การสำรวจเชื้อไข้หวัดใหญ่ชนิดเอในเป็ดไล่ทุ่งในภาคเหนือตอนล่างของประเทศไทย

นางสาวสุภณัฐ บุญญาภิสิทธิ์โสภา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

SURVEY OF INFLUENZA A VIRUSES IN FREE-GRAZING DUCKS IN LOWER NORTHERN PART OF THAILAND

Miss Supanat Boonyapisitsopa

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2013

Thesis Title	SURVEY OF INFLUENZA A VIRUSES IN FREE-GRAZING DUCKS IN LOWER NORTHERN PART OF THAILAND
By	Miss Supanat Boonyapisitsopa
Field of Study	Veterinary Pathobiology
Thesis Advisor	Associate Professor Kanisak Oraveerakul, D.V.M., Ph.D
Thesis Co-Advisor	Associate Professor Alongkorn Amonsin, 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 Roongroje Thanawongnuwech, D.V.M., Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Anudep Rungsipipat, D.V.M., Ph.D.)

.....Thesis Advisor

(Associate Professor Kanisak Oraveerakul, D.V.M., Ph.D)

.....Thesis Co-Advisor

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

.....Examiner

(Associate Professor Somsak Pakpinyo, D.V.M., Ph.D.)

.....External Examiner

(Associate Professor Taweesak Songserm, D.V.M., Ph.D.)

สุภณัฐ บุญญาภิสิทธิ์โสภา : การสำรวจเชื้อไข้หวัดใหญ่ชนิดเอในเป็ดไล่ทุ่งในภาคเหนือตอนล่างของประเทศไทย. (SURVEY OF INFLUENZA A VIRUSES IN FREE-GRAZING DUCKS IN LOWER NORTHERN PART OF THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร.คณิศักดิ์ อรวีระกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร.อลงกร อมรศิลป์, 52 หน้า.

เชื้อไวรัสไข้หวัดใหญ่ชนิดเอ (influenza A virus) สามารถก่อให้เกิดโรคไข้หวัดใหญ่ได้ในสัตว์หลายสายพันธุ์ ทั้งในสัตว์ปีก สัตว์เลี้ยงลูกด้วยนม รวมถึงมนุษย์ ในประเทศไทยพบว่าเป็ดไล่ทุ่งมีส่วนสำคัญในการแพร่กระจายของเชื้อไวรัสชนิดนี้ เนื่องจากเป็ดไล่ทุ่งมีการอพยพเคลื่อนย้ายที่อยู่ตลอดเวลาและมีแหล่งหากินร่วมกับนกป่าหลากหลายชนิดรวมถึงสัตว์ปีกหลังบ้านที่อาศัยในบริเวณใกล้เคียง การศึกษาวิจัยในครั้งนี้เป็นการสำรวจเชื้อไวรัสไข้หวัดใหญ่ชนิดเออย่างต่อเนื่องตลอดระยะเวลา 4 เดือนในฝูงเป็ดไล่ทุ่งจำนวน 2 ฝูง (ฝูงเอและบี) ที่เลี้ยงในเขตพื้นที่จังหวัดพิจิตรและพิษณุโลก จากผลการทดสอบตัวอย่างตรวจพบเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ 2 สายพันธุ์ในฝูงบีคือ สายพันธุ์ H4N6 จำนวน 1 ตัวอย่างจากเป็ดอายุ 13 สัปดาห์ขณะที่เลี้ยงในพื้นที่จังหวัดพิษณุโลก และสายพันธุ์ H3N8 จำนวน 5 ตัวอย่างจากเป็ดอายุ 15 สัปดาห์ขณะที่เลี้ยงในพื้นที่จังหวัดพิจิตร ซึ่งเป็ดที่เพาะแยกเชื้อไวรัสได้ดังกล่าวพบว่ามีอาการซึมและมีสารคัดหลั่งในตา การวิเคราะห์ลักษณะทางพันธุกรรมของไวรัสไข้หวัดใหญ่ที่เพาะแยกได้พบว่าเชื้อไวรัสทั้งสองสายพันธุ์เป็นไวรัสชนิดก่อโรคไม่รุนแรงและถูกจัดอยู่ในกลุ่มของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอที่แยกได้ในเขตยุโรปและเอเชีย สำหรับฝูงเอตรวจไม่พบเชื้อไวรัสไข้หวัดใหญ่ชนิดเอใดๆ ในส่วนของการตรวจหาภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่ชนิดเอพบว่าเป็ดในฝูงเอให้ผลบวกต่อการตรวจด้วยวิธี ELISA เมื่ออายุ 9 สัปดาห์ ในขณะที่ฝูงบีให้ผลบวกเมื่ออายุ 13 สัปดาห์ซึ่งตรงกับเวลาที่เพาะแยกเชื้อไวรัสได้ แต่เมื่อนำตัวอย่างที่ให้ผลบวกด้วยวิธี ELISA ไปตรวจหาภูมิคุ้มกันที่จำเพาะต่อสายพันธุ์ของเชื้อที่เพาะแยกได้ด้วยวิธี HI พบว่าผลการตรวจเป็นลบ จากผลการศึกษาวิจัยในครั้งนี้พบว่าเป็ดไล่ทุ่งเป็นแหล่งที่มีเชื้อไข้หวัดใหญ่ชนิดเอวนเวียนอยู่และมีความสามารถในการแพร่กระจายเชื้อได้ ดังนั้นการสำรวจติดตามเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในเป็ดไล่ทุ่งจะเป็นประโยชน์ต่อการวางแผนป้องกันและควบคุมเชื้อไข้หวัดใหญ่ชนิดเอที่อาจเกิดระบาดในอนาคตได้

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5375563031 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: FREE-GRAZING DUCK / INFLUENZA A VIRUS / SURVEY

SUPANAT BOONYAPISITSOPA: SURVEY OF INFLUENZA A VIRUSES IN FREE-GRAZING DUCKS IN LOWER NORTHERN PART OF THAILAND. ADVISOR: ASSOC. PROF. KANISAK ORAVEERAKUL, D.V.M., Ph.D, CO-ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 52 pp.

Influenza A viruses cause influenza in multiple species of avian and mammals, including human being. Due to the frequently movement and sharing the same habitat with wild birds and other backyard poultry, free-grazing ducks in Thailand have potential to spread influenza A viruses. In this study, a 4-months longitudinal survey of influenza A viruses was conducted in two of free-grazing duck flocks (flock A and B) raised in Phichit and Phitsanulok. Two subtypes of influenza A viruses, H4N6 (n=1) and H3N8 (n=5), were isolated from flock B at the age of 13 and 15 weeks, respectively. It noted that both virus subtypes were collected from the different location. The signs of depression and ocular discharge were observed in the virus isolated ducks. Phylogenetic analysis and genetic characterization indicated that all virus isolates were clustered in the Eurasian lineage and indicated as low pathogenic avian influenza viruses. It noted that no virus was isolated from flock A. Serological analysis showed that the seropositive ducks were detected at 9 and 13 weeks old in flock A and B, respectively. Interestingly, all virus isolated ducks were seropositive for ELISA, but none was positive in the HI test with homologous viruses. In summary, our results indicated that free-grazing ducks have potential to be the reservoir and transmitting influenza A viruses. The continued survey of influenza A virus in free-grazing ducks can be benefit for the prevention and control strategy of the next influenza outbreak.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2013	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and grateful thanks to the following individuals who helped in making this thesis possible;

First of all, Associate Professor Dr. Kanisak Oraveerakul, my advisor for his kindness support, advice and excellent instruction throughout the period of this study.

Associate Professor Dr. Alongkorn Amonsin, my co-advisor for his excellent supervision, suggestion and encouragement throughout my study period.

Dr. Pravina Kitikoon and Dr. Juthathip Kaewcharoen, my former advisors for their kindness support, constructive advice and encouragement.

Associate Professor Dr. Taweesak Songserm for his kindly help and excellent instruction of free-grazing duck study, also for his valuable suggestions and criticisms as the external committee.

Associated Professor Dr. Anudep Rungsipipat, the chairman of thesis committee and Associate Professor Somsak Pakpinyo, the member of thesis committee for their constructive criticisms and valuable suggestions.

Dr. Rachod Tantilertcharoen, Dr. Napawan Bunpapong, Dr. Nutthawan Nonthabenjenwan, Dr. Waleemas Jairak and the staffs of Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals, Faculty of Veterinary Science, Chulalongkorn University for their kindly help, support, encouragement and friendship throughout my study period.

All staffs of the Virology unit, Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University for their help and support on this study.

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

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LIST OF ABBREVIATIONS

AI	Avian Influenza
bp	base pair
cDNA	conplementary deoxyribonucleic acid
ºC	degree Celsius
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	et alibi, and other
HA	Hemagglutinin
HI	Hemagglutination inhibition test
HPAI	Highly Pathogenic Avian Influenzaμ
LPAI	Low Pathogenic Avian Influenza
M	Matrix
mg	milligram(s)
ml	milliliter(s)
μl	microliter
mM	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

CHAPTER 1
INTRODUCTION

Influenza virus is an enveloped single-stranded RNA virus in the *Orthomyxoviridae* family. This virus can be divided into three types as influenza A, B and C. Influenza A can be classified into several subtypes and causes influenza in multiple species of avian and mammals. The outbreaks of highly virulence subtype such as H5N1 avian influenza are fatal in many animal species including poultry, cats (Kuiken et al., 2004; Songserm et al., 2006[a]), dog (Songserm et al., 2006[b]), tigers (Keawcharoen et al., 2004; Thanawongnuwech et al., 2005), leopards, waterfowls and wild birds (Ellis et al., 2004) as well as in human (Peiris et al., 2004; Tran et al., 2004; Chotpitayasunondh et al., 2005; Adisasmito et al., 2013). The widespread outbreaks of H5N1 avian influenza virus can affect to the global economy and public health. Recently, early 2013, the outbreak of low pathogenic avian influenza (LPAI) virus was also reported. The H7N9 outbreak in human have been reported in many provinces of China (Li et al., 2014) and Taiwan. This virus caused severe respiratory illness and mortality in human (Gao et al., 2013; Li et al., 2014). As of February 28th 2014, 375 confirmed human cases of H7N9 influenza virus infection, including 115 deaths, were reported (WHO, 2014).

Ducks and waterfowls were documented as natural reservoirs for Influenza A viruses and had potential to spread viruses to other animal species (Hinshaw et al., 1980; Webster et al., 1992; Munster et al., 2007). Olsen et al. (2006) reported that wild ducks and waterfowls, especially the Anseriformes and Charadriiformes had highest prevalence of LPAI viruses. The wild ducks, especially mallard ducks could carry and shed highly pathogenic avian influenza (HPAI) virus without clinical or pathological signs of the disease (Keawcharoen et al., 2008; Gaidet et al., 2010). Domestic duck could also be infected with HPAI-H5N1 asymptomatically and shed virus to the contact ducks

from both oral and cloacal routes (Wibawa et al., 2014). However, some HPAI-H5N1 infected ducks showed some clinical signs such as depression, mild respiratory distress and mild diarrhea (Kwon et al., 2005).

