GENETIC CHARACTERISTICS OF AVIAN INFLUENZA A VIRUSES ISOLATED FROM THAI FREE-GRAZING DUCKS AND THEIR PATHOGENICITY POTENTIAL IN ANIMAL MODELS

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ลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่สัตว์ปีกในเป็ดไล่ทุ่งไทยและศักยภาพในการก่อโรคในสัตว์ทดลอง

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สุพัศมา ไชยวงษ์ : ลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่สัตว์ปีกในเป็ดไล่ทุ่งไทยและศักยภาพในการก่อโรคในสัตว์ทดลอง. (GENETIC CHARACTERISTICS OF AVIAN INFLUENZA A VIRUSES ISOLATED FROM THAI FREE-GRAZING DUCKS AND THEIR PATHOGENICITY POTENTIAL IN ANIMAL MODELS) อ.ที่ปรึกษาหลัก : ศ. น.สพ.ดร.อลงกร อมรศิลป์

เชื้อไวรัสไข้หวัดใหญ่ชนิดเอ (influenza A virus) สามารถก่อให้เกิดโรคไข้หวัดใหญ่ได้ในสัตว์หลายชนิดรวมถึงมนุษย์ ในประเทศไทยเป็ดไล่ทุ่งเป็นโฮสต์ที่สำคัญของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ โดยเป็ดไล่ทุ่งมีความสำคัญเนื่องจากเป็ดไล่ทุ่งสัมผัสกับทั้งนกป่าและสัตว์ปีกที่เลี้ยงตามบ้าน ซึ่งทำให้เกิดการติดต่อของเชื้อไวรัสไข้หวัดใหญ่ข้ามชนิดสัตว์ การศึกษาวิจัยในวิทยานิพนธ์นี้มี 3 ขั้นตอน ประกอบด้วย ขั้นตอนที่ 1 สำรวจเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในเป็ดไล่ทุ่ง ขั้นตอนที่ 2 วิเคราะห์รหัสพันธุกรรม และความหลากหลายทางสายพันธุ์ของเชื้อไวรัสในเป็ดไล่ทุ่ง ขั้นตอนที่ 3 ทดสอบศักยภาพในการก่อโรคของเชื้อไวรัสจากเป็ดไล่ทุ่งในสัตว์ทดลองชนิดไก่และนกกระทา สำหรับขั้นตอนที่ 1 การสำรวจเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในเป็ดไล่ทุ่งเป็นเวลาหลายปี โดยเก็บตัวอย่างจากพื้นที่ภาคกลางและภาคเหนือตอนล่างของประเทศไทย ผลการตรวจพบเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ จากตัวอย่างป้ายปาก จำนวน 2,012 ตัวอย่าง และ ตัวอย่างป้ายทวาร จำนวน 2,012 ตัวอย่าง คิดเป็น 1.77% และจากตัวอย่างซีรัม (n=1,959) ตรวจพบแอนติบอดีต่อเชื้อไวรัสไข้หวัดใหญ่คิดเป็น 70.24% และแอนติบอดีจำเพาะต่อเชื้อไวรัสสายพันธุ์ H5 คิดเป็น 6.40% จากผลการตรวจพิสูจน์เชื้อไวรัสไข้หวัดนกจำนวน 71 ตัวอย่าง พบตัวอย่างที่ให้ผลบวกเชื้อไวรัสและจำแนกสายพันธุ์ได้ 15 สายพันธุ์ ซึ่งสายพันธุ์ที่พบบ่อยที่สุดคือ H3N8 และ H4N6 ในขั้นตอนที่ 2 ได้เลือกเชื้อไวรัสไข้หวัดนกจำนวน 26 สายพันธุ์มาวิเคราะห์รหัสพันธุกรรมทั้งหมด ผลการวิเคราะห์โดยวิธี phylogenetic analysis พบเชื้อไวรัสลูกผสม (reassortant viruses) จำนวน 3 สายพันธุ์ (H11N9, H4N6 และ H11N7) โดยพบว่าเชื้อไวรัสไข้หวัดใหญ่ในไทยมีต้นกำเนิดจากสายพันธุ์ที่อยู่ในทวีปเอเชียและยุโรป (Avian Eurasian lineage I) ในการวิเคราะห์ของแต่ละยีนของเชื้อไวรัสที่เป็นตัวแทนจำนวน 26 สายพันธุ์ พบว่ามีรูปแบบของยีนที่แตกต่างกันจำนวน 24 รูปแบบ ในขั้นตอนที่ 3 เชื้อไวรัสสายพันธุ์ H1N3 และ H11N9 ได้นำมาทดสอบศักยภาพในการก่อโรค พบว่าเชื้อไวรัสทั้ง 2 สายพันธุ์สามารถทำให้ไก่และนกกระทาเกิดการติดเชื้อได้ โดยไก่และนกกระทาที่ติดเชื้อนั้นมีการตอบสนองทางภูมิคุ้มกันในระหว่างการทดลอง นอกจากนี้เชื้อไวรัสทั้ง 2 สายพันธุ์สามารถติดต่อไปยังไก่และนกกระทาที่สัมผัสกับสัตว์ที่ได้รับเชื้อไวรัส ผลของการศึกษานี้ยืนยันว่าเป็ดไล่ทุ่งเป็นโฮสต์ที่สำคัญของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ และเชื้อไวรัสสายพันธุ์ H1N3 และ H11N9 ที่พบในเป็ดไล่ทุ่งสามารถก่อให้เกิดการติดเชื้อและแพร่กระจายได้ในไก่และนกกระทา ดังนั้นผลของการสำรวจเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในเป็ดไล่ทุ่ง การวิเคราะห์รหัสพันธุกรรม และการทดสอบศักยภาพในการก่อโรคในสัตว์ทดลอง เป็นประโยชน์สำหรับการป้องกันโรคไข้หวัดใหญ่ และวางแผนการควบคุมโรคทั้งในสัตว์และมนุษย์ในอนาคต

