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LONGITUDINAL SURVEY OF INFLUENZA A VIRUSES IN LIVE BIRD MARKETS IN BANGKOK

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ตลาดค้าสัตว์ปีกมีชีวิตเป็นสถานที่ซึ่งรวบรวมสัตว์ปีกหลายชนิดจากต่างแหล่งที่มาไว้ด้วยกัน สภาพแวดล้อมเช่นนี้จึงเอื้อต่อการแพร่กระจายและการกลายพันธุ์ของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ โดยพบว่าผู้ป่วยซึ่งติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ ส่วนใหญ่มีประวัติการสัมผัสกับสัตว์ปีกในตลาดค้าสัตว์ปีกมีชีวิต การศึกษาครั้งนี้เป็นการสำรวจระยะยาวของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิต 3 แห่งในกรุงเทพมหานครเป็นระยะเวลา 1 ปี ตั้งแต่เดือนพฤษภาคม 2557 ถึงเดือนเมษายน 2558 เพื่อตรวจหาเชื้อไวรัส ระบุชนิดสายพันธุ์และลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในตลาดค้าสัตว์ปีกมีชีวิตในกรุงเทพมหานคร การศึกษาครั้งนี้ประกอบไปด้วย 3 ขั้นตอนได้แก่ การเลือกกำหนดตลาดและการเก็บตัวอย่าง การเพาะแยกเชื้อไวรัสและการตรวจพิสูจน์เชื้อไวรัส และการวิเคราะห์ลักษณะทางพันธุกรรมของเชื้อไวรัส โดยเก็บตัวอย่างจากการป้ายปากและทวารร่วมจากเป็ดและไก่มีชีวิตในตลาดค้าสัตว์ปีกเดือนละ 1 ครั้งและทบสอบหาเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ ผลการทดสอบพบว่า จากตัวอย่างทั้งหมด 1,374 ตัวอย่าง มีตัวอย่างให้ผลบวกต่อเชื้อไวรัสไข้หวัดใหญ่ชนิดเอโดยวิธี real-time RT-PCR จำนวน 18 ตัวอย่าง (1.31%) และเมื่อจำแนกสายพันธุ์ของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ พบสายพันธุ์ H2N3 จำนวน 13 ตัวอย่าง และH2N8 จำนวน 5 ตัวอย่างจากเป็ดในตลาดค้าสัตว์ปีก 1 แห่ง ผลการวิเคราะห์ทางพันธุกรรมและการจัดกลุ่มเชื้อไวรัสพบว่าเป็นเชื้อไวรัสไข้หวัดใหญ่ชนิดรุนแรงต่ำ และถูกจัดกลุ่มอยู่ในสายพันธุ์ Eurasian จากผลการศึกษาครั้งนี้สามารถบ่งบอกได้ว่า มีเชื้อไวรัสไข้หวัดใหญ่ชนิดเอหลายสายพันธุ์ที่แพร่กระจายหมุนเวียนในตลาดค้าสัตว์ปีกมีชีวิตในกรุงเทพมหานคร ดังนั้นการสำรวจเชื้อไวรัสไข้หวัดใหญ่ชนิดเออย่างต่อเนื่องในตลาดค้าสัตว์ปีกมีชีวิตจึงเป็นสิ่งสำคัญ เพื่อตรวจสอบหาการแพร่กระจายและการกลายพันธุ์ของเชื้อไวรัส และการมีสุขอนามัยของผู้ค้าสัตว์ปีกรวมถึงสุขศาสตร์ที่ดีในตลาดจะสามารถลดความเสี่ยงในการแพร่กระจายของเชื้อไวรัสชนิดนี้ได้

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สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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WITTAWAT WECHTAISONG: LONGITUDINAL SURVEY OF INFLUENZA A VIRUSES IN LIVE BIRD MARKETS IN BANGKOK. ADVISOR: PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 66 pp.

Live bird markets (LBMs) are places housing multiple poultry species from different sources and suitable environment for transmission and genetic reassortment of Influenza A viruses (IAVs). Most of human cases with IAVs infection had been reported with the history of exposure with poultry in LBMs. In this study, longitudinal survey was conducted in 3 LBMs in Bangkok during May 2014 to April 2015 to determine the occurrence of IAVs and to identify the subtypes and characteristics of IAVs in LBMs in Bangkok. This study was composed of 3 phases including live bird markets selection and sample collection, virus isolation and identification and genetic characterization of the viruses. In this study, oropharyngeal and cloacal swabs were collected monthly from each selected LBM and tested for IAVs. The result showed that 18 out of 1,374 swab samples (1.31%) were positive for IAVs identification by real-time RT-PCR. Two subtypes of IAVs were identified from ducks in a LBM including H2N3 (n=13) and H2N8 (n=5). Phylogeneic and genetic analysis of the viruses suggested that H2N3 and H2N8 posed low pathogenic avian influenza (LPAI) characteristics and grouped into the Eurasian lineage. This result suggested that several subtypes of LPAI were circulating in LBMs in Bangkok, Thailand. In summary, continuous survey of IAVs in LBMs is very important to investigate transmission and evolution of IAVs. Proper sanitation in LBMs and hygiene of vendors or workers' hygiene can reduce risk of IAVs transmission in LBMs.

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List of abbreviations

AI	Avian Influenza
BHI	Brain-Heart infusion broth
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	Complementary deoxyribonucleic acid
CS	cloacal swab
ct	cycle threshold
HA	Hemagglutinin
HPAI	High Pathogenic Avian Influenza
IAV	Influenza A virus
IVPI	Intravenous pathogenicity index
LBM	live bird market
LPAI	Low Pathogenic Avain Influenza
M	Matrix
NA	Neuraminidase
NP	Nucleoprotein
NS	Nonstructural protein
OIE	World Organization for Animal Health

OP	Oropharyngeal
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PCR	Polymerase Chain Reaction
RBS	Receptor binding site
RNA	Ribonucleic acid
rRT-PCR	Real-time Reverse Transcription Polymerase Chain Reaction
WHO	World Health Organization

Chapter 1 Introduction

Influenza viruses are the member of Family *Orthomyxoviridae*. This family compose of 6 genera including *Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Quaranfilvirus and Thogotovirus* (Perez D, 2011). Influenza A viruses (IAVs) can infect a wide range of host including mammals, poultry and wild birds (Webster et al., 1992). Influenza B and C viruses commonly infect human and have been isolated from seals and pigs, respectively (Kimura et al., 1997; Osterhaus et al., 2000). World organization for animal health (OIE) defines avian influenza as an infection of poultry caused by any Infuenza A viruses with high pathogenicity (HPAI) or low pathogenicity (LPAI) (OIE, 2014). Outbreaks of IAV subtype H5N1 have been reported in several countries such as, South Korea in December 2003 and Southeast Asia including Lao PDR, Cambodia, Japan, Malaysia, Thailand, Indonesia, Vietnam and Eastern Asia (China and Japan) in 2004 (Lee et al., 2005). The H5N1 outbreaks spread westward from China through Europe and Africa continents. The first H5N1 human cases in Turkey was confirmed to have H5N1 infection in January 2006 (WHO, 2015b). Moreover, the H5N1 outbreak was reported from poultry farm in Nigeria (Joannis et al., 2006) and spread to other African countries (Cattoli et al., 2009) and in March 2006, the first H5N1 human case was confirmed in Egypt (WHO, 2015b). The H5N1 outbreaks caused diseases in poultry with more than 100 million deaths and culling of poultry (Sims et al., 2005). Since 2003 to April 2016, the H5N1 outbreaks in

poultry were reported in 55 countries and 850 H5N1 confirmed human cases and 449 deaths (OIE, 2015; WHO, 2015a).

Several subtypes of influenza A viruses have been isolated from live bird markets (LBMs) (Indriani et al., 2010; Lee et al., 2010; Nishi et al., 2014; Wang et al., 2014). Since LBMs could be the housing places of multiple poultry species from different sources (Cardona et al., 2009). The poultry are placed with high density setting. This condition increases chance for the transmission of Influenza A Viruses (Lee et al., 2010). In addition, LBMs are the liable areas for viral transmission and genetic reassortment (Choi et al., 2005; Moon et al., 2010; Wisedchanwet et al., 2011). For example, H7N9 viruses, new subtype of influenza A viruses silently spread in LBMs in China. These viruses are LPAI in poultry but they can infect humans and cause severe respiratory illness. Up to date, 786 with 307 deaths of H7N9 confirmed cases have been reported.

In Thailand, H5N1 outbreaks emerged at least 7 waves since 2004 to 2008 (Tiensin et al., 2005; Amonsin et al., 2006a) and caused at least 25 confirmed human cases with 17 deaths and over 62 million poultry death and culling (Tiensin et al., 2009; WHO, 2015a). The HPAI H5N1 viruses were isolated from multiple animal species in Thailand including domestic poultry, tigers, leopards and cats (Keawcharoen et al., 2004; Viseshakul et al., 2004; Thanawongnuwech et al., 2005; Amonsin et al., 2006b; Songserm et al., 2006a; Songserm et al., 2006b). From July

2006 to August 2007, the active surveillance of IAVs in live bird markets and local food markets in the central part of Thailand reported HPAI-H5N1 circulation in these markets (Amonsin et al., 2008). In 2009, IAVs subtypes H4N6, H4N9 and H10N3 were reported in LBMs in Bangkok (Wisedchanwet et al., 2011).

However, Thailand has limited information of IAVs survey in LBMs especially longitudinal survey. Continuous survey of IAVs in LBMs is a very important strategy and should be done to investigate the circulation and evolution of IAVs. For this study, one year longitudinal survey was conducted in three live bird markets in different districts of Bangkok. Results from this study provided valuable information about the circulation, evolution and transmission of IAVs in live bird markets in Bangkok.

Research questions

1. What is the occurrence of Influenza A viruses in LBMs in Bangkok during May 2014 to April 2015?
2. What are the genetic characteristic and diversity of Influenza A viruses in LBMs in Bangkok?

Objectives of study

1. To determine the occurrence of IAVs circulating in LBMs in Bangkok during May 2014 to April 2015.
2. To identify the subtypes and characteristics of IAVs in LBMs in Bangkok.

Chapter 2 Literature review

2.1 Influenza A viruses

Influenza A viruses (IAVs) are belonged in Family *Orthomyxoviridae*. This family composed of six genera which are *Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus, Isavirus* and *Quaranfilvirus* (Perez D, 2011). Structural diameter of influenza A viruses is around 80 – 120 nm with roughly spherical. Eight gene segments encode at least 10 proteins in genome structure. Envelope surface of viruses composed 2 types of glycoproteins. There are hemagglutinin protein for attachment and cell fusion between viral particles and host cells and neuraminidase protein that associates in viruses release from host cells. Lipid bilayer membranes are abundant of matrix proteins including viral membrane protein and membrane ion channel. Internal ribonucleoprotein consist of 4 proteins including nucleoprotein as predominant protein for RNA synthesis and 3 transcriptase-associated proteins including polymerase basic protein 1, polymerase basic protein 2 and polymerase acidic protein. IAVs have 2 nonstructural proteins as NS1 and NS2 (Webster et al., 1992; Shaw, 2013). In addition, IAVs are classified into subtype based on antigenic variations of hemagglutinin (H1 – H16) and neuraminidase (N1 – N9) on the viral surface (Fouchier et al., 2005). Influenza A viruses can infect in wide host range including mammals, poultry and wild birds (Webster et al., 1992).

World organization for animal health (OIE) defines avian influenza as an infection of poultry caused by any IAVs with high pathogenicity (HPAI) or low pathogenicity (LPAI). OIE provided criteria for determining pathogenicity of IAVs following by 1) High pathogenicity (HPAI) are the any IAVs that cause at least 75% mortality in 4 to 8 weeks old chickens infected intravenously or any IAVs that has an intravenous pathogenicity index (IVPI) greater than 1.2 in 6 weeks old chickens. In addition, for all H5/H7 LPAI that have the hemagglutinin molecule amino acids sequences are similar to other HPAI isolates, will be considered to be HPAI. 2) H5 and H7 subtypes with low pathogenicity (H5/H7 LPAI) are H5 and H7 isolates from poultry that are not high pathogenicity for chickens and do not have HA_0 cleavage site amino acids sequences similar to any HPAI. 3) Non H5 or H7 subtypes such as H1 - 4, H6, H8 - 16 are not avian influenza and not notified. 4) IAVs of high pathogenicity in another avian species such as wild birds are notified (OIE, 2014).

2.2 Influenza A virus outbreaks

Outbreaks of IAVs in poultry could raise public health concerns especially H5N1 outbreaks. Several countries were effected on H5N1 outbreaks such as, South Korea, Southeast Asia in 2004 including Lao PDR, Cambodia, Japan, Malaysia, Thailand, Indonesia, Vietnam and Eastern Asia (China and Japan) (Lee et al., 2005; Sims et al., 2005). H5N1 outbreaks spread westward from China through Europe and Africa continents. The H5N1 outbreak was reported in poultry in Russia as the first

outbreak in Europe and the first H5N1 human cases in Turkey were confirmed to H5N1 infection in January 2006 (WHO, 2015b). The H5N1 outbreak was reported in poultry farm in Nigeria in February 2006 and spread to other African countries (Joannis et al., 2006; Cattoli et al., 2009). In March 2006, the first H5N1 human case were confirmed in Egypt (WHO, 2015b). From December 2003 to May 2016, H5N1 outbreaks in poultry were reported in 55 countries infection and with 850 H5N1 confirmed human cases and 449 deaths (OIE, 2015; WHO, 2015a).

In Thailand, H5N1 outbreaks emerged at least 7 waves since 2004 to 2008 (Tiensin et al., 2005; Amonsin et al., 2006a) and caused at least 25 confirmed human cases with 17 deaths (WHO, 2015a) and over 62 million poultry death and culling (Tiensin et al., 2009). The H5N1 subtype viruses were isolated from multiple animal species in Thailand.

H7N9 subtype is reassortant IAVs. As the LPAI virus in poultry but it caused severe diseases in human. HA gene of this virus was derived from H7N3 in ducks, NA gene was transferred from H7N9 in wild birds and other 6 internal genes were derived from H9N2 in poultry in China (Liu et al., 2013). The evolution of H7N9 subtype has been related to adaptation in human and also provided virus replication in human respiratory tract (Chen et al., 2013; Gao et al., 2013). Three Chinese people presented with rapidly respiratory illness and were confirmed with H7N9 infection as the first cases reported (Gao et al., 2013). Since February 2013 to May 2016, a total

number of H7N9 confirmed human cases is 786 with 307 deaths were reported in 3 countries including China, Malaysia and Canada.