In Thailand, wild ducks and domestic ducks are the prime suspects of the influenza A virus transmission and HPAI-H5N1 outbreaks. Domestic ducks, especially free-grazing ducks are suspected as the reservoir of this virus due to sharing the same habitat with backyard poultry. The previous study showed that the distribution and the number of free-grazing ducks were found to be the important risk factor of the HPAI presence in the HPAI outbreaks of Thailand (Gilbert et al., 2006). In Southeast Asia especially in Thailand, free-grazing duck production is practiced to reduce the production or the feeding cost. Most of the free-grazing ducks are egg-laying ducks, while meat ducks are rarely practiced in this system. These ducks are raised in the brooder since one-day old duckling and then are released to free range in rice paddy fields after harvesting. Ducks are free-grazing to be fed with rice, snails and insects in the fields. When they are 4-5 months old, some ducks start to lay the eggs. Some free-grazing duck flocks are moved to raise in the farm system at this age, while some flocks still freely range in the field. The ducks are raised for one and a half or two years until they almost stop laying, then they are sent to the slaughter houses. With their grazing behavior, these ducks are frequently moved from one rice field to another depend on the food source in the field.

Since free-grazing ducks can be infected with influenza virus with mild or no clinical signs and they can move from one place to the others. Free-grazing ducks have potential to spread influenza A viruses from one area to the others. The experimental study in the mallard ducks showed that ducks shed the HPAI-H5N1 viruses for 17 days after infection (Hulse-Post et al., 2005). Since the free-grazing duck's habitats are often located within or around the rural villages, the influenza A viruses

from free-grazing ducks can be cross-transmitted to backyard poultry or local wild birds. The transmission can be either by direct contact from ducks or contaminated materials such as persons, duck-transporting trucks, water (Rohani et al., 2009) and environment. It has been reported that the free-grazing ducks could shed the virus through tracheal and cloacal content and be contaminated into the water sources of the villages (Hulse-Post et al., 2005). This condition might increase the risk of virus transmission to the backyard poultry or the villagers.

At the present, about 9 million free-grazing ducks are raised in Thailand with high density in the central and lower northern parts of the country. Up to date, most of the studies of influenza A viruses in free-grazing ducks in Thailand were conducted during HPAI-H5N1 outbreaks in 2004-2008. While the data of the influenza A viruses circulating in free-grazing ducks in Thailand after the 2008 HPAI-H5N1 outbreaks are limited. The information of the current status of influenza A infection in free-grazing ducks will be benefit for the prevention and control of this diseases. In this study, 4 months-longitudinal survey of influenza A virus during December 2010 to April 2011 was conducted in free-grazing duck flocks raised in the lower northern part of Thailand. Virus isolation, identification and genetic characterization were performed. Serological analysis was also performed to determine seroconversion to influenza A viruses of free-grazing ducks. The results from the study demonstrated the current influenza A viruses status circulating in free-grazing ducks of Thailand.

Hypothesis

Multiple subtypes of influenza A viruses circulate in free-grazing ducks in lower northern part of Thailand.

Objectives of Study

1. To determine the occurrence of influenza A viruses in free-grazing duck flocks in longitudinal survey.
2. To characterize the influenza A viruses isolated from free-grazing ducks.
3. To determine the time of seroconversion to influenza A viruses of free-grazing ducks.

CHAPTER II
REVIEW LITERATURE

1. Influenza A virus

Influenza virus is an enveloped virus, belonging in the *Orthomyxoviridae* family. This virus can be divided into three types as influenza A, B and C. Influenza A virus can be differentiated from influenza B and C viruses by two proteins, the nucleoprotein (NP) and matrix (M) (Webster et al., 1992). Influenza B can infect humans and seals (Osterhaus et al., 2000), while influenza C can infect humans and pigs (Guo et al., 1983).

Influenza A virus is a spherical virus with 80-120 nm in diameter. This virus contains 8 segments of single-stranded RNA, including Polymerase Basic protein 1 and 2 gene (PB1 and PB2), Polymerase gene (PA), Hemagglutinin gene (HA), Nucleoprotein gene (NP), Neuraminidase gene (NA), Matrix gene (M) and Nonstructural protein gene (NS). These genes encode 10 proteins as, PB1, PB2, PA, HA, NP, NA, M1, M2, NS1 and NS2 (Webster et al., 1992). The functions of each encoded protein were described in table 1. Influenza virus has two types of glycoproteins on the envelope, Hemagglutinin (HA) and Neuraminidase (NA). These glycoproteins were used to classify the influenza A virus into different subtypes (Lamb and Krug, 2001). Up to date, the subtypes of influenza A viruses are now classified into 18 HA and 11 NA subtypes (Tong et al., 2013)

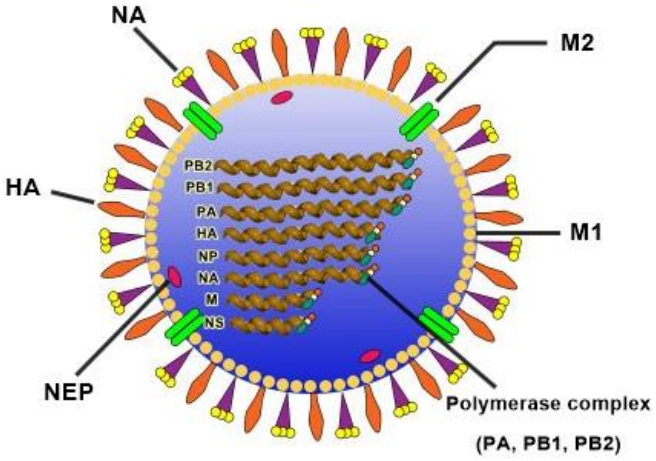

Figure 1. The structure of influenza virus

Table 1. Functions of protein encoded by 8 RNA segment of influenza A virus.

(modified from Swayne and Halvorson, 2008)

Segment	Protein	Length (bp)	Functions
1	PB2	2,341	Component of Endonuclease
2	PB1	2,341	Component of RNA transcriptase
3	PA	2,233	Viral RNA replication, Proteolytic activity
4	HA	1,778	Virus attachment to sialyloligosaccharide cell recepto, antibody mediated viral neutralization
5	NP	1,565	Cytoplasmic to nuclear protein: transport of viral RNP, and target for cytotoxic T cells
6	NA	1,413	Cell receptor-destroying enzyme and antibody-mediated virus neutralization
7	M1	1,027	Membrane protein: role in virus budding
	M2		Ion channel
8	NS1	890	Inhibit processing of cellular mRNA, enhance of cytoplasmic translation of viral mRNA, and inhibition of interferon pathways
	NS2		Nuclear export of viral RNP

Influenza A virus can infect in many species of animals, including humans with the different combinations of HA and NA subtypes. For example, the highly pathogenic influenza A virus such as H5N1 subtype can cause a severe fatal disease in many avian species (Ellis et al., 2004), also in dog (Songserm et al., 2006[b]) , cat (Kuiken et al., 2004; Songserm et al., 2006[a]) , tigers (Keawcharoen et al., 2004; Thanawongnuwech et al., 2005), leopards (Keawcharoen et al., 2004) and humans (Peiris et al., 2004; Tran et al., 2004; Chotpitayasunondh et al., 2005; Adisasmito et al., 2013)

2. Influenza A virus in waterfowls and ducks

Waterfowls and ducks were recorded as natural reservoirs of influenza A virus (Hinshaw et al., 1980; Webster et al., 1992; Munster et al., 2007). All influenza A virus subtypes, except H17, have been founded in wild waterfowls, particularly in migrated waterfowls (Webster et al., 1992). Low pathogenic avian influenza (LPAI) could be isolated from 105 wild bird species of 26 families (Olsen et al., 2006). H1 to H12 subtypes of influenza A virus could be isolated from the migrating wild ducks, while H1 to H13 subtypes could be isolated from the shorebirds (Krauss et al., 2004). The dabbling ducks in the genus *Anus*, particularly the mallards were found to be infected with influenza more frequently than other birds, including the diving ducks (Olsen et al., 2006).

As the reservoir of influenza A viruses, ducks showed no clinical signs after HPAI (H5N1) infection (Shortridge et al., 1998). Even though, the HPAI-H5N1 replicated in several organs of the experimental infected ducks, the ducks did not show any clinical signs during the observation period. However, some of infected ducks showed neurological signs such as blindness and head-shaking due to the lesions in brain (Kishida et al., 2005).

Since late 2002, some HPAI-H5N1 viruses were reported causing a severe fatal disease in wild birds and waterfowls (Ellis et al., 2004). This evidence raised a question

whether the waterfowls and ducks could still be the reservoir of influenza virus. From previous study, twenty-three H5N1 influenza virus isolates were inoculated in the juvenile mallard ducks. Eight from twenty-three virus isolates produced mild clinical sign (low pathogenic characteristics), but the virus could be transmitted to the contact ducks (Sturm-Ramirez et al., 2005). In another experimental HPAI-H5N1 infection in wild ducks, two of diving ducks in the genus *Aythya* showed difficulty breathing, severe neurological signs and died. In contrast, the infected dabbling ducks in the genus *Anus* showed virus shedding without any clinical signs. These findings indicated that the wild dabbing ducks had potential to transmit HPAI-H5N1 virus to the other species. In addition, the mallard had a potential to carry the HPAI virus for long-distance since it was the only species that showed viral shedding without clinical or pathological signs of the disease (Keawcharoen et al., 2008).

3. Influenza A viruses in free-grazing ducks

Free-grazing ducks in Thailand are domestic ducks raised by free ranging in the rice paddy fields. These ducks frequently move from one harvested rice-field to another. Mostly, free-grazing ducks are laying ducks such as, Khaki Campbell or cross-breed of Khaki Campbell and native breed of laying ducks. However, some of free-grazing ducks are raised for meat such as, Pekin, Cherry Valley or male laying ducks (Songserm et al., 2006[c]). Size of free-grazing duck flock can be ranging from 800 to 10,000 ducks. The one-day old ducklings are raised in the brooder until 3 weeks old, then move to freely range in rice fields until at one and a half or two years old. According to the free-grazing ducks database of Thailand, Department of livestock development (DLD) in 2011, there are approximately 9 million free-grazing ducks in Thailand. Most of free-grazing ducks are distributed in the central and lower northern part of Thailand, such as Suphanburi, Phichit, Phitsanulok, Ang Thong and Kamphaeng Phet.

Since the HPAI H5N1 outbreaks in poultry of Thailand, free-grazing ducks were suspected as the risk factor of HPAI viruses. The distribution and density of free-grazing ducks were found associated with the occurrence of HPAI from the previous outbreaks (Gilbert et al., 2006). HPAI infection in poultry was commonly found in the central and northern part of Thailand (Tiensin et al., 2005). The density of free-grazing ducks was also high in these areas. From the previous studies, the density of free-grazing ducks was associated with the rice agriculture system. The areas produced two-crops per year as in the central and northern parts of were found high density of free-grazing ducks, since feeding in the field were available all year (Gilbert et al., 2006; Gilbert et al., 2007).

According to Thailand's national surveillance of influenza virus in 2004, cloacal swabs were collected from sixty-one free-grazing duck flocks for virus isolation. The result showed that twenty-eight flocks (45.9%) were detected for H5N1 influenza virus infection. Ten free-grazing ducks flocks in 3 provinces of the central part of Thailand were selected for the further study of H5N1 infection. The result showed that H5N1 influenza virus was detected in all flocks, but less than 1% of infected ducks were showed clinical signs. This H5N1 infection was detected only after the ducks were moved to the rice fields. None of free-grazing ducks infected with H5N1 while they were in the brooders (Songserm et al., 2006[c]).

