concentration avoiding embryonated egg or MDCK cell inoculation in order to prevent adaptation associated with alteration of receptor binding properties (Widjaja et al., 2006). Of 302 specimens, 33 (10.92%) were positive for influenza A virus and other respiratory viruses detected by Chieochansin et al. (2007). Those specimens included 20 (6.62%) positive for HBoV, 48 (15.89%) positive for RSV, 28 (9.27%) positive for hMPV, 18 (5.9%) positive for adenovirus, 14 (4.63%) positive for parainfluenza and 1 (0.33%) positive for influenza B virus. We characterized the respective subtypes of influenza A virus by preliminary sequencing using primers specific for the 5'- and 3'-ends of the HA and NA gene and extending the inner nucleotide of the HA or NA gene by using overlap primers specific for each subtype.

To date, molecular and phylogenetic analysis of influenza virus spanning an entire year has not been reported in Thailand. Based on our results, two subtypes of influenza A virus, H1N1 and H3N2, alternatively infected pediatric patients throughout the year. Each year, WHO recommends the most suitable composition of influenza vaccine strains for the Northern and Southern Hemispheres, respectively. Comparison of the nucleotide and amino acid sequences of the HA1 region and NA genes between both H1N1 and H3N2 isolates and the vaccine strains showed they are closely related to the vaccine strains recommended for the Northern Hemisphere 2007–2008.

HA gene characterization showed higher variation in HA1 than HA2, 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 as previously reported (Parrish and Kawaoka, 2005; Skehel and Wiley, 2000). The same amino acid substitution had been described by Lekcharoensuk et al. (2006) in

H3N1 swine influenza virus. Since Leu, Ile, and Val are similar neutral non-polar amino acids, substitution between them most likely maintains hydrophobic interactions and proper conformation of the binding pocket. Furthermore, the substitution of V226I had been found in the adaptation of A/Fujian/411/2002 (H3N2) in eggs during serial passages in the amniotic then allantoic cavities aimed at augmenting growth (Widjaja et al., 2006). Variations in H1N1 and H3N2 isolates were predominantly located at the antigenic site, which is of interest for developing suitable vaccine strains. In this context, Wilson and Cox (1990) proposed that epidemiologically important drift variants usually display four or more amino acid substitutions located at two or more antigenic sites on the HA1 protein. The N-linked glycosylation is conserved among various HA subtypes of influenza A viruses. Its presence or absence may cause increase or loss of function of the glycoprotein (Panda et al., 2004) because N-linked glycosylation can initiate and maintain folding, stability, solubility, transportation, antigenicity, and immunogenicity of the protein. HA1 H3 amino acid characterization (Figure 13) also showed a probable N-linked glycosylation site at amino acids 45–47 of two isolates (A/Thailand/CU272/07 and A/Thailand/CU280/07) which may affect the protein's function.

As for the NA gene, we discovered more amino acid substitutions in N1 than in N2. None were at the catalytic or framework sites of the neuraminidase enzyme. These changes may occur because the virus requires NA to be more active whenever HA has undergone substitutions as suggested in the previous report on molecular changes associated with adaptation of human influenza A virus in embryonated chicken eggs (Widjaja et al., 2006).

In conclusion, the present study has confirmed conserved sequences as well as discovered variations due to amino acid substitutions at the receptor-binding site and hence, the antigenic site including the potential N-linked glycosylation site of HA, and at the catalytic and framework sites of NA in both H1N1 and H3N2 subtypes of 20 influenza A virus isolates from Thailand. The phylogenetic tree also showed close similarities to the current vaccine strains for the Northern Hemisphere 2007–2008.

CHAPTER VII

GENETIC CHARACTERIZATION OF H1N1, H1N2 AND H3N2 SWINE INFLUENZA VIRUS IN THAILAND

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Swine have been known to be a suitable host for influenza A virus. In Thailand, phylogenetic analysis on swine influenza virus (SIV) has as yet not been attempted. The present report presents molecular and phylogenetic analysis performed on SIV in Thailand. In this study, 12 SIV isolates from the central and eastern part of Thailand were subtyped and the molecular genetics of hemagglutinin and neuraminidase were elucidated. Three subtypes, H1N1, H1N2 and H3N2, are described. Phylogenetic analysis of the SIV hemagglutinin and neuraminidase genes shows individual clusters with swine, human or avian influenza virus at various global locations. Furthermore, amino acid substitutions were detected either at the receptor binding site or the antigenic sites of the hemagglutinin gene.

1. Introduction

Swine influenza virus (SIV) has first been detected during the Spanish influenza pandemic in the USA during 1918–1919. In 1930, the porcine virus has first been isolated from humans and classified as a member of the family *Orthomyxoviridae*, species influenza A virus (Myers et al., 2007). Three different SIV subtypes, H1N1, H3N2 and H1N2, have been circulating in swine worldwide. H1N1 SIV known as classical SIV, having spread throughout the major swine populations of the world. In the USA, the predominant H1N1 SIV has remained antigenically conserved as classical SIV, whereas the virus found in Europe is entirely of avian origin (van Reeth, 2007). Subsequent to the human "Hong Kong flu" pandemic, the human H3N2 virus has been transmitted to pigs. Due to re-assortment, the newly resulting virus is a human-like swine H3N2 virus with the HA and NA of the human virus and the internal proteins either derived from classical swine H1N1 as detected in the USA (van Reeth, 2007) or by re-assortment obtained from the avian-like swine H1N1 virus as found in Italy (Castrucci et al., 1993). In addition, re-assorted human-like swine H1N2 virus has become increasingly important. H1N2

subtype viruses have also originated from genetic re-assortment between the H1N1 and H3N2 viruses. In Europe, the virus resulting from re-assortment contains H1 HA closely related to that of human H1N1 viruses circulating in the early 1980s, a human-like N2 NA and other internal genes corresponding to avian-like H1N1 viruses (Marozin et al., 2002) whereas in the US, the virus has been derived from re-assortment between a classical swine H1N1 virus and a swine-human-avian re-assorted H3N2 virus (Karasin et al., 2000b). Recently, newly re-assorted subtypes of swine influenzas have been identified as for example, subtypes H3N1 (Ma et al., 2006; Shin et al., 2006), H4N6 (Karasin et al., 2000a) and H9N2 (Cong et al., 2007).

In Asia, particularly in China, classical H1N1 SIV apparently constitutes the predominant influenza A virus infecting pigs and in south China, an independent transfer of H1N1 virus from birds to pigs has been reported (Guan et al., 1996). Moreover, some of the H3N2 viruses isolated from pigs in China since 1970s have been entirely avian-like (Kida et al., 1988). In Thailand, there have been a few reports on SIV. Since 1978, H3N2 SIV in Thailand has been reported displaying serological relatedness to contemporary human strains (Nerome et al., 1981) and since 1988, H1N1 SIV has been the first virus reported showing serological and antigenic relatedness to the isolate from the USA (Kupradinun et al., 1991). Until 2004, serological studies of SIV H1N1 H1N2 and H3N2 were conducted on farmed pigs in Thailand (Damrongwatanapokin et al., 2006). In addition, the recent study (Komadina et al., 2007) has demonstrated that since 2005 H1N1 SIV isolates from humans from Thailand have displayed the highest degree of similarity to the classical swine H1 viruses circulating in Asia and the USA and that the N1 protein is related to viruses circulating in European swine. This present study presents molecular characterization and phylogenetic analysis of SIV in Thailand. Both the HA and NA gene of SIV subtypes H1N1, H1N2 and H3N2 were characterized and the amino acids at the receptor binding site and antigenic sites were evaluated.

2. Materials and Methods

2.1. Clinical swine samples

Clinical swine samples were collected from the central part - Saraburi, Nakhon Pathom and Ratchaburi provinces - and the eastern part - Chachoengsao and Chon Buri provinces - of Thailand. All nasal swabs were incubated using specific antibody negative embryonated chicken eggs according to the method described by OIE (Office International des Epizooties), subsequently the hemagglutination (HA) test was performed.

