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MONITORING OF INFLUENZA A VIRUSES AND USING A SENTINEL BIRD MODEL IN A LIVE BIRD MARKET

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ตลาดค้าสัตว์ปีกมีชีวิตเป็นสถานที่ที่มีปัจจัยเสี่ยงในการแพร่ระบาดของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในมนุษย์ เนื่องจากมีการรายงานการพบเชื้อไวรัสไข้หวัดหวัดใหญ่ชนิดเอหลายสายพันธุ์ในสัตว์ปีกจากตลาดค้าสัตว์ปีกมีชีวิต การศึกษานี้ได้เฝ้าระวังการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิตหนึ่งแห่งในจังหวัดพิษณุโลก ตั้งแต่เดือนมีนาคม 2553 ถึงเดือนกุมภาพันธ์ 2554 โดยเก็บตัวอย่างการป้ายปากและป้ายทวารของสัตว์ปีกในตลาดค้าสัตว์ปีกมีชีวิตแห่งนี้ทุกๆสองสัปดาห์เป็นเวลาหนึ่งปี นอกจากนี้ได้ศึกษาการใช้ระบบสัตว์ปีกยาม โดยนำเป็ดพันธุ์มัสโควี่ (Cairina moschata) จำนวน 20 ตัว มาใช้เป็นสัตว์ปีกยามเพื่อตรวจติดตามการแพร่กระจายและการติดต่อของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิตแห่งนี้ โดยเก็บตัวอย่างการป้ายปากและป้ายทวาร และตัวอย่างเลือดจากสัตว์ปีกยามระหว่างเดือนพฤศจิกายน 2553 ถึงเดือนมกราคม 2554 ผลการศึกษาในการเฝ้าระวังการติดเชื้อไวรัสไข้หวัดหวัดใหญ่ชนิดเอ ต่อเนื่องเป็นเวลา 1 ปี โดยการตรวจตัวอย่างด้วยวิธี real-time RT-PCR พบอุบัติการณ์ของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิตเท่ากับร้อยละ 1.64 (22/1338) จากจำนวนตัวอย่างดังกล่าวสามารถแยกเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ สายพันธุ์ H7N6 ได้จำนวน 20 ตัวอย่าง การศึกษานี้เป็นรายงานแรกที่สามารถแยกเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ สายพันธุ์ H7N6 ได้ในประเทศไทย ผลการศึกษานี้แสดงให้เห็นว่าตลาดค้าสัตว์ปีกมีชีวิตเป็นแหล่งของการติดเชื้อวนเวียนและการแพร่กระจายของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในประเทศไทย และจากผลการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในระบบสัตว์ปีกยามเป็นเวลา 70 วัน พบว่าร้อยละ 45 (9/20) ของสัตว์ปีกยามมีภูมิคุ้มต่อเชื้อไวรัสไข้หวัดใหญ่ชนิดเอโดยการตรวจด้วยวิธี NP-ELISA แต่ไม่สามารถแยกเชื้อไวรัสไข้หวัดใหญ่ชนิดเอได้จากสัตว์ปีกยามในช่วงเวลาดังกล่าว ผลการศึกษานี้แสดงให้เห็นว่าการศึกษาทางซีรั่มวิทยาในสัตว์ปีกยามสามารถช่วยเพิ่มประสิทธิภาพในการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิตได้ การเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิตอย่างต่อเนื่องนั้นมีความสำคัญเพื่อติดตามวิวัฒนาการและความสามารถในการแพร่กระจายติดต่อของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในอนาคต

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5475320031 : MAJOR VETERINARY PUBLIC HEALTH
KEYWORDS: INFLUENZA A VIRUS / LIVE BIRD MARKET / MONITORING / SENTINEL

WALEEMAS JAIRAK: MONITORING OF INFLUENZA A VIRUSES AND USING A SENTINEL BIRD MODEL IN A LIVE BIRD MARKET. ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 56 pp.

Live bird market (LBM) has been identified as a risk of human infection with Influenza A viruses (IAVs), since several subtypes of IAVs can circulate in poultry in LBMs. In this study, an LBM in Phitsanulok province was monitored for IAVs circulation and transmission. During March 2010 to February 2011, one year IAV monitoring program was conducted by collecting oropharyngeal and cloacal swabs from market poultry every two weeks to determine IAV infection in the LBM. In addition, during November 2010 to January 2011, sentinel birds, 20 Muscovy ducks (Cairina moschata) were placed in the LBM to determine IAVs transmission in the LBM. Swab and blood samples were collected from the sentinel birds at designated time. All swab and blood samples were subjected to virus isolation and serological test respectively. For one year IAV monitoring in an LBM, the occurrence of IAV infection was 1.64% (22/1338) by real-time RT-PCR. Twenty IAVs could be isolated and subtyped as H7N6. This finding is the first to report influenza virus subtype H7N6 circulating in Muscovy ducks in LBM in Thailand. These results indicated that LBM was a potential source of IAV circulation and transmission. For sentinel bird model, IAVs could not be isolated during 70 days of monitoring program, while 45 % (9/20) of sentinel birds were seropositive for IAV infection by NP-ELISA. These results indicated that the serological study in sentinel bird system was capable to detect IAV transmission and could improve the effectiveness of IAV monitoring in LBM. In conclusion, routine monitoring of IAV in LBMs is necessary to observe the evolution and transmission ability of IAVs in the future.

Department: Veterinary Public Health Student's Signature .....
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List of Abbreviations

AIV	Avian Influenza Virus
bp	base pair
cDNA	Complementary deoxyribonucleic acid
°C	degree Celcius
dNTP	Deoxynucleotide triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	et alibi, and other
HA	Hemagglutinin
HI	Hemagglutination inhibition
HPAI	Highly Pathogenic Avian Influenza
HPNAI	High Pathogenicity Notifiable Avian Influenza
LPAI	Low Pathogenic Avian Influenza
LPNAI	Low Pathogenicity Notifiable Avian Influenza
IAV	Influenza A Virus
LBM	Live Bird Market
M	Matrix
mg	milligram(s)
ml	milliliter(s)
µl	microliter
nm	nanometer

NA	Neuraminidase
NP	Nucleoprotein
NP-ELISA	Nucleoprotein-based Enzyme-Linked Immunosorbent Assay
NS	Nonstructural protein
OIE	Office Internationale des Epizooties
PA	Polymerase Acidic protein
PCR	Polymerase Chain Reaction
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
PBS	Phosphate Buffered Saline
RNA	Ribonucleic acid
RBC	Red blood cell
rRT-PCR	Real-time Reverse Transcription-Polymerase Chain Reaction
SPF	Specific Pathogen Free
SAN	Specific Antibody Negative
U	Unit
WHO	World Health Organization

Chapter 1 Introduction

Influenza A virus (IAV) is an enveloped, single strand RNA virus in the family Orthomyxoviridae and can cause disease in avian and mammal species. Influenza A viruses (IAVs) have eight RNA segments including PB2, PB1, PA, HA, NP, NA, M and NS genes. The viruses can be classified into subtypes based on their surface antigens; hemagglutinin antigen (HA) and neuraminidase antigen (NA). Up to date, there are 18 hemagglutinin antigens (H1-H18) and 11 neuraminidase antigens (N1- N11) (Tong et al., 2013). In the late 2003, the outbreaks of highly pathogenic avian influenza viruses (HPAI) subtype H5N1 occurred in East and Southeast Asia and caused severe avian disease with over 100 million poultry death and culling (Sims et al., 2005). In 2006, further outbreaks of HPAI-H5N1 were reported in Europe, Africa, the Middle East and South Asia. From 2003 to 2014, 70 countries around the world reported HPAI-H5N1 infection in poultry, and 650 H5N1 confirmed human cases with 386 deaths in 16 countries (OIE, 2014; WHO, 2014a). The HPAI-H5N1 viruses can transmit from poultry to humans (Suarez et al., 1998; Tran et al., 2004) and there are limited evidences of potential of human-to-human transmission (Webby and Webster, 2001; Bridges et al., 2002). In the recent year, 2013, the avian-origin IAV-H7N9 had emerged and infected in human population in main-land China. This virus has low pathogenic in avian, in contrast with the severity of respiratory disease and fatal in human (Cowling et al.,

2013). As of 27 June 2014, World Health Organization reported 450 confirmed cases with H7N9 infection, including 165 deaths (WHO, 2014b).

Live bird market (LBM) is a place for sell live poultry species. Live birds can be slaughtered on site or sold live to customers. LBMs have been determined as the potential sources of avian influenza viruses because of close contact and mingle of birds from different sources and several avian species (Cardona et al., 2009). Several IAVs both LPAI and HPAI circulating in LBMs were reported in many countries such as USA (Suarez et al., 1999; Cardona et al., 2009), Egypt (Abdelwhab et al., 2010), Hong Kong (Shortridge et al., 1998; Butt et al., 2005), China (Liu et al., 2003; Su et al., 2013), Korea (Kim et al., 2006), Vietnam (Nguyen et al., 2005; Jadhao et al., 2009), Indonesia (Santhia et al., 2009) and Thailand (Amonsin et al., 2008; Wisedchanwet et al., 2011a). In addition, several studies have suggested that exposure to LBMs or direct contact with freshly slaughtered poultry in LBMs may be major risk factors for human infection with IAVs including HPAI-H5N1 (Mounts et al., 1999; Yu et al., 2007; Wan et al., 2011), H9N2 (Butt et al., 2005) and H7N9 (Han et al., 2013). Furthermore, LBMs have been identified as suitable environment for viral reassortment because multiple influenza subtypes and reassortants could be circulated in LBMs (Liu et al., 2003; Moon et al., 2010; Wisedchanwet et al., 2011a). Since, the reassortment of IAVs can generate novel viruses with high virulence or pandemic potential, therefore, the continuous monitoring of IAVs in LBMs is important.

IAVs monitoring can be conducted by passive surveillance in dead or sick birds and by active surveillance in live or subclinical birds. Sentinel bird practice is the cost-effective method of active surveillance (McCluskey, 2003). It has been used successfully for early detection of avian influenza viruses in wild bird population and commercial poultry flocks around the world (Halvorson et al., 1983; Globig et al., 2009). The surveillance of IAVs in LBMs usually conducts by collecting samples from live poultry, bird meats and environments (Choi et al., 2005; Amonsin et al., 2008; Indriani et al., 2010); however, there are limit information about sentinel bird method in LBMs especially in Thailand.

The objectives of this study were to determine the occurrence and transmission of IAV in an LBM by monitoring poultry in the market and sentinel bird model. For LBM monitoring, the swab samples were collected from poultry in the LBM every 2 weeks for one year. For sentinel bird model, a sentinel bird model was established in a LBM by placing 20 Muscovy ducks (*Cairina moschata*) and examined swab and blood samples from the birds for 2 months to determine the possible transmission of IAVs in the LBM. The results provided information of IAV occurrence and the effectiveness of a sentinel model for IAV monitoring in LBM. The data of influenza A viruses circulation and transmission in LBM can be used to improve the influenza prevention and control program in LBMs in the future.