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Supassama Chaiyawong : GENETIC CHARACTERISTICS OF AVIAN INFLUENZA A VIRUSES ISOLATED FROM THAI FREE-GRAZING DUCKS AND THEIR PATHOGENICITY POTENTIAL IN ANIMAL MODELS. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Influenza A viruses (IAVs) can infect several animal species and humans. In Thailand, free-grazing ducks are reservoir hosts of influenza A virus. The free-grazing ducks are potentially interface with wild birds and domestic birds and subsequently cause interspecies transmission of IAVs. This thesis composes of three study phases. Phase 1 is surveillance of influenza A in free-grazing ducks. Phase 2 is genetic characterization and diversity analysis of influenza A viruses from free-grazing ducks. Phase 3 is challenge of influenza A viruses isolated from free-grazing ducks in animal models. For phase 1, multi-years surveillance of IAVs in free-grazing ducks was conducted in central and lower northern parts of Thailand. Our results found 1.77% of influenza A virus from oropharyngeal swabs (n=2,012) and cloacal swabs (n=2,012), 70.24% of influenza A antibody and 6.40% of specific H5 subtype antibody from serum samples (n=1,959). Of 71 IAV positive samples, 15 subtypes of IAVs could be identified and the predominant subtypes were subtypes H3N8 and H4N6. For phase 2, representative viruses (n=26) were characterized by whole genome sequencing. The results from phylogenetic analysis of 8 genes of Thai influenza viruses revealed new reassortant viruses (n=3) (H11N9, H4N6 and H11N7). While the origin of most Thai IAVs was from Avian Eurasian lineage I. According to the origins of each gene, the gene constellation of 26 representative viruses was identified to 24 patterns. For phase 3, influenza A virus subtype H1N3 (n=1) and H11N9 (n=1) were selected for animal challenge study. Both H1N3 and H11N9 viruses can infect chickens and quails. Chickens and quails can shed the viruses and develop antibody response during the experiment period. The viruses can also transmit to contacted chickens and quails. In summary, our results confirmed that free-grazing ducks are important reservoir hosts of influenza A virus and the H1N3 and H11N9 viruses from free grazing ducks were able to infect and transmit to chickens and quails. Thus, the results from multi-years influenza A surveillance in free-grazing ducks, the genetic characteristics and the pathogenicity of the viruses in animal models provided useful information for influenza prevention and control measures both in animals and humans in the future.