2.2. RNA extraction and cDNA synthesis

RNA was extracted from 140 µl allantoic fluid of each sample using the QIAmp viral RNA mini kit (Qiagen, GmbH, Germany) according to the manufacturer's specifications. cDNAs were synthesized at 37°C for 2 hours using the M-MLV reverse-trancription system (Promega, Madison, WI) comprising 200 units of M-MLV reverse transcriptase, 5 µl of 5X M-MLV reaction buffer, 5 µl of 10mM dNTP, 25 units of RNasin® ribonuclease Inhibitor, 1 µM of universal primer (Uni12 primer 5'-AGCAAAAGCAGG -3') as described by Hoffmann et al (2001). Twelve µl of RNA were heated to 70°C for 5 min, then cooled on ice and nuclease-free water was added to a final volume of 25 µl.

2.3. Full length PCR amplification of HA and NA

HA positive samples were confirmed by PCR using primers specific for the Matrix (M) gene as described elsewhere (Payungporn et al., 2004). Subsequently, full length HA and NA genes were amplified using the forward and reverse primers published by Hoffmann et al (2001) with modifications. The HA gene was amplified using 0.5 µM of HAF5': 5'-CAGGGAGCAAAAGCAGGGG-3' and 0.5 µM of HAR3': 5'-CCAGTAGAAACAAGGGTGTTTT-3', the NA gene was amplified using 0.5 µM of NAF5': 5'-CAGGGAGCAAAAGCAGGAGT-3' and 0.5 µM of NAR3': 5'- CCAGTAGAAACAAGGA GTTTTTT-3' with 10 µl of 2.5X Eppendorf MasterMix (Eppendorf, Hamburg, Germany), 2 µl of cDNA and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following

conditions: Denaturation at 94°C for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 2 min, and concluded by a final extension step at 72°C for 7 min.

2.4. Sequencing and phylogenetic analysis

The resulting amplicons were analyzed by 2% agarose gel electrophoresis and purified with the Perfectprep Gel Cleanup Kit (Eppendorf, Hamburg, Germany). DNA sequencing was carried out using the Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction kit (ABI, Foster City, CA) together with the respective inner primer which was designed de novo to ascertain specificity for each sequence (Primers are available upon request). Subsequently, any unincorporated labeled ddNTPs were removed by ethanol precipitation. The reactions were resolved on an ABI-Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT), sequences were edited by Chromas Lite version 2.01 (Technelysium Pty Ltd, Australia), SeqMan program (DNASTAR, Madison, WI) and the Bioedit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999). Finally, sequences were aligned using ClustalX (Thompson et al., 1997). Phylogenetic trees were constructed based on continuous nucleotide sequence alignment and genetic distances were calculated applying Kimura's two parameter method using MEGA3.1 (Kumar et al., 2004), and applied to construct neighbour-joining (NJ) trees. Confidence values for the tree topologies were evaluated by bootstrap analysis of 1000 pseudo-replicate datasets.

3. Results

3.1. SIV subtype and BLAST analysis

Clinical swine samples were collected from the central part - Saraburi, Nakhon Pathom and Ratchaburi provinces - and the east - Chachoengsao and Chon Buri provinces - of Thailand. In total, 12 extracted RNA samples were sent to the Center of Excellence in Clinical Virology, Chulalongkorn University, Bangkok, Thailand. In each sample, the hemagglutinin (HA) and neuraminidase (NA) genes were amplified and sequenced. SIV subtypes were analyzed using the BLAST (Basic Local Alignment

Search Tool) program available at http://www.ncbi.nlm.nih.gov/blast/. Three SIV subtypes, H1N1, H1N2 and H3N2 were identified and their respective distribution in each area is shown in Figure 14. Strain name and subtype of each virus, accession number, year and homology analysis performed on the HA and NA genes are depicted in table 8.

3.2. H1N1 swine influenza virus

Four of 12 SIV isolates were characterized as H1N1 subtype. Phylogenetic analysis of the HA and NA genes of SIV H1N1 from Thailand in comparison with other nucleotide sequences is shown in Figure 15 and 16, respectively. The HA sequences of 4 strains, A/Swine/Chachoengsao/NIAH587/2005 (NIAH587), A/Swine/Chonburi/NIAH589/2005 (NIAH589), A/Swine/Chonburi/05CB1/2005 (05CB1) and A/Swine/Chonburi/06CB2/2006 (06CB2), isolated from Thailand clustered with the classical SIV sequences determined for the American and Asian SIV along with the swine-like human influenza virus strain A/Thailand/271/05(H1N1) (Komadina et al., 2007). In contrast, the NA gene sequences of SIV H1N1 isolated from Thailand clustered with the avian-like SIV from Europe. The HA genes of all 4 isolates showed the highest homology to A/Thailand/271/05(H1N1). However, the NA genes of NIAH587 and NIAH589 were distinctly homologous to A/Swine/England/195852/92(H1N1) whereas the NA genes of 05CB1 and 06CB2 showed pronounced homology to A/Thailand/271/05(H1N1).

Table 8 BLAST subtype and homology analysis of the HA and NA genes of 12 swine influenza viruses in Thailand

Virus strains	Subtype	Province	Year	Accession number		Hemagglutinin (HA) gene			Neuraminidase (NA) gene		
				HA	NA	Virus with highest homology	Nucleotide analyzed	%[a]	Virus with highest homology	Nucleotide analyzed	%[b]
A/Swine/Chonburi/NIAH589/05	H1N1	Chon Buri	2005	EU296599	EU296600	A/Thailand/271/05(H1N1)	1-1032	95	A/Swine/England/195852/92(H1N1)	22-1381	93
A/Swine/Chachoengsao/NIAH587/05	H1N1	Chachoengsao	2005	EU296601	EU296602	A/Thailand/271/05(H1N1)	1-1032	95	A/Swine/England/195852/92(H1N1)	22-1381	93
A/Swine/Chonburi/05CB1/05	H1N1	Chon Buri	2005	EU296603	EU296604	A/Thailand/271/05(H1N1)	1-1032	97	A/Thailand/271/05(H1N1)	22-1381	97
A/Swine/Chonburi/06CB2/06	H1N1	Chon Buri	2006	EU296605	EU296606	A/Thailand/271/05(H1N1)	1-1032	97	A/Thailand/271/05(H1N1)	22-1381	97
A/Swine/Saraburi/NIAH13021/05	H1N2	Saraburi	2005	EU296607	EU296608	A/Thailand/271/05(H1N1)	46-1032	94	A/swine/UK/119404/91(H3N2)	23-1382	92
A/Swine/Chachoengsao/NIAH-Nongwa/03	H3N2	Chachoengsao	2003	EU296609	EU296610	A/swine/Italy/729/88(H3N2)	49-1032	89	A/swine/Gent/1/84(H3N2)	34-1380	92
A/Swine/Chachoengsao/NIAH586/05	H3N2	Chachoengsao	2005	EU296611	EU296612	A/Bilthoven/2600/75(H3N2)	49-1032	88	A/Albany/20/74(H3N2)	34-1380	93
A/Swine/Nakhon pathom/NIAH586-1/05	H3N2	Nakhon pathom	2005	EU296613	EU296614	A/Bilthoven/2600/75(H3N2)	49-1032	89	A/Albany/20/74(H3N2)	34-1377	89
A/Swine/Nakhon pathom/NIAH586-2/05	H3N2	Nakhon pathom	2005	EU296615	EU296616	A/Bilthoven/2600/75(H3N2)	49-1032	89	A/Victoria/4/72(H3N2)	34-1377	90
A/Swine/Ratchaburi/NIAH874/05	H3N2	Ratchaburi	2005	EU296617	EU296618	A/Caracas/422/97(H3N2)	49-1032	96	A/Christchurch/1/96(H3N2)	34-1380	96
A/Swine/Ratchaburi/NIAH9426/05	H3N2	Ratchaburi	2005	EU296619	EU296620	A/Caracas/422/97(H3N2)	49-1032	96	A/Christchurch/1/96(H3N2)	34-1380	95
A/Swine/Chonburi/05CB2/05	H3N2	Chon Buri	2005	EU296621	EU296622	A/Bilthoven/2600/75(H3N2)	49-1032	89	A/Albany/20/74(H3N2)	34-1377	89

