การตรวจติดตามเชื้อไวรัสไข้หวัดนก H5N1 จากสัตว์ปีกในบริเวณชายแดนระหว่างประเทศไทยและประเทศเพื่อนบ้าน (ลาว และพม่า)

นายจิรเดช ลาภขุนทด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MONITORING OF INFLUENZA A H5N1 VIRUS FROM AVIAN SPECIES IN BORDER AREAS BETWEEN THAILAND AND NEIGHBORING COUNTRIES (LAOS AND MYANMAR)

Mr. Jiradej Lapkuntod

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	MONITORING OF INFLUENZA A H5N1 VIRUS FROM AVIAN SPECIES IN BORDER AREAS BETWEEN THAILAND AND NEIGHBORING COUNTRIES (LAOS AND MYANMAR)
By	Mr. Jiradej Lapkuntod
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จิรเดช ลาภชุนทด : การตรวจติดตามเชื้อไวรัสไข้หวัดนก H5N1 จากสัตว์ปีกในบริเวณชายแดนระหว่างประเทศไทยและประเทศเพื่อนบ้าน (ลาว และพม่า) (MONITORING OF INFLUENZA A H5N1 VIRUS FROM AVIAN SPECIES IN BORDER AREAS BETWEEN THAILAND AND NEIGHBORING COUNTRIES (LAOS AND MYANMAR)) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.น.สพ.ดร. อลงกร อมรศิลป์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.สพ.ญ.ดร. รุ่งทิพย์ ชวนชื่น 100 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจติดตามเชื้อไวรัสไข้หวัดนก H5N1 จากสัตว์ปีกในบริเวณชายแดนระหว่างประเทศไทยและประเทศเพื่อนบ้าน (ลาว และพม่า) ระหว่างเดือนกันยายน พ.ศ. 2550 ถึง มิถุนายน พ.ศ. 2551 โดยเก็บตัวอย่างจำนวน 2,175 ตัวอย่าง แบ่งเป็นตัวอย่างจากสัตว์ปีกมีชีวิตจำนวน 2,139 ตัวอย่าง และอวัยวะภายในของสัตว์ปีกจำนวน 36 ตัวอย่าง จากนั้นนำตัวอย่างทั้งหมดมาเพาะแยกเชื้อไวรัสไข้หวัดนก H5N1 ด้วยวิธีการฉีดเข้าไข่ไก่ฟัก และตรวจพิสูจน์ด้วยวิธี Hemagglutination test Multiplex RT-PCR Realtime RT-PCR และ PCR-ELISA จากนั้นศึกษาลักษณะทางพันธุศาสตร์ด้วยวิธีการถอดรหัสพันธุกรรมของยีนทั้งหมดของเชื้อไวรัส วิเคราะห์รหัสพันธุกรรมด้วยวิธี phylogenetic analysis และวิเคราะห์การเปลี่ยนแปลงของกรดอะมิโนในตำแหน่งต่างๆ ที่มีความสำคัญบนยีนทั้ง 8 ยีน (PB2, PB1, PA, HA, NP, NA, M, และ NS) ผลการศึกษาพบอุบัติการณ์ของเชื้อไข้หวัดนกสายพันธุ์ H5N1 คิดเป็น 0.69% (15/2,175) โดยเชื้อไวรัสไข้หวัดนกที่พบเป็นเชื้อไวรัสไข้หวัดนกชนิดก่อโรครุนแรง (Highly Pathogenic Avian Influenza; HPAI) ซึ่งพบการเรียงตัวของกรดอะมิโนชนิดเบสหลายตัวที่ HA cleavage site และการลดจำนวนของกรดอะมิโน 20 ตัว ที่ NA stalk region รวมถึงไม่พบการเปลี่ยนแปลงของกรดอะมิโนในตำแหน่งที่มีความสำคัญ การศึกษาทาง phylogenetic analysis พบว่าเชื้อไวรัสไข้หวัดนกที่พบจัดอยู่ในกลุ่มเดียวกันกับที่แยกได้ในประเทศไทย อยู่ใน genotype Z หรือ clade 1 ดังนั้นไวรัสไข้หวัด-นกที่พบในบริเวณชายแดนระหว่างประเทศไทยและประเทศเพื่อนบ้าน (ลาว และพม่า) มีความใกล้เคียงกับไวรัสไข้หวัดนกที่แยกได้ในประเทศไทยในปี พ.ศ. 2547-2549 การศึกษาครั้งนี้แสดงให้เห็นว่ามีการแพร่ระบาดของเชื้อไวรัสไข้หวัดนกในบริเวณชายแดนระหว่างประเทศไทยและประเทศเพื่อนบ้าน (ลาว และพม่า) ดังนั้นการเฝ้าระวังเชื้อไวรัสไข้หวัดนกในบริเวณชายแดนระหว่างประเทศไทยและประเทศเพื่อนบ้าน (ลาว และพม่า) จะช่วยควบคุมและป้องกันการติดเชื้อไวรัสไข้หวัดนกสายพันธุ์ H5N1 ในคนได้

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต.....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2552..... ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : AVIAN INFLUENZA VIRUS MONITORING cDNA SEQUENCING

JIRADEJ LAPKUNTOD: MONITORING OF INFLUENZA A H5N1 VIRUS FROM AVIAN SPECIES IN BORDER AREAS BETWEEN THAILAND AND NEIGHBORING COUNTRIES (LAOS AND MYANMAR). THESIS ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., THESIS CO-ADVISOR: ASST. PROF. RUNGTIP CHUANCHUEN, D.V.M., Ph.D., 100 pp.

The purpose of this study was to monitor Influenza A H5N1 virus from avian species in border areas between Thailand and neighboring countries (Laos and Myanmar) from September 2007 to June 2008. Two-thousand one hundred seventy five samples, including 2,139 live birds and 36 visceral organs, were collected. The H5N1 viruses were isolated and identified using embryonated egg inoculation, Hemagglutination test, Multiplex RT-PCR, Realtime RT-PCR, and PCR-ELISA. Then, the viruses were genetically characterized by using sequencing of whole genome avian influenza H5N1 viruses, phylogenetic analysis, and analysis of key determinant residue changes of 8 genes (PB2, PB1, PA, HA, NP, NA, M, and NS). The results revealed that the evidence of avian influenza H5N1 virus was 0.69% (15/2,175). The viruses had common genetic characteristics of Highly Pathogenic Avian Influenza (HPAI), with multiple basic amino acids in the HA cleavage site and a 20-amino acid deletion in NA stalk region. No point mutations were identified in the key determinant residues of those genes. Phylogenetic analysis of whole genes showed that the viruses clustered within the lineage of H5N1 avian isolates from Thailand-Vietnam lineage, genotype Z or clade 1. These indicate that avian influenza H5N1 virus circulating in border areas between Thailand, Laos and Myanmar were genetically related to avian influenza H5N1 virus in 2004-2006 in Thailand. In summary, this study presented the evidence of HPAI spreading in border area between Thailand and neighboring countries (Laos and Myanmar). Therefore, monitoring and surveillance of avian influenza virus along the border areas will be beneficial for prevention and control of H5N1 infection in humans.

Department: Veterinary Public Health Student's Signature Jiradej Lapkuntod

Field of Study: Veterinary Public Health Advisor's Signature

Academic Year: 2009 Co-Advisor's Signature

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Finally for my family, I am deeply grateful for their love, supports and encouragement.

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LIST OF ABBREVIATIONS

AI	Avian Influenza
bp	base pair
^{o}C	degree Celsius
cDNA	Complementary deoxyribonucleic acid
et al.	et alibi, and other
g	gram (s)
HA	Hemagglutinin
HPAI	Highly Pathogenic Avian influenza
h	hour (s)
M	Matrix
mg	milligram (s)
min	minutes (s)
µl	micro liter
µM	micro molar
NA	Neuraminidase
NP	Nucleoprotein
NS	Nonstructural protein
PA	Polymerase acidic protein
PCR	Polymerase Chain Reaction
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sec	second (s)

CHAPTER I

INTRODUCTION

Avian influenza H5N1 virus is a Highly Pathogenic Avian Influenza (HPAI) virus. The virus is highly contagious and causes disease in several species of pet birds, wild birds, humans and food producing birds (e.g. chickens, ducks, turkeys, quails, and guinea fowls) (Abdel-Ghafar et al., 2008). World Organization for Animal Health or Office International des Epizooties (OIE) has included Avian Influenza (AI) in OIE listed diseases that are characterized by causing severe disease, fast spreading and promoting serious threats to economy and public health worldwide (OIE, 2000). The diseases in this category are required by law to be reported to government authorities. In Thailand, outbreaks of avian influenza H5N1 virus have caused both economical losses and the public health problems (Amonsin et al., 2008; Buranathai et al., 2007; Tiensin et al., 2005). In 2004, emergence of avian influenza H5N1 virus affected poultry production and exporting industries due to the eradication of poultry in the radius of outbreaks, banning exportation of chicken products to various countries and reduction of consumption within the country (Tiensin et al., 2005). From previous reports in Thailand, avian influenza H5N1 virus infected 17 persons with 12 deaths in 2004, infected 5 persons with 2 deaths in 2005, and infected 3 persons with 3 deaths in 2006. No human cases reported during 2007-2009. As of July 1, 2009, avian influenza H5N1 virus infected 25 persons with 17 deaths. During the same period of time, the virus infected 436 persons with 262 deaths in 15 countries worldwide (World Health Organization, 2008).

Based on nucleotide changes in hemagglutinin gene, avian influenza H5N1 viruses are classified into 10 main groups termed “clades” (WHO/OIE/FAO H5N1 Evolution Working Group, 2007). Clade 0 includes the ancestor of all avian influenza H5N1 viruses that spread in Hong Kong in 1997. Clade 1 comprises the viruses that expanded in Thailand, Southern Vietnam, Malaysia and Cambodia during 2003-2006 (Boltz et al., 2006; Li et al., 2004). Clade 2 contains 5 subclades including clade 2.1

covers the viruses that caused outbreaks in Indonesia in 2003-2007; clade 2.2 are those that spread in Europe and Africa in 2005-2007; clade 2.3 includes the viruses that spread in southern China and neighboring countries; clade 2.4 consists of the avian influenza H5N1 viruses distributed in China in 2002-2005 and clade 2.5 includes those detected in China and spread to Korea and Japan in 2003-2004. Clades 3 to 9 are avian influenza H5N1 viruses mostly detected in China (Webster and Govorkova, 2006).

Classification of avian influenza H5N1 virus genotypes can be performed by comparing genetic relatedness of their eight genes (Duan et al., 2008; Li et al., 2004)). This could be successfully accomplished when all genetic data of the genes are available. Genetic characteristics of avian influenza H5N1 viruses from Thailand were found to be similar to avian influenza viruses in Vietnam; therefore, the viruses have been classified as Thailand-Vietnam lineage or genotype Z (Amonsin et al., 2006a; Li et al., 2004; Viseshakul et al., 2004; Webster and Govorkova, 2006). A new genotype (genotype V or clade 2.3.4) of avian influenza H5N1 viruses "A/chicken/Thailand/NP-172/2006" was reported in Nakhon Phanom province during August 2006 - February 2007(Chutinimitkul et al., 2007). The virus was different from avian influenza H5N1 viruses recovered from most outbreaks in the country based on phylogenetic analysis of PA gene (NP-172). The virus was also classified into the same group of avian influenza viruses in southeastern China, Laos, and northern Vietnam (Chutinimitkul et al., 2007; Puthavathana et al., 2009). Currently, there are at least 2 clades (i.e. clade 1 and 2.3.4) or 2 genotypes (i.e. genotype Z and V) of avian influenza H5N1 viruses found to emerge and cause the avian influenza outbreaks in Thailand (Figure1) (Chutinimitkul et al., 2007). The genotype V was first detected in border areas and identified into the same group of viruses in neighbor countries (Laos and Myanmar), suggesting that the viruses may be disseminated from neighbor countries to Thailand (Chutinimitkul et al., 2007). The presence of two different clades/genotypes also indicates the multiple introductions of avian influenza H5N1virus into the country. Therefore, it is essential to conduct avian influenza virus surveillance and monitor for the emergence of novel viruses or genotypes along border areas of Thailand.

In this study, we isolated, identified and genetically characterized avian influenza H5N1 viruses from avian species around the border areas of Thailand between Thailand and neighboring countries (Laos and Myanmar). The results obtained from this study will provide insight information of the evolutionary history and the possible pathways of transmission of the viruses. Also, this study will demonstrate the avian influenza H5N1 evidence among avian Influenza A viruses isolates in the border areas of Thailand and distinguish clades/genotypes of avian influenza A viruses that have spread in Thailand.

Objectives of Study.

1. To collect samples, isolates, identify avian influenza H5N1 viruses from avian species from villages and fresh markets around border areas between Thailand and neighboring countries (Laos and Myanmar)
2. To analyze genetic relatedness and identify genotypes of the avian influenza H5N1 viruses.

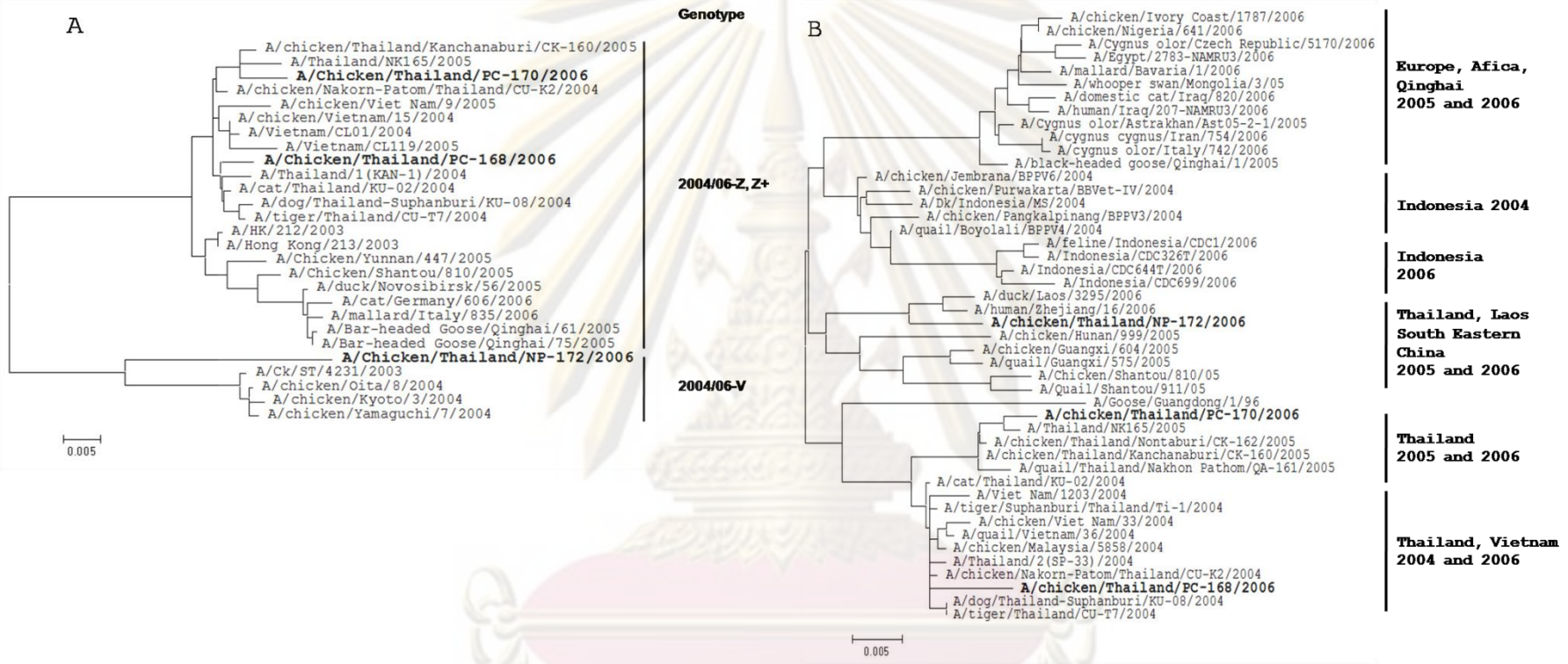


Figure1: A) Phylogenetic relationships of the polymerase acid protein gene comparing genotype Z, Z+, and V.

B) Hemagglutinin gene of avian influenza A H5N1 viruses in Thailand 2006 compared with several other strains worldwide (Chutinimitkul et al., 2007).

CHAPTER II

REVIEW LITERATURES

A. Morphology of avian influenza H5N1 virus

Influenza virus belongs to the Orthomyxoviridae family. The virus can be divided into three types A, B and C. Influenza A virus can infect humans, mammals, and avian species. Influenza B virus can infect human only. Influenza C virus can infect human and rarely infect swine (Webster et al., 1992). Influenza A virus is an enveloped virus that has 2 glycoproteins, haemagglutinin (HA) protein and neuraminidase (NA) protein. Virions are spherical to pleomorphic and 80-120 nm in diameter. HA caries rod-shaped spike and NA contains mushroom-shaped spike on enveloped (De Jong et al., 2000)). Within enveloped, the virus has single-stranded RNA of negative polarity containing 8 segments with different molecular weight; i.e. Polymerase Basic protein 1 and 2 gene (PB1 and PB2), Polymerase gene (PA), Hemagglutinin gene (HA), Nucleoprotein gene (NP), Neuraminidase gene (NA), Matrix protein gene (M), and Nonstructural protein gene (NS), respectively (Figure2).

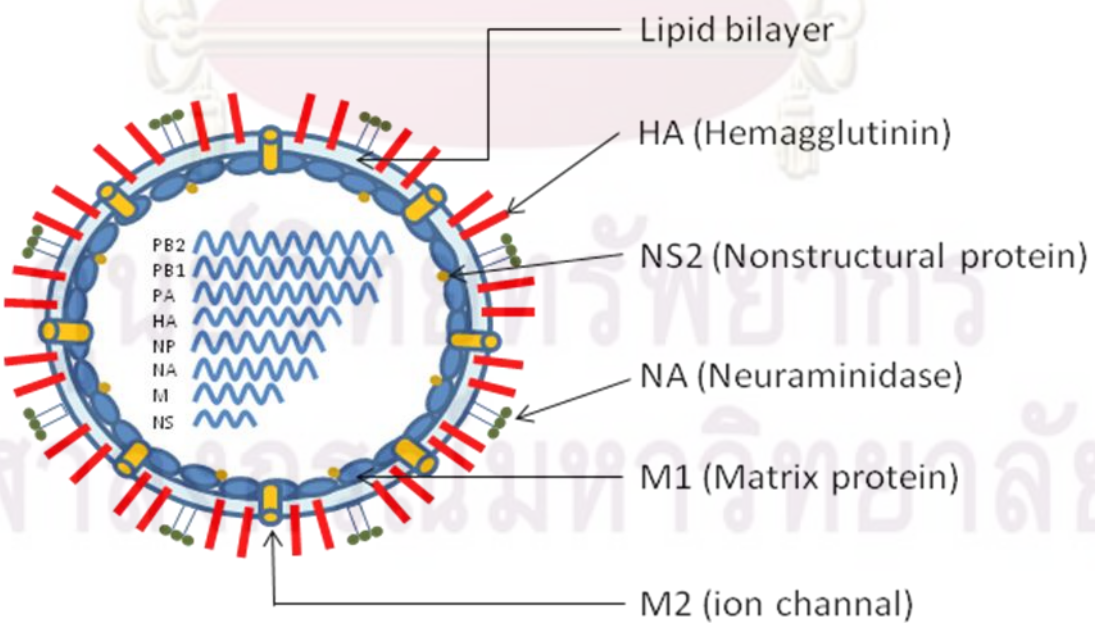


Figure 2. Diagram of avian influenza H5N1 virus structure.

RNA segments are contained in the viral core. Protein synthesized from 8 RNA segments have 10 types such as PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NS2. Functions of these proteins are shown in Table1 (Lamb and Choppin, 1983). Influenza A viruses are classified based on their haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. At present, 16 HA (H1-16) and 9 NA (N1-9) subtypes have been identified (Fouchier et al., 2005).

Table 1: RNA segments, encoding protein and their function. (Lamb and Choppin, 1983)

Segment	Encoded polypeptide	Nucleotide length (bp)	Function
1	PB2	2,341	Host-cell RNA cap binding: component of RNA transcriptase
2	PB1	2,341	Initiation of transcription: possibly endonuclease activity: component of RNA transcriptase
3	PA	2,233	Elongation of mRNA chains: component of RNA transcriptase
4	HA	1,778	Surface glycoprotein; major antigenic determinant
5	NP	1,565	Associated with RNA segment to form ribonucleoprotein: structural component of RNA transcriptase
6	NA	1,413	Surface glycoprotein: neuraminidase activity.
7	M1	1,027	Major protein component of virus: underlies lipid bilayer
	M2		Spliced mRNA, nonstructural protein: ion channel
8	NS1	890	Nonstructural protein: function unknown
	NS2		Spliced mRNA, nonstructural protein: function unknown

B. Genotype classification for H5N1

Influenza A virus subtype H5N1 (Avian Influenza H5N1 virus) is the important subtype that caused AI in many animal species and humans. In 2001 – 2004, avian influenza H5N1 virus can be further classified into genotypes A-E, V-Z and Z+ (Li et al., 2004). Since 2002, genotype Z viruses are the main virus that cause outbreaks in Asia (Guan et al., 2002). Avian Influenza H5N1 viruses outbreaking in China and eastern Asia in 2001-2006 have been classified in genotype Z. Furthermore, genotype V viruses were classified in southern China in 2003-2006 and have been detected in VietNam and Laos in 2006-2007 (Boltz et al., 2006; Duan et al., 2008; Li et al., 2004). The genotype classification of avian influenza H5N1 virus can be performed when genetic data of 8 genes of viruses are available for comparing the genetic relatedness in each gene. The genotype classification steps including preparation of complete nucleotide sequence of each avian influenza H5N1 virus gene, comparison of nucleotide sequence by using computer program to analyze genetic relationship which displays in phylogenetic tree, classification of lineage of each gene of avian influenza H5N1 virus, arrangement of the sequential combination of the lineage of each gene, and assignment the genotype of avian influenza H5N1 virus (Duan et al., 2008; Li et al., 2004).

C. Clade nomenclature system for H5N1

The avian influenza H5N1 viruses have appeared at least in 60 countries and continued to evolve and be diversed. Dissimilar names have been used in publications to explain emerging lineages of highly pathogenic avian influenza A (H5N1) viruses (WHO/OIE/FAO H5N1 Evolution Working Group, 2007). This generates difficulty for discussion and comparison of the various lineages. Since 1996, avian influenza A H5N1 viruses have experienced reassortment into many different genotypes. It is only the hemagglutinin protein that has not been replaced in the variant isolates. Evolution of hemagglutinin protein has an initial constant that the strains may be efficiently assessed. WHO/OIE/FAO suggested to improve a clade nomenclature system based on the evolutions of hemagglutinin protein; for reason unify the system so that interpretation of sequence and surveillance data from different laboratory becomes easier, removing stigmatizing labelling of clades by geographical reference, providing clade for easy

future expansion of the phylogenetic tree, and providing a starting point for a more extensive system to follow antigenic variation and reassortment.

Clade descriptions (WHO/OIE/FAO H5N1 Evolution Working Group, 2007)

0 = early progenitors; predominately 1996-2002 from Hong Kong (HK) and China (mostly avian, few human)

1 = 2002/2003 progenitors from HK; 2003-2006 from Vietnam, Cambodia, Thai, Laos, Malaysia (mixed A/H)

2.1 = 2003-2007 from Indonesia (mixed avian/human)

2.2 = 2005 progenitors from Qinghai Lake outbreak and Mongolia; 2005-2007 isolates from Eastern and Western Europe, the Middle East, and Africa (mixed avian/human)

2.3 = 2003-2006 from China, HK, Vietnam, Thailand, Laos, and Malaysia (mixed avian/human)

2.4 = 2002-2005 from China (predominately Yunnan and Guangxi Provinces) (all avian)

2.5 = 2003/2004 from Korea, Japan, China; 2006 lineage from Shantou Prov. (all avian)

3 = 2000-2001 from HK, China, Vietnam (all avian)

4 = 2002/2003 lineage from HK and China; 2005/2006 from Guiyang Prov. (all avian)

5 = 2000-2003 from China and Vietnam; 2004 lineage from Guangxi Province (all avian)

6 = 2002/2004 from China (all avian)

7 = 2002/2004 from China; 2005/2006 from Yunnan, Hebei, Shanxi Provinces (all avian)

8 = 2001-2004 from HK and China (all avian)

9 = 2003-2005 from China (all avian)

D. Avian influenza (H5N1) outbreaks in Thailand

In Thailand, outbreaks of avian influenza H5N1 virus caused both economic losses and public health problems. Avian influenza H5N1 virus infected 17 persons with 12 deaths in 2004, infected 5 persons with 2 deaths in 2005, infected 3 persons with 3 deaths in 2006 and no human cases reported in 2007-2009. As of July 1, 2009, avian influenza H5N1 virus infected 25 persons with 17 deaths while avian influenza H5N1 virus infected 436 persons with 262 deaths in 15 countries worldwide (World Health Organization, 2008). In Thailand, outbreaks of avian influenza H5N1 virus in avian species since 2004 were reported at least 7 waves (Amonsin et al., 2006a; Buranathai et al., 2007; Suwannakarn et al., 2009; Tiensin et al., 2007; Viseshakul et al., 2004). For example, outbreaks in January-March 2004, July-December 2004 (Tiensin et al., 2005), October-December 2005, January-March 2006, November 2006–March 2007, January 2008 and November 2008 (Amonsin et al., 2008; Chaichoune et al., 2009) respectively.