Field of Study:	Veterinary Public Health	Student's Signature .....
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LIST OF ABBREVIATIONS

AI	Avian Influenza
bp	base pair
cDNA	conplementary deoxyribonucleic acid
ºC	degree Celsius
CS	Cloacal swab
dNTPs	Deoxynucleotide triphosphates ELISA Enzyme-Linked Immunosorbent Assay
EID_{50}	50% Egg Infective Dose
et al.	et alibi, and other
HA	Hemagglutinin
HI	Hemagglutination inhibition test
HPAI	Highly Pathogenic Avian Influenza
IAV	Influenza A virus
IVPI	Intravenous Pathogenicity Index
LPAI	Low Pathogenic Avian Influenza
M	Matrix
mg	milligram(s)
ml	milliliter(s)
µl	microliter

mM	micromolar
NA	Neuraminidase
NP	Nucleoprotein
NS	Nonstructural protein
OP	Oropharyngeal swab
PA	Polymerase acidic protein 4049754705
PCR	Polymerase Chain Reaction
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Standard Deviation

CHAPTER 1

INTRODUCTION

Influenza virus is a RNA virus of the family Orthomyxoviridae. The virus can be classified into 5 genera: Influenza A, B and C, Thogotovirus and Isavirus. Influenza A virus (IAV) can infect several animal species including humans and can transmit interspecies. Influenza A virus contains eight gene segments including PB2, PB1, PA, HA, NP, NA, M and NS (Webster et al., 1992). IAV can be subtyped, based on hemagglutinin and neuraminidase proteins on surface of the virus, into 18 HA subtypes and 11 NA subtypes (Fouchier et al., 2005; Tong et al., 2012; Tong et al., 2013). The first influenza pandemic outbreak in 1918 was caused by IAV subtype H1N1. Since then, two other pandemic IAV outbreaks were reported in 1957 and 1968. In the 21th century, a pandemic IAV outbreak caused by pdmH1N1-2009 was reported in 2009. During the past ten to fifteen years, IAV epidemics have also been reported to affect animals and humans including H5N1 and recently H7N9 (Garten et al., 2009; Gibbs and Gibbs, 2006; Kageyama et al., 2013; Worobey et al., 2014). Both IAV-H5N1 and IAV-H7N9 viruses can directly transmit from poultry to human (de Jong et al., 1997; Gao et al., 2013). Genetic of IAV can change by reassortment and point mutation process. Due to the segmented genome of IAV, the virus can exchange the genetic material with another virus strain, when infect in the same host cell and generate reassortant virus (Steinhauer and Holland, 1987). Quail is one of the intermediate hosts for IAVs due to it appropriate for both avian and mammal influenza virus infections. Thus, the intermediate host can generate mixed origins-reassortant virus (Wan and Perez, 2006).

Natural reservoir of influenza A virus is aquatic bird including ducks. Aquatic birds have been reported to infect with several IAV subtypes such as H1-H16 and N1-N9. In general, IAVs found in aquatic birds are low pathogenicity avian influenza

(LPAI). LPAI causes subclinical signs in infected birds (Yoon et al., 2014). When LPAI transmits and infects in poultry, the virus can rapidly evolve to high pathogenicity avian influenza virus (HPAI) and sometimes high virulence in poultry (Suarez et al., 2004). Therefore, LPAI viruses in aquatic birds can be ancestral viruses of HPAI viruses of poultry (Claas et al., 1998; Peiris et al., 1999). Some LPAI such as IAV subtype H7N9 cause subclinical sign in poultry, however it can cause severe respiratory disease and death in human (Lam et al., 2013).