Research questions

1. What are occurrences and subtypes of influenza A viruses circulating in an LBM in Thailand?
2. How effective of sentinel bird system be used as influenza A virus monitoring in an LBM?

Objectives of Study

1. To monitor influenza A virus infection in an LBM in Thailand
2. To determine the possible transmission of influenza A viruses in an LBM by using sentinel bird mode

Chapter 2 Literature review

Influenza A virus

Influenza viruses are members of the family *Orthomyxoviridae* which are enveloped, single stranded, negative-sense RNA viruses. Influenza viruses are classified into three genera: Influenza virus A, Influenza virus B and Influenza virus C. Influenza A viruses (IAVs) are infectious in many avian and mammalian species (Alexander, 1982; Webster et al., 1992). Influenza B viruses are predominantly infectious in human and can infect seal (Osterhaus et al., 2000; Bodewes et al., 2013). Influenza C viruses are commonly pathogenic in humans; however, there are many reports of pigs and dogs with influenza C viruses infection (Guo et al., 1983; Ohwada et al., 1986; Hause et al., 2013). IAVs are 80-120 nm in diameter with lipid bilayer envelope containing spikes on surface. The spikes consist two types of glycoproteins; rod-shaped hemagglutinin (HA) and mushroom shaped neuraminidase (NA) (Wilson et al., 1981; Varghese et al., 1983), which are used to classify the viruses into subtypes (WHO, 1980). Currently, there are 18 hemagglutinin antigens (H1 – H18) and 11 neuraminidase antigens (N1-N11) (Tong et al., 2013). The genome of IAV comprises eight RNA segments encoding at least 10 different viral proteins including polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), neuraminidase (NA), neucleoprotein (NP), hemagglutinin (HA), matrix protein (M1), membrane ion channel

(M2), non-structural protein 1 (NS1) and non-structural protein 2 (NS2) (Webster et al., 1992). The protein of function of each gene is summarized in table 1.

Table 1. RNA segments and encoded proteins of Influenza A virus (Webster et al., 1992; Brown, 2000).

RNA segments	Protein	Length (bp)	Functions
1	PB2	2,341	Host cap binding and endonuclease
2	PB1	2,341	Viral mRNA elongation and viral RNA synthesis
3	PA	2,233	Viral RNA synthesis; genome replication
4	HA	1,778	Receptor binding and fusion to host cell
5	NP	1,565	Viral replication; encapsidate the cRNA and synthetic the viral RNA progeny and target of cytotoxic T cells
6	NA	1,413	Cell receptor-destroying enzyme to free virus particles from host cell receptors for virus spread and antibody-mediated virus neutralization
7	M1	1,027	initiating progeny virus assembly
	M2		ion channel
8	NS1	890	interferon response inhibitor
	NS2		Viral ribonucleocapsids exportation from nucleus to cytoplasm for viral replication

Avian influenza virus

IAVs infecting birds are designated as avian influenza viruses (AIVs). The natural reservoirs of AIV are birds especially the Anseriformes (ducks, geese and swan) and Charadiformes (shorebirds, gull, terns and auks) (Webster et al., 1992; Stallknecth, 1997). AIV are highly host adapted viruses and can transmit to other hosts, including mammals and domestic poultry. To date, the Office Internationale des Epizooties (OIE) defines pathogenicity of AIV as Notifiable avian influenza (NAI). The NAI viruses are classified into high pathogenicity notifiable avian influenza (HPNAI) viruses and low pathogenicity notifiable avian influenza (LPNAI) viruses. HPNAI viruses have intravenous pathogenicity index (IVPI) greater than 1.2 in 6-week-old chickens or cause at least 75% mortality in 4-to-8-week-old chickens infected intravenously or present of multiple basic amino acids at the cleavage site of hemagglutinin molecule (HA0). LPNAI viruses are H5 or H7 subtypes that are not HPNAI viruses. HPNAI viruses are synonymous with HPAI viruses, while LPAI viruses are LPNAI viruses and all AIV subtypes that are not HPNAI (OIE, 2012c).

Since 2003, HPAI-H5N1 outbreaks have been reported in several countries. The HPAI-H5N1 infected not only in poultry, but also in mammal species, including tiger, leopard (Keawcharoen et al., 2004), domestic cat (Songserm et al., 2006a; Klopfleisch et al., 2007a), palm civet (Roberton et al., 2006), dog (Songserm et al., 2006b), stone marten (Klopfleisch et al., 2007b) and human (Chotpitayasunondh et al., 2005). Since

the outbreaks of HPAI-H5N1 in 2003, poultry over 50 countries around the world have been affected from HPAI-H5N1 viruses (OIE, 2014). The continued circulation of HPAI-H5N1 can increase chance for the viruses to adapt to human to human transmission resulting in a human pandemic (Taubenberger and Morens, 2009).

In March 2013, there was the first report of human infection with novel reassortant AIV subtype H7N9 in China. Within 3 months since the first report, at least 130 human cases with H7N9 AIV infection were documented (Cowling et al., 2013). As of 20 February 2014, total 355 confirmed cases with 112 deaths have been reported for human infection with AIV H7N9 (WHO, 2014c). It is interesting to note that the viruses caused mild disease in poultry but caused severe respiratory disease and fatal in human (Gao et al., 2013). Many studies suggested that direct contact with poultry in live bird markets could be a risk factor of AIV H7N9 infection in human (Chen et al., 2013; Qiu et al., 2014). Moreover, there are some evidences indicated that AIV H7N9 probably transmit from human to human (Qi et al., 2013; Liu et al., 2014). Therefore, the AIV H7N9 has potential to be the pandemic agent in human population.

Live Bird Market

Live bird market (LBM) is a place for sell live poultry species. Live birds can be slaughtered on site or sold alive to customers. Various species of birds from different ages and sources are housed together in a cage or a stack. Therefore, LBM is an ideal

environment for virus transmission, intraspecies and interspecies, due to its suitable conditions for virus infection, transmission and genetic reassortment (Nguyen et al., 2005).

The investigation of AIVs in LBMs has been conducted in several countries in Asia, Africa as well as North America. In 1994-1998, low-pathogenic H7 AIVs have been reported in LBMs from multi-states in the Northeast United States. The evidences of multiple viral genes reassortment have also been obviously observed (Suarez et al., 1999). In 2001, four subtypes of AIVs were isolated from LBMs in Hanoi, Vietnam. H4N6, H5N2 and H9N3 subtypes were isolated from healthy ducks as well as HPAI-H5N1 viruses were isolated from healthy geese (Nguyen et al., 2005). In 2005, outbreak of LPAI H6N2 occurred in 3 markets in the Southern California. After the outbreak, those markets have implemented the influenza control plan and resulted in AIV free in LBMs system of Southern California since December 2005 (Cardona et al., 2009). From July 2006 to August 2007, H5N1 AIVs were reported in live birds and bird meats from LBMs in Thailand. The sources of AIV were quails, moorhens and water cocks and HPAI-H5N1 were isolated from healthy live poultry; chicken and ducks (Amonsin et al., 2008). In 2009, AIV isolates in LBMs have been reported in many countries such as China, Egypt and Thailand. In Egypt, the result of AIV monitoring in LBMs showed that waterfowl were the major species of H5N1 positive by real-time PCR (Abdelwhab et al., 2010). In eastern China, H3N2 and H4N2 were isolated from ducks in LBMs in

Shanghai and H11N2 were isolated from chicken in LBM in Nanjing. In Thailand, influenza A viruses subtype H4N6, H4N9 and H10N3 were firstly isolated from one LBM in Thailand. In addition, influenza A viruses subtype H4N6 and H4N9 were isolated from the same duck, indicating possibility of two influenza A viruses infection and genetic reassortment in the bird. This report indicated that LBMs are potential sources of AIV transmission and genetic reassortment (Wisedchanwet et al., 2011b).

There were many reports about human infection with AIV from LBM such as H5N1, H7N9 and H9N2. In 1997, case-control study in 15 patients with AIV-H5N1 infection in Hong Kong revealed that exposure to live poultry in LBM was a risk factor of AIV H5N1 infection in human (Mounts et al., 1999). Moreover, the study of H5N1 human isolates in China from 2005 to 2010 discovered that the genetic of H5N1 human isolates were highly similar to H5N1 from environment in LBMs (Wan et al., 2011). In 1999 to 2003, AIVs H9N2 were isolated from children in Hong Kong and the genetic of H9N2 human isolates were similar to AIVs H9N2 from poultry in LBM (Peiris et al., 1999; Butt et al., 2005). From 2013 to 2014, AIVs H7N9 have caused of over 300 human cases with respiratory disease in China (WHO, 2014c). Besides most of patients with AIV H7N9 infection had history of recent exposure to live poultry in LBM, the genetic of H7N9 from patients were closely related to AIV H7N9 from poultry and environment in LBMs (Chen et al., 2013; Li et al., 2014). These results showed evidences of LBMs as an important risk factor of human avian influenza virus infection.

Sentinel bird system

Since HPAI-H5N1 outbreaks in 2003, surveillance for avian influenza virus in birds has been focusing. In general, the animal disease surveillance systems could be divided to two main systems, passive surveillance and active surveillance. Passive surveillance involves examining clinical cases that usually rely on the report of suspicious cases from veterinarians or farmers. In contrast, active surveillance usually has routine sampling on clinical or normal animals such as mass screening method and sentinel system (Racloz et al., 2006). For HPAI, the virus infection induces severe clinical signs and high mortality in avian species, thus the virus detection has been successfully based on passive surveillance. However, for LPAI, a mild or no clinical sign and low mortality may under notice by passive surveillance, thus an early detection of LPAI infection has been performed by active surveillance (Comin et al., 2012). In addition, sentinel system was used successfully for AI monitoring in wild birds in central Europe in 2007 (Globig et al., 2009).

The epidemiological term, the sentinel herd is defined as a cohort of animals for specific disease agent with specific time period of monitoring (Ward et al., 1995). According to the Office Internationale des Epizooties (OIE), sentinel units are described as a group of animals whose geographic location and immune status are known for specific disease detection (OIE, 2012a). Sentinel herds have been conducted for parasitic, viral, bacterial diseases, toxicological screening and animal welfare issues over

the past few decades. The basis of sentinel herd remains two parts: firstly, establishing the objective of the surveillance, and secondly, designing criteria and using animals depending on the nature of the disease and condition in the questions (Racloz et al., 2006). The sentinel framework has three components; pathogen, target population and sentinel population. All of sentinel surveillance, sentinel population must always interact with both pathogen and target population (Halliday et al., 2007).