Percent homology of hemagglutinin[a] and neuraminidase[b] gene of swine influenza viruses in Thailand from BLAST analysis

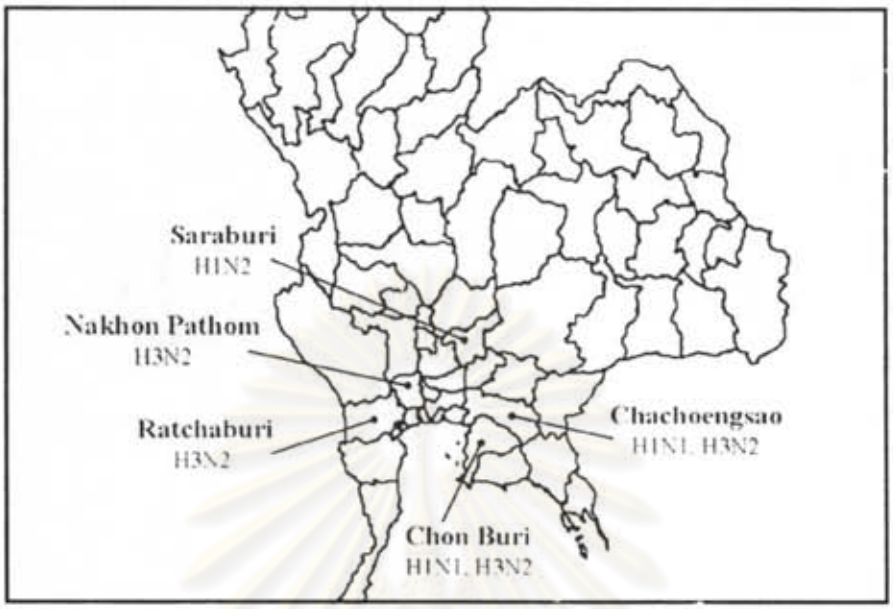

Figure 14 Map of Thailand highlighting five provinces, Saraburi, Nakhon Pathom and Ratchaburi province in the center and Chachoengsao and Chon Buri province in the east where swine influenza viruses were isolated.

3.3. H1N2 swine influenza virus

SIV H1N2 was initially isolated in Europe and North America in 1994 and 1998, respectively (van Reeth, 2007). SIV H1N2 isolated in this study has for the first time been reported in Thailand for the A/Swine/Saraburi/NIAH13021/2005 (NIAH13021) strain in Thailand. Phylogenetic analysis performed on the HA and NA genes of SIV H1N2 comparing between the Thai isolate and other SIV H1N2 subtypes is shown in Figures 17 and 18, respectively. The H1N2 HA sequence of the NIAH13021 strain clustered with the American and Asian SIV H1N2 whereas the NA sequence of the same strain clustered with the European SIV group. The HA gene of the NIAH13021 isolate was predominantly homologous to the human isolate A/Thailand/271/05(H1N1) whereas the NA gene showed pronounced homology to SIV A/swine/UK/119404/91(H3N2). In addition, comparisons between H1N2 and H1 of H1N1 as well as N2 of H3N2 are shown in Figures 15 and 20, respectively. The H1N2 HA of NIAH13021 clustered with SIV H1N1

isolated from Thailand and also with A/Thailand/271/05(H1N1). The H1N2 NA of NIAH13021 clustered with 4 SIV H3N2 isolates from Thailand.

3.4. H3N2 swine influenza virus

Seven of the 12 SIV isolates displayed the H3N2 subtype. Phylogenetic analysis performed on the HA and NA genes of SIV H3N2 from Thailand in comparison with other nucleotide sequences is shown in Figures 19 and 20. The phylogram of HA and NA shows that two H3N2 isolates, A/Swine/Ratchaburi/NIAH874/2005 (NIAH874) and A/Swine/Ratchaburi/NIAH9426/2005 (NIAH9426), cluster with the American and Asian SIV and that their HA genes are predominantly homologous to A/Caracas/422/97(H3N2) whereas their NA genes are more profoundly homologous to A/Christchurch/1/96(H3N2). One sample, A/Swine/Chachoengsao/NIAH-Nongwa/2003 (Nongwa), was grouped with the European SIV for both HA and NA and showed the highest homology to A/swine/Italy/729/88 for HA and A/swine/Gent/1/84 for NA. As for the remaining 4 samples, A/Swine/Chachoengsao/NIAH586/2005 (NIAH586), A/Swine/Nakhon pathom/NIAH586-1/2005 (NIAH586-1), A/Swine/Nakhon pathom/NIAH586-2/2005 (NIAH586-2) and A/Swine/Chonburi/05CB2/2005 (05CB2), the HA genes were grouped with H3N2 human influenza virus from the 1970s whereas the NA genes clustered on a separate branch of European SIV although they displayed the highest homology for both HA and NA to H3N2 human influenza virus from the 1970s.

3.5. Receptor-binding and antigenic sites analysis

Hemagglutinin (HA) constitutes the receptor-binding site and the target for neutralizing antibodies. Terminal sialic acid (SA) residues of glycoproteins and glycolipids represent the cellular receptors for influenza A virus. Alignments of the HA1 amino acids of H1 and H3 SIV isolates are shown in Figures 21A and B. Five conserved amino acid residues, Tyr(Y)-98, Ser(S)-136, Trp(W)-153, His(H)-183 and Tyr(Y)-195 (numbering according to H3 structure) at the hemagglutinin receptor binding site of H1 and H3 SIV which have been described by Skehel and Wiley (2000) are highlighted.

In contrast to the previous report (Skehel and Wiley, 2000) one amino acid residue at position 136 of the SIV H1 isolates was Thr (T) instead of Ser (S). Nevertheless, SIV H3 isolates were conserved. With H1, SA binding was specific to the NeuAcα2,6Gal linkage due to amino acids Asp(D)-190 and Asp(D)-225 although the SIV H1 isolates had Gly(G) instead of D at position 225. As for H3, amino acids Ile(I)-226 and Ser(S)-228 were specific to the NeuAcα2,6Gal linkage but the SIV H3 isolates had Ile(I) or Leu(L) at amino acid position 226. The antigenic sites of the H1 and H3 isolates were related to the sites already defined in a previous study of HA1 (Caton et al., 1982; Wiley et al., 1981). As for H1, antibodies are directed to each of the two strain-specific (Sa and Sb) and common antigenic sites (Ca and Cb) of the virus hemagglutinin (Caton et al., 1982). Upon comparison between the various SIV isolates, we detected several different amino acids at the H1 HA1 antigenic sites (Figure 21A). As for H3 and its antigenic sites A – E described by Wiley et al. (1981), several distinct amino acids were found at each distinctive antigenic site (Figure 21B).