2.3 Live bird markets

Live bird markets (LBMs) are housing multiple poultry species from different sources and slaughtering live poultry for fresh meat (Cardona et al., 2009). Many people prefer fresh meat slaughtered in LBMs because of consumer taste, traditions and religious beliefs. The poultry are housed in cages with high density setting provided the optimal conditions for transmission and evolution of pathogens such as IAVs (Lee et al., 2010). In addition, LBMs were reported to being the reservoirs and spread for IAVs (Wan et al., 2011). The poultry especially domestic ducks possibly are the mixing vessels to generate reassortant of IAVs in LBMs (Lee et al., 2010).

Epidemiological survey is the important strategy to investigate the circulation and evolution of IAVs in LBMs. The HPAI subtypes were reported to isolate from poultry and environment in LBMs in several countries. In Asian countries, such as Hong Kong, eleven H5N1 were isolated from fecal samples in cages and cloacal swab from chicken carcasses in LBMs in 2001 (Guan et al., 2002). Six IAVs were isolated from swab samples of domestic poultry in 10 LBMs in Hanoi, Vietnam including H5N1, H5N2, H4N6, H9N3 (Nguyen et al., 2005). Thirteen H5N1 were isolated from environmental samples in 83 LBMs in Indonesia (Indriani et al., 2010). Ten H5N1

were isolated from environmental samples in 4 LBMs in Cambodia (Horm et al., 2013). In African country, LBMs in Egypt were positive for H5N1 detecting by real time quantitative PCR (Abdelwhab et al., 2010).

Moreover, LPAI viruses were isolated from LBMs in several countries. Ten LPAI subtypes were isolated from poultry, water fowl and environment in LBMs of the northeastern US since 1993 to 2000 (Panigrahy et al., 2002), and in 2005, LPAI subtype H6N2 were isolated from swab samples of poultry in 3 LBMs in California (Yee et al., 2011). In South Korea, from September 2006 to March 2008, 65 LPAI were isolated swab samples in LBMs including H3N2, H3N8, H4N2, H4N6, H6N2, H9N2 and others unclear subtypes (Lee et al., 2010). In Vietnam from October 2010 to 2012, 198 IAVs were isolated from LBMs including H5N1 subtype and other LPAI (Okamatsu et al., 2013).

In Thailand, the active surveillance of IAVs in live bird markets and live food markets in central part of Thailand reported 12 HPAI-H5N1 were isolated from healthy chicken, health ducks and visceral organs in the markets in July 2006 to August 2007 (Amonsin et al., 2008). In 2009, IAVs subtypes H4N6, H4N9 and H10N3 were firstly reported in a LBM in Bangkok (Wisedchanwet et al., 2011). In addition, two IAV isolates (H4N6 and H4N9) were found in the same duck, possible multiple subtypes infection or genetic reassortment in duck.

Chapter 3 Materials and Methods

This study consisted of 3 phases including; Phase 1, live bird market selection and sample collection, Phase 2, virus isolation and identification and Phase 3, genetic characterization of Influenza A viruses. The conceptual framework of this study was shown in figure 1.

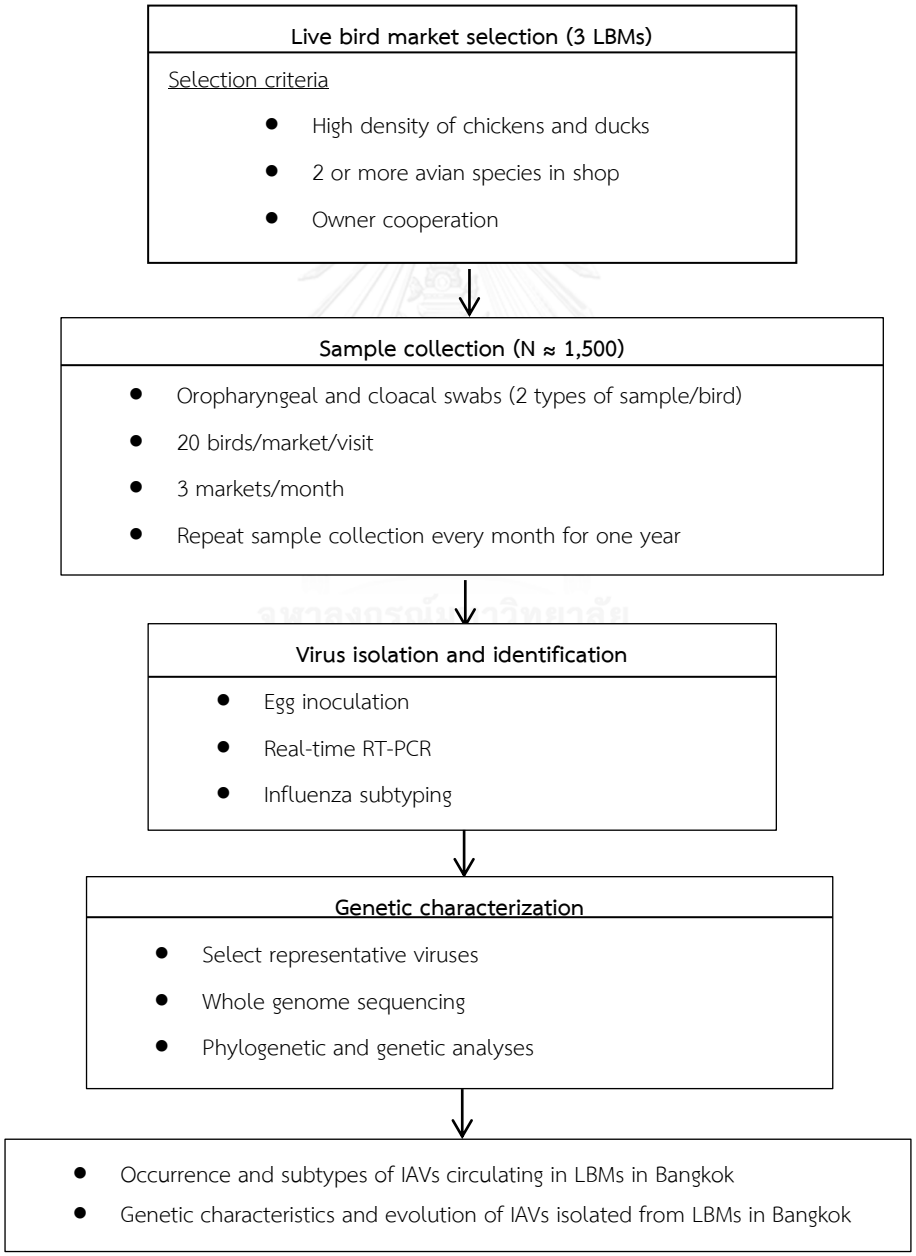

Figure 1 The conceptual framework of this study

Phase 1: LBM selection and sample collection

Three live bird markets (Market A, B, and C) from different districts in Bangkok were selected in this study. The LBMs selection criteria were high density of live birds especially chickens and ducks in the markets, multiple avian species in participating LBM shops and cooperation of the shop owners.

Oropharyngeal and cloacal swabs were collected monthly from 20 chickens or ducks in each selected LBM. Visiting in Market A, B and C were planned in the second week, third week and fourth week of each month, respectively. A total sample was approximately 1,500 samples. The swab samples were placed in 2 ml of Brain-Heart Infusion broth (BHI) on ice and transported to laboratory within 24 hours. All samples were stored at -80°c immediately until tested.

Phase 2: Virus isolation and identification

All samples were subjected to virus isolation following OIE Terrestrial Manual 2014 (OIE, 2014). In brief, supernatant of swab from each sample was inoculated into allantoic cavity of 3 embryonated chicken eggs (9-11 days old). All eggs were then incubated at 37°c for 72 hours. After 72 hours of incubation, allantoic fluid was collected from each inoculated egg and processed for influenza antigen screening by using hemagglutination test. Both, virus isolation and HA test were performed in biosafety laboratory level 3.

For virus identification, the HA positive samples were processed for RNA extraction using QIAamp® Viral RNA Mini kit (QIAamp®, QIAGEN Group, Germany).

Briefly, 150 µl allantoic fluid was mixed to 560 µl buffer AVL containing carrier RNA and then incubated at room temperature (25°C) for 10 minutes. 560 µl of ethanol was added and then applied solution to QIAamp spin column. After two washing steps with buffer AW1 and AW2 respectively, RNA was eluted in 50 µl of AVE elution buffer.

RNA samples were tested for Matrix (M) gene of IAVs by using real-time RT-PCR with a Rotor-Gene RG-3000 machine (Corbett Research, Australia). The primers and probe used were described in previous report (Spackman et al., 2002) (APPENDIX). The results were interpreted by cycle threshold (Ct) value, samples with Ct value less than 36 were considered as positive for IAVs. For influenza subtyping, the RNA of M gene positive samples were reverse transcribed into cDNA and processed to PCR subtyping with subtype specific primers (Tsukamoto et al., 2008; Tsukamoto et al., 2009) (APPENDIX). The PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide and visual analyzed under UV light to identify influenza subtypes.

Phase 3: Genetic characterization of influenza viruses

All IAV isolates were selected for nucleotide sequencing. Some IAVs were selected as representative viruses and subjected for whole genome sequencing based on their subtypes and collection date. All eight genes of IAVs isolates were

amplified by PCR using oligonucleotide primer sets from our primer inventories or newly designed primer sets by using Primer3 Input (v.0.4.0) (APPENDIX). The PCR products were prepared by agarose gel electrophoresis and purified by using Nucleospin® PCR clean up kit. The cleaned PCR products were then subjected for nucleotide sequencing at First BASE laboratories Sdn Bhd, Selangor, Malaysia. Nucleotide sequences were validated and assembled using DNASTAR software (DNASTAR, Inc., WI, USA).

The validated sequence of each gene of the IAV-H2 was analyzed for genetic relationship with the reference nucleotide sequences of IAVs from GenBank database using NCBI nucleotide BLAST tool. Reference sequences included viruses from North American and Eurasian lineages. For phylogenetic analysis, the sequences were aligned using Muscle v.3.6 (Tempe, AZ, USA) (Edgar, 2004). The phylogenetic analysis was performed using the MEGA v.6.06 with neighbor-joining algorithm apply bootstrap method with 1,000 replications (Tamura et al., 2007). For genetic analysis of IAVs, nucleotide sequences and deduced amino acids of each gene were aligned and compared using by MegAlign v.5.03 (DNASTAR, Inc., WI, USA). The deduced amino acids at significant positions were analyzed for genetic characteristic and virulent determinants as shown in table 1.

Table 1 Significant amino acid residues for genetic analysis

Viral protein	Amino acid substitutions	Phenotypic consequence
PB2	E627K	Mammalian host adaptation (Steel et al., 2009)
HA	Multiple basic amino acids at HA cleavage site	HPAI (Horimoto and Kawaoka, 1994)
HA	Q226L	Increased virus binding to human-type receptors (Connor et al., 1994)
HA	G228S	Increased virus binding to human-type receptors (Connor et al., 1994)
NA (N3)	56-78 deletion	Viruses from domesticated poultry (Campitelli et al., 2004)
M1	N30D	Increased virulent in mice (Fan et al., 2009)
NS1	E92D	Increased virulent in mice (Seo et al., 2004)

Chapter 4 Results

In this study, the IAVs survey in LBMs in Bangkok was conducted during May 2014 to April 2015. 1,374 samples were collected from chickens and ducks in three different LBMs in different districts of Bangkok. All swab samples were processed for virus isolation by using egg inoculation and haemagglutination (HA) test for screening IAVs. The real-time RT-PCR and subtyping of IAVs were performed for virus identification and subtyping. Whole genome sequencing and phylogenetic analysis of some IAVs were performed for genetic characterization of IAVs.

4.1 Sample collection from three LBMs in Bangkok

The sample collection was conducted during May 2014 to April 2015. Three LBMs from different districts of Bangkok were selected in this study including Samphanthawong (Market A), Khlong San (Market B) and Khlong Toei (Market C) districts. All selected LBMs are local food markets where poultry meat, pork, beef, fish, fruits, vegetables and other ingredients are sold (Table 2 and Figure 2). Oropharyngeal and cloacal swabs were collected from each animal. A total of 1,374 swab samples were collected from 687 animals including 60.55% (416/687) from chickens and 39.44% (271/687) from ducks (Table 3 and Table 4).

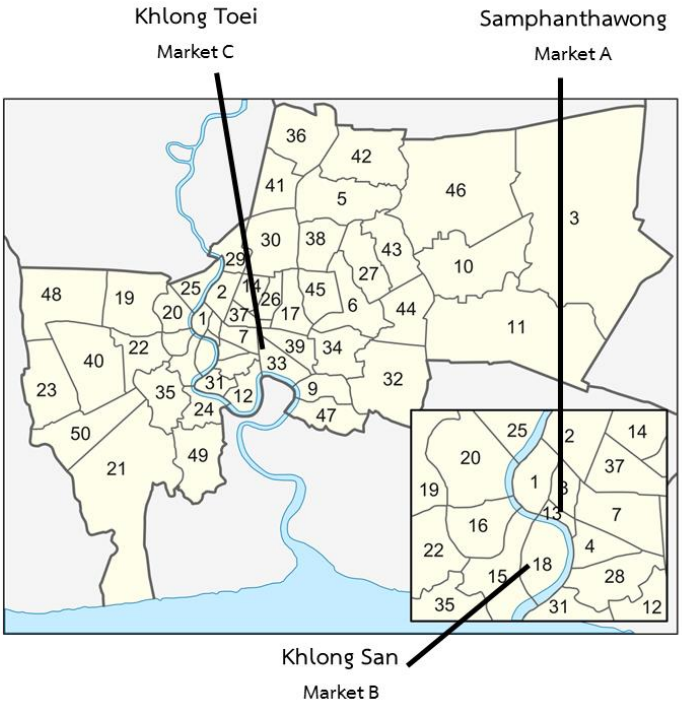

Figure 2 Map of districts of Bangkok where sample collection from LBMs

Table 2 Description of LBMs in this study

Market	Province	District	Type of market
A	Bangkok	Samphanthawong	Local food market
B	Bangkok	Khlong San	Local food market
C	Bangkok	Khlong Toei	Local food market

Table 3 Number of avian species and swab samples collected in this study by market

Market	Chickens	Ducks	Total by market
A	235 (470)	0 (0)	235 (470)
B	53 (106)	84 (168)	137 (274)
C	128 (256)	187 (374)	315 (630)
Total by species	416 (832)	271 (542)	687 (1,374)

Numbers in parentheses are number of swab samples

Table 4 Number of avian species and swab samples collected in this study by month

Month	Chickens	Ducks	Total by month
May-14	36 (72)	16 (32)	52 (104)
Jun-14	36 (72)	16 (32)	52 (104)
Jul-14	35 (70)	15 (30)	50 (100)
Aug-14	35 (70)	15 (30)	50 (100)
Sep-14	41 (82)	21 (42)	62 (124)
Oct-14	36 (72)	26 (52)	62 (124)
Nov-14	36 (72)	25 (50)	61 (122)
Dec-14	36 (72)	27 (54)	63 (126)
Jan-15	34 (68)	23 (46)	57 (114)
Feb-15	30 (60)	27 (54)	57 (114)
Mar-15	31 (62)	28 (56)	59 (118)
April-15	30 (60)	32 (64)	62 (124)
Total by species	416 (832)	271 (542)	687 (1,374)

Numbers in parentheses are number of swab samples

4.2 Influenza A viruses isolation and identification

All swab samples were subjected to virus isolation by using egg inoculation. After virus isolation, all samples were processed for IAVs antigen screening using haemagglutination (HA) test. A total of HA positive samples were 30.49% (419/1,374). The highest percentage of HA positive samples by markets was market B (35.76%) (Table 5). By month, the highest percentage of HA positive samples was in March 2015 (69.49%) (Table 6). The HA positive samples were tested for Influenza A identification by real-time RT-PCR. Of 419 samples, 18 samples were positive for

real-time RT-PCR. 18 Influenza viruses were detected from market C in November 2014, March 2015 and April 2015 (Table 7). The percentage of Influenza A viruses isolated from market C was 2.85% (18/630) (Table 5). By month, the percentages of Influenza A viruses were 4.09% (5/122) in November 2014, 6.78% (8/118) in March 2015 and 4.03% (5/124) in April 2015 (Table 6).