Sentinel birds have been used as a surveillance tool of IAV investigation in the past few decades especially in wild birds. The domestic ducks have been used as a sentinel flock to influenza detection in pelagic bird colonies and have been isolated the viruses higher than wild birds (Sinnecker et al., 1982a; Sinnecker et al., 1982b). Moreover, sentinel ducks have been used to determine avian influenza infection in turkeys regarding the arrival of wild migratory waterfowls in Minnesota (Halvorson et al., 1983; Halvorson et al., 1985). Recently, sentinel ducks for avian influenza in wild birds were conducted in the wetlands in Austria, Germany and Switzerland. The result showed that AIV detection rate in sentinel ducks was higher than monitoring in trapped wild birds. This result demonstrated the effectiveness of sentinel system on AIV detection (Globig et al., 2009).

Influenza A virus diagnostic techniques

1. Identification of influenza A virus

The suitable IAV isolation from birds is viral isolation from cloacal swabs (wild birds and poultry) and tracheal swabs (poultry) (OIE, 2012b). The samples from birds including oropharyngeal and cloacal swabs should be placed in isotonic phosphate-buffered saline (PBS) with antibiotics or a solution containing protein (e.g. brain heart infusion, 5% [v/v] cattle serum, 5% [v/v] bovine albumen) with antibiotics. The reference standard for propagation of IAVs from birds is specific pathogen free (SPF) embryonated chicken eggs inoculation or specific antibody negative (SAN) eggs inoculation. The swab supernatant is inoculated into allantoic cavity of 9-11 days embryonating chicken eggs and then incubated at 37 °c for 4-7 days (OIE, 2012b). The presence of IAV is screened by haemagglutination (HA) test using allantoic fluid and chicken or turkey red blood cells. The HA positive sample is confirmed by real-time RT-PCR for viral identification (Spackman et al., 2002).

2. Detection of the antibody

The IAV antibodies detection has included many test formats such as agar gel immunodiffusion (AGID) assay, haemagglutination inhibition (HI) assay and commercial enzyme-linked immunoassay (ELISA) (Spackman et al., 2008). The AGID assay can detect M and NP proteins of IAV infection. This assay has been widely used for IAV antibodies detection in chicken and turkey flocks, but rarely used for antibodies detection in other avian species (OIE, 2012b). The HI test can be used to identify HA

subtype of IAV antibodies in serum, plasma and egg yolk by using known subtype virus as an antigen. The HI assay can detect antibodies to the viruses for longer period than AGID and can test in many avian species including chickens, turkeys and ducks. The HI assay is considered as a labour intensive technique when used as an antibody screening test because all of 16 HA subtype of antigens and antiserums are required (OIE, 2012b). To date, several commercial ELISA kits are available for avian influenza virus antibodies detection in serum, plasma and chicken egg yolk. Generally, ELISA can detect antibodies only to the NP protein of all IAV subtypes. This assay has two different formats, an indirect ELISA (species-dependent) and a competitive ELISA (species-independent) format (Suarez and Schultz-Cherry, 2000). The advantages of ELISA test are more sensitive and faster than AGID and HI test (Spackman et al., 2008).

Chapter 3 Materials and methods

This study consists of 4 phases; **phase 1**, LBM selection in Thailand; **phase 2**, Sample collection from sentinel ducks and avian species in the LBM; **phase 3**, Isolation and identification of influenza A virus and serological investigation; **phase 4**, Genetic characterization of influenza A virus.

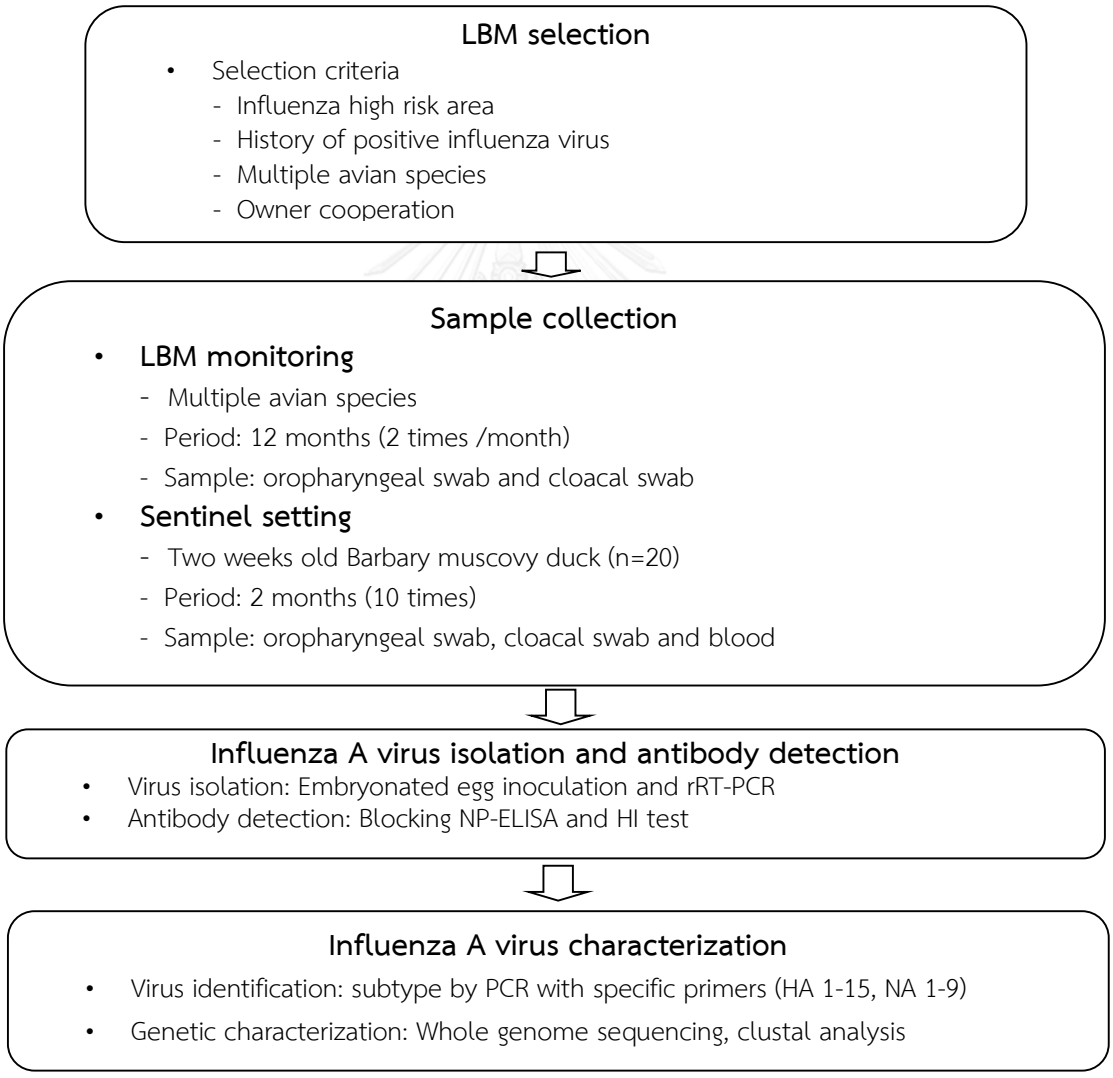

Figure 1. The conceptual framework of this study

Phase 1: Live bird market selection

In this study, an LBM in Phitsanulok province was selected for longitudinal monitoring and sentinel bird setting. This LBM usually keep various poultry species such as Khaki Campbell duck (*Anas platyrhynchos*), Muscovy duck (*Cairina moschata*) and Chicken (*Gallus gallus domesticus*) from different sources in the same pen. Approximate 100 birds were slaughtered and sold in this LBM every day. It is noted that this LBM received poultry from Phitsanulok and lower northern provinces of Thailand where considered as influenza high risk areas. Moreover this LBM had a history of influenza virus positive in the previous surveillance. During the course of the study, the owner collaborated well with the research team.

Phase 2: Sample collection

2.1 LBM monitoring

In this study, approximately 20 birds in the LBM were selected for oropharyngeal and cloacal swab collection every 2 weeks for one year IAV monitoring. The samples were collected using sterile polyester tip swabs and kept in 2 ml of Brain Heart Infusion (Bacto™ BD diagnostics, USA) with Penicillin G 1,000 U/ml, Streptomycin 1 mg/ml, Gentamycin 0.25 mg/ml and Kanamycin 0.5 mg/ml as viral transport media. The swab samples were transported to laboratory at 4ºC within 24 hours and stored at -80ºC immediately upon arrival until tested.

2.2 Sentinel bird model

In this study, Twenty Barbary muscovy ducks (*Cairina moschata*) at 14-day-old were placed in the LBM as the sentinel birds. All ducks were negative for antibody against IAV by nucleoprotein antibody ELISA (FlockCheck® AI MultiS-Screen diagnostic kit, IDEXX Laboratories, USA) before placing in a pen. The sentinel ducks were then placed in a pen (estimate 10 square meters) nearby poultry pens in the market. The sentinel ducks were shared the environment with the market poultry such as the pond where receives drain water from the slaughter site and the workers in the LBM.

Oropharyngeal swabs, cloacal swabs and blood samples were collected from individual sentinel birds at 1, 3, 5, 7, 14, 21, 28, 42, 56 and 70 days post placing (Figure 2). The swab samples were processed as the same protocol as the swab samples from LBM monitoring. Approximately 2 ml of blood sample was collected from jugular vein from each sentinel bird and kept in the sterile 2 ml microtubes without blood anticoagulant. Blood samples were transported to laboratory at 4ºC within 24 hours and were centrifuged to separate serum immediately upon arrival then the sera were stored at -20ºC until tested.

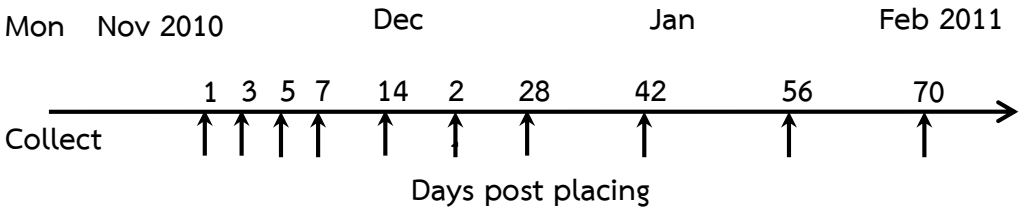

Figure 2. Sample collection dates of the sentinel birds.