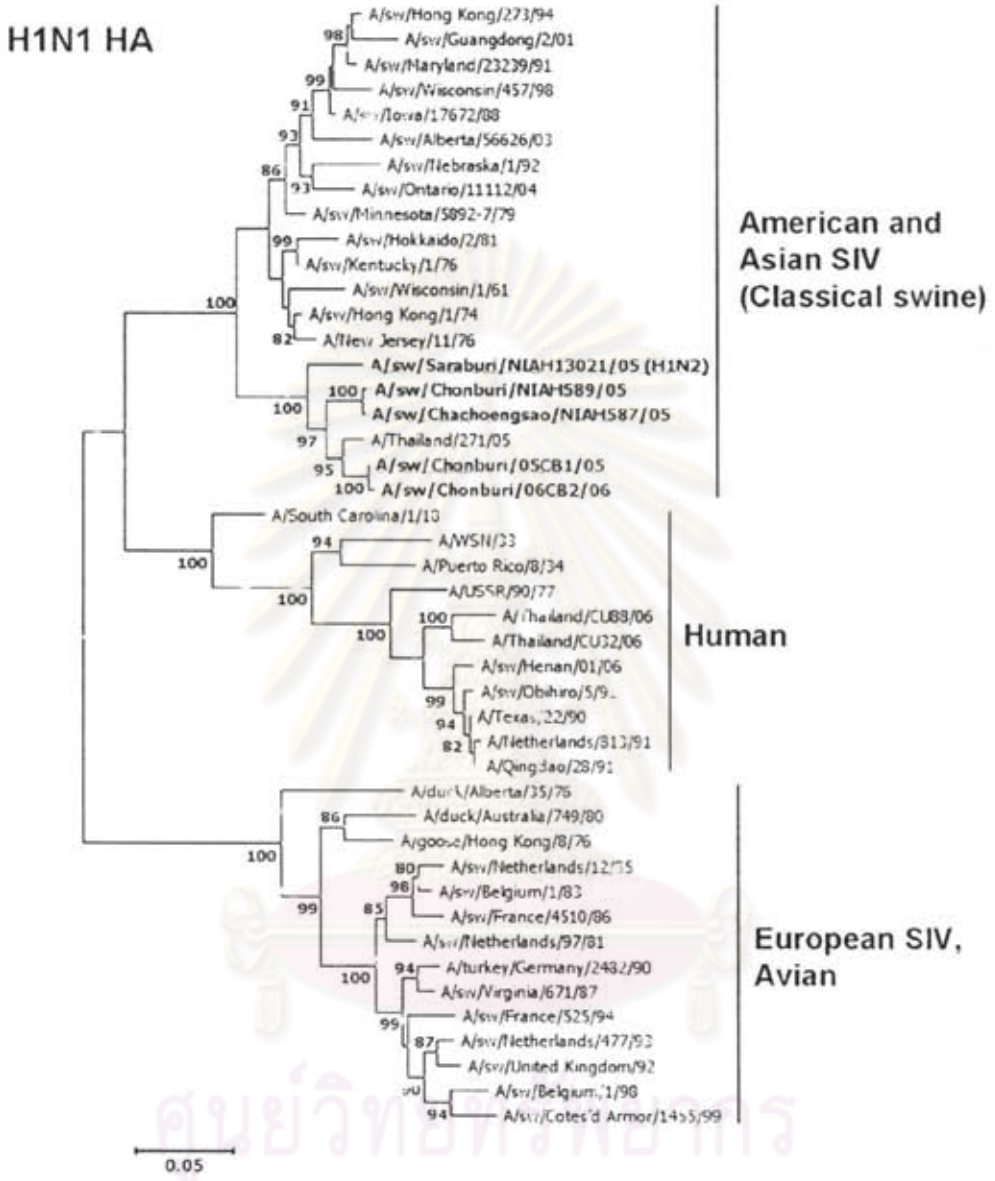

Figure 15 Phylograms of the hemagglutinin (HA) 1 region of the HA gene of SIV H1N1 isolates from Thailand with nucleotide sequences determined for each subtype of avian, human and swine influenza virus from several areas. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. Only bootstrap values above 80 are shown.

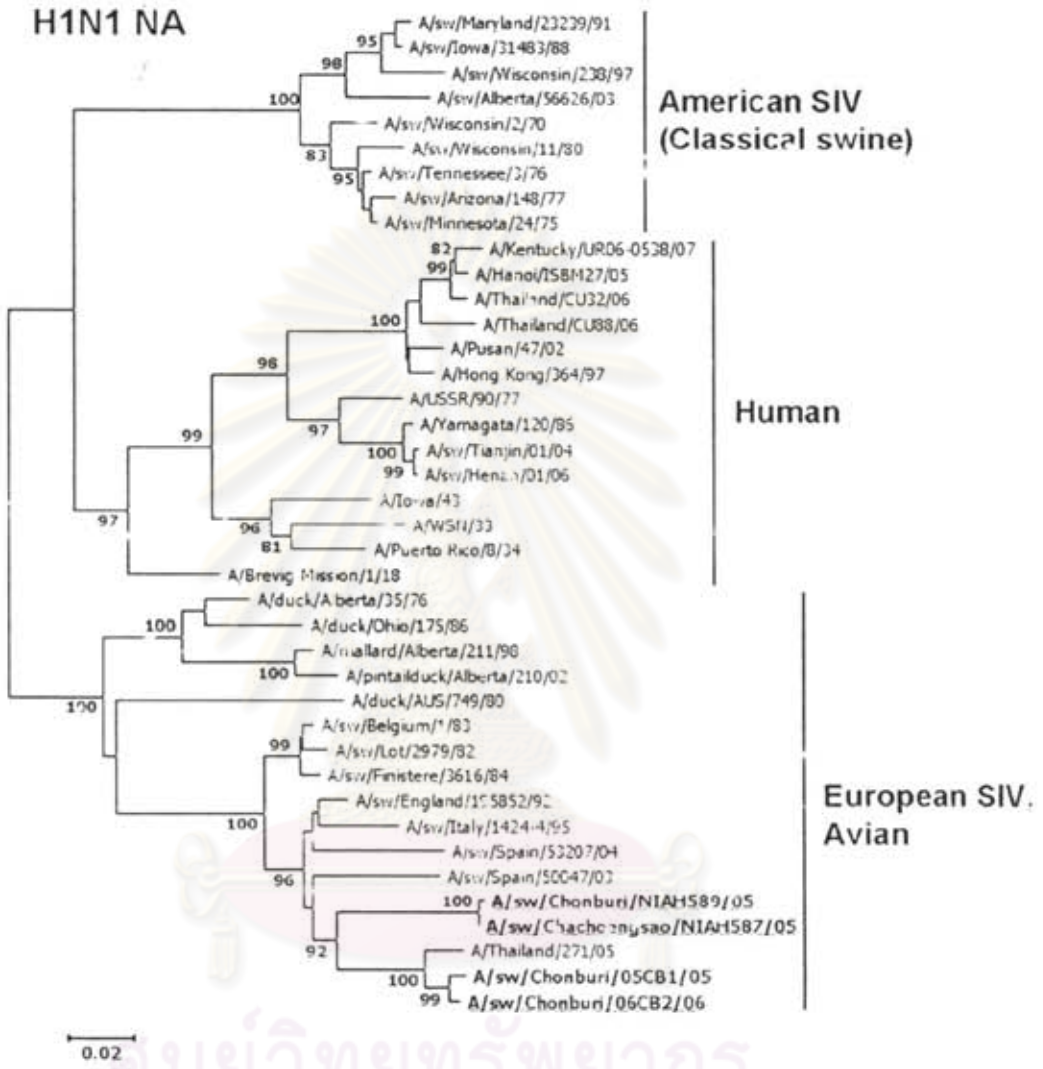

Figure 16 Phylograms of the NA gene of SIV H1N1 isolates from Thailand with nucleotide sequences determined for each subtype of avian, human and swine influenza virus from several areas. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. Only bootstrap values above 80 are shown.