These results implied that the occurrence of Influenza A viruses in this study is 1.31% by samples (18/1,374) or 2.18% by animals (15/687). In this study, 18 Influenza A viruses were isolated from 15 ducks (5 isolates from 4 ducks in November 2014, 8 isolates from 6 ducks in March 2015 and 5 isolates from 5 ducks in April 2015). It is noted that 3 ducks were positive for IAVs by both oropharyngeal and cloacal swabs (Table 7). Analysis by species of animals, the results showed occurrence of IAVs in ducks is 5.53% (15/271), while chickens were all negative for IAVs.

In this study, two subtypes of Influenza A isolates were identified by PCR subtyping with specific primers as H2N3 (n=13) and H2N8 (n=5) (Figure3, 4 and 5). All H2N8 viruses were isolated from ducks in November 2014, while H2N3 viruses were isolated from ducks in March (8/13) and April 2015 (5/13). Twelve out of 18 Influenza A isolates were isolated from cloacal swabs, while the others (n=6) were isolated from oropharyngeal swabs (Table 7).

Table 5 HA positive and Influenza A positive samples from each market

Market	Samples	HA positive samples (%)	Influenza A viruses (%)
A	470	168 (35.74)	0
B	274	98 (35.76)	0
C	630	153 (24.28)	18 (2.85)
Total	1,374	419 (30.49)	18 (1.31)

Numbers in parentheses are percentage of positive samples in each market

Table 6 HA positive and Influenza A positive samples in each month

Month	Samples	HA positive samples (%)	Influenza A viruses (%)
May-14	104	13 (12.50)	0
Jun-14	104	6 (5.76)	0
Jul-14	100	7 (7.00)	0
Aug-14	100	22 (22.00)	0
Sep-14	124	11 (8.87)	0
Oct-14	124	38 (30.64)	0
Nov-14	122	60 (49.18)	5 (4.09)
Dec-14	126	29 (23.01)	0
Jan-15	114	33 (28.94)	0
Feb-15	114	49 (42.98)	0
Mar-15	118	82 (69.49)	8 (6.78)
Apr-15	124	69 (55.64)	5 (4.03)
Total	1,374	419 (30.49)	18 (1.31)

Numbers in parentheses are percentage of positive samples in each month

Table 7 Description of 18 Influenza A viruses isolated in this study

Sample numbers	Species of animals	Type of samples	Month and year	Subtype
CU-15550C	duck	Cloacal swab	November 2014	H2N8
CU-15551T	duck	Oropharyngeal swab	November 2014	H2N8
CU-15555C	duck	Cloacal swab	November 2014	H2N8
CU-15556T	duck	Oropharyngeal swab	November 2014	H2N8
CU-15556C	duck	Cloacal swab	November 2014	H2N8
CU-16697C	duck	Cloacal swab	March 2015	H2N3
CU-16698C	duck	Cloacal swab	March 2015	H2N3
CU-16699T	duck	Oropharyngeal swab	March 2015	H2N3
CU-16699C	duck	Cloacal swab	March 2015	H2N3
CU-16700T	duck	Oropharyngeal swab	March 2015	H2N3
CU-16700C	duck	Cloacal swab	March 2015	H2N3
CU-16701C	duck	Cloacal swab	March 2015	H2N3
CU-16703C	duck	Cloacal swab	March 2015	H2N3
CU-16810C	duck	Cloacal swab	April 2015	H2N3
CU-16813T	duck	Oropharyngeal swab	April 2015	H2N3
CU-16818C	duck	Cloacal swab	April 2015	H2N3
CU-16819C	duck	Cloacal swab	April 2015	H2N3
CU-16823T	duck	Oropharyngeal swab	April 2015	H2N3

Sample numbers ; T are oropharyngeal swab ; C are cloacal swab

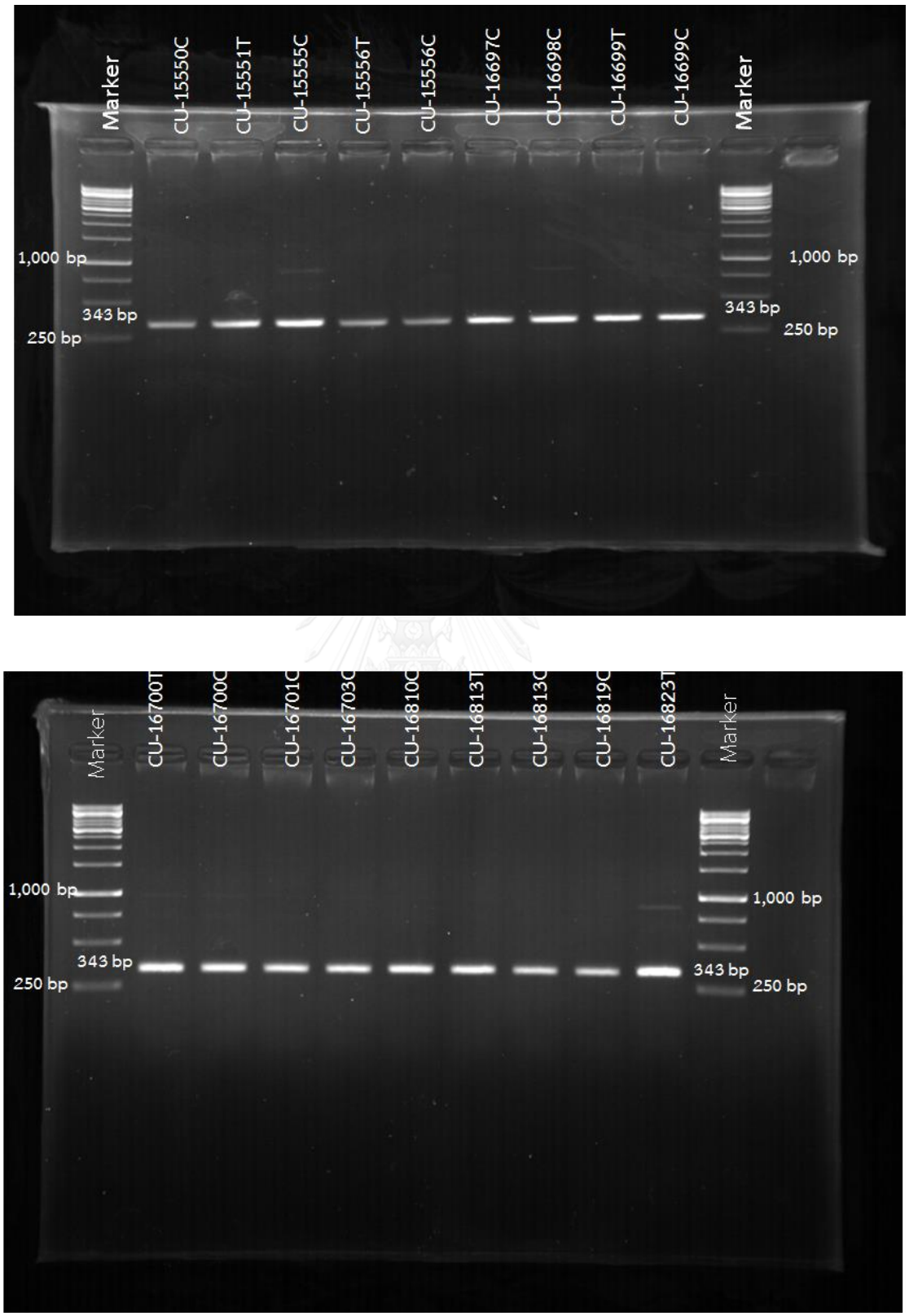

Figure 3 H2 subtype identification by PCR assay using subtype specific primers; Marker: 1,000 bp marker, H2: expected PCR product 343 bp

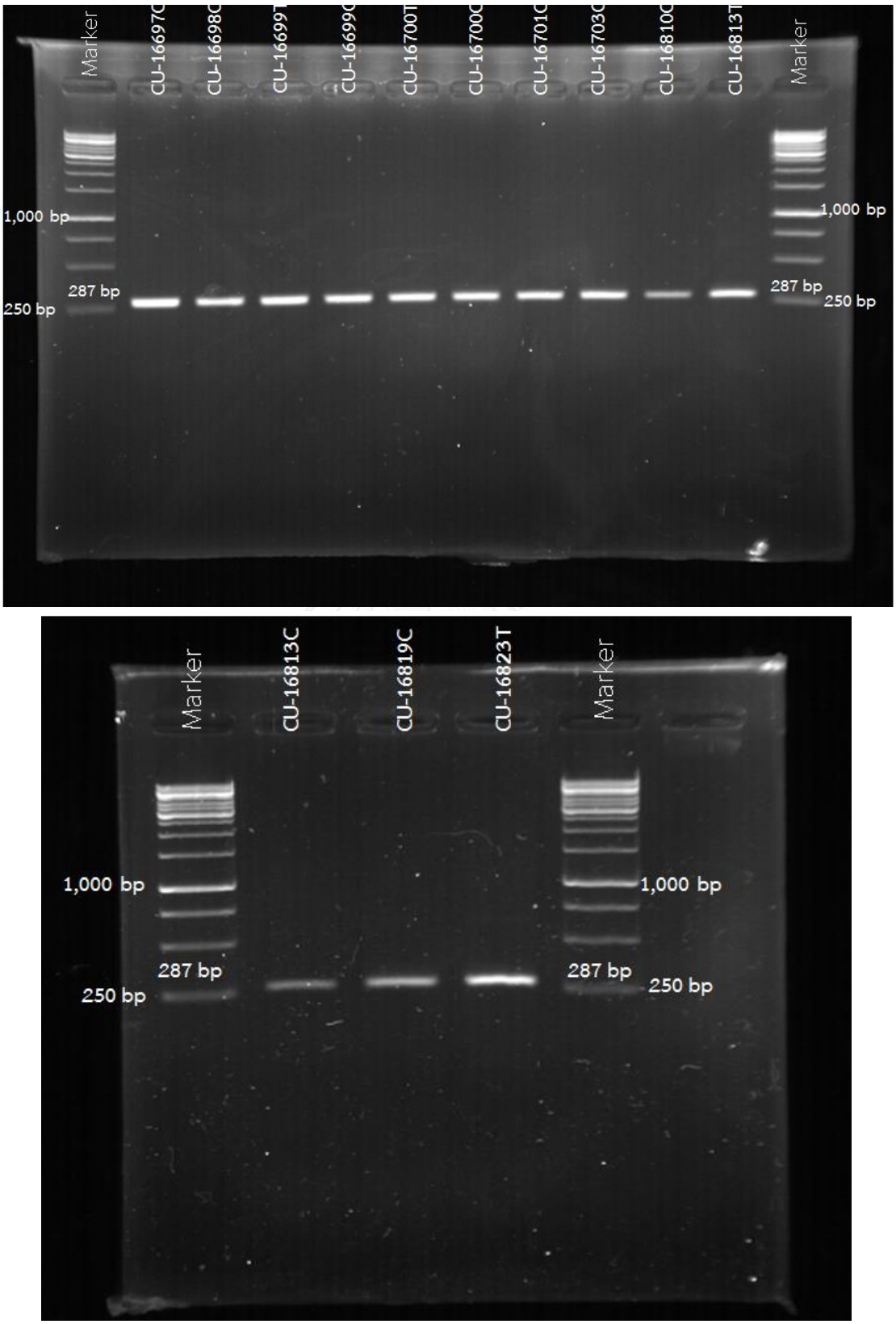

Figure 4 N3 subtype identification by PCR assay using subtype specific primers; Marker: 1,000 bp marker, N3: expected PCR product 287 bp

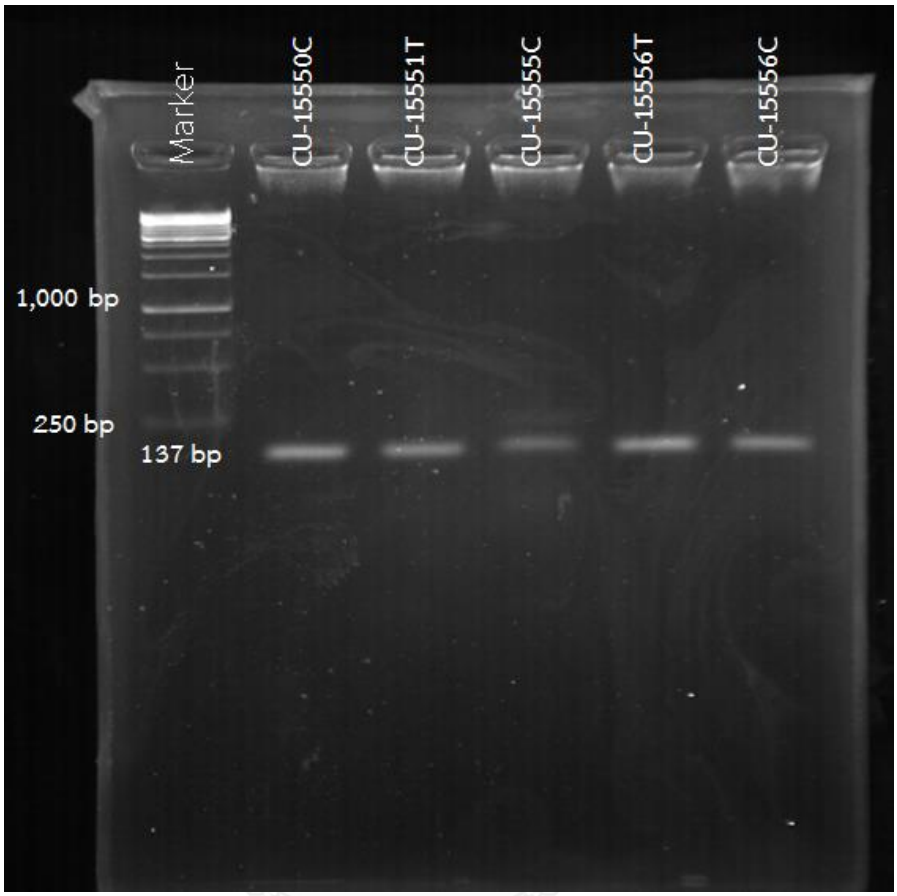

Figure 5 N8 subtype identification by PCR assay using subtype specific primers; Marker: 1,000 bp marker, N8: expected PCR product 137 bp

4.3 Genetic characterization of the Influenza A viruses isolates

4.3.1 Whole genome sequencing of Influenza A viruses

In this study, two representative viruses, CU-16701C (H2N3) and CU-15555C (H2N8) were selected for whole genome sequencing. In addition, other 16 Influenza A isolates were subjected to HA and NA gene sequencing (Table 8).