Phase 3: Isolation and identification of Influenza A virus and serological investigation

3.1 IAV isolation and identification

3.1.1 IAV isolation

Oropharyngeal swab and cloacal swab samples were subjected to viral isolation for influenza A virus following OIE Terrestrial Manual 2008 (OIE, 2008). The 750 µl of supernatant of each swab sample was inoculated into three eggs of 9-11 days old embryonated chicken egg (250 µl per egg) and then was incubated at 37ºC for 72 hours. After 72 hours of incubation, the allantoic fluid was collected from each egg and examined by hemagglutination test (HA test) by using 1% chicken RBC suspension. The samples that showed hemagglutination activity were considered as positive HA test. The allantoic fluid of positive HA test samples were kept at -80ºC until IAV identification.

3.1.2 Influenza A virus identification

The positive HA activity allantoic fluid were subject to real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay for confirmation of IAV infection and then PCR assay with specific primers for subtype identification of IAV infection. The detail of IAV identification protocol is conducted including:

3.1.2.1 RNA extraction

The HA positive allantoic fluid were subjected to RNA extraction using Nucleospin viral RNA mini kit (Nucleospin®, Macherey-Nagel, Germany). Briefly, 150 µl allantoic fluid was mixed to 600 µl RNA carrier and then incubated at 70ºC for 5 minutes. 600 µl of ethanol was added to the lysate and then applied the entire mixture to Nucleospin® spin column. After two washing steps according to manufacturer's protocol, RNA was eluted in 50 µl of RNase-free water.

3.1.2.2 Real-time reverse transcription-polymerase chain reaction (rRT-PCR)

For identification of IAV, the RNA was examined for a presence of Matrix (M) gene of IAV by real-time RT –PCR (Spackman et al., 2002). The rRT-PCR reaction was performed using SuperScript III Platinum® One-step RT-PCR system (Invitrogen, Carlsbad, CA, USA) with specific IAV primers and probe (Spackman et al., 2002). Total 15 µl of rRT-PCR mixture contained; 4 µl of RNA, 7.5 µl of 2X reaction mix, 0.6 µl of each forward and reverse primer (10 µmol of each), 0.6 µl of probe (2.5 µM), 0.3 µl of SuperScript III RT Platinum® Taq Mix, 0.1 µl of 50 mM $MgSO_4$ and 1.3 µl of RNase-free

water. The rRT-PCR was processed using thermo cycler (Corbett Research Rotor-Gene 3000, Sydney, Australia) with PCR conditions including 50ºC for 30 minutes, 95ºC for 15 minutes follow by 40 cycles of amplification; 95ºC for 15 seconds and 60ºC for 30 seconds. The results were interpreted by cycle threshold (Ct) value. The samples with Ct value greater than 40 were considered negative and the samples with Ct value less than 36 were considered positive. The samples with Ct value between 36 and 40 were considered as suspected. The suspected samples were retested from second egg inoculation samples.

3.1.2.3 cDNA synthesis

Before IAV subtype by PCR assay, the positive RNA from real-time RT-PCR was reverse transcribed to cDNA using Improm-II™ Reverse Transcription System and random primer (Promega®, USA). From the manufacturer's protocol, the mixture of 5 µl of viral RNA and 5 µl of random primers (100 µM) was heat at 70 ºC for 15 minutes and then chill on ice for 5 minutes. Then, the RNA-primer mixture was added with 12 µl of reverse transcriptase enzyme contained 4 µl of 5X cDNA buffer, 1 µl of 0.5 mM dNTP mix, 2 µl of 2.5 mM $MgCl_2$, 0.3 µl of RNase inhibitor, 1 µl of ImProm-II™ Reverse Transcriptase and 3.7 µl of distilled water. Then, the mixture for cDNA synthesis was loaded into thermocycler under the condition of 25ºC for 5 minutes, 42ºC for 60 minutes and 72ºC for 15 minutes. The cDNA was kept at -20ºC until further use.

3.1.2.4 PCR assay

The cDNA were then subjected to PCR amplification for subtyping with specific primers for IAV H1-H15 and N1-N9 (Tsukamoto et al., 2008; Tsukamoto et al., 2009). In brief, total 10 µl of PCR mixture contained 0.5 µl of cDNA, 0.5 µl of each forward and reverse primer (10 µM), 5 µl of 2X KAPA Taq Master Mix (KAPA Biosystems, MA, USA) and 3 µl of distilled water. Then, the PCR mixture was loaded into thermocycler with PCR conditions; initial denaturation at 94ºC for 3 minutes, denaturation at 94ºC for 30 seconds, annealing at 50ºC for HA primers or 45ºC or NA primers for 30 seconds, extension at 72ºC for 30 seconds and final extension at 72ºC for 7 minutes. The PCR products were then examined by gel electrophoresis using 1.2% of agarose gel in 0.5x Tris Borate EDTA (TBE). The presence banding on agarose gel was interpreted corresponding to IAV primer suptypes.

3.2 Serological investigation

3.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

For screening of IAV infection, the sera were examined for antibody against the NP protein by blocking NP-ELISA (FlockCheck® AI MultiS-Screen diagnostic kit, IDEXX Laboratories, USA). The blood samples were undergone centrifugation for serum separation at 3000 rpm for 10 minutes. Then, each serum sample was subjected to ELISA test following the manufacturer's protocol. The serum sample was diluted ten-

fold by diluting 15 µl of serum with 135 µl of sample diluent. 100 µl of mixture, negative and positive control was dispensed into the avian influenza (AI) viral antigen-coated plate and incubated at room temperature for 60 minutes. After washing with 350 µl of wash solution for 3 times, 100 µl of Anti-AI: Horseradish Peroxidase Conjugate was added into each well and incubated at room temperature for 30 minutes. After washing, TMB substrate solution was added into each well and incubated at room temperature for 15 minutes. The reaction was stopped by adding 100 µl of stop solution. For results validation, the serum sample of each well was measure the absorbance value at A650 nm by ELISA reader. The results were interpreted by the sample to negative (S/N) ratio of each sample. The sera with an S/N ratio > 0.7 were considered negative for the presence of IAV antibody. The sera with an S/N ratio between 0.6 and 0.7 were considered suspected. The sera with S/N ratio <0.6 were considered positive (Brown et al., 2009).

3.2.2 Hemagglutination Inhibition (HI) Test

For confirming subtype of IAV infection, the ELISA positive and suspected sera were subjected to Hemagglutination Inhibition (HI) assay. In this study, three subtypes of Thai IAVs including, H5 (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004), H7 (A/duck/Thailand/CU-LM7279T/2010) and H10 (A/duck/Thailand/LM-CU4747/2009) at 4 HAU were used as the antigens for HI test. In brief, the sera were treated by heat at 56ºC for 30 minutes, 20% Kaolin for 30 minutes and then 50% RBC for 60 minutes to

remove non-specific inhibitor before HI test. The procedure of HI test was done as described of OIE protocol (OIE, 2008). Briefly, 25 µl of each treated serum was two-fold diluted by phosphate buffered saline (PBS) from 1:10 to 1:2560. Then, 25 µl of 4 HAU virus was mixed in each well and incubated the plate at room temperature for 60 minutes. After incubation, 50 µl of 1% chicken red blood cells was added into each well and incubated the mixture at room temperature for 60 minutes. The HI titer was interpreted by the reciprocal at the last dilution that completely hemagglutination inhibition. The sera with HI titer less than 40 were considered negative. Conversely, the sera with HI titer greater than or equal to 40 were considered positive (OIE, 2008).

Phase 4: Influenza A virus characterization

For whole genome sequencing of IAV, the viruses with subtype identification were subjected to whole genome sequencing. All eight genes of IAV isolates were amplified by PCR using oligonucleotide primer sets either from our primer inventories or newly designed primer sets by using Primer3 Input (v.0.4.0). The primer list for whole genome sequencing designed in this study is shown in table 2. The PCR products were determined by agarose gel electrophoresis as previous described. The PCR products were then subjected for nucleotide sequencing at Molecular Informatics Laboratory Limited, Shatin, N.T., Hong Kong. The nucleotide sequences of each gene were then validated and aligned using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI, USA).

For phylogenetic analysis, HA and NA gene segment were compared with the reference IAVs available at the GenBank database. The reference nucleotide sequences (n=50) were included in the analysis to represent the avian influenza viruses from both Eurasian and North American lineages. Nucleotide sequences of each gene were aligned using Muscle v.3.6 (Edgar, 2004). The phylogenetic analysis was performed using the MEGA v.4.0 with neighbor-joining algorithm apply bootstrap analysis with 1,000 replications and Mr.Bayes software with Bayesian Markov chain Monte Carlo (BMCMC) with 40,000 generations and an average standard deviation of split frequencies <0.05 (Drummond and Rambaut, 2007; Tamura et al., 2007) . To analyze viruses genetic characteristics, the nucleotide sequences and deduced amino acids of each gene of the viruses were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc., Madison, WI, USA).

Table 2. List of oligonucleotide primers for IAVs (H7N6) designed in this study

Gene	Primer name	Size (bp)	Primer sequence (5'-3')
PB2	PB2-1F	19	agc raa agc agg tca awt a
	PB2-1R	18	ccc att gct gcy ttg cat
	PB2-2F	18	gga tgg trg aca tyc tta
	PB2-2R	22	ggt tca aay tcc atc tta ttg t
	PB2-3F	19	caa tga tgt ggg ara tca a
	PB2-3R	19	tgg cca tca gta gaa aca a
PB1	PB1-1F	21	tga atg gat gtc aay ccg act
	PB1-1R	19	gat ttg cat tcc ggg tgt t
	PB1-2F	22	kca ctg aca ctg aac aca wtg a
	PB1-2R	20	aac atg ccc atc mtc att cc
	PB1-3F	23	cat gtt cga aag taa gag cat ga
	PB1-3R	21	cca tcy gaa acc amc agt cct
	PB1-4F	21	tgg att tgt rgc caa ttt cag
	PB1-4R	20	tga gyt ctt caa tgg tgg aa
	PB1-5F	21	tcc tct nat nat ttc nct ctc
	PB1-5R	21	ant ana aac aan nca ttt ttt
PA	PA-1F	20	ttg tgc gac aat gct tca at
	PA-1R	20	ttt cct ccc ctg tga atg ag
	PA-2F	20	gag tga cac gga ggg aag tt
	PA-2R	20	act cgg gtc ttc gat gct ta
	PA-3F	20	acg ccc tct cag act acc tg
	PA-3R	20	gac gta gct cca att gag ca
	PA-4F	22	cac tag caa gct gga ttc aga g
	PA-4R	19	aga agg cat cgc ctc att t
	PA-5F	20	tgc agt agg cca agt ttc aa
	PA-5R	18	cag tgc atg tgc gag gaa
H7	H7-1F	19	caa aag cag ggg ata caa a
	H7-1R	21	ctc tgc ata gaa tga aga tcc
	H7-2F	20	caa tgg gat tca cat aca gc
	H7-2R	20	gca tca acc tgt act cca ct
	H7-3F	18	tga tgc taa acc cca atg
	H7-3R	20	gct ttt caa cct cag tga ac
	H7-4F	19	att gaa aat gga tgg gaa g
	H7-4R	24	cga ctc act ata agt aga aac aag
NP	NP-1F	20	ctc aag gca cca aac gat ct
	NP-1R	20	gtt gcg tcc tct cca ttg tt
	NP-2F	21	tgg gtg aga gag cta att ctg
	NP-2R	20	ctt tgt gct gct gtt tgg aa
	NP-3F	20	ttt ctg gag agg cga aaa tg
	NP-3R	20	tcc tct tgg gac cac tct tg
	NP-4F	24	gag aat cca gca cat aag agt caa
	NP-4R	23	gca ttg tct ccg aag aaa taa ga
NA	N6-1F	20	gca aaa gca ggg tga aaa tg
	N6-1R	20	agc tcg gaa tgg gct tct at
	N6-2F	20	ctg cag gat gtt tgc tct ga
	N6-2R	20	cgg tta gga cct ttg agc ac
	N6-3F	20	tcc tga aat gat gac cca ca
	N6-3R	24	aga aac aag ggt gtt ttt ctt aaa
M	M-1F	20	gga gcr aaa gca ggt aga tr
	M-1R	21	tct gct cca tag cct twg cyg
	M-2F	18	cca gtg arc gag gac tgc
	M-2R	20	tgt tga caa aat gac cat cg
NS	NS-1F	20	agc raa agc agg gtg aca aa
	NS-1R	20	agt aga aac aag ggt gtt tt

