การสำรวจเชื้อไข้หวัดใหญ่ในตลาดสัตว์ปีกมีชีวิตในประเทศไทย

นายตรอง วิเศษชาญเวทย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาสัตวแพทยศาสตรมหาบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

SURVEY OF INFLUENZA A VIRUSES IN LIVE BIRD MARKETS IN THAILAND

Mr. Trong Wisedchanwet

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Public Health

Department of Veterinary Public Health

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2009

Thesis Title	SURVEY OF INFLUENZA A VIRUSES IN LIVE BIRD MARKETS IN THAILAND
By	Mr. Trong Wisedchanwet
Field of Study	Veterinary Public Health
Thesis Advisor	Associate Professor Alongkorn Amonsin, D.V.M., Ph.D.
Thesis Co-Advisor	Assistant Professor Rungtip Chuanchuen, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science
(Professor Mongkol Techakumphu, D.V.M., Doctorate de 3e cycle)

THESIS COMMITTEE

..... Chairman
(Assistant Professor Suphachai Nuanualsuwan, D.V.M., Ph.D.)

..... Thesis Advisor
(Associate Professor Alongkorn Amonsin, D.V.M., Ph.D.)

..... Thesis Co-Advisor
(Assistant Professor Rungtip Chuanchuen, D.V.M., Ph.D.)

..... External Examiner
(Associate Professor Thaveesak Songserm, D.V.M., Ph.D.)

ตรอง วิเศษชาญเวทย์ : การสำรวจเชื้อไข้หวัดใหญ่ในตลาดสัตว์ปีกมีชีวิตในประเทศไทย.(SURVEY OF INFLUENZA A VIRUSES IN LIVE BIRD MARKETS IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.น.สพ.ดร. อลงกร อมรศิลป์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. สพ.ญ. ดร. รุ่งทิพย์ ชวนชื่น, 74 หน้า.

ตลาดสัตว์ปีกมีชีวิต (live bird market) หมายถึง ตลาดสดที่มีการค้าขายสัตว์ปีกที่มีชีวิตเพื่อนำไปเป็นอาหารหรือเป็นสัตว์เลี้ยง โดยมีการเก็บขังสัตว์อย่างหนาแน่น หลากหลายชนิดสัตว์ รวมถึงการนำสัตว์ที่มาจากหลากหลายแหล่งมาไว้รวมกัน ทำให้อาจมีการแพร่กระจายของโรคต่างๆได้ง่าย เป็นที่ทราบกันดีว่า สัตว์ปีกเป็นแหล่งรังโรคของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ (Influenza A virus)โดยเฉพาะสัตว์ปีกในตระกูลนกน้ำและเป็ด การศึกษาครั้งนี้เป็นการสำรวจเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ ในตลาดสัตว์ปีกมีชีวิตในปี พ.ศ. 2552 เป็นระยะเวลา 12 เดือน จากตลาดสัตว์ปีกมีชีวิตจำนวน 10 ตลาด ผลการทดสอบตัวอย่างจำนวน 5,304 ตัวอย่าง จากสัตว์ปีก 2,652 ตัว ตรวจพบเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ สายพันธุ์ H4N6 จำนวน 2 ตัวอย่าง, H4N9 จำนวน 1 ตัวอย่าง และ H10N3 จำนวน 16 ตัวอย่าง คิดเป็น 0.36 % (19/5,304) ตัวอย่างเชื้อไวรัสที่ตรวจพบทั้งหมดได้มาจากเป็ดเทศในตลาดแห่งหนึ่งในกรุงเทพมหานคร ซึ่งเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ ทั้ง 3 สายพันธุ์นี้ ไม่พบว่าเคยมีรายงานในประเทศไทยมาก่อน และในการสำรวจครั้งนี้ ไม่พบว่ามีเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ ชนิดก่อโรครุนแรง เช่น เชื้อไข้หวัดนกสายพันธุ์ H5N1 ซึ่งสอดคล้องกับรายงานที่ไม่พบการระบาดของเชื้อไวรัสไข้หวัดนกในปี พ.ศ. 2552 การศึกษาครั้งนี้ คณะผู้วิจัยสามารถแยกเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ สองสายพันธุ์ คือ สายพันธุ์ H4N6 และ H4N9 ได้จากสัตว์ตัวเดียวกัน ซึ่งบ่งบอกถึงความเป็นไปได้ในการแลกเปลี่ยนสารพันธุกรรมของเชื้อไวรัส 2 สายพันธุ์ในตลาดสัตว์ปีกมีชีวิต ดังนั้นการสำรวจโรคในตลาดสัตว์ปีกมีชีวิตจึงมีความสำคัญ และเป็นการเตือนภัยล่วงหน้าถึงโอกาสในการเกิดการกลายพันธุ์และแพร่ระบาดของเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ สู่สัตว์ปีกและสัตว์ชนิดอื่นๆรวมถึงมนุษย์ อันอาจก่อให้เกิดความเสียหายทั้งทางเศรษฐกิจและการสาธารณสุขในอนาคตได้

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....
สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5175556531 : MAJOR VETERINARY PUBLIC HEALTH

KEYWORDS : LIVE BIRD MARKET / INFLUENZA A VIRUS / SURVEY

TRONG WISEDCHANWET : SURVEY OF INFLUENZA A VIRUS IN LIVE BIRD MARKETS IN THAILAND. THESIS ADVISOR : ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., THESIS CO-ADVISOR : ASSIST. PROF. RUNGTIP CHUANCHEUN, D.V.M., Ph.D., 74 pp.

Live bird markets (LBMs) are places where the birds are sold as pet or poultry meat. LBMs settings usually appear with dense population, multiple species and different sources in order to supply the customers demand. With these conditions, many diseases can transmit and spread within LBMs while some species such as waterfowls and ducks are known as reservoir of influenza A virus entering LBMs everyday. In this study, we conducted a 12 months survey of influenza A virus in 10 LBMs in Thailand, 2009. A total of 5,304 samples from 2,652 animals were collected and tested for influenza A virus. Influenza A viruses subtype H4N6 (n=2), H4N9 (n=1) and H10N3 (n=16) were identified with 0.36% positive (19/5,304). All influenza A isolates were collected from Muscovy ducks in a market located in Bangkok. In Thailand, none of these 3 influenza A subtypes had ever been reported in the country. In this study, H5N1 subtype was not detected which concordant with the report of none influenza A H5N1 outbreak in Thailand during 2009. Our results showed that two influenza A subtypes (H4N6 and H4N9) can be isolated from the same bird at the same collecting time. This result suggested that the reassortment of influenza A virus can occurs in LBMs. In summary, survey of influenza A virus in LBMs is very important and can serves as an early warning system for viral reassortment and spreading of the virus to various avian and mammal species which can cause economical losses and public health problems in the future.

Department :	Veterinary Public Health	Student's Signature	T. Wisedchanwet
Field of Study :	Veterinary Public Health	Advisor's Signature	
Academic Year :	2009	Co-Advisor's Signature	Rungtip Chuancheun

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to the following individuals who helped in making this thesis possible:

First of all, Associate Professor Dr. Alongkorn Amonsin, my advisor for his excellent instruction, advice and encouragement throughout the period of this study.

Assistant Professor Dr. Rungtip Chuanchuen, my co-advisors for all of their support, excellent instruction, suggestion and kind assistance throughout my study period.

Associate Professor Dr. Suphachai Nuanualsuwan, the chairman of thesis committee ; Associate Professor Dr. Thaweesak Songserm the member of thesis committee for their constructive criticisms and valuable suggestions.

Dr. Rachod Tantilertcharoen, Dr. Napawan Bunpapong, Dr. Supanat Boonyapisitsopa, Dr. Nutthawan Nonthabenjawan, Dr. Waleemas Jairak , students in Dr. Amonsin Lab, students in Dr. Chuanchuen Lab and all of my friends for their help, supports and encouragement throughout my study period.

All staffs of the Department of Veterinary Public Health and the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University for their kindness, encouragement and friendship throughout my study period.

All staffs of the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University for their kindly help and support on this work.

Finally for my family, I am deeply thankful for their encouragement, support and love.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	Avian Influenza
bp	base pair
cDNA	complementary deoxyribonucleic acid
°C	degree Celsius
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DW	distilled water
et al.	et alibi, and other
HA	Hemagglutinin
H&E	hematoxylin & eosin
HPAI	Highly Pathogenic Avian Influenza
LBM	Live-bird market
LFM	Local food market
LPAI	Low Pathogenic Avian Influenza
M	Matrix
mg	milligram(s)
ml	milliliter(s)
µl	microliter
µM	micromolar
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

U	unit(s)
VTM	viral transport media

CHAPTER I

INTRODUCTION

Influenza A virus is an enveloped, single-stranded RNA virus of the family *Orthomyxoviridae*. The virus can cause a serious disease in humans, mammals, poultry and wild birds. The virus is categorized by its surface proteins, hemagglutinin (HA) and neuraminidase (NA) into 16 HA and 9 NA subtypes (Fouchier et al., 2005). Some influenza A subtypes are very important and can cause a severe, fatal disease such as avian influenza subtype H5N1 in humans (Tran et al., 2004), cats (Songserm et al., 2006a), dogs (Songserm et al., 2006b), tigers (Thanawongnuwech et al., 2005) and several avian species (Alexander, 2007). 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, 2005).

Live-bird markets (LBMs) are where wild birds, pet birds and poultry meat are sold to households; therefore, these markets are implicated as the major sources of influenza A virus widespread (Cardona et al., 2009; Webster, 2004). Due to the cultural preference to consume the freshly slaughtered poultry, LBMs are usually located in suburban area or the centre of the communities for their convenience. Local community activities are often involved in the LBMs; thus, bringing crowds of people each day. In these markets, thousands of animals such as poultry, wild birds and exotic mammals that are from different sources and sold in wire stack cages with dense population. These conditions provide excellent environments for animal-animal and animal-human transmissions and may result in an outbreak of influenza A (Yee et al., 2009). In China, there were five H5N1 patients with no history of exposure to diseased or dead birds before the onset of illness. Four of these five patients visited markets with live birds. The surveillance of influenza A was performed in LBMs where the patients visited and the virus was isolated from goose. These findings indicated that LBMs could be a

source for distribution of avian influenza in humans (Wang et al., 2006). LBMs are not present only in Asia but also other continents around the world. A survey by the U.S. Department of Agriculture supported that there are up to 104 LBMs all over the U.S. (Panigrahy et al., 2002).

Up to date, many studies on influenza A in LBMs from different countries have been conducted. During 2000-2001, the presence of influenza A viruses was investigated in LBMs in China and 6 subtypes with 9 genotypes of low-pathogenic avian influenza (LPAI) were identified (Liu et al., 2003). In 2001, three LPAI subtypes (i.e. H4N6, H5N2 and H9N3) including one highly pathogenic avian influenza (HPAI) subtype H5N1 were demonstrated in Vietnam (Nguyen et al., 2005). Two years later, three LPAI subtypes were reported in Korea (Choi et al., 2005). In Thailand, LBMs and local food markets (LFMs) surveillance program was conducted in 2006-2007. The H5N1 Influenza A virus was identified in 12 of 930 samples tested. Three samples were obtained from healthy birds at the same time with the 5th avian influenza outbreak in Thailand during 2007. These findings supported that the movement of birds in the avian influenza outbreak area may introduce the virus into markets and play a role in emergence or re-emergence of influenza A in animals in Thailand (Amonsin et al., 2008). In 1983-1989, many H5N2 LPAI viruses have been isolated from LBMs of several states in the US (Suarez and Senne, 2000). Another study in the U.S. examined the occurrence of avian influenza virus in LBMs in 1993-2000 and could isolate up to 10 LPAI subtypes (Panigrahy et al., 2002). The results indicated that many subtypes of influenza A virus can be found in LBMs. In this case, the co-infection of multiple subtypes in animals may occur and lead to genetic reassortment or adaptation of viruses into new host. Such reassortment will generate the new influenza A virus strains with high potential to infect humans (Panigrahy et al., 2002; Sharp et al., 1997). For example, triple reassortment from human, avian and swine occurred in early 2009. This reassortant virus resulted in a pandemic H1N1 of swine origin influenza that caused a widespread of influenza and killed many people all over the world (Smith et al., 2009).

Since LBMs play an important role in a wide distribution of avian influenza virus, active surveillance of influenza A virus in these places are required in order to develop the prevention strategy for influenza A outbreaks. If multiple subtypes or genetic reassortment are found in LBMs this can be an important early warning system for the public and also benefits for selection of the seasonal influenza vaccine for humans in the future (Webster, 2004). In this study, active surveillance of influenza A viruses among avian species was performed in LBMs from several areas of Central part of Thailand and Bangkok to identify the subtype and to characterize the genetic of influenza A viruses. The results obtained from this study demonstrated the occurrence and the subtypes of influenza A viruses that circulating in LBMs in Thailand. In addition, the molecular information from whole genome analysis will provide insight information of the viruses and the evidence of reassortment of the viruses. The information gained from this study will provide a better understanding of influenza A virus evolution in avian species in Thailand.

CHAPTER II

REVIEW LITERATURES

1. Morphology of influenza A virus

Influenza virus belongs to the Orthomyxoviridae family. Influenza virus can be divided into three types A, B and C. Influenza A virus can infect humans, mammals, and avian species. Influenza B virus reported that can infect only in human, while influenza C virus can infect in human and rarely infect in swine (Webster et al., 1992). Influenza A virus is an enveloped virus that has 2 glycoproteins, haemagglutinin (HA) and neuraminidase (NA) (figure 1). Virions are spherical to pleomorphic, 80-120 nm in diameter. HA figure is rod-shaped spike and NA figure is mushroom-shaped spike on enveloped (De Jong et al., 2000).