Table 8 Influenza A isolates selected for nucleotide sequencing in this study

Sample number	Month and year	Genes for sequencing
CU-15555C (H2N8)	November 2014	Whole genome sequencing
CU-15550C (H2N8)	November 2014	HA and NA gene sequencing
CU-15551T (H2N8)	November 2014	HA and NA gene sequencing
CU-15556T (H2N8)	November 2014	HA and NA gene sequencing
CU-15556C (H2N8)	November 2014	HA and NA gene sequencing
CU-16701C (H2N3)	March 2015	Whole genome sequencing
CU-16697C (H2N3)	March 2015	HA and NA gene sequencing
CU-16698C (H2N3)	March 2015	HA and NA gene sequencing
CU-16699T (H2N3)	March 2015	HA and NA gene sequencing
CU-16699C (H2N3)	March 2015	HA and NA gene sequencing
CU-16700T (H2N3)	March 2015	HA and NA gene sequencing
CU-16700C (H2N3)	March 2015	HA and NA gene sequencing
CU-16703C (H2N3)	March 2015	HA and NA gene sequencing
CU-16810C (H2N3)	April 2015	HA and NA gene sequencing
CU-16813T (H2N3)	April 2015	HA and NA gene sequencing
CU-16818C (H2N3)	April 2015	HA and NA gene sequencing
CU-16819C (H2N3)	April 2015	HA and NA gene sequencing
CU-16823T (H2N3)	April 2015	HA and NA gene sequencing

Table 9 Nucleotide sequences of each gene of Influenza A isolates in this study

Genes (position)	CU-16701C (H2N3)	CU-15555C (H2N8)
PB2	1-2280	1-2280
PB1	1-2274	1-2271
PA	1-2151	1-2151
HA	1-1689	1-1689
NP	1-1497	1-1497
NA	1-1410	1-1413
M	1-957	1-955
NS	1-816	1-838

4.3.2 Genetic characterization of H2 from ducks

In HA gene, BLAST analysis showed that H2 genes of Thai H2N3 and H2N8 viruses were closely related to H2N1 virus isolated from Japan (A/duck/Hokkaido/162/2013(H2N1) with 97% and 98% similarity, respectively (Table 10 and Table 11). Phylogenetic analysis showed that all H2 viruses from this study were grouped with viruses of Eurasian lineage (Figure 6). For genetic analysis, the deduced amino acids of IAV-H2 were compared with those H2 viruses as shown in table 12 including human pandemic viruses (A/Japan/305/57, A/Korea/426/68), H2 swine virus which derived from avian viruses (A/swine/Missouri/2124514/2006), H2 virus which were highest percent similarity (A/duck/Hokkaido/162/2013) and H5-HPAI and LPAI for comparing pathogenic characteristics. HA cleavage sites of H2N3 and H2N8 were PQIESR and PQTESR, respectively. There were no multiple basic amino

acids indicating low pathogenic characteristics. The receptor binding sites of H2 genes were Q226 and G228 suggesting preferential binding with avian-type receptor (2,3-linked sialic receptor). The right and left edges of receptor binding sites of Thai H2 viruses at position 224-229 and 134-138 were NGQGGR and GGSRA, respectively. These observations were similar to other avian viruses of Eurasian and North American lineages (Table 12).

4.3.3 Genetic characterization of N3 from ducks

The N3 gene of 13 H2N3 viruses was compared with the reference sequences of IAVs available in GenBank database. BLAST analysis showed that N3 gene of Thai viruses was closely related to H2N3 virus isolated from Georgia (A/tufted duck/Republic of Georgia/1/2012(H2N3) with 98% similarity (Table 10). As shown in Figure 7, phylogenetic tree showed that all N3 isolates were grouped into the Eurasian lineage, similar to other Thai N3 isolates from previous studies. For genetic analysis of N3 gene, amino acid deletion was not found at the NA stalk region (position 56 to 78; N3 numbering) suggesting wild bird origin. For example, amino acid deletions of N3 gene were reported in viruses from domestic aquatic birds but not in wild species (Campitelli et al., 2004).

4.3.4 Genetic characterization of N8 from ducks

For the N8 gene, five H2N8 isolates were compared with the reference sequences from GenBank database. BLAST analysis showed that N8 genes were closely related to H3N8 virus isolated from Mongolia (A/duck/Mongolia/30/2015(H3N8) with 99% similarity (Table 11). Phylogenetic analysis of N8 genes showed that the viruses were grouped in Eurasian lineage which similar to the Thai viruses from previous studies (Figure 8).

In addition, 3 ducks were positive for IAVs by both oropharyngeal and cloacal swabs. Comparison of nucleotide and amino acid similarities of both swab samples from same duck showed 99.8-100% nucleotide similarity and 99.4-100% amino acid similarity (Table 13). Point mutation was observed among swab samples in each duck, it changed some nucleotides or base pairs of nucleotide sequences and affected amino acid sequence of the protein products.

4.3.5 Internal genes of Influenza A viruses isolates in this study

As the representative viruses for whole genome sequencing, CU-16701C (H2N3) and CU-15555C (H2N8) were selected. The other 6 internal genes (PB2, PB1, PA, NP, M, NS) from each isolate were compared with the reference sequences of IAVs available in GenBank database. BLAST analysis of internal genes was shown in Tables 10 and 11. Phylogenetic analysis of internal genes of both H2N3 and H2N8

showed that internal genes were grouped with viruses in the Eurasian lineage except NS1 gene of Thai H2N3 virus which was grouped into North American lineage. Genetic analysis of internal genes, Glutamic acid (E) residue at position 627 of PB2 genes were observed in Thai H2 viruses. Aspartic acid (D) was found in Thai viruses at M1 and NS1 genes (position 30 and 92, respectively). Amino acid substitution at M1 gene (N30D) and NS1 gene (E92D) associated with high virulence of the viruses in mammal hosts were observed (Seo et al., 2004; Fan et al., 2009).

Table 10 BLAST result present nucleotide identity of CU-16701C (H2N3) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	1-2280	LC121409	A/duck/Mongolia/543/2015(H4N6)	99%
PB1	1-2274	LC108118	A/duck/Mongolia/66/2015(H10N2)	99%
PA	1-2151	AB916666	A/Muscovy duck/Vietnam/ LBM529/2013(H3N8)	99%
HA	1-1689	LC041995	A/duck/Hokkaido/162/2013(H2N1)	97%
NP	1-1497	LC121341	A/duck/Mongolia/211/2015(H3N8)	99%
NA	1-1410	CY185342	A/tufted duck/Republic of Georgia/1/2012(H2N3)	98%
M	1-957	LC121239	A/duck/Mongolia/30/2015(H3N8)	99%
NS	1-816	KP286881	A/duck/Jiangxi/27793/2013(mixed)	99%

Table 11 BLAST result present nucleotide identity of CU-15555C (H2N8) compared to reference virus sequences available in GenBank

Gene	Position	GenBank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	1-2280	JX454720	A/duck/Korea/DY104/2007(H4N6)	99%
PB1	1-2271	KJ764786	A/environment/Korea/UPO218/ 2008(H1N6)	99%
PA	1-2151	CY185582	A/mallard/Republic of Georgia/13/2011(H6N2)	99%
HA	1-1689	LC041995	A/duck/Hokkaido/162/2013(H2N1)	98%
NP	1-1497	LC121341	A/duck/Mongolia/211/2015(H3N8)	99%
NA	1-1413	LC121238	A/duck/Mongolia/30/2015(H3N8)	99%
M	1-955	LC121311	A/duck/Mongolia/179/2015(H3N8)	99%
NS	1-838	JX454717	A/duck/Korea/DY104/2007(H4N6)	99%

Table 12 Genetic analysis of H2 gene of CU-16701C (H2N3) and CU-15555C (H2N8) at the HA cleavage site and receptor binding sites comparing to H2 and H5 viruses

Virus strains	Pathotype	HA cleavage	HA receptor binding sites						Left edge of RBS	Right edge of RBS
H3 system		320-329	138	190	194	225	226	228	224-229	134-138
H2 position		335-340	148	200	204	235	236	238	234-239	144-148
CU-16701C (H2N3)	LPAI	PQIESR	A	E	L	G	Q	G	NGQGGR	GGSRA
CU-15555C (H2N8)	LPAI	PQTESR	A	E	L	G	Q	G	NGQGGR	GGSRA
A/duck/Hokkaido/162/2013 (H2N1)	LPAI	PQIESR	A	E	L	G	Q	G	NGQGGR	GGSRA
A/duck/Wuxi/2/2013 (H2N2)	LPAI	PQIKSR	A	E	L	G	Q	G	NGQGGR	GGSRA
A/gull/MD/19/77 (H2N8)	LPAI	PQIESR	A	E	L	G	Q	G	NGQGGR	GGSQA
A/chicken/OH/494832/2007 (H2N3)	LPAI	PQIESR	A	E	L	G	Q	G	NGQGGR	GGSRA
A/Japan/305/57 (H2N2)	LPAI	PQIESR	A	E	L	G	Q	G	NGQGGR	GGSRA
A/Korea/426/68 (H2N2)	LPAI	PQIESR	A	E	L	G	L	S	NGLGSR	GGSMA
A/swine/Missouri/2124514/2006 (H2N3)	LPAI	PQIESR	A	E	L	G	L	G	NGLGGR	GGSRA
H5 position		321-331	150	202	206	237	238	240	236-241	146-150
A/duck/Yunnan/435/2002 (H5N3)	LPAI	PQRETR	A	E	L	G	Q	G	NGQSGR	GVSSA
A/chicken/Mexico/31381_991/1994 (H5N2)	LPAI	PQRETR	A	E	L	G	Q	G	NGQSGR	GVSSA
A/quail/Thailand/CU-332/2006 (H5N1)	HPAI	PQRERRRKKR	A	E	L	G	Q	G	NGQSGR	GVSSA
A/goose/Taiwan/TNC1/2015 (H5N8)	HPAI	PLRERRRKR	A	E	L	G	Q	G	NGQRGR	GVSAA
A/goose/Zhejiang/925108/2014 (H5N6)	HPAI	PLRERRRKR	A	E	L	G	Q	G	NGQRGR	GVSAA
A/tiger/Suphanburi/Thailand/Ti-1/04 (H5N1)	HPAI	PQRERRRKKR	A	E	L	G	Q	G	NGQSGR	GVSSA

Table 13 Percentage of nucleotide and amino acid similarities of oropharyngeal and cloacal swabs of 3 ducks (CU-15556, CU-16699 and CU-16700) that were positive for IAVs by both swab samples

Sample no.	Percent similarity of HA genes		Percent similarity of NA genes	
	Nucleotides	Amino acids	Nucleotide	Amino acids
CU-15556	99.9%	100.%	99.8%	99.4%
CU-16699	99.9%	99.9%	99.9%	99.8%
CU-16700	100.%	100.%	99.8%	100.%