Free-grazing duck production is a common practice in Southeast Asia countries including Thailand. The animals are raised to free-graze in rice field after harvesting and frequently moved among rice fields for new food sources. During the epidemic of HPAI-H5N1 in Thailand, free-grazing ducks have been reported in association with H5N1 outbreaks (Gilbert et al., 2006; Waicharoen et al., 2008). Infected free-grazing ducks with un-observed clinical signs increase risk of virus to spread widely area (Hulse-Post et al., 2005). Moreover, free-grazing ducks are potentially interface with wild birds and domestic birds such as backyard ducks and chickens and subsequently cause interspecies transmission of IAVs. In 2004, Thailand's national influenza surveillance reported 45.6% (28/61) of HPAI-H5N1 infection in free-grazing duck flocks (Songserm et al., 2006). In 2010, serological survey found 85% of free-grazing ducks posed antibody against IAV by NP-ELISA and 12% by H5 by HI test (Beaudoin et al., 2014).

The outbreak of H5N1 has not been reported in Thailand since 2008. However, sero-surveillance of IAV revealed that antibody against IAV subtype H5 can be detected in free-grazing ducks and wild birds (Beaudoin et al., 2014; Poltep et al., 2018). There are several reports of IAV-H5 reassortants with different NA subtypes, including N1, N2, N3, N5, N6, and N8, and subsequently the viruses can cause

outbreaks in poultry and in Asia, e.g. H5N5 and H5N8 in China, South Korea and Japan, H5N6 in China, Japan, Korea, Laos, and Vietnam (Gu et al., 2011; Kanehira et al., 2015; Lee et al., 2017; Okamatsu et al., 2017; Song et al., 2015; Wong et al., 2015)

In this thesis, we conducted the multi-years influenza A surveillance in free-grazing ducks to identify subtypes and determine potential virulence of the viruses in Thai free-grazing ducks. Moreover, the genetic characteristics and their pathogenicity in animal models were investigated. This study provided useful information for influenza prevention and control measures both in animals and humans.

Research questions

The research questions of the thesis "genetic characteristics of avian influenza A viruses isolated from Thai free-grazing ducks and their pathogenicity potential in animal models" included

1. What is the occurrence of influenza A viruses in free-grazing ducks in Thailand during 2011-2015?

2. What are the genetic characteristic and diversity of influenza A viruses isolated from Thai free-grazing ducks?

3. What are the pathogenicity in animal models of influenza A viruses isolated from Thai free-grazing ducks?

Objectives

To answers the research questions. We have formulated the objectives of this thesis as:

1. To determine the occurrence of influenza A viruses in free-grazing ducks in Thailand.
2. To determine the genetic characteristics and diversity of influenza A viruses isolated from free-grazing ducks in Thailand.
3. To investigate the pathogenicity in animal models of influenza A viruses isolated from free-grazing ducks in Thailand.

Literature review

Influenza A virus

Influenza virus is an enveloped negative sense RNA virus of the Orthomyxoviridae family. Influenza A, B and C are classified base on neucleoprotein (NP) and matrix (M) proteins (Webster et al., 1992). Influenza A virus can extensively infect several species of avian and mammalian. Influenza B virus is isolated from humans and seals (Osterhaus et al., 2000) and influenza C virus is isolated from humans, pigs and dogs (Guo et al., 1983; Webster et al., 1992).

Influenza A virus contains 8 single-stranded RNA segments encoding 12 proteins. The PB2 (2,341 bp), PB1 (2,341 bp) and PA (2,233 bp) genes encode RNA transcriptase. Moreover, PB1 gene also encodes PB1-F2 relating to pro-apoptotic virulence and PB1-N40 relating to virus replication factor. HA and NA genes encode surface glycoproteins, haemagglutinin and neuraminidase. M gene encodes matrix protein (M1), a major component of the virus and integral membrane protein (M2). NS gene encodes NS1 and NS2/NEP, non-structural proteins (Webster (Chen et al., 2001; Steinhauer and Skehel, 2002; Webster et al., 1992; Wise et al., 2009).

Surface glycoproteins, haemagglutinin binds to host cell receptors. The protein contains epitopes for host immunity. On the other hand, surface glycoprotein, neuraminidase helps virus releasing from host cell. These surface glycoproteins, haemagglutinin and neuraminidase are used for influenza subtype classification. To date, influenza A viruses are classified into 18 HA and 11 NA subtypes. Latest two subtypes, H17N10 and H18N11 have been reported in bats (Fouchier et al., 2005; Tong et al., 2012; Tong et al., 2013).