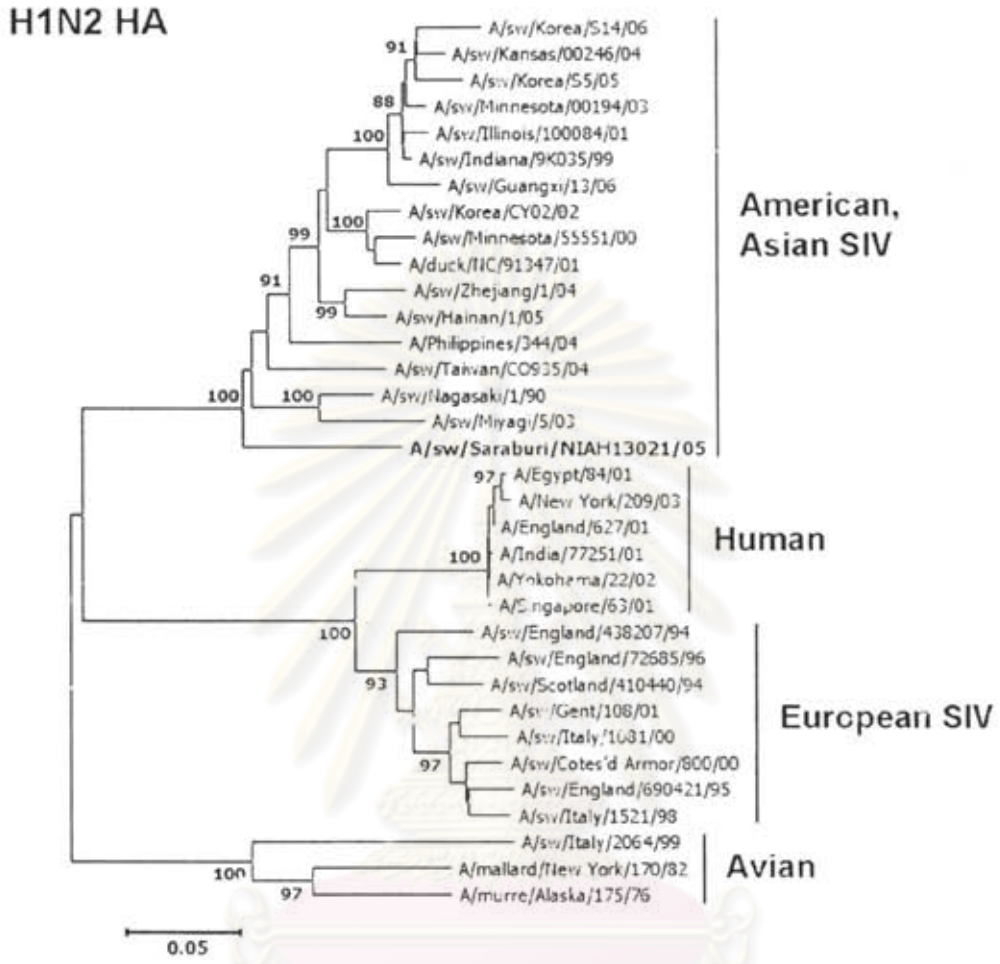

Figure 17 Phylograms of the hemagglutinin (HA) 1 region of the HA gene of SIV H1N2 isolates from Thailand with nucleotide sequences determined for each subtype of avian, human and swine influenza virus from several areas. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. Only bootstrap values above 80 are shown.

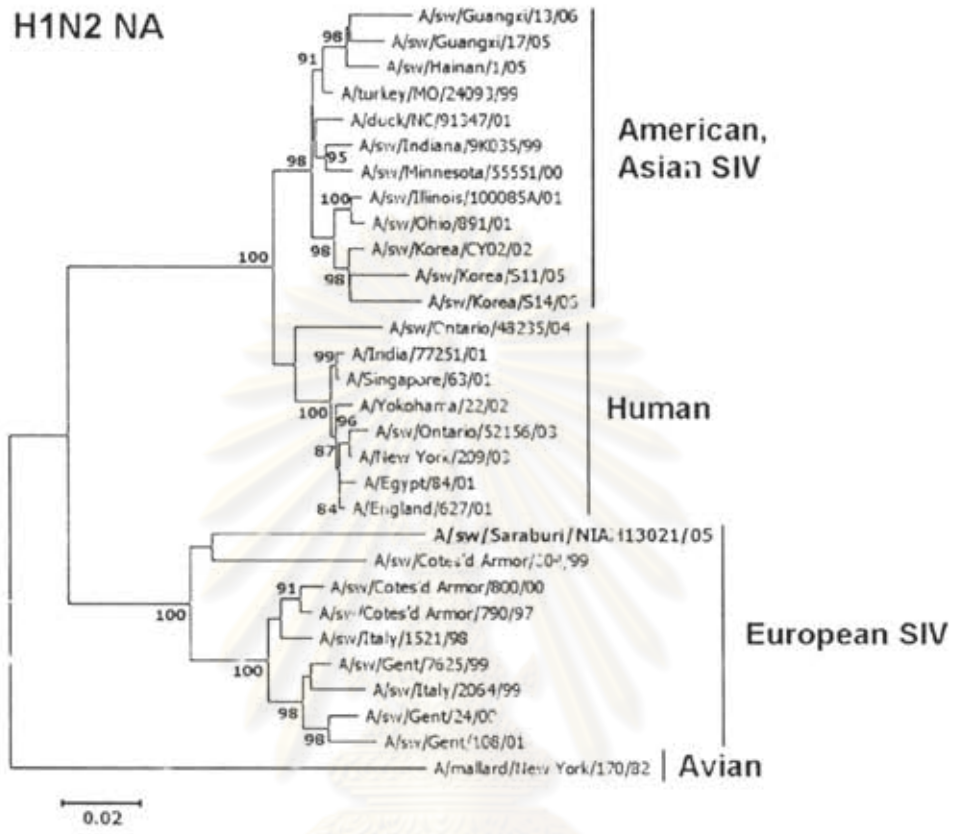

Figure 18 Phylograms of the NA gene of H1N2 isolates from Thailand with nucleotide sequences determined for each subtype of avian, human and swine influenza virus from several areas. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. Only bootstrap values above 60 are shown.