In 2010, sero-surveillance of free-grazing ducks in Suphanburi showed that 97% (194/201) of free-grazing duck flock were positive for antibodies against influenza A virus by NP-ELISA. 85% (5305/6254) of ducks were positive for influenza A antibodies. From the NP-ELISA positive sera, 39% (553/1423) and 4% (57/1423) were positive and suspected for H5 ELISA, respectively. 12% (74/610) of positive/suspect sera had HI (H5) titer more than 1:20 (Beaudoin et al., 2012). It is interesting that no HPAI-H5N1 outbreak in Thailand since 2008.

Up to date, most of the study of influenza A virus in free-grazing duck were concentrated on HPAI-H5N1 and conducted during the HPAI-H5N1 outbreaks. The information about the other influenza A viruses, both HPAI and LPAI, in free-grazing ducks of Thailand are limited. The information about the current subtype of influenza A virus circulated in free-grazing ducks would be benefit of the preparedness for the next influenza outbreaks, in terms of the prevention and control strategies.

CHAPTER III

MATERIAL AND METHODS

This study was divided into 3 parts. The first part was the sample collection from free-grazing ducks raising in the influenza high risk areas. The second part was the virus isolation, identification and genetic characterization of influenza A viruses isolate from free-grazing ducks. The third part was the serological analysis to detect influenza A viruses antibody. The overall process of this study is showed in figure 2.

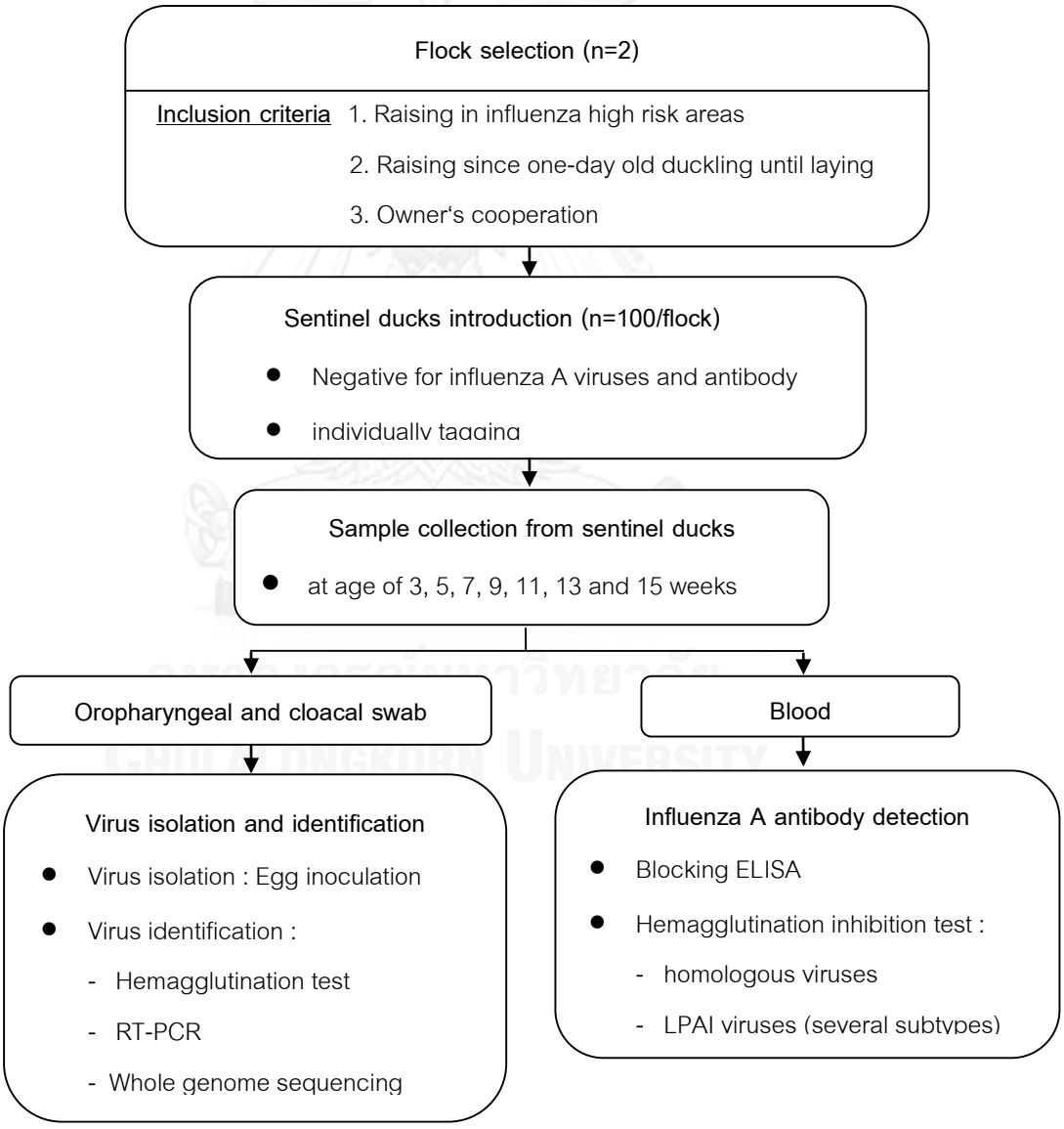

Figure 2. Conceptual framework of this study

Part 1: Sample collection from free-grazing ducks raising in the influenza high risk areas

A longitudinal survey were conducted in two selected free-grazing duck flocks. One hundred sentinel ducks were added into each original flock as the representative for sample collection.

1.1 Free-grazing duck flock selection

In this study, two flocks free-grazing ducks raised in Phichit and Phitsanulok were selected. The number of ducks was 2500 and 4000 ducks per flock. The flock selection criteria are 1) raising in the influenza high risk areas 2) raising since one-day old ducklings until laying and 3) participation of the flock's owner. These selected flocks were raised since one-day old duckling in the backyard stable, then moved for grazing when reached 3 weeks old. In the field, the ducks grazed in the harvested rice-paddy fields and stayed in the movable pen near the grazing field at night with 2-3 caretakers. Both flocks were frequently moved to the new rice fields in Phichit and Phitsanulok areas within 5-7 days by rental trucks, except the first two months of age that rarely moved.

1.2 Sentinel ducks introduction

In this study, one hundred of 2-weeks old ducks were added into each flock as the sentinel ducks when the ducks of the original flocks were at the same age. These sentinel ducks were monitored to represent the ducks in the flock and used for sample collection. Before adding into the original flocks, all sentinel ducks were sampled for oro-pharyngeal swabs, cloacal swabs and blood samples. Swabs samples were tested for influenza A virus by realtime-reverse transcriptase PCR (rRT-PCR) test and serum samples were tested for influenza A virus specific antibody by blocking Enzyme-Linked Immunosorbent Assay, *ELISA* (FlockChek® AI MultiS-Screen Ab Test Kit, IDEXX Laboratories, USA). The sentinel ducks must be negative for influenza A virus and

influenza A antibody. Each sentinel duck was marked with individually number at the right leg to differentiate them from the ducks in original flocks (Figure 3).

Figure 3. The individually number marker attached to the right leg of sentinel ducks. Sentinel ducks can be differentiated from the ducks in original flocks.

1.3 Sample collection

Oro-pharyngeal swabs (OP), cloacal swabs and blood samples were collected from all sentinel ducks every 2 weeks since start grazing at 3, 5, 7, 9, 11, 13 and 15 weeks old (figure 4). Swabs were collected in the sterile tube contained 2 ml viral transport media (Brain-Heart Infusion broth supplemented with Penicillin G 1,000 U/ml, Streptomycin 1 mg/ml, Gentamycin 0.25 mg/ml and Kanamycin 0.5 mg/ml) and kept on ice until reach the laboratory. Two ml of blood samples were also obtained from jugular vein and collected in the sterile 2.2 ml-microtubes. Swab samples were processed within 24 hours after collected at the Biosafety level 3 laboratory. Viral transport media of swab samples were aliquot and kept at -80°C until use for virus isolation. Blood samples were centrifuged at 3,000 g for 10 minutes to separate sera then kept at -20°C for the next process.

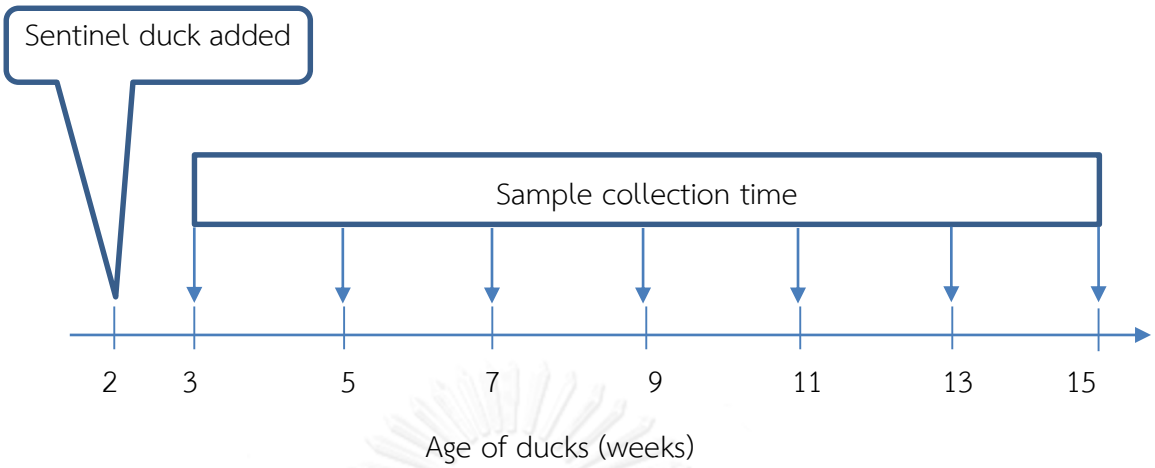

Figure 4. Sample collection timeline of this study

Part 2: virus isolation, identification and genetic characterization of influenza A viruses isolated from free-grazing ducks.

2.1 Virus isolation

All swab samples were subjected for influenza A virus isolation by egg inoculation method. Three of 9 to 11 day-old, specific antibody negative-embryonated chicken eggs were used for each sample. Two hundred µl of suspension from swab samples were inoculated into each embryonated eggs by the allantoic route (WHO, 2002). The inoculated eggs were incubated at 37°C for 48 hours. After incubation period, the eggs were chilled at 4°C overnight, then harvested for allantoic fluid. The harvested allantoic fluid was tested by Hemagglutination (HA) test (WHO., 2002). The serial 2-fold dilutions of allantoic fluid were mixed with 1% of chicken red blood cell. After one hour of incubation period, the sample that showed hemagglutination was interpreted as positive and the highest dilution that showed complete hemagglutionation was considered as HA titer. The HA positive samples were stored at -80°C until the next process.