One of the main causes of avian influenza H5N1 virus's outbreak is the migratory birds that excreted numerous viruses from intestine. Migratory birds received viruses from their reservoirs or somewhere in the migratory way (Liu et al., 2005). Backyard chicken and free-grazing ducks can play a role as H5N1 hosts (Tiensin et al., 2005). Other studies reported that avian influenza H5N1 virus infected other mammals such as tigers, leopards (Amonsin et al., 2006b; Thanawongnuwech et al., 2005), cat (Amonsin et al., 2007; Songsermn et al., 2006), and dog (Amonsin et al., 2007; Songserm et al., 2006).

F. Identification and diagnosis methods of avian influenza virus in laboratory

One of molecular methods used to detect H5N1 subtypes is polymerase chain reaction (PCR) assays. A reverse transcriptase polymerase chain reaction (RT-PCR) was developed to detect the avian influenza H5N1 virus using specific primers. The specificity and sensitivity of the assay was shown by testing with subtypes of influenza A virus (Payungporn et al., 2004). Another detection method World organization for animal health has recommended, is virus isolation by embryonated egg inoculation (high sensitive and standard method) (WHO, 2002). Avian influenza viruses were isolated using specific antibody negative, embryonated chicken eggs. The supernatant fluid from

viral transport media (VTM) suspension was inoculated into allantoic sacs of the eggs. Samples yielding positive hemagglutination activity (HA test) were tested for influenza genes (WHO, 2002). Total RNA was extracted and purified from the allantoic fluid. Then, viral RNA was reverse transcribed into cDNA. To identify the virus subtype, a RT-PCR was performed using the primers specific for avian influenza H5N1 virus (WHO, 2002). Antigen tests using PCR-ELISA technique was claimed greater than conventional PCR according to its specificity and sensitivity. In addition, it needs less expense equipment than real-time PCR (Chaharaein et al., 2009).

G. Molecular characterization of avian influenza virus (H5N1) in Thailand

Molecular characteristics of avian influenza H5N1 virus in Thailand were previously observed such as determinant residues relating to virulence, oseltamivir resistance, amantadine resistance, typical characteristics of both avian-like and human-like viruses etc.

In HA gene, the cleavage site has multiple basic amino acids of highly pathogenic characteristics. These basic amino acids affected hemagglutinin molecule that is sensitive to protease enzyme. Since protease enzyme can be found in all organs, the H5N1 virus can spread to every organs and causes human or animal death (Horimoto and Kawaoka, 2005). Receptor binding sites retain amino acid residues at 222Q (Glutamine) and 224G (Glycine). The viruses isolated from mammal and poultry in Thailand contain Glutamine in position 226 and Glycine in position 228. These positions indicate preferencial binding of the virus to receptor sialic acid α 2, 3-Gal- terminated saccharide more than sialic acid α 2, 6-Gal- terminated saccharide (Stevens et al., 2006).

Sequence of the NA gene contains 20-amino acid deletion from position 49 to 68. This deletion causes the adaptation of virions on cell membrane (Guan et al., 2002). The change of amino acids at NA stalk region was the result of the adaptation or evolution occuring from the infection in wild aquatic birds to domestic poultry (Matrosovich et al., 1999). The position 275 of NA gene contain amino acid Y (Tyrosine), indicating oseltamivir sensitive viruses (Gubareva et al., 2000).

M2 protein at position 31 (serine; S) can appear in amantadine resistance. Viruses have the S31N substitution (Serine; S to Asparagine; N) that might present amantadine or rimantadine resistance (Puthavathana et al., 2005).

Single amino acid substitution at position 627 of PB2 protein (Glutamic acid; E to Lysine; K) found in isolates from mammal or human. This substitution produces high violence in human (Shinya et al., 2004). However, this substitution can be found in avian species from Qinghai, Eurasia, and Africa.

C-terminal sequence in NS gene of HPAI H5N1 virus had substitution from Arg-Ser-Lys-Val (RSKV) to Glu-Ser-Glu-Val (ESEV) and had 5 amino acid deletions (position 80 to 84). These will increase the virulence and pathogenicity of the virus, interrupt immunity and cause extensive pathology in mammals (Jackson et al., 2008; Lipatov et al., 2005).

CHAPTER III

MATERIALS AND METHODS

The aim of this study was to monitor of Influenza A H5N1 virus from avian species in border areas between Thailand and neighboring countries (Laos and Myanmar) from September 2007 to June 2008. The experimental study included 3 phases; phase 1, collection of samples from avian species in the border areas between Thailand and neighboring countries (Laos and Myanmar); phase 2, isolation and identification of avian influenza H5N1 viruses and phase 3, genetic characterization of avian influenza H5N1 viruses using cluster analysis and identification of virus genotypes. The conceptual framework of this study is shown in Figure 3.

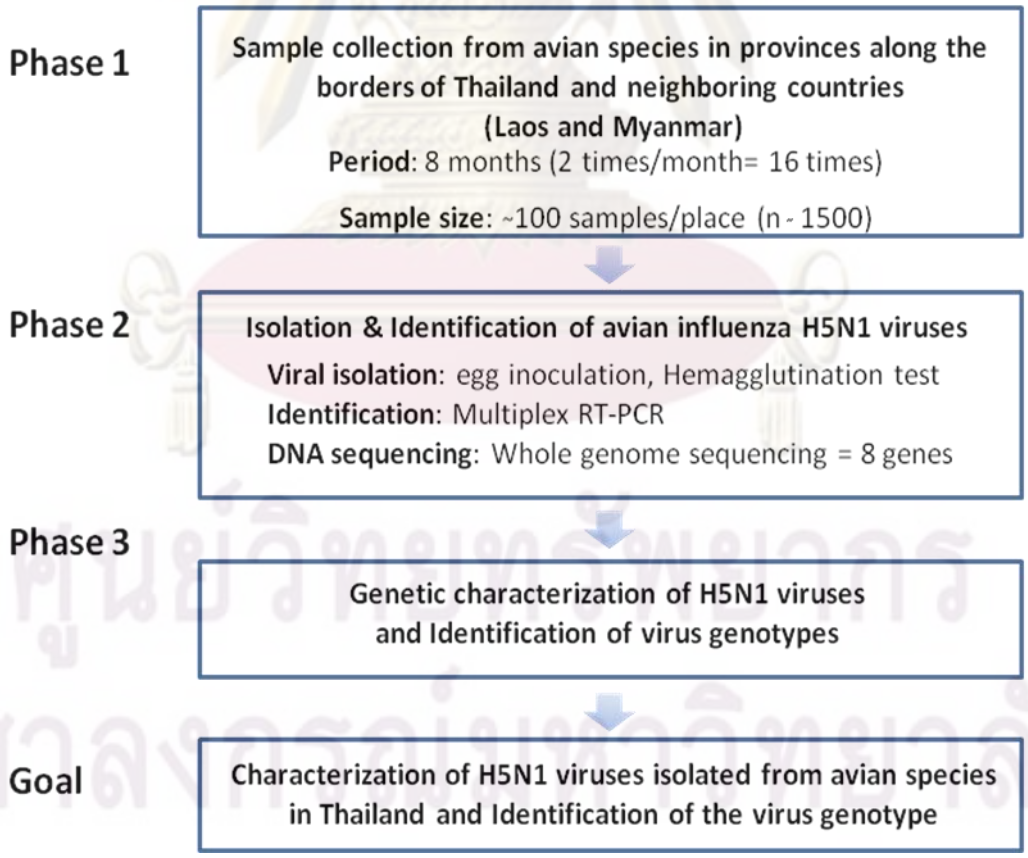


Figure 3: The conceptual framework in this study

Phase 1: Collection of samples from avian species and visceral organs in provinces along the borders between Thailand and neighboring countries (Laos and Myanmar)

Location and type of samples

Samples were collected in provinces along the borders of Thailand and neighboring countries, Myanmar and Laos. Selection of places for sample collection depended on history of outbreaks of avian influenza virus, mortality rate, and animal movement. The samples were collected from villages and fresh markets in Chiang Rai, Chiang Mai, Mae Hong Son, Tak, Kanchanaburi, Ratchaburi, Prachuap Khiri Khan, Ranong, Loei, Nong Khai, Nakhon Phanom, Mukdahan, and Ubon Ratchathani (Figure 4). Sample collections were carried out twice a month for 8 months (16 times).

Two thousands one hundreds and seventy five samples, including 2,139 live birds and 36 visceral organs, were collected from border areas between Thailand, Laos and Myanmar. The samples included feces, cloacal contents, tracheal exudates and internal organs e.g. trachea, lung, liver, spleen and heart. Sterile cotton swabs were used to obtain samples from choanal-slit, trachea and cloaca. Each collected sample was placed in a sterile plastic tube containing 2 ml-viral transport media (VTM) and kept on ice. Then, the samples were transferred to Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. All samples were kept at -80^{o}C immediately after arrival.

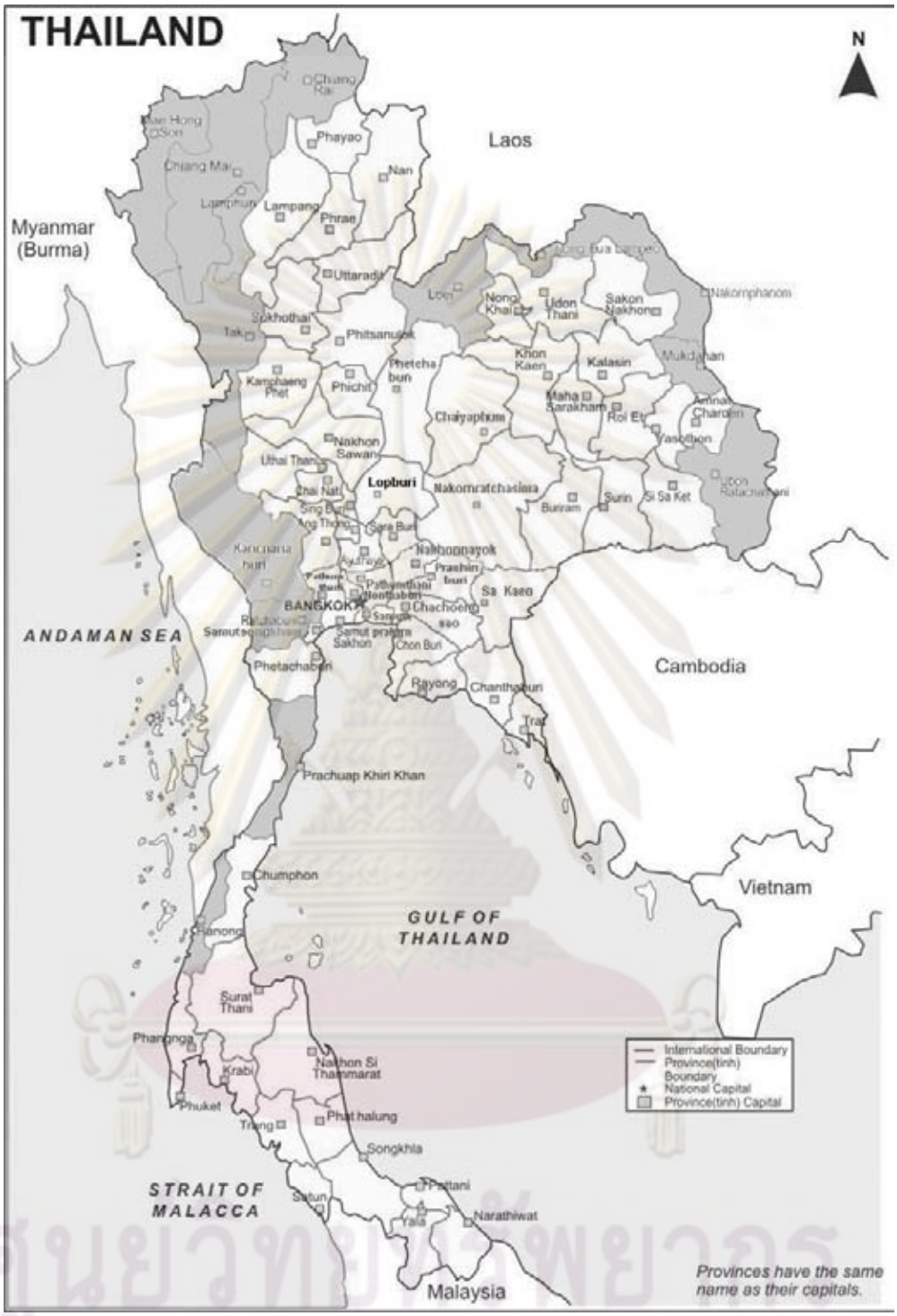


Figure 4: Locations for sample collection (gray areas) along the border of Thailand, Laos and Myanmar in this study, including Chiang Rai, Chiang Mai, Mae Hong Son, Tak, Kanchanaburi, Ratchaburi, Prachuap Khiri Khan, Ranong, Loei, Nong Khai, Nakhon Phanom, Mukdahan, and Ubon Ratchathani.

Phase 2: Isolation and identification of HPAI H5N1 virus

Virus isolation

Avian Influenza viruses were isolated from samples using inoculation of embryonated chicken eggs (WHO, 2002). The specific antibody negative-embryonated chicken eggs at the age of 9-11 days were used. The supernatant fluid from VTM suspension were inoculated into allantoic sacs of the eggs and incubated at 37°C. After the incubation period of 24-96 hour, the inoculated-embryonated eggs showing infected lesions or death were collected and chilled at 4°C. The allantoic fluid were harvested and tested for Hemagglutination activity (HA test) (WHO, 2002). The samples yielding positive hemagglutination activities were frozen at -80°C until needed (David et al., 1998). Virus isolation by inoculated embryonated eggs were accomplished at Biosafety level 2+ laboratory, Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University.

Virus identification

Total RNA were extracted from the allantoic fluid with positive HA test using QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany). At the beginning, buffer AVL containing carrier RNA was pipetted 560 µl into a 1.5 ml microcentrifuge tube. The 140 µl portion of allantoic fluid was added into the buffer AVL–carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 sec. Then, the mixture was incubated at room temperature (15–25°C) for 10 min. 560 µl of ethanol (96–100%) was added to the sample, and the mixture was mixed by pulse-vortexing for 15 sec. The tube was further briefly centrifuged to remove drops from inside the lid. The 630 µl solution was applied carefully into the QIAamp Mini column (collection tube) without wetting the rim, and centrifuged at 8000 rpm for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp Mini column was opened carefully, and procedure was repeated with remainder solution. Then, the five hundred µl buffer AW1 was added and centrifuged at 8000 rpm for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube,

and the tube containing the filtrate was discarded. Five hundred µl of Buffer AW2 was added and the column was centrifuged at full speed (14,000 rpm) for 3 min. The QIAamp Mini column was placed in a new 2 ml collection tube, and centrifuged at full speed for 1 min. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube. Sixty µl of Buffer AVE equilibrated to room temperature was added and incubated at room temperature for 1 min. The column was finally centrifuged at 8000 rpm 1 min for viral RNA collection.

The viral RNA was reverse transcribed into cDNA as previously described by random primer (Promega, Madison, WI, USA) (Viseshakul et al., 2004). The 4 µl RNA was mixed with 0.1 µg Random primers and incubated at on 70°C for 15 min for combination between RNA and random primers. Then, 20 µl cDNA synthesis reaction mixture comprising 5.0 µl RNA was mixed with Random primers, 1x Improm-II™ Reaction buffer (Promega®) 4.0 µl, 2.5 mM $MgCl_2$ 2.0 µl (Promega®), 0.5 mM dNTPs 2.0 µl (Fermentas®, Marryland, USA), 40 U/µl Ribonuclease Inhibitor 0.3 µl (Promega®), 1U Improm-II™ Reverse Transcriptase 1.0 µl (Promega®), and RNase-free water to a final volume of 20 µl. The condition step included 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min. cDNA from the reverse transcription reaction was collected and chilled at -20°C before identified in the next step.

To identify the virus subtype, a multiplex reverse transcriptase polymerase chain reaction (multiplex RT-PCR) was performed using the previously-published primers specific for M, HA, and NA genes of Influenza A (Payungporn et al., 2004). The 25 µl multiplex PCR amplification reaction mixture comprised 1x Master mix 10 µl (Eppendorf®, Hamburg, Germany), 0.5 µM of each primer 1.5 µl, 1.0 µl of cDNA from the previous reverse transcription reaction, 1.0 mM $MgCl_2$ 1 µl (Promega®) and RNase-free water to a final volume of 25 µl. The amplification cycles included an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min (Payungporn et al., 2004).

A 5 µl volume of the PCR products was mixed with 2 µl of loading buffer (0.2% Orange G in 50% glycerol) and loaded into 0.2% agarose gel. The mixture was exposed to electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5µg/ml). The gel was photographed under UV light with a gel documentation system (Vilber Lourmat®, Lavalle Cedex, France). The expected size of the multiplex PCR products for the M, H5 and N1 genes was 125, 148 and 110 bp, respectively. Also cDNA sequencing of whole genome avian influenza H5N1 viruses were performed.

To identify the virus subtype, one step multiplex real-time RT-PCR was performed using the previously-published specific primers and probes for M, HA, and NA genes of Influenza A (Payungporn et al., 2006). The 10 µl one step multiplex real-time RT- PCR reaction mixture comprised 2x Reaction mix 5 µl (Invitrogen®, USA) and 0.2 µl SuperScript. III RT/Platinum® *Taq* Mix, 0.5 µM of each primer in 0.25 µl, 1.0 µl of RNA sample, 0.25 µl Probe for M, HA, and NA genes (0.5 µM) and RNase-free water to a final volume of 10 µl. The PCR reaction included a reverse transcription step at 50°C for 30 min, followed by initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 30 sec (Payungporn et al., 2004).

Polymerase chain reaction enzyme linked immunoassays (PCR-ELISA) technique was performed using the previously-published specific primers and probes for M, HA, and NA genes of Influenza A (Chaharaein et al., 2009). PCR-ELISA contained 2 steps including multiplex PCR and ELISA step. The viral RNA was reverse transcribed into cDNA. Multiplex PCR was performed using specific primers for M, HA, and NA genes. The 50 µl Multiplex PCR reaction mixture comprised Taq DNA polymerase 0.5 µl, 50 µM of each primer in 0.5 µl, 2.0 µl of cDNA from the previous reverse transcription reaction, 5 µl PCR DIG labeling mix, 5 µl 10x buffers $MgCl_2$ and RNase-free water to a final volume of 50 µl. The amplification reaction included an initial denaturation step at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec, concluded by a final extension step at 72°C for 5 min (Chaharaein et al., 2009). And the ELISA step, the samples were submitted to avian influenza researcher, faculty of veterinary science, Chulalongkorn University who

developed this method.This step was preceded with PCR ELISA for DIG detection (Roche®, Switzerland).

Phase 3: Genetic characterization of H5N1 virus and identification of virus genotypes

Genetic characterization of H5N1 viruses

After RNA extraction and cDNA preparation in phase 2, PCR amplification using specific primers for PB2, PB1, PA, HA, NP, NA, M, and NS was performed. The 50 µl multiplex PCR amplification reaction mixture comprised 1x Master mix 20 µl (Eppendorf®), 0.2 µM of each primer in 1.0 µl, 2.0 µl of cDNA from the previous reverse transcription reaction, 1.0 mM $MgCl_2$ 2 µl (Promega®) and RNase-free water to a final volume of 50 µl. The amplification cycles included an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 50-55°C for 30 sec and extension at 72°C for 90 sec, and concluded by a final extension step at 72°C for 7 min

PCR products were examined by agarose gel electrophoresis. A 25 µl volume of the PCR products was mixed with 2 µl of loading buffer. The products were further gel-purified using QIAquick gel extraction kit (QIAGEN®). The DNA fragment was excised from the agarose gel and placed into an eppendorf tube. These volumes of Binding buffer was added 1 volume of gel. The mixture was incubated at 50°C for 10 min and mixed by vortex every 2–3 min. One gel volume of isopropanol was added to the sample and mixed. The liquid was applied to the QIAquick column, and centrifuged for 1 min at 13,000 rpm. The 0.5 ml binding buffer was added to QIAquick column and centrifuged for 1 min at 13,000 rpm. To wash, The 0.75 ml Wash buffer was added to QIAquick column and centrifuged for 1 min at 13,000 rpm. The 50 µl elution buffer was added to the center of the QIAquick membrane and centrifuged the column for 1 min at 13,000 rpm.

Purified PCR products were sent to Molecular Informatrix Laboratory Limited, Shatin N.T. in Hong Kong for DNA sequencing. The DNA sequence data were validated by using Chromas V1.45 (Griffith University, Queensland, Australia) and aligned using

BioEdit program (Carlsbad, CA, USA). The phylogenetic analysis was performed by the clustal analysis using the MEGA 3.1 program (Tempe, AZ, USA).

Identification of viral genotypes

Steps of genotype classification included 1) preparation of complete nucleotide sequence of each genes of avian influenza virus, 2) comparison of nucleotide sequences using computer MEGA 3.1 program (Tempe, AZ, USA) to analyze genetic relationship, 3) classification of lineage of each gene of avian influenza virus, 4) arrangement of the sequential combination of the lineage of each gene, and 5) assignment the genotype of avian influenza H5N1 virus (Duan et al., 2008; (Li et al., 2004)). Bootstrapping support for tree topologies was operated using NJ methods with 1000 replicates performed by MEGA 3.1 program. A distinct phylogenetic lineage was then identified based on NJ bootstrap support of $\geq$70% or Bayesian posterior probability of $\geq$95%. A genotype was assigned using the 8 gene sequential combinations.

Instruments and chemical substances

PCR assay

- 2,3-dideoxynucleoside triphosphate (dNTPs), 5mM (Fermentas®, USA)
- Improm-II™ Reverse Transcriptase (Promega®, Madison, WI, USA)
- Improm-II™ 5x Reaction buffer (Promega®, Madison, WI, USA)
- $MgCl_2$, 25 mM (Promega®, Madison, WI, USA)
- Random primers, 0.5 µg (Promega®, Madison, WI, USA)
- Recombinant RNAsin® Ribonuclease Inhibitor, 40 u/ µl (Promega®, Madison, WI, USA)
- Ultrapure™ Distilled water DNase, RNase free (GIBCO®, USA)
- 2.5x Master Mix (Eppendorf®, Hamburg, Germany)
- Mg^{2+} solution, 25 mM (Eppendorf®, Hamburg, Germany)
- GeneRuler™ 100 bp DNA ladder (Fermentas®, USA)
- Agarose gel (Molecular grade)
- Ethidium Bromide 10 mg/ml (Sigma Aldrich Inc., USA)
- 0.2% Orange G loading dye in 50% glycerol (Carlo Ebra Reagent®, USA)
- 40x Tris-boric acid –EDTA (TBE) powder (Bio Basic Inc®, USA)
- 2x Reaction mix 5 µl (Invitrogen®, USA)
- SuperScript. III RT/Platinum® *Taq* (Invitrogen®, USA)

Viral Transport Media (VTM)

QIAamp® Viral RNA mini kit (Qiagen®, Hilden, Germany)

QIAquick Gel Extraction Kit (Qiagen®, Hilden, Germany)

PCR tube 0.2 ml (Axygen Scientific®, CA, USA)

Microcentrifuge tube 2 ml

Micropipette 0.5-2, 2-20, and 100-1000 µl (Gilson®, France)

Micropipette tip 2, 200 and 1,000 µl

Thermo cycler (Thermo electron corporation®, Cambridge, UK)

Gel electrophoresis system (OWL Scientific Inc®, USA)

UV transilluminator (Vilber Lourmat®, Lavalle Cedex, France)

Centrifuge (Denville Scientific Inc®, USA)

Refrigerator -20°C and -80°C

CHAPTER IV

RESULTS

In this study, we monitored Influenza A H5N1 virus from avian species in border areas between Thailand and neighboring countries (Laos and Myanmar) from September 2007 to June 2008. Two thousands one hundreds and seventy five samples were collected from the border areas. The samples were subjected to influenza A isolation, identification, and genetically characterization using embryonated egg inoculation, hemagglutination assay, multiplex RT-PCR, realtime RT-PCR, PCR-ELISA, nucleotide sequencing and phylogenetic analysis.

The 2,175 samples (2,139 samples from live poultry and 36 samples from visceral organs) were collected from 42 districts of 13 provinces along the borders of Thailand and neighboring countries, Myanmar and Laos. The samples were collected from villages and fresh markets of Chiang Rai, Chiang Mai, Mae Hong Son, Tak, Kanchanaburi, Ratchaburi, Prachuap Khiri Khan, Ranong, Loei, Nong Khai, Nakhon Phanom, Mukdahan, and Ubon Ratchathani provinces (Table 2). Selection criteria of locations for sample collection depended on history of outbreaks of avian influenza, mortality rate, and animal movements.

Table 2: List of locations, number of samples and date of sample collection in this study.

Province	District	No. samples	total	Date of sample collection
Chiang Rai	Muang	37	84	Sep 07
	Mae Sai	20		
	Chiang Saen	24		
	Phan	3		

Loei	Muang	12	69	Oct 07
	Chiang Khan	57		
Nong Khai	Sangkhom	1	57	Oct 07
	Si Chiang Mai	56		
Mae Hong Son	Pai	1	165	Dec 07
	Pang Ma Pha	6		
	Khun Yuam	158		
Nakhon Phanom	Muang	81	196	Dec 07
	Tha Phanom	92		
	Tha Uthen	23		
Ratchaburi	Suan Phueng	87	87	Dec 07
Ubon Ratchathani	Khong Chiam	225	229	Feb 08
	Warin Chamrap	4		
Kanchanaburi	Sai Yok	67	104	Mar 08
	Thong Pha Phum	12		
	Sangkhla Buri	25		
Tak	Mae Sot	132	328	Mar 08
	Mae Ramat	152		
	Ban Tak	44		
Mukdahan	Muang	58	196	April 08
	Don Tan	66		
	Nikom	72		
Prachuap Khiri Khan	Thap Sakae	45	201	April 08
	Kui Buri	11		
	Muang	145		
Ranong	Muang	57	176	May 08
	La Un	46		

	Kra Buri	73		
Chiang Mai	Mae Rim	1	283	June 08
	Chiang Dao	45		
	Hang Dong	61		
	Saraphi	102		
	San Sai	50		
	San Kamphaeng	25		
Total			**2175**	

List of samples collected from different avian species is shown in Table 3. Of the 2,175 samples, 2,139 live birds (Chicken n= 1,907, Duck n= 179, quail n= 31, Pigeon n= 6, Baya weaver n= 5, Turkey n= 3, Red-whiskered bulbul n= 3, Spotted dove n= 2, each kind of peafowl, white-rumped sham, and hill myna n= 1) and 36 visceral organs (Chicken n= 22, Duck n= 1, Watercock n= 3, and Moorhen n= 10) were collected from 42 districts of 13 provinces in border areas between Thailand and neighboring countries (Laos and Myanmar).

Table 3: List of samples collected from different avian species. (*n*=2,175)

Host	No. samples	
	Live birds	Food market
Chicken	1,907	22
Duck	179	1
Quail	31	-
Watercock	-	3
Moorhen	-	10
Pigeon	6	-
Baya weaver	5	-
Turkey	3	-
Red-whiskered Bulbul	3	-

Spotted Dove	2	-
other*	3	-
Total	**2,139**	**36**

Other*: peafowl, white-rumped sham, and hill myna

Isolation and identification of Influenza A H5N1 virus

In this study, the 2,175 samples were subjected for virus isolation using egg inoculation at the Biosafety level 2+ laboratory. Of 2,175 samples, 68 (3.13 %) were positive for Hemagglutination activity. Among these positive samples, 60 samples were from chicken (88.2%) and 8 samples were from Duck (11.8%). In partition, provinces that have most positive HA samples were Prachuap Khiri Khan (35.3%) and Mukdahan (16.2 %) (Table 4).

Table 4: List of hemagglutination activity (HA) positive samples by species and location. (*n*=68)

Province	Chicken	Duck	Total	Percentage %
Loei	2	2	4	5.88
Nong Khai	1	1	2	2.94
Chiang Rai	5	-	5	7.35
Nakhon Phanom	2	-	2	2.94
Kanchanaburi	9	-	9	13.23
Tak	8	2	10	14.71
Ubon Ratchathani	1	-	1	1.47
Mukdahan	10	1	11	16.17
Prachuap Khiri Khan	22	2	24	35.29
Total	**60**	**8**	**68**	**100**

The HA-positive samples were then examined by multiplex RT-PCR , realtime RT-PCR, and PCR-ELISA for influenza A (M gene) and subtype H5N1 (H5 and N1 genes) Of

68 HA positive samples, 15 samples were positive for M, H5, and N1 by multiplex RT-PCR (Table 5). The samples were recovered from chicken (*n*=13) and duck (*n*=2). These 15 samples were confirmed as H5N1 viruses by realtime RT-PCR and PCR-ELISA. Subtype identification of HPAI H5N1 virus by realtime RT-PCR and PCR-ELISA is now shown in Table 6.

Table 5: List of H5N1 viruses identify by multiplex RT-PCR

Province	Host	Sample		
		Influenza A (M gene)	Subtype H5 (HA gene)	Subtype N1 (NA gene)
Loei	chicken	2	2	2
	duck	2	2	2
Chiang Rai	chicken	1	1	1
Prachuap Khiri Khan	Chicken	10	2	7
Total		**15**	**7***	**12***

*The samples were tested positive for H5N1 subtype either by Realtime RT-PCR or PCR ELISA.

Table 6: Subtype identification of HPAI H5N1 virus by realtime RT-PCR and PCR-ELISA

ID sample	Results	
	HA gene (H5)	NA gene (N1)
177	H5	N1
205	H5	N1
212	H5	N1
219	H5	N1
31	H5	N1
13/6	H5[a]	N1
15/6	H5[a]	N1
23/6	H5	N1

27/6	H5	N1
45/6	H5[a]	N1
48/6	H5[a]	N1
97/6	H5[a]	N1
98/6	H5[a]	N1[a]
100/6	H5[a]	N1[a]
101/6	H5[a]	N1[a]

[a] Positive samples by PCR-ELISA only

The results of influenza A virus identification (positive matrix gene) by RT-PCR is shown in Figure 5. Positive Matrix gene samples yielded PCR product with 125 bp insize. These results confirmed the identification of 15 samples as influenza A virus. The results of subtype H5 identification (Hemagglutinin gene positive) by RT-PCR is shown in Figure 6. Positive Hemagglutinin gene samples generated 148 bp- PCR product. Again, these results were in agreement with the result of the identification of those Influenza A virus subtype H5.

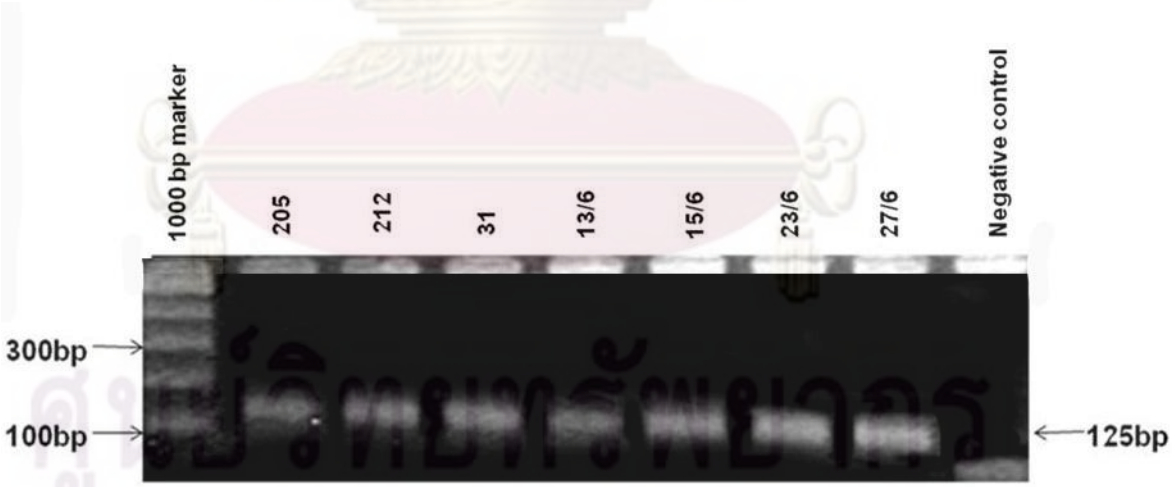


Figure 5: Identification of influenza A viruses (matrix gene positive) by multiplex RT-PCR. The size of expected PCR product is 125 bp.

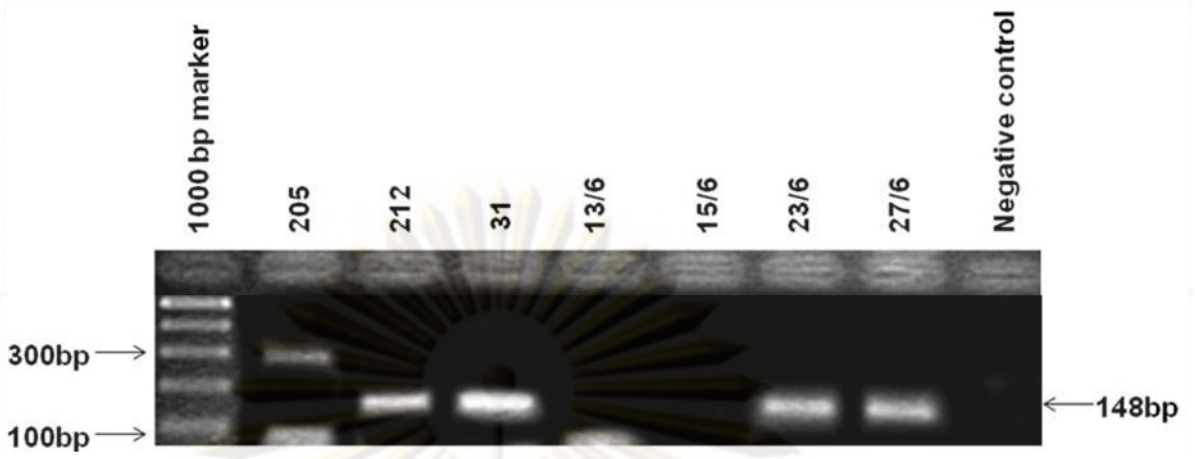


Figure 6: Identification of influenza A subtype H5 (hemagglutinin gene positive) by multiplex RT-PCR. The expected product size is 148 bp.

In this study, we also confirmed the HA positive samples using realtime RT-PCR and PCR-ELISA. The results were consistented with those from multiplex RT-PCR. The viruses were identified as avian influenza H5N1 virus by Realtime RT-PCR. Identification of M gene (Matrix gene), H5 (Hemagglutinin gene), and N1 (Neuraminidase gene) by realtime RT-PCR is shown in Figure 7, 8, and 9, respectively.

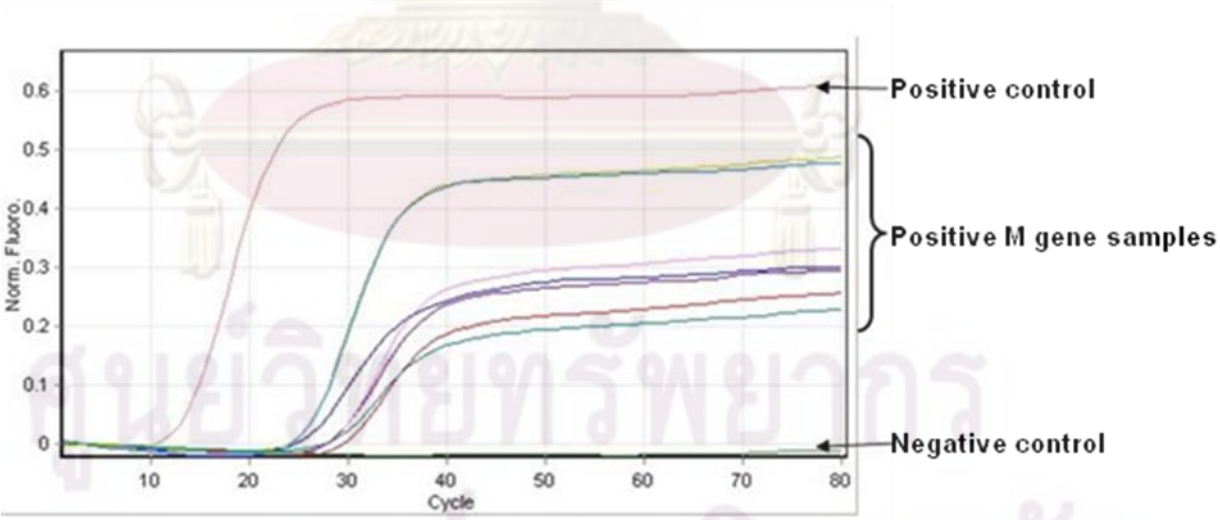


Figure 7: Identification of M gene of influenza A virus by realtime RT-RCR. Positive results of PCR amplification were Ct value of < 40 Ct.

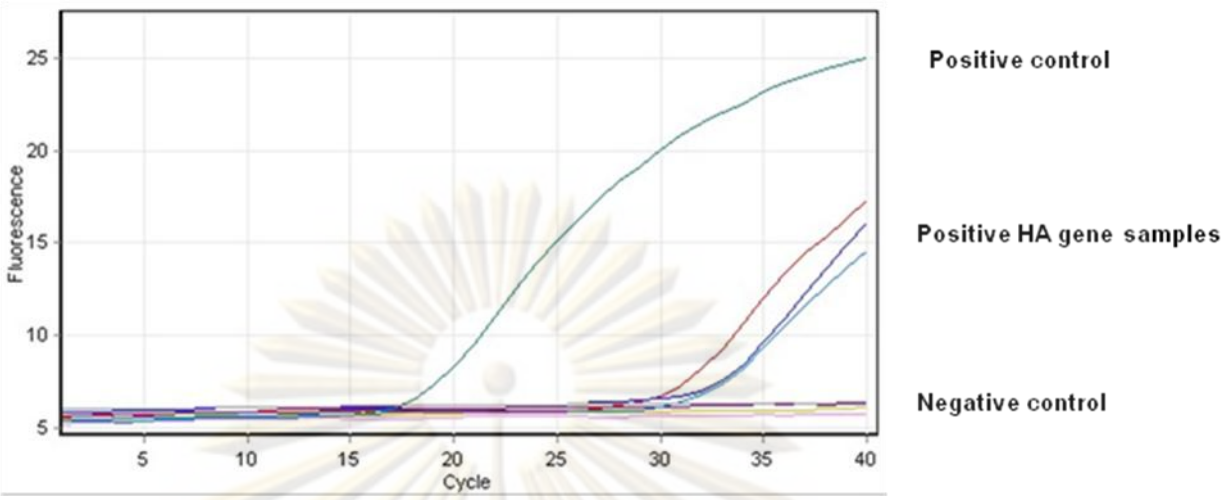


Figure 8: Identification of HA gene of influenza A virus by realtime RT-RCR. Positive results of PCR amplification were Ct value of < 40 Ct.

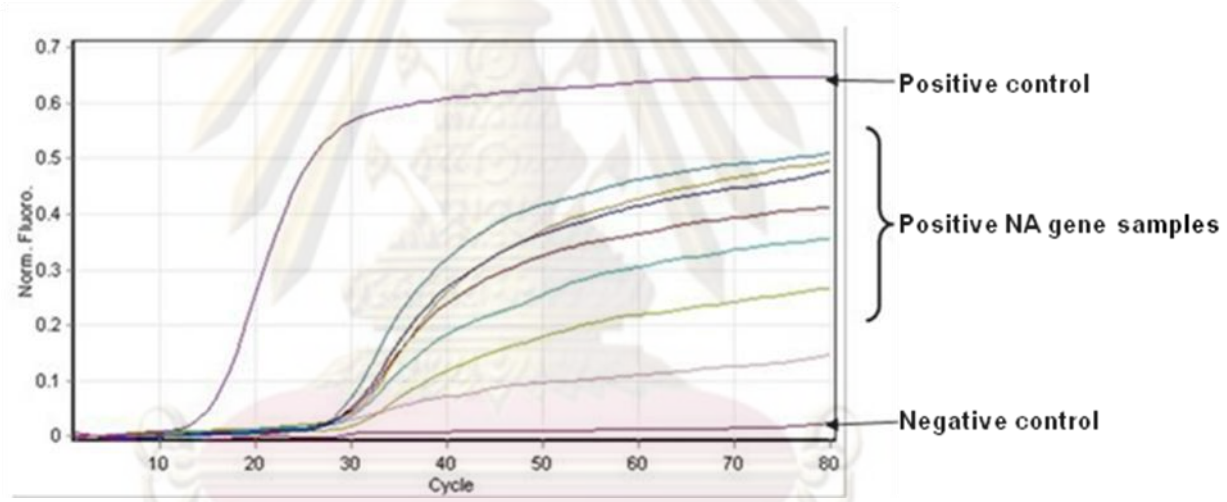


Figure 9: Identification of NA gene of influenza A virus by realtime RT-RCR. Positive results of PCR amplification were Ct value of < 40 Ct.

Influenza A H5N1 virus was also confirmed by PCR-ELISA. This method was developed by a researcher from the faculty of Veterinary Science, Chulalongkorn University. PCR-ELISA technique is considered to be a high specificity and sensitivity method. The technique also provides consistent result with multiplex RT-PCR and realtime PCR technique (Chaharaein et al., 2009). The results of PCR-ELISA are shown in Figure 10.

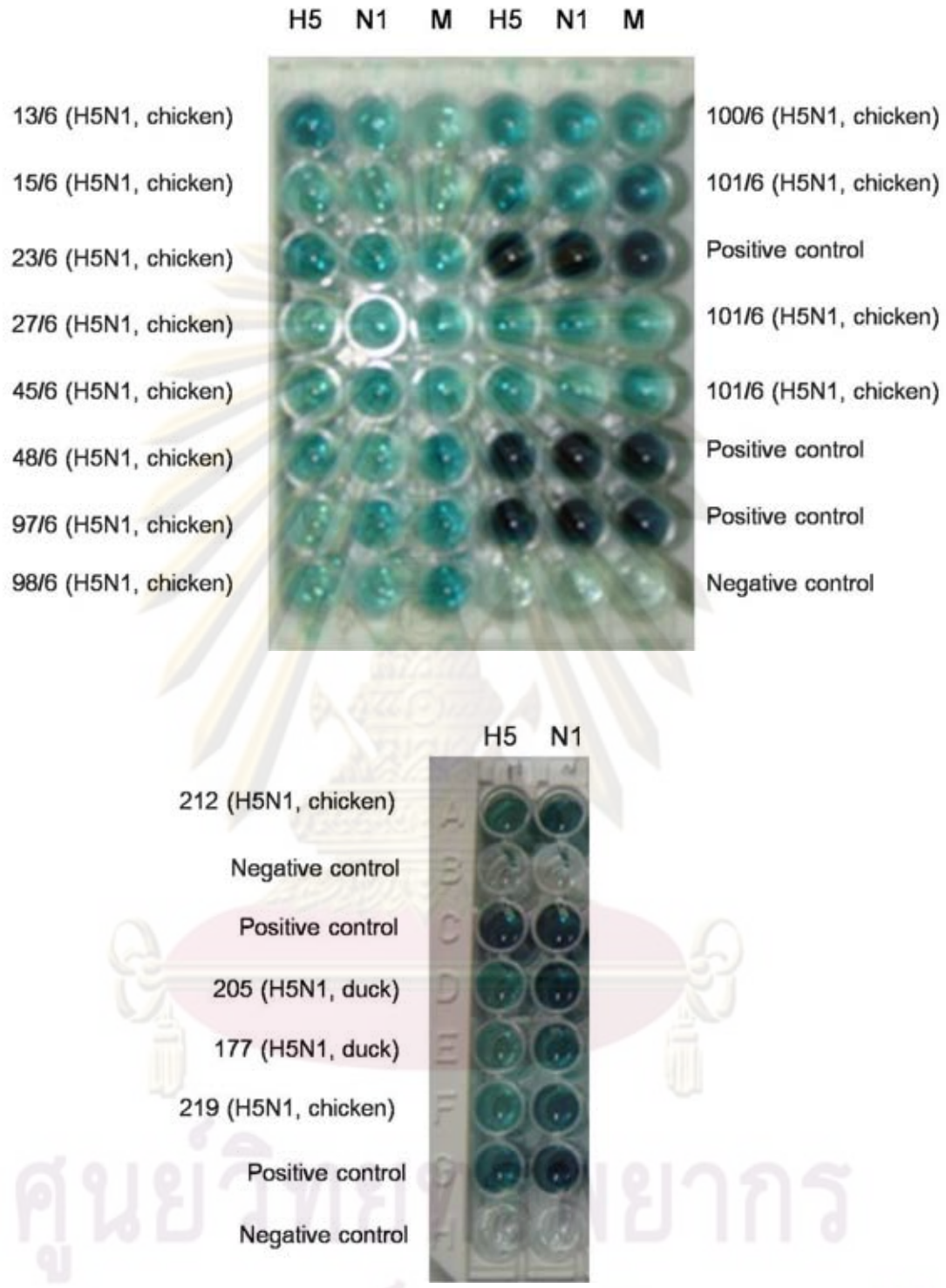


Figure 10: Identification of influenza A virus subtype H5N1 by PCR-ELISA. Positive M, H5, and N1 genes were identified as increasing of color intensity ($O.D._{405}$-$O.D._{492}$> 0.3).

DNA sequencing of avian influenza H5N1 viruses

In this study, we have selected 3 avian influenza H5N1 viruses which were the representatives from each province of Loey, Chiang Rai, and Prachuap Khiri Khan for whole gene sequencing. We have sequenced whole genome of 3 avian influenza H5N1 viruses, CU-345 (ID- sample: 219), CU-346 (ID- sample: 31) and CU-347 (ID- sample: 23/6). The description of avian influenza H5N1 virus samples characterized in this study is shown in Table 7. Nucleotide sequences of 8 genes (PB2, PB1, PA, HA, NP, NA, M, and NS) of 3 avian influenza H5N1 viruses is shown in Table 8.

Table 7: Host, location, and year of avian influenza H5N1 virus samples sequenced in this study.

CU-ID samples*	Description	Host	Location	year
CU345	A/duck/Loei/Thailand/CU-345/07	Duck	Loei	2550
CU346	A/chicken/Chiang Rai/Thailand/CU-346/07	Chicken	Chiang Rai	2550
CU347	A/ck/Prachuap Khiri Khan/Thailand/CU-347/08	Chicken	Prachuap Khiri Khan	2551

*CU-345 (ID- sample: 219), CU-346 (ID- sample: 31) and CU-347 (ID- sample: 23/6)

Table 8: Available nucleotide sequence of 8 genes (PB2, PB1, PA, HA, NP, NA, M, and NS) of 3 avian influenza H5N1 viruses characterized in this study.

CU-ID samples	Nucleotide sequence (bp)							
	PB2 gene	PB1 gene	PA gene	HA gene	NP gene	NA gene	M gene	NS gene
CU345	-*	-*	517	456	555	1353	-*	825
CU346	-*	-*	-*	755	555	1381	-*	-*
CU347	2282	2294	2221	1717	1523	1382	985	854

-* : N/A not available

Genetic relatedness and genotype analysis of avian influenza H5N1 virus

Genetic relatedness of avian influenza H5N1 virus can be analyzed by sequence comparison of these viruses with the viruses isolated from Thailand and foreign countries. Results of genetic relatedness of 3 avian influenza H5N1 viruses are shown in phylogenetic tree (Figure 12- 31). The polymorphisms of key determinant residues of 8 genes of 3 avian influenza H5N1 viruses were analyzed and compared with the original avian influenza H5N1 virus "Goose/Guangdong/1/96" and other avian influenza viruses from in Thailand and other countries (Table 9-16).

Genotype analysis

The genotype of avian influenza H5N1 virus can be classified when genetic data of 8 genes of viruses are available for comparing the genetic relatedness. Genotype analysis of avian influenza H5N1 virus in this study was performed in only 1 sample (CU-347) with the sequences of the 8 genes. The nucleotide sequences from each gene segment analyzed were as follows: PB2 1-2277 bp, PB1 1-2271 bp, PA 2151 bp, HA 1-1676 bp, NP 1-1481 bp,NA 1-1386 bp, M 1-960 bp, and NS 1-690 bp. A distinct phylogenetic lineage was recognized based on NJ bootstrap support of $\geq$70% or Bayesian posterior probability of $\geq$95% as indicating the origin. A genotype was assigned when the 8 genes lineage resulted in a unique gene constellation. Nevertheless, genotypes were also named in cases where viruses had the same gene lineage but diverged by some key determinant, for example, an amino-acid deletion, e.g. genotypes Z and Z+ diverge only in the presence or absence of a 20-aa deletion in the neuraminidase (NA) protein (Guan et al., 2002).

Previous studies have shown that 2 clades (clade 1 and 2.3.4) or 2 genotypes (genotype Z and V) of avian influenza H5N1 viruses were found to emerge and cause the avian influenza outbreaks in Thailand (Chutinimitkul et al., 2007). Genotypes of avian influenza H5N1 viruses in Thailand, 2004-2008 were analyzed and compared with the original avian influenza H5N1 virus "Goose/Guangdong/1/96" and other avian influenza viruses from other countries and are shown in Figure 11. In this study, phylogenetic

analysis of whole genome (CU-347) showed that the viruses clustered within the lineage of H5N1 avian isolates from Thailand-Vietnam lineage, genotype Z or clade 1. In contrast, the viruses isolated in Indonesia and China formed a separate lineage. In Thailand 2004-2008, genotypes were detected including genotype Z and genotype V. Genotypes Z and V have only HA and NA genes origin from Goose/Guangdong/1/96-lingeage (Figure 11). Genotypes Z and V have seven segmented genes from a common source whereas their PA genes have unlike origins (Chen et al., 2006). Results of genetic analysis of 3 avian influenza H5N1 viruses are shown in phylogenetic tree (Figure 12-31).

Currently, the classification of clade system of avian influenza H5N1 virus was developed based on the evaluation of the HA gene. This classification system was developed by the group of scientists and relevant international organizations such as World Health Organization (WHO), World Animal Health Organization (OIE) and the Food and Agriculture Organization (FAO). In this study, the 3 avian influenza H5N1 viruses (CU-345, CU-346 and CU-347) belonged to clade 1 that was considered as an important clade and the cause's outbreaks of avian influenza in Thailand and Vietnam as well as Cambodia, Laos and Malaysia. The results of clade classification can infer the evolution of virus from the same original virus group (same ancestor).

In summary, avian influenza H5N1 viruses isolated from border areas of Thailand, Laos and Myanmar were classified as genotype Z or clade 1. The viruses belonged to the common avian influenza H5N1 genotype or clade that has caused the outbreaks of avian influenza in Thailand.

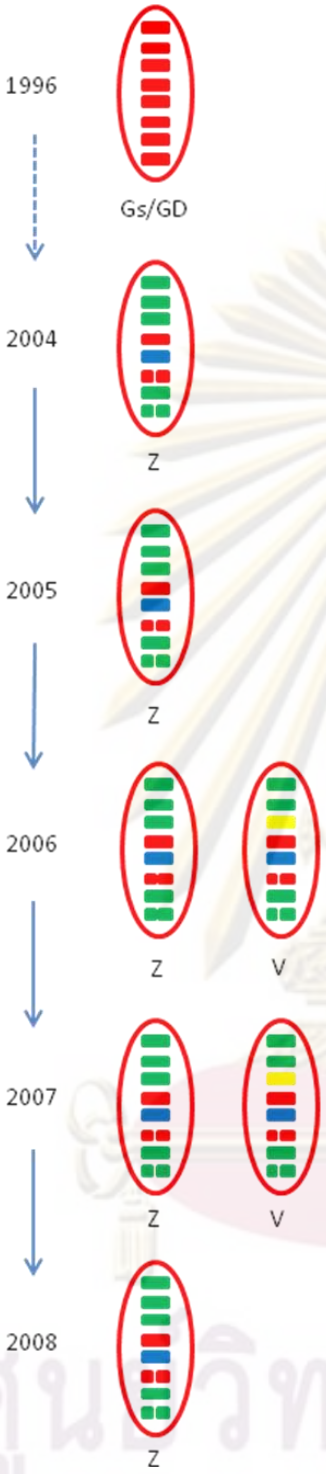


Figure 11: Genotype of avian influenza H5N1 viruses in Thailand, 2004-2008. The eight gene segments, represented by horizontal bars are, from top to bottom, polymerase (PB2, PB1, PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural (NS) genes. A different color represents each

Hemagglutinin gene

Avian influenza H5N1 viruses from this study (CU-345, CU-346, and CU-347) are grouped in Clade 1 which is a group related to the avian influenza outbreaks in the last 4-5 years in Thailand (Figure 12). Connecting peptide sequences or HA cleavage site of those viruses harbor multiple basic amino acids (RERRRKK), similar to the majority of HPAI H5N1 isolates outbreaks. CU-346 and CU-347 isolates had a glutamine at position 222 and a glycine at position 224, which are related to receptor binding sites for avian species (Matrosovich et al., 1999; Shinya et al., 2006; Webster et al., 1997). The polymorphisms of Hemagglutinin protein of avian influenza viruses at key determinant residues such as HA cleavage site, receptor binding site, glycosylation, and antigenic sites are shown in Table 9, 10, and Figure 13, 14. In this study, avian influenza H5N1 viruses (CU-345, CU 346, and CU347) have no mutations in any key determinants of Hemagglutinin gene.

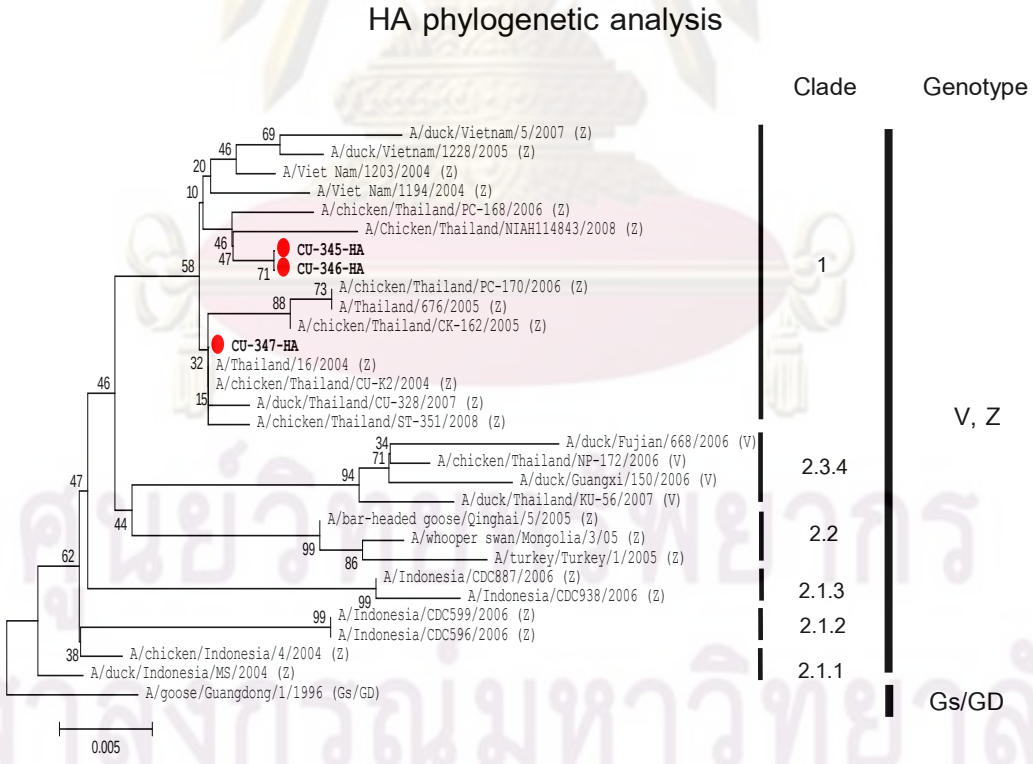


Figure 12: Phylogenetic analysis of HA gene of H5N1 viruses isolated in this study comparing with other H5N1 viruses isolated from Thailand and other countries. The analysis was based on nucleotides 1-1676 of the HA gene.

Table 9: Genetic analysis of deduced amino acid of HA gene at key determinant residues in cleavage sites and receptor binding sites.

Virus	HA gene				
	Connecting peptide Sequences	Receptor binding site			
	323-329[a]	129[b]	175[b]	222[c]	224[c]
A/chicken/Thailand/CU-347/2008	RERRRKK	L	L	Q	G
A/duck/Thailand/CU-345/2007	RERRRKK	-*	-*	-*	-*
A/chicken/Thailand/CU-346/2007	RERRRKK	-*	L	Q	G
A/Thailand/16/2004	RERRRKK	L	L	Q	G
A/chicken/Thailand/CU-K2/2004	RERRRKK	L	L	Q	G
A/Thailand/676/2005	REKRRKK	V	L	Q	G
A/chicken/Thailand/CK-162/2005	REKRRKK	L	L	Q	G
A/chicken/Thailand/NP-172/2006	RERRRK-	S	L	Q	G
A/chicken/Thailand/PC-168/2006	RERRRKK	L	L	Q	G
A/chicken/Thailand/PC-170/2006	REKRRKK	L	L	Q	G
A/duck/Thailand/CU-328/2007	RERRRKK	L	L	Q	G
A/duck/Thailand/KU-56/2007	REKRRK-	S	L	Q	G
A/chicken/Thailand/ST-351/2008	RERRRKK	L	L	Q	G
A/chicken/NIAH114843/2008	RERRRKK	L	L	Q	G
A/Viet Nam/1194/2004	RERRRKK	L	L	Q	G
A/Viet Nam/1203/2004	RERRRKK	L	L	Q	G
A/duck/Vietnam/1228/2005	RERRRKK	M	M	Q	G
A/duck/Vietnam/5/2007	REGRRKK	M	M	Q	G
A/chicken/Indonesia/4/2004	RERRRKK	S	L	Q	G
A/duck/Indonesia/MS/2004	RERRRKK	S	L	Q	G
A/Indonesia/CDC596/2006	RERRRKK	L	L	Q	G
A/Indonesia/CDC599/2006	RERRRKK	L	L	Q	G
A/Indonesia/CDC887/2006	RESRRKK	S	L	Q	G
A/Indonesia/CDC938/2006	RESRRKK	S	L	Q	G
A/turkey/Turkey/1/2005	GERRRKK	A	L	Q	G
A/whooper swan/Mongolia/3/2005	GERRRRK	S	L	Q	G
A/goose/Guangdong/1/1996	RERRRKK	S	L	Q	G
A/bar-headed goose/Qinghai/5/2005	GERRRKK	S	L	Q	G
A/duck/Guangxi/150/2006	RERRRK-	S	L	Q	G
A/duck/Fujian/668/2006	RERRRK-	S	L	Q	G

[a] Avian influenza viruses in this study have connecting peptide sequences that are RERRRKK.

[b] Amino acid at position 129 and 175 of HA gene: S (Serine)/L: (Leucine) and L (Leucine) respectively.

[c] Amino acid at position 222 and 224 of HA gene: Q (Glutamine), G (Glycine) respectively.

-*: N/A not available

Table 10: Genetic analysis of deduced amino acid of HA gene at key determinant residues in glycosylation sites.

Virus	Glycosylation sites[a]						
	10-12	11-13	23-25	154-	165-	193-	286-
A/chicken/Thailand/CU-347/2008	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Thailand/CU-345/2007	-*	-*	-*	-*	-*	-*	N-S
A/chicken/Thailand/CU-346/2007	-*	-*	-*	N-T	N-T	N-T	N-S
A/Thailand/16/2004	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Thailand/CU-K2/2004	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Thailand/676/2005	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Thailand/CK-162/2005	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Thailand/NP-172/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Thailand/PC-168/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Thailand/PC-170/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Thailand/CU-328/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Thailand/KU-56/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Thailand/ST-351/2008	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/NIAH114843/2008	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Viet Nam/1194/2004	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Viet Nam/1203/2004	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Vietnam/1228/2005	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Vietnam/5/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/chicken/Indonesia/4/2004	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Indonesia/MS/2004	N-S	N-T	N-T	(N-A)[b]	N-T	N-T	N-S
A/Indonesia/CDC596/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Indonesia/CDC599/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Indonesia/CDC887/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Indonesia/CDC938/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/turkey/Turkey/1/2005	N-S	N-T	N-T	(D-A)[b]	N-T	N-T	N-S
A/whooper swan/Mongolia/3/2005	N-S	N-T	N-T	(D-A)[b]	N-T	N-T	N-S
A/goose/Guangdong/1/1996	N-S	N-T	N-T	(N-A)[b]	N-T	N-T	N-S
A/bar-headed goose/Qinghai/5/2005	N-S	N-T	N-T	N-A	N-T	N-T	N-S
A/duck/Guangxi/150/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/duck/Fujian/668/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S

[a] Amino acid at glycosylation site of HA gene: N (Asparagine), S (Serine), T (Threonine)

[b] Amino acid at position 154-156 (N-A) and (D-A) do not have glycosylation properties.

-*: N/A not available

Table 11: Genetic analysis of deduced amino acid of HA gene at key determinant residues in antigenic sites.

Virus	Antigenic sites[a]				
	Antigenic site E		Antigenic site A		
	83	86	138	140	141
A/chicken/Thailand/CU-347/2008	A	V	Q	K	S
A/duck/Thailand/CU-345/2007	-*	-*	-*	-*	-*
A/chicken/Thailand/CU-346/2007	-*	-*	-*	-*	L
A/Thailand/16/2004	A	V	Q	K	S
A/chicken/Thailand/CU-K2/2004	A	V	Q	K	S
A/Thailand/676/2005	P	A	Q	K	S
A/chicken/Thailand/CK-162/2005	A	A	Q	K	S
A/chicken/Thailand/NP-172/2006	A	A	Q	T	P
A/chicken/Thailand/PC-168/2006	A	V	L	K	S
A/chicken/Thailand/PC-170/2006	P	A	Q	K	S
A/duck/Thailand/CU-328/2007	A	A	Q	K	S
A/duck/Thailand/KU-56/2007	A	A	Q	T	P
A/chicken/Thailand/ST-351/2008	A	V	Q	K	S
A/chicken/NIAH114843/2008	A	V	L	R	S
A/Viet Nam/1194/2004	A	V	Q	K	S
A/Viet Nam/1203/2004	A	V	Q	K	S
A/duck/Vietnam/1228/2005	A	V	Q	K	S
A/duck/Vietnam/5/2007	A	V	Q	R	S
A/chicken/Indonesia/4/2004	A	A	Q	K	S
A/duck/Indonesia/MS/2004	A	A	Q	K	S
A/Indonesia/CDC596/2006	A	A	L	R	S
A/Indonesia/CDC599/2006	A	A	L	R	S
A/Indonesia/CDC887/2006	A	T	L	S	P
A/Indonesia/CDC938/2006	A	T	L	S	P
A/turkey/Turkey/1/2005	I	A	Q	R	S
A/whooper swan/Mongolia/3/2005	I	A	Q	R	S
A/goose/Guangdong/1/1996	A	A	H	R	S
A/bar-headed goose/Qinghai/5/2005	I	A	Q	R	S
A/duck/Guangxi/150/2006	A	A	Q	T	P
A/duck/Fujian/668/2006	A	A	Q	T	P

[a] Amino acid at position 83, 86, 138, 140, 141 of HA gene: A (Alanine), V (Valine), H (Histidine), Q (Glutamine), L (Leucine), R (Arginine), K (Lysine), N (Asparagine), S (Serine), P (Proline)

-*: N/A not available

HA analysis

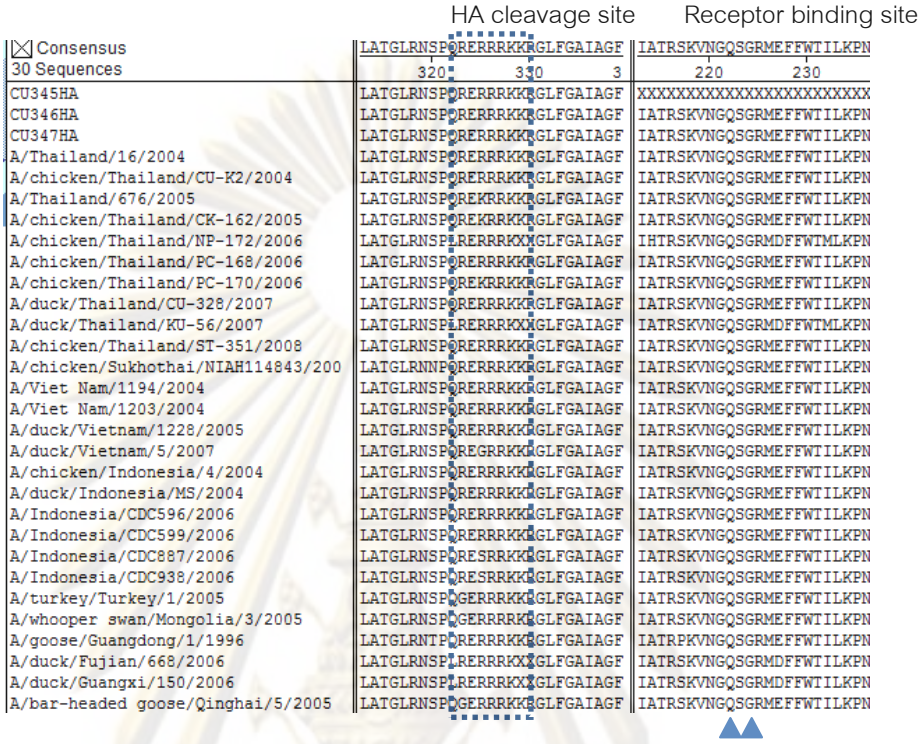


Figure 13: Comparison of deduced amino acid of HA protein at HA cleavage site at position 323-329 and receptor binding site at position 222-224

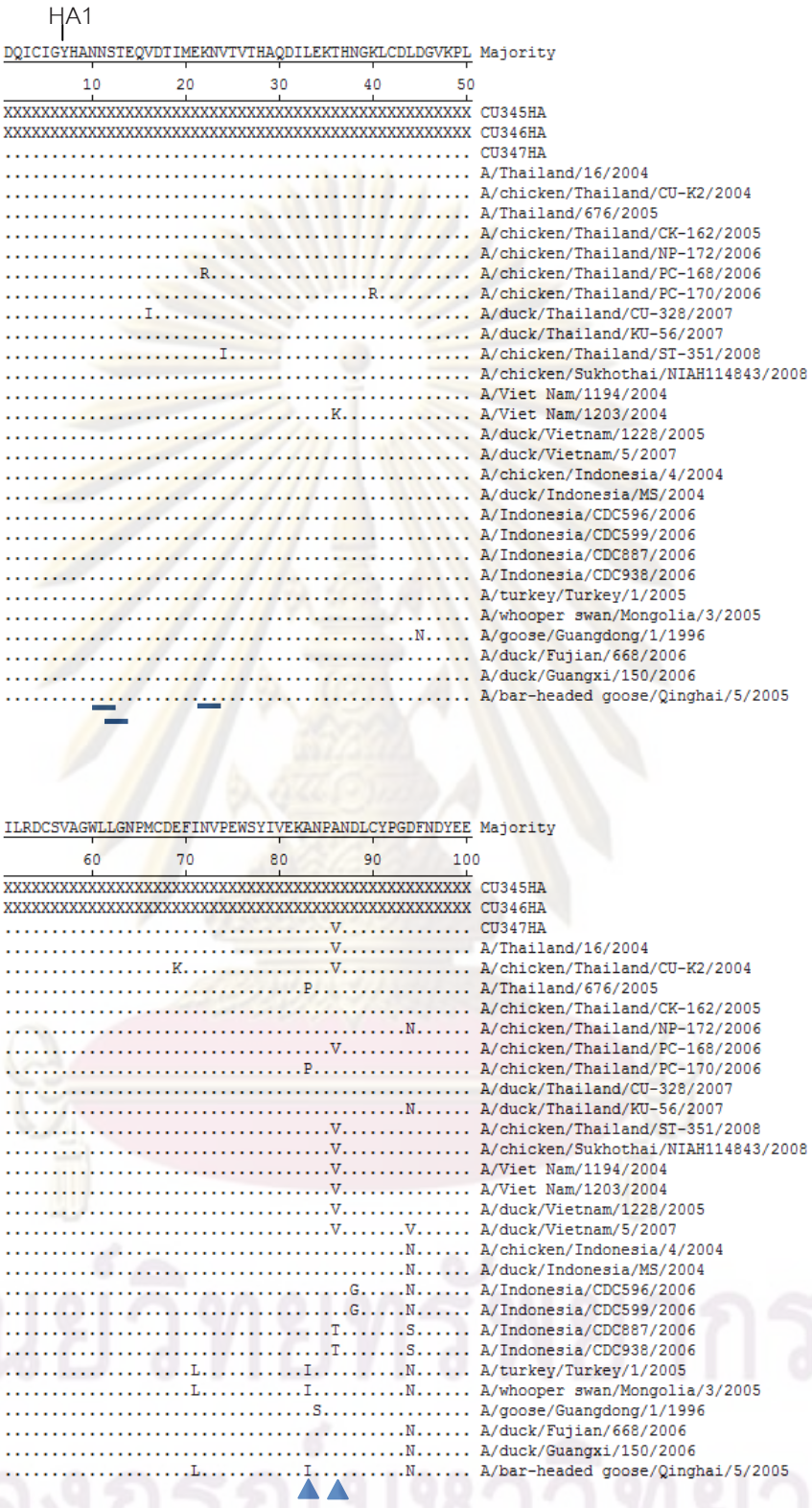


Figure 14: Comparison of deduced amino acid of HA protein at glycosylation sites and antigenic sites (underlines represent glycosylation sites and triangles represent antigenic sites).

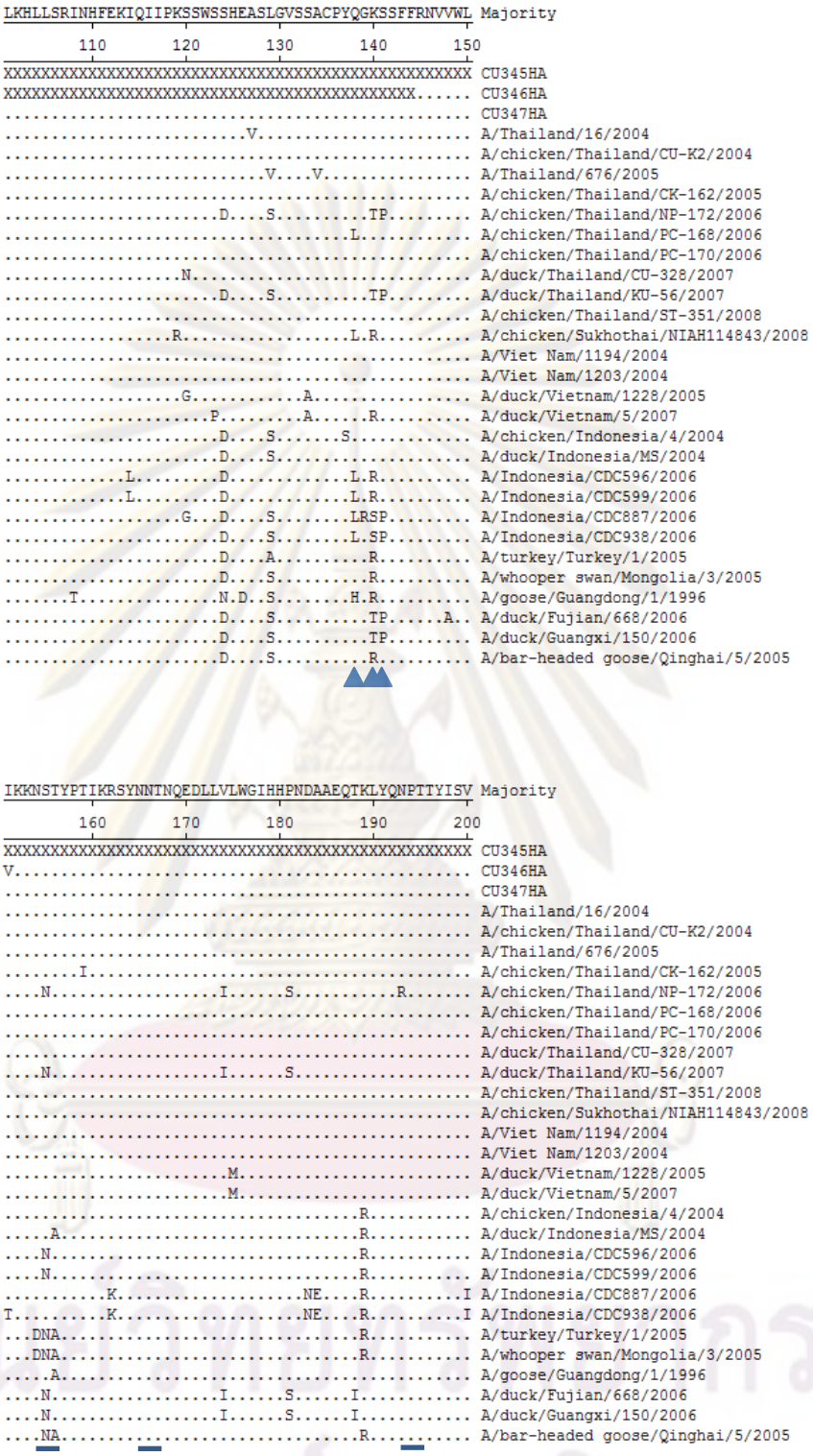


Figure 14 (Cont): Comparison of decuded amino acid of HA protein at glycosylation sites and antigenic sites (underlines represent glycosylation sites and triangles represent antigenic sites).

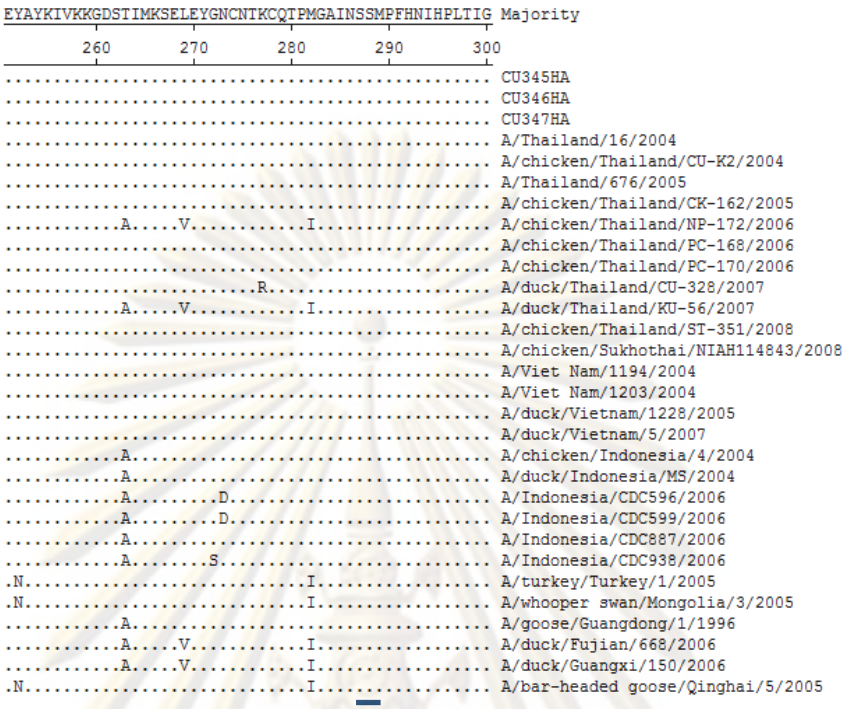


Figure 14 (Cont): Comparison of deduced amino acid of HA protein at glycosylation sites and antigenic sites (underlines represent glycosylation sites and triangles represent antigenic sites). (Cont)

Neuraminidase gene

Genetic relatedness of NA gene of avian influenza H5N1 viruses was determined (Figure 15). Avian influenza H5N1 viruses from this study (CU-345, CU 346, and CU347) are grouped into Genotype Z. Sequence analysis of those viruses revealed that all three isolates contained a 20 amino acid deletion in the NA stalk (position 49-68). On the other hand, no amino acid deletion was detected in the Goose/Guangdong/1/96 isolate. Genetic analysis of NA protein at key determinant residues including NA stalk region, and Oseltamivir resistance residues are shown in Table 12 and Figure 16, 17. Avian influenza H5N1 viruses (CU-345, CU 346, and CU347) were found to have no mutation at NA stalk region and were sensitive to Oseltamivir.

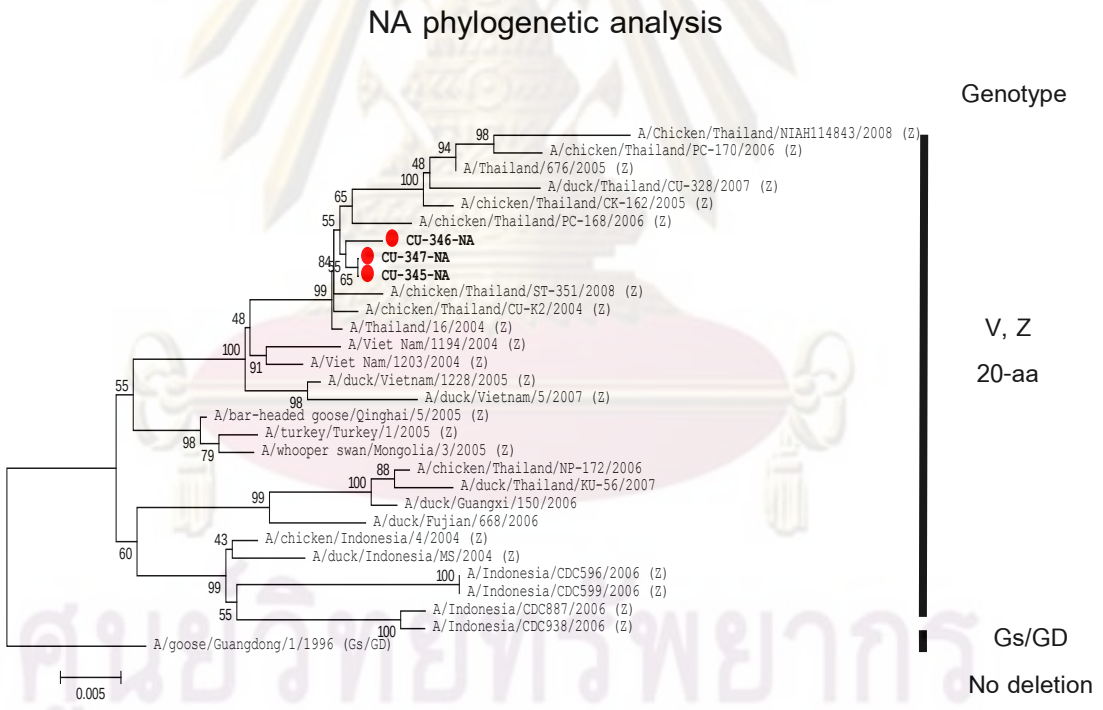


Figure 15: Phylogenetic analysis of NA gene of H5N1 viruses isolated in this study compared to that of other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-1386 of the NA gene.

Table 12: Genetic analysis of NA protein at key determinant residues in NA stalks region and Oseltamivir resistance residues.

Virus	NA gene				
	NA stalk region	Oseltaivir resistant residues			
	49-68[a]	119[b]	275[c]	293[d]	295[e]
A/chicken/Thailand/CU-347/2008	20 aa deletion	E	H	R	N
A/duck/Thailand/CU-345/2007	20 aa deletion	E	H	R	N
A/chicken/Thailand/CU-346/2007	20 aa deletion	E	H	R	N
A/Thailand/16/2004	20 aa deletion	E	H	R	N
A/chicken/Thailand/CU-K2/2004	20 aa deletion	E	H	R	N
A/Thailand/676/2005	20 aa deletion	E	H	R	N
A/chicken/Thailand/CK-162/2005	20 aa deletion	E	H	R	N
A/chicken/Thailand/NP-172/2006	20 aa deletion	E	H	R	N
A/chicken/Thailand/PC-168/2006	20 aa deletion	E	H	R	N
A/chicken/Thailand/PC-170/2006	20 aa deletion	E	H	R	N
A/duck/Thailand/CU-328/2007	20 aa deletion	E	H	R	N
A/duck/Thailand/KU-56/2007	20 aa deletion	E	H	R	N
A/chicken/Thailand/ST-351/2008	20 aa deletion	E	H	R	N
A/chicken/NIAH114843/2008	20 aa deletion	E	H	R	N
A/Viet Nam/1194/2004	20 aa deletion	E	H	R	N
A/Viet Nam/1203/2004	20 aa deletion	E	H	R	N
A/duck/Vietnam/1228/2005	20 aa deletion	E	H	R	N
A/duck/Vietnam/5/2007	20 aa deletion	E	H	R	N
A/chicken/Indonesia/4/2004	20 aa deletion	E	H	R	N
A/duck/Indonesia/MS/2004	20 aa deletion	E	H	R	N
A/Indonesia/CDC596/2006	20 aa deletion	E	H	R	N
A/Indonesia/CDC599/2006	20 aa deletion	E	H	R	N
A/Indonesia/CDC887/2006	20 aa deletion	E	H	R	N
A/Indonesia/CDC938/2006	20 aa deletion	E	H	R	N
A/turkey/Turkey/1/2005	20 aa deletion	E	H	R	N
A/whooper swan/Mongolia/3/2005	20 aa deletion	E	H	R	N
A/goose/Guangdong/1/1996	no deletion	E	H	R	N
A/bar-headed goose/Qinghai/5/2005	20 aa deletion	E	H	R	N
A/duck/Guangxi/150/2006	20 aa deletion	E	H	R	N
A/duck/Fujian/668/2006	20 aa deletion	E	H	R	N

[a] Amino acid at position 49-68 of NA gene

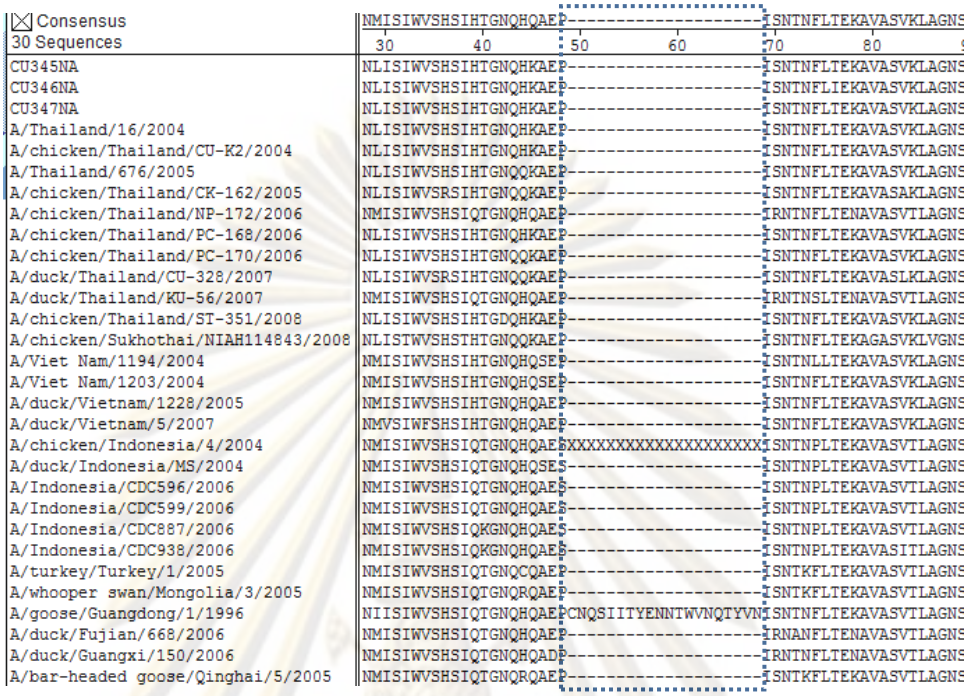
[b] Amino acid at position 119 can result in Oseltamivir resistance when contains E119V mutation

[c] Amino acid at position 275 can result in Oseltamivir resistance when contains H275Y mutation

[d] Amino acid at position 293 can result in Oseltamivir resistance when contains R293K mutation

[e] Amino acid at position 295 can result in Oseltamivir resistance when contains N295S mutation

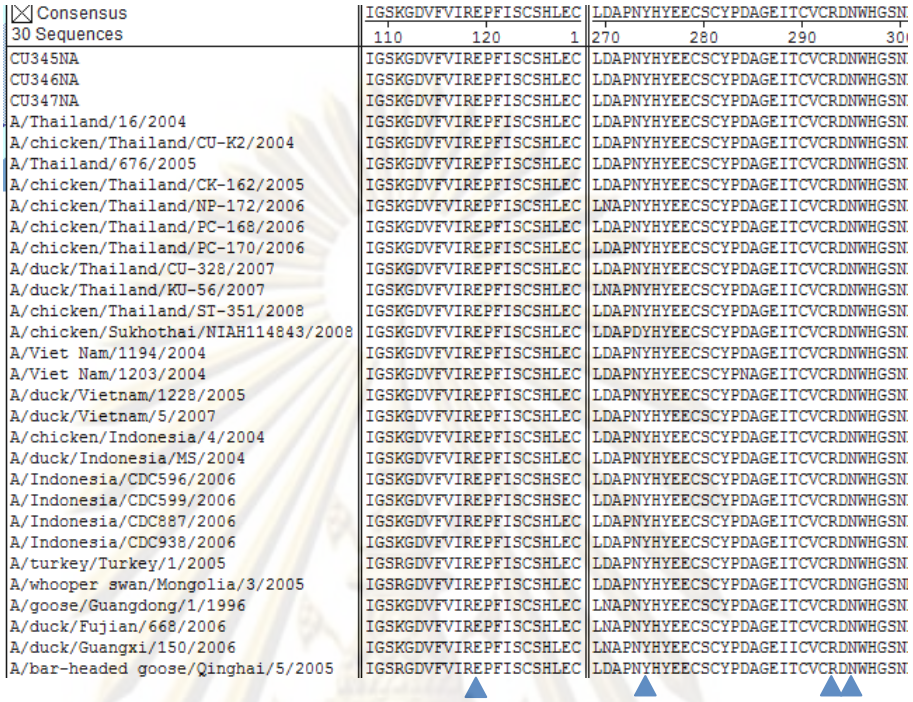
NA stalk region



Note: 20 amino acid deletions at NA stalk regions is the position at 49-68.

Figure 16: Comparison of deduced amino acid of NA protein at NA stalk region.

Oseltamivir resistance amino acids



Note: N2 numbering system, amino acids at position 119, 275, 293, and 295 are related to Oseltamivir resistant/sensitive (Moscona, 2005)

Figure 17: Comparison of deduced amino acid of NA protein at position 119, 275, 293, and 295 relating to Oseltamivir resistance.

Matrix gene

Genetic relatedness of Matrix gene of avian influenza H5N1 viruses isolated was investigated (Figure 18). Avian influenza H5N1 virus (CU-347) was grouped into the group related to the outbreaks in Thailand and separated from China and Indonesia lineage. Matrix gene at key determinant residues such as amantadine resistance residues and characterization of avian-like and human-like amino acids are shown in Table 13 and Figure 19. In this study, avian influenza H5N1 virus (CU347) was found to have mutation at position L26I (Leucine to Isoleucine) and S31N (Serine to Asparagine) which have been shown to be involved in resistance to amantadine (Cheung et al., 2006). In addition, avian influenza H5N1 viruses (CU347) had typical characteristics of both avian-like and human-like viruses at E16, L55, and V28, respectively.

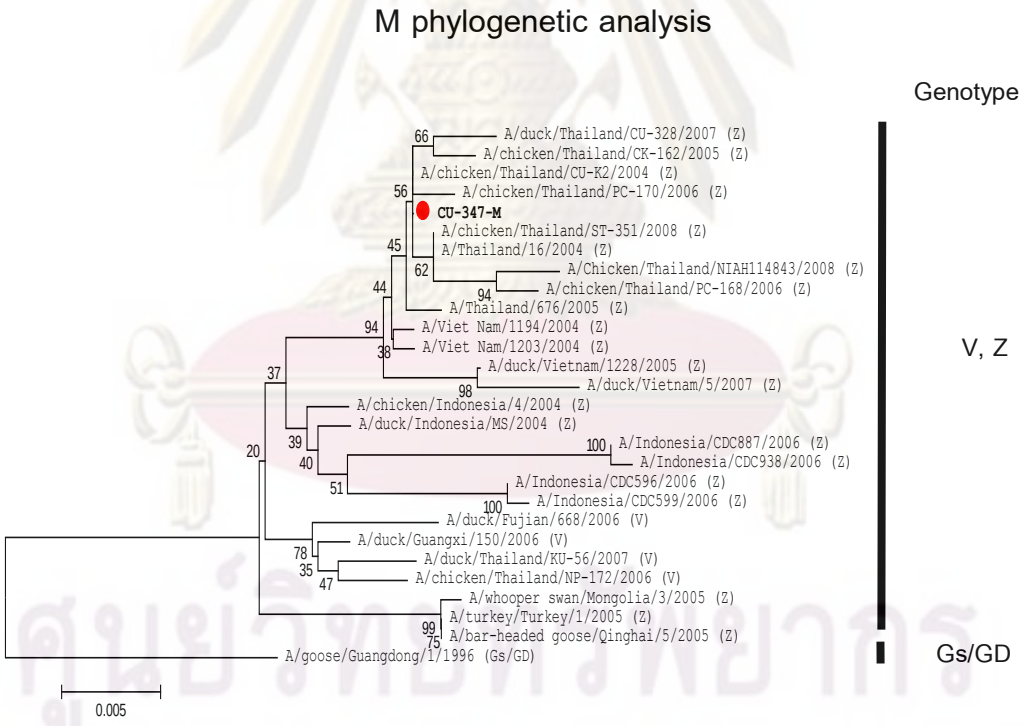


Figure 18: Phylogenetic analysis of M gene of H5N1 viruses isolated in this study compared to other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-960 of the M gene.

Table 13: Genetic analysis of M2 protein at amantadine resistance residues and avian-like and human-like amino acids.

Virus	M gene								
	Amantadine resistance						Human/ avian like		
	26[b]	27[b]	30[b]	31[b]	64[b]	66[b]	16[c]	28[d]	55[c]
A/chicken/Thailand/CU-347/2008	I	V	A	N	A	A	E	V	L
A/duck/Thailand/CU-345/2007	-*	-*	-*	-*	-*	-*	-*	-*	-*
A/chicken/Thailand/CU-346/2007	-*	-*	-*	-*	-*	-*	-*	-*	-*
A/Thailand/16/2004	I	V	A	N	A	A	E	V	L
A/chicken/Thailand/CU-K2/2004	I	V	A	N	A	A	E	V	L
A/Thailand/676/2005	I	V	A	N	A	A	E	V	L
A/chicken/Thailand/CK-162/2005	I	V	A	N	A	A	E	V	L
A/chicken/Thailand/NP-172/2006	L	V	A	S	S	E	E	V	L
A/chicken/Thailand/PC-168/2006	I	V	A	N	A	A	E	V	L
A/chicken/Thailand/PC-170/2006	I	V	A	N	A	A	E	V	L
A/duck/Thailand/CU-328/2007	I	V	A	N	A	A	E	V	L
A/duck/Thailand/KU-56/2007	L	V	A	S	S	E	E	V	L
A/chicken/Thailand/ST-351/2008	I	V	A	N	A	A	E	V	L
A/chicken/NIAH114843/2008	I	V	A	N	A	A	E	V	L
A/Viet Nam/1194/2004	I	V	A	N	A	A	E	V	L
A/Viet Nam/1203/2004	I	V	A	N	A	A	E	V	L
A/duck/Vietnam/1228/2005	I	V	A	N	A	A	E	V	L
A/duck/Vietnam/5/2007	I	V	A	N	A	A	E	V	L
A/chicken/Indonesia/4/2004	L	V	A	S	S	A	E	V	L
A/duck/Indonesia/MS/2004	L	V	A	S	S	A	E	V	L
A/Indonesia/CDC596/2006	L	V	A	N	S	A	E	V	L
A/Indonesia/CDC599/2006	L	V	A	N	S	A	E	V	L
A/Indonesia/CDC887/2006	L	A	A	S	S	A	E	V	L
A/Indonesia/CDC938/2006	L	A	A	S	S	A	E	V	L
A/turkey/Turkey/1/2005	L	V	A	S	S	E	E	V	L
A/whooper swan/Mongolia/3/2005	L	V	A	S	S	E	E	V	L
A/goose/Guangdong/1/1996	L	V	A	S	S	E	E	V	L
A/bar-headed goose/Qinghai/5/2005	L	V	A	S	S	E	E	V	L
A/duck/Guangxi/150/2006	L	V	A	S	S	E	E	V	L
A/duck/Fujian/668/2006	L	V	A	S	S	E	E	F	L

[a] genetic analysis is based on M2 protein numbering system

[b] antiviral resistance when contain mutation at L26I, V27A, A30, S31N, A64S, and A66E

[c] characteristics of avian-like amino acid at E16 and L55

[d] characteristics of human-like amino acid at V28

-*: N/A not available

M2 analysis

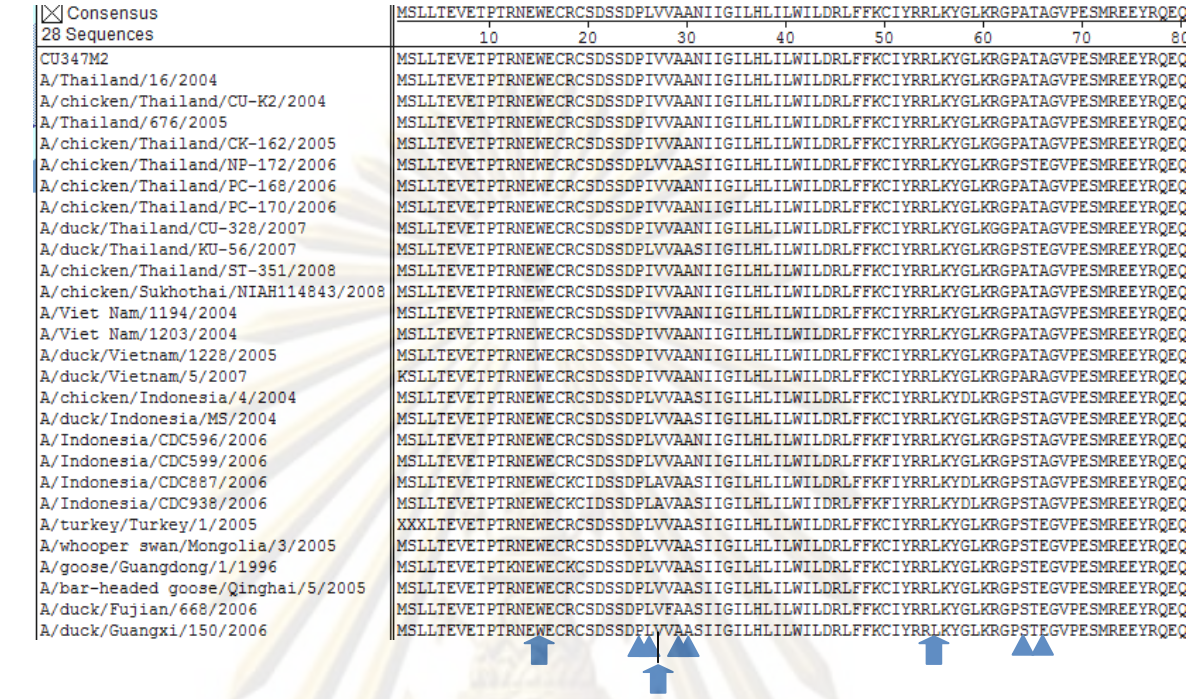


Figure 19: Comparison of deduced amino acid of M2 protein at position 26, 27, 30, 31, 64, and 66 involving amantadine resistance (grey triangles) and at position 16, 28, and 55 representing avian-like and human-like characteristics (grey arrows).

Polymerase gene (PB2, PB1, PA)

Figure 20, 21, and 22 show genetic relatedness of PB2, PB1, and PA gene of avian influenza H5N1 viruses isolated in this study and comparing to other viruses from Thailand and other countries. Genetic relatedness of PB2, PB1, and PA gene were consistented with genetic relatedness of other genes and are clustered in the same group of Vietnam-Thailand lineage and separated from China and Indonesia lineages. Table 14 and Figure 23, 24, and 25 show genetic analysis of three polymerase proteins of avian influenza virus.

In this study, genetic analysis of PB2 protein at positions 627 and 355 of CU-347 isolate were E627 and R355 indicating the characteristic of non-virulence in mammal. Some polymorphisms at amino acid residues 199, 661, 667 and 702 of CU-347 contains avian like amino acids; A119, A661, V667 and K702 respectively. Based on the analysis of PB1 protein at position 198 of the CU-347 isolate is K198 representing non-virulence in mammal. Sequence analysis of PA gene at position 409 of the CU-347 isolate has characterization of avian-like amino acids (S409).

PB2 phylogenetic analysis

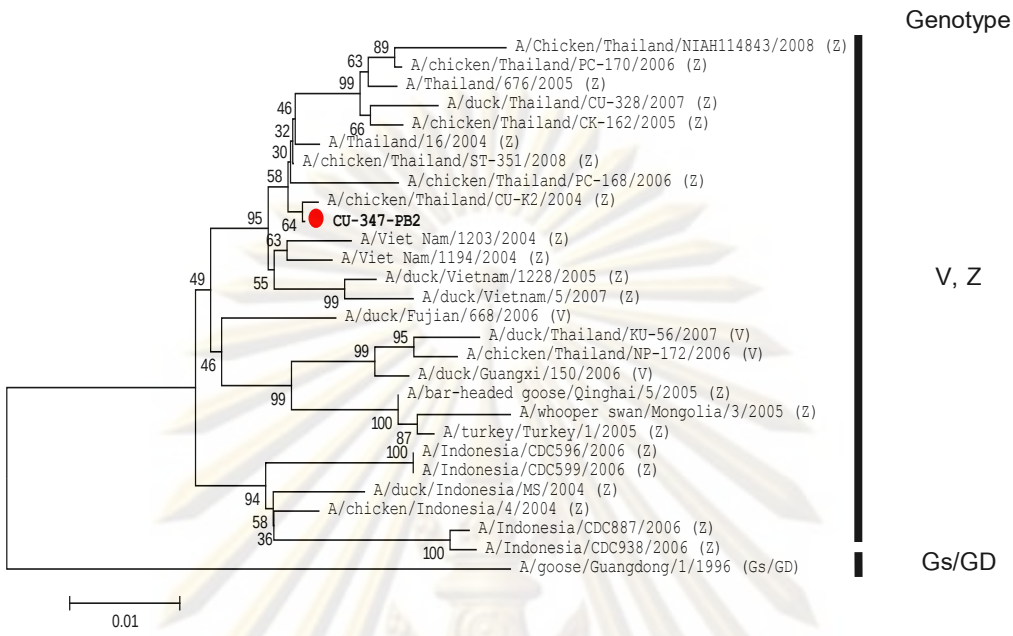


Figure 20: Phylogenetic analysis of PB2 gene of H5N1 viruses isolated in this study compared to other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-2277 of the PB2 gene.

PB1 phylogenetic analysis

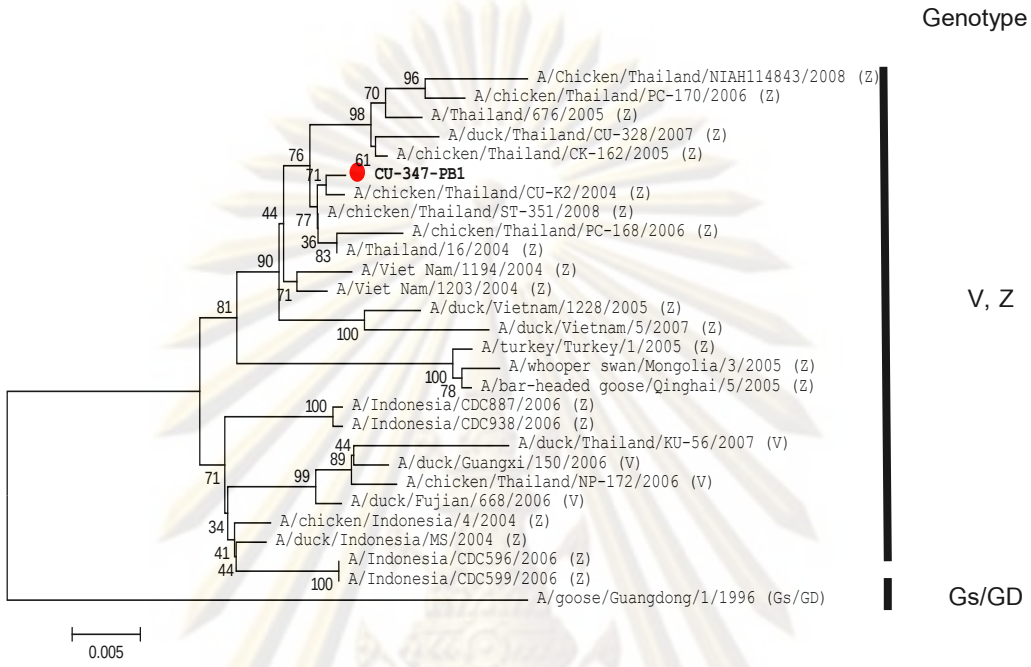


Figure 21: Phylogenetic analysis of PB1 gene of H5N1 viruses isolated in this study compared to other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-2271 of the PB1 gene.

PA phylogenetic analysis

Figure 22: Phylogenetic analysis of PA gene of H5N1 viruses isolated in this study compared to other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-2151 of the PA gene.

Table 14: Genetic analysis of deduced amino acid of PB2, PB1, and PA genes at key determinant residues relating to human or avian-like and virulence characteristics.

Virus	PB2[a,b]						PB1[c]	PA[d]
	H/A like[a]				Virulence[b]		Virulence	H/A like
	199	661	667	702	627	355	198	409
A/chicken/Thailand/CU-347/2008	A	A	V	K	E	R	K	S
A/duck/Thailand/CU-345/2007	-*	-*	-*	-*	-*	-*	-*	S
A/chicken/Thailand/CU-346/2007	-*	-*	-*	-*	-*	-*	-*	-*
A/Thailand/16/2004	A	A	V	K	K	R	K	S
A/chicken/Thailand/CU-K2/2004	A	A	V	K	E	R	K	S
A/Thailand/676/2005	A	A	V	K	K	R	K	S
A/chicken/Thailand/CK-162/2005	A	A	V	K	E	R	K	S
A/chicken/Thailand/NP-172/2006	A	A	I	K	E	R	K	S
A/chicken/Thailand/PC-168/2006	A	A	V	K	E	R	K	S
A/chicken/Thailand/PC-170/2006	A	A	V	K	E	R	K	S
A/duck/Thailand/CU-328/2007	A	A	V	K	E	R	K	S
A/duck/Thailand/KU-56/2007	A	A	I	K	E	R	K	S
A/chicken/Thailand/ST-351/2008	A	A	V	K	E	R	K	S
A/chicken/NIAH114843/2008	A	A	V	K	E	R	K	S
A/Viet Nam/1194/2004	A	A	V	K	K	R	K	S
A/Viet Nam/1203/2004	A	A	V	K	K	R	K	S
A/duck/Vietnam/1228/2005	A	A	V	K	E	R	K	S
A/duck/Vietnam/5/2007	A	A	V	K	E	R	K	S
A/chicken/Indonesia/4/2004	A	A	V	K	E	R	K	S
A/duck/Indonesia/MS/2004	A	A	V	K	E	R	K	S
A/Indonesia/CDC596/2006	A	T	V	K	E	R	K	S
A/Indonesia/CDC599/2006	A	T	V	K	E	R	K	S
A/Indonesia/CDC887/2006	A	A	V	K	E	R	K	N
A/Indonesia/CDC938/2006	A	A	V	K	E	R	K	S
A/turkey/Turkey/1/2005	A	A	V	K	K	R	K	S
A/whooper swan/Mongolia/3/2005	A	A	V	K	K	R	K	S
A/goose/Guangdong/1/1996	A	A	V	K	E	K	K	S
A/bar-headed goose/Qinghai/5/2005	A	A	V	K	K	R	K	S
A/duck/Guangxi/150/2006	A	A	V	K	E	R	K	S
A/duck/Fujian/668/2006	A	A	V	K	E	R	K	S

[a] Amino acid at position 199, 611, 667, 702: A (Alanine), V (Valine), K (Lysine)

[b] Amino acid at position 627, 355: E (Glutamic acid), K (Lysine), R (Arginine)

[c] Amino acid at position 198: K (Lysine)

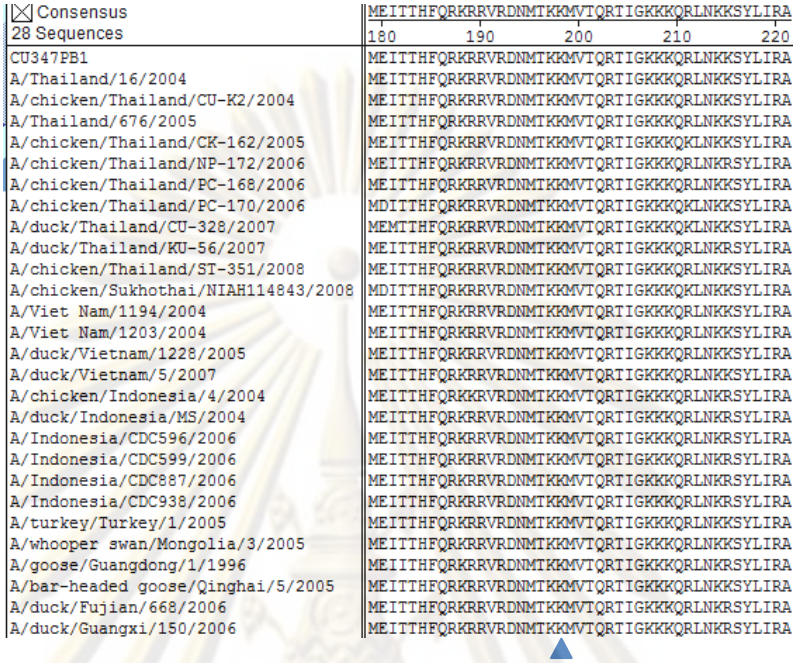
[d] Amino acid at position 409: S (Serine)

-*: N/A not available

PB2 analysis

Figure 23: Comparison of deduded amino acid of PB2 protein at position 355 and 627 which involving with virulence determinant amino acids of virus (triangles) and at position 199, 661, 667, and 702 which represent of avian-like and human-like characteristics (arrows).

PB1 analysis



Consensus / 28 Sequences	180 190 200 210 220
	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
CU347PB1	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/Thailand/16/2004	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/chicken/Thailand/CU-K2/2004	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/Thailand/676/2005	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/chicken/Thailand/CK-162/2005	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQKLNKKSYLIRA
A/chicken/Thailand/NP-172/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/chicken/Thailand/PC-168/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/chicken/Thailand/PC-170/2006	MDITTHFQRKRRVRDNMTKKMVTQRTIGKKKQKLNKKSYLIRA
A/duck/Thailand/CU-328/2007	MEMTTHFQRKRRVRDNMTKKMVTQRTIGKKKQKLNKKSYLIRA
A/duck/Thailand/KU-56/2007	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/chicken/Thailand/ST-351/2008	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/chicken/Sukhothai/NIAH114843/2008	MDITTHFQRKRRVRDNMTKKMVTQRTIGKKKQKLNKKSYLIRA
A/Viet Nam/1194/2004	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/Viet Nam/1203/2004	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/duck/Vietnam/1228/2005	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/duck/Vietnam/5/2007	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/chicken/Indonesia/4/2004	MEITTHFQRKKRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/duck/Indonesia/MS/2004	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/Indonesia/CDC596/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/Indonesia/CDC599/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/Indonesia/CDC887/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/Indonesia/CDC938/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/turkey/Turkey/1/2005	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/whooper swan/Mongolia/3/2005	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/goose/Guangdong/1/1996	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/bar-headed goose/Qinghai/5/2005	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKKSYLIRA
A/duck/Fujian/668/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA
A/duck/Guangxi/150/2006	MEITTHFQRKRRVRDNMTKKMVTQRTIGKKKQRLNKRSYLIRA

▲ Virulence determinant amino acid

Figure 24: Comparison of deduced amino acid of PB1 protein at position 198 which involving with virulence of virus (triangle).

PA analysis

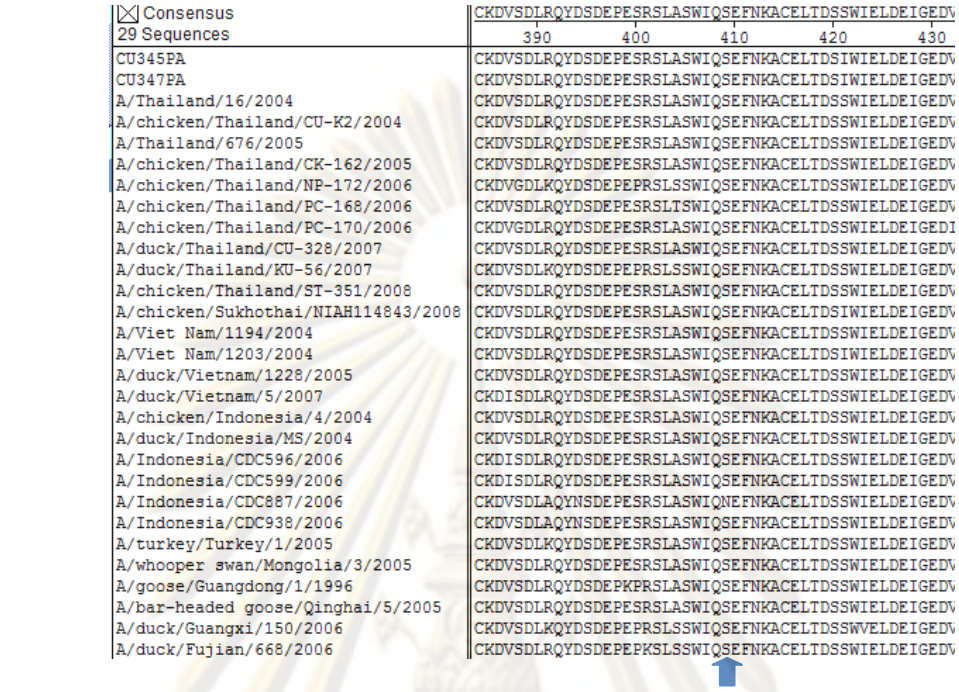


Figure 25: Comparison of decuded amino acid of PA protein at position 409 which involving with characterization of avian-like and human-like characteristics (arrow).

Nonstructural protein gene

Genetic relatedness of Nonstructural protein gene of avian influenza H5N1 viruses is shown in Figure 26. Avian influenza H5N1 viruses (CU-345 and CU347) are clustered in the same group as Vietnam-Thailand lineage. Mutation analysis of Nonstructural protein gene of avian influenza viruses at virulence determinant are demonstrated in Table 15 and Figure 27. In this study, avian influenza H5N1 viruses (CU-345 and CU347) carried 5 amino acid deletions at position 80-84 and have C-terminal ESEV that are virulence features. However, position 92 poseses Aspartic acid (D) which indicating non- virulence in mammals (Seo et al., 2004).

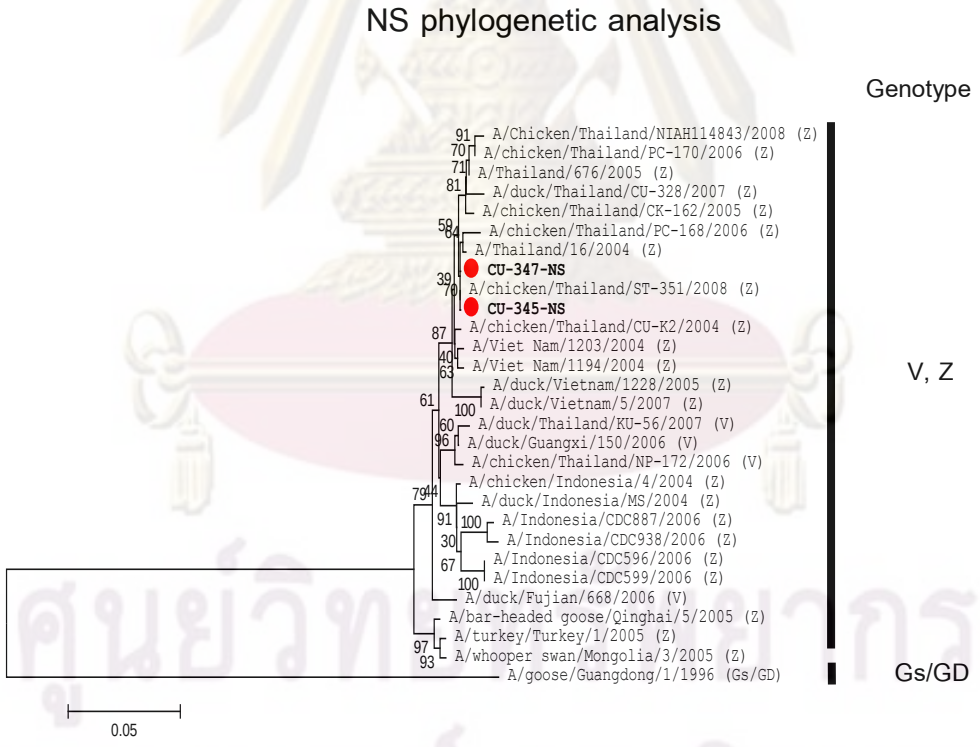


Figure 26: Phylogenetic analysis of NS gene of H5N1 viruses isolated in this study comparing to other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-690 of the NS gene.

Table 15: Genetic analysis of deduced amino acid of NS1 gene at key determinant residues related to virulence characteristics.

Virus	NS1 gene[a]		
	5 amino acid deletion[b]	Virulence determinant	
	80-84[b]	92[c]	Carboxyl-terminal[d]
A/chicken/Thailand/CU-347/2008	5 aa deletion	D	ESEV
A/duck/Thailand/CU-345/2007	5 aa deletion	D	ESEV
A/chicken/Thailand/CU-346/2007	-*	-*	-*
A/Thailand/16/2004	5 aa deletion	D	ESEV
A/chicken/Thailand/CU-K2/2004	5 aa deletion	D	ESEV
A/Thailand/676/2005	5 aa deletion	D	ESEV
A/chicken/Thailand/CK-162/2005	5 aa deletion	D	ESEV
A/chicken/Thailand/NP-172/2006	5 aa deletion	D	ESEV
A/chicken/Thailand/PC-168/2006	5 aa deletion	D	ESEV
A/chicken/Thailand/PC-170/2006	5 aa deletion	D	ESEV
A/duck/Thailand/CU-328/2007	5 aa deletion	D	ESEV
A/duck/Thailand/KU-56/2007	5 aa deletion	D	ESEV
A/chicken/Thailand/ST-351/2008	5 aa deletion	D	ESEV
A/chicken/NIAH114843/2008	5 aa deletion	D	ESEV
A/Viet Nam/1194/2004	5 aa deletion	D	ESEV
A/Viet Nam/1203/2004	5 aa deletion	D	ESEV
A/duck/Vietnam/1228/2005	5 aa deletion	D	ESEV
A/duck/Vietnam/5/2007	5 aa deletion	D	ESEV
A/chicken/Indonesia/4/2004	5 aa deletion	D	ESEV
A/duck/Indonesia/MS/2004	5 aa deletion	D	ESEI
A/Indonesia/CDC596/2006	5 aa deletion	D	ESEV
A/Indonesia/CDC599/2006	5 aa deletion	D	ESEV
A/Indonesia/CDC887/2006	5 aa deletion	D	ESEV
A/Indonesia/CDC938/2006	5 aa deletion	D	ESEV
A/turkey/Turkey/1/2005	5 aa deletion	D	ESKV
A/whooper swan/Mongolia/3/2005	5 aa deletion	D	ESKV
A/goose/Guangdong/1/1996	no deletion	D	ESEV
A/bar-headed goose/Qinghai/5/2005	5 aa deletion	D	ESEV
A/duck/Guangxi/150/2006	5 aa deletion	D	ESEV
A/duck/Fujian/668/2006	5 aa deletion	D	ESEV

[a] Region of analysis is based on NS1 protein

[b] amino acid at position 80-84 of NS1 gene have 5 amino acid deletion

[c] amino acid at position 92 of NS1: D (Aspartic acid)

[d] amino acid at carboxy terminal of NS1: ESEV (Glutamic acid, Serine, Glutamic acid, and Valine)

NS1 analysis

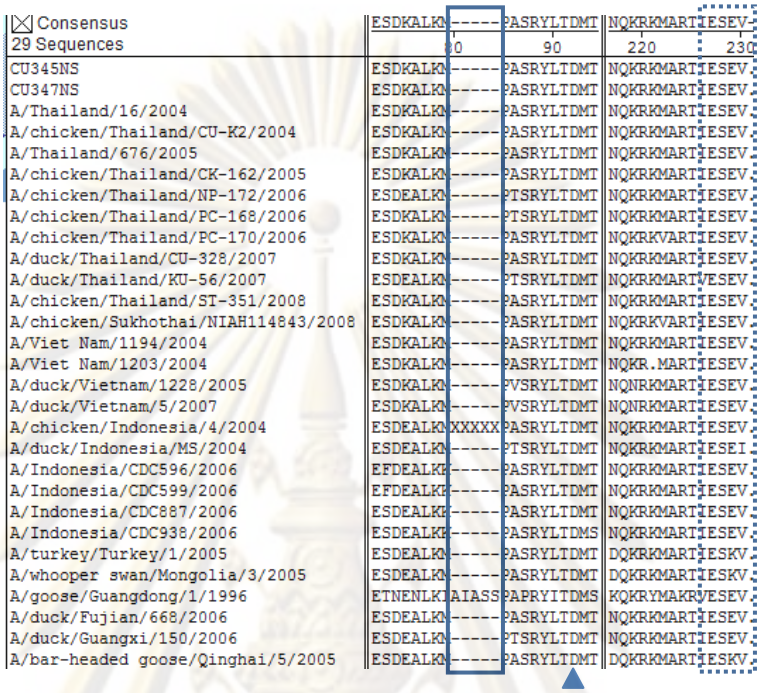


Figure 27: Comparison of deduced amino acid of NS1 protein at position 80-84 which involving virulence determinant amino acid of virus (indicated in box), 92 (triangle) and C-terminal (dashed).

Nucleoprotein gene

Genetic relatedness of Nucleoprotein gene of avian influenza H5N1 viruses is shown in Figure 28. Avian influenza H5N1 viruses (CU-345, CU 346, and CU347) are clustered in group related to the outbreaks in Thailand and separated from China and Indonesia lineages. Genetic analysis of Nucleoprotein gene of avian influenza viruses at key determinant residues such as of avian-like and human-like characteristics is shown in Table 16 and Figure 29. In this study, avian influenza H5N1 viruses (CU-345, CU 346, and CU347) carried Leucine (L) indicating avian-like characteristics.

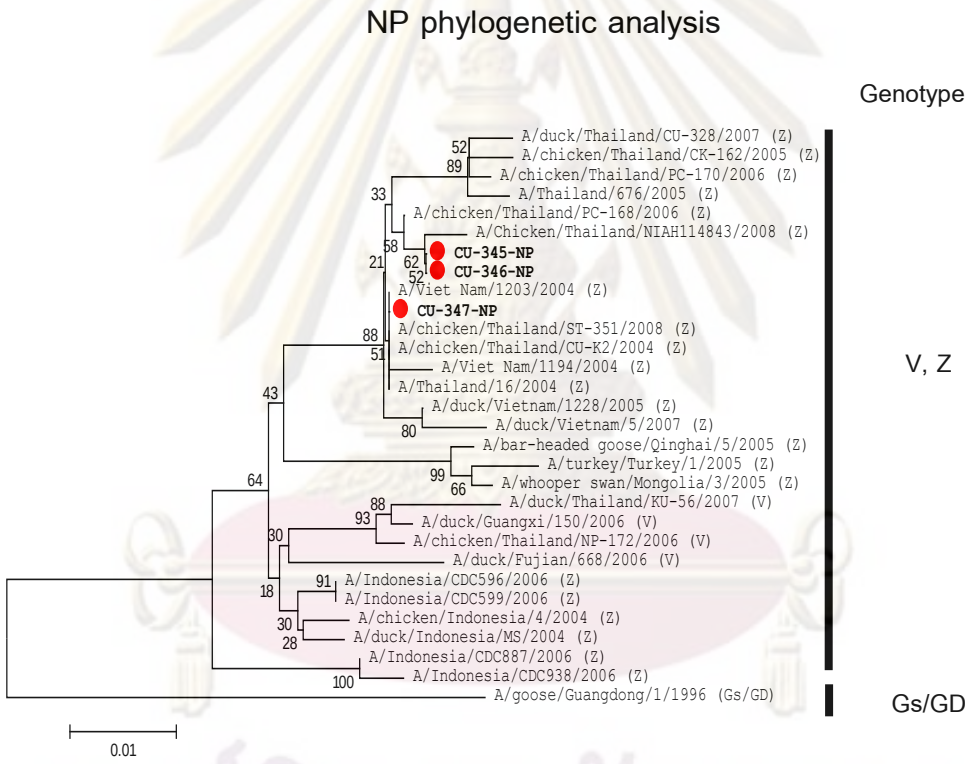


Figure 28: Phylogenetic analysis of NP gene of H5N1 viruses isolated in this study compared to other H5N1 viruses from Thailand and other countries. The analysis was based on nucleotides 1-1481 of the NP gene.

Table 16: Genetic analysis of deduced amino acid of NP gene at key determinant residues relating to human or avian-like and virulence characteristics.

Virus	NP gene
	Human/Avian like characteristics
	136[a]
A/chicken/Thailand/CU-347/2008	L
A/duck/Thailand/CU-345/2007	L
A/chicken/Thailand/CU-346/2007	L
A/Thailand/16/2004	L
A/chicken/Thailand/CU-K2/2004	L
A/Thailand/676/2005	L
A/chicken/Thailand/CK-162/2005	L
A/chicken/Thailand/NP-172/2006	L
A/chicken/Thailand/PC-168/2006	L
A/chicken/Thailand/PC-170/2006	L
A/duck/Thailand/CU-328/2007	L
A/duck/Thailand/KU-56/2007	L
A/chicken/Thailand/ST-351/2008	L
A/chicken/NIAH114843/2008	L
A/Viet Nam/1194/2004	L
A/Viet Nam/1203/2004	L
A/duck/Vietnam/1228/2005	L
A/duck/Vietnam/5/2007	L
A/chicken/Indonesia/4/2004	L
A/duck/Indonesia/MS/2004	L
A/Indonesia/CDC596/2006	L
A/Indonesia/CDC599/2006	L
A/Indonesia/CDC887/2006	L
A/Indonesia/CDC938/2006	L
A/turkey/Turkey/1/2005	L
A/whooper swan/Mongolia/3/2005	L
A/goose/Guangdong/1/1996	M
A/bar-headed goose/Qinghai/5/2005	L
A/duck/Guangxi/150/2006	L
A/duck/Fujian/668/2006	L

[a] Amino acid at position 136 of Nucleoprotein gene: M (Methionine), L (Leucine)

NP analysis

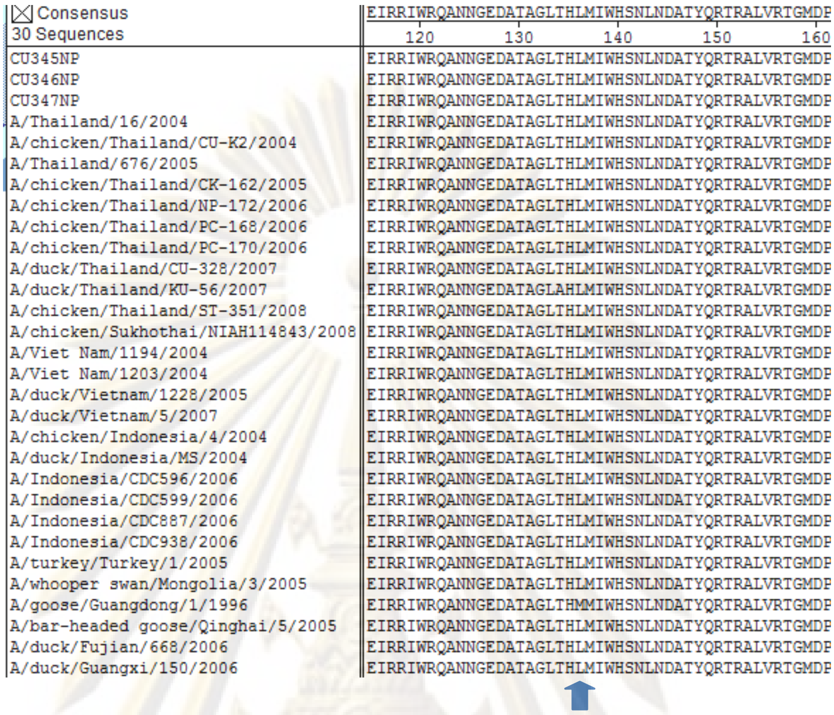


Figure 29: Comparison of deduced amino acid of NP protein at position 136 which represents avian-like and human-like characteristics (indicated by arrows).

CHAPTER V

DISCUSSION

In Thailand, avian influenza H5N1 virus is a new emerging virus, which was first reported at the beginning of year 2004. To date, avian influenza virus has spread periodically with at least 7 waves of AI outbreaks reported in the country. The outbreaks of avian influenza in Thailand are mostly caused by avian influenza H5N1 virus classified as Genotype Z or clade 1 (Li et al., 2004; Viseshakul et al., 2004). Genetic characteristics of avian influenza H5N1 viruses from Thailand were found to be similar to those in Vietnam; therefore, the viruses have been classified as Thailand-Vietnam lineage or genotype Z (Amonsin et al., 2006; Li et al., 2004; Viseshakul et al., 2004; Webster and Govorkova, 2006). WHO/OIE/FAO and team (2008) have summarized avian influenza H5N1 virus outbreaks in many countries around the world. They reported that avian influenza H5N1 virus can be divided into several groups or clades by using the comparison of genetic sequence of HA gene. For example, avian influenza H5N1 virus from Thailand can be classified into in clade 1 that is the virus spreading in Thailand, Vietnam, and Malaysia (Boltz et al., 2006; Li et al., 2004). The outbreaks of avian influenza H5N1 in Thailand were previously caused by clade 1 viruses. Currently, there are at least 2 clades (i.e. clade 1 and 2.3.4) or 2 genotypes (i.e. genotype Z and V) of avian influenza H5N1 viruses found to emerge and cause the outbreaks in the country. Chutinimitkul and team (2007) found that avian influenza H5N1 virus "A/chicken/Thailand/NP-172/2006" that is a virus isolated from chickens at Nakhon Phanom and has the genetic characteristics of genotype V. The virus, NP-172 (genotype V) is different from the genotype Z that causes most AI outbreaks in Thailand. It is noted that the genotype V virus has PA gene that exhibited low nucleotide similarity from the genotype Z (Chutinimitkul et al., 2007).

In this study, we have collected the samples from 42 districts of 13 provinces in the border of Thailand, Myanmar and Laos. These locations were selected based on the criteria as follows: 1) an area that has a joint border of Thailand with Myanmar and Laos

2) an area that has an international crossing point, a temporary border crossing, a temporarily permitted between Thailand and neighboring countries, which are essential for disease surveillance and poultry movement) and 3) an area with the outbreaks of avian influenza previously reported in the last 4-5 years. Provinces that are located along the borders of Thailand and neighboring countries, Myanmar and Laos with the report of avian influenza outbreaks are Nakorn Phanom, Nong Khai and Chiang Mai, etc. (Amonsin et al., 2006; Chutinimitkul et al., 2007). In this study, 2,175 samples were collected from several poultry species from 42 districts of 13 provinces between September 2007 and June 2008. Sixty-eight samples (3.13% (68 / 2,175)) were tested positive for the HA. Out of 68 HA positive samples, 15 samples were tested positive as influenza A H5N1 virus using multiplex RT-PCR, realtime RT-PCR and PCR-ELISA assays. We have selected only 3 avian influenza H5N1 viruses which are the representatives from each province of Loey, Chiang Rai, and Prachuap Khiri Khan for whole gene sequencing of the virus.

In the present study, we used 3 PCR-based assays to identify influenza A H5N1 virus. The samples that yield positive results by using multiplex RT-PCR samples were further confirmed by realtime RT-PCR using M, HA, and NA gene specific primers (Payungporn et al., 2006; Payungporn et al., 2004; Suwannakarn et al., 2008) and PCR-ELISA using H5N1 specific probes (Chaharaein et al., 2009). Multiplex RT-PCR can be identified 15 samples as influenza A virus. Multiplex RT-PCR can also further identify 15 influenza A into H5 (7 samples) and N1 (12 samples).When the realtime RT-PCR and PCR-ELISA were used to all isolates were confirmed as influenza A subtype H5N1. Test results showed that multiplex PCR methods may not be sensitive enough to find subtype viruses. Therefore, test methods together with realtime RT-PCR and PCR-ELISA could be identifying as H5N1.

In this study, only 3 avian influenza H5N1 viruses (CU-345, CU-346 and CU-347) were selected for whole gene sequencing. The genetic information from whole genome sequences were used for phylogenetic analysis and genotype analysis of the viruses. We were able to elucidate whole genome sequences (8 genes) of only one virus (CU-

347). Due to the limitation of RNA quality, only 4 genes of the other two viruses (CU-345 and CU-346) were sequenced. Reasons for the unsuccessful whole gene sequencing may be spend many times to handle or using RNA in any PCR method that RNA could be degrade or the low virus titers may be affect the amount of extracted RNA which was not good enough for whole genome sequencing of the viruses.

Genetic relatedness of avian influenza H5N1 viruses isolated in this study was evaluated by phylogenetic analysis. Our analysis indicated that avian influenza H5N1 viruses (CU-345, CU 346 and CU-347) in this study are arranged in the same group of Vietnam-Thailand lineage, which was related to most of the AI outbreaks in Thailand, Vietnam, and Malaysia. The phylogenetic analysis of 8 genes of the viruses showed that the genetic relatedness of 3 viruses in each gene were generally consistent. The result of this study indicated that avian influenza H5N1 viruses in Thailand derived from the ancestor "Goose/Guangdong/96-lineage" (Chen et al., 2006). In clade classification system, the CU-345, CU-346 and CU-347 avian influenza H5N1 viruses belonged to clade 1, which was considered as an important clade and caused the outbreaks of avian influenza in Thailand and Vietnam as well as Cambodia, Laos and Malaysia.

We determined the mutations in key determinant residues of 8 genes of the viruses and compared with the avian influenza H5N1 virus circulating in Thailand and other countries. The analysis of HA cleavage site at the position 323-329 in HA gene showed the characteristics of multiple basic amino acids insertion. This characteristic designated the virus as highly pathogenic avian influenza (HPAI) (Claas et al., 1998). The avian influenza H5N1 viruses in this study had the characteristics of HA cleavage site type "RERRRKK". The cleavage site can be found in other avian influenza H5N1 viruses in Thailand that have multiple insertion of basic amino acids at different types such as "RERKRKK", "REKRRKK" and "RERRRKK" (Amonsin et al., 2006). In this study, avian influenza H5N1 viruses contained avian specific-receptor binding properties at position 222-224 (Q222 and G224). The presence of Q222 and G224 can affect the ability of the virus to bind the host receptor (α-2,3 linkage), typical for avian but not the human virus (Connor et al., 1994). The mutation of amino acids at these positions may

affect the virulence of infection particularly in mammals (Matrosovich et al., 1999; Shinya et al., 2006; Webster et al., 1997). Amino acids related to the receptor binding pockets were also analyzed. These amino acids had highly positive selection pressure (Smith et al., 2006). We found no mutations of amino acids at position 129 and 175 (L129 and L175), that was related to the virulence of avian influenza H5N1 virus infection. The analysis of glycosylation sites of avian influenza viruses showed that the viruses contain all 7 glycosylation sites related to the virulence of avian influenza H5N1 virus (Matrosovich et al., 1999; Shortridge et al., 1998). In addition, the comparative analysis of amino acids of 5 locations of the HA1 protein including antigenic site A-E at amino acids positions 83, 86, 138, 140, and 141 were analyzed due to their highly positive selection pressure (Smith et al., 2006). The analysis showed that avian influenza H5N1 virus had amino acid mutations at position 86, 138, 140, and 141. These mutations was previously shown to result in the changes of antigenic epitopes, which reflect the adaptation of the virus to escape host immunity (de Jong and Hien, 2006).

The previous studies demonstrated the presence of 20 amino acid deletion at NA stalk region of NA gene in avian influenza H5N1 viruses from Thailand, Vietnam, Indonesia and many countries in Asia and Europe during the year 2003-2007 (Amonsin et al., 2006; Chen et al., 2006; Li et al., 2004; Salzberg et al., 2007; Viseshakul et al., 2004). This 20 amino acid deletion is missing in avian influenza H5N1 virus isolated from goose “Goose/Guangdong/1/196". The change of amino acids at NA stalk region was the result of the adaptation or evolution occuring from the infection in wild aquatic birds to domestic poultry (Matrosovich et al., 1999). In this study, 3 viruses have 20 amino acid deletion at position 49-68 of NA stalk region, which were consistent with avian influenza H5N1 viruses circulating in Thailand. Amino acids associated with Oseltamivir resistance were previously identified at positions 119 (E to V), 293 (R to K), and 295 (N to S) (Kiso et al., 2004) and at position 275 (H to Y) (Gubareva et al., 2000). However, such amino acids were not found in this study.

The particular amino acid positions or concensus in M gene were found to be specific to avian virus (avian-like amino acids) or mammal virus (human-like amino

acids) (Matrosovich et al., 1999), for example, amino acids in M2 protein at position 16 (E /G) (avian/human-like), position 28 (I/V) and position 55 (L/F). The results of this study found that amino acids at position 16 and 55 were E and L which were avian-like amino acids. Amino acid at position 28 was V, the human-like amino acid. In addition, amino acid associated with amatadine resistance I 26 and 31 N were identified. In general, the amantadine resistances were related to the mutations of amino acids at position 26, 27, 30, 31, 64, 66 of M2 protein (Cheung et al., 2006). These mutations are involved in the structure of protein that may affect the ability of antiviral drugs (Cheung et al., 2006; Pinto et al., 1992).

Amino acids of the PB2 gene at position 199, 661, 667 and 702 were Alanine (A), Alanine (A), Valine (V) and Lysine (K) respectively. These amino acid represented the characteristics of the virus in avian (avian-like amino acids), while the characteristics of virus in mammal (human-like amino acids) had the amino acid as Serine (S), Threonine,(T) Glutamic acid (E), and Arginine (R) (Puthavathana et al., 2005). The analysis of amino acids in polymerase gene (PB2) can indicate virulence characteristics of viruses in mammals (Shinya et al., 2004) such as in the PB2 gene at positions 627 and 355. However, in this study the virus (CU-347) contained E627 and R355 which were the characteristics of non-virulence in mammals. The amino acid of the PB1 protein at position 198 was Lysine (K) indicating non-virulence characteristics. Moreover, the amino acid of PA protein at position 409 was Serine (S), which is the characteristic of the avian-like amino acids. Therefore, genetic analysis of the polymerase genes of avian influenza H5N1 viruses isolated in this study indicated the characteristics of viruses both in avian and mammal. However, single position of amino acids was not the only a marker representing the virulence of avian influenza.

Previous study revealed that some amino acid deletion in NS gene may increase the virulence of avian influenza H5N1 virus (Lipatov et al., 2005). The current study revealed that avian influenza H5N1 virus had 5 amino acid deletions at positions 80-84. This characteristic was previously found in avian influenza H5N1 viruses in Thailand, Vietnam, Indonesia, China, Europe and Africa. However, no deletion was found in avian

influenza H5N1 virus from China "Goose/Guangdong/1/96" (Duan et al., 2008). The mutation of amino acids at position 92 from aspartic acid (D) to glutamic acid (E) may affect the virulence of the virus, especially in mammal strains (Seo et al., 2004). Avian influenza H5N1 virus in this study possed D92 indicating non-virulent characteristics in mammal. This amino acid of the NS1 gene is one of several markers that reflect the virulence of virus. Another virulence determinant in the NS1 protein was carboxyl-terminal. This virulence determinant was related to the capture of host proteins in PDZ domain (Obenauer et al., 2006). Avian influenza H5N1 virus mostly contained c-terminal motif ESEV, whereas non virulent avian influenza H5N1 virus may has c-terminal motif as RSKV (Obenauer et al., 2006). In this study, avian influenza H5N1 virus contained c-terminal motif of the NS1 protein commonly found in virulent avian influenza H5N1 in poultry. In NP gene, the genetic analysis revealed avian-like characteristics of the virus that was the position of L136. In contrast, the "Goose/Guangdong/1/96" virus had Methionine (M), human-like amino acid (Reid et al., 2004).

Genotype analysis of avian influenza H5N1 virus in this study was done in a H5N1 virus (CU-347) that had whole genome sequenced. It can be concluded that avian influenza H5N1 virus isolated from provinces located at joint border of Myanmar, Laos, Thailand was avian influenza H5N1 viruses, genotype Z. Currently, the classification system named “clade” of avian influenza H5N1 virus is developed. Clade nomenclature system is based on principles of the HA gene evolutions. The analysis of HA gene of three H5N1 viruses (CU-345, CU-346 and CU-347) indicated that the viruses belonged to clade 1 responsible for the outbreaks of avian influenza in Thailand and Vietnam including Cambodia, Laos and Malaysia.

Conclusion and suggestion

The present study collected 2,715 samples from the several poultry species in 42 districts of 13 provinces that joint border of Thailand, Myanmar and Laos between September 2007 and June 2008. Sixty eight samples were tested positive for the HA test (3.13% (68 / 2,175)). Out of 68 samples, 15 samples were identified as avian influenza H5N1 by multiplex RT-PCR, realtime RT-PCR and PCR-ELISA methods. Three avian influenza H5N1 viruses from Loey (CU-345), Chiang Rai (CU-346), and Prachuap Khiri Khan (CU-347) were selected for whole gene sequencing and genotype classification. Total numbers of sequence of avian influenza H5N1 viruses available in this study were 16 sequences.

Genetic relatedness and genotype analysis of avian influenza H5N1 viruses revealed that avian influenza H5N1 viruses isolated from border of Thailand, Laos and Myanmar were classified as genotype Z or clade1. These viruses were closely related and arranged in the same group with the viruses from avian and human in Vietnam-Thailand lineage, which is responsible for most AI outbreaks in Thailand, Vietnam, Cambodia, and Malaysia. Genetic analysis of these viruses demonstrated no mutations at key determinant residues such as HA cleavage site, receptor binding site in HA protein, NA stalk region, antiviral drug resistant residues in NA protein, antiviral drug resistant residues in M2 protein, and virulence determinants in PB2, PB1, and NS protein. The H5N1 viruses had characteristics of both avian-like and Human-like viruses.

According to the results of present study, monitoring of influenza A H5N1 virus from avian species in border area between Thailand and neighboring countries should be following up continuously. The genetic characterizations of the virus to examine mutations or genetic changes of avian influenza H5N1 virus in Thailand are required.

Data obtained could be beneficially used as follows:

1. To know evidence of avian influenza H5N1 viruses in the border areas between Thailand and neighboring countries (Laos and Myanmar).
2. To be published genetic data in GenBank database.
3. To explain spreading and mutations of avian influenza H5N1 virus.
4. To be used as a resource of an origin or a primary cause of outbreak of avian influenza A viruses.
5. To be used as a fundamental data to select and develop a candidate influenza (H5N1) vaccine strain.

From the results, the suggestion for further studies could be as follows:

1. Information of this outbreak will be used to plan control and prevention strategies of AI. Therefore, intensive active surveillances are recommended.
2. Genetic of avian influenza H5N1 virus could be mutating easily, genetic characterizations of the virus to examine are required.
3. Avian influenza surveillance should require cooperation from various parties and have adequate equipment. Therefore, coordination with local agencies before the surveillance will be work easier.
4. Researcher should be vaccinated against influenza before they will collect the sample. In field, researcher should wear protective equipment such as mask, glove, and Tyvec suite etc.

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APPENDICES

APPENDIX A

Standard amino acid abbreviations

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

APPENDIX B

Reagents and preparations

1. Phosphate Buffer Saline (PBS)

Sodium chloride (NaCl)	8	g
Potassium chloride (KCl)	0.2	g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.2	g
Di-sodium hydrogen phosphate (Na2HPO4)	1.15	g

Gentle stir on stirrer for 30 min and adjust pH to 7.2 and sterilize immediately by autoclave

Reagents for agarose gel electrophoresis

1. 10 mg/ml Ethidium bromide

Ethidium bromide	1	g
Distilled water	1,000	ml

Stir few hours for dye has dissolved, wrap container in aluminum foil and transfer to a dark bottle and store at $4^{o}C$

2. 2% Agarose gel

Agarose (ultrapure)	0.3	g
1X TBE	20.0	ml
10 mg/ml Ethidium bromide	1.0	µl

APPENDIX C

Nucleotide sequences of A/duck/Loei/Thailand/CU-345/07

CU-345-PA: Polymerase acidic gene (PA)

```
LOCUS       CU-345-PA 516 bp DNA linear 23-DEC-2008
DEFINITION  Influenza A virus strain A/duck/Loei/Thailand/CU-345/07(H5N1)
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
ORGANISM    Influenza A virus Unclassified.
REFERENCE   1  (bases 1 to 516)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Monitoring of influenza A virus from avian species in border
            areas of Thailand
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 516)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Direct Submission
JOURNAL     Submitted (23-DEC-2008) Chulalongkorn University, Department  of
Veterinary Public Health, Rama 4 Rd. Pathumwan, Bangkok     10330, Thailand
FEATURES    Location/Qualifiers
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            /strain="A/duck/Loei/Thailand/CU-345/07(H5N1)"
            /serotype="H5N1"
            /segment="PA"
            /country="Thailand"
BASE COUNT      177 a    101 c    129 g    109 t
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     61 ggatgcccct aaattaagca tcgaagaccc gagtcatgag ggggagggga taccactata
    121 cgatgcaatc aaatgcatga agacattttt cggatggaaa gagcccaaca tcgtgaaacc
    181 acatgaaaag ggtgttaact ccaattacct cctggcttgg aagcaggtgc tggcagaact
    241 ccaagatatt gaaaatgagg agaaaatccc aaaaacaaag aacatgaaaa aaacaagcca
    301 gttgaagtgg acactcggtg agaacatggc accagagaaa gtagactttg aggactgcaa
    361 agatgttagc gacctaagac agtatgacag tgatgaacca gagtctagat cactagcaag
    421 ctggattcag agtgaattca acaaggcatg tgaattgaca gattcgattt ggattgaact
    481 tgatgaaata ggagaagacg tagctccaat tgagca//
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CU-345-HA: Hemagglutinin gene (HA)

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LOCUS       CU-345-HA 455 bp DNA linear 23-DEC-2008
DEFINITION  Influenza A virus strain
A/duck/Loei/Thailand/CU345/07(H5N1)
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
ORGANISM    Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 455)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Monitoring of influenza A virus from avian species in
            border areas of Thailand
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 455)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Direct Submission
JOURNAL     Submitted (23-DEC-2008) Chulalongkorn University,
            Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
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            /country="Thailand"
BASE COUNT      164 a     81 c    113 g     97 t
ORIGIN
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 tatgaaaagt gaattggaat atggtaactg caacaccaag tgtcaaactc caatgggggc
121 gataaactct agtatgccat tccacaatat acaccctctc accatcgggg aatgccccaa
181 atatgtgaaa tcaaatagat tagtccttgc gactgggctc agaaatagcc ctcaaagaga
241 gagaagaaga aaaaagagag gattatttgg agctatagca ggttttatag agggaggatg
301 gcagggaatg gtagatggtt ggtatgggta ccaccatagc aatgagcagg ggagtgggta
361 cgctgcagac aaagaatcca ctcaaaaggc aatagatgga gtcaccaata aggtcaactc
421 gataattgac aaaatgaaca ctcagtttga ggccg//
```

CU-345-NP: Nucleoprotein gene (NP)

```
LOCUS       CU-345-NP 554 bp DNA linear 23-DEC-2008
DEFINITION  Influenza A virus strain
            A/duck/Loei/Thailand/CU345/07(H5N1)
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
ORGANISM    Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 554)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
Poovorawan,Y. and Amonsin,A.
TITLE       Monitoring of influenza A virus from avian species in
            border areas of Thailand
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 554)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Direct Submission
JOURNAL     Submitted (23-DEC-2008) Chulalongkorn University,
             Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330,Thailand
FEATURES    Location/Qualifiers
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            /strain="A/duck/Loei/Thailand/CU-345/07(H5N1)"
            /serotype="H5N1"
            /segment="NP"
            /country="Thailand"
BASE COUNT      175 a    108 c    163 g    108 t
ORIGIN
  1 atggcgtctc aaggcaccaa acgatcttat gaacagatgg aaactggtgg ggaacgccag
 61 aatgctactg agatcagggc atctgttgga agaatggtta gtggcattgg gaggttctac
121 atacagatgt gcacagaact caaactcagt gactatgaag ggaggctgat ccagaacagc
181 ataacaatag agagaatggt actctctgca tttgatgaaa gaaggaacag atacctggaa
241 gaacacccca gtgcgggaaa ggacccgaag aagactggag gtccaattta tcggaggaga
301 gacgggaaat gggtgagaga actaattctg tacgacaaag aggagatcag gaggatttgg
361 cgtcaagcga acaatggaga ggacgcaact gctggtctta cccacctgat gatatggcat
421 tccaatctaa atgatgccac atatcagaga acgagagctc tcgtgcgtac tggaatggac
481 ccaaggatgt gctctctgat gcaagggtca actctcccta ggagatctgg agctgccggt
541 gcagcagtaa aggg//
```

CU-345-NA: Neuraminidase gene (NA)

```
LOCUS       CU-345-NA    1352 bp    DNA     linear       23-DEC-2008
DEFINITION  Influenza A virus strain A/duck/Loei/Thailand/CU-
            345/07(H5N1)
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
ORGANISM    Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 1352)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Monitoring of influenza A virus from avian species in
            border areas of Thailand
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 1352)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Direct Submission
JOURNAL     Submitted (23-DEC-2008) Chulalongkorn University,
            Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..1352
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/duck/Loei/Thailand/CU-345/07(H5N1)"
            /serotype="H5N1"
            /segment="NA"
            /country="Thailand"
BASE COUNT      400 a    245 c    343 g    364 t
ORIGIN
   1 atgaatccaa ataagaagat aataaccatc ggatcaatct gtatggtaac tggaatggct
  61 agcttaatgt tacaaattgg gaacttgatc tcaatatggg tcagtcattc aattcacaca
 121 gggaatcaac acaaagctga accaatcagc aatactaatt ttcttactga gaaagctgtg
 181 gcttcagtaa aattagcggg caattcatct ctttgcccca ttaatggatg ggctgtatac
 241 agtaaggaca acagtataag gatcggttcc aaggggatg tgtttgttat aagagagcca
 301 ttcatctcat gctcccactt ggaatgcaga actttctttt tgactcaggg agccttgctg
 361 aatgacaagc actccaatgg gactgtcaaa gacagaagcc ctcacagaac attaatgagt
 421 tgtcctgtgg gtgaggctcc ctccccatat aactcaaggt ttgagtctgt tgcttggtca
 481 gcaagtgctt gccatgatgg caccagttgg ttgacaattg gaatttctgg cccagacagt
 541 ggggctgtgg ctgtattgaa atacaatggc ataataacag acactatcaa gagttggagg
 601 aataacatac tgagaactca agagtctgaa tgtgcatgtg taaatggctc ttgctttact
 661 gtaatgactg acggaccaag taatggtcag gcatcacata agatcttcaa aatggaaaaa
 721 gggaaagtgg ttaaatcagt cgaattggat gctcctaatt atcactatga ggaatgctcc
 781 tgttatcctg atgccggcga aatcacatgt gtgtgcaggg ataattggca tggctcaaat
 841 cggccatggg tatctttcaa tcaaaatttg gagtatcaaa taggatatat atgcagtgga
 901 gttttcggag acaatccacg ccccaatgat ggaacaggta gttgtggtcc ggtgtcctct
 961 aacggggcat atggggtaaa agggttttca tttaaatacg gcaatggtgt ctggatcggg
1021 agaacaaaaa gcactaattc caggagcggc tttgaaatga tttgggatcc aaatgggtgg
1081 actgaaacgg acagtagctt ttcagtgaaa caagatatcg tagcaataac tgattggtca
1141 ggatatagcg ggagttttgt ccagcatcca gaactgacag gactagattg cataagacct
1201 tgtttctggg ttgagttgat cagagggcgg cccaaagaga gcacaatttg gactagtggg
1261 agcagcatat ctttttgtgg tgtaaatagt gacactgtgg gttggtcttg gccagacggt
1321 gctgagttgc cattcaccat tgacaagtag tt//
```

CU-345-NS: Nonstructural gene (NS)

```
LOCUS       CU-345-NS     824 bp    DNA     linear       23-DEC-2008
DEFINITION  Influenza A virus strain A/duck/Loei/Thailand/CU-
            345/07(H5N1)
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
ORGANISM    Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 824)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Monitoring of influenza A virus from avian species in
            border areas of Thailand
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 824)
AUTHORS     Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
TITLE       Direct Submission
JOURNAL     Submitted (23-DEC-2008) Chulalongkorn University,
            Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330,Thailand
FEATURES    Location/Qualifiers
source      1..824
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/duck/Loei/Thailand/CU-345/07(H5N1)"
            /serotype="H5N1"
            /segment="NS"
            /country="Thailand"
BASE COUNT      262 a    168 c    200 g    194 t
ORIGIN
  1 atggattcca acactgtgtc aagctttcag gtagactgct ttctttggca tgtccgcaaa
 61 cgatttgcag accaagaact gggtgatgcc ccattccttg accggcttcg ccgagatcag
121 aagtccctaa gaggaagagg caacactctt ggtctggaca tcgaaacagc tactcgcgca
181 ggaaagcaga tagtggagcg gattctggag gaggagtctg ataaggcact taaaatgccg
241 gcttcacgct acctaactga catgactctc gaagaaatgt caagggactg gttcatgctc
301 atgcccaagc agaaagtggc aggttccctt tgcatcaaaa tggaccaggc aataatggat
361 aaagtcatca tattgaaagc aaacttcagt gtgatttttg accggttgga aaccctaata
421 ctacttagag ctttcacaga agaaggagca atcgtgggag aaatctcacc attaccttct
481 cttccaggac atactggtga ggatgtcaaa aatgcaattg gcgtcctcat cggaggactt
541 gaatggaatg ataacacagt tcgagtcact gaaactatac agagattcgc ttggagaagc
601 agtgatgagg atgggagact tccactccct ccaaatcaga aacggaaaat ggcgagaaca
661 attgagtcag aagtttgaag aaataaggtg gctgattgaa gaagtaagac atagattgaa
721 aattacagaa aacagcttcg aacagataac gtttatgcaa gccttacaac tactgcttga
781 agtggagcaa gagataagag ccttctcgtt tcagcttatt taat//
```

Nucleotide sequences of A/chicken/Chiang Rai/Thailand/CU-346/07

CU-346-HA: Hemagglutinin gene (HA)

```
LOCUS       CU-346-HA     754 bp    DNA     linear       23-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Chiang
            Rai/Thailand/CU-346/07(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 754)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in
            border areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 754)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2008) Chulalongkorn University,
            Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330,Thailand
FEATURES    Location/Qualifiers
source      1..754
            /organism="Influenza A virus
            /mol_type="genomic DNA"
            /strain="A/chicken/Chiang Rai/Thailand/CU-346/07(H5N1)"
            /serotype="H5N1"
            /segment="HA"
            /country="Thailand"
BASE COUNT      276 a    138 c    175 g    165 t
ORIGIN
  1 agaaatgtgg tatggcttgt caaaaagaac agtacatacc caacaattaa gaggagctac
 61 aataatacca accaagaaga tcttttggta ctgtgggggа ttcaccatcc taatgatgcg
121 gcagagcaga caaagctcta tcaaaaccca accacctata tttctgttgg gacatcaaca
181 ctaaaccaga gattggtacc aagaatagct actagatcca aagtaaacgg gcaaagtgga
241 aggatggagt tcttttggac aattttaaaa ccgaatgatg caatcaactt tgagagtaat
301 ggaaatttca ttgctccaga atatgcatac aaaattgtca agaaagggga ctcaacaatt
361 atgaaaagtg aattggaata tggtaactgc aacaccaagt gtcaaactcc aatgggggcg
421 ataaactcta gtatgccatt ccacaatata caccctctca ccatcgggga atgccccaaa
481 tatgtgaaat caaatagatt agtccttgcg actgggctca gaaatagccc tcaaagagag
541 agaagaagaa aaaagagagg attatttgga gctatagcag gttttataga gggaggatgg
601 cagggaatgg tagatggttg gtatgggtac caccatagca atgagcaggg gagtgggtac
661 gctgcagaca aagaatccac tcaaaaggca atagatggag tcaccaataa ggtcaactcg
721 ataattgaca aaatgaacac tcagtttgag gccg//
```

CU-346-NP: Nucleoprotein gene (NP)

```
LOCUS       CU-346-NP    554 bp    DNA     linear       23-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Chiang
            Rai/Thailand/CU-346/07(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 554)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
Poovorawan,Y. and
            Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in
            border areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 554)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2008) Chulalongkorn University,
            Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..554
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Chiang Rai/Thailand/CU-346/07(H5N1)"
            /serotype="H5N1"
            /segment="NP"
            /country="Thailand"
BASE COUNT      175 a    108 c    163 g    108 t
ORIGIN
  1 atggcgtctc aaggcaccaa acgatcttat gaacagatgg aaactggtgg ggaacgccag
 61 aatgctactg agatcagggc atctgttgga agaatggtta gtggcattgg gaggttctac
121 atacagatgt gcacagaact caaactcagt gactatgaag ggaggctgat ccagaacagc
181 ataacaatag agagaatggt actctctgca tttgatgaaa gaaggaacag atacctggaa
241 gaacacccca gtgcgggaaa ggacccgaag aagactggag gtccaattta tcggaggaga
301 gacgggaaat gggtgagaga actaattctg tacgacaaag aggagatcag gaggatttgg
361 cgtcaagcga acaatggaga ggacgcaact gctggtctta cccacctgat gatatggcat
421 tccaatctaa atgatgccac atatcagaga acgagagctc tcgtgcgtac tggaatggac
481 ccaaggatgt gctctctgat gcaagggtca actctcccta ggagatctgg agctgccggt
541 gcagcagtaa aggg//
```

CU-346-NA: Neuraminidase gene (NA)

```
LOCUS       CU-346-NA   1380 bp    DNA     linear       23-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Chiang
            Rai/Thailand/CU-346/07(H5N1).
ACCESSIONVERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 1380)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in
            border areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1380)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2008) Chulalongkorn University,
            Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..1380
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Chiang Rai/Thailand/CU-346/07(H5N1)"
            /serotype="H5N1"
            /segment="NA"
            /country="Thailand"
BASE COUNT      410 a    249 c    343 g    378 t
ORIGIN
   1 atgaatccaa ataagaagat aataaccatc ggatcaatct gtatggtaac tggaatggtt
  61 agcttaatgt tacaaattgg gaacttgatc tcaatatggg tcagtcattc aattcacaca
 121 ggaaatcaac acaaagctga accaatcagc aatactaatt ttcttattga gaaagctgtg
 181 gcttcagtaa aattagcagg caattcatct ctttgcccca ttaatggatg ggctgtatac
 241 agtaaggaca acagtataag gatcggttcc aaggggatg tgtttgttat aagagagcca
 301 ttcatctcat gctcccactt ggaatgcaga actttctttt tgactcaggg agccttgctg
 361 aatgacaagc actccaatgg gactgtcaaa gacagaagcc ctcacagaac attaatgagt
 421 tgtcctgtgg gtgaggctcc ctccccatat aactcaaggt ttgagtctgt tgcttggtca
 481 gcaagtgctt gccatgatgg caccagttgg ttgacaattg gaatttctgg cccagacagt
 541 ggggctgtgg ctgtattgaa atacaatggc ataataacag acactatcaa gagttggagg
 601 aataacatac tgagaactca agagtctgaa tgtgcatgtg taaatggctc ttgctttact
 661 gtaatgactg acggaccaag taatggtcag gcatcacata agatcttcaa aatggaaaaa
 721 gggaaagtgg ttaaatcagt cgaattggat gctcctaatt atcactatga ggaatgctcc
 781 tgttatcctg atgccggcga aatcacatgt gtgtgcaggg ataattggca tggctcaaat
 841 cggccatggg tatctttcaa tcaaaatttg gagtatcaaa taggatatat atgcagtgga
 901 gttttcggag acaatccacg ccccaatgat ggaacaggta gttgtggtcc ggtgtcctct
 961 aacggggcat atggggtaaa agggttttca tttaaatacg gcaatggtgt ctggatcggg
1021 agaacaaaaa gcactaattc caggagcggc tttgaaatga tttgggatcc aaatgggtgg
1081 actgaaacgg acagtagctt ttcagtgaaa caagatatcg tagcaataac tgattggtca
1141 ggatatagcg ggagttttgt ccagcatcca gaactgacag gactagattg cataagacct
1201 tgtttctggg ttgagttgat cagagggcgg cccaaagaga gcacaatttg gactagtggg
1261 agcagcatat ctttttgtgg tgtaaatagt gacactgtgg gttggtcttg gccagacggt
1321 gctgagttgc cattcaccat tgacaagtag tttgttcaaa aaactccttt gtttctacta
```

Nucleotide sequences of A/chicken/Prachuap Khiri Khan/Thailand/CU-347/08

CU-347-PB2: Polymerase basic 2 gene (PB2)

```
LOCUS       CU-347-PB2    2281 bp    DNA     linear       17-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 2281)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2281)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES             Location/Qualifiers
source      1..2281
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1)"
            /serotype="H5N1"
            /segment="PB2"
            /country="Thailand"
CDS         1..2281
            /codon_start=1

BASE COUNT      755 a    429 c    595 g    502 t
ORIGIN
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 61 acaaaaacca ctgtggacca tatggccata atcaagaaat acacatcagg aagacaagag
121 aagaaccctg ctctcagaat gaaatggatg atggcaatga aatatccaat cacagcggac
181 aagagaataa tagagatgat tcctgaaagg aatgaacaag ggcagacgct ctggagcaag
241 acaaatgatg ctggatcgga cagggtgatg gtgtctcccc tagctgtaac ttggtggaat
301 aggaatgggc cggcgacaag tgcagttcat tatccaaagg tttacaaaac atactttgag
361 aaggttgaaa ggttaaaaca tggaaccttc ggtcccgttc atttccgaaa ccaagttaaa
421 atacgccgcc gagttgatat aaatcctggc catgcagatc tcagtgctaa agaagcacaa
481 gatgtcatca tggaggtcgt tttcccaaat gaagtgggag ctagaatatt gacatcagag
541 tcgcaattga caataacgaa agagaagaaa gaagagctcc aagattgtaa gattgctccc
601 ttaatggttg catacatgtt ggaaagggaa ctggtccgca aaaccagatt cctaccggta
661 gcaggcggaa caagcagtgt gtacattgag gtattgcatt tgactcaagg gacctgctgg
721 gaacagatgt acactccagg cggagaagtg agaaatgacg atgttgacca gagtttgatc
781 atcgctgcca gaaacattgt taggagagca acggtatcag cggatccact ggcatcactg
841 ctggagatgt gtcacagcac acaaattggt gggataagga tggtggacat ccttaggcaa
901 aatccaactg aggaacaagc tgtggatata tgcaaagcag caatgggtct gaggatcagt
961 tcttccttta gctttggagg cttcactttc aaaagaacaa gtggatcatc cgtcaagaag
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1021 gaagaggaag tgcttacagg caacctccaa acattgaaaa taagagtaca tgagggatat
1081 gaggaattca caatggttgg gcggagggca acagctatcc tgaggaaagc aactagaagg
1141 ctgattcagt tgatagtaag tggaagagac gaacaatcaa tcgctgaggc aatcattgta
1201 gcaatggtgt tctcacagga ggattgcatg ataaaggcag tccgaggcga tctgaatttc
1261 gtaaacagag caaaccaaag attaaacccc atgcatcaac tcctgagaca ttttcaaaag
1321 gatgcaaaag tgctatttca gaattgggga attgaaccca ttgataatgt catggggatg
1381 atcggaatat tacctgacat gactcccagc acagagatgt cactgagagg agtaagagtt
1441 agtaaaatgg gagtggatga atattccagc actgagagag tagttgtaag tattgaccgt
1501 ttcttaaggg ttcgagatca gcgggggaac gtactcttat ctcccgaaga ggtcagcgaa
1561 acccagggaa cagagaaatt gacaataaca tattcatcat caatgatgtg ggaaatcaac
1621 ggtcctgagt cagtgcttgt taacacttat cagtggatca tcagaaactg ggagactgtg
1681 aagattcaat ggtctcaaga ccccacgatg ctgtacaata agatggagtt tgaaccgttc
1741 caatccttgg tacccaaggc tgccagaggt caatacagtg gatttgtgag aacattattc
1801 caacaaatgc gtgacgtact ggggacattt gatactgtcc agataataaa gctgctacca
1861 tttgcagcag ccccaccgga gcagagcaga atgcagtttt cttctctaac tgtgaatgtg
1921 agaggctcag gaatgagaat actcgtaagg ggcaattccc ctgtgttcaa ctataataag
1981 gcaaccaaaa ggcttaccgt tcttggaaag gacgcaggtg cattaacaga ggatccagat
2041 gaggggacag ccggagtgga atctgcagta ctgaggggat tcttaattct aggcaaggag
2101 gacaaaaggt atggaccagc attgagcatc aatgaactga gcaatcttgc gaagggggag
2161 aaagctaatg tgctgatagg gcaaggagac gtggtgttgg taatgaaacg aaaacgggac
2221 tctagcatac ttactgacag ccagacagcg accaaaagaa ttcggatggc catcaattag
2281 t//
```

CU-347-PB1: Polymerase basic 1 gene (PB1)

```
LOCUS       CU-347-PB1 2293 bp    DNA     linear       17-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 2293)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2293)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..2293
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1)"
            /serotype="H5N1"
            /segment="PB1"
            /country="Thailand"
 CDS        1..2293
            /codon_start=1

BASE COUNT      798 a    450 c    529 g    516 t
ORIGIN
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  61 acattccctt atactggaga ccctccatac agccatggaa cagggacagg atacaccatg
 121 gacacagtca acagaacaca ccaatattca gaaaagggga agtggacaac aaacacagag
 181 actggagcac cccaactcaa cccgattgat ggaccactgc ctgaggataa tgagcccagt
 241 gggtatgcac aaacagattg tgtattggaa gcaatggctt tccttgaaga atcccaccca
 301 gggatctttg aaaactcgtg tctagaaaca atggaaattg ttcaacaaac aagagtggat
 361 aaactgaccc aaggtcgcca gacctatgac tggacattga atagaaacca accggctgca
 421 actgctttgg ccaacactat agaaatcttc agatcaaacg gtctaacagc caatgaatcg
 481 ggacggctaa tagatttcct caaggatgtg atggaatcaa tggataagga agaaatggag
 541 ataacaacac atttccagag aaagagaagg gtgagggaca acatgaccaa aaaaatggtc
 601 acacaaagaa caatagggaa gaaaaaacaa aggctgaaca aaaagagcta cctgataaga
 661 gcactgacac tgaacacaat gacaaaagat gcagaaagag gcaaattgaa gaggcgagcg
 721 attgcaacac ccggaatgca aatcagagga ttcgtgtact ttgttgaaac actagcgagg
 781 agtatctgtg agaaacttga gcaatctgga ctcccagtcg gagggaatga gaagaaggct
 841 aaattggcaa acgtcgtgag gaagatgatg actaactcac aagatactga actctccttt
 901 acaattactg gagacaatac caaatggaat gagaatcaga atcctaggat gtttctggca
 961 atgataacgt acatcacaag gaaccagcca gaatggtttc ggaatgtctt aagcattgct
1021 cctataatgt tctcaaacaa gatggcgaga ctaggaaaag gatacatgtt cgaaagtaag
1081 agcatgaagt tacgaacaca aataccagca gaaatgcttg caaacattga tcttaaatac
1141 ttcaatgaat taacgaaaaa gaaaattgag aaaataaggc ctctattaat agatggtaca
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1201 gcctcattga gccctggaat gatgatgggc atgttcaaca tgctgagtac agtcctagga
1261 gtttcaatcc tgaatcttgg acagaaaagg tacaccaaaa ccacatattg gtgggacgga
1321 ctccaatgct ctgatgattt cgctctcatc gtaaatgcac cgaatcatga gggaatacaa
1381 gcaggagtgg ataggtttta taggacttgt aaactagttg gaatcaatat gagcaagaag
1441 aagtcttaca taaatcggac agggacattt gaattcacga gctttttcta ccgctatgga
1501 tttgtagcca atttcagtat ggagctgccc agttttggag tgtctggaat taatgaatcg
1561 gccgacatga gcattggtgt tacagtgata aaaaacaata tgataaacaa cgaccttggg
1621 ccagcaacag ctcagatggc tcttcagtta ttcatcaagg actacagata cacataccga
1681 tgccacagag gggatacgca aatccaaaca aggagatcat tcgagctgaa gaagctgtgg
1741 gagcaaaccc gttcaaaggc aggactgttg gtttcagatg gaggaccaaa tctatacaat
1801 atccgaaatc tccatattcc tgaagtctgc ttaaaatggg aattgatgga tgaagattac
1861 cagggcagac tgtgtaatcc tctgaatcca ttcgtcagcc ataaggaaat tgaatctgtc
1921 aacaatgctg tagtaatgcc agctcatggc ccggccaaga gtatggaata tgatgccgtt
1981 gcaactacac attcatggat tcctaaaagg aaccgttcca ttctcaatac gagtcaaagg
2041 ggaattcttg aggatgaaca gatgtaccag aagtgctgca atttattcga gaaattcttc
2101 cccagcagtt catatcggag gccagttgga atttccagca tggtggaggc catggtgtct
2161 agggcccgaa ttgacgcacg aattgacttc gagtctggaa ggattaagaa agaagagttt
2221 gctgagatca tgaagatctg ttccaccatt gaagaactca gacggcaaaa atagtgaatt
2281 tagcttgtcc ttc//
```

CU-347-PA: Polymerase acidic gene (PA)

```
LOCUS       CU-347-PA  2220 bp    DNA     linear       17-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 2220)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2220)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan,Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..2220
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1)"
            /serotype="H5N1"
            /segment="PA"
            /country="Thailand"
CDS         1..2220
            /codon_start=1

BASE COUNT      730 a    438 c    533 g    519 t
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 121 cacttggagg tctgtttcat gtattcggat tttcatttta ttgatgaacg gagtgaatca
 181 ataattgtag aatctggaga tccgaatgca ttattaaaac accgatttga aataattgaa
 241 ggaagagacc gaacgatggc ctggactgtg gtgaatagta tctgcaacac cacaggagtt
 301 gagaaaccta aatttctccc agatttgtat gactacaaag agaaccgatt catcgaaatt
 361 ggagtgacac ggagggaagt tcatacatac tatctggaga aagccaacaa gataaaatcc
 421 gagaagacac atattcacat attctcattc acaggggagg aaatggccac caaagcggac
 481 tataccttg atgaagagag cagggcaaga attaaaacca ggctgttcac cataaggcag
 541 gaaatggcca gtaggggtct atgggattcc tttcgtcaat ccgagagagg cgaagagaca
 601 attgaagaaa aatttgaaat cactggaacc atgcgcagac ttgcagacca aagcctccca
 661 ccgaacttct ccagccttaa aaactttaga gcctatgtgg atggattcga accgaacgga
 721 tgcattgagg gcaagctttc tcaaatgtca aaagaagtga atgctagaat tgagccattt
 781 ttgaagacaa cgccacgccc tctcagacta cctgatgggc ctccttgctc tcagcggtcg
 841 aagtttttgc tgatggatgc ccttaaatta agcatcgaag acccgagtca tgagggggag
 901 gggataccac tatacgatgc aatcaaatgc atgaagacat ttttcggatg gaaagagccc
 961 aacatcgtga aaccacatga aaagggtgtt aactccaatt acctcctggc ttggaagcag
1021 gtgctggcag aactccaaga tattgaaaat gaggagaaaa tcccaaaaac aaagaacatg
1081 aaaaaaacaa gccagttgaa gtggacactc ggtgagaaca tggcaccaga gaaagtagac
1141 tttgaggact gcaaagatgt tagcgaccta agacagtatg acagtgatga accagagtct
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1201 agatcactag caagctggat tcagagtgaa ttcaacaagg catgtgaatt gacagattcg
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1321 atgagaagga attattttac ggcggaagta tcccattgca gggccactga atacataatg
1381 aagggagtgt acataaacac agccctgttg aatgcatcct gtgcagccat ggatgacttt
1441 caactaattc caatgataag caaatgcaga accaaagaag gaagacggaa aactaatctg
1501 tatggattca ttataaaagg gagatcccac ttgagaaatg ataccgatgt ggtgaatttt
1561 gtgagtatgg aattctctct tactgatccg aggctggagc cacacaagtg ggaaaagtac
1621 tgtgtcctcg agataggaga catgctcctc cggactgcag taggccaagt ttcaaggccc
1681 atgttcctgt atgtaagaac caatggaacc tccaagatca aaatgaaatg gggcatggaa
1741 atgaggcgat gccttcttca atcccttcaa caaattgaaa gcatgattga agccgagtct
1801 tctgtcaaag agaaggacat gaccaaagaa ttctttgaaa acaaatcaga aacatggccg
1861 attggagagt cccccaaggg agtggaggaa ggctccatcg gaaaggtgtg cagaaccttg
1921 ctggcgaagt ctgtgttcaa cagtttatat gcatctccac aactcgaggg gttttcagct
1981 gaatcaagaa aattgcttct cattgctcag gcacttaggg acaacctgga acctgggacc
2041 ttcgatcttg gagggctata tgaagcaatt gaggagtgcc tgattaacga tccctgggtt
2101 ttgcttaatg cgtcttggtt caactccttc ctcgcacatg cactgaaata gttgtggcaa
2161 tgctactatt tgctatccat actgtccaaa aaagtacctt gtttctacta atacgagacg//
```

CU-347-HA: Hemagglutinin gene (HA)

```
LOCUS       CU-347-HA   1716 bp    DNA     linear       17-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 1716)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1716)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..1716
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1)"
            /serotype="H5N1"
            /segment="HA"
            /country="Thailand"
CDS         1..1716
            /codon_start=1

BASE COUNT      596 a    311 c    397 g    412 t
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   61 attggttacc atgcaaacaa ctcgacagag caggttgaca caataatgga aaagaacgtt
  121 actgttacac atgcccaaga catactggaa aagacacaca acgggaagct ctgcgatcta
  181 gatggagtga agcctctaat tttgagagat tgtagtgtag ctggatggct cctcggaaac
  241 ccaatgtgtg acgaattcat caatgtgccg gaatggtctt acatagtgga gaaggccaat
  301 ccagtcaatg acctctgtta cccaggggat ttcaatgact atgaagaatt gaaacaccta
  361 ttgagcagaa taaaccattt tgagaaaatt cagatcatcc ccaaaagttc ttggtccagt
  421 catgaagcct cattaggggt gagctcagca tgtccatacc agggaaagtc ctccttttc
  481 agaaatgtgg tatggcttat caaaaagaac agtacatacc caacaataaa gaggagctac
  541 aataatacca accaagaaga tcttttggta ctgtggggga ttcaccatcc taatgatgcg
  601 gcagagcaga caaagctcta tcaaaaccca accacctata tttccgttgg gacatcaaca
  661 ctaaaccaga gattggtacc aagaatagct actagatcca aagtaaacgg gcaaagtgga
  721 aggatggagt tcttctggac aattttaaaa ccgaatgatg caatcaactt cgagagtaat
  781 ggaaatttca ttgctccaga atatgcatac aaaattgtca agaaagggga ctcaacaatt
  841 atgaaaagtg aattggaata tggtaactgc aacaccaagt gtcaaactcc aatgggggcg
  901 ataaactcta gtatgccatt ccacaatata caccctctca ccatcgggga atgccccaaa
  961 tatgtgaaat caaacagatt agtccttgcg actgggctca gaaatagccc tcaaagagag
 1021 agaagaagaa aaaagagagg attatttgga gctatagcag gttttataga gggaggatgg
 1081 cagggaatgg tagatggttg gtatgggtac caccatagca atgagcaggg gagtgggtac
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1381 gtcaagaacc tttacgacaa ggtccgacta cagcttaggg ataatgcaaa ggagctgggt
1441 aacggttgtt tcgagttcta tcataaatgt gataatgaat gtatggaaag tgtaagaaac
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1561 ggagtaaaat tggaatcaat aggaatttac caaatactgt caatttattc tacagtggcg
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1681 tctttacaat gcagaatttg catttaaatc aggagt//
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CU-347-NP: Nucleoprotein gene (NP)

```
LOCUS       CU-347-NP   1522 bp    DNA     linear       17-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 1522)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R., Poovorawan,Y.
and
            Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1522)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..1522
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1)"
            /serotype="H5N1"
            /segment="NP"
            /country="Thailand"
CDS         1..1522
            /codon_start=1

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 121 atacagatgt gcacagaact caaactcagt gactatgaag ggaggctgat ccagaacagc
 181 ataacaatag agagaatggt actctctgca tttgatgaaa gaaggaacag atacctggaa
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 301 gacgggaaat gggtgagaga gctaattctg tacgacaaag aggagatcag gaggatttgg
 361 cgtcaagcga acaatggaga ggacgcaact gctggtctta cccacctgat gatatggcat
 421 tccaatctaa atgatgccac atatcagaga acgagagctc tcgtgcgtac tggaatggac
 481 ccaaggatgt gctctctgat gcaagggtca actctcccga ggagatctgg agctgccggt
 541 gcagcagtaa agggggtagg gacaatggtg atggagctga ttcggatgat aaaacgaggg
 601 atcaacgacc ggaatttctg gagaggcgaa aatggaagaa gaacaaggat tgcatatgag
 661 agaatgtgca acatcctcaa agggaaattc caaacagcag cacaaagagc aatgatggat
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 781 cggtctgcac tcatcctgag aggatcagtg gcccataagt cctgcttgcc tgcttgtgtg
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 961 aatccagcac ataagagtca attagtgtgg atggcatgcc actctgcagc atttgaggac
1021 cttagagtct caagtttcat cagagggaca agagtggtcc caagaggaca gctatccacc
1081 agaggggttc aaattgcttc aaatgagaac atggaggcaa tggactccaa cactcttgaa
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1261 gaaagagcga ccattatggc agcatttaca ggaaatactg agggcagaac gtctgacatg
1321 aggactgaaa tcataagaat gatggaaagt gccagaccag aagatgtgtc attccagggg
1381 cggggagtct tcgagctctc ggacgaaaag gcaacgaacc cgatcgtgcc ttcctttgac
1441 atgaataatg aaggatctta tttcttcgga gacaatgcag aggagtatga caattaaaga
1501 aaaataccct tgtttctact at //
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CU-347-NA: Neuraminidase gene (NA)

```
LOCUS       CU-347-NA               1381 bp    DNA     linear       17-DEC-
2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 1381)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. andAmonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1381)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..1381
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
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            Khan/Thailand/CU-347/08(H5N1)"
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            /country="Thailand"
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            /codon_start=1

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 121 gggaatcaac acaaagctga accaatcagc aatactaatt ttcttactga gaaagctgtg
 181 gcttcagtaa aattagcggg caattcatct ctttgcccca ttaatggatg ggctgtatac
 241 agtaaggaca acagtataag gatcggttcc aaggggatg tgtttgttat aagagagcca
 301 ttcatctcat gctcccactt ggaatgcaga actttctttt tgactcaggg agccttgctg
 361 aatgacaagc actccaatgg gactgtcaaa gacagaagcc ctcacagaac attaatgagt
 421 tgtcctgtgg gtgaggctcc ctccccatat aactcaaggt ttgagtctgt tgcttggtca
 481 gcaagtgctt gccatgatgg caccagttgg ttgacaattg gaatttctgg cccagacagt
 541 ggggctgtgg ctgtattgaa atacaatggc ataataacag acactatcaa gagttggagg
 601 aataacatac tgagaactca agagtctgaa tgtgcatgtg taaatggctc ttgctttact
 661 gtaatgactg acggaccaag taatggtcag gcatcacata agatcttcaa aatggaaaaa
 721 gggaaagtgg ttaaatcagt cgaattggat gctcctaatt atcactatga ggaatgctcc
 781 tgttatcctg atgccggcga aatcacatgt gtgtgcaggg ataattggca tggctcaaat
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 901 gttttcggag acaatccacg ccccaatgat ggaacaggta gttgtggtcc ggtgtcctct
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1261 agcagcatat ctttttgtgg tgtaaatagt gacactgtgg gttggtcttg gccagacggt
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1381 g //
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CU-347-M: Matrix gene (MA)

```
LOCUS       CU-347-M    984 bp    DNA     linear       17-DEC-2008
DEFINITION  Influenza A virus strain A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1).
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 984)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 984)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
FEATURES    Location/Qualifiers
source      1..984
            /organism="Influenza A virus"
            /mol_type="genomic DNA"
            /strain="A/chicken/Prachuap Khiri
            Khan/Thailand/CU-347/08(H5N1)"
            /serotype="H5N1"
            /segment="M"
            /country="Thailand"
CDS         1..984
            /codon_start=1

BASE COUNT      280 a    208 c    263 g    233 t
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  121 gctctcatgg agtggctaaa gacaagacca atcctgtcac ctctgactaa agggattttg
  181 ggatttgtat tcacgctcac cgtgcccagt gagcgaggac tgcagcgtag acgctttgtc
  241 cagaatgccc taaatggaaa tggagatcca aataatatgg atagggcagt taagctatat
  301 aagaagctga aaagagaaat aacattccat ggggctaagg aggtcgcact cagctactca
  361 accggtgcac ttgccagttg catgggtctc atatacaaca ggatgggaac ggtgactacg
  421 gaagtggctt ttggcctagt gtgtgccact tgtgagcaga ttgcagattc acagcatcgg
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CU-347-NS: Nonstructural gene (NS)

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ACCESSION
VERSION
KEYWORDS    .
SOURCE      Influenza A virus
  ORGANISM  Influenza A virus
            Unclassified.
REFERENCE   1  (bases 1 to 853)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Monitoring of influenza A virus from avian species in border
            areas of Thailand
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 853)
  AUTHORS   Lapkuntod,J., Chuatakul,C., Tantilertcharoen,R.,
            Poovorawan,Y. and Amonsin,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-DEC-2008) Chulalongkorn University, Bangkok,
            1Department of Veterinary Public Health, Rama 4 Rd.
            Pathumwan, Bangkok 10330, Thailand
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BIOGRAPHY

Mr. Jiradej Lapkuntod was born on November 13, 1983 in Bangkok, Thailand. He graduated from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2006. After that, he enrolled the Master degree of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2009.