Chapter 4 Results

In this study, an LBM in Phitsanulok was monitored for IAV in market poultry during March 2010 to February 2011 and in sentinel birds during November 2010 to January 2011. The poultry included backyard poultry (chickens and ducks) and free grazing ducks gathered and transported for selling in the LBM by the vendors and farmers. Approximately 100 birds of various species from different sources were housed together for 1-2 days before slaughtering. In general, customers, farmers and vendors visit this LBM for selling their live poultry or buying poultry products. In this study, we collected oropharyngeal swabs, cloacal swabs and blood samples from market poultry and sentinel birds. The swab samples were subjected to virus isolation by egg inoculation and subtype identification by PCR assay. Then, whole genome sequencing was conducted for genetic characterization by bioinformatics software. For serological results, blood samples were subjected to blocking-ELISA and HI tests for determination of Influenza A infection status in sentinel birds.

Sample collection

Sample collection in LBM monitoring

During March 2010 to February 2011, swab samples were collected from market poultry every 2 weeks for 12 months. A total of 1,338 swab samples were collected from 669 animals including Muscovy ducks (349 birds) Khaki Campbell ducks (146 birds) and chickens (174 birds). The samples included oropharyngeal swabs

(n=669) and cloacal swab (n=669) from avian species. The description of swab samples by each month and each avian species is shown in table 3.

Table 3. Description of samples collected in LBM monitoring program during March 2010 – February 2011

Month	Chicken *(Gallus gallus domesticus)*		Muscovy duck *(Cairina moschata)*		Khaki campbel duck *(Anas platyrhynchos)*		Total
	OP	CS	OP	CS	OP	CS	
Mar 2010	18	18	0	0	30	30	**96**
Apr 2010	12	12	25	25	0	0	**74**
May 2010	0	0	30	30	0	0	**60**
Jun 2010	14	14	0	0	23	23	**74**
Jul 2010	15	15	28	28	7	7	**100**
Aug 2010	15	15	34	34	0	0	**98**
Sep 2010	15	15	35	35	35	35	**170**
Oct 2010	30	30	60	60	0	0	**180**
Nov 2010	15	15	60	60	0	0	**150**
Dec 2010	20	20	27	27	24	24	**142**
Jan 2011	11	11	33	33	16	16	**120**
Feb 2011	9	9	17	17	11	11	**74**
Total	**174**	**174**	**349**	**349**	**146**	**146**	**1338**

Sample collection from sentinel bird model

In this study, twenty Barbary muscovy ducks (*Cairina moschata*) at 14-day-old were used as the sentinel birds. Before placing in the LBM, the sentinel ducks were tested negative for antibody against IAV by blocking ELISA. Oropharyngeal swabs, cloacal swabs and blood samples were collected from the sentinel ducks at day 1, 3, 5, 7, 14, 21, 28, 42, 56 and 70 post placing. A total of 545 samples including 366 swab samples and 179 blood samples were collected from sentinel ducks during sentinel model setting. The description of samples in sentinel model is shown in table 4.

Table 4. Description of samples collected from sentinel ducks

Date sampled (day post placing)	Oropharyngeal swab	Cloacal swab	Blood	Total
1	20	20	19	59
3	19	19	18	56
5	19	19	19	57
7	18	18	17	53
14	18	18	18	54
21	18	18	18	54
28	18	18	17	53
42	18	18	18	54
56	18	18	18	54
70	17	17	17	51
Total	**183**	**183**	**179**	**545**

Influenza A virus isolation and identification

Influenza A virus in LBM monitoring

From March 2010 through February 2011, total 1,338 swab samples were subjected to virus isolation by egg inoculation and were then tested for HA activity by HA test. In total, 87 swab samples were positive to HA test. It is noted that the HA positive was high in November 2010 (Table 5). For IAV identification, 22 out of 87 HA positive samples were positive for M gene representing 1.64% (22/1338) of swab samples IAV positive by rRT-PCR. In details, the highest occurrence of IAV in the LBM was in November 2010. The 22 IAV positive samples were obtained from oropharyngeal swab (n=12) and cloacal swab (n=10). One IAV was isolated from Khaki Campbell ducks in June 2010 and twenty one IAVs were isolated from 15 Muscovy ducks in November 2010 (Table 5).

For virus identification, 20 out of 22 IAV positive samples could be subtyped by PCR assay with specific primers. The result showed that all twenty virus isolates were IAV subtype H7N6 (Figure 3 and Figure 4). It is noted that all twenty H7N6 IAVs were isolated from Muscovy ducks in LBM in November 2010. The H7N6 viruses were isolated from oropharyngeal swabs (n=11) and cloacal swabs (n=9).

Table 5. Description of IAV isolation and identification from avian species in LBM monitoring

Date sampled	Muscovy duck *(Cairina moschata)*					Khaki Campbell ducks *(Anas platyrhynchos)*					Chicken *(Gallus gallus domesticus)*				
	No. bird	No. HA positive (%)		No. IAV positive (%)[a]		No. bird	No. HA positive (%)		No. IAV positive (%)[a]		No. bird	No. HA positive (%)	No. IAV positive (%)[a]		
		OPS	CS	OPS	CS		OPS	CS	CS	OPS		OPS	CS	OPS	CS
March 2010	0	0	0	0	0	30	2 (6.67)	4 (13.33)	0	0	18	0	0	0	0
April 2010	25	0	0	0	0	0	0	0	0	0	12	1 (8.33)	0	0	0
May 2010	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Jun 2010	0	0	0	0	0	23	1 (4.35)	4 (17.39)	0	1 (4.35)	14	0	0	0	0
July 2010	28	0	1 (3.57)	0	0	7	0	1 (14.28)	0	0	15	0	0	0	0
August 2010	34	0	4 (11.76)	0	0	0	0	0	0	0	15	0	0	0	0
September 2010	35	3 (8.57)	2 (5.71)	0	0	35	5 (14.28)	3 (8.57)	0	0	15	2 (13.33)	0	0	0
October 2010	60	0	2 (3.33)	0	0	0	0	0	0	0	30	0	0	0	0
November 2010	60	15 (25.0)	14 (23.33)	12 (20.0)	9 (15.0)	0	0	0	0	0	15	1 (6.67)	0	0	0
December 2010	27	2 (7.41)	2 (7.41)	0	0	24	2 (8.33)	1 (4.17)	0	0	20	0	1 (5.0)	0	0
January 2011	33	1 (3.03)	2 (6.06)	0	0	16	1 (6.25)	1 (6.25)	0	0	11	0	0	0	0
February 2011	17	0	0	0	0	11	0	1 (9.09)	0	0	9	5 (5.55)	3 (33.33)	0	0
Total	**349**	**21 (6.02)**	**27 (7.74)**	**12 (3.44)**	**9 (2.58)**	**146**	**11 (7.53)**	**15 (10.27)**	**0**	**1 (0.68)**	**174**	**9 (5.17)**	**4 (2.30)**	**0**	**0**

[a]Samples positive to M gene of IAV using rRT-PCR

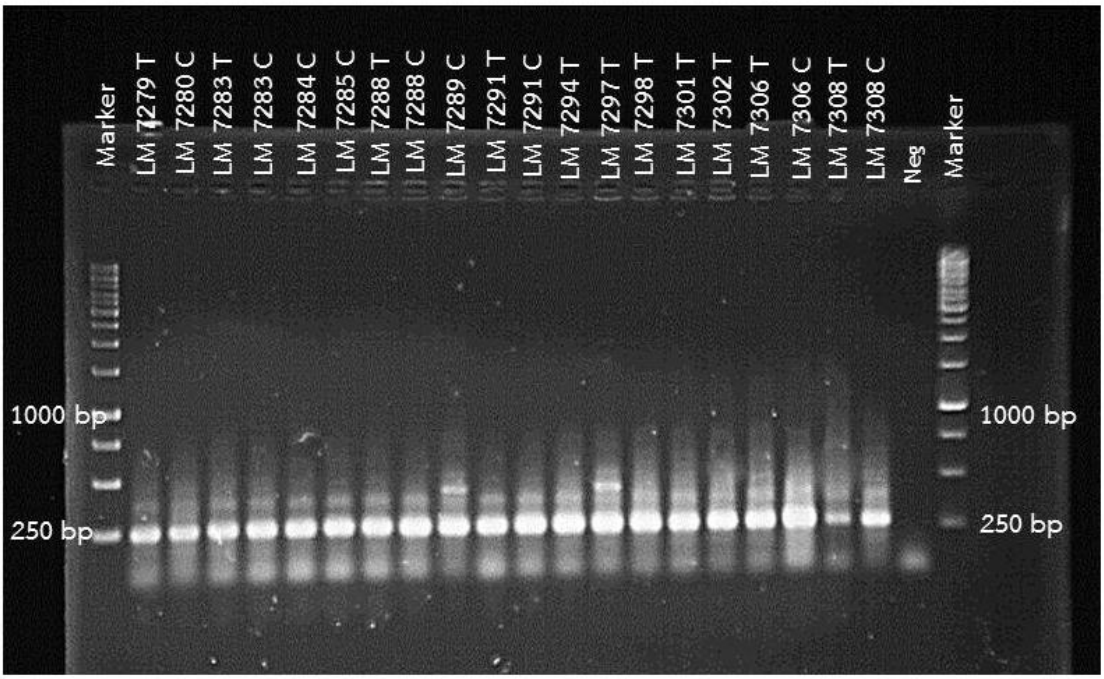

Figure 3. H7 identification by PCR assay using subtype specific primer; Marker: 1,000-bp marker, H7: expected PCR product 241 bp