2.2 Virus identification

2.2.1 RNA extraction

The HA-positive samples were extracted for RNA by a commercial RNA extraction kit, Nucleospin® RNA virus (Macherey-Nagel, Germany). In brief, 600 µl of lysis buffer containing RNA carrier was added into 150 µl then incubated at 70°C for 5 minutes to lyse virus. 600 µl ethanol was added to clear lysis solution then loaded on silica gel membrane column then centrifuged at 8,000 g for 1 minute to bind viral RNA. The wash buffers were added to wash silica membrane including, 500 µl Buffer RAW then centrifuged at 8,000 g for 1 minute, 600 µl RAV3 then centrifuged at 8,000 g for 1 minute, and 200 µl then centrifuged at 11,000 g for 5 minutes for dry silica membrane. The flow through liquid was discard in every step. The final step was elution of viral RNA by place the column in 1.5 ml micro-centrifuge tube then add 50 µl of pre-heated RNase-free water into column. After centrifuged at 11,000 g for 1 minute, the column was discarded and the flow through contained viral RNA was kept at -20°C for the next process.

2.2.2 Realtime reverse transcriptase polymerase chain-reaction (rRT-PCR)

Realtime RT-PCR was performed to detect Matrix (M) gene of influenza A virus (Spackman et al., 2002). In brief, a single step was perform by using one-step quantitative rRT-PCR system (Invitrogen®, USA). Total 12.5 µl mixture of each PCR reaction contained 1x Master Mix (Invitrogen®), 0.25 µl Superscript III RT, 0.4 µM of specific primer for M gene, 4 µl of RNA, 0.1 µM of probe, 0.08 µl of $MgSO_4$,and 0.42 µl of Distilled water. rRT-PCR was performed by using the rRT-PCR thermo cycler with the set conditions as reverse transcription at 50ºC for 30 minutes, initial denaturation at 95ºC for 15 minutes, then denaturation for 50 cycles at 95ºC for 15 seconds and annealing-extension at 60ºC for 30 seconds. The sample with cycle threshold (Ct) value less than 36 was considered positive for M gene of influenza A virus which also

mean positive for influenza A virus. The sample with Ct value more than 40 was considered negative. However, the sample with Ct value between 36 and 40 was suspected sample that need to propagate sample for next passage and re-test.

2.2.3 cDNA synthesis

The viral RNA of influenza A virus isolates were reverse transcribed into cDNA (Viseshakul et al., 2004). In brief, the cDNA synthesis was performed by prepared a mixture of 4 µl of viral RNA and 4 µl of random primer. The mixture in PCR tube was loaded into Thermal cycler with the set conditions as 70ºC for 15 minutes, then 4ºC for 5 minutes. Total 12 µl of mixture of reverse transcriptase enzyme was prepared with 1 µl of Impromp II™ reverse transcriptase, 4 µl of Impromp II™ 5x buffer, 2.5 mM of $MgCl_2$, 0.3 µl of RNase inhibitor, 1 µM of dNTP and 2.7 of distrilled water, then added in the mixture of RNA and random primer. The mixture for cDNA synthesis was loaded into the thermo cycler with the set condition at 25ºC for 5 minutes, 42ºC for 60 minutes, and 72ºC for 15 minutes. The cDNA were stored at -20ºC for the next process.

2.2.4 Virus subtype identification

A multiplex RT-PCR was performed to identify subtype of virus, using the primers specific for influenza A virus H1 to H15 and N1 to N9 as previous described (Tsukamoto et al., 2008; Tsukamoto et al., 2009). In brief, each PCR mixture contained 1x KAPA Taq Master Mix, 0.47 µM of each specific primers, 0.7 µl of cDNA and 5.4 µl distilled water. The PCR conditions were set as the initial denaturation at 94ºC for 3 minutes, then denaturation at 94ºC for 30 seconds, annealing at 50ºC (for H1-H15) or 46ºC (for N1-N9) for 30 seconds, extension at 72ºC for 30 seconds, and final extension at 72ºC for 7 minutes.

Gel electrophoresis was used to examine PCR products. 1.2% of agarose gel in 0.5x Tris Borate EDTA (TBE) was used as gel for loading 4 µl of PCR product. DNA was separated in agarose gel in 100 voltage for 40 minutes. Agarose gel was bathed

in 0.5 µg/µl Ethidium bromide solution and read the result under Ultraviolet light of Gel documentation system.

2.2.5 Virus characterization

After subtype identification, whole genome sequencing of virus isolate was conduct. PCR amplification was performed to amplify each gene segment (PB2, PB1, PA, HA, NP, NA, M and NS) of isolated viruses, using previous design specific primers (Amonsin et al., 2006). PCR products were examined by agarose gel electrophoresis as previous described in the subtype identification process. These products were purified by using a commercial kit, Nucleospin® PCR clean up and gel extraction (Macherey-Nagel, Germany). The pure PCR products were submitted for DNA sequencing at Ward Medic Ltd., part. in Malaysia.

The nucleotide sequences of each gene were assembled by using Seqman program software v.5.03 (DNASTAR Inc., Madison, WI, USA). Nucleotide sequence of each gene was compared similarity with the reference nucleotide sequence in GenBank database by using nucleotide BLAST tool of GenBank database. Phylogenetic analysis was performed using MEGA v.6 (Tamura et al., 2007). The deduced amino acid sequence was compared and aligned by using MegAlign program v.5.03 (DNASTAR Inc., Madison, WI, USA).

Part 3: the serological analysis to detect influenza A antibody

After separated sera from blood samples, sera were tested for antibodies against influenza A virus by using blocking ELISA. The ELISA positive and suspected sera were subjected for Hemagglutination inhibition (HI) test with several subtype of LPAI antigen.

3.1 Blocking Enzyme-Linked Immunosorbent Assay (ELISA)

Sera were tested for antibodies against NP protein of influenza A virus by using blocking *ELISA* (FlockChek® AI MultiS-Screen Ab Test Kit, IDEXX Laboratories, USA)

following commercial procedure. Briefly, each samples was diluted tenfold (1/10) with sample diluent and was added into each well of the avian influenza (AI) viral antigen-coated plate, including negative and positive control. The plate was incubated at room temperature for 60 minutes for AI-specific antibody forming complex with the coated antigen then wash with 1x wash buffer. Anti-AI: Horseradish Peroxidase conjugate was added, then incubated at room temperature for 30 minutes. After wash, TMB substrate solution was added, then incubated at room temperature for 15 minutes. Stop solution was added, then solution of each well was measure the absorbance value at A650 nm by ELISA reader. S/N ratio of each sample was calculated by compared with the negative control. The sample with S/N ratio $\geq$ 0.7 considered as negative. The sample with S/N ratio between 0.6 and 0.7 considered as suspected. The sample with S/N ratio <0.6 considered as positive (Brown et al., 2009).

3.2 Hemagglutination Inhibition (HI) test

The ELISA positive and suspected sera were subjected for HI test with several influenza subtypes isolated from avian species in Thailand including, H3 (A/duck/Thailand/CU-7518C/2010), H4 (A/muscovy duck/Thailand/CU-LM1983/2009), H5 (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004), H7 (A/duck/Thailand/CU-LM7279T/2010) subtypes and the homologous viruses isolated in this study (WHO, 2002). Before performed HI test, sera were treated to remove non-specific inhibitor by heat inactivation at 56 ºC for 30 minutes, 20% Kaolin for 30 minutes and 50% RBC for 60 minutes. In brief of HI test, 25 µl of each treated serum was 2-fold diluted with PBS in 96-well plate (V-bottom). 25 µl of 4HAU virus was added to each well and incubated at room temperature for 60 minutes. 50 µl of 1% chicken RBC was added into each well and incubated at room temperature for 60 minutes. For interpretation, the HI titer was the reciprocal of the last dilution that completely inhibit the hemagglutination. The serum with HI titer more than 40 was considered positive.

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CHAPTER IV
RESULTS

A longitudinal survey of influenza A viruses in free-grazing ducks was conducted during December 2010 – April 2011. OP swabs, cloacal swabs and blood samples were collected from 200 sentinel ducks for 4 months. Swab samples were subjected for influenza A virus isolation and identified for HA activity. Realtime RT-PCR was used to confirm influenza A virus (M gene). The virus isolates were subtyped by RT-PCR and characterized by gene sequencing. In addition, serum samples were tested for antibody against influenza A virus by blocking ELISA.

1. Samples collection from sentinel free-grazing ducks

In this study, two flocks of 2-weeks old free-grazing ducks (flock A and B) raised in Phichit and Phitsanulok were selected. These two provinces are the influenza highly risk areas due to the history of the avian influenza outbreaks and the high density of free-grazing ducks. One hundred of 2-weeks old duckling were added in each flocks as the sentinel ducks. Before mingling, all one hundred sentinel ducks were tested for influenza A virus by rRT-PCR (M gene) in swab samples and antibody against influenza A virus by blocking ELISA in serum samples. The results showed that none of sentinel ducks were positive for influenza A virus or antibody before added into the original flocks.

OP swabs , cloacal swabs and blood samples were collected from the sentinels ducks since the ducks start grazing at 3, 5, 7, 9, 11, 13 and 15 weeks of age. At start, samples were collected from total one hundred sentinel ducks of each flock, then the sentinels gradually lost or died. The number of sentinel ducks in both flocks in each collection time were showed in Table 2. It noted that at the week of 13, approximately 60% of total ducks in flock B gradually sick and died due to the severe rainstorm in the raising area. Some of sentinel ducks showed clinical signs such as depressed,

ocular discharge. However, the necropsy of the dead ducks was not performed. In total 1,809 samples (603 of each OP swab, clocal swab and serum samples) and 1,311 samples (437 of each OP swab, clocal swab and serum samples) were collected from sentinel ducks of flock A and B respectively (Table 3).

Table 2. Total number of sentinel ducks in each collection time

Age of ducks	Number of sentinel ducks	
	Flock A	Flock B
3 weeks	100	100
5 weeks	99	86
7 weeks	83	83
9 weeks	84	70
11 weeks	75	56
13 weeks	81	23
15 weeks	81	19
Total	603	437

Table 3. Total number of samples collected in longitudinal follow up of 2 free-grazing duck flocks

Age of ducks	Number of sample collected							
	Flock A				Flock B			
	OP	C	Blood	Total	OP	C	Blood	Total
3 weeks	100	100	100	300	100	100	100	300
5 weeks	99	99	99	297	86	86	86	258
7 weeks	83	83	83	249	83	83	83	249
9 weeks	84	84	84	252	70	70	70	210
11 weeks	75	75	75	225	56	56	56	168
13 weeks	81	81	81	243	23	23	23	69
15 weeks	81	81	81	243	19	19	19	57
Total	603	603	603	1809	437	437	437	1311

2. Influenza A virus isolation and identification

In this study, total 1,206 and 874 of swab samples from flock A and B were subjected for influenza A virus isolation by egg inoculation. HA test was further performed to confirm the HA activity of the virus. The HA test result showed that 9 samples from 3, 13 and 15 weeks old ducks of flock B were positive. Out of 9 HA positive samples, 6 samples were positive for influenza A viruses by using rRT-PCR (M gene) assay confirming the present of influenza A virus. In total 6 virus isolated from flock B, 1 virus was isolated from OP swab of 13 weeks old duck. The other 5 viruses were isolated from OP swab (n = 2) and cloacal swabs (n = 3) of 15 weeks old duck (Table 5). It noted that all virus isolates were isolated from the different ducks as showed in table 4. It is noted that, none of virus was isolated from the sentinel duck of flock A.