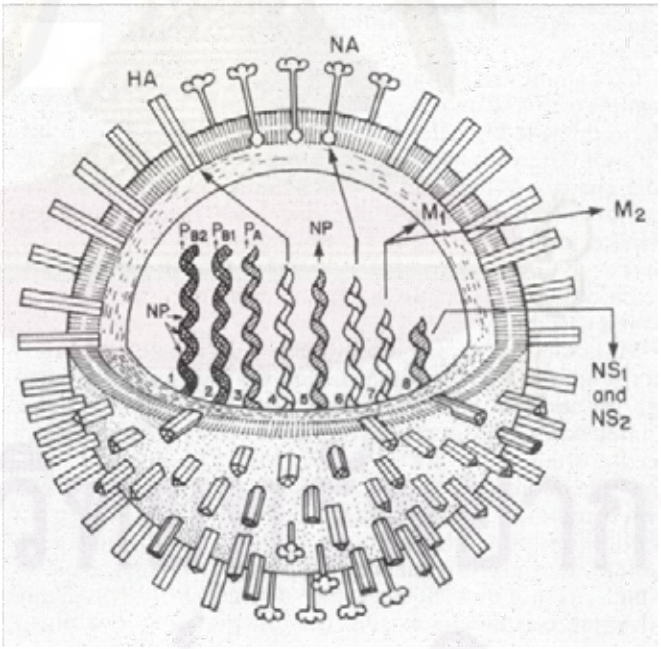


Figure 1. Structure of Influenza virus. (Webster et al., 1992)

The virus has single-stranded RNA of negative polarity 8 gene segments in its envelop with different molecular weight as Polymerase Basic protein 2 and 1 gene (PB2 and PB1), Polymerase gene (PA), Hemagglutinin gene (HA), Nucleoprotein gene (NP), Neuraminidase gene (NA), Matrix protein gene (M), and Nonstructural protein gene (NS), respectively. RNA segments are contained in the viral core. Ten types of proteins Protein (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NS2) were synthesized from 8 RNA segments (table 1) (Lamb and Choppin, 1983). Influenza A viruses are classified based on their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). At present, 16 HA (H1-16) and 9 NA (N1-9) subtypes have been identified (Fouchier et al., 2005).

Table 1. Functions of polypeptides encoding by 8 RNA segments of Influenza A virus.

Segments	Encoding polypeptide	Nucleotide Length (bp)	Functions
1	PB2	2,341	Host-cell RNA cap binding : Component of RNA transcriptase
2	PB1	2,341	Component of RNA transcriptase
3	PA	2,233	Component of RNA transcriptase
4	HA	1,778	Surface glycoprotein : attaches to cell surface sialic receptors
5	NP	1,565	Structural component of RNA transcriptase
6	NA	1,413	Surface glycoprotein : neuraminidase activity
7	M1	1,027	Membrane protein
	M2		Ion channel
8	NS1	890	TNFα response
	NS2		

2. Live bird markets system

Live bird markets (LBMs) are places where live birds are sold as a pet bird or slaughtered as a poultry meat. LBMs are usually located in suburban areas but in some developing country LBMs are placed in center of the town. LBMs provide such a favorable environment for influenza A virus because of variety of bird species were placed together with high population in small stack cages, this environment provides both direct and indirect contact to newly susceptible animals (Choi et al., 2005; Nguyen et al., 2005; Panigrahy et al., 2002; Shortridge et al., 1998; Yee et al., 2009). .Animals of various ages which gathered from different sources (i.e. backyard poultry, commercial poultry, other LBMs, shows and ornamental poultry/game farms) are found in LBMs. Before entering the LBMs, the birds may pass through many channels; producers, dealers, wholesalers and distributors. Because of these many channels, it is difficult to identify the origin of birds and influenza A virus infection. In the US, LBMs be involved in movement of 24,000 in one market day with 80% of chickens, 8% of guinea fowl, 6% of ducks, 3% of turkeys, 1% of quails and 1% in total of peafowl, ring-necked pheasants, chukar partridges and geese (Panigrahy et al., 2002). This information proved that multiple avian species were circulated in LBMs. However, in Asian country including Thailand, number of species in LBMs was unknown. With these conditions (various ages, multiple species, different sources and housed together), influenza A virus can easily transmit to new, naive animals and/or other species and may results in a small outbreak in the LBMs with or without the clinical signs.

3. Occurrence of influenza A viruses in live bird markets

In Thailand, LBMs and LFMs surveillance program was conducted in 2006-2007. Twelve viral isolates out of 930 samples tested (0.01%) were isolated and identified as influenza A (H5N1) in avian species sold in the markets. Three samples were collected from animals without any clnical signs of disease on the collection day which were in the same period of H5N1 outbreak in the country (Amonsin et al., 2008).

In Vietnam, 3.2% (6 from 189 samples) of avian influenza were isolated from LBMs in Hanoi. Four subtypes were isolated and found both HPAI (H5N1) and LPAI (H9N3, H4N6 and H5N2). Four of six avian influenza viruses were isolated from ducks while the others were isolated from goose (Nguyen et al., 2005).

In China, 1% of LPAI were isolated (63 of 6360 samples) from the 16 months surveillance program of LBMs in 2000-2001. Of the 7 subtypes isolated, H3N6 predominated at 27 from 63 samples, followed by H9N2(16/63), H4N6(10/63), H2N9(6/63), H3N3(2/63), H3N2 (1/63) and H1N1 (1/63) (Liu et al., 2003).

In South Korea, 16 of 281 samples (6%) were isolated from LBMs during 2003. The viruses were isolated from chickens (n = 8), ducks (n = 6) and doves (n = 2) and were subtyped as H9N2 (9 isolates), H3N2 (6 isolates) and H6N1 (1 isolate) (Choi et al., 2005).

In the U.S., many studies about avian influenza circulated in LBMs have been conducted. In 1983-1989, H5N2 LPAI viruses have been isolated from LBMs in several states in the US (Suarez and Senne, 2000). In 1993-2000, 10 LPAI subtypes of avian influenza virus in LBMs were isolated (Panigrahy et al., 2002).

From these studies, LBMs in many parts of the world contain many subtypes of influenza A virus. When multiple subtypes of influenza A virus infected in birds, reassortment of the viruses may occurs in the host and may generates the new subtype or new strain of influenza A virus. The new subtype or new strain of influenza A virus can be potential pandemic virus with the ability to infect to other species including human (Sharp et al., 1997). In example, triple reassortment of influenza A virus occurred in swine in 2009. After this virus transmit to human, it resulted in pandemic H1N1 of swine origin influenza that killed many people all over the world in 2009 (Smith et al., 2009).

4. Standard methods for influenza A virus isolation and identification.

Highly sensitive and standard detection method recommended by World organization for animal health (OIE) is virus isolation by embryonated egg inoculation (WHO, 2002). 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 (Swayne et al., 1998 and WHO, 2002).

Modern methods for the detection of influenza genes is polymerase chain reaction (PCR) assay. A reverse transcriptase polymerase chain reaction (RT-PCR) was developed to detect viral RNA using specific primers for influenza A virus (Payungporn et al., 2004). 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 previously published primers with high sensitivity and specificity for influenza A viruses H1-15 at 98% sensitivity (Tsukamoto et al., 2008) and N1-9 at 99.2% sensitivity (Tsukamoto et al., 2009). After subtyped identification, genetic character-rization of influenza virus by whole genome sequencing can be performed by using the universal primer sets for each gene of influenza A virus (Hoffmann et al., 2001).

CHAPTER III

MATERIALS AND METHODS

This study consists of 3 phases: **phase 1**, collection of samples from avian species in LBMs in Bangkok and Central part of Thailand; **phase 2**, isolation and identification of influenza A viruses and **phase 3**, genetic characterization of avian influenza viruses using cluster analysis. The conceptual framework of this study is shown in figure 2.

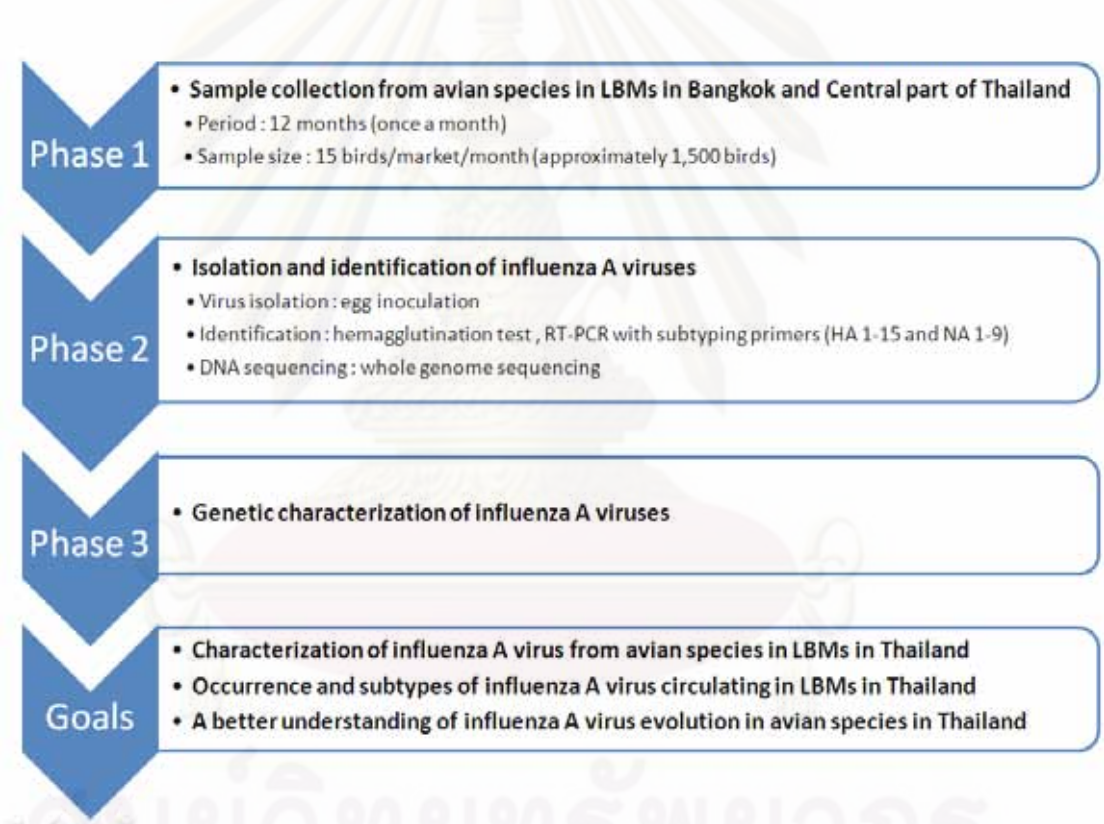


Figure 2 . Diagram shows the conceptual framework of the study.

Phase 1: Collection of samples from avian species in LBMs in Bangkok and Central part of Thailand

Ten LBMs from 5 provinces in Central Part of Thailand and Bangkok were selected for this study. The selection criteria are 1). history of outbreaks of avian influenza virus in central part of Thailand and 2). location in the center of the town and 3). collaboration of the vendors in the LBMs. The samples included cloacal contents by cloacal swabs and tracheal exudates by oropharyngeal swabs. Sterile cotton swab was used to obtain samples from choanal-slit, trachea and cloaca. The samples were placed in a sterile plastic tube containing 2 ml-viral transport media (VTM) and kept on ice. Total 5,304 samples (2,652 of oropharyngeal and 2,652 of clocal swabs) were collected from 2,652 animals. The samples were then transferred to the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. All samples were kept at -80^{o}C immediately after arrival.

Phase 2: Isolation and identification of Influenza A virus

1. Isolation of influenza A virus

Avian Influenza viruses were isolated from samples using embryonated egg inoculation (WHO, 2002). The specific antibody negative embryonated chicken eggs at the age of 9-11 days were used. The supernatant fluid from VTM suspension was inoculated into allantoic sacs of the eggs and was incubated at 37^{o}C. After the incubation period of 24-72 hours, the inoculated embryonated eggs showing infected lesions or death were collected and chilled at 4^{o}C. The allantoic fluid was harvested and tested for HA test. The samples yielding positive HA test were frozen at -80^{o}C until needed (Swayne et al., 1998).

2. Identification of influenza A virus

2.1 RNA extraction

Total RNA was extracted from the allantoic fluid using QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany). By using silica gel membrane technique, total RNA was extracted with more than 1 microgram at 60 µl final concentration.

2.2 cDNA synthesis

The viral RNA was reverse transcribed into cDNA as previously described (Viseshakul et al., 2004). In brief, cDNA synthesis was performed by preparing of the mixture of RNA and random primers as follow.

	Volume	Final concentration
RNA	4 µl	>1 µg
0.5 µg random primers	1 µl	0.1 µg
Final volume	5 µl	

After finish preparing RNA with random primers, the PCR tube was placed in Thermal cycler at 70°C for 15 minutes and 4°C for 5 minutes so that the RNA and random primers start annealing. Then the mixture of reverse transcriptase enzyme was prepared with the following formula.

	Volume	Final concentration
Improm-II™ 5x reaction buffer	4 µl	1x
25 mM $MgCl_2$	2 µl	2.5 mM
5 mM dNTPs	2 µl	0.5 mM
Recombinant RNAsin® Ribonuclease inhibitor	0.3 µl	40 U/µl
Improm-II™ reverse transcriptase	1 µl	1U
DW	5.7 µl	
Final volume	15 µl	

The mixture of RNA, random primers and the mixture of reverse transcriptase enzyme for cDNA synthesis were mixed and placed into Thermal cycler. The cDNA synthesis was performed at temperature 25°C for 5 minutes, 42°C for 60 minutes and 70°C for 15 minutes. The final product was kept in -20°C.

2.3 Identification of influenza A subtype using PCR amplification with subtyping primers

To identify the virus subtype, a reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the previously-published primers specific for influenza A virus (Tsukamoto et al., 2008; Tsukamoto et al., 2009).

RT-PCR was performed by using the protocol for amplification described below.

	Volume	Final concentration
cDNA	1 µl	
10 µM of each primers	1.5 µl	0.5 µM
25 mM $MgCl_2$	1 µl	1 mM
2.5x Eppendorf® Mastermix	10 µl	1x
DW	11.5 µl	
Final volume	25 µl	

Appropriate condition for HA subtyping PCR amplification is described below

Step		Temperature	
1	Initial denaturation	94°C	180 seconds
2	Denaturation	94°C	30 seconds
	Annealing	50°C	30 seconds
	Extension	72°C	30 seconds
	Repeat step 2 for 40 cycles		
3	Final extension	72°C	420 seconds
	Holding temperature	25°C	

Appropriate condition for NA subtyping PCR amplification is described below

Step		Temperature	
1	Initial denaturation	94°C	180 seconds
2	Denaturation	94°C	30 seconds
	Annealing	45°C	30 seconds
	Extension	72°C	30 seconds
	Repeat step 2 for 40 cycles		
3	Final extension	72°C	420 seconds
	Holding temperature	25°C	

2.4 Confirmation of PCR product sizes by using agar gel electrophoresis

Agarose gel at 2% concentration in 1x Tris borate EDTA (TBE) was used for gel electrophoresis. Ten µl of PCR product was mixed with 2 µl of 0.2% orange G loading dye in 50% glycerol (Carlo Ebra Reagent®). DNA was separated in agarose gel under 100 voltage electrical field for 30 minutes. Agarose gel was stained in 10 mM Ethidium bromide solution and confirmed the subtype of PCR product under Ultra violet light at 365 nanometres.

2.5 Whole gene amplification of influenza A virus.

After subtype of influenza A virus was known. Whole gene PCR amplification were processed by using new designed primers of each gene subtypes. Primer 3 program (v 0.4.0) was used for primer designing.