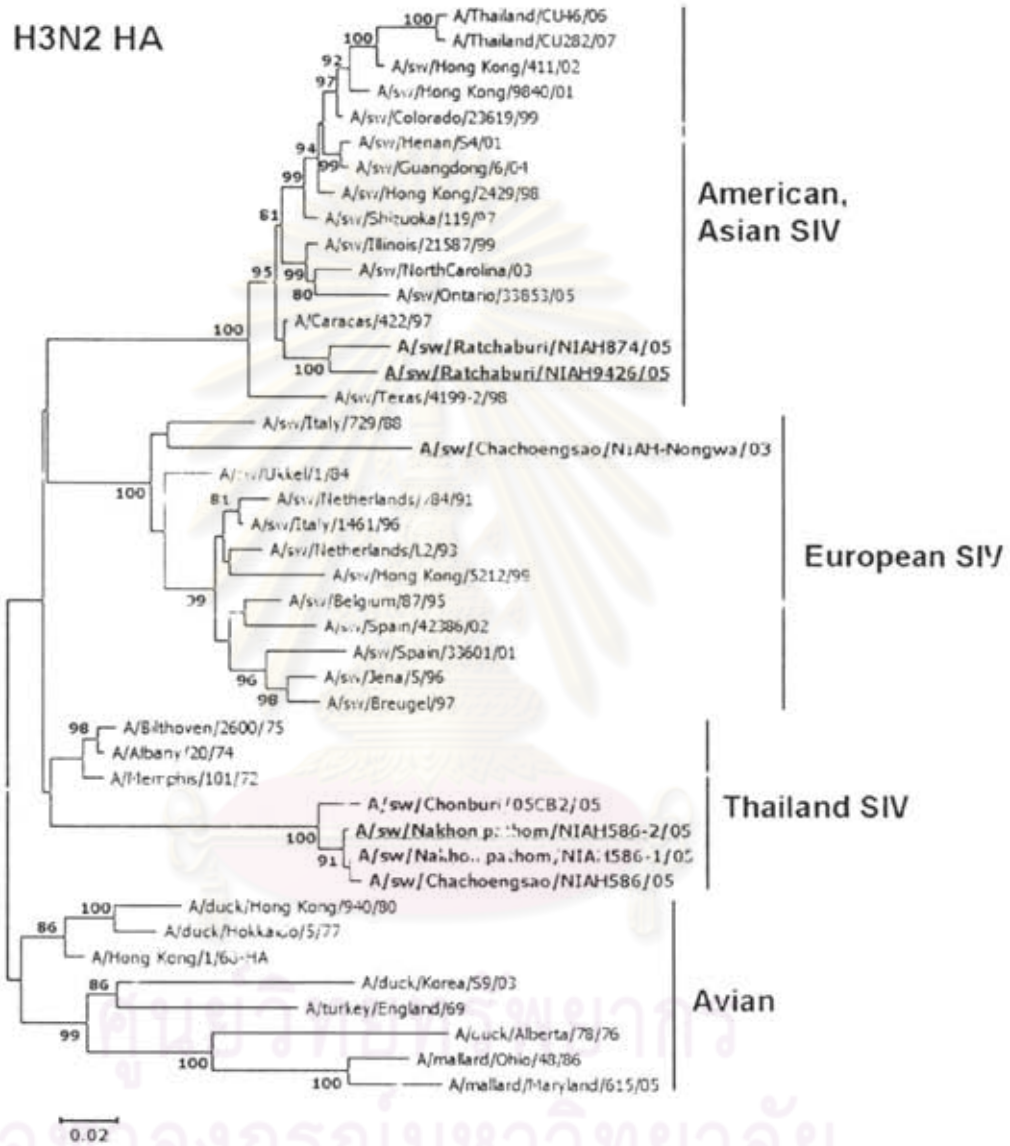

Figure 19 Phylograms of the hemagglutinin (HA) 1 region of the HA gene of SIV H3N2 isolates from Thailand with nucleotide sequences determined for each subtype of avian, human and swine influenza virus from several areas. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. Only bootstrap values above 80 are shown.

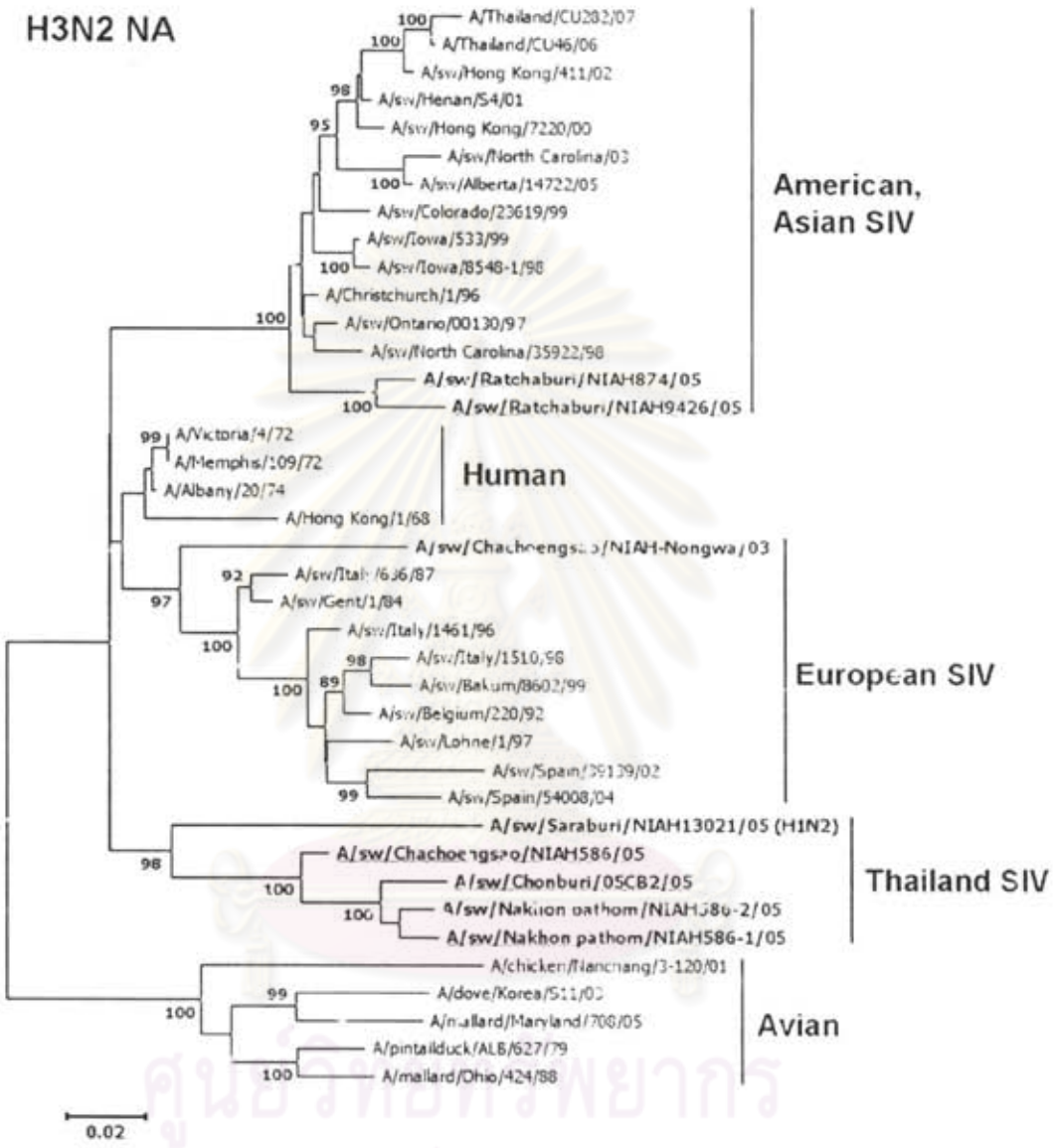

Figure 20 Phylograms of the NA gene of SIV H3N2 isolates from Thailand with nucleotide sequences determined for each subtype of avian, human and swine influenza virus from several areas. The tree was created by Neighbor-Joining method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. Only bootstrap values above 80 are shown.