Table 4. Total HA positive samples and influenza A virus isolates

Age of ducks	Flock A			Flock B		
	Total sample tested	HA positive	Influenza A viruses	Total sample tested	HA positive	Influenza A viruses
3 wks	200	0	0	200	3	0
5 wks	198	0	0	172	0	0
7 wks	166	0	0	166	0	0
9 wks	168	0	0	140	0	0
11 wks	150	0	0	112	0	0
13 wks	162	0	0	46	1	1
15 wks	162	0	0	38	5	5
Total	1,206	0	0	874	9	6

Table 5. Description of influenza A virus isolated in this study

Age	Sentinel duck ID	Sample ID	Type of specimen	Month	Year	Location	Subtype
13 wks	B056	CU-11655T	OP	March	2011	Phichit	H4N6
15 wks	B015	CU-11671C	C	April	2011	Phitsanulok	H3N8
	B042	CU-11676C	C	April	2011	Phitsanulok	H3N8
	B071	CU-11679T	OP	April	2011	Phitsanulok	H3N8
	B087	CU-11682C	C	April	2011	Phitsanulok	H3N8
	B099	CU-11686T	OP	April	2011	Phitsanulok	H3N8

For virus subtyping, the result showed that the virus isolate from 13 weeks old duck of flock B was identified as influenza A subtype H4N6 (table 5, figure 5 and 6). The other five viruses from 15 weeks old ducks of flock B were identified influenza A subtype as H3N8 (table 5, figure 7 and 8).

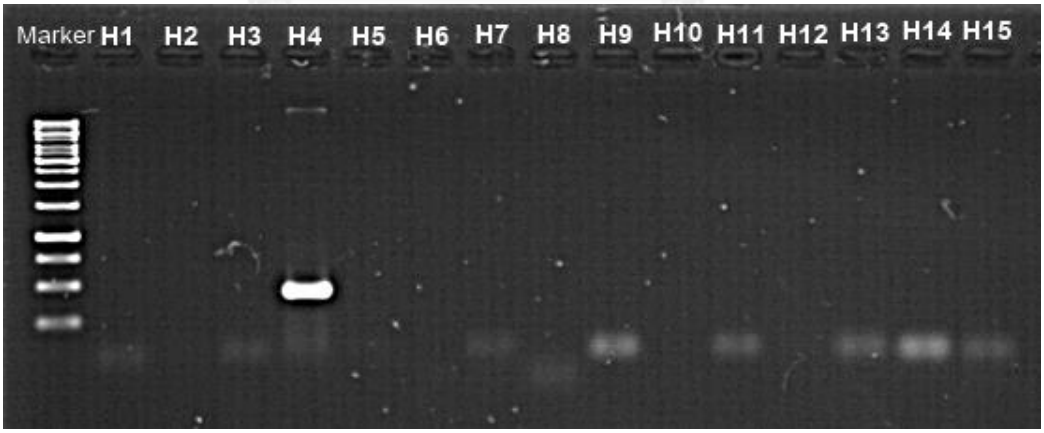

Figure 5. Subtype identification of CU-11655T by HA subtype specific primer. The virus isolate was positive for H4 subtype.

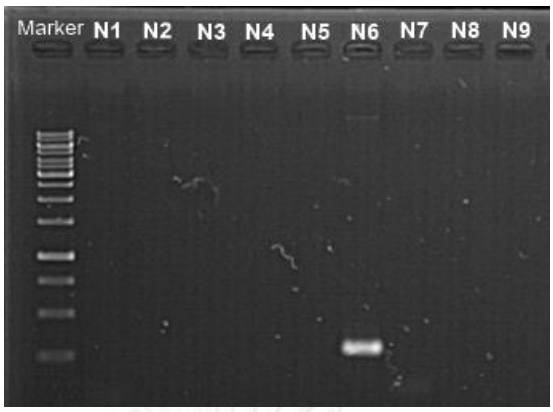

Figure 6. Subtype identification of CU-11655T by NA subtype specific primer. The virus isolate was positive for N6 subtype.

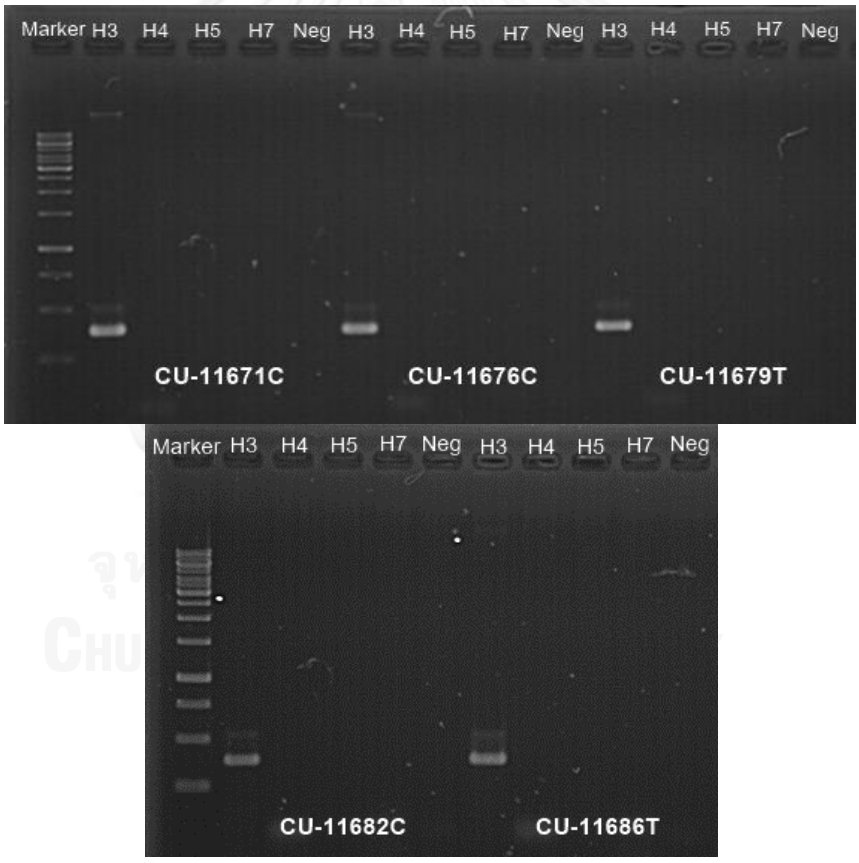

Figure 7. Subtype identification of CU-11671C, CU-11676C, CU-11679T, CU-11682C and CU-11686T by HA subtype specific primer. These virus isolates were positive for H3 subtype.

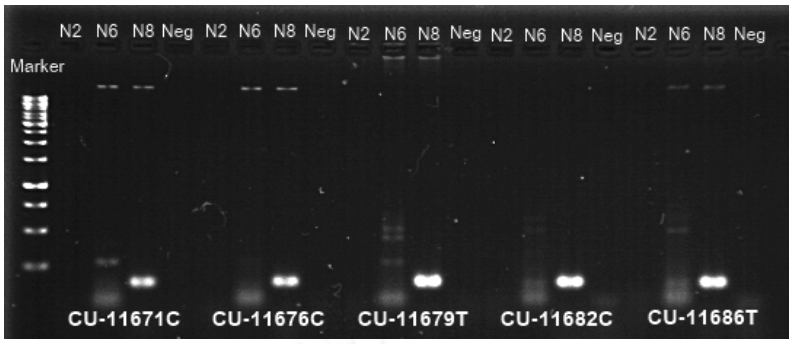

Figure 8. Subtype identification of CU-11671C, CU-11676C, CU-11679T, CU-11682C and CU-11686T by NA subtype specific primer. These virus isolates were positive for N8 subtype.

3. Whole genome sequencing of influenza A viruses

After subtype identification, whole genome sequencing was performed by using previous design specific primers for each gene of influenza A virus. In this study, one of H4N6 isolate and 2 from 5 of H3N8 isolates were selected for whole genome sequencing. Table 6 show the nucleotide position of each gene of influenza A virus isolates sequenced in this study.

Table 6: Nucleotide position of each gene of influenza A virus isolates in this study

Sample ID	Subtype	Gene (position)							
		PB2	PB1	PA	HA	NP	NA	M	NS
CU-11655T	H4N6	23-2280	11-2274	21-2151	22-1634	1-1520	14-1403	22-959	1-845
CU-11671C	H3N8	69-2192	41-2274	21-2166	31-1698	1-1475	7-1364	17-945	22-818
CU-11676C	H3N8	69-2262	38-2274	22-2151	62-1699	1-1462	4-1391	25-961	22-832
CU-11679T	H3N8	N/A	N/A	N/A	47-1698	N/A	13-1391	N/A	N/A
CU-11682C	H3N8	N/A	N/A	N/A	30-1697	N/A	4-1391	N/A	N/A
CU-11686T	H3N8	N/A	N/A	N/A	35-1698	N/A	13-1391	N/A	N/A

4. Genetic characterization of influenza A viruses isolated in this study

4.1 Genetic characterization of influenza A virus subtype H4N6

The H4 gene of influenza A H4N6 (CU-11655T) was sequenced and then compared with the H4 gene of reference influenza viruses in the GenBank database. Nucleotide sequences of influenza A (H4 subtype) from both Eurasian and North American linages were included for phylogenetic analysis (Figure 9). The result showed that CU-11655T was grouped in the Eurasian linage (figure 9).

Genetic similarity of H4 gene of the virus was analyzed, nucleotide sequences of H4 gene of CU-11655T was compared with those of the reference H4 gene in the GenBank database by using the NCBI nucleotide BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST result showed that CU-11655T has the highest percent nucleotide similarity to A/wild duck/Korea/CSM20-5/2009(H4N6) at 98% nucleotide identity (Table 7). This virus and the H4N6 isolate of this study were also grouped in the Eurasian linage.

To further analyze the genetic characteristics of HA gene, the deduced amino acids of H4 gene of CU-11655T was compared with the reference H4 gene available in the GenBank database by using MegAlign program. The result showed that the HA cleavage site sequences of CU-11655T is PEKASR, which is similar to most isolates in Eurasian lineage, while the isolates in North American lineage are PEKATR (Table 8). The CU-11655T virus does not contain the multiple basic similar to the other reference strain, which indicated low pathogenic characteristics of influenza A virus. The amino acids at the receptor binding sites were also analyzed and showed in Table 8. The amino acids at the position 226 and 288 (H3 numbering system) of the receptor binding sites were Q and G, respectively (Table 8), which were similar to the other avian strains in both Eurasian and North American lineage. These amino acids were differenced from A/swine/ON/01911-1/1999 which were Q226L and G228S.

In NA gene, phylogenetic analysis of N6 gene of CU-11655T compared with the N6 of reference influenza virus in the GenBank database was conducted. The result showed that the N6 gene of CU-11655T were clustered in Eurasian Lineage (Figure 10). The nucleotide identity of N6 gene of this virus compared with the reference N6 gene available in the GenBank database was also conducted by the NCBI nucleotide BLAST tool. The BLAST result showed that the highest nucleotide similarity to the N6 gene of CU-11655T was A/duck/Jiangxi/25134/2009(H7N6) at 99% nucleotide similarity.