2.6 Whole genome sequencing of influenza A virus

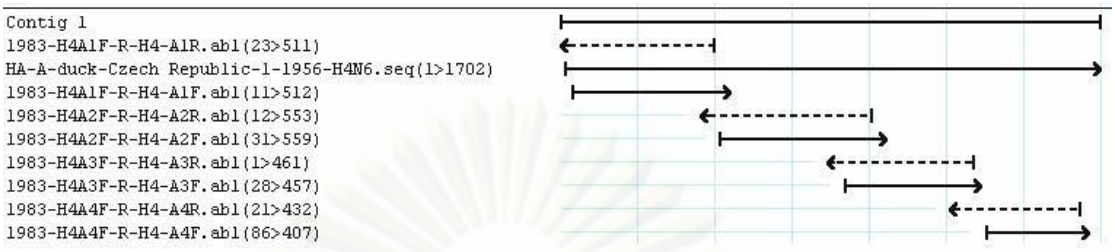
After HA and NA gene were sequenced. Whole genome amplifications for M, NP, NS, PA, PB1 and PB2 gene were performed by using primers and conditions previously described (Hoffmann et al., 2001).

Phase 3: Genetic characterization of avian influenza virus using cluster analysis

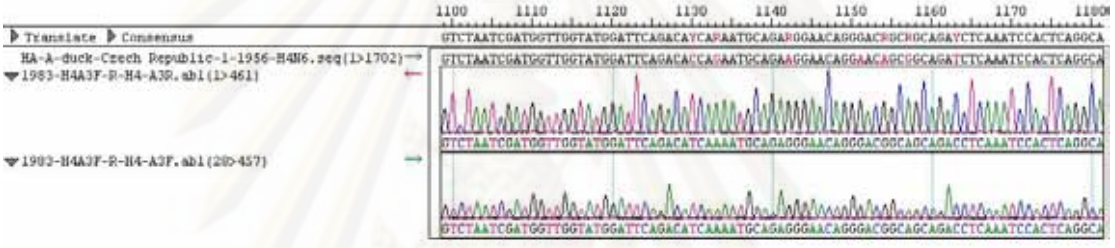
After PCR products were examined by agarose gel electrophoresis. The products were submitted for DNA sequencing at Molecular Informatics Laboratory Limited, Shatin, N.T. in Hong Kong.

The sequence results were aligned and assembled by using Seqman program (DNASTAR, Madison, WI, USA) (Figure 3a and 3b). Nucleotide similarities of each gene were analyzed by using nucleotide BLAST in GenBank database in order to identify genetic relatedness of each gene (Figure 3c). Phylogenetic analysis was performed by the clustal analysis using the MEGA 4.1 program (Tempe, AZ, USA) (Figure 4). Genetic relatedness of the viruses was determined by using phylogenetic analysis.

(a)



(b)



(c)

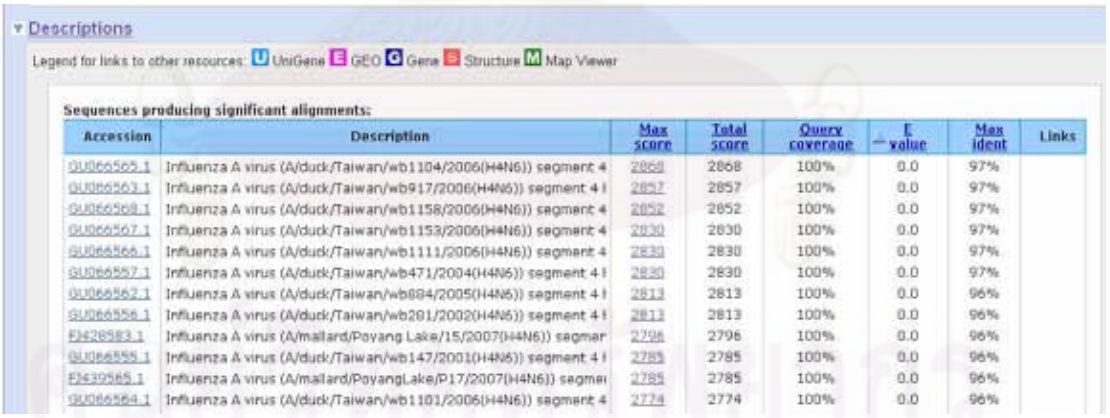


Figure 3 : Example of each steps of genetic analysis. a) Assemble of nucleotide sequences by using Seqman program. b) Chromatogram of sequence results showed in Seqman. c) Nucleotide similarity was performed using BLAST tools in GenBank database.

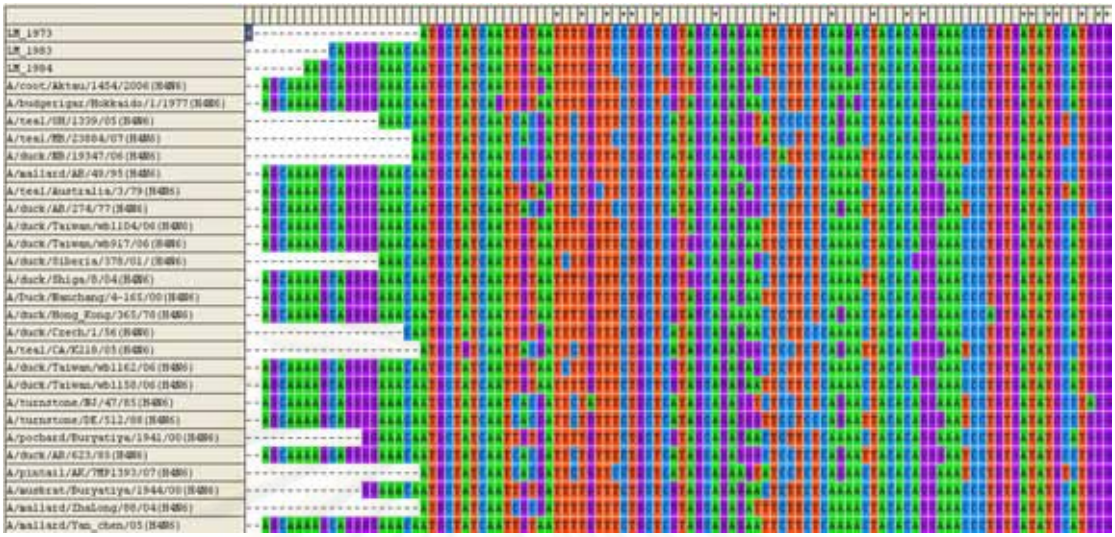


Figure 4 : The alignment of nucleotide sequences with represent isolates from each region of the world was performed by using MEGA 4.1 in order to identify the genetic relatedness.

CHAPTER IV

RESULTS

In this study, one year surveillance program in live bird markets was conducted during January 2009 to December 2009. The total of 5,304 samples from 2,652 animals were collected and examined during the course of the study. Virus isolation was conducted by using embryonated egg inoculation. Influenza A virus identification and subtype identification were done by using Hemagglutination test (HA test) and Real time RT-PCR assays. Genetic characterization of the virus was conducted by using whole genome sequencing and phylogenetic analysis.

1. Sample collected from LBMs during January – December 2009.

Sample collections from 10 LBMs were done during January – December 2009 (Fig. 5 and Table 2). Eight LBMs from 4 provinces in central part of Thailand (Nakhonsawan, Pichit, Phitsanulok and Sukhothai) with prior outbreaks of H5N1 were also selected. These provinces are known as one of the main areas of rice production in Thailand for a long time because of the main river of Thailand runs through them. In the migration period, these rice fields are served as temporary habitats for wild birds which are known as a reservoir of influenza A virus. With wild birds around these areas, transmission of an influenza A viruses from wild birds to avian species in these area can occur, therefore, 8 LBMs (two LBMs from each provinces in central part of Thailand) were selected. In addition, two LBMs in Bangkok were selected. One LBM is a local food market located in the center of Bangkok which selling poultry meat, vegetables, fruits and other ingredients thus bringing thousands of people everyday while another is a pet bird market. This pet bird market sells not only birds but also other animals such as dogs, cats, rabbits, squirrel and some reptiles. With multiple species circulating in the market, possibility of influenza A virus transmission between animals is high.

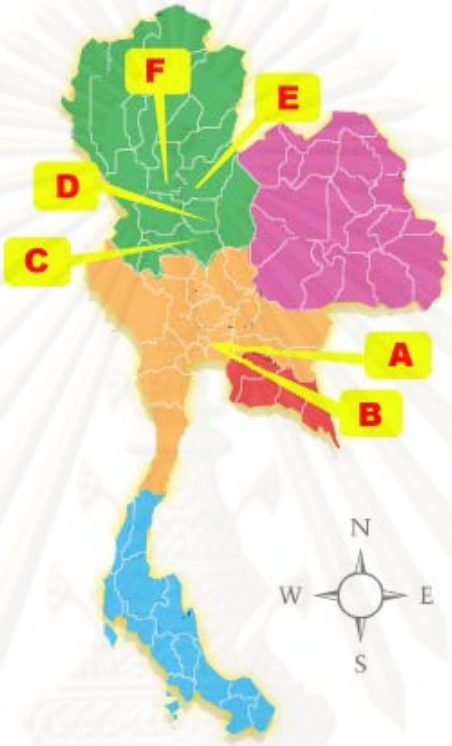


Figure 5 : Map of LBMs location in Thailand which were selected in this study

\

Table 2 : Description of LBMs selected in this study.

Market	Province	Region	Types of Markets
A	Bangkok	Bangkok	Local food market
B	Bangkok	Bangkok	Pet Birds Market
C1	Nakhonsawan	Central	Local food market
C2	Nakhonsawan	Central	Local food market
D1	Pichit	Central	Local food market
D2	Pichit	Central	Local food market
E1	Phitsanulok	Central	Local food market
E2	Phitsanulok	Central	Local food market
F1	Sukhothai	Central	Local food market
F2	Sukhothai	Central	Local food market

Table 3: Total number of avian species collected during January – December 2009

Market	Chicken	Duck	Quail	Dove	Pigeon	Goose	Total (by market)
A	214	271	0	0	0	0	485
B	33	96	80	62	45	5	321
C1	227	0	0	0	0	0	227
C2	70	31	0	0	0	0	101
D1	140	0	0	0	0	0	140
D2	181	0	0	0	0	0	181
E1	244	268	0	0	0	0	512
E2	233	13	0	0	0	0	246
F1	215	0	0	0	0	0	215
F2	224	0	0	0	0	0	224
Total(by species)	1781	679	80	62	45	5	2652

Figure 6 : Total number of samples collected and HA positive samples in each market

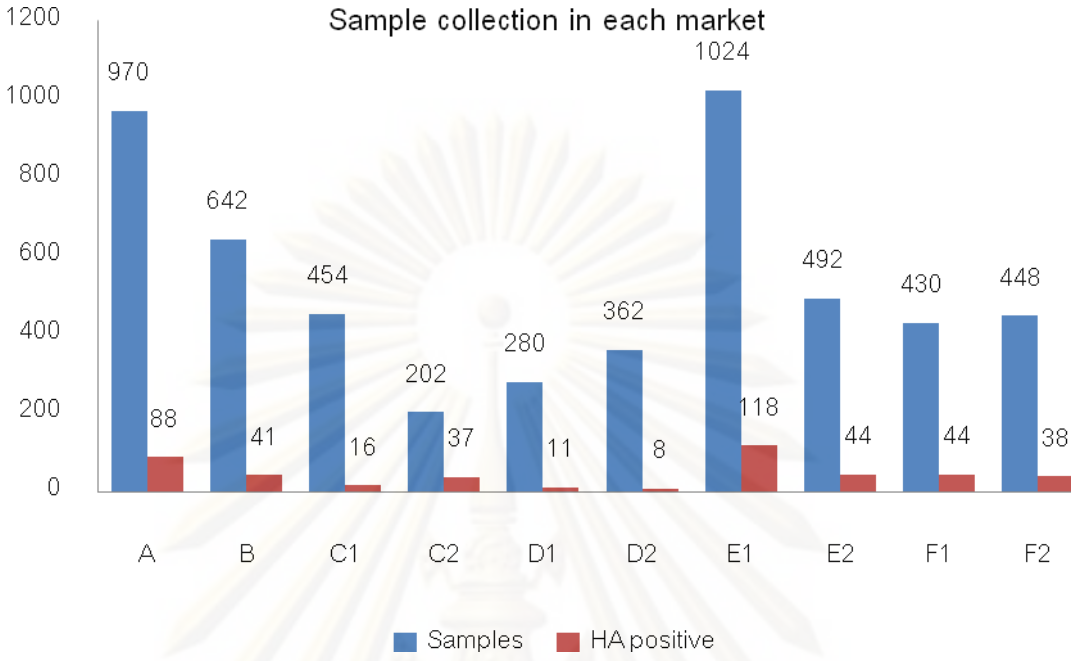


Table 4: HA positive and influenza A isolates of samples collected from each market