Influenza A virus can infect several animal species. Specific influenza A subtypes can preferentially replicate and circulate in different hosts. For example, seasonal influenza in humans are influenza A subtypes H1N1 and H3N2, while swine influenza in pigs are Influenza A subtypes H1N1, H1N2 and H3N2. Equine influenza in horses are influenza A subtypes H3N8 and H7N7. While, avian Influenza are caused by almost every influenza subtypes including H1-16 and N1-9 (Yoon et al., 2014).

Avian influenza virus

Avian influenza virus, almost all influenza A subtypes (H1-16 and N1-9) can infect and circulate in birds. Severities of avian influenza in birds depend on virulence of the virus. Low pathogenic avian influenza (LPAI) viruses cause absent or mild clinical signs in the animals. On the other hand, highly pathogenic avian influenza (HPAI) viruses cause severe systemic disease in birds and also occasionally mammals. HPAI include influenza A subtypes H5 and H7, but not all strain of these subtypes. HPAI can cause many avian influenza outbreaks in poultry including H5N2 (Mexico 1994, Italy 1997 and Texas 2004), H7N1 (Italy 1999), H7N3 (Australia 1994, Pakistan 1994, Chili 2002 and Canada 2003), H7N4 (Australia 1997) (Alexander (Alexander, 2000; Munster et al., 2005), and can transmit to humans; H7N7 (Fouchier et al., 2004; Koopmans et al., 2004) as well as H5N1 (de Jong et al., 1997). Not only HPAI but LPAI

such as H7N9 can infect in humans and cause death (Kageyama et al., 2013). Additional LPAI H9N2 can infect human but most cases are mild.

Avian influenza especially H5N1 (since 2003) and H7N9 (since 2013) causes human death and affect poultry industry in many countries specially Asia. Both avian influenza subtypes can cross infect from poultry to human (Herfst et al., 2014). It is noted that subtype H7N9 is low virulence in birds with mild clinical signs. Thus, H7N9 outbreak in poultry could be missed and potential high risk to infect humans.

Avian influenza in aquatic birds and free-grazing ducks

Aquatic birds and ducks are natural reservoirs of influenza viruses including H1-16 and N1-9 subtypes (Fouchier et al., 2005). Since aquatic birds can be infected with several influenza subtypes, the viral gene pool can evolve through adaptive mutation and reassortment. Avian influenza virus can adaptively infect birds, poultry, and mammals. In general, Influenza A virus circulating in aquatic birds are LPAI. Infected birds show un-observed clinical signs. Influenza A subtypes H5 and H7 founded in natural reservoirs are mostly LPAI. However, some H5 and H7 can be HPAI especially in wild birds; H5N3 in South Africa 1961 (Becker, 1966) and H5N1 (Chen et al., 2005).

Free-grazing duck production is a common practice in many countries in Southeast Asia including Thailand. The ducks are raised for free-grazing for food in rice field. The animals are frequently moved among rice fields for new food sources. Most of the free-grazing ducks are egg laying ducks that are Khaki Campbell breed or cross-breed of Khaki Campbell and native breed of laying ducks. The other breeds of the free grazing ducks are Pekin and Cherry Valley that are meat ducks. Young ducks are raised in housing for 3 weeks and moved to rice fields. Ducks are free-grazing in rice fields for 5-6 months (Songserm et al., 2006).

During epidemics of HPAI-H5N1 in Thailand, free-grazing ducks were related to H5N1 outbreaks (Gilbert et al., 2006). Free-grazing ducks can be infected with influenza A virus without clinical signs and able to spread the viruses (Hulse-Post et al., 2005). It has been known that free-grazing ducks have an opportunity of animal-animal interface with wild birds and backyard ducks and chickens. In Thailand, based on information from Department of livestock development (DLD), there are approximately 9 million free-grazing ducks in 2011. High densities free-grazing areas in Thailand are central and lower northern parts. In 2004, Thailand's national surveillance of influenza virus detected HPAI-H5N1 infection in free-grazing duck flocks (45.9%; 28/61) and only few flocks (<1%) showed clinical signs (Songserm et al., 2006). The later in 2010, serological surveillance showed 85% (5,305/6,254) of free-grazing ducks in Suphanburi province posed antibody against influenza virus (Beaudoin et al., 2014).