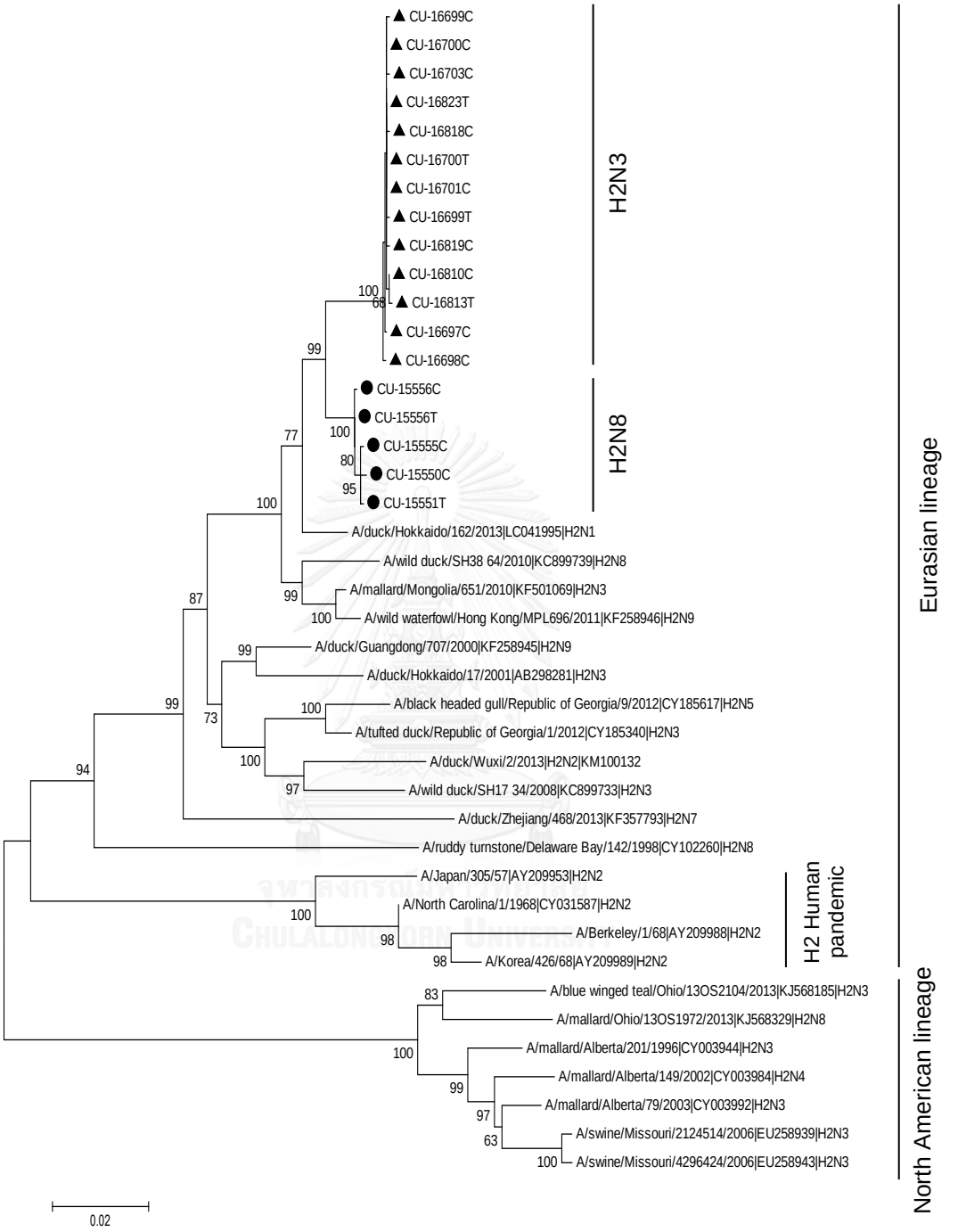

Figure 6 Phylogenetic tree of H2 genes

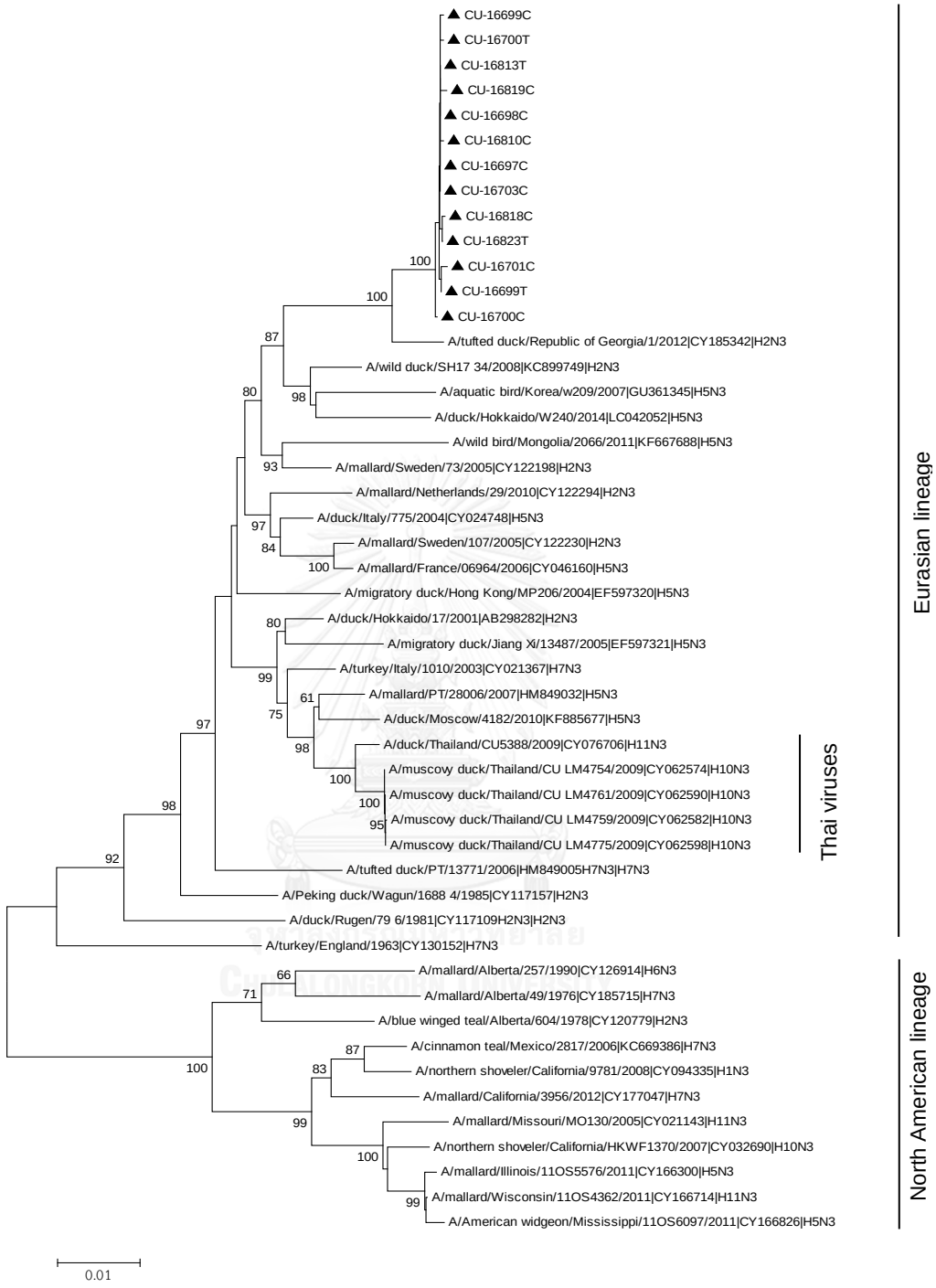

Figure 7 Phylogenetic tree of N3 genes

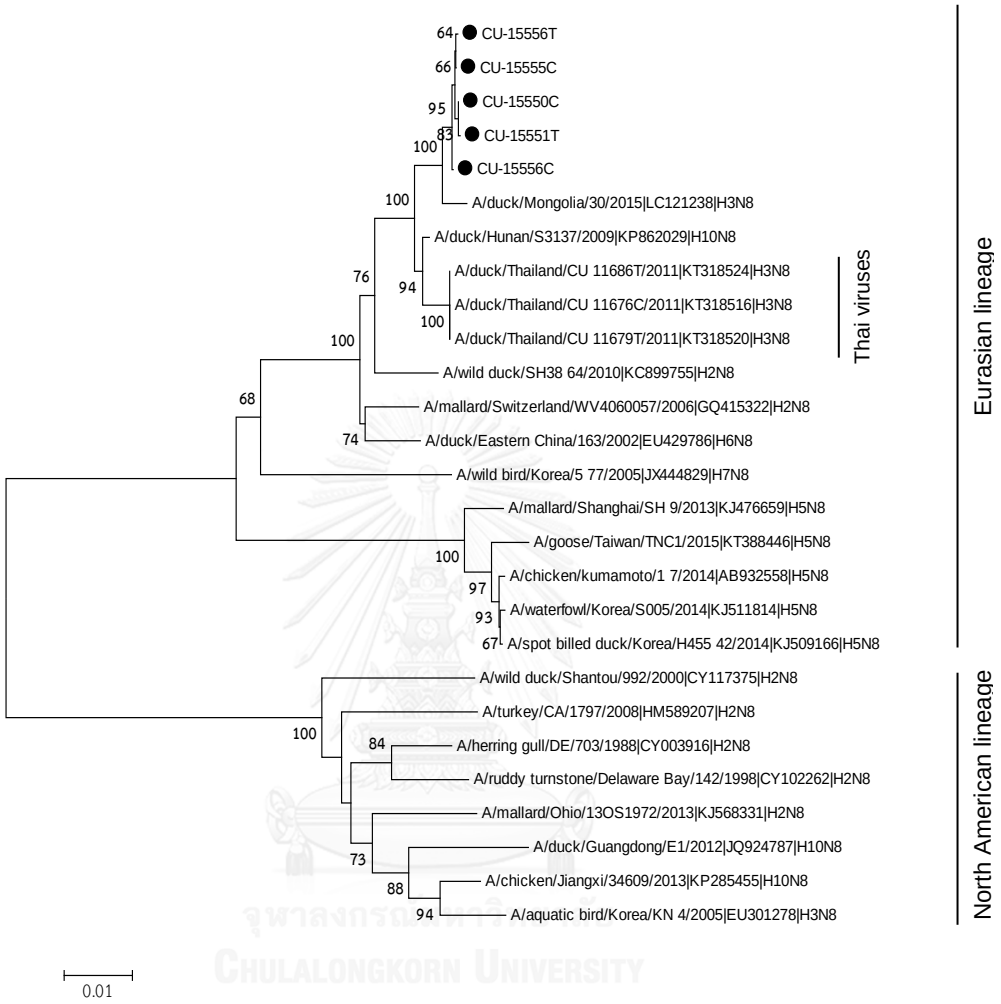

Figure 8 Phylogenetic tree of N8 genes

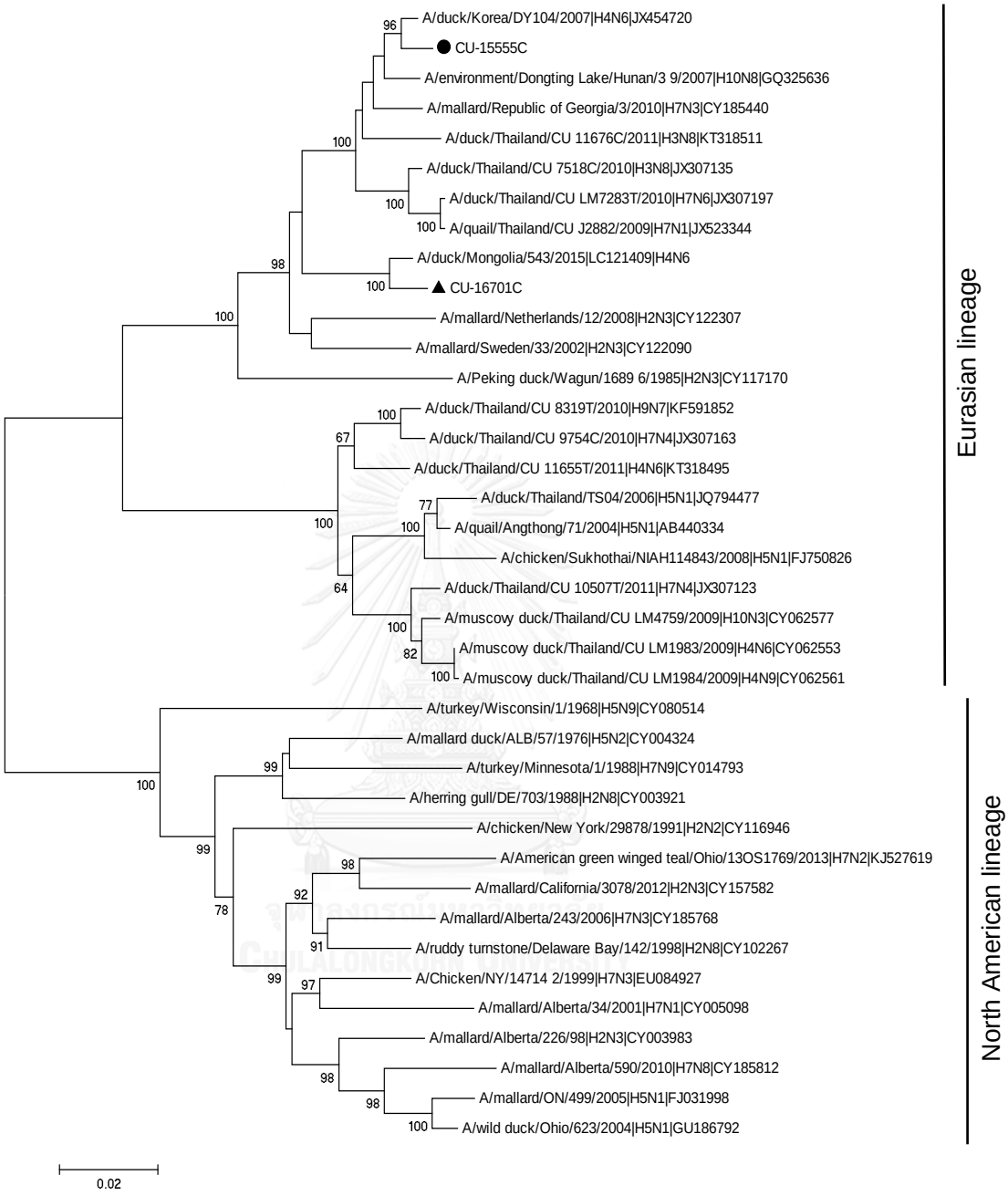

Figure 9 Phylogenetic tree of PB2 genes

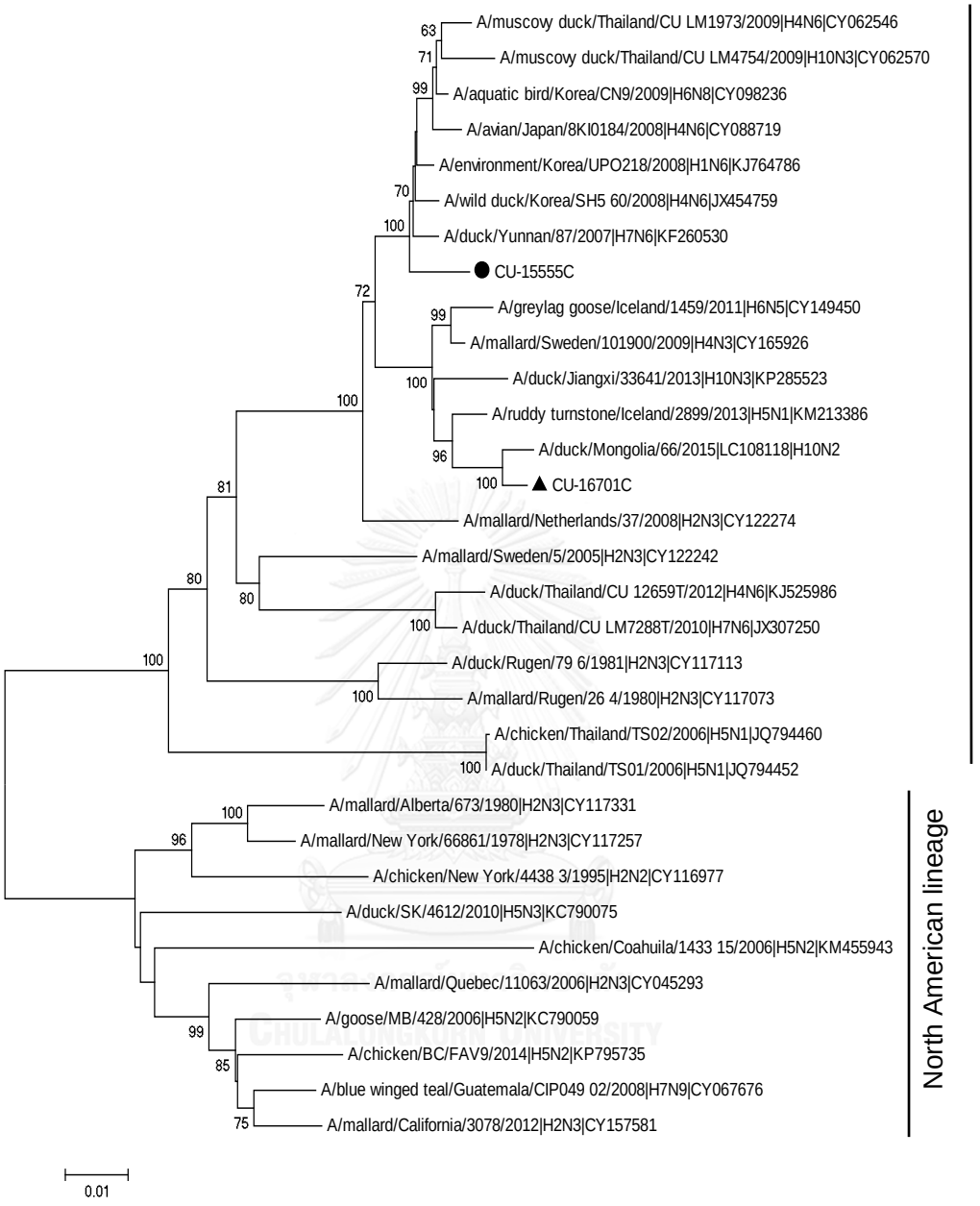

Figure 10 Phylogenetic tree of PB1 genes

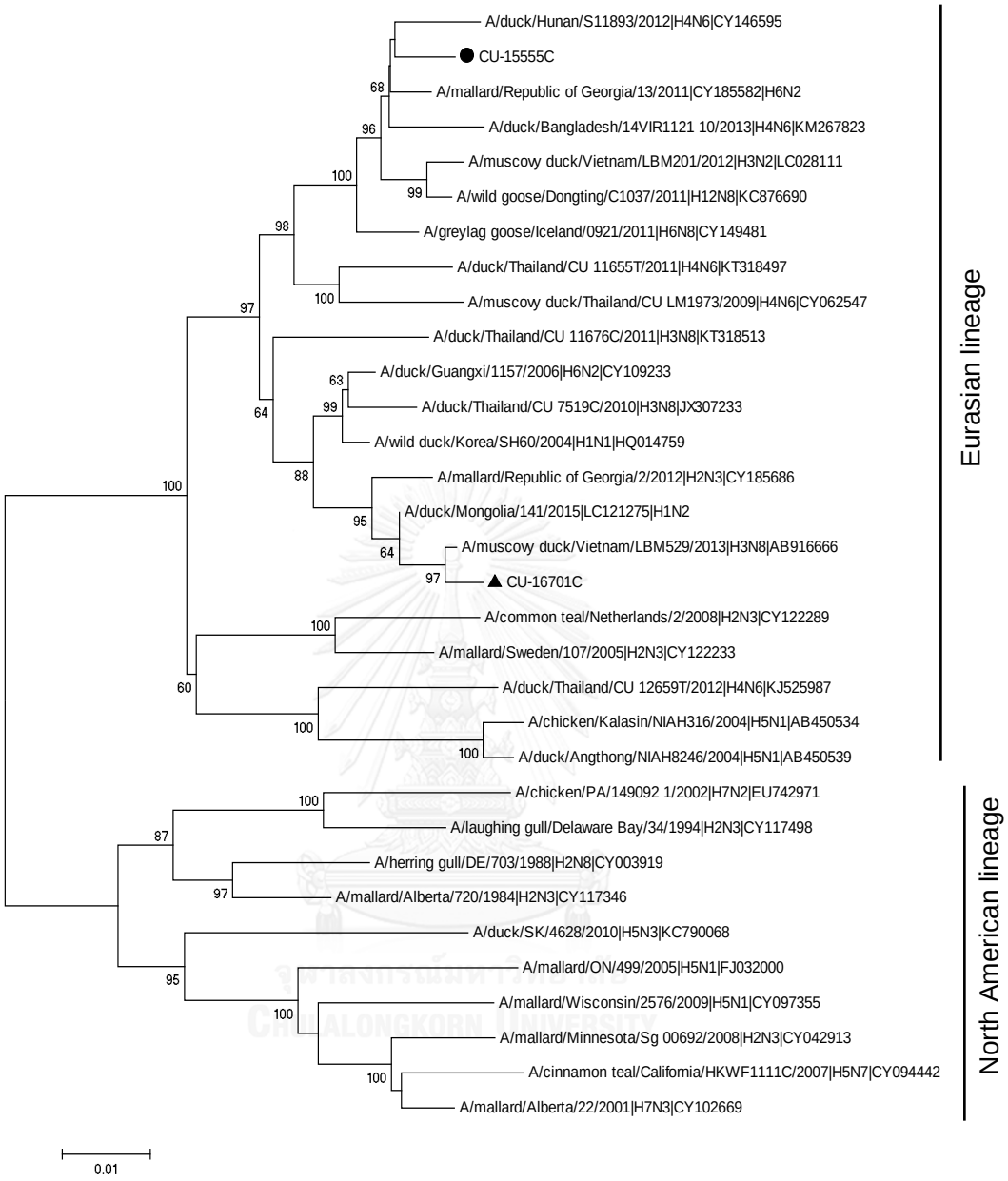

Figure 11 Phylogenetic tree of PA genes

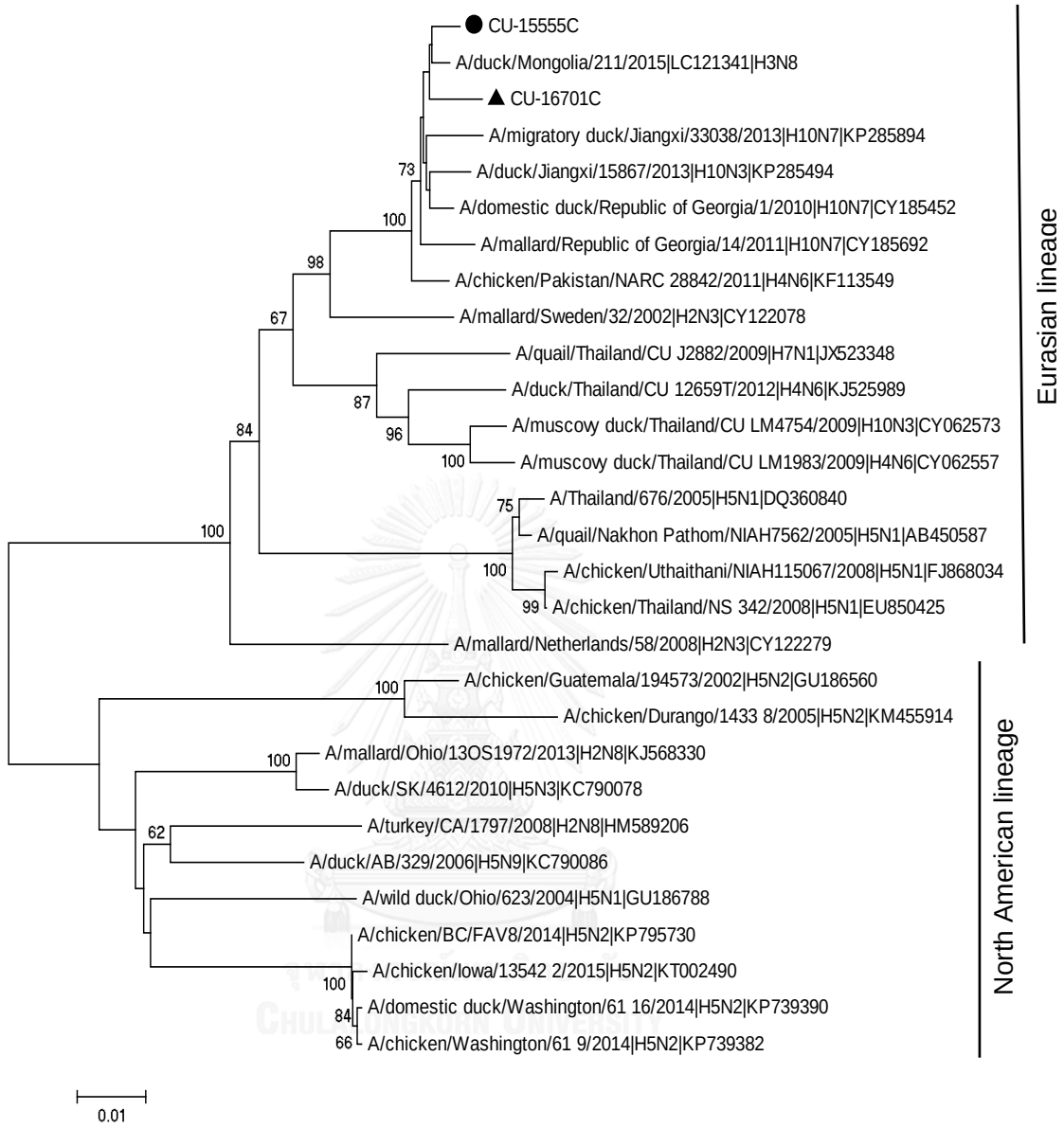

Figure 12 Phylogenetic tree of NP genes

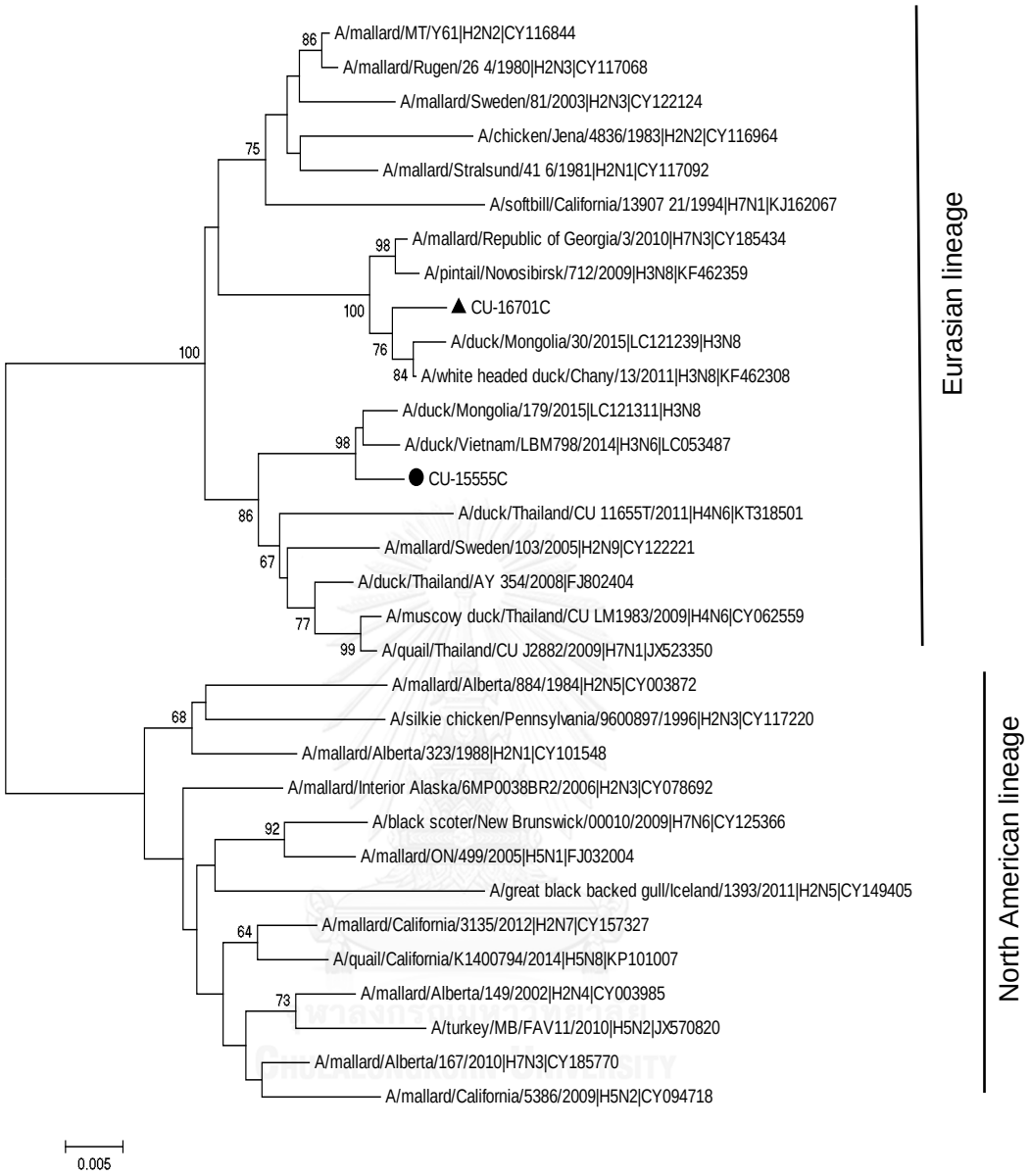

Figure 13 Phylogenetic tree of M genes

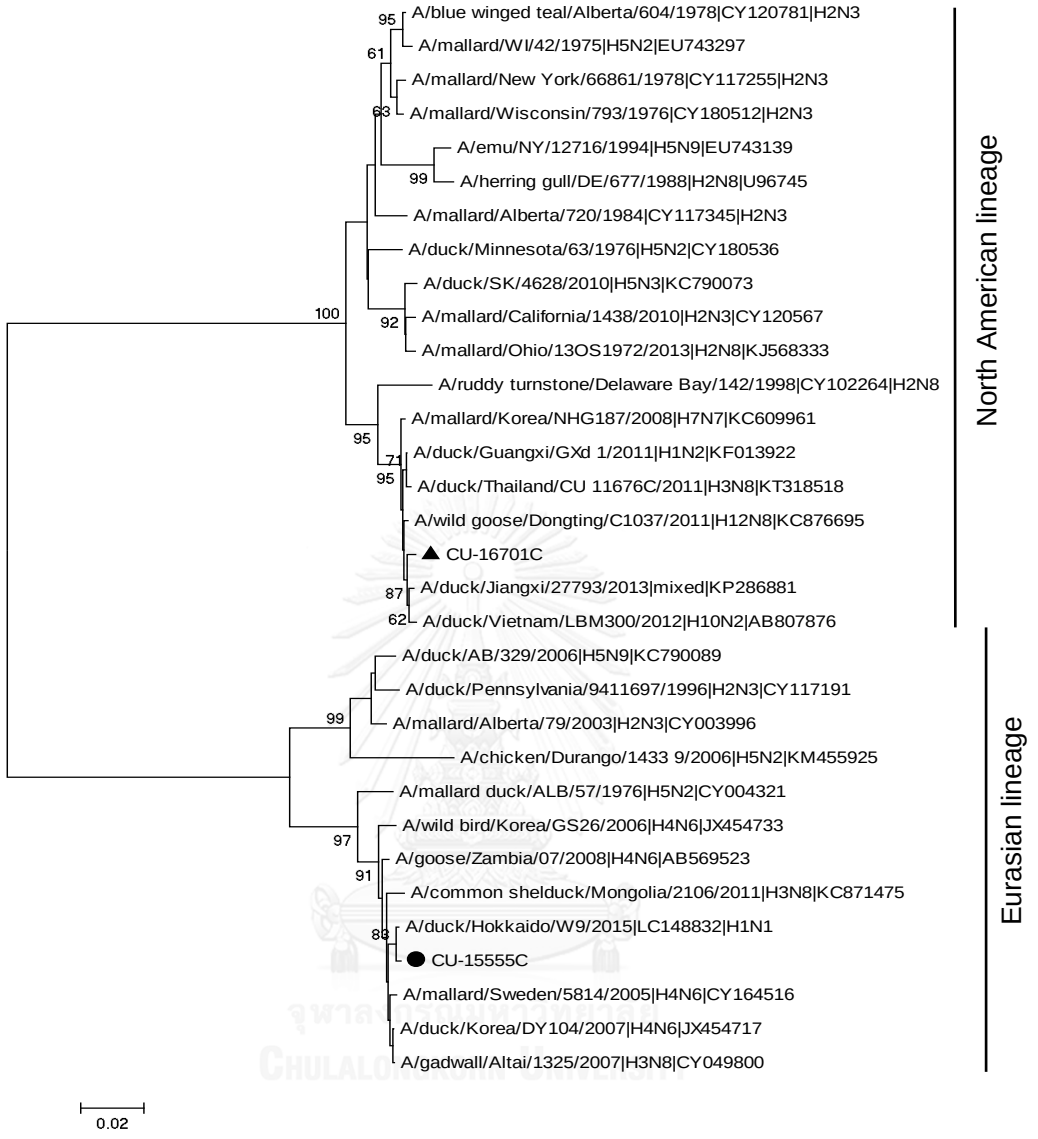

Figure 14 Phylogenetic tree of NS genes

Chapter 5 Discussion

5.1 Epidemiology of influenza A viruses isolated from LBMs

The occurrence of influenza A isolated from this study was 1.31% (18/1,374) which was higher than those in the previous studies in LBMs in 2009 (0.36%) and 2006-2007 (1.30%) in Thailand (Amonsin et al., 2008; Wisedchanwet et al., 2011). However, this study was surveyed in Bangkok area, while the previous studies in 2006-2007 focused on HPAI H5N1 survey in 10 provinces of central Thailand. Previous study in 2009 focused on 4 provinces of central Thailand. In this study, 18 isolates were isolated from 15 healthy mature ducks from a LBM (Market C) located in Khlong-Toei district, Bangkok. It is noted that this LBM housed many live poultry vendor shops and received poultry from different sources from central and eastern provinces of Thailand.

In this study, all viruses were isolated from ducks in a LBM. Ducks and other aquatic birds are known as the reservoir of IAVs. Survey of IAVs in ducks and aquatic birds have revealed high prevalence of low pathogenic avian influenza (LPAI) primarily in juvenile birds (Webster et al., 1992). However, in this study, most ducks housed in LBMs were mature ducks and slaughtered for fresh meat every day. Thus, it could reflect low prevalence of IAVs in LBMs in this study. On the other hand, all chickens were tested negative for IAVs. This result suggested low rate of IAVs infection in chickens in LBMs and correlated with previous study that chickens have

lower susceptibility to LPAI than other avian species (Tonnessen et al., 2011). Another reason, only healthy chickens were usually sold in LBMs, while HPAI infected chickens present severe clinical signs. It is noted that HPAI-H5N1 was not detected from selected LBMs in this study. This finding correlated with no HPAI-H5N1 outbreaks reported in Thailand since 2008 until now.

5.2 Live bird markets

Our results correlated with other reports that LBMs are the potential sources of IAVs transmission and evolution. Previous study reported that two IAV isolates (H4N6 and H4N9) were found in the same duck, possible multiple subtypes infection or genetic reassortment in duck in LBM in Thailand (Wisedchanwet et al., 2011).

In this study, three LBMs in Bangkok were surveyed monthly during May 2014 to April 2015. All selected LBMs were local food market located near community areas. Domestic poultry were housed in cages with high density setting and slaughtered on site for fresh meat. From our observations in each vendor shops, they usually housed poultry from dealers for 1-7 days depending on supply and demand. Some shops receive poultry from dealers daily, causing more poultry from different sources in the shops during housing periods. In this study, market C in Khlong-Toei district, Bangkok house many live poultry vendor shops and receive poultry from different sources every day, this environment could lead more chances

of IAVs transmission than other 2 LBMs which located in Samphanthawong and Khlong-San districts in Bangkok.

5.3 Genetic characterization of influenza A viruses isolated from LBMs

In this study, two IAV subtypes were identified as H2N3 and H2N8. Genetic characterization of representative viruses showed that viruses from this study pose low pathogenic characteristics (LPAI). Phylogenetic analysis showed that viruses were closely related to Eurasian avian viruses.

Genetic analysis of representative viruses, the receptor binding sites of H2 genes were Q226 and G228, the viruses prefer to bind with avian receptor (2,3-linked sialic acid receptors) (Connor et al., 1994). In human H2 and H3 viruses, the amino acid at position 226 and 228 change to leucine (Q226L) and serine (G228S), correlated with the adaptation from avian to mammalian hosts (Connor et al., 1994). The right and left edges of receptor binding site of the representative viruses at position 144-148 and 234-239, were GGSRA and NGQGGR, which is similar to those of H2 of avian Eurasian lineage (Nobusawa et al., 1991).

In N3 gene, the 23 amino acid deletion (position 56-78) was not observed at NA stalk region, implied that N3 genes from this study could be derived from Eurasian wild birds. The amino acid deletion of N3 gene was found in IAVs from domestic or terrestrial poultry but not wild species (Campitelli et al., 2004).