Market	Samples	HA positive (%)	Influenza A (%)
A	970	88 (9.07)	19 (1.96)
B	642	41 (6.39)	0
C1	454	16 (3.52)	0
C2	202	37 (18.32)	0
D1	280	11 (3.93)	0
D2	362	8 (2.21)	0
E1	1024	118 (11.52)	0
E2	492	44 (8.94)	0
F1	430	44 (10.23)	0
F2	448	38 (8.48)	0
Total	5304	445 (8.38)	19 (0.36)

note ; number in parentheses are percentage of positive samples in each market

Figure 7 : Total sample collected and HA positive samples in each month

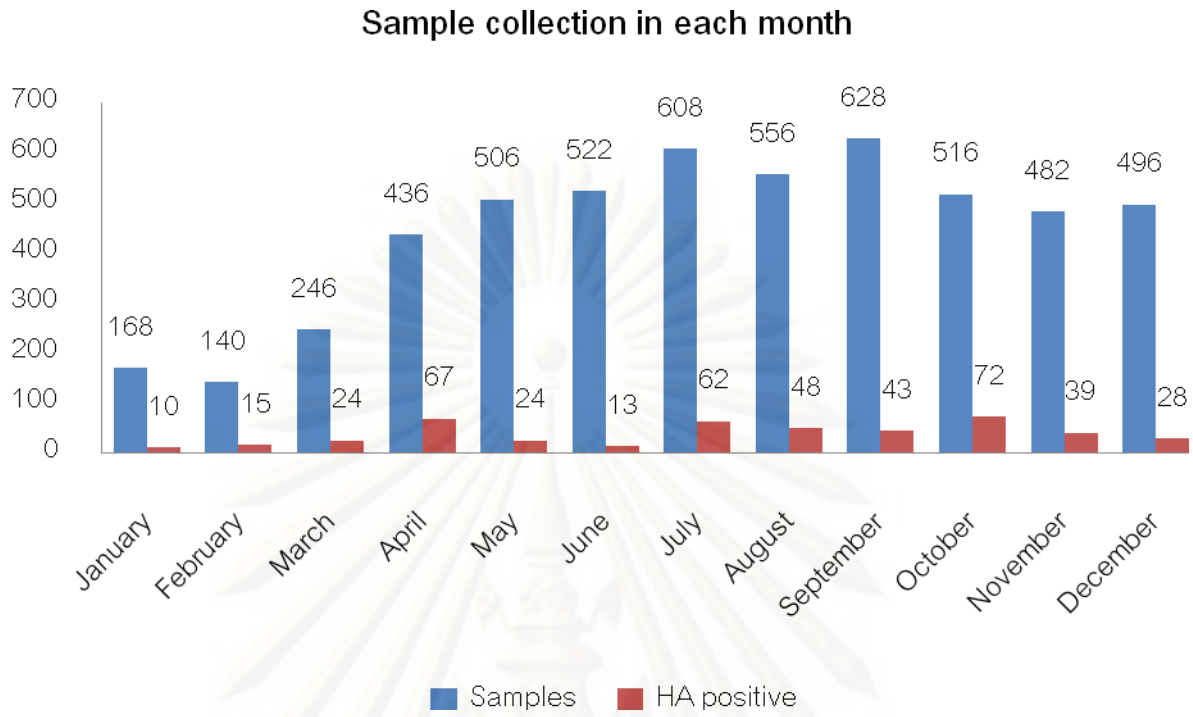


Table 5 : HA positive and influenza isolates of samples collected in each month

Months	Samples	HA positive (%)	Influenza A (%)
January	168	10 (5.95)	0
February	140	15 (10.71)	0
March	246	24 (9.76)	0
April	436	67 (15.37)	0
May	506	24 (4.74)	0
June	522	13 (2.49)	3 (0.57)
July	608	62 (10.20)	0
August	556	48 (8.63)	0
September	628	43 (6.85)	0
October	516	72 (13.95)	0
November	482	39 (8.09)	16 (3.32)
December	496	28 (5.65)	0
Total	5304	445 (8.38)	19 (0.36)

Note ; number in parentheses are percentage of samples in each month

Table 3 shows the total number of animals (n = 2,652) sampled in this study. Each animal was sampled by both oropharyngeal and cloacal swab which resulted in a total of 5,304 samples. The 2,652 animals (5,304 samples) were collected from chickens (layers and fighting cocks) 67.16 % (1781/2652), ducks 25.60 % (679/2652), quails 3.02 % (80/2652), doves 2.34 % (62/2652), pigeons 1.70% (45/2652) and geese 0.18 % (5/2652).

As shown in table 4, number of samples collected by province were 30.39% (1,612/ 5,304) in Bangkok, 12.37% (656/5,304) in Nakhonsawan, 12.11% (642/5,304) in Pichit, 28.58% (1,516/5,304) in Phitsanulok and 16.55% (878/5,304) in Sukhothai.

2. Influenza A virus isolation and identification

After virus isolation using embryonated eggs, all samples were subjected for HA test. The total HA positive samples were 8.38 % (445/5,304) (table 4 and 5). The highest percentage of HA positive samples by markets was market C2 of Nakhonsawan province (18.32%) (table 4). By month, the highest percentage of HA positive samples was in April (15.37%) (table 5). Out of 5,304 samples, 19 influenza A viruses were isolated from LBMs. It is noted that influenza A viruses were isolated from only one market (market A, Bangkok) in the month of June and November. The percentage of influenza A virus isolated from market A was 1.96 % (19/970) (table 4). The percentages of influenza A virus isolated by month were 0.57 % (3/552) in June and 3.32 % (16/482) in November (table 5).

The occurrence of Influenza A virus in this study is 0.36% by samples (19/5,304 samples) or 0.49% by animals (13/2,652 animals). We found that 6 out of 13 animals provided viral isolates in both oropharyngeal and cloacal swabs, therefore influenza A virus were isolated as 19 influenza A isolates from 13 animals (3 isolates from 2 animals in June and 16 isolates from 11 animals in November) (table 5). All 19 influenza A isolates from avian species were recovered from muscovy duck in the same LBM located in Bangkok (market A) as shown in Table 4. Analysis based on animal species, we found that the occurrence of the influenza A virus in muscovy duck in this study is

1.9 % (13/679) while other species were all negative. Analysis by months, the occurrence of the influenza A virus in market A in June and November were 5 % (2/40 animals) and 36.67 % (11/30 animals) respectively.

In this study, virus identification was performed by using PCR subtyping with specific primers (Tsukamoto et al., 2008; Tsukamoto et al., 2009). Three influenza A subtypes were identified as H4N6 (n=2) (figure 8 and 9), H4N9 (n=1) (figure 10) and H10N3 (n=16) (figure 11 and 12). Two subtypes (H4N6 and H4N9) were isolated from samples collected in June while H10N3 subtype was isolated in November (table 6). Twelve out of 19 influenza isolates were isolated from oropharyngeal swab while the others (n=6) were isolated from cloacal swab (table 6).

Figure 8 : Subtype identification by PCR with H1-15 subtyping primers of sample No. LM 1973. The sample was positive as H4 subtype (421 bp).

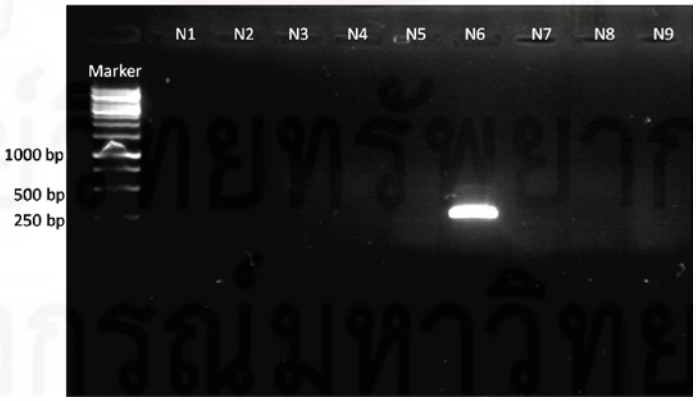


Figure 9 : Subtype identification by PCR with N1-9 subtyping primers of sample No. LM 1983. The sample was positive as N6 subtype (264 bp).

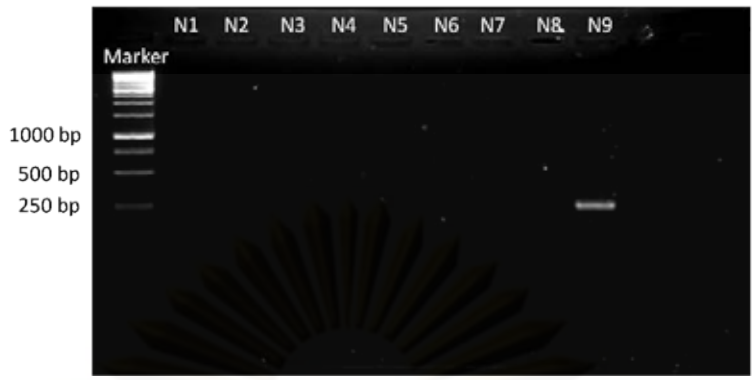


Figure 10 : Subtype identification by PCR with N1-9 subtyping primers of sample No. LM 1984. The sample was positive as N9 subtype (227 bp)

Figure 11 : Subtype identification by PCR with H1-15 subtyping primers of sample No. LM 4754. The sample was positive as H10 subtype (311 bp).

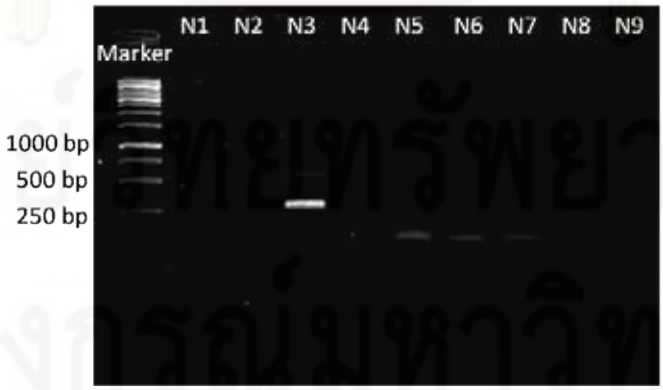


Figure 12 : Subtype identification by PCR with H1-15 subtyping primers of sample No. LM 4759. The sample was positive as N3 subtype (287 bp).

Table 6 : Description of Influenza A viruses isolated from this study

Sample No.	Month	Taxon name	Subtype
1973	June	A/Muscovy duck/Bangkok/Thailand/LM1973/2009	H4N6
1983[a]	June	A/Muscovy duck/Bangkok/Thailand/LM1983/2009	H4N6
1984[a]	June	A/Muscovy duck/Bangkok/Thailand/LM1984/2009	H4N9
4747	November	A/Muscovy duck/Bangkok/Thailand/LM4747/2009	H10N3
4752	November	A/Muscovy duck/Bangkok/Thailand/LM4752/2009	H10N3
4753[b]	November	A/Muscovy duck/Bangkok/Thailand/LM4753/2009	H10N3
4754[b]	November	A/Muscovy duck/Bangkok/Thailand/LM4754/2009	H10N3
4759	November	A/Muscovy duck/Bangkok/Thailand/LM4759/2009	H10N3
4761[c]	November	A/Muscovy duck/Bangkok/Thailand/LM4761/2009	H10N3
4762[c]	November	A/Muscovy duck/Bangkok/Thailand/LM4762/2009	H10N3
4763[d]	November	A/Muscovy duck/Bangkok/Thailand/LM4763/2009	H10N3
4764[d]	November	A/Muscovy duck/Bangkok/Thailand/LM4764/2009	H10N3
4767	November	A/Muscovy duck/Bangkok/Thailand/LM4767/2009	H10N3
4769[e]	November	A/Muscovy duck/Bangkok/Thailand/LM4769/2009	H10N3
4770[e]	November	A/Muscovy duck/Bangkok/Thailand/LM4770/2009	H10N3
4773[f]	November	A/Muscovy duck/Bangkok/Thailand/LM4773/2009	H10N3
4774[f]	November	A/Muscovy duck/Bangkok/Thailand/LM4774/2009	H10N3
4775	November	A/Muscovy duck/Bangkok/Thailand/LM4775/2009	H10N3
4777	November	A/Muscovy duck/Bangkok/Thailand/LM4777/2009	H10N3

* a, b, c, d, e and f are collected from same duck; odd numbers are oropharyngeal swab and even numbers are cloacal swab

3. Whole genome sequencing of influenza A virus

After subtype of influenza A virus was identified. Specific primers of HA and NA gene were designed while the other genes (M, NP, NS, PA ,PB1 and PB2) sequencing were performed by using primers that previously described (Hoffmann et al., 2001). In table 7, nucleotide position of influenza A virus genes obtained from whole genome sequencing were shown.

Table 7 : Nucleotide position of each gene of influenza A virus isolated in this study.

	Genes (position)							
	PB2	PB1	PA	HA	NP	NA	M	NS
LM 1973 (H4N6)	28-2266	17-2299	63-2157	1-1679	12-1507	1-1405	26-1007	27-843
LM 1983 (H4N6)	28-2238	21-2289	33-2157	1-1682	12-1507	1-1406	26-1007	27-845
LM 1984 (H4N9)	82-2239	29-2295	33-2157	1-1684	11-1507	25-1368	26-983	27-844
LM 4754 (H10N3)	70-2257	44-2271	46-2163	18-1667	39-1507	1-1400	21-984	14-842
LM 4759 (H10N3)	69-2257	44-2272	46-2163	16-1667	38-1507	13-1400	20-984	13-842
LM 4761 (H10N3)	69-2257	44-2268	46-2141	16-1667	38-1507	2-1400	19-985	18-842
LM 4775 (H10N3)	69-2257	44-2267	46-2163	16-1654	39-1507	2-1400	13-982	14-842

Note : Only 4 isolates of H10N3 were selected for whole genome sequencing. Number of position was compared by using position of influenza A genes previously described (Karasin et al., 2000a)

4. Genetic characterization of the influenza virus isolates

4.1 Genetic characterization H4N6 and H4N9 from duck

In HA gene, H4 gene of H4N6 (n=2) and H4N9 (n=1) from this study were compared with the H4 gene available in GenBank database. Phylogenetic analysis of the H4 gene showed that the H4 gene can be divided into 2 major lineages, Eurasian and North American lineages (figure 13). Our results showed that all 3 H4 influenza A isolates were grouped in the Eurasian lineage.

Amino acid of HA gene of H4 influenza A isolates were aligned and compared with other H4 isolates published in GenBank database by using Bioedit program. Regarding to previous study of H4 gene, amino acid at cleavage site in all three isolates are PEKASR which is similar to the most avian isolates in Eurasian lineage while the North American avian lineage including swine H4N6 isolates are PEKATR (table 8)(Karasin et al., 2000a). In addition, only the muskrat H4N6 isolate contains PEKAPR at cleavage site. Amino acid at receptor binding site at position 226 and 228 were Q and G respectively. These amino acids were similar to all H4N6 in both Eurasian and North American lineage. Only the swine H4N6 were L and S at position 226 and 228 respectively (Bateman et al., 2008). For N-linked glycosylation site, four sites in HA1 and one site in HA2 were observed (Donis et al., 1989). It is noted that one position (18-20) in HA1 of all three H4 influenza A isolates in this study was absent. At amino acid position 18 of HA1, change of amino acid from N to D was observed while the other positions still conserved.

When compare the nucleotide of H4 gene of all 3 H4 isolates in this study with the H4 gene in GenBank database using BLAST tools. The highest nucleotide similarity was A/duck/Taiwan/wb1104/2006(H4N6) at 97% in all three isolates and these viruses were grouped in the same cluster of Eurasian lineage (table 9-11).

Table 8 : Amino acid of significant location of H4 genes compared with representative strains from 2 major lineages.

	Taxon name	HA gene		
		Cleavage site	Receptor binding site	
			226	228
This study	A/Muscovy duck/ Bangkok/Thailand/LM1973/2009 (H4N6)	PEKASR	Q	G
	A/Muscovy duck/ Bangkok/Thailand/LM1983/2009 (H4N6)	PEKASR	Q	G
	A/Muscovy duck/ Bangkok/Thailand/LM1984/2009 (H4N6)	PEKASR	Q	G
North American lineage	A/ruddy turnstone/New Jersey/47/1985 (H4N6)	PEKATR	Q	G
	A/mallard/Alberta/49/1995(H4N6)	PEKATR	Q	G
	A/swine/Ontario/01911-1/1999 (H4N6)	PEKATR	L	S
Eurasian lineage	A/duck/Czech republic/1/1956 (H4N6)	PEKASR	Q	G
	A/muskrat/Buryatiya/1944/2000 (H4N6)	PEKASR	Q	G
	A/mallard/Yan Chen/2005 (H4N6)	PEKASR	Q	G

In NA gene, two isolates were subtyped as N6 (LM 1973 and LM 1983) while only LM 1984 isolate was N9. Interestingly, the LM 1983 and LM 1984 were isolated from the same duck.