CHAPTER 2

MATERIAL AND METHODS

This thesis composes of three study phases. Phase 1 is surveillance of influenza A in free-grazing ducks. Phase 2 is genetic characterization and diversity analysis of influenza A viruses from free-grazing ducks. Phase 3 is challenge of influenza A viruses isolated from free-grazing ducks in animal models. The conceptual framework of this thesis is shown in figure 1.

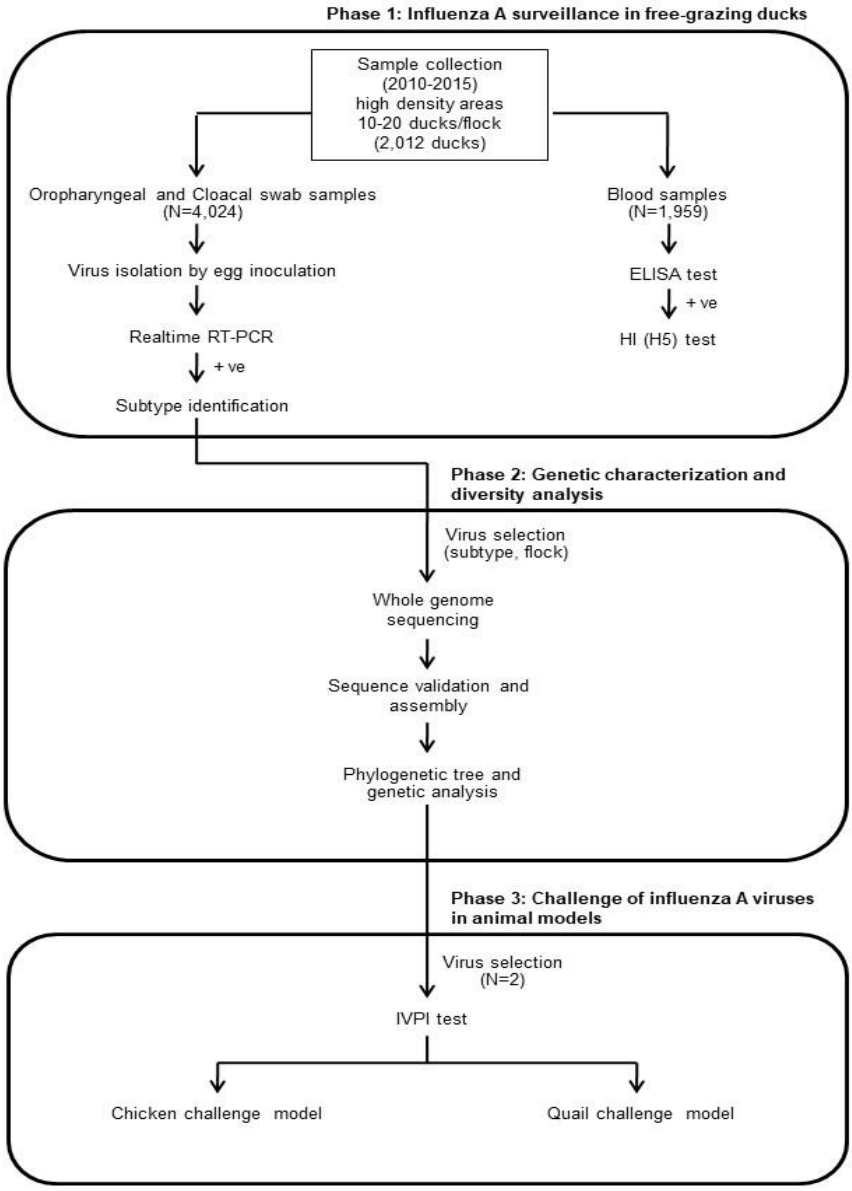

Figure 1. The conceptual framework of this thesis

1. Phase 1: Influenza A surveillance in free-grazing ducks

Influenza A surveillance program in free-grazing ducks was conducted during 2010-2015. The study areas for influenza A surveillance in free-grazing ducks were central and lower northern parts of Thailand where high density of free-grazing duck production areas (Gilbert et al., 2006). It was found from previous study that avian influenza infection correlated with late rainy and winter seasons in Thailand, therefore the sample collection was performed in October to March (Tiensin et al., 2005).