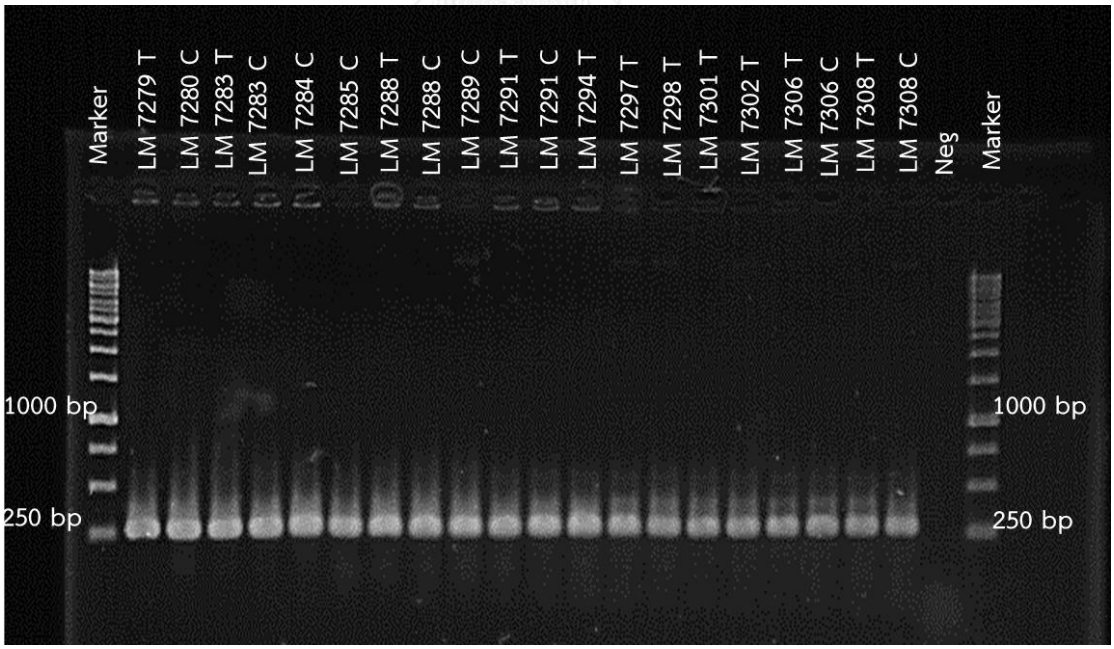

Figure 4. N6 identification by PCR assay using subtype specific primer; Marker: 1,000-bp marker, N6: expected PCR product 264 bp

Influenza A virus in sentinel ducks

From November 2010 through January 2011 of sentinel bird monitoring, total 366 swab samples were subjected to virus isolation by egg inoculation and tested for HA activity by HA test. In total 6 swab samples were HA positive after 28 days post placing until the end of monitoring but none of IAV virus could be isolated from sentinel ducks by rRT-PCR (M gene detection) (Table 6). All 6 HA positive samples were collected from 5 sentinel ducks including ducks number 5, 9, 12, 16 and 18. It is noted that, sentinel duck number 9 was positive for HA test at 28 and 70 days post placing.

Table 6. Details of Influenza A virus isolation from sentinel bird model during November 2010 – January 2011

Date sampled (day post placing)	Sample tested	HA positive	% HA positive	IAV isolates
1	40	0	0	0
3	38	0	0	0
5	38	0	0	0
7	36	0	0	0
14	36	0	0	0
21	36	0	0	0
28	36	1	2.78	0
42	36	1	2.78	0
56	36	2	5.55	0
70	34	2	5.88	0
Total	**366**	**6**	**1.64**	**0***

*None of IAV could be isolated from sentinel ducks

Serological investigation

In this study, serological test was conducted in sentinel bird model but not in market poultry since the market poultry were not allowed to collect blood samples. In total 179 blood samples were collected from sentinel birds at day 1, 3, 5, 7, 14, 21, 28, 42, 56 and 70 after placing from November 2010 to January 2011. All 179 serum samples were screened of antibodies against IAV for all subtype by blocking NP-ELISA. From our results, it is interesting to note that 4 out of 19 birds posed IAV antibody at 3 days post placing. Then, 6 out of 18 birds showed seropositive at 7 days post placing and 1 out of 18 birds showed seropositive at 14 days post placing. However, all birds were seronegative after 21 days post placing until the end of study (70 days) (Table 7). In conclusion, 9 out of 20 ducks were seropositive for ELISA, while the others were negative until 70 days of the study. For specific subtype serological analysis, the ELISA seropositive samples by were tested for specific IAV subtype infection by HI test using H5, H7 and H10 antigens. Our result showed that all 11 positive and suspected ELISA sera were negative for H5, H7 and H10 infection by HI test (Table 8).

Table 7. Details of seropositive ducks by blocking NP-ELISA in sentinel bird monitoring during November 2010 to January 2011

Day post placing sentinel duck ID	NP-ELISA result									
	1	3	5	7	14	21	28	42	56	70
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	+	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	+	-	-	-	-	-	-
5	-	+	-	+	-	-	-	-	-	-
6	-	+	-	-	-	-	-	-	-	-
7	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	-	-	-	N/A	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12	-	-	-	+	-	-	-	-	-	-
13	-	N/A	-	+	+	-	-	-	-	-
14	N/A	+	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	+	-	-	-	-	-	-	-	N/A
17	-	-	-	-	-	-	N/A	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	+	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-

N/A: Blood sample were not collected from duck because of missing or dead birds

Table 8. HI results of seropositive ELISA samples from sentinel bird monitoring

Sentinel duck ID	3 days				7 days				14 days			
	ELISA	HI test			ELISA	HI test			ELISA	HI test		
		H5	H7	H10		H5	H7	H10		H5	H7	H10
2	-	N/A	N/A	N/A	+	<10	<10	<10	-	N/A	N/A	N/A
4	-	N/A	N/A	N/A	+	<10	<10	<10	-	N/A	N/A	N/A
5	+	<10	<10	<10	+	<10	<10	<10	-	N/A	N/A	N/A
6	+	<10	<10	<10	-	N/A	N/A	N/A	-	N/A	N/A	N/A
12	-	N/A	N/A	N/A	+	<10	<10	<10	-	N/A	N/A	N/A
13	-	N/A	N/A	N/A	+	<10	<10	<10	+	<10	<10	<10
14	+	<10	<10	<10	-	N/A	N/A	N/A	-	N/A	N/A	N/A
16	+	<10	<10	<10	-	N/A	N/A	N/A	-	N/A	N/A	N/A
19	-	N/A	N/A	N/A	+	<10	<10	<10	-	N/A	N/A	N/A

N/A: the negative ELISA samples were not subjected to HI test.

Influenza A virus characterization

In this study, 20 IAVs subtype H7N6 isolated from Muscovy ducks in a LBM in November 2010 were characterized. In detail, eight H7N6 viruses (CU-LM7279T - CU-LM7288C) were selected as the representatives and subjected to whole genome sequencing. The remaining twelve H7N6 viruses (CU-LM7289C - CU-LM7308C) were subjected for HA and NA gene sequencing. The nucleotide sequences of all twenty H7N6 IAVs were submitted to the GenBank and the details of these viruses are shown in table 7.

For H7 gene, the nucleotide sequences were analyzed using the NCBI nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the genetic relatedness of H7 from this study with other reference IAVs available at the GenBank database. The results showed that the HA gene of H7N6 from this study (CU-LM7279T as a representative to BLAST) was closely related to that of IAV subtype H7N7 from duck from Japan (A/duck/Shiga/B149/2007(H7N7)) with 97% nucleotide identity (Table 10). Moreover, the nucleotide sequences of twenty H7 genes of this study and the other H7 subtype nucleotide sequences from Eurasian and North American lineages were subjected to phylogenetic analysis. The phylogenetic tree shows that the viruses in Eurasian lineage and North American lineage were obviously clustered in separated groups. All twenty H7 viruses in this study were clustered together in a group of Eurasian lineage. It is noted that H7N6 of this study were grouped into separated subgroup from those of novel virulence H7N9 from China outbreaks in 2013

(Figure 5). For genetic analysis, the deduced amino acids of H7 of CU-LM7279T were compared with the HPAI and LPAI H7 viruses in GenBank using MegAlign software (DNASTAR). The amino acids at HA cleavage site, receptor-binding residues, left edge of receptor binding pockets and right edge of receptor binding pockets were analyzed. The result showed that amino acids at the HA cleavage site of the H7N6 virus (CU-LM7279T) were P-E-I-P-K-G-R/G which was not present polybasic amino acids. Thereby, the H7N6 virus of this study was considered as LPAI virus. The right edge of receptor binding pockets of H7N6 of this study contained G-A-T-S-A amino acid sequences which were similar to LPAI-H7 viruses but different from HPAI-H7 (G-V/T-T-S-A/T). The amino acids sequence at receptor-binding residues and left edge of receptor binding pockets of CU-LM7279T consisted W/L/H/Q/L and N-G-Q-S-G-R subsequently which were indifferent in both LPAI and HPAI. The presence of glycosylation sequences at position 123, 149 and 188 were not found in HA gene of H7N6 viruses (Table 11).

For NA gene analysis, the NA gene segment of H7N6 (CU-LM7279T) was blasted using the NCBI nucleotide BLAST program to identify genetic relatedness with the reference N6 gene available at the GenBank database. H7N6 from duck from China (A/duck/Jiangxi/25134/2009) was closely similar to Thai H7N6 with 99% nucleotide identity (Table 10). All twenty N6 nucleotide sequences of the viruses in this study were subjected to phylogenetic analysis to evaluate relationship with the other N6 viruses from Eurasian lineage and North American lineage. The tree of NA gene analysis

showed that twenty H7N6 viruses of this study were grouped in one branch and clustered into the Eurasian lineage (Figure 6).