However, ducks that infected with H2N3 and H2N8 viruses could not be traced back to origin sources. Therefore, we do not know whether these ducks were housed with or without contact from wild birds.

In PB2 gene, E627 was observed in Thai H2N3 and H2N8 viruses. However, mutation of E627K in PB2 gene enhance the virus replication in mammal hosts as previously found in HPAI-H5N1 from mammals and human in Thailand and H7N9 isolated from human in China (Amonsin et al., 2006b; Gao et al., 2013). Moreover, aspartic acid (D) at position 30 in M1 gene and position 92 in NS1 gene were found in Thai H2N3 and H2N8 viruses, it was noted that D30 in M1 and D92 in NS1 gene associated with high virulence of virus in mammals as previously found in HPAI-H5N1 isolated in Thailand (Seo et al., 2004; Amonsin et al., 2006b; Fan et al., 2009).

5.4 Influenza A viruses subtype H2

IAV subtype H2 have never been reported from animals or humans in Thailand (according to data from GenBank database). Our result is the first report of H2N3 and H2N8 subtypes in Thailand**.** It should be noted that IAV subtype H2 were first isolated in human from 1957 to 1968 as the pandemic H2 human viruses and then disappeared. This H2 human pandemic strain was derived from avian Eurasian strains (Mulder et al., 1958). Even though, H2 viruses have not been detected in

human for a long period of time, H2 viruses have commonly circulated in domestic poultry and aquatic birds (Shortridge, 1979; Sinnecker et al., 1983).

In 1900-1991, the National Veterinary Services Laboratories in Ames, USA, reported H2N2 viruses in healthy chickens, ducks, guinea fowl, and environment in live poultry markets (Schafer et al., 1993). In 2013, novel H2N2 isolated from a healthy duck in LBM in China, this virus emerged from genetic reassortant viruses circulating in Eurasian ducks (Ma et al., 2014).

During the past decades, only small numbers of avian H2 viruses were reported from domestic poultry and wild birds in North America, Sweden, the Netherlands, Japan and Hong Kong but not in Thailand. In general, H2 subtypes of IAVs are isolated not only from avian species, but also from swine. The researchers in the US isolated novel H2N3 viruses from swine herd in 2006, the genetic analysis showed that these viruses were originated from American avian lineage. The swine H2N3 viruses pose receptor binding residues which increase affinity for 2,6-linked sialic acid receptors, suggesting it ability to replicate and transmit in mammalian hosts (Ma et al., 2007). Swine could be infected with both human and avian influenza viruses. It is possible to promote novel reassortant viruses, the resulting those viruses would likely be able to infect human and spread from person to person (Castrucci et al., 1993; Ito et al., 1998). Thus, active surveillance and genetic characterization of avian H2 viruses are important for monitoring of mammalian

adaptation, antiviral susceptibility and candidate vaccine strain selection. All of which are critical components in public health response to Influenza.

In addition, we found some limitations of this study. First, this study was conducted for one year (during May 2014 to April 2015). Even though H2 viruses could be found circulating in LBMs but the correlation of seasonal pattern or other factors indicating the risk of IAVs circulation and transmission in LBMs could not be concluded. Second, the origin of H2 viruses could not be identified. Infection of H2 viruses in poultry could be before or after entering LBMs. Thus H2 viruses may or may not be introduced from wild birds to domestic poultry and brought into LBMs. Third, H2 viruses had never been reported in LBMs in Thailand. Further studies on the pathogenicity and transmission of H2 viruses should be conducted.

Based on our results, several subtypes of IAV in this study reveal diversity of viruses circulating in Bangkok. The valuable information about occurrence, circulation and transmission of IAVs can help the authorities to utilize this knowledge for disease preventive measures. Good sanitary and personal hygiene of vendors or workers can reduce risk of IAV circulation in LBMs. Thus, continuous survey of IAV in LBMs should be routinely conducted.

Conclusions and suggestions

In this study, H2N3 and H2N8 were found circulating in LBMs in Bangkok during May 2014 to April 2015. Our findings could be summarized as following

- H2N3 and H2N8 had never been reported in LBM in Thailand. The isolation of H2N3 and H2N8 subtypes in this study implied that H2 viruses introduced to domestic poultry and brought into LBM.
- H2N3 viruses were isolated from LBM in March and April in 2015. In this situation, the viruses could be separately introduced from poultry farms into LBM or continuously circulate in LBM. Unfortunately, information of the source of H2 viruses was limited.
- From genetic analysis, both H2N3 and H2N8 pose low pathogenic characteristics. Based on receptor binding sites of H2 viruses suggested that these viruses preferential binding with avian-type receptor.