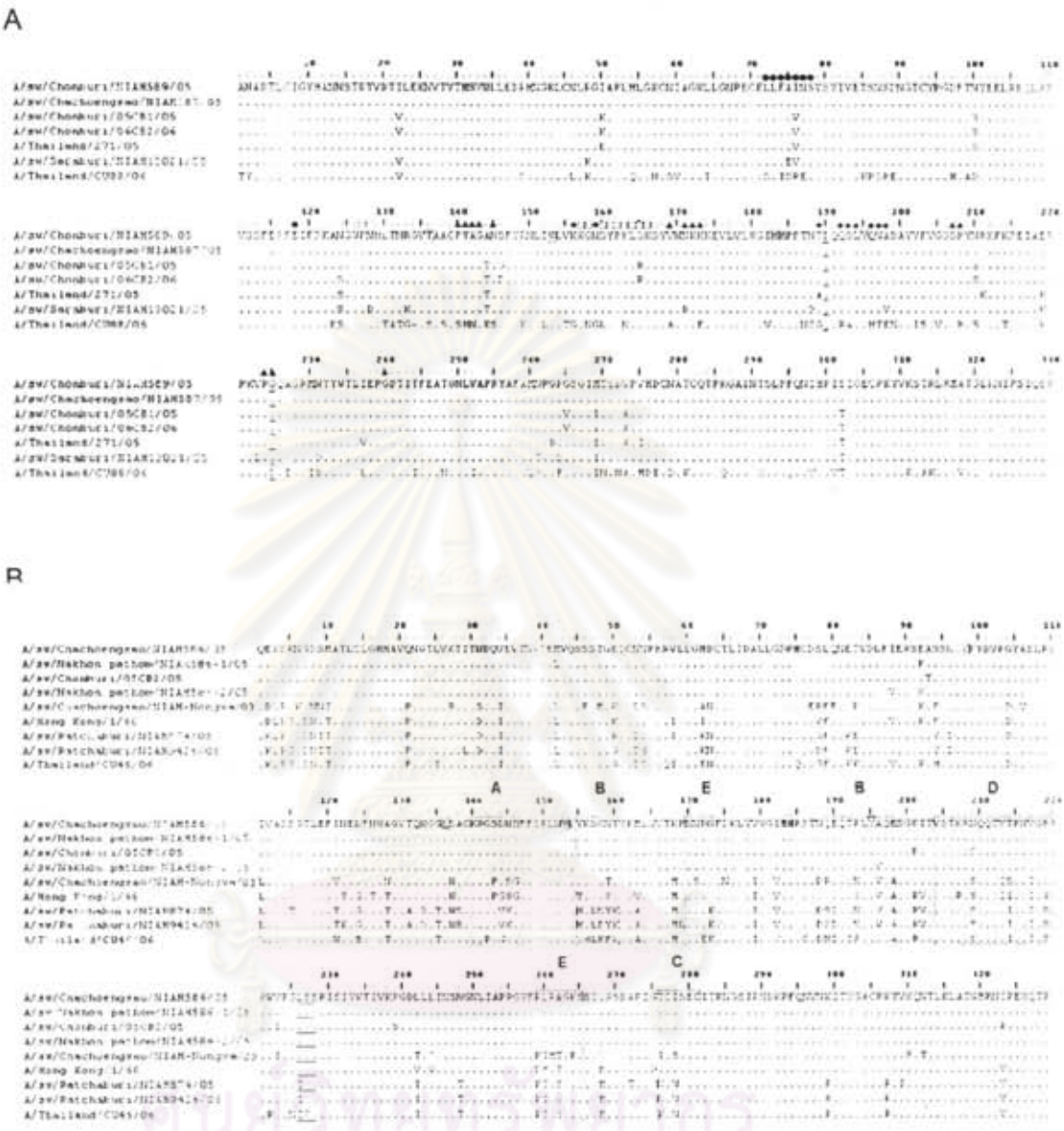

Figure 21 Amino acid comparison at the HAI domains of SIV H1 (A) and H3 (B) isolates. Dots represent amino acids similar to the consensus. The conserved amino acid residues at the receptor-binding site are shown as small rectangles. The HA control binding to alternative forms and linkages of sialic acid is underlined. The amino acid residues mapped at previously defined antigenic sites are shown as follows: site Sa (□), site Sb (◆), site Ca (▲), and site Cb (●) for H1 and sites A-E are shown as large rectangles for H3.

4. Discussion

Pigs are believed to play a vital role in interspecies transmission of influenza, since they harbor receptors to both avian and human influenza virus strains. For that reason, pigs have been considered as a possible "mixing vessel" in which genetic material can be exchanged, with the potential to result in novel progeny viruses to which humans are immunologically naïve and highly susceptible (Webster et al., 1992). In Thailand, subsequent to previous studies on SIV subtype H3N2 in 1978 (Nerome et al., 1981) and on serological and antigenic relatedness of SIV subtype H1N1 in 1988 (Kupradinun et al., 1991) no further molecular investigation of SIV has been reported. Thus, the present study represents the first attempt at molecular investigation of SIV in Thailand. Based on the results the SIV subtype in Thailand can actually be divided into 3 subtypes, H1N1, H1N2 and H3N2. SIV H1N1 can be isolated from east Thailand, Chachoengsao and Chon Buri, and displays the highest homology of both the HA and NA genes to the A/Thailand/271/05(H1N1) strain which constitutes the swine-like influenza virus recently isolated from a 4 year old male suffering from rhinorrhea, fever and myalgia (Komadina et al., 2007). The phylogenetic tree of SIV H1N1 also shows the HA gene clustering with the classical SIV which is related to virus prevalent in the USA, Japan and China and the NA gene clustering with European SIV that originated from avian influenza virus. This finding supports a previous study on SIV isolated from humans in Thailand (Komadina et al., 2007)

SIV subtype H1N2 was first isolated from swine in Saraburi province in the center of Thailand. Based on 4 phylogenetic tree patterns and upon comparison with H1N2 for both HA and NA, as well as with H1 of H1N1 and N2 of H3N2, the SIV H1N2 HA gene is similar to HA of the American and Asian SIV H1N2 whereas the NA gene is more closely related to the European SIV. In the H1N1 group, the H1N2 HA isolate clustered with classical SIV, identical to the SIV H1N1 isolate from Thailand and also, A/Thailand/271/05(H1N1), a swine-like human influenza virus. The H1N2 NA isolate clustered with the SIV H3N2 isolate from Thailand.

Surprisingly, the SIV subtype H3N2 isolates from Thailand display similarity to American, Asian and European SIV and also, to H3N2 human influenza virus having

circulated in the 1970s. Noticeably, this subtype has spread through both central and east Thailand. Specifically in Chachoengsao province, 2 patterns of SIV subtype H3N2 emerged, with both the HA and NA genes showing similarity to human influenza virus spreading in the 1970s and to European SIV.

Comparison between the receptor binding site and antigenic sites of the hemagglutinin gene of H1 and H3 SIV, showed some differences between the amino acids at the receptor binding site of H1 SIV. Furthermore, the amino acids at the antigenic sites of both H1 and H3 SIV were slightly different from the antigenic sites of H1 and H3 human influenza virus. But with reference to the previous report (Kupradinun et al., 1991), the amino acid sequence of swine-like human influenza virus A/Thailand/271/05(H1N1) at the receptor binding site and antigenic sites was similar to H1 SIV isolates from Thailand. Hence, although there are some amino acids differences between identical subtypes of swine and human influenza virus, swine influenza virus may potentially be transmitted to humans. However, the 7 other genes of SIV ought to be considered and consequently investigated in the course of any transmission experiment and receptor specificity test.

This report draws attention to an SIV subtype which has never been investigated in Thailand. With the present dissemination of human and avian influenza virus, swine may constitute a crucial intermediate host. In order to prevent a potential future influenza pandemic, in depth studies on SIV re-assortment are required. Thus, it might be feasible to better understand the variation continuously occurring in this virus and to monitor the genetic drift causing swine-like influenza virus transmission to humans.

CHAPTER VIII

SUMMARIZING DISCUSSION

When there is an outbreak of disease, the first important thing to do is to study the etiology of that disease for prompt treatment, effective management and to successfully defend against recurrence of disease. The spread of avian influenza A virus subtype H5N1 in Thailand was a good example to draw attention to research collaboration. The Center of Excellence in Clinical Virology is one of the laboratories that explored and published the data of the spread of H5N1 influenza virus in Thailand in collaboration with Kasetsart University, Srinakharinwirot University, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam and Department of Livestock Development, National Institute of Animal Health, Bangkok. This resulted in several publications, some of which are part of this thesis.

The studies in this thesis mainly focused on molecular analysis of influenza A virus strains. Unfortunately, these studies were limited by the availability of samples and sample processing techniques. These molecular analyses of genetic materials of influenza A virus may not answer all research questions, however, they can be the basis for analyzing and monitoring changes in the virus that may result in antiviral drug resistance or even a pandemic.

Nucleotide and amino acid sequence analysis

The first goal of this thesis was to determine the importance of specific nucleotide and amino acid sequences in each influenza A virus gene. As mentioned above, influenza A virus is a negative strand RNA virus composed of 8 gene segments. Influenza A virus is divided into subtypes based on antigenic differences in the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Up until now, there are 16 HA and 9 NA subtypes (Fouchier et al., 2005). In this thesis, only subtypes H5N1, H1N1, H3N2 and H1N2 isolated from mammalian and H5N1 from avian species were analyzed.

In chapter III, V, VI and VII, sequences of influenza A virus strains were analyzed and variability of nucleotide sequences and the resulting amino acid changes were studied.