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Table 9. Influenza A virus isolated from LBM monitoring in this study

Virus	Subtype	Swab sample type	Date sampled	Species	Location	GenBank Accession No.	sequence
A/duck/Thailand/CU-LM7279T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307169- JX307176	Complete
A/duck/Thailand/CU-LM7280C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307153- JX307160	Complete
A/duck/Thailand/CU-LM7283T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307195- JX307202	Complete
A/duck/Thailand/CU-LM7283C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307217- JX307224	Complete
A/duck/Thailand/CU-LM7284C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307141-JX307148	Complete
A/duck/Thailand/CU-LM7285C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307183- JX307190	Complete
A/duck/Thailand/CU-LM7288T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307249- JX307256	Complete
A/duck/Thailand/CU-LM7288C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307239- JX307246	Complete
A/duck/Thailand/CU-LM7289C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	KF591860- KF591861	HA&NA
A/duck/Thailand/CU-LM7291T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307117- JX307118	HA&NA
A/duck/Thailand/CU-LM7291C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307151- JX307152	HA&NA
A/duck/Thailand/CU-LM7294T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307191- JX307192	HA&NA
A/duck/Thailand/CU-LM7297T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307227- JX307228	HA&NA
A/duck/Thailand/CU-LM7298T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307181- JX307182	HA&NA
A/duck/Thailand/CU-LM7301T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307119- JX307120	HA&NA
A/duck/Thailand/CU-LM7302T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307215- JX307216	HA&NA
A/duck/Thailand/CU-LM7306T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307225- JX307226	HA&NA
A/duck/Thailand/CU-LM7306C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307177- JX307178	HA&NA
A/duck/Thailand/CU-LM7308T/2010	H7N6	Oropharyngeal	November 2010	*Cairina moschata*	Phitsanulok	JX307149- JX307150	HA&NA
A/duck/Thailand/CU-LM7308C/2010	H7N6	Cloacal	November 2010	*Cairina moschata*	Phitsanulok	JX307129- JX307130	HA&NA

Table 10. Genetic similarity of eight gene segments of CU-LM7279T (H7N6) with reference nucleotide sequences available on GenBank database

Gene	Position	GenBank accession No.	Strain name	Percent nucleotide identity
PB1	7-2249	JN817597.1	A/pintail/Korea/188/2009(H10N4)	99%
PB2	58-2297	JX523344.1	A/quail/Thailand/CU-J2882/2009(H7N1)	99%
PA	22-2133	JX523346.1	A/quail/Thailand/CU-J2882/2009(H7N1)	99%
HA	1-1683	AB558257.1	A/duck/Shiga/B149/2007(H7N7)	97%
NA	7-1419	KF259615.1	A/duck/Jiangxi/25134/2009(H7N6)	99%
NP	40-1491	JX454701.1	A/wild duck/Korea/CSM20-5/2009(H4N6)	99%
M	15-985	JN982530.1	A/lesser whistling-duck/Thailand/CU-W3947/2010(H12N1)	99%
NS	1-845	KF260019.1	A/duck/Jiangxi/21714/2011(H11N9)	99%

Table 11. Deduced amino acid sequences of HA gene of H7N6 (CU-LM7279T) compared with references H7 sequences available in the GenBank database

Virus name	Pathotype	Right edge of RBS	Receptor binding site					Glycosylation position			Left edge of RBS	HA cleavage site	GenBank Accession No.
		124-128	142	143	175	182	185	123	149	188	215-220 (224-229)	315-329 (333-339)	
Chicken/Rostock/8/1934(H7N1)	HPAI	G-T-T-S-A	W	L	H	Q	L	+	+	-	N-G-Q-S-G-R	P-E-P-S-K-K-R-K-K-R/G	M24457
Chicken/Italy/444/99 (H7N1)	HPAI	G-T-T-S-T	W	L	H	Q	L	+	-	-	N-G-Q-S-G-R	P-E-I-P-K-G-S-R-V-R-R/G	AJ704810
Turkey/Italy/4617/1999 (H7N1)	HPAI	G-T-T-S-T	W	L	H	Q	L	+	-	-	N-G-Q-S-G-R	P-E-I-P-K-G-S-R-V-R-R/G	CY025141
Netherlands/219/03 (H7N7)	HPAI	G-T-T-S-A	W	L	H	Q	L	+	-	-	N-G-Q-S-G-R	P-E-I-P-K-R-R-R-R/G	AY338459
Mallard/Sweden/56/02 (H7N7)	LPAI	G-A-T-S-A	W	L	H	Q	L	-	-	-	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	AY999977
Mallard/Korea/21_9/2008 (H7N8)	LPAI	G-A-T-S-A	W	L	H	Q	L	-	-	-	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	JX444843
Duck/Thailand/CU_10507T/2011 (H7N4)	LPAI	G-A-T-S-A	W	L	H	Q	L	-	-	-	N-G-Q-S-G-R	P-E-L-P-K-G-R/G	JX307127
Duck/Shiga/B149/2007 (H7N7)	LPAI	G-A-T-S-A	W	L	H	Q	L	-	-	-	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	AB558257
Chicken/Guangdong/SD641/2013 (H7N9)	LPAI	G-A-T-S-A	W	L	H	Q	L	-	-	-	N-G-L-S-G-R	P-E-I-P-K-G-R/G	CY146908
Duck/Thailand/CU-LM7279T/2010 (H7N6)[a]	ND	G-A-T-S-A	W	L	H	Q	L	-	-	-	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	JX307175

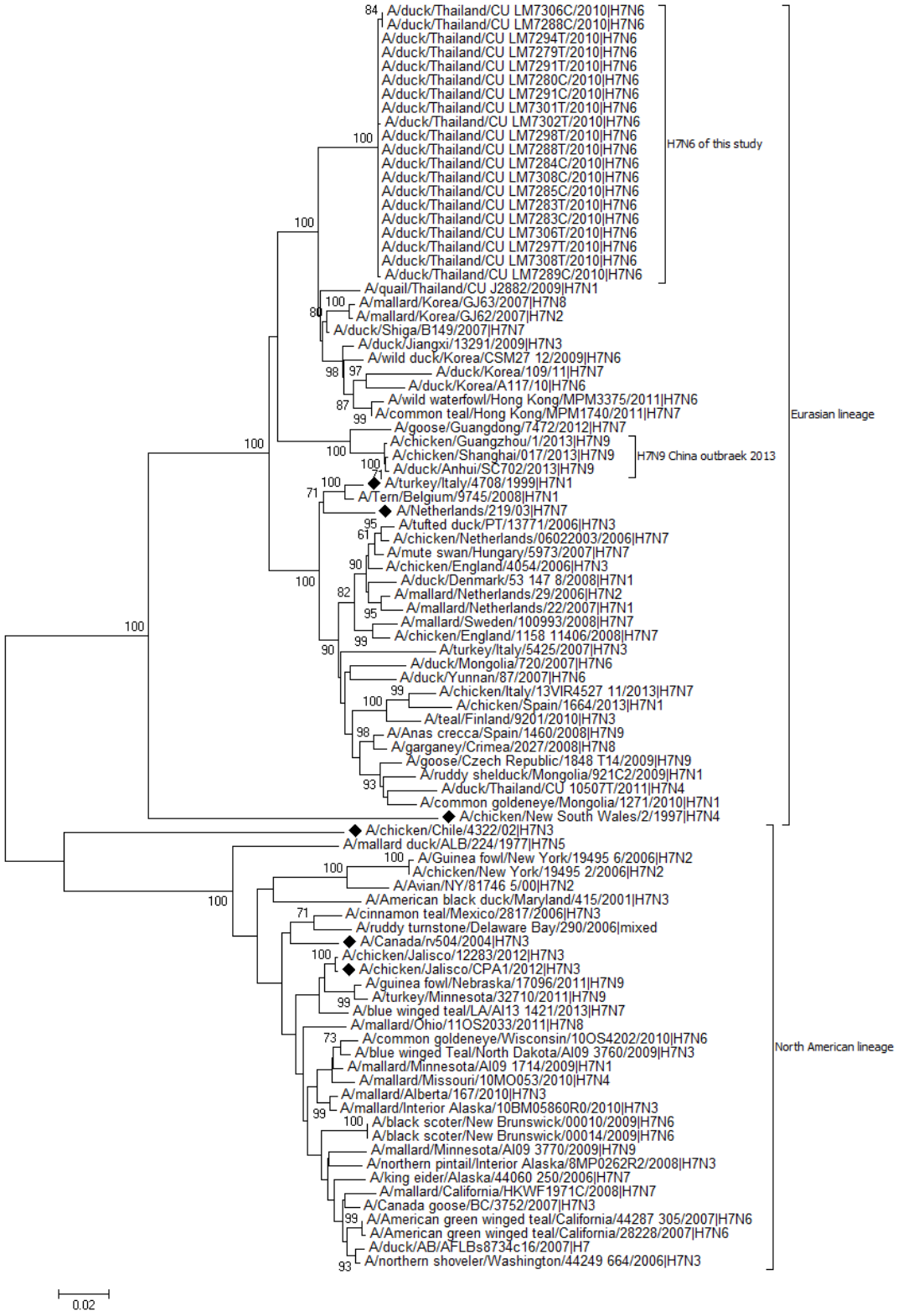

Figure 5. Phylogenetic analysis of the H7 gene of all twenty H7N6 IAVs of this study; Diamonds indicate HPAI H7