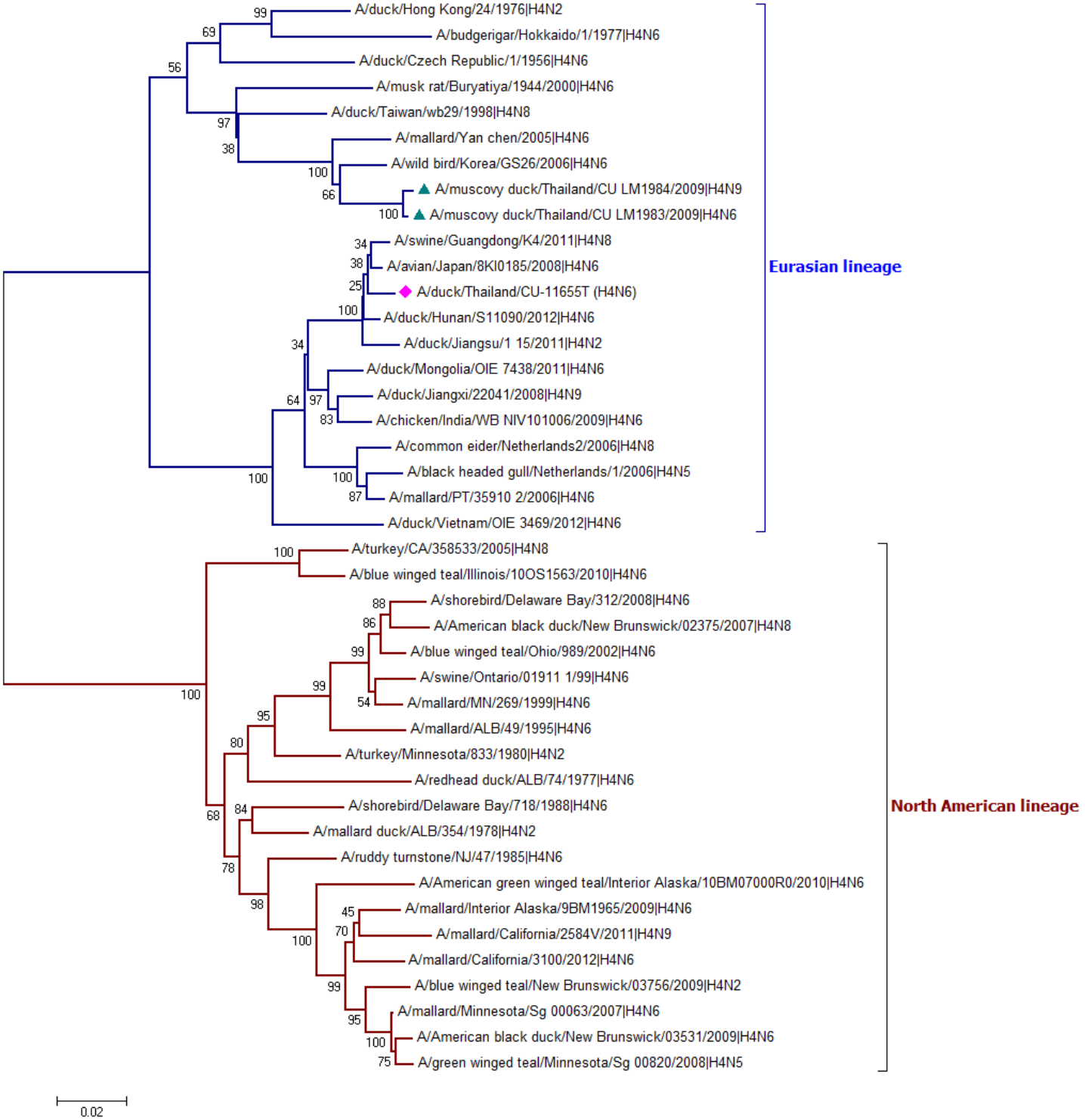

Figure 9. Phylogenetic analysis of H4 gene of H4N6 (CU-11655T) isolate in this study compared with the H4 genes in GenBank database

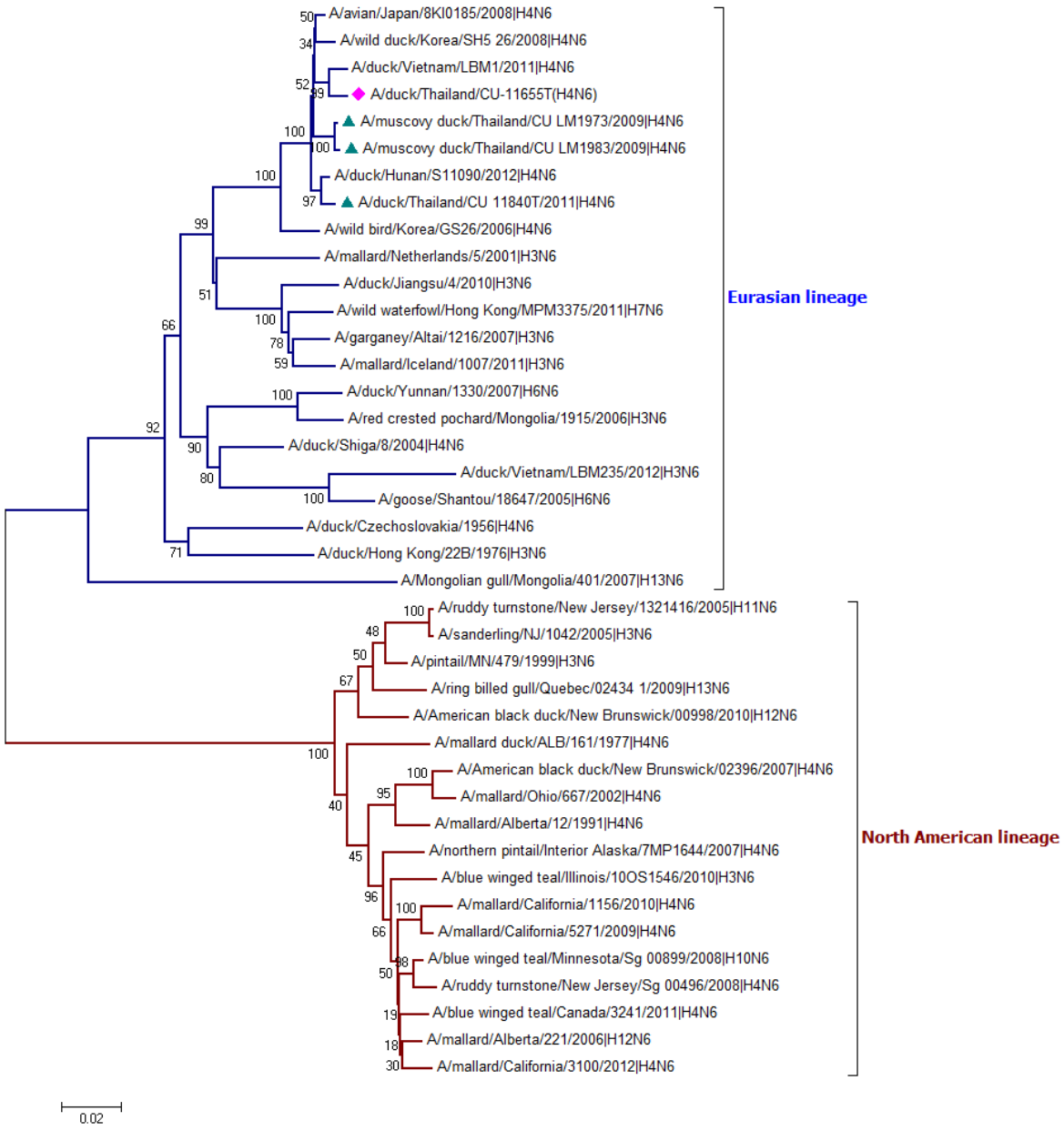

Figure 10. Phylogenetic tree of N6 gene of H4N6 (CU-11655T) isolate in this study

Table 7. Sequence homology of whole genome of CU-11655T (H4N6) isolate in this study compared to reference nucleotide sequences in GenBank database

Gene	Position	GenBank accession no.	Virus with the highest percentage of nucleotide identity	Percent nucleotide identity
PB2	23-2280	KJ161954.1	A/duck/Thailand/CU-11869C/2011(H1N9)	99%
PB1	11-2274	JN029621.1	A/pintail/Mongolia/2-65/2007(H3N8)	99%
PA	21-2151	JN244181.1	A/wild bird/Korea/A9/2011(H7N9)	99%
HA	42-1653	JX454698.1	A/wild duck/Korea/CSM20-5/2009(H4N6)	98%
NA	14-1403	KF259615.1	A/duck/Jiangxi/25134/2009(H7N6)	99%
NP	1-1565	JX465641.1	A/aquatic bird/Korea/CN5/2009(H6N5)	99%
M	22-959	JX236007.1	A/wild bird/Korea/A278/2009(H5N2)	99%
NS	12-878	JX454757.1	A/wild duck/Korea/SH5-60/2008(H4N6)	99%

Table 8 Deduced amino acid at the HA cleavage site and receptor binding sites of the H4 isolate in this study compared with the reference H4 subtype viruses in GenBank database.

Virus name	HA cleavage	Receptor binding site							Left edge of receptor binding site					Right edge of receptor binding site				
	320-329	98	153	155	183	190	194	195	224	225	226	227	228	134	135	136	137	138
A/duck/Thailand/CU-11655T (H4N6)	PEKASR	Y	W	V	H	E	L	Y	R	G	Q	S	G	G	G	S	G	A
A/duck/Czech Republic/1/1956 (H4N6)	PEKASR	Y	W	V	H	E	L	Y	R	G	Q	S	G	G	G	S	G	A
A/budgerigar/Hokkaido/1/77 (H4N6)	PEKASR	Y	W	V	H	E	L	Y	R	G	Q	S	G	G	G	S	G	A
A/mallard/Yan Chen/2005 (H4N6)	PEKASR	Y	W	V	H	E	L	Y	R	G	Q	S	G	G	G	S	G	A
A/turnstone/NJ/47/1985 (H4N6)	PEKATR	Y	W	T	H	E	L	Y	R	G	Q	S	G	G	G	S	G	A
A/swine/ON/01911-1/1999 (H4N6)	PEKATR	Y	W	T	H	E	L	Y	R	G	L	S	S	G	G	S	G	A
A/mallard/California/2584V/2011 (H4N6)	PEKATR	Y	W	T	H	E	L	Y	R	G	Q	S	G	G	G	S	G	A

4.2 Genetic characterization of influenza A virus subtype H3N8

In this study, all five of the H3 gene of H3N8 isolates (CU-11671C, CU-11676C, CU-11679T, CU-11682C and CU-11686T) were compared with the other H3 gene available in the GenBank database. The reference influenza H3 viruses of the Eurasian lineage and North American lineage were included for phylogenetic analysis. The result showed that H3 genes of Eurasian and North American lineage were distinctively divided. All five of H3 genes from this study were clustered in Eurasian lineage (Figure 11).

Nucleotide of H3 gene of all H3N8 isolates were compared with the nucleotide of other H3 genes available in the GenBank database by using NCBI nucleotide BLAST program. The BLAST result showed that all five H3N8 isolates have the highest percent of nucleotide similarity to A/ruddy shelduck/Mongolia/1-26/2007(H3N8) at 99% nucleotide similarity.