Cluster analysis was conducted by using phylogenetic tree analysis. In phylogenetic trees of N6 (figure 13) and N9 (figure 14) influenza A isolates, both N6 and N9 genes were grouped and clustered into Eurasian lineage. Because the H4N9 influenza A virus in GenBank database was present only the viruses from North American lineage, therefore, the phylogenetic tree of N9 gene was conducted with N9 from other HA subtype but the result of N9 gene are still conserved in each lineage.

Nucleotide identity of both N6 and N9 genes were demonstrated using BLAST tools. The highest nucleotide similarity of all three isolates was the isolate from Eurasian lineage. In N6, A/duck/Eastern China/01/2007(H4N6) was the highest similarity at 97% to both N6 isolates (table 9-10), while A/duck/Mongolia/119/2008(H7N9) was highest similarity at 97% to N9 isolate (table 11). This result may suggest that N9 gene were not derived from H4N9 isolate but was a result from reassortment of HxN9 and H4Nx.

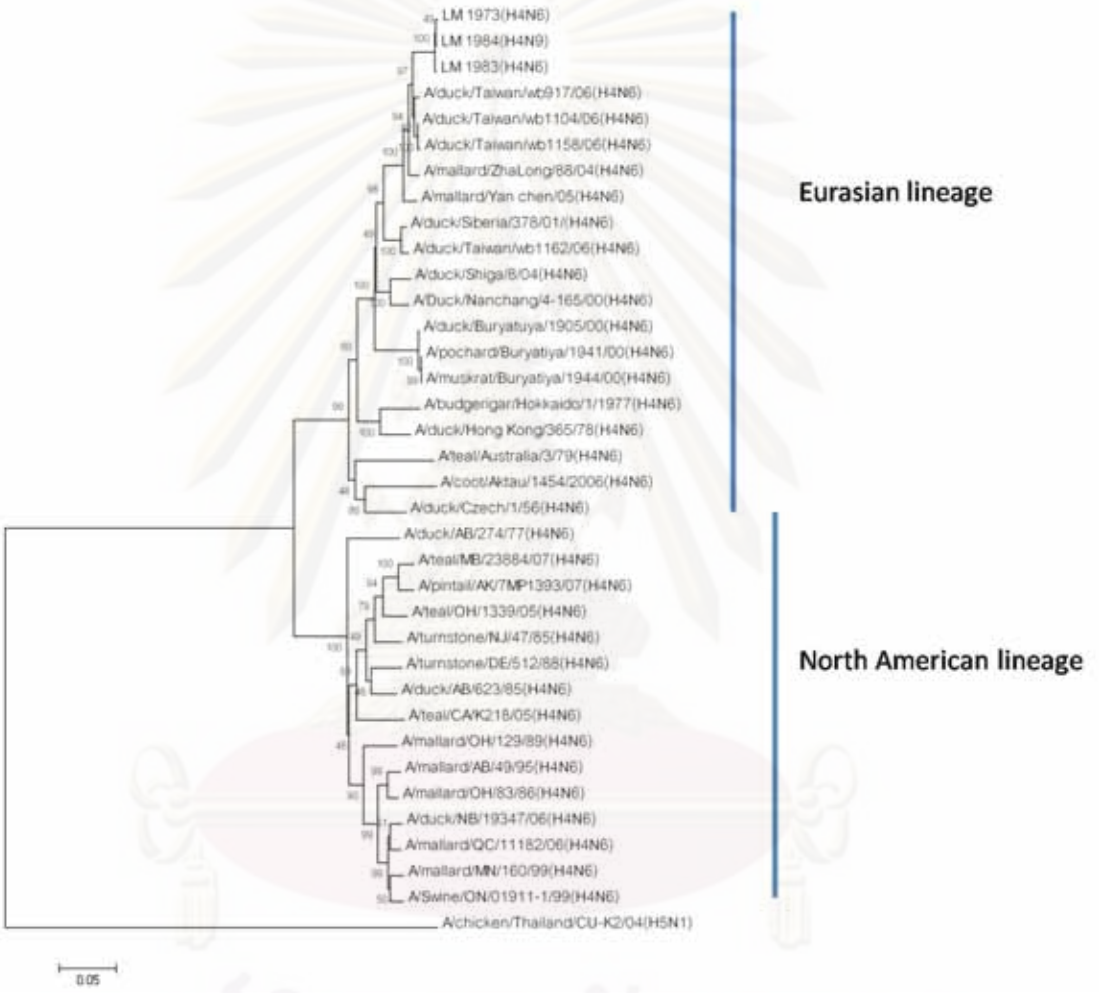


Figure 13 : Phylogenetic analysis of H4 gene compare with 2 major distinct lineages.

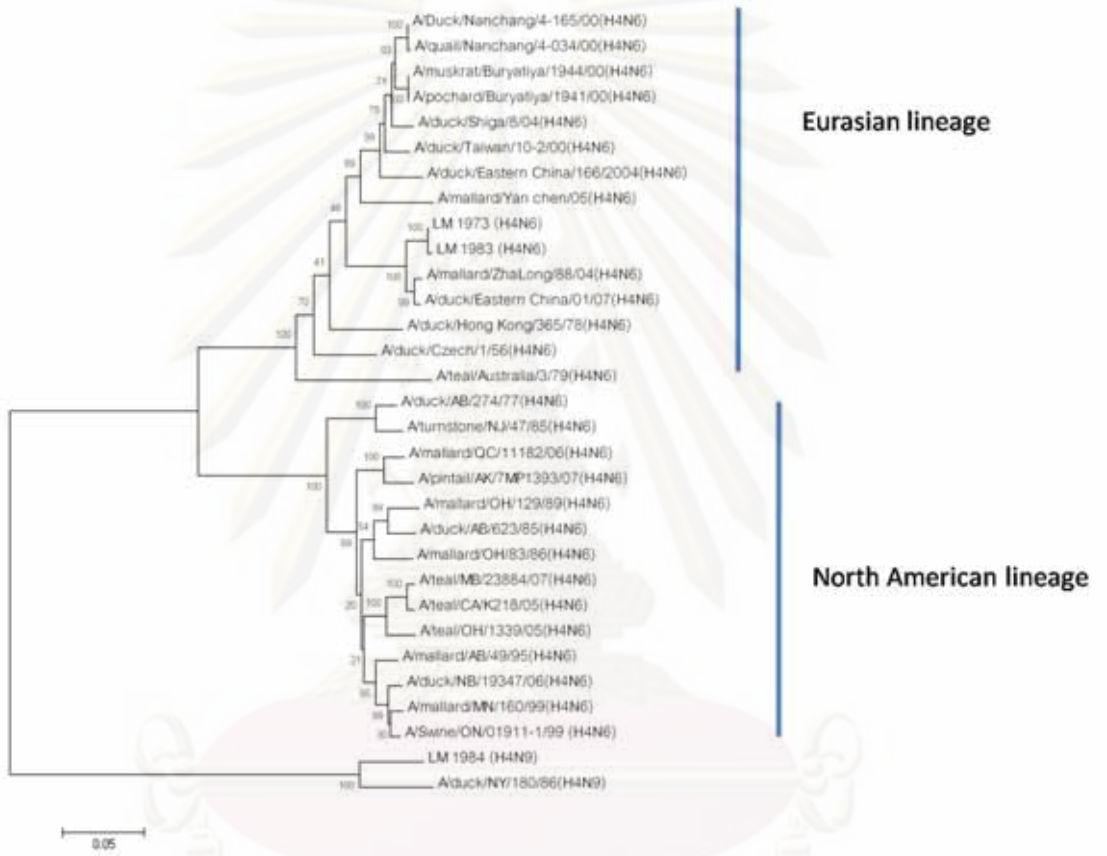


Figure 14 : Phylogenetic analysis of N6 gene compare with 2 major distinct lineages.

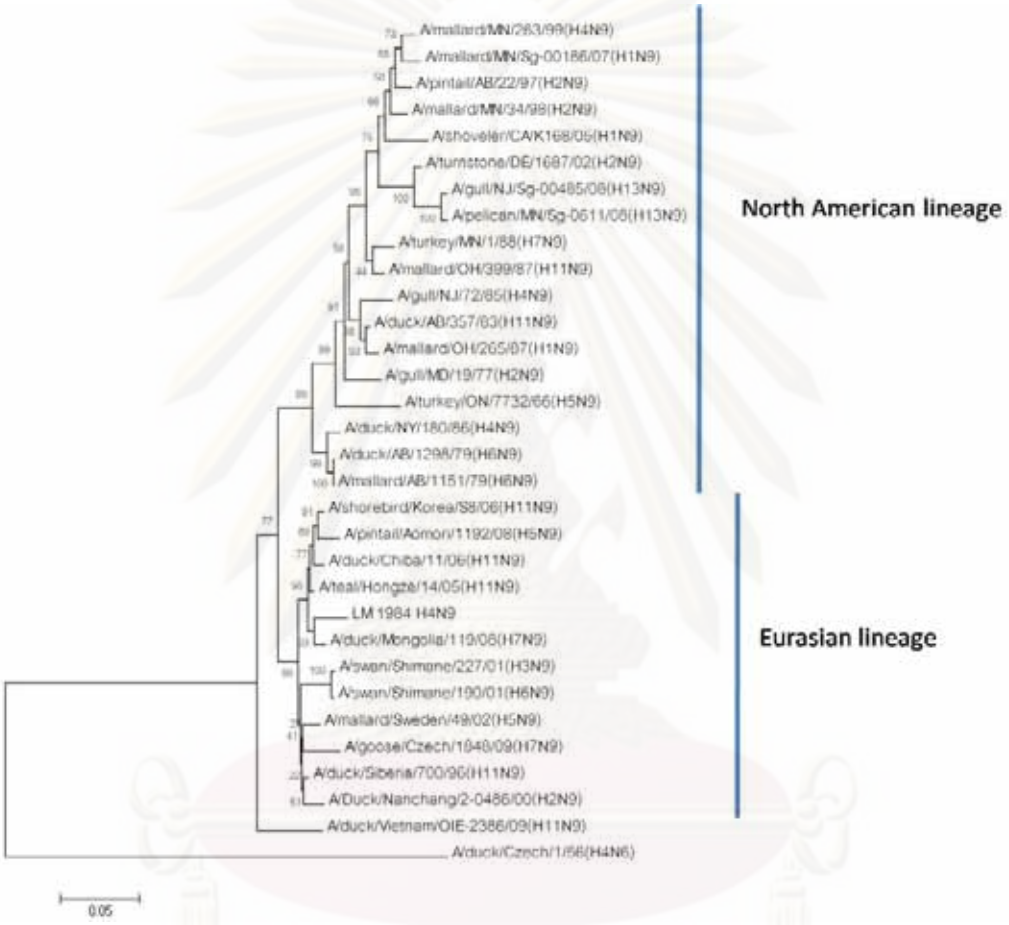


Figure 15 : Phylogenetic analysis of N9 gene compare with 2 major distinct lineages.

Table 9 : Sequence homology of each gene from LM 1973 (H4N6) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	28-2266	AB355926.1	A/duck/Hokkaido/Vac-3/2007(H5N1)	98%
PB1	17-2299	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	98%
PA	63-2157	GQ325646.1	A/environment/Dongting Lake/Hunan/ 3-9/2007(H10N8)	99%
HA	1-1679	GU066565.1	A/duck/Taiwan/wb1104/2006(H4N6)	97%
NP	12-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	97%
NA	1-1405	EU429790.1	A/duck/Eastern China/01/2007(H4N6)	97%
M	26-1007	GU052229.1	A/gull/Astrakhan/1846/1998(H13N6)	99%
NS	27-843	EU599315.1	A/teal/Egypt/912908/2005(H10N7)	98%

Table 10 : Sequence homology of each gene from LM 1983 (H4N6) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	28-2238	EF597476.1	A/migratory duck/Hong Kong/MP206/2004(H5N2)	97%
PB1	21-2289	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	98%
PA	33-2157	GQ325646.1	A/environment/Dongting Lake/Hunan/ 3-9/2007(H10N8)	98%
HA	1-1682	GU066565.1	A/duck/Taiwan/wb1104/2006(H4N6)	97%
NP	12-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	98%
NA	1-1406	EU429790.1	A/duck/Eastern China/01/2007(H4N6)	97%
M	26-1007	GU052229.1	A/gull/Astrakhan/1846/1998(H13N6)	99%
NS	27-845	EU599315.1	A/teal/Egypt/912908/2005(H10N7)	99%

Table 11 : Sequence homology of each gene from LM 1984 (H4N9) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	82-2239	EF597476.1	A/migratory duck/Hong Kong/MP206/2004(H5N2)	97%
PB1	29-2295	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	98%
PA	33-2157	GQ325646.1	A/environment/Dongting Lake/Hunan/ 3-9/2007(H10N8)	99%
HA	1-1684	GU066565.1	A/duck/Taiwan/wb1104/2006(H4N6)	97%
NP	11-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	98%
NA	25-1368	AB481213.1	A/duck/Mongolia/119/2008(H7N9)	97%
M	26-983	GU052229.1	A/gull/Astrakhan/1846/1998(H13N6)	99%
NS	27-844	EU599315.1	A/teal/Egypt/912908/2005(H10N7)	99%

4.2 Genetic characterization H10N3 from duck

Out of 16 isolates, 4 isolates were chosen for genetic analysis. In HA gene, phylogenetic tree of H10N3 was conducted by using MEGA 4.1 program with other H10 subtypes because H10N3 in GenBank database was not enough to construct a phylogenetic tree. All H10 isolates from this study were grouped in the Eurasian lineage which was clearly distinct from the North American lineage (figure 16). Amino acid alignment of all H10 isolates in this study was conducted with Bioedit program. The cleavage site of H10 influenza A isolates was compared and resulted in high genetic diversity among isolates as shown in table 12. Amino acids at receptor binding site of all H10 isolates were Q226 and G228 (table 12). These results were conserved and were similar to all other H10 selected in the phylogenetic tree. For N-linked glycosylation sites in H10 gene, there were four sites in HA1 and two sites in HA2 domain described previously (Feldmann et al., 1988). When compare the nucleotide identity with other H10 isolates in GenBank database. The highest nucleotide similarity of all H10 isolates was A/duck/Hokkaido/W87/2007 (H10N2) at 97% and was grouped in the same cluster of Eurasian avian lineage (table 13-16).