- All viruses were isolated from ducks in LBM where housed many poultry vendor shops and received poultry from the central and eastern provinces in Thailand. It is noted that LBMs could be potential sources for transmission and evolution of IAVs.

From our conclusions, continuous survey of IAVs in LBMs is very important and should be conducted to reveal influenza A viruses circulation and evolution.

The results from this study could lead to suggestions on IAVs prevention and control in LBMs as following

- Authorities or veterinarians should know information about occurrence, circulation and transmission of IAVs to utilize this knowledge for disease preventive measures.
- Vendors or workers should wear proper personal protective equipment (PPE), careful attention to hand hygiene and avoid unprotected contact with sick or death poultry in LBMs.
- LBMs need appropriate sanitation and hygiene. Cleaning of environment in LBMs with disinfectant can reduce the risk of IAVs circulation.
- Farm workers should maintain sanitation standards in poultry farms, provide protective clothes to those who visit farm, house poultry indoor to avoid contact with wild birds which have been known as natural reservoirs of IAVs.
- In rural areas, poultry were raised in backyard with other animals. This environment creates suitable surroundings for IAVs transmission and reassortment. To reduce the risk of IAVs transmission to human, reduction of human-animal interface should be recommended.

To date, there is no evidence of human infection with H2 viruses from LBMs in Thailand. However, good sanitation of LBMs, provident information of IAVs and

continuous surveys of IAVs in LBMs are very important to prevent outbreaks or transmission of H2 viruses from animals to human.

REFERENCES

Abdelwhab EM, Selim AA, Arafa A, Galal S, Kilany WH, Hassan MK, Aly MM and Hafez MH. 2010. Circulation of avian influenza H5N1 in live bird markets in Egypt. Avian Dis. 54(2): 911-914.

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

Amonsin A, Chutinimitkul S, Pariyothorn N, Songserm T, Damrongwantanapokin S, Puranaveja S, Jam-On R, Sae-Heng N, Payungporn S, Theamboonlers A, Chaisingh A, Tantilertcharoen R, Suradhat S, Thanawongnuwech R and Poovorawan Y. 2006a. Genetic characterization of influenza A viruses (H5N1) isolated from 3rd wave of Thailand AI outbreaks. Virus Res. 122(1-2): 194-199.

Amonsin A, Payungporn S, Theamboonlers A, Thanawongnuwech R, Suradhat S, Pariyothorn N, Tantilertcharoen R, Damrongwantanapokin S, Buranathai C, Chaisingh A, Songserm T and Poovorawan Y. 2006b. Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. Virology. 344(2): 480-491.

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

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

Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y and Webster RG. 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology. 193(1): 503-506.

Cattoli G, Monne I, Fusaro A, Joannis TM, Lombin LH, Aly MM, Arafa AS, Sturm-Ramirez KM, Couacy-Hymann E, Awuni JA, Batawui KB, Awoume KA, Aplogan GL, Sow A, Ngangnou AC, El Nasri Hamza IM, Gamatie D, Dauphin G, Domenech JM and Capua I. 2009. Highly pathogenic avian influenza virus subtype H5N1 in Africa: a comprehensive phylogenetic analysis and molecular characterization of isolates. PLoS One. 4(3): e4842.

Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, Yao H, Wo J, Fang Q, Cui D, Li Y, Yao X, Zhang Y, Wu H, Zheng S, Diao H, Xia S, Zhang Y, Chan KH, Tsoi HW, Teng JL, Song W, Wang P, Lau SY, Zheng M, Chan JF, To KK, Chen H, Li L and Yuen KY. 2013. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. Lancet. 381(9881): 1916-1925.

Choi YK, Seo SH, Kim JA, Webby RJ and Webster RG. 2005. Avian influenza viruses in Korean live poultry markets and their pathogenic potential. Virology. 332(2): 529-537.

Connor RJ, Kawaoka Y, Webster RG and Paulson JC. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology. 205(1): 17-23.

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 5: 113.

Fan S, Deng G, Song J, Tian G, Suo Y, Jiang Y, Guan Y, Bu Z, Kawaoka Y and Chen H. 2009. Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 avian influenza viruses in mice. Virology. 384(1): 28-32.

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

Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T,

Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z and Shu Y. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 368(20): 1888-1897.

Guan Y, Peiris JS, Lipatov AS, Ellis TM, Dyrting KC, Krauss S, Zhang LJ, Webster RG and Shortridge KF. 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc Natl Acad Sci U S A. 99(13): 8950-8955.

Horimoto T and Kawaoka Y. 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J Virol. 68(5): 3120-3128.

Horm SV, Sorn S, Allal L and Buchy P. 2013. Influenza A(H5N1) virus surveillance at live poultry markets, Cambodia, 2011. Emerg Infect Dis. 19(2): 305-308.

Indriani R, Samaan G, Gultom A, Loth L, Irianti S, Adjid R, Dharmayanti NL, Weaver J, Mumford E, Lokuge K, Kelly PM and Darminto. 2010. Environmental sampling for avian influenza virus A (H5N1) in live-bird markets, Indonesia. Emerg Infect Dis. 16(12): 1889-1895.

Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG and Kawaoka Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol. 72(9): 7367-7373.

Joannis T, Lombin LH, De Benedictis P, Cattoli G and Capua I. 2006. Confirmation of H5N1 avian influenza in Africa. Vet Rec. 158(9): 309-310.

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

Kimura H, Abiko C, Peng G, Muraki Y, Sugawara K, Hongo S, Kitame F, Mizuta K, Numazaki Y, Suzuki H and Nakamura K. 1997. Interspecies transmission of influenza C virus between humans and pigs. Virus Res. 48(1): 71-79.

Lee CW, Suarez DL, Tumpey TM, Sung HW, Kwon YK, Lee YJ, Choi JG, Joh SJ, Kim MC, Lee EK, Park JM, Lu X, Katz JM, Spackman E, Swayne DE and Kim JH. 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J Virol. 79(6): 3692-3702.

Lee HJ, Kwon JS, Lee DH, Lee YN, Youn HN, Lee YJ, Kim MC, Jeong OM, Kang HM, Kwon JH, Lee JB, Park SY, Choi IS and Song CS. 2010. Continuing evolution and interspecies transmission of influenza viruses in live bird markets in Korea. Avian Dis. 54(1 Suppl): 738-748.

Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, Bi Y, Wu Y, Li X, Yan J, Liu W, Zhao G, Yang W, Wang Y, Ma J, Shu Y, Lei F and Gao GF. 2013. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet. 381(9881): 1926-1932.

Ma MJ, Yang XX, Qian YH, Zhao SY, Hua S, Wang TC, Chen SH, Ma GY, Sang XY, Liu LN, Wu AP, Jiang TJ, Gao YW, Gray GC, Zhao T, Ling X, Wang JL, Lu B, Qian J and Cao WC. 2014. Characterization of a novel reassortant influenza A virus (H2N2) from a domestic duck in Eastern China. Sci Rep. 4: 7588.

Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, Gauger PC, Patnayak DP, Webby RJ and Richt JA. 2007. Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci U S A. 104(52): 20949-20954.

Moon HJ, Song MS, Cruz DJ, Park KJ, Pascua PN, Lee JH, Baek YH, Choi DH, Choi YK and Kim CJ. 2010. Active reassortment of H9 influenza viruses between wild birds and live-poultry markets in Korea. Arch Virol. 155(2): 229-241.

Mulder J, Masurel N and Hers JF. 1958. [The Asian influenza pandemic of 1957]. Ned Tijdschr Geneeskd. 102(41): 1992-1999.

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

Nishi T, Okamatsu M, Sakurai K, Chu HD, Thanh LP, van Nguyen L, van Hoang N, Thi DN, Sakoda Y and Kida H. 2014. Genetic analysis of an H5N2 highly pathogenic avian influenza virus isolated from a chicken in a live bird market in Northern Vietnam in 2012. J Vet Med Sci. 76(1): 85-87.

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

OIE. 2014. "Subject: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" (online). Available: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.

OIE. 2015. "Subject: Update on Highly Pathogenic Avian Influenza in Animals (TYPE H5 and H7)" (online). Available: http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/.

Okamatsu M, Nishi T, Nomura N, Yamamoto N, Sakoda Y, Sakurai K, Chu HD, Thanh LP, Van Nguyen L, Van Hoang N, Tien TN, Yoshida R, Takada A and Kida H. 2013. The genetic and antigenic diversity of avian influenza viruses isolated from domestic ducks, muscovy ducks, and chickens in northern and southern Vietnam, 2010-2012. Virus Genes. 47(2): 317-329.

Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM and Fouchier RA. 2000. Influenza B virus in seals. Science. 288(5468): 1051-1053.

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

Perez D RE, Smith G, et al. 2011. Orthomyxoviridae. In: Virus Taxonomy. Elsevier, Oxford. 749-762.

Schafer JR, Kawaoka Y, Bean WJ, Suss J, Senne D and Webster RG. 1993. Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology. 194(2): 781-788.

Seo SH, Hoffmann E and Webster RG. 2004. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. Virus Res. 103(1-2): 107-113.

Shaw M. 2013. Fields Virology. Vol. 1. 6th ed. In: Orthomyxoviridae. Lippincott Williams & Wilkins, Philadephia, PA, USA.

Shortridge KF. 1979. H2N2 influenza viruses in domestic ducks. Lancet. 1(8113): 439.

Sims LD, Domenech J, Benigno C, Kahn S, Kamata A, Lubroth J, Martin V and Roeder P. 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. Vet Rec. 157(6): 159-164.

Sinnecker R, Sinnecker H, Zilske E and Kohler D. 1983. Surveillance of pelagic birds for influenza A viruses. Acta Virol. 27(1): 75-79.

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

Songserm T, Jam-on R, Sae-Heng N, Meemak N, Hulse-Post DJ, Sturm-Ramirez KM and Webster RG. 2006b. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerg Infect Dis. 12(4): 575-581.

Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT and Suarez DL. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 40(9): 3256-3260.

Steel J, Lowen AC, Mubareka S and Palese P. 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog. 5(1): e1000252.

Tamura K, Dudley J, Nei M and Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24(8): 1596-1599.

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

Tiensin T, Ahmed SS, Rojanasthien S, Songserm T, Ratanakorn P, Chaichoun K, Kalpravidh W, Wongkasemjit S, Patchimasiri T, Chanachai K, Thanapongtham W, Chotinan S, Stegeman A and Nielen M. 2009. Ecologic risk factor investigation of clusters of avian influenza A (H5N1) virus infection in Thailand. J Infect Dis. 199(12): 1735-1743.

Tiensin T, Chaitaweesub P, Songserm T, Chaisingh A, Hoonsuwan W, Buranathai C, Parakamawongsa T, Premashthira S, Amonsin A, Gilbert M, Nielen M and Stegeman A. 2005. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg Infect Dis. 11(11): 1664-1672.

Tonnessen R, Valheim M, Rimstad E, Jonassen CM and Germundssond A. 2011. Experimental inoculation of chickens with gull-derived low pathogenic avian influenza virus subtype H16N3 causes limited infection. Avian Dis. 55(4): 680-685.

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

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

Viseshakul N, Thanawongnuwech R, Amonsin A, Suradhat S, Payungporn S, Keawchareon J, Oraveerakul K, Wongyanin P, Plitkul S, Theamboonlers A and Poovorawan Y. 2004. The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. Virology. 328(2): 169-176.

Wan XF, Dong L, Lan Y, Long LP, Xu C, Zou S, Li Z, Wen L, Cai Z, Wang W, Li X, Yuan F, Sui H, Zhang Y, Dong J, Sun S, Gao Y, Wang M, Bai T, Yang L, Li D, Yang W, Yu H, Wang S, Feng Z, Wang Y, Guo Y, Webby RJ and Shu Y. 2011. Indications that live poultry markets are a major source of human H5N1 influenza virus infection in China. J Virol. 85(24): 13432-13438.

Wang C, Wang J, Su W, Gao S, Luo J, Zhang M, Xie L, Liu S, Liu X, Chen Y, Jia Y, Zhang H, Ding H and He H. 2014. Relationship between domestic and wild birds in live poultry market and a novel human H7N9 virus in China. J Infect Dis. 209(1): 34-37.

Webster RG, Bean WJ, Gorman OT, Chambers TM and Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 56(1): 152-179.

WHO. 2015a. "Subject: Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO" (online). Available: http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/.

WHO. 2015b. "Subject: H5N1 avian influenza: timeline of major events" (online). Available: http://www.who.int/influenza/human_animal_interface/en/.

Wisedchanwet T, Wongpatcharachai M, Boonyapisitsopa S, Bunpapong N, Jairak W, Kitikoon P, Sasipreeyajun J and Amonsin A. 2011. Influenza A virus surveillance in live-bird markets: first report of influenza A virus subtype H4N6, H4N9, and H10N3 in Thailand. Avian Dis. 55(4): 593-602.

Yee KS, Novick CA, Halvorson DA, Dao N, Carpenter TE and Cardona CJ. 2011. Prevalence of low pathogenicity avian influenza virus during 2005 in two U.S. live bird market systems. Avian Dis. 55(2): 236-242.

APPENDICES

1. **Description of each LBMs in this study**

Market A

Location	Samphanthawong district, Bangkok
Type of market	Local food market
Poultry sold in market	Chickens and ducks
Number of poultry shops	3
Number of participating shop	1


Market B

Location	Khlong-San district, Bangkok
Type of market	Local food market
Poultry sold in market	Chickens and ducks
Number of poultry shops	2
Number of participating shop	1


Market C

Location	Khlong-Toei district, Bangkok
Type of market	Local food market
Poultry sold in market	Chickens, ducks and geese
Number of poultry shops	Approximately 20
Number of participating shops	2

2. **Protocol for real-time reverse transcription-polymerase chain reaction (rRT-PCR)**

Reagents	**Volume**
10 µM of forward and reverse primers	4 µl
2.5 µM 64 probe	0.5 µl
2x Master mix	6.25 µl
Superscript III	0.25 µl
Distilled water	0.42 µl
50 µM $MgSO_4$	0.08 µl
Final volume	**12.0 µl**

The PCR condition for real-time RT-PCR

Step1; 50°C for 30 minutes

Step2; 95°C for 15 minutes follow by 50 cycles

Step3; 95°C for 15 seconds

Step4; 60°C for 30 seconds

3. Protocol for cDNA synthesis

First step

Reagents	**Volume**
RNA	5 µl
Random primer	5 µl
Second step	
Distilled water	3.7 µl
5X cDNA buffer	4 µl
2.5 mM MgCl2	2 µl
0.5 mM dNTP	1 µl
RNase inhibitor	0.3 µl
ImProm-II™ Reverse Transcriptase	1 µl
Final volume	**22 µl**

4. Protocol for PCR subtyping assay

Reagents	**Volume**
cDNA	0.5 µl
10 µM of forward and reverse primers	0.5 µl
2X KAPA Taq Master Mix	5 µl
Distilled water	3.5 µl
Final volume	**10 µl**

The PCR condition for IAVs subtyping assay

Step1; 94°C for 2 minutes (Initial denaturation)

Step2; 94°C for 30 seconds (Denaturation)

Step3; 50°C (HA primers) and 45°C (NA primers) for 30 seconds (Annealing)

Step4; 72°C for 30 seconds (Extension)

Repeat step 2 to 4 for 40 cycles

Step5; 72°C for 7 minutes (Final extension

5. List of oligonucleotide primers for IAVs designed in this study

Gene	Primer name	Size (bp)	Primer sequence (5'-3')
PB2	PB2 F5'	24	agc aaa agc agg tca att ata ttc
	PB2 R1+	20	ttt gga ggt tgc ctg taa gc
	PB2 F2	21	agc aca aga tgt cat cat gga
	PB2 R3	21	cga cca ctc ttt cag tgc tag
	PB2 F41	21	gta gca atg gtg ttc tca cag
	PB2 R3'	25	agt aga aac aag gtc gtt ttt aaa c
	PB2 11F	19	agc raa agc agg tca awt a
	PB2 11R	18	ccc att gct gcy ttg cat
	PB2 12F	18	gga tgg trg aca tyc tta
	PB2 12R	22	ggt tca aay tcc atc tta ttg t
	PB2 13F	19	caa tga tgt ggg ara tca a
	PB2 13R	19	tgg cca tca gta gaa aca a
PB1	PB1 F5'	23	agc aaa agc agg caa acc att tg
	PB1 R2	21	agc tct gta tct tgt gag tta
	PB1 F3	21	gca tct gtg aaa aac ttg agc
	PB1 R4	21	atc act gta act cca atg ctc
	PB1 F3'	21	tcc tct gat gat ttc gct ctc
	PB1 R3'	23	agt aga aac aag gca ttt ttt ca
PA	PA F5'	22	agc aaa agc agg tac tga tcc g
	PA R1	19	gtc tct tcg cct ctc tcg g
	PA F2	20	gat gaa gag agc agg gca ag
	PA R3	21	ctc att tcc atg ccc cat ttc
	PA F3	19	tgg aag cag gtg ctg gca g
	PA R3'	24	agt aga aac aag gta ctt ttt tgg
HA	H2 A162F	24	cca tag aca atc aaa agc aag aca
	H2 A162R	20	agc att tgt tcc cca ctt gt
	H2 A163F	20	ttg ccc aaa gat agg tgg ac
	H2 A163R	20	tcc ata cca acc atc aac ca
	H2 A164F	22	ttg gca aca gga tta aga aat g
	H2 A164R	20	ttc atg cat tca tca tca ca
	H2 A165F	21	tgc aaa aga act agg gaa tgg
	HA HoffR	35	ata tcg tct cgt att agt aga aac aag ggt gtt tt
NP	NP F5'	22	agc aaa agc agg gta gat aat c
	NP R1	21	cca tcg tcc cga ctc cct tta
	NP F2	21	tga tgc cac ata cca gag aac
	NP R3'	23	agt aga aac aag ggt att ttt ct

List of oligonucleotide primers for IAVs designed in this study (Continued)

Gene	Primer name	Size (bp)	Primer sequence (5'-3')
NA	N3 F1	21	tga atc caa atc aga aga taa
	N3 R1	20	tag ttc cya gya wwg cyc ct
	N3 F2	20	ttc aca agg aca atg caa ta
	N3 R2	20	aca aca car gtc ccr tca at
	N3 F3	20	tgc atg acw ggr aat gay aa
	N3 R3	19	ayr ttr ctt ggr gar tca c
	N3 F4	20	ayt rga aac agg gta tgt rt
	N3 R4	21	tct rtt act tgg gca taa acc
	N8 A31F	22	aaa agc agg agt tta aaa tga a
	N8 A31R	18	gtc aca cca acc gtc atc
	N8 A32F	18	act cga atg gga cgg taa
	N8 A32R	20	tca atg tgt cct cca tta aa
	N8 A33F	20	tag ttc att atg ggg gag tg
	N8 A33R	22	gac caa ttc aaa tta tca aca a
	N8 A34F	20	gat act cca aga ggg gaa ga
	N8 A34R	21	tga cca gta gaa aca agg agt
M	M 71F	20	gga gcr aaa gca ggt aga tr
	M 71R	21	tct gct cca tag cct twg cyg
	M72F	18	cca gtg arc gag gac tgc
	M 72R	20	tgt tga caa aat gac cat cg
NS	NS 1F	20	agc raa agc agg gtg aca aa
	NS 890R	20	agt aga aac aag ggt gtt tt

6. Single letter amino acid code

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

VITA

Mr. Wittawat Wechtaisong was born on September 28, 1988 in Nakhon Ratchasima, Thailand. He graduated from the Faculty of Veterinary Medicine, Khon Kaen University, Thailand in 2012. After that, he enrolled in the Master degree of Science at the department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand since academic year 2013.