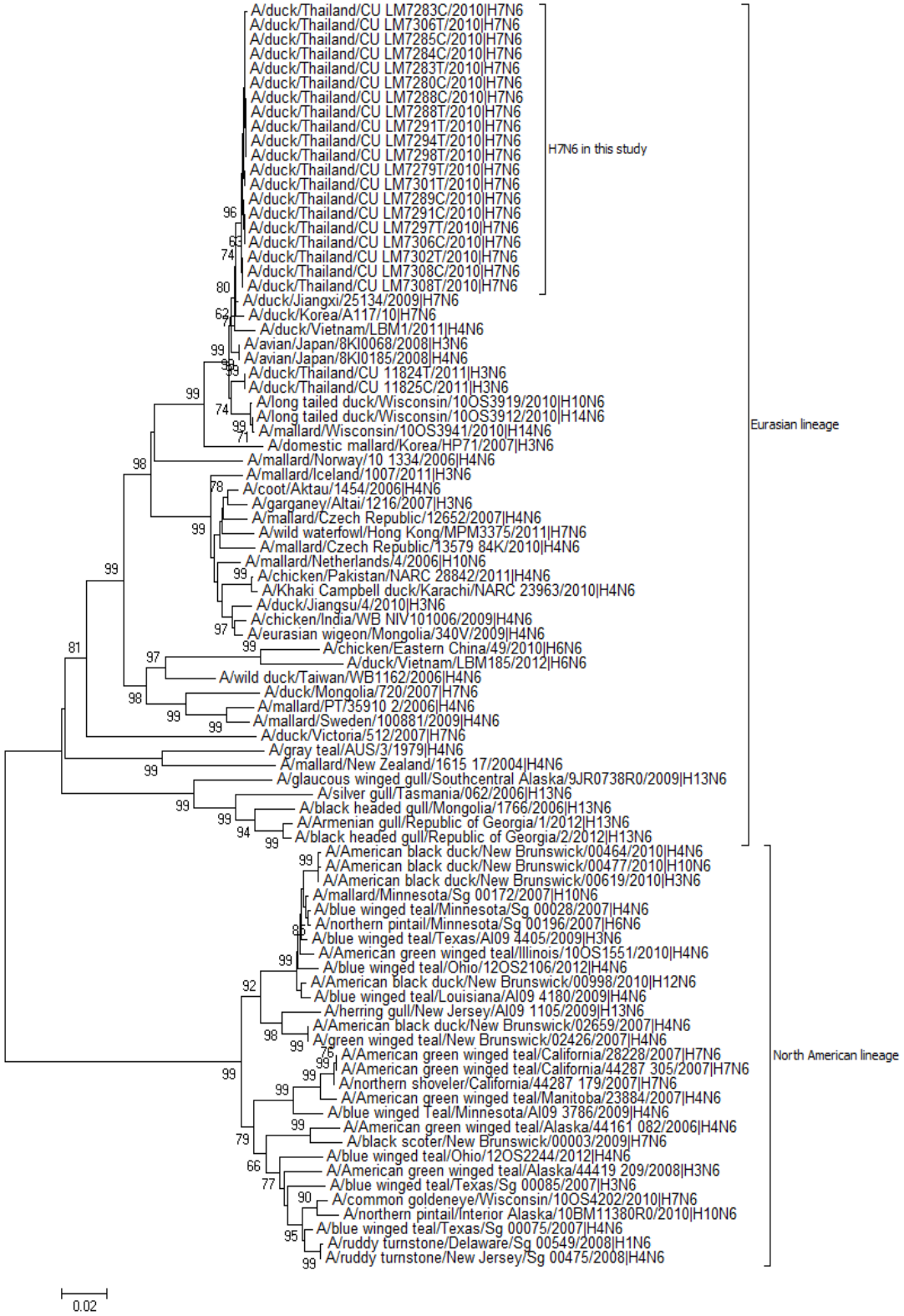

Figure 6. Phylogenetic analysis of the N6 gene of all twenty H7N6 IAVs of this study

Chapter 5 Discussion

In this study, the IAV monitoring in market poultry and sentinel birds were conducted in an LBM in Phitsanulok province. The market poultry were investigated for IAV infection for one year monitoring program. Almost poultry in this LBM were backyard poultry and free grazing ducks from Phitsanulok province and vicinity. Various species of birds from several sources were mingled and kept in a pen for 1-2 days until slaughter by conventional method. The sentinel birds were 14 day-old Muscovy ducks which were monitored of IAV infection for 2 months of monitoring program.

For monitoring of IAV infection in poultry market, the occurrence of IAV in the LBM in 2010 was 1.64% which was higher than the occurrence of HPAI-H5N1 in LBMs during 2006-2007 (1.3%) (Amonsin et al., 2008) and of IAV in LBMs in 2009 (0.36%) (Wisedchanwet et al., 2011a). The IAV monitoring in LBMs in 2009 was conducted in many parts of Thailand including low and high risk influenza areas, and IAV monitoring in LBMs during 2006-2007 focused particular HPAI-H5N1. In this study, HPAI and LPAI were monitored in a large scale LBM in Influenza high risk area due to the high occurrence of IAV than the previous study. H7N6 IAVs were isolated from a Muscovy flock in the LBM in November 2010. From previous study, several strains of IAV have been isolated in winter season including AIV (Wisedchanwet et al., 2011a; Wongphatcharachai et al., 2012) and SIV (Kupradinun et al., 1991; Sreta et al., 2010). In Thailand, IAV occurrence have been correlated with season because the seasonal

effect influence the spread and transmission ability of influenza virus (Lowen et al., 2007). In addition, the close contact behavior of backyard poultry lead to more easily and rapidly spread of IAV in winter season.

In this study, twenty H7N6 IAVs were isolated from Muscovy ducks while chickens and Khaki Campbell ducks could not be isolated of any IAV. The occurrence of IAVs infection in Muscovy duck was 2.87% (20/698). The result of this study tallied to the LBM surveillance for IAVs in 2009 that all IAVs were collected from Muscovy ducks (Wisedchanwet et al., 2011a). In chicken, the previous experiments show that the susceptibility of LPAI in chicken was lower than the other avian species (Zarkov, 2008; Tonnessen et al., 2011), and HPAI infection in chicken usually shows severe clinical signs and high mortality rate (Swayne and Pantin-Jackwood, 2006; Swayne, 2007). The chickens in the LBM were sold in healthy for persuading the consumer, its might be the reason of low rate of IAVs isolation from chicken in LBM of this study. However, IAVs in chickens in LBM should be continuously monitored because they have been identified as a risk factor of human infection of avian influenza in mane reports (Mounts et al., 1999; Butt et al., 2005; Chen et al., 2013; Cowling et al., 2013; Han et al., 2013). For Khaki Campbell duck (*Anas Platyrhynchos*), It has been known that this species are natural reservoir of IAVs (Webster et al., 1992) and this duck breed is commonly raised as free grazing ducks in Thailand (Songserm et al., 2006c). The high rate of IAVs infection should be found in Khaki Campbell ducks due to the conditions

of IAV susceptibility and raising system. Previous study shown juvenile ducks are more susceptible to IAVs infection than the mature ducks (Webster et al., 1992). Khaki Campbell ducks are egg-laying ducks, therefore only mature or senescent ducks which post egg production are sold in LBM. Hence, the age of ducks led to none of IAV isolation from Khaki Campbell duck in this study.

Twenty isolates of H7N6 IAVs were obtained from one flock of Muscovy ducks (*Cairina moschata*). The IAV infection rate in this flock was 66.67% (20/30) that might imply an IAV outbreak in this flock. These Muscovy ducks were possibly infected in farm level because they were sampled in the LBM when just arrival. This Muscovy flock was not raised in rice fields but in backyards. From previous study, the domestic ducks in Thailand were infected with influenza viruses after the ducks were released into rice fields because they had chance to contact with influenza virus natural reservoir such as waterfowl and wild migratory birds (Songserm et al., 2006c). The source of IAV infection in these ducks remains unclear but an inadequate biosecurity management in backyard system might lead to IAVs exposure in backyard poultry.

From genetic characterization, the HA and NA genes of Thai H7N6 virus were highest similar to H7N7 from duck from japan and H7N6 from duck from China, respectively. These results suggested that Thai H7N6 virus was closely related to AIV circulated in duck in Asia. The phylogenetic analysis revealed that HA and NA genes of H7N6 IAVs in this study were clustered into the Eurasian lineage and closely related

with LPAI H7 IAVs circulation in poultry and waterfowl in Asia. Genetic analysis of H7 gene from one isolate (CU-LM7279T) showed PEIPKGR as amino acid sequences at cleavage site. This H7 isolate possessed one amino acid of arginine (R) residue at HA cleavage site which have been suggested to LPAI (OIE, 2012b). In contrast, H7N1 (Chicken/Italy/444/99) possessed PEIPKGSRVRR as multiple basic amino acid at HA cleavage site and it has been pathotyped as HPAI (Capua et al., 2000). However the pathogenicity identification of Thai H7N6 isolate should be further confirmed by intravenous pathogenicity index test (IVPI) according the OIE protocol (OIE, 2012b). The receptor binding site of Thai H7 isolate was N-G-Q-S-G-R while novel AIV H7N9 from China was N-G-L-S-G-R. Amino acid residue at 226 and 228 of HA gene of Thai H7N6 were glutamine (Q) and glycine (G), respectively (H3 numbering). These results suggested that this virus has susceptible to binding to 2,3-linked sialic acid receptors as receptors specificity in avian species (Connor et al., 1994). The substitution at 226 residue from Q to L and at 228 residue from G to S could increase the binding affinity to humans receptor (Srinivasan et al., 2013). Therefore, novel AIV H7N9 from China could transmit to humans but Thai H7N6 has lacked this affinity. Moreover, *N*-link glycosylation analysis of HA gene showed that Thai H7N6 did not possess additional glycosylation sequon at position 123,149 and 188 of HA gene while HPAI H7N1 from Italy in 1999 and 1934, and HPAI H7N7 from Netherland in 2003 consisted at least one glycosylation site at those positions. The correlation of additional glycosylation and

pathogenicity of virus remains unclear (Banks and Plowright, 2003) but some study suggested that the presence of additional glycosylation due to adaptation of infection from waterfowl to poultry (Perdue and Suarez, 2000). It might be implicated that Thai H7N6 preferentially circulated in duck or waterfowl.

In sentinel bird model, there were 45% (9/20) seropositive ducks for IAV infection by NP-ELISA, but no IAV was isolated during 2 months of monitoring program. The subtype of IAV infection could not be identified due to the limited IAV subtypes used in our HI test. NP-ELISA is highly sensitive to detection of all subtype of IAV infection while HI test is subtype specific test. However, NP-ELISA can use as screening method for IAV detection, then the positive ELISA sera should be confirmed IAV subtype infection by HI test (Marche and van den Berg, 2010). The sentinel ducks were seropositive with no any clinical signs and no any influenza isolation. The sentinel ducks might be infected with low load or low pathogenicity of IAV lead to lack of viral shedding. The possible routes of IAV transmission of sentinel ducks were indirect contact including aerosol, pond and contaminated fomites of LBM workers. The indirect exposures especially aerosol were importance mode of transmission of IAV among poultry in LBM setting (Yee et al., 2009). During 10 weeks of sentinel monitoring, No IAV was isolated in market poultry monitoring program while sentinel ducks showed seropositive of IAV infection. These results indicated that the sentinel bird could be an effective model for monitor the status of IAVs in the LBM especially by serological

study. However, the sentinel system should be conducted in the study environment over one year to increase the probability of IAV detection.

Chapter 6 Conclusions and Suggestions

In this study, the IAV infection and transmission among poultry in an LBM were investigated by one year longitudinal monitoring during March 2010-February 2011 and sentinel bird model during November 2010-January 2011. This study focused on an LBM in Phitsanulok province; northern part of Thailand. Twenty H7N6 subtype of IAVs were isolated from market poultry, Muscovy ducks, in November 2010. Moreover, the sentinel birds were seropositive for IAV infection during 2 months of monitoring program, while no IAV could be isolated in this period. These results indicated that the LBM can be the source of IAVs circulation and transmission among LBM poultry and the sentinel birds can improve the effectiveness of IAV monitoring in LBM especially by serological study.

The control measures of IAVs in LBMs need the efficient biosecurity systems. Mingling different poultry species from several sources in LBM should be avoided to reduce the risk of IAVs reassortment. All-in and all-out system of poultry and cleaning of environment with efficient disinfectant possibly keep the LBM from sustaining of IAVs circulations.

The LBMs have been identified as the sources of IAVs infection in humans and the sustainment of IAV circulation in LBM may produce the reassortment viruses due to the IAV outbreak. IAV monitoring in LBMs can provide the information about current status, evolution and transmission ability among poultry in LBM and to humans.

Therefore, the continuous monitoring of IAV in LBMs in Thailand is important for helpful the preparedness of future IAVs outbreak in Thailand.

REFERENCES

APPENDIX

VITA

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