In NA gene, phylogenetic tree was constructed with other N3 isolates from GenBank database. All N3 influenza A genes isolated in this study were grouped in Eurasian lineage (figure 17). The nucleotide identity of all N3 influenza A isolates was compared with other N3 isolates in GenBank database with BLAST tools. The highest nucleotide similarity of all N3 isolates was A/mallard/Italy/43/01(H7N3) at 97% (table 13-16). Amino acids of N3 gene of influenza A isolates were aligned by using Bioedit program. In this study, Twenty-three amino acids deletion at position 56-78 of N3 gene was not observed in all N3 isolates and all receptor binding sites of N3 gene (position 14, 57, 66, 72 ,146 and 308) were conserved.

Table 12 : Amino acid of significant location of H10 genes compared with representative strains from 2 major lineages..

	Taxon name	HA gene		
		Cleavage site	Receptor binding site	
			226	228
Isolates in this study	A/Muscovy duck/ Bangkok/Thailand/LM4754/2009	PEIIQGR	Q	G
	A/Muscovy duck/ Bangkok/Thailand/LM4759/2009	PEIIQGR	Q	G
	A/Muscovy duck/ Bangkok/Thailand/LM4761/2009	PEIIQGR	Q	G
	A/Muscovy duck/ Bangkok/Thailand/LM4775/2009	PEIIQGR	Q	G
North American lineage	A/blue-winged teal/ Alberta/778/1978 (H10N3)	PEIIQER	Q	G
	A/northern shoveler/ California/JN587/2006 (H10N3)	PEVVQGR	Q	G
	A/mallard/ Minnesota/Sg-00194/2007 (H10N3)	PEVVQGR	Q	G
Eurasian lineage	A/chicken/Germany/N/1949 (H10N7)	PEVVQGR	Q	G
	A/duck/Hong Kong/786/1979 (H10N3)	PEIMQGR	Q	G
	A/duck/Hokkaido/24/2004 (H10N5)	PEIMQGR	Q	G

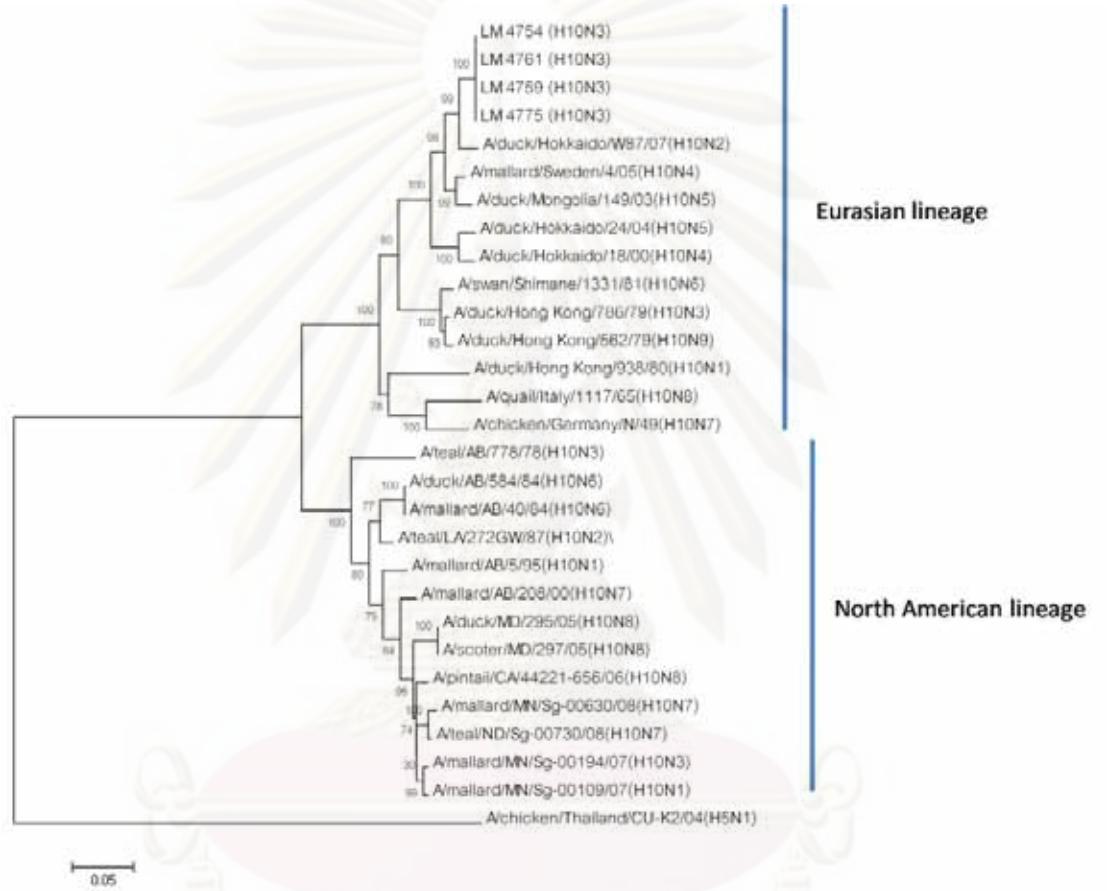


Figure 16 : Phylogenetic analysis of H10 gene compare with 2 major distinct lineages.

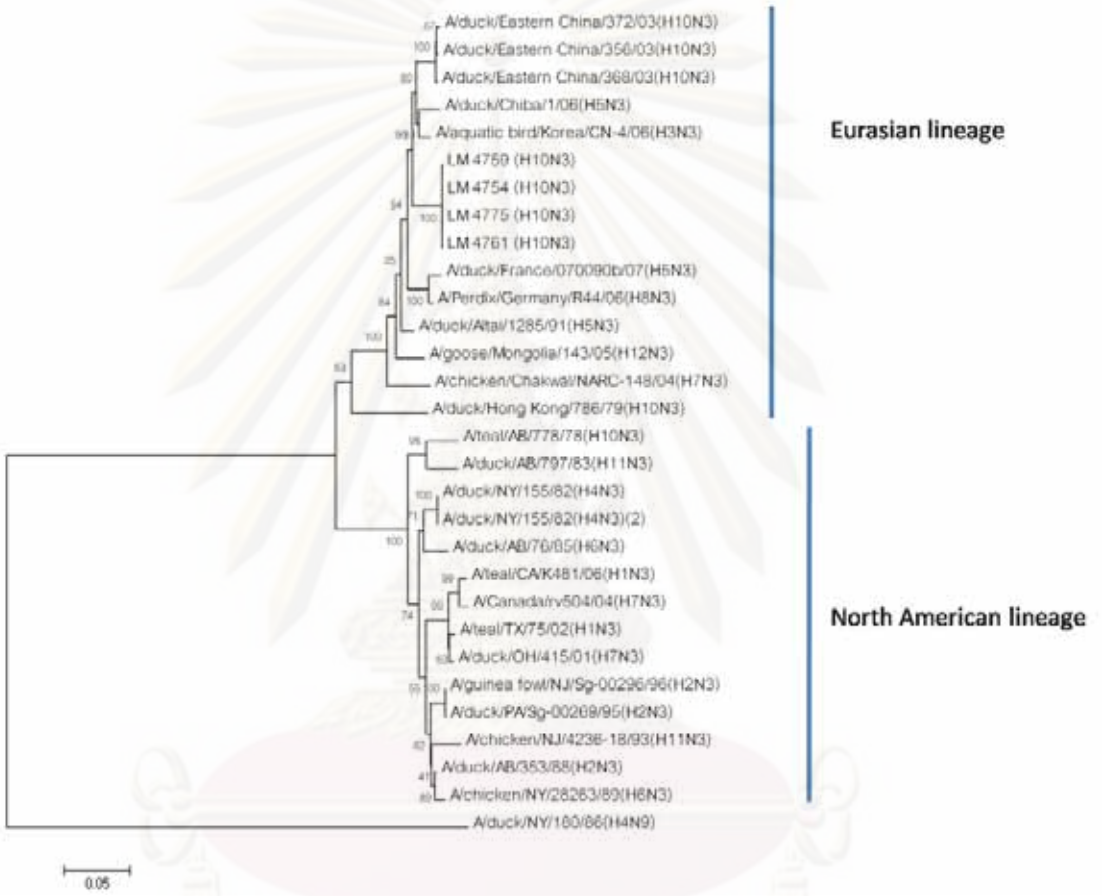


Figure 17 : Phylogenetic analysis of N3 gene compare with 2 major distinct lineages.

Table 13 : Sequence homology of each gene from LM 4754 (H10N3) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	70-2257	EF597476.1	A/migratory duck/Hong Kong/MP206/2004(H5N2)	97%
PB1	44-2271	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	97%
PA	46-2163	EF597421.1	A/duck/Jiang Xi/1850/2005(H5N2)	97%
HA	18-1667	AB450443.1	A/duck/Hokkaido/W87/2007(H10N2)	97%
NP	39-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	98%
NA	1-1400	AY586416.1	A/mallard/Italy/43/01(H7N3)	97%
M	21-984	FJ959087.1	A/mallard/Korea/GH171/2007(H7N7)	99%
NS	14-842	GQ227609.1	A/duck/Primorie/2633/2001(H5N3)	98%

Table 14 : Sequence homology of each gene from LM 4759 (H10N3) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	69-2257	EF597476.1	A/migratory duck/Hong Kong/MP206/2004(H5N2)	97%
PB1	44-2272	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	97%
PA	46-2163	EF597421.1	A/duck/Jiang Xi/1850/2005(H5N2)	97%
HA	16-1667	AB450443.1	A/duck/Hokkaido/W87/2007(H10N2)	97%
NP	38-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	98%
NA	13-1400	AY586416.1	A/mallard/Italy/43/01(H7N3)	97%
M	20-984	FJ959087.1	A/mallard/Korea/GH171/2007(H7N7)	99%
NS	13-842	GQ227609.1	A/duck/Primorie/2633/2001(H5N3)	98%

Table 15 : Sequence homology of each gene from LM 4761 (H10N3) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	69-2257	EF597476.1	A/migratory duck/Hong Kong/MP206/2004(H5N2)	97%
PB1	44-2268	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	97%
PA	46-2141	EF597421.1	A/duck/Jiang Xi/1850/2005(H5N2)	97%
HA	16-1667	AB450443.1	A/duck/Hokkaido/W87/2007(H10N2)	97%
NP	38-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	98%
NA	2-1400	AY586416.1	A/mallard/Italy/43/01(H7N3)	97%
M	19-985	FJ959087.1	A/mallard/Korea/GH171/2007(H7N7)	99%
NS	18-842	GQ227609.1	A/duck/Primorie/2633/2001(H5N3)	98%

Table 16 : Sequence homology of each gene from LM 4775 (H10N3) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	69-2257	EF597476.1	A/migratory duck/Hong Kong/MP206/2004(H5N2)	97%
PB1	44-2267	AB530990.1	A/mallard/Hokkaido/24/2009(H5N1)	97%
PA	46-2163	EF597421.1	A/duck/Jiang Xi/1850/2005(H5N2)	97%
HA	16-1654	AB450443.1	A/duck/Hokkaido/W87/2007(H10N2))	97%
NP	39-1507	FJ802402.1	A/duck/Thailand/AY-354/2008(H3N2)	98%
NA	2-1400	AY586416.1	A/mallard/Italy/43/01(H7N3)	97%
M	13-982	FJ959087.1	A/mallard/Korea/GH171/2007(H7N7)	99%
NS	14-842	GQ227609.1	A/duck/Primorie/2633/2001(H5N3)	98%

4.3 Internal gene of all influenza A isolates in this study

In all seven isolates chosen for genetic analysis, the highest nucleotide similarity of NP and PB1 of all isolates were A/duck/Thailand/AY-354/2008(H3N2) and A/mallard/Hokkaido/24/2009(H5N1) respectively, while the other genes (PA, M and NS) were similar in each month of sample collection. The highest nucleotide similarity of PA, M and NS genes isolated in June were different from genes isolated in November. Only PB2 gene of LM 1973 isolate that collected in June was different from other isolates in the same month. In LM 1973, the highest nucleotide similarity was A/duck/ Hokkaido/Vac-3/2007(H5N1) at 97% while the other 6 isolates were A/migratory duck/Hong Kong/MP206/2004(H5N2) at 97%. But when compare deduced amino acids of PB2 gene, the similarity between LM 1973 and other isolates was ranked at 98.9 - 99.2 % while the nucleotide similarity were ranked at 87.6 - 87.7%. (Appendix B)

The comparison of the amino acids of each gene of the viruses in this study was conducted and resulted in 97.1-100% similarity at NS gene while the other genes were 98.7-100% similarity (Appendix B). This result showed that some of the internal genes (NP and PB1) of the viruses in this study were highly conserved although the viruses were isolated by 5 months interval.

CHAPTER V

DISCUSSION

1. Occurrence of influenza A virus in LBMs in Thailand

The occurrence of influenza A virus surveyed in LBMs during January 2009 to December 2009 was 0.36% (19/5304). The occurrence of influenza A virus in the study was lower than those in previous study of influenza A virus H5N1 in LBMs during 2006-2007 which was 1.3% (Amonsin et al., 2008). The previous study was aimed to identify only HPAI H5N1 during the period of HPAI H5N1 outbreak so that the true occurrence of influenza A virus both HPAI and LPAI was still unknown. In the 2009 LBMs surveillance program, no HPAI H5N1 outbreak was reported, this resulted in HPAI H5N1 was not isolated from LBMs during the surveillance year 2009. In this study, we were able to identify 3 subtypes of LPAI including, H4N6, H4N9 and H10N3, however the status of LPAI outbreak in Thailand during 2009 was unknown. It is noted that H10N3 infected animals (11/30) were identified from only one market in Bangkok in November. This high occurrence of LPAI in ducks in one sampling day may suggest that LPAI outbreak may occur. However the origin of the ducks could not be traced back. In addition, the sources of H10N3 viruses could not be concluded that they either from the same duck farm or the results of infection at LBMs. The LPAI subtypes in LBMs in this study may or may not represent the LPAI status in other parts of Thailand but this data provide the information of the LPAI subtypes circulating in Thailand.

When we compared with the other study of influenza A virus in LBMs, many differences were observed. In Nanchang, China, sixteen months study during 2000-2001, 1% of influenza A virus was isolated in LBMs (Liu et al., 2003). In total, 7 subtypes of influenza A virus were isolated from six avian species in 12 out of 16 months during 2000-2001 surveillance program. Comparing to this study, only three subtypes of

influenza A virus were isolated from one avian species (Muscovy ducks) in June and November. The LBMs in Nanchang sold many avian species including aquatic birds (geese and wild waterfowls), while the LBMs in Thailand mostly sold chickens and ducks. Only market B in LBMs in Thailand that sold more than three species. This difference in species sold in LBMs between these two studies may be an important reason in the subtypes diversity and number of subtypes isolated in LBMs.