1.1 Sample collection

Free-grazing duck flocks were selected based on the following criteria, 1).Ducks were raised in provinces of high density of free-grazing ducks, 2).The ducks grazed in rice field. 3). Owner collaboration. In this study, 2,012 free-grazing ducks from 126 flocks in 10 provinces, were randomly selected and sampled. Approximately, 10-30 ducks per flock were sampled including oropharyngeal swabs, cloacal swabs and blood. The oropharyngeal swabs (n=2,012) and cloacal swabs (n=2,012) were collected and tested for influenza A virus antigen. The blood samples (n=1,959) were collected and tested for influenza A antibody. In total, 4,024 swabs samples and 1,959 blood samples were collected from 2,012 ducks.

1.2 Sample preparation

During sample collection, the swabs were deposited in 2 ml of viral transport media (Brain-Heart Infusion broth supplemented with antibiotics) at 4°C and transported to laboratory within 24 hours. The viral transport media with swab sample was mixed by vortex for 20 seconds and aliquoted into 3 portions; 1) 150 ul for RNA extraction, 2) 750 ul for egg inoculation, 3) for stock sample. All samples were kept in -80°C. The serum samples were separated and kept at -20°C until use.

1.3 Influenza A virus isolation

All swab samples were processed for virus isolation by egg inoculation method. Each sample was inoculated into 9 to 11 day-old embryonated chicken eggs, free of specific antibody. In brief, 200 ul of suspension from each swab sample was inoculated in allantoic cavity of each egg (3 eggs per sample) and incubated at 37°C for 48 hours. Afterwards the eggs were chilled at 4°C overnight and harvested for allantoic fluid. The hemagglutination (HA) test were performed by using 1% of chicken red blood cell and serial 2-fold dilutions of the allantoic fluid. Hemagglutination of RBC were observed after one hour of incubation period. The positive samples showed the hemagglutination and titer ≥2 HA unit (WHO, 2002). The HA positive samples were collected and stored at 80°C for next process.

1.4 Influenza A virus detection and subtype identification

1.4.1 RNA extraction

The HA positive samples from step 1.3 were subjected to RNA extraction by RNA extraction kit (Nucleospin® RNA virus, Macherey-Nagel, Germany). In brief, 150 µl of the HA positive samples were added into 600 µl of lysis buffer with RNA carrier. The mixture was incubated at 70°C for 5 minutes to lyse virus. After incubation, 600 µl of ethanol was added and loaded to silica gel membrane column. The column contained the solution was centrifuged at 8,000 g for 1 minute to bind viral RNA. The column was washed by adding wash buffer 3 time by 500 µl of RAW and centrifuged at 8,000 g for 1 minute, then 600 µl of RAV3 and centrifuged at 8,000 g for 1 minute, and final 200 µl of RAV3 and centrifuged at 11,000 g for 5 minutes to dry silica membrane. To elute RNA, the dry column was placed into 1.5 ml tube and 30 µl of 70°C of RNase-free water was added into column. The column was incubated at room temperature for 1 minute and centrifuged at 11,000 g for 1 minute. After centrifugation, the elution contained viral RNA was kept in the 1.5 ml tube. The viral RNA was kept at -20°C until use.