To further analysis, the genetic characteristics of HA gene was conducted. The deduced amino acid of H3 gene of all H3N8 isolates were compared with the reference H3 gene in the GenBank database by using MegAlign program. The alignment result showed that the HA cleavage site sequences of all H3N8 isolates in this study are PEKQTR (Table 10) similar to the other avian influenza virus in Eurasian and North American lineages. It is noted that all H3N8 isolates in this study showed no addition of multiple basic amino acids at the HA cleavage site. Furthermore, the amino acids at the receptor binding sites were also analyzed and showed in table 10. The amino acids at the receptor binding sites were similar to all H3 of avian influenza in both Eurasian and North American lineages, especially Q226 and G228 (Table 10).

In NA gene, phylogenetic tree was constructed compare with the nucleotide of the reference N8 genes in the GenBank database. The result showed that the N6 gene of CU-11655T were clustered in Eurasian Lineage (Figure 12). Nucleotide of N8 gene of all H3N8 isolates were compared with the nucleotide of other N8 genes available in the GenBank database by NCBI nucleotide BLAST tool. The BLAST result showed CU-11676C, CU-11679T, CU-11682C and CU-11686T have the highest percent nucleotide similarity to A/mallard/Sweden/80057/2008(mixed) at 97% nucleotide identity (Table 9). Only CU-11671C was different from the other isolates, which has the highest nucleotide similarity to A/velvet scoter/Mongolia/879V/2009(H10N8) at 97% nucleotide identity (Table 9). Both highest nucleotide similarity viruses and all H3N8 isolates in this study were the avian influenza viruses in Eurasian lineage.

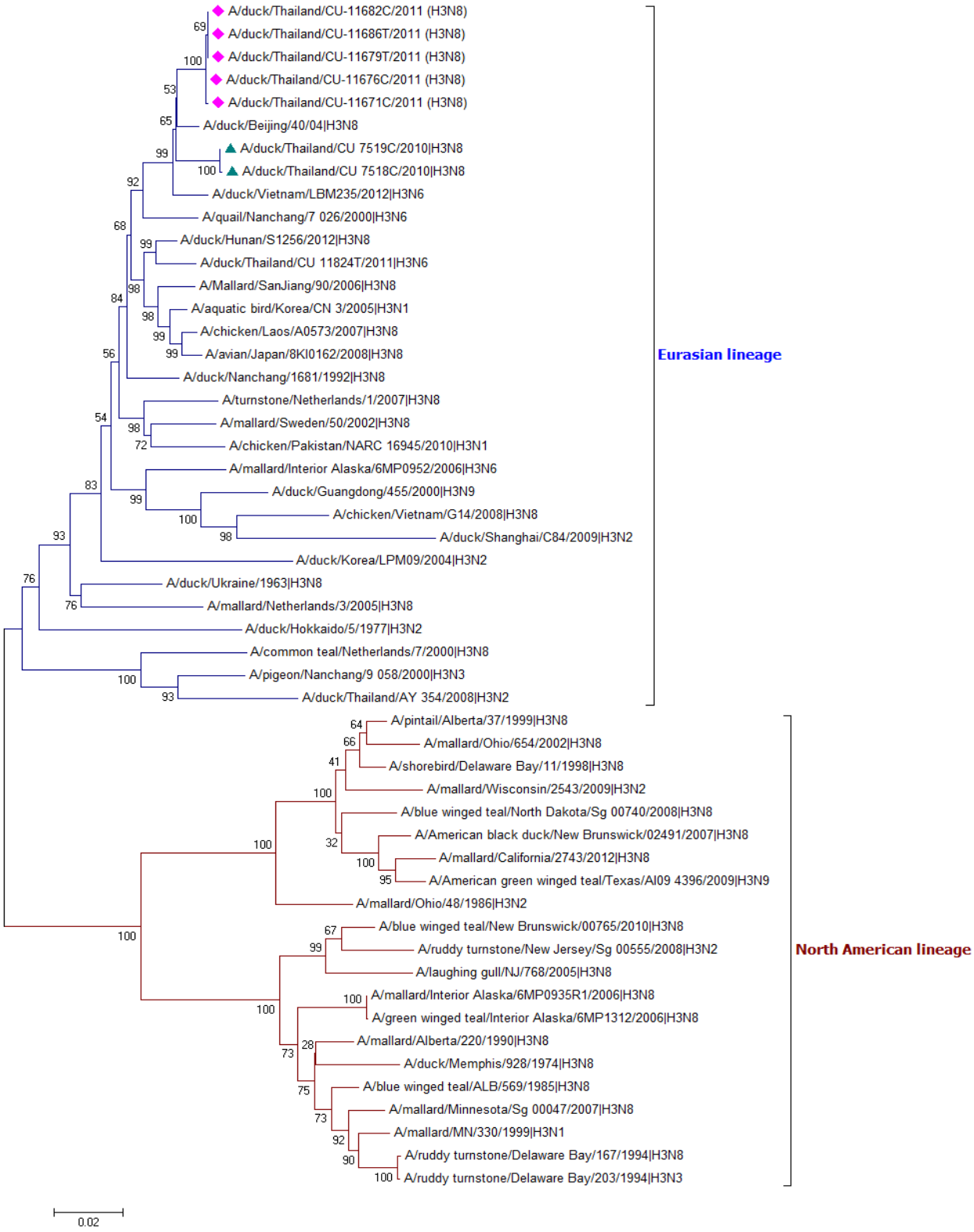

Figure 11. Phylogenetic analysis of H3 gene of CU-11671C, CU-11676C, CU-11679T, CU-11682C and CU-11686T

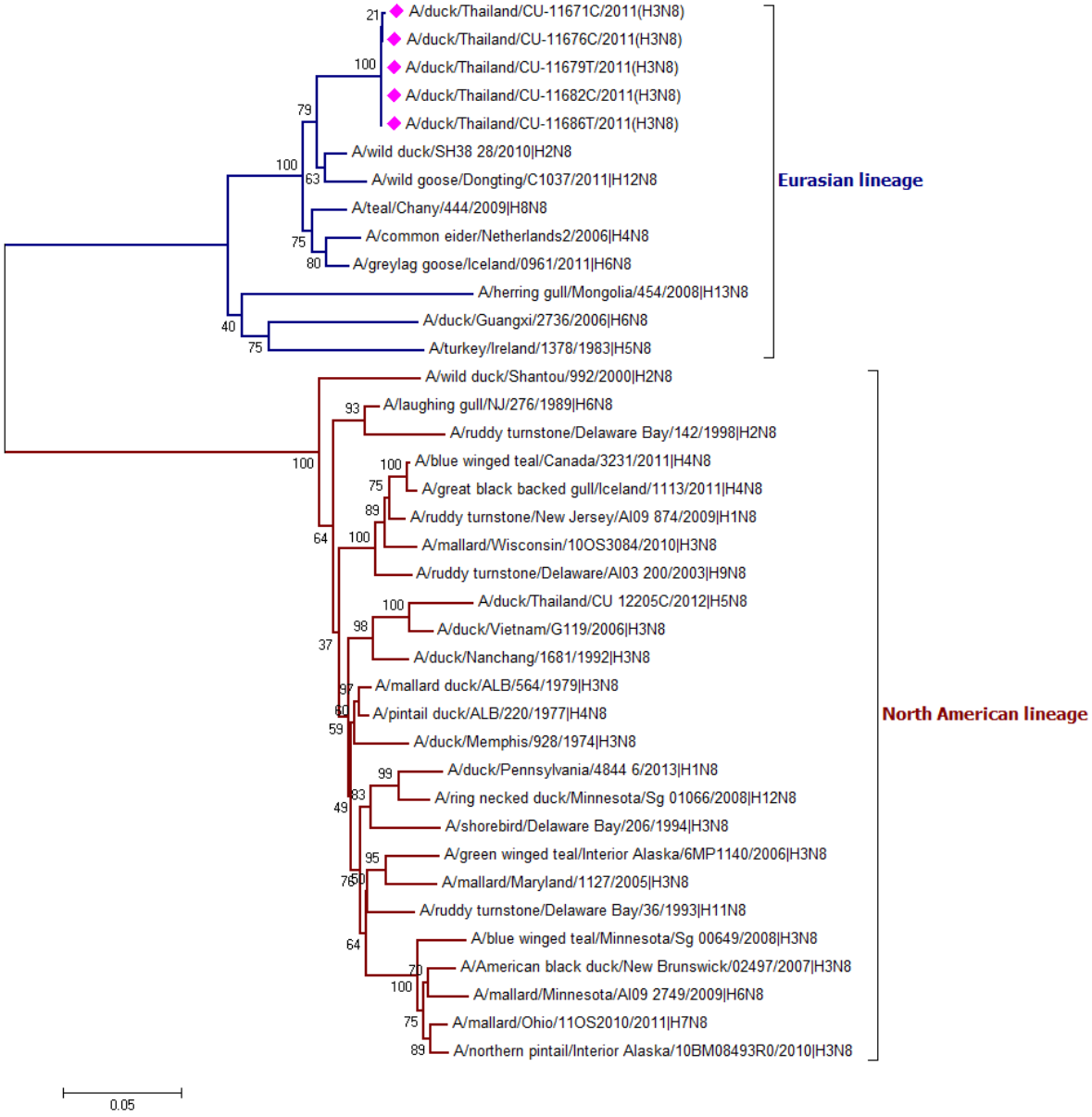

Figure 12. Phylogenetic analysis of N8 gene of CU-11671C, CU-11676C, CU-11679T, CU-11682C and CU-11686T

Table 9. Sequence homology of CU-11671C, CU-11676C, CU-11679T, CU-11682C and CU-11686T compared to reference nucleotide sequences in GenBank database

Gene	Virus with the highest percentage of nucleotide identity	GenBank accession no.	Nucleotide identities (%)				
			CU-11671C	CU-11676C	CU-11679T	CU11682C	CU-11686T
PB2	A/duck/Thailand/CU-12326T/2012(H11N2)	KJ161962.1	99%	99%	-	-	-
PB1	A/wild bird/Korea/A02/2011(H10N4)	JN817608.1	99%	99%	-	-	-
PA	A/wild duck/Korea/UP122/2007(H1N1)	HQ014823.1	99%	-	-	-	-
	A/wild duck/Jiangxi/9157/2005(H7N8)	KF260297.1	-	99%	-	-	-
HA	A/ruddy shelduck/Mongolia/1-26/2007(H3N8)	JN029588.1	99%	99%	99%	99%	99%
NP	A/chicken/Taiwan/TC149/2010(H4)	KC693609.1	98%	98%	-	-	-
NA	A/velvet scoter/Mongolia/879V/2009(H10N8)	KF667733.1	97%	-	-	-	-
	A/mallard/Sweden/80057/2008(mixed)	CY164186.1	-	97%	97%	97%	97%
M	A/wild waterfowl/Dongting/PC2574/2012(H9N2)	KF972120.1	99%	99%	-	-	-
NS	A/duck/Guangxi/GXd-4/2011(H1N2)	KF013930.1	99%	99%	-	-	-

Table 10. Deduced amino acid at the HA cleavage site and receptor binding sites of the H3 isolate in this study compared with the H3 subtype viruses in GenBank database.