All influenza A viruses in this study were collected from Muscovy ducks at 1.9% (13/679) without any clinical signs observed on the collecting time. While the all other avian species were negative in influenza A virus. It has been known that ducks and other aquatic birds are the reservoirs of influenza A virus which almost all HA subtypes and all NA subtypes can be isolated from wild ducks without any observed clinical signs (Webster et al., 1992). The presence of the ducks in LBMs significantly increases the diversity of influenza A viruses but does not impact the virus prevalence (Liu et al., 2003). In this study, five markets house and sell ducks, however, only in market A where 3 subtypes of influenza A virus were isolated from ducks.

Influenza A viruses are easily isolated from juvenile ducks more than mature ducks (Webster et al., 1992). In this study, no influenza A virus isolated from juvenile ducks. All influenza viruses were isolated from mature ducks from only one market (market A). Samples from juvenile ducks were collected from one pet bird market (market B) contains, while the samples from other markets were from mature ducks which are sold as a food in terms of poultry meat.

2. Genetic characterization of influenza A viruses isolated from LBMs

2.1 HA gene of H4 and H10 subtypes isolated from LBMs

Geographic distribution of influenza A virus was influenced by flyways and migratory routes of wild waterfowls and migratory birds which are known as reservoir of influenza A virus (Donis et al., 1989). It was noted that most of the H4 and H10 isolates

published in GenBank database was isolated from wild ducks. Result from phylogenetic analysis of HA gene of both H4 and H10 viruses from LBMs showed that the HA gene were grouped in the Eurasian avian lineage which explained that H4 and H10 isolated from this study were distributed and influenced by the flyway of migratory birds.

In all influenza A isolates, amino acids at cleavage site were compare by using positions previously described (Nobusawa et al., 1991). Amino acids at cleavage site of H4 viruses were PEKASR which were similar to Eurasian avian lineage while the North American avian lineage was PEKATR. In all H10 isolates, the cleavage site was PEIIQGR. However in other H10 isolates from GenBank, many changes of the cleavage site of H10 isolates were observed such as PEIMQGR or PEVVQGR. One study proved that the difference of pathogenicity of the H10 influenza A virus may not be determined by the changes in the cleavage site (Wood et al., 1996). After the intravenous pathogenicity index (IVPI) test had been conducted with influenza A virus H10N5 A/mandarin duck/Singapore/805/F-72/7/1993 that possessed the same amino acid sequence at cleavage site (PEIMQGR) as A/chicken/England/378/1985 (H10N4), highly pathogenicity was considered in A/mandarin duck/Singapore/805/F-72/7/1993 while A/chicken/England/378/1985 was low pathogenicity (Wood et al., 1996). From these results, it can be indicated that the high genetic diversity and different in pathogenicity were observed in influenza A virus subtype H10. Therefore, further investigation needs to be done in order to describe the ability and pathogenicity of the H10N3 influenza A virus isolated from this study.

Receptor binding site of H4 and H10 isolates contains amino acids representing the preferential binding to avian receptor characteristics (Nobusawa et al., 1991). The amino acids at position 226 and 228 of both H4 and H10 were Q226 and G228 respectively. In Swine, influenza A subtype H4N6 was isolated in Ontario, Canada, 2000

(Karasin et al., 2000b). The H4 gene contains amino acid changes at position 226 (Q226L) and 228 (G228S) (Bateman et al., 2008). Further study of the virulence of this swine H4 virus showed that the virus has a higher affinity of infection in SA2,6gal receptors which were dominant in human and other mammalian hosts than avian H4 Q226 and G228 (Bateman et al., 2008). Therefore, the ability of H4 and H10 viruses in this study to infect mammalian hosts may be less important.

In H10 isolates, all N-linked glycosylation sites were conserved in all positions compared with the previous study (Feldmann et al., 1988). While in H4 isolates, from four N-linked glycosylation site in HA1 described in previous study (Donis et al., 1989), we found that one glycosylation site in all three isolates is absent (amino acid position 18-20 of HA1). This finding could indicate that the mutation at the N-linked glycosylation site may affect the function of the protein of virus. The main functions of N-linked glycosylation site are promotion of proper folding, modulation of biological activities, protection against denaturation including proteolysis and maintenance of protein conformation and stability (Roberts et al., 1993). The study of site-directed mutagenesis by induced elimination of one N-linked glycosylation site showed a significant reduction of the rate of transport, increasing the temperature sensitivity and affection of the polypeptide folding step (Roberts et al., 1993). However, the position of the absent N-linked glycosylation site gave a different results (Roberts et al., 1993), therefore, in these H4 isolates, further study need to be done in order to explain the ability of virus with the absent of the N-linked glycosylation site at position 18.

2.2 NA genes of N3, N6 and N9 subtypes isolated from LBMs

In one muscovy duck, two subtypes of influenza A were isolated. H4N6 subtype was isolated from oropharyngeal swab while H4N9 was isolated from cloacal swab. This

result indicated that the reassortment of influenza A virus may occurred in this duck and resulted in reassortant of NA gene. In previous study, reassortment of two avian influenza isolates were observed in cloacal samples of feral ducks after inoculated with two virus subtypes. Because the influenza virus can replicate in the intestinal tract of the ducks and shred the virus in the feces after 30 days post inoculation (Hinshaw et al., 1980) and in order to reassort the virus, multiple subtypes of avian influenza A infection in the same host must occurred (Sharp et al., 1997). In this study, there might be more than one influenza subtypes (H4N? and H?N9) replicated in the intestinal tract of the duck at the time of collection, therefore, the viruses were reassorted and resulted in the new influenza A virus (H4N9) while the tracheal sample possessed only one avian influenza subtype (H4N6).

The 23 amino acids deletion of NA gene is observed in N1, N2 and N3 genes isolated from terrestrial or raised aquatic birds but usually absent in wild avian species (Campitelli et al., 2004). In this study, this amino acid deletion was not observed which is similar to other isolates from GenBank database. However, the muscovy ducks that possessed the H10N3 influenza A virus could not be traced back to the origin, therefore, we do not know whether these ducks were raised with or without contact from wild avian species.

3. Important information of influenza A viruses in LBMs

Although the time of infection of influenza A virus isolated in this study cannot be identified, there are two main reasons that can reflect the important of LBMs in influenza A virus widespread and evolution. First, in June, two subtypes were isolated from the same duck at the same collecting time. However, we could not identify that the duck infected with these two subtypes before or after entering LBMs. If the infection occurs

after entering LBMs, it will be a good evidence that reassortment of influenza A virus can occurs in LBMs. But if the infection occurs before entering LBMs, it will prove that multiple influenza A subtypes entering to LBMs at the same period of time and this will create a risk of reassortment of influenza A virus in LBMs. Second, in November, from 30 animals sampled in this month, 11 animals were positive with influenza A virus. If the infection occurs after entering LBMs, this will prove that LBMs are suitable environments for transmission and widespread of influenza A virus. But if the infection occurs before entering LBMs, it will prove the LPAI outbreaks. In addition, it will prove that high rate of infected animals are entering LBMs and may transmit to other animals in LBMs. Based on these two reasons, LBMs can play an important role in widespread and evolution of influenza A virus.

LBMs provide suitable environment for viral reassortment and possible generating a new influenza A virus. This may be due to the nature of LBMs settings (stack cages, dense population, different sources, various ages and species and mixing of the birds) (Campitelli et al., 2004; Cardona et al., 2009; Panigrahy et al., 2002; Yee et al., 2009) as well as the circulating of multiple subtypes of avian influenza A virus in the markets (Choi et al., 2005; Liu et al., 2003; Nguyen et al., 2005; Panigrahy et al., 2002). The vendors in the markets always mixed the birds from different sources together in the same wire stack cage. Therefore, if influenza A infected birds entering to LBM and mixing with other birds, it may lead to viral transmission and potential to viral reassortment. In the situation of this study that H4N6 and H4N9 were isolated from the same host, the host (Muscovy duck) might be infected with H?N9 prior or after coming in LBM and still replicated in an intestinal tract at the time of infection with H4N?. Then it resulted in a new reassorted influenza A virus H4N9.

However, previous studies showed that immunity of the animals can protect them from second influenza A infection. After H9N2 influenza A virus was inoculated in chickens for 5 days, chickens were challenged with HPAI H5N1 and resulted in 100% survival rate but all chickens still shed the H5N1 virus (Khalenkov et al., 2009). Another experimental study is the result of vaccination with H5N2 against H5N1 influenza A virus which resulted in 100% survival rate and no virus was isolated from vaccinated chickens in this study (Poetri et al., 2009). Because the serum profiles of the animal in this study were not performed in order not to reduce the quality of poultry meat. We cannot determine the immunity status of the ducks in this study. Therefore, the duck with H4N6 and H4N9 might be infected with multiple subtypes nearly the same time so that the immunity response of the duck is not developed and the virus is not eliminated by the immunity response from the host itself. This period with the lack of protection from another subtype might lead to mix infection which might resulted in genetic reassortment of the virus.

The new reassorted influenza A virus occurred in LBMs might be a potential risk to human and birds. Because the new reassorted virus were shredded in feces, the wire stack cages in LBM provide an appropriate condition to tranmit the new reassorted virus to other birds living in the cage below (Yee et al., 2009). Consequently, the small outbreak of a new reassorted influenza A virus in the birds in LBM might occur. If the new reassorted virus circulating in LBMs has high virulence or ability to infect human, it will be a threat to human nearby, vendors, buyers or people who passing by. One study in China, a patient infected by HPAI H5N1 had visited many LBMs before he showed the flu-like clinical signs (Wang et al., 2006). This study showed the evidence that LBMs can be a potential source of an influenza A in a local community and the infection of an influenza A virus in human can occurs not only by direct but also by indirect contact.

Based on this information, providing the knowledge to public will be a good public awareness in understanding and controlling of influenza A virus. Veterinarians, officers or epidemiologists who take control in this field should create a plan for control of influenza A virus such as creating a rest day as an intervention of the LBMs system because it can significantly reduce the occurrence of the virus in LBMs (Lau et al., 2007). Disinfection of fomites on rest day of LBMs should be performed as much as possible because influenza A virus is usually presented in fomites (Boone and Gerba, 2005).Vendors should be informed to understand how to prevent themselves and customers from the infection of influenza A virus while the customers should be informed to cook and not to feed on other animals with raw meat like the outbreak of HPAI H5N1 in tigers in Thailand, 2004 which resulted in a high economic losses and threat to one of the most endanger species in the world (Keawcharoen et al., 2004).

Surveillance of influenza A virus in LBMs can serves as a good early warning system (Webster, 2004). Subtypes of influenza A virus isolated in this LBMs can reflect the influenza A virus that circulating in Thailand. The continuing surveillance of influenza A virus in LBMs should be performed in order to explain the influenza virus evolution in Thailand, thus, understanding the potential of LBMs in an evolution of influenza A virus in Thailand. With this knowledge, prevention and protection strategies of influenza A virus outbreak in avian species and in human can be prepared with a better understanding.

Conclusions and suggestions

In this study, we focused on the occurrence of the influenza A virus from avian species in LBMs in Thailand. Two areas were selected- Bangkok and Central part of Thailand. Bangkok is the most crowded area in Thailand while the Central part of Thailand is the temporary habitat of migratory birds in Thailand. LBMs in many studies showed that the occurrence and diversity of influenza A virus subtype are important for an outbreak or transmission to human. Our study showed

1. Animals especially ducks in LBMs can be infected with influenza A virus. In this study we cannot explain that the animals in LBMs infected with influenza A virus before or after entering to LBMs. However, to prevent the outbreak or infection from this influenza A virus, this information should be provided to vendors, customers and veterinarians for better understanding and to prevent public from influenza A virus.

2. Even though influenza A virus were not isolated from other LBMs in this study. Survey of the influenza A virus in these LBMs should be performed in order to monitor these potential risk areas.

3.Genetic reassortment might occurs in LBMs and might be a risk to public health. If the reassortant virus is a highly pathogenic or human pandemic strain, it will create a higher or more danger than the mother strains. Intervention or strategy to control the reassortment process should be prepared and provided to the vendors to protect themselves and public against this virus.

4. Three subtypes isolated in this study had never been reported in Thailand before. Therefore, study and investigation of these subtypes isolated should be done to further to explain the pathogenicity and ability of viruses for better understanding of influenza A virus circulating in Thailand.

References

Alexander, D.J. 2007. An overview of the epidemiology of avian influenza. Vaccine. 25(30): 5637-5644.

Amonsin, A., Choatrakol, C., Lapkuntod, J., Tantilertcharoen, R., Thanawongnuwech, R., Suradhat, S., Suwannakarn, K., Theamboonlers, A. and Poovorawan, Y. 2008. Influenza virus (H5N1) in live bird markets and food markets, Thailand. Emerg Infect Dis. 14(11): 1739-1742.

Bateman, A.C., Busch, M.G., Karasin, A.I., Bovin, N. and Olsen, C.W. 2008. Amino acid 226 in the hemagglutinin of H4N6 influenza virus determines binding affinity for alpha2,6-linked sialic acid and infectivity levels in primary swine and human respiratory epithelial cells. J Virol. 82(16): 8204-8209.

Boone, S.A. and Gerba, C.P. 2005. The occurrence of influenza A virus on household and day care center fomites. J Infect. 51(2): 103-109.

Campitelli, L., Mogavero, E., De Marco, M.A., Delogu, M., Puzelli, S., Frezza, F., Facchini, M., Chiapponi, C., Foni, E., Cordioli, P., Webby, R., Barigazzi, G., Webster, R.G. and Donatelli, I. 2004. Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. Virology. 323(1): 24-36.

Cardona, C., Yee, K. and Carpenter, T. 2009. Are live bird markets reservoirs of avian influenza? Poult Sci. 88(4): 856-859.

Choi, Y.K., Seo, S.H., Kim, J.A., Webby, R.J. and Webster, R.G. 2005. Avian influenza viruses in Korean live poultry markets and their pathogenic potential. Virology. 332(2): 529-537.

De Jong, J.C., Rimmelzwaan, G.F., Fouchier, R.A. and Osterhaus, A.D. 2000. Influenza virus: a master of metamorphosis. J Infect. 40(3): 218-228.

Donis, R.O., Bean, W.J., Kawaoka, Y. and Webster, R.G. 1989. Distinct lineages of influenza virus H4 hemagglutinin genes in different regions of the world. Virology. 169(2): 408-417.