In chapter III, we described a virus isolated in 2005 from a fatal human case of H5N1 infection. Genetic analysis showed that this virus belonged to clade 1 of HPAI H5N1 viruses circulating in Southeast Asia. HA, NA and PB2 of the virus were studied in more detail. The sequence analyses of the viruses showed that the HA cleavage site differed from the 2004 H5N1 influenza virus by an arginine to lysine substitution at position 341. Besides, a 20–amino acid deletion at the NA stalk region was observed and the amino acid residues (E119, H274, R292, and N294) of the NA active site were conserved, which suggests that the virus was sensitive to oseltamivir. In addition, a single amino acid substitution from glutamic acid to lysine at position 627 of PB2 showed increased virus replication efficiency in mammals (Shinya et al., 2004).

In chapter V, we described HPAI H5N1 viruses isolated in different regions in Thailand in 2006. Genetic analysis showed that the viruses isolated from Phichit belonged to genotype Z, whereas virus isolated from Nakhon Phanom belonged to genotype V, which differs from genotype Z in the PA gene. Futhermore, they were also different in clade based on sequence analysis of the HA gene. The viruses isolated from Phichit belonged to clade 1, while virus isolated from Nakhon Phanom belonged to clade 2. These findings are important because viruses from different clades differ in antiviral drug resistance and because they can indicate the origin of the fourth wave of H5N1 outbreaks in Thailand. In addition, this finding raises concern for development of new candidate influenza (H5N1) vaccine strains which have high potential for cross protection.

In chapter VI, we showed that two subtypes of influenza A virus, H1N1 and H3N2, alternatively infected pediatric patients throughout the year. From these isolates, the variations due to amino acid substitutions at the receptor-binding site, the antigenic site including the potential N-linked glycosylation site of HA were discovered, which is of interest for developing suitable vaccine strains. Besides, the catalytic and framework sites of NA in both H1N1 and H3N2 subtypes of 20 influenza A virus isolates from Thailand were revealed.

In chapter VII, the first attempt at molecular investigation of swine influenza virus (SIV) in Thailand is described. Three subtypes, H1N1, H1N2 and H3N2 were detected. SIV of subtype H1N1 displayed the highest homology of both HA and NA genes to the swine-like human influenza virus, A/Thailand/271/05(H1N1), which was isolated from a 4-year-old boy. For the H1N2 SIV, HA and NA gene clustered with SIV H1N1 and SIV H3N2 isolate from Thailand, respectively. The SIV subtype H3N2 isolates, both HA and NA, displayed similarity to American, Asian and European SIV and also, to H3N2 human influenza virus having circulated in the 1970s. Surveillance and genetic characterization of SIV circulation is important because upon transmission to humans it may cause severe infections (Rimmelzwaan et al., 2001; Komadina et al., 2007).

Besides using molecular techniques for epidemiological analysis of circulating strains, it can also be used to study more specific virus characteristics. For instance, we can predict; the receptor binding specificity from the specific amino acids in the HA gene. This is important because avian viruses use α-2,3 – linked sialic acids as a receptor whereas human viruses bind to α-2,6 – linked sialic acids (Ito and Kawaoka, 2000). Changes in receptor specificity of avian viruses that increase binding to α-2,6 – linked sialic acids may thus indicate increased risk of transmission to and between humans. Moreover, we can predict the susceptibility to antiviral drugs such as oseltamivir from the amino acid changes in the NA gene or amantadine in matrix (M) gene which is important for treatment of patients. The efficiency of viral replication in mammals may be increased by a single amino acid substitution from glutamic acid to lysine at position 627 of PB2 (Subbarao et al., 1993; Massin et al., 2001; Shinya et al., 2004; Mase et al., 2006). Finally, the sensitivity to treatment with interferon and tumor necrosis factor-alpha can be predicted from amino acid ESEV residues in the C-terminal and Asp92 of non-structural (NS) gene (Krug 2006).

Presence of live virus in plasma

The presence of live H5N1 influenza A virus in plasma is unusual (Naficy, 1963; Lehmann and Gust, 1971). However, in chapter III it was shown that H5N1 virus could be isolated from plasma of an infected patient on day 10 after symptoms developed

which raiseds concern about transmission among humans. Because viable virus has been detected in blood samples, handling, transportation, and testing of blood samples should be performed in a biosafety (category III) containment laboratory to prevent the spread of the virus to healthcare and laboratory workers. Moreover, it would be interesting to study why this strain caused systemic disease.

Applied molecular technique

The second goal of this thesis was to distinguish drug-resistant from wild type H5N1 influenza A viruses rapidly. There are several molecular methods for discriminating wild type from drug-resistant viruses by cell culture based assays or cloning and sequencing, however, they are time consuming. Since oseltamivir remains the primary recommended antiviral treatment, observational data on treatment with oseltamivir in the early stages of the disease suggest its usefulness in reducing H5N1 influenza A virus infection-associated mortality. Since it is important to start antiviral drug treatment as early as possible in order for the treatment to be effective, thus, a fast method to detect oseltamivir-resistant viruses was developed. Accordingly, real-time PCR was chosen and probes were designed in chapter IV. The problem of designing probes for H5N1 influenza A virus was its' sequence variability. hence, the shortest and most specific probes had to be designed. In this thesis, minor groove binding with degenerated nucleotide probes are described that had both high specificity and sensitivity. It was demonstrated that, using only 2 probes, drug-resistant viruses can be distinguished from a mixture of wild type and resistant-viruses with an efficiency as high as those achieved by cloning into a vector and subsequent sequencing. Thus, the real-time PCR assay described in this thesis had equal efficiency to other assays but can be performed within hours of obtaining the virus isolate.

Future perspectives

The data presented in this thesis can be used as the basis for future research. First, the rapid oseltamivir resistance assays could be developed for the resistance N2 influenza A viruses. However, this might be complicated by the fact that there are

several resistance mutations in N2 gene. Second, genetic characterization of circulating strains is important to monitor changes that require updating probes or changes other than the H274Y substitution that lead to oseltamivir resistance. Third, it may be possible to develop similar tests to determine resistance to other antiviral drugs such as the adamantane drug group, zanamivir and peramivir. Fourth, it would be beneficial to combine molecular characterization of HA and NA described in this thesis with antigenic characterization of strains to study vaccine efficacy.

Fifth, the surveillance of swine influenza virus should be maintained because pigs are thought to play an important role in interspecies transmission of influenza A virus. Pigs have receptors to both avian and human influenza A viruses, therefore they have been considered a possible "mixing vessel" in which genetic material can be exchanged. This could potentially result in novel progeny viruses to which humans are immunologically naive and highly susceptible.

Sixth, surveillance for presence of influenza A virus in children should be upgraded to include other hospitals thereby increasing sample size. Also, it would be interesting to include clinical specimens from adults because the immunological response and viral exposure in adults might be different from that of children. Finally, the H5N1 influenza A virus surveillance in Thailand should be continued, since this virus is highly pathogenic strain. Monitoring the variation in these strains can predict the fundamental characteristic of the H5N1 influenza virus and may be important for vaccine development.

In conclusion, the data described in this thesis are important for developing laboratory diagnostic techniques on human and animal health, for studying in inter-species transmission and may lead to vaccine development for both humans and animals.

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BIOGRAPHY

Miss Salin Chutinimitkul was born on July 29, 1981 in Bangkok, Thailand. She studied in primary and secondary school at Khema Siri Memorial School. She received her Bachelor of Science degree (2nd class honours) in the field of Biochemistry in 2002 from Faculty of Science, Chulalongkorn University. She has enrolled in the Royal Golden Jubilee Ph.D Program of Thailand Research Fund in graduate program for Doctor of Philosophy Program in Biomedical Sciences, Inter-Department of Biomedical Sciences, Faculty of Graduate School, Chulalongkorn University since 2003 as an advisee of Prof. Yong Poovorawan, MD. She has been trained at department of virology, Erasmus medical center, Rotterdam, The Netherlands during her Ph.D program. She has the Publications from her thesis as follow;

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