1.4.2 Influenza A virus detection by realtime reverse transcriptase polymerase chain-reaction (rRT-PCR)

The viral RNA samples from step 1.4.1 were confirmed for influenza A virus by rRT-PCR specific to Matrix (M) gene (Spackman et al., 2002). In brief, the rRT-PCR was performed in a Rotor-Gene RG-3000 thermo cycler machine (Corbett Research, Australia) using the SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen®). The sample reagent mixture containing with 1x master mix buffer, 0.6 mM $MgSO_4$, 1 unit of Superscript III reverse transcriptase, 0.8 µM of specific primer for M gene, 0.2 µM of specific probe for M gene and RNase-free water. Lastly, 1 µl RNA template was added, Condition for rRT-PCR reaction of the realtime thermo cycler machine was setting in 3 steps, 1) reverse transcription at 50ºC for 30 minutes, 2) pre-denaturation at 95ºC for 15 minutes, 3) two step cycling of denaturation for 50 cycles at 95ºC for 15 seconds and annealing-extension at 60ºC for 30 seconds. The signal was detected at end of each cycle until 50 cycles. The result from the realtime thermo cycler machine was interpreted to cycle at the signal higher than threshold, cycle threshold (Ct). The viral RNA samples with Ct value less than 36 was considered as positive for influenza A virus. The negative sample was Ct value more than 40 and the suspected sample was Ct between 36 and 40. Second passage of egg inoculation and virus detection by rRT-PCR was performed on suspected sample.

1.4.3 cDNA synthesis

The positive samples from step 1.4.2 were selected for cDNA synthesis. In this study the RNA of positive samples were reverse transcribed to cDNA for subtype identification. To synthesize cDNA, the Improm-II Reverse Transcription System (Promega, Madison, WI, USA) and random primer (10 µM) were used. In brief, one reaction of cDNA synthesis contained 1 µg of viral RNA sample and 5 µM of random primer. The mixture was placed in a thermalcycler with the condition at 70ºC for 15 minutes and then 4ºC for 5 minutes. The mixture was mixed with 12 µl of reagents

containing with 1 unit of Impromp II™ reverse transcriptase, 5x Impromp II™ buffer, 2.5 mM of $MgCl_2$, 1 µM of dNTP, 1 unit of RNase inhibitor and distilled water. The mixture was placed in a thermalcycler with the condition at 25ºC for 5 minutes, 42ºC for 60 minutes, and 72ºC for 15 minutes. The cDNA samples were kept at -20ºC until use.

1.4.4 Influenza A virus subtype identification

The cDNA of positive samples from step 1.4.3 (n=71) were subjected to influenza A virus subtype identification. The specific primers of each influenza subtypes; H1-H15 and N1- N9, were used for influenza subtyping by conventional PCR (Tsukamoto et al., 2008; Tsukamoto et al., 2009; VanDalen et al., 2008). For each sample, 15 PCR tubes and 9 PCR tubes were prepared for each primer set for H1-H15 and N1-N9 subtypes. A mixture contained 1x master mix (Toptaq™) and distilled water. We divided the mixture for each PCR tube and added 0.8 µM of primers for each subtype. One µl of the cDNA sample was added in each tube. The PCR conditions were 94ºC for 3 minutes and 35 cycles of 94ºC for 30 seconds, 50ºC (for H1-H15) or 45ºC (for N1-N9) for 30 seconds, and 72ºC for 30 seconds. The PCR product was examined by gel electrophoresis. The PCR product was mixed with 10x loading dry and loaded in 1.2% of agarose gel with Red safe in 0.5x Tris Borate EDTA (TBE). The gel was run at 100 voltage for 40 minutes and monitored under ultraviolet light in gel documentation system.

1.5 Influenza A antibody detection

Serum was separated from blood sample and kept at -20 ºC until testing. The serum samples were examined for the antibodies against influenza A virus by using blocking ELISA and the antibodies against H5 subtype by Hemagglutination Inhibition (HI).

1.5.1 Blocking Enzyme-Linked Immunosorbent Assay (ELISA)

All serum samples were tested for antibodies against NP protein of influenza by blocking enzyme-linked immunosorbent assay (ELISA) (FlockChek® AI MultiS-Screen Ab Test Kit, IDEXX Laboratories, USA). Following ELISA procedure, ELISA plate of commercial kit was coated with avian influenza (AI) viral antigen (NP protein). Each sample was tenfold (1/10) diluted with diluent and was added into each well of the plate including negative and positive control. The plate was incubated for 1 hour at room temperature for complex forming between influenza antibody and coated influenza antigen. The plate was washed by using 1x