Virus name	HA cleavage	Receptor binding site							Left edge of receptor binding site						Right edge of receptor binding site				
	320-329	98	153	155	183	190	194	195	224	225	226	227	228	229	134	135	136	137	138
A/duck/Thailand/CU-11671C (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/duck/Thailand/CU-11676C (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/duck/Thailand/CU-11679T (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/duck/Thailand/CU-11682C (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/duck/Thailand/CU-11686T (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/duck/Hong Kong/7/1975 (H3N2)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/chicken/Laos/A0573/2007 (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/blue winged teal/ALB/569/85 (H3N8)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A
A/mallard/MN/330/1999 (H3N1)	PEKQTR	Y	W	T	H	E	L	Y	R	G	Q	S	G	R	G	G	S	G	A

5. Serological analysis

In flock A, total 603 serum samples were subjected for blocking ELISA to detect antibodies against influenza A virus. The ELISA result showed that 22.6% (19/84) of serum samples from 9 weeks old ducks were positive. However, all serum samples from 11 weeks old were negative except one duck (no. A011) that was suspected at 11 and 13 weeks old (Table 11). To further analyze, the ELISA positive and suspect samples were subjected for HI test with H3, H4, H5 and H7 subtype of influenza viruses. The HI test result showed that only duck no. A040 that was positive for H7 subtype with HI titer of 40 (Table 11).

In flock B, total 437 serum samples were subjected for blocking ELISA. The result showed that 91.3% (21/23) and 100% (19/19) of serum samples from were positive from 13 and 15 weeks old ducks, respectively (Table 12). For further serological analysis, the ELISA positive samples were subjected for HI test with H5, H7 subtype and inactivated of homologous viruses, H3N8 (CU-11676C) and H4N6 (CU-11655C) isolated in this study. The HI test results showed that none of serum sample was HI positive, even for homologous viruses (Table 12). It should be noted that no serum sample was collected from ducks after 15 weeks of age due to the death of all sentinel ducks.

Table 11. Serological analysis results of serum samples from flock A

Sentinel	9 weeks					11 weeks					13 weeks				
ID	ELISA	HI (H3)	HI (H4)	HI (H5)	HI (H7)	ELISA	HI (H3)	HI (H4)	HI (H5)	HI (H7)	ELISA	HI (H3)	HI (H4)	HI (H5)	HI (H7)
A007	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A008	+	< 10	< 10	< 10	10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A010	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A011	+	< 10	< 10	< 10	< 10	+/-	< 10	< 10	< 10	< 10	+/-	< 10	< 10	< 10	< 10
A012	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A020	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A023	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A033	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A035	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A039	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A040	+	< 10	< 10	< 10	40	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A043	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A044	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A058	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A065	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A073	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A084	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A091	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10
A096	+	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10

Table 12. Serological analysis results of serum samples from flock B

Sentinel	11 weeks					13 weeks					15 weeks				
ID	ELISA	HI (H3)	HI (H4)	HI (H5)	HI (H7)	ELISA	HI (H3)	HI (H4)	HI (H5)	HI (H7)	ELISA	HI (H3)	HI (H4)	HI (H5)	HI (H7)
B009	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B012	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B013	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B014	+/-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B015	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B016	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	N/A	N/A	N/A	N/A	N/A
B021	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B026	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B037	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B042	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B056	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B059	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B060	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	N/A	N/A	N/A	N/A	N/A
B067	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	N/A	N/A	N/A	N/A	N/A
B071	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B072	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B075	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B086	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B087	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	10	< 10	< 10	< 10
B092	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B097	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	N/A	N/A	N/A	N/A	N/A
B098	-	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10	+	< 10	< 10	< 10	< 10
B099	-	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10	< 10	+	10	< 10	< 10	< 10

CHAPTER V
DISCUSSION

In Thailand, free-grazing ducks were speculated to associated with the HPAI-H5N1 outbreaks (Gilbert et al., 2006). There were many studies of the influenza infection in free-grazing ducks. However, Most of them were focus on HPAI-H5N1 and conducted during the HPAI outbreaks. The current status of influenza A viruses, both HPAI and LPAI, circulating in free-grazing duck was unknown.

During December 2010 to April 2011, the 4-months longitudinal surveillance was conducted to monitor the influenza A virus infection in free-grazing ducks. Our results showed that six influenza viruses were isolated in the study. The influenza viruses were isolated from free-grazing ducks since 13 weeks old. The previous study showed that the HPAI-H5N1 infected ducks were found only after ducks started to graze in the rice field. No infected duck was found in the brooding house. Although, the influenza virus were detected earlier at 6 weeks of age, 45.9% of free-grazing ducks flocks were detected HPAI-H5N1 infection during the outbreak (Songserm et al., 2006[c]). However, there was no published data about the occurrence of the LPAI viruses in free-grazing ducks. In this study, two subtype of LPAI viruses, H4N6 (n=1) and H3N8 (n=5), were isolated from the same flock, but in the different time and locations. The H4N6 subtype of influenza virus were isolated when ducks raised in Phitsanulok at 13 weeks old, while H3N8 viruses were isolated in Phichit at 15 weeks old. These suggested that multiple subtypes of influenza A viruses can circulated in the same free-grazing duck flock. However, no evidence of co-infection of both subtypes in the same host was found in this study.

It is noted that in this study, influenza viruses were isolated from the samples collected in the summer season of Thailand (March and April). In contrast, the previous

study was showed that influenza virus maintained low-level transmission during summer and re-emerged in the rainy season (Chaichoune et al., 2009). However, this longitudinal surveillance was conducted only 4 months during the winter to early summer. To further confirm this observation, one-year longitudinal surveillace should be performed to further confirm. In addition, it should be noted there was severe storm occurred during the week that the viruses were isolated. Approximately 60% of free-graizing duck flock were dead due to the cluster of ducks during the storm. The signs of depression and ocular discharge were observed in some survived sentinel ducks at 13 and 15 weeks old. None of sentinel duck survived after the 16 weeks of age. In the previous studies, the influenza infected ducks were showed only asymthomatic or mild clinical signs (Keawcharoen et al., 2008; Gaidet et al., 2010). However, the clinical signs and dead of sentinels ducks could not be concluded due to influenza viruses infection.

The source of the influenza viruses in this study could not be identified. The possible source of influenza viruses in this study might be from wild bird, transportation truck or the nearby free-grazing duck flock. The previous study in China showed that the free-grazing ducks and wild birds shared the same habitat and might increase the risk of influenza virus transmission between their population (Cappelle et al., 2014). The same LPAI viruses were found in both wild birds and domestic ducks (Duan et al., 2011). Based on our observation, in our study areas, many species of wild bird were found sharing the same feeding area in rice-paddy field with our free-grazing ducks, including little egret, Asian open-bill stork, white-breasted waterhen, lesser-whistling duck and swallows. In Thailand, the influenza surveillance in 16 species of wild bird was conducted during the HPAI outbreak period. The low-level of HPAI-H5N1 virus infection was detected in wild birds (Siengsanan et al., 2009). The previous study showed that the H12N1 influenza viruses were isolated from 2 species of wild bird,

watercock and lesser-whistling duck (Wongphatcharachai et al., 2012). Another possible source of virus transmission, the transportation truck could not be ignored. In this study, both free-grazing duck flocks were moved from one area to another by the rental multilevel truck. Due to the transport of various free-grazing duck flocks, the rental truck may be contaminated with influenza viruses shedding from free-grazing ducks. Furthermore, there were several free-grazing duck flocks raising surrounded our studied flocks in this study.

Base on phylogenetic analysis, our results showed that all H3N8 and H4N6 influenza viruses in this study were clustered in the Eurasian lineage. The deduced amino acid at the cleavege site of all influenza virus isolates were compared with the reference HA gene in GenBank database. The amino acid at the HA cleavage site of all H3N8 viruses were PEKQTR. In the H4N6 virus, the amino acid at the HA cleavge site was PEKASR, which similar to the other reference viruses in the Eurasian lineage and also similar to the previous reported H4N6 and H4N9 viruses in Thailand (Wisedchanwet et al., 2011). It noted that there was no addition of multiple basic amino acids at the HA cleavage site of all virus isolates in this study. This indicated that all virus isolates in this study were low-pathogenic influenza A viruses (Horimoto and Kawaoka, 1994). However, the intravenous pathogenicity index (IVPI) test should be further performed to confirm the pathogenicity of the viruses in this study (WHO, 2002). In addition, the receptor binding site of all virus isolates at the positiion 226 and 228 were Q226 and G228. This indicated that the viruses in this study prefered to bind to 2,3-linked sialic acid receptors, which predominantly in avian species (Connor et al., 1994).

In serological analysis, the blocking NP-ELISA was performed to detect antibodies against influenza A virus. The previous sero-surveillance in free-grazing ducks showed that the seropositive ducks were found since 2 weeks old (Beaudoin et

al., 2012). In this study, one of free-grazing duck flock had seropositive at 13 weeks old. Interestingly, the seropositive ducks were detected at the same time the viruses were isolated. The previous experimental study showed that the ducks sheded the virus at 1-7 dpi and had seropositive at 8 dpi (Wibawa et al., 2014). This might be indicated that there was another influenza virus infection earlier than the viruses isolated in this study. The HI test also supported this suggestion. The result of HI test with the homologous virus revealed that no HI positive duck (titer $\geq$ 40) at the 13 and 15 weeks old. In contrast, the serological result of another flocks was different. The seropositive ducks were detected only at 9 weeks old. No seropositive duck was found after that age. From the previous study, the serological response can be prolonged for at least 8 months (Curran et al., 2013). The contrast of serological result should be further investigated.

It has been documented that free-grazing ducks were the important factor of the HPAI-H5N1 persistance and transmission (Gilbert et al., 2006). Not only Thailand, the free-grazing or scavenging ducks in many countries of Asia were considered as the important reservoir of the influenza A viruses (Henning et al., 2010; Khatun et al., 2013). The results of this study may suggested that multiple subtypes of influenza A virus circulated in lower northern part of Thailand. Due to the shared habitats with wild birds and backyard poultry, the free-grazing ducks might be transmitted the influenza viurses to the other avian species or human. The current status of influenza A viruses circulating in free-grazing ducks would be benefit for the prevention and control strategies of the next influenza A outbreak.

Conclusion and suggestion

In this study, we focused on the longitudinal survey of influenza A viruses circulated in free-graizing ducks in hight risk areas and the serological response to influenza A virus infection in free-grazing ducks. Two of free-grazing duck flocks in lower northern part of Thailand (Phichit and Phitsanulok) were selected for this study. Our results showed

1. There were multiple subtypes of influenza A viruses circulated in free-grazing ducks. Both subtypes of influenza virus (H4N6 and H3N8) were infected the same free-grazing duck flock, but at the different time and location. This indicated that the frequently movement of free-grazing ducks could potentially spread the influenza A viruses.

2. Eventhough all influenza virus isolates were genetic charactized as LPAI, influenza A surveillance in free-grazing ducks should be continued. The recently outbreak of LPAI as H7N9 is an example that the information of the current status of influenza virus circulating in poultry would be benefit for the prevention and control strategies for influenza outbreaks.

3. Serological analysis showed that free-grazing ducks in this study posed seropositive since 13 weeks old. Due to the viruses were isolated from the seropositive ducks suggested that the serological response may not prevented the free-grazing ducks from influenza infection. The serum neutralization test should be performed for further analysis of protective immunity.

4. One-year longitudinal surveillance should be performed for additional information of the influenza A virus in free-grazing ducks such as the status of influenza virus circulating in one year or season of high influenza transmission.

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VITA

Miss Supanat Boonyapisitsopa was born on November 2nd, 1983 in Samut Prakan, Thailand. She completed the D.V.M. from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2008. After that, she enrolled the Master degree of Science in Veterinary Pathobiology program, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University since academic year 2010.