Feldmann, H., Kretzschmar, E., Klingeborn, B., Rott, R., Klenk, H.D. and Garten, W. 1988. The structure of serotype H10 hemagglutinin of influenza A virus: comparison of an apathogenic avian and a mammalian strain pathogenic for mink. Virology. 165(2): 428-437.

Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B. and Osterhaus, A.D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 79(5): 2814-2822.

Hinshaw, V.S., Bean, W.J., Webster, R.G. and Sriram, G. 1980. Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology. 102(2): 412-419.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G. and Perez, D.R. 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol. 146(12): 2275-2289.

Karasin, A.I., Brown, I.H., Carman, S. and Olsen, C.W. 2000a. Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol. 74(19): 9322-9327.

Karasin, A.I., Olsen, C.W., Brown, I.H., Carman, S., Stalker, M. and Josephson, G. 2000b. H4N6 influenza virus isolated from pigs in Ontario. Can Vet J. 41(12): 938-939.

Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R.A., Amonsin, A., Payungporn, S., Noppornpanth, S., Wattanodorn, S., Theambooniers, A., Tantilertcharoen, R., Pattanarangsan, R., Arya, N., Ratanakorn, P., Osterhaus, D.M. and Poovorawan, Y. 2004. Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis. 10(12): 2189-2191.

Khalenkov, A., Perk, S., Panshin, A., Golender, N. and Webster, R.G. 2009. Modulation of the severity of highly pathogenic H5N1 influenza in chickens previously inoculated with Israeli H9N2 influenza viruses. Virology. 383(1): 32-38.

Lamb, R.A. and Choppin, P.W. 1983. The gene structure and replication of influenza virus. Annu Rev Biochem. 52467-506.

Lau, E.H., Leung, Y.H., Zhang, L.J., Cowling, B.J., Mak, S.P., Guan, Y., Leung, G.M. and Peiris, J.S. 2007. Effect of interventions on influenza A (H9N2) isolation in Hong Kong's live poultry markets, 1999-2005. Emerg Infect Dis. 13(9): 1340-1347.

Liu, M., He, S., Walker, D., Zhou, N., Perez, D.R., Mo, B., Li, F., Huang, X., Webster, R.G. and Webby, R.J. 2003. The influenza virus gene pool in a poultry market in South central china. Virology. 305(2): 267-275.

Nguyen, D.C., Uyeki, T.M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H., Xu, X., Balish, A., Klimov, A., Tumpey, T.M., Swayne, D.E., Huynh, L.P., Nghiem, H.K., Nguyen, H.H., Hoang, L.T., Cox, N.J. and Katz, J.M. 2005. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J Virol. 79(7): 4201-4212.

Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y. and Nakajima, K. 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology. 182(2): 475-485.

OIE (World Organization of Animal Health). 2005. Old Classification of Diseases Notifiable to the OIE (update on 25/1/2005). [Online] Available : http://www.oie.int/eng/maladies/en_oldclassification.htm#ListeA

Panigrahy, B., Senne, D.A. and Pedersen, J.C. 2002. Avian influenza virus subtypes inside and outside the live bird markets, 1993-2000: a spatial and temporal relationship. Avian Dis. 46(2): 298-307.

Payungporn, S., Phakdeewirot, P., Chutinimitkul, S., Theamboonlers, A., Keawcharoen, J., Oraveerakul, K., Amonsin, A. and Poovorawan, Y. 2004. Single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. Viral Immunol. 17(4): 588-593.

Poetri, O.N., Bouma, A., Murtini, S., Claassen, I., Koch, G., Soejoedono, R.D., Stegeman, J.A. and van Boven, M. 2009. An inactivated H5N2 vaccine reduces transmission of highly pathogenic H5N1 avian influenza virus among native chickens. Vaccine. 27(21): 2864-2869.

Roberts, P.C., Garten, W. and Klenk, H.D. 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J Virol. 67(6): 3048-3060.

Sharp, G.B., Kawaoka, Y., Jones, D.J., Bean, W.J., Pryor, S.P., Hinshaw, V. and Webster, R.G. 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. J Virol. 71(8): 6128-6135.

Shortridge, K.F., Zhou, N.N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K.G., Norwood, M., Senne, D., Sims, L., Takada, A. and Webster, R.G. 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology. 252(2): 331-342.

Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghwani, J., Bhatt, S., Peiris, J.S., Guan, Y. and Rambaut, A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature. 459(7250): 1122-1125.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A. and Poovorawan, Y. 2006a. Avian influenza H5N1 in naturally infected domestic cat. Emerg Infect Dis. 12(4): 681-683.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., Chutinimitkul, S., Thanawongnuwech, R. and Poovorawan, Y. 2006b. Fatal avian influenza A H5N1 in a dog. Emerg Infect Dis. 12(11): 1744-1747.

Suarez, D.L. and Senne, D.A. 2000. Sequence analysis of related low-pathogenic and highly pathogenic H5N2 avian influenza isolates from United States live bird markets and poultry farms from 1983 to 1989. Avian Dis. 44(2): 356-364.

Swayne D.E., Dennis, A., Senne and Beard, W.C., 1998. A Laboratory manual for the isolation and identification of avian pathogens. 4(29):150-154.

Thanawongnuwech, R., Amonsin, A., Tantilertcharoen, R., Damrongwatanapokin, S., Theamboonlers, A., Payungporn, S., Nanthapornphiphat, K., Ratanamungklanon, S., Tunak, E., Songserm, T., Vivatthanavanich, V., Lekdumrongsak, T., Kesdangsakonwut, S., Tunhikorn, S. and Poovorawan, Y. 2005. Probable tiger-to-tiger transmission of avian influenza H5N1. Emerg Infect Dis. 11(5): 699-701.

Tran, T.H., Nguyen, T.L., Nguyen, T.D., Luong, T.S., Pham, P.M., Nguyen, V.C., Pham, T.S., Vo, C.D., Le, T.Q., Ngo, T.T., Dao, B.K., Le, P.P., Nguyen, T.T., Hoang, T.L., Cao, V.T., Le, T.G., Nguyen, D.T., Le, H.N., Nguyen, K.T., Le, H.S., Le, V.T., Christiane, D., Tran, T.T., Menno de, J., Schultsz, C., Cheng, P., Lim, W., Horby, P. and Farrar, J. 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med. 350(12): 1179-1188.

Tsukamoto, K., Ashizawa, H., Nakanishi, K., Kaji, N., Suzuki, K., Okamatsu, M., Yamaguchi, S. and Mase, M. 2008. Subtyping of avian influenza viruses H1 to H15 on the basis of hemagglutinin genes by PCR assay and molecular determination of pathogenic potential. J Clin Microbiol. 46(9): 3048-3055.

Tsukamoto, K., Ashizawa, T., Nakanishi, K., Kaji, N., Suzuki, K., Shishido, M., Okamatsu, M. and Mase, M. 2009. Use of reverse transcriptase PCR to subtype N1 to N9 neuraminidase genes of avian influenza viruses. J Clin Microbiol. 47(7): 2301-2303.

Wang, M., Di, B., Zhou, D.H., Zheng, B.J., Jing, H., Lin, Y.P., Liu, Y.F., Wu, X.W., Qin, P.Z., Wang, Y.L., Jian, L.Y., Li, X.Z., Xu, J.X., Lu, E.J., Li, T.G. and Xu, J. 2006. Food markets with live birds as source of avian influenza. Emerg Infect Dis. 12(11): 1773-1775.

Webster, R.G. 2004. Wet markets--a continuing source of severe acute respiratory syndrome and influenza? Lancet. 363(9404): 234-236.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 56(1): 152-179.

World Health Organization (WHO). 2002. Laboratory procedures. WHO Manual on Animal Influenza Diagnosis and Surveillance. 2002(5): 15-66.

World Health Organization (WHO). 2009. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1). Reported to WHO, [Online] Available: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_09_24/en/index.html

Wood, G.W., Banks, J., Strong, I., Parsons, G. and Alexander, D.J. 1996. An avian influenza virus of H10 subtype that is highly pathogenic for chickens, but lacks multiple basic amino acids at the haemagglutinin cleavage site. Avian Pathol. 25(4): 799-806.

Yee, K.S., Carpenter, T.E., Farver, T.B. and Cardona, C.J. 2009. An evaluation of transmission routes for low pathogenicity avian influenza virus among chickens sold in live bird markets. Virology. 394(1): 19-27.

APPENDICES

APPENDIX A

Description of each LBMs in this study

1. Market A

Location	Bangkok
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks), ducks and geese
Number of shops	Approximate 15 shops that sell poultry (live and meat)
Slaughter area	In market

2. Market B

Location	Bangkok
Type of market	Pet bird market
Species sold in market	Chickens (layers and fighting cocks), ducks, geese, quails, pigeons, pet birds (i.e. budgerigar, parrots, finches), mammals (i.e. ferrets, dogs and cats), rodent (rabbits, squirrels, hamsters and guinea pigs) and reptiles (i.e. turtoise, iguana and chameleon)
Number of shops	Approximate 10 shops (only shops that sell avian species)
Slaughter area	No

3. Market C1

Location	Nong-bua district, Nakhonsawan
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks)
Number of shops	Approximate 3 shops that sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

4. Market C2

Location	Chum-saeng district, Nakhonsawan
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks) and ducks
Number of shops	Approximate 5 shops that sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

5. Market D1

Location	Bung-narang district, Pichit
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks)
Number of shops	Only one shop that sells poultry (meat)
Slaughter area	At vendor's home (sell live birds)

6. Market D2

Location	Dong-chareon district, Pichit
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks)
Number of shops	Approximate 2 shops sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

7. Market E1

Location	Muang district, Phitsanulok
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks) and ducks
Number of shops	Approximate 5 shops sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

8. Market E2

Location	Prom-piram district, Phitsanulok
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks) and ducks
Number of shops	Approximate 3 shops sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

9. Market F1

Location	Muang district, Sukhothai
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks)
Number of shops	Approximate 3 shops sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

10. Market F2

Location	Sawan-kalok district, Sukhothai
Type of market	Local food market
Species sold in market	Chickens (layers and fighting cocks)
Number of shops	Approximate 4 shops sell poultry (meat)
Slaughter area	At vendor's home (sell live birds)

APPENDIX B

Table : Percent similarity of nucleotide and amino acid (bracket) of M gene

M	LM 1973	LM 1983	LM 1984	LM 4754	LM 4759	LM 4761	LM 4775
LM 1973		97.5	97.5	97.2	97.2	97.2	97.2
LM 1983	(99.1)		98.4	98.1	98.1	98.1	98.1
LM 1984	(99.1)	(100)		98.1	98.1	98.1	98.1
LM 4754	(98.7)	(99.7)	(99.7)		98.4	98.4	98.4
LM 4759	(98.7)	(99.7)	(99.7)	(100)		98.4	98.4
LM 4761	(98.7)	(99.7)	(99.7)	(100)	(100)		98.4
LM 4775	(98.7)	(99.7)	(99.7)	(100)	(100)	(100)	

Table : Percent similarity of nucleotide and amino acid (bracket) of NP gene

NP	LM 1973	LM 1983	LM 1984	LM 4754	LM 4759	LM 4761	LM 4775
LM 1973		98.5	98.5	98.4	98.4	98.4	98.4
LM 1983	(99.6)		99.9	98.8	98.8	98.8	98.8
LM 1984	(99.6)	(100)		98.7	98.7	98.7	98.7
LM 4754	(99.4)	(99.8)	(99.8)		100	100	100
LM 4759	(99.4)	(99.8)	(99.8)	(100)		100	100
LM 4761	(99.4)	(99.8)	(99.8)	(100)	(100)		100
LM 4775	(99.4)	(99.8)	(99.8)	(100)	(100)	(100)	

Table : Percent similarity of nucleotide and amino acid (bracket) of NS gene

NS	LM 1973	LM 1983	LM 1984	LM 4754	LM 4759	LM 4761	LM 4775
LM 1973		97.9	97.8	97.6	97.6	97.6	97.4
LM 1983	(97.8)		99.6	98.2	98.2	98.2	98
LM 1984	(98.2)	(99.6)		98	98	98	97.9
LM 4754	(97.4)	(97.1)	(97.4)		100	99.8	99.9
LM 4759	(97.4)	(97.1)	(97.4)	(100)		99.8	99.9
LM 4761	(97.8)	(97.4)	(97.8)	(99.6)	(99.6)		99.9
LM 4775	(97.4)	(97.1)	(97.4)	(100)	(100)	(99.6)	

Table : Percent similarity of nucleotide and amino acid (bracket) of PA gene

PA	LM 1973	LM 1983	LM 1984	LM 4754	LM 4759	LM 4761	LM 4775
LM 1973		99.7	99.7	95	95	95	95
LM 1983	(99.4)		99.9	95	95	95	95
LM 1984	(99.3)	(99.9)		95	95	95.1	95
LM 4754	(99.4)	(99.7)	(99.6)		100	100	100
LM 4759	(99.4)	(99.7)	(99.6)	(100)		100	100
LM 4761	(99.4)	(99.7)	(99.6)	(100)	(100)		100
LM 4775	(99.4)	(99.7)	(99.6)	(100)	(100)	(100)	

Table : Percent similarity of nucleotide and amino acid (bracket) of PB1 gene

PB1	LM 1973	LM 1983	LM 1984	LM 4754	LM 4759	LM 4761	LM 4775
LM 1973		99.2	99.2	98.7	98.7	98.7	98.7
LM 1983	(99.6)		100	98.7	98.7	98.7	98.7
LM 1984	(99.6)	(100)		98.7	98.7	98.7	98.7
LM 4754	(99.5)	(99.6)	(99.6)		100	100	100
LM 4759	(99.5)	(99.6)	(99.6)	(100)		100	100
LM 4761	(99.5)	(99.6)	(99.6)	(100)	(100)		100
LM 4775	(99.5)	(99.6)	(99.6)	(100)	(100)	(100)	

Table : Percent similarity of nucleotide and amino acid (bracket) of PB2 gene

PB2	LM 1973	LM 1983	LM 1984	LM 4754	LM 4759	LM 4761	LM 4775
LM 1973		87.7	87.7	87.6	87.6	87.6	87.6
LM 1983	(99.2)		100	99	99	99	98.9
LM 1984	(99.2)	(100)		99	99	99	98.9
LM 4754	(99)	(99.4)	(99.4)		100	100	100
LM 4759	(99)	(99.4)	(99.4)	(100)		100	100
LM 4761	(99)	(99.4)	(99.4)	(100)	(100)		100
LM 4775	(98.9)	(99.3)	(99.3)	(99.9)	(99.9)	(99.9)	

APPENDIX C

Snigle letter amino acid code

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

BIOGRAPHY

Mister Trong Wisedchanwet was born on September 6th , 1982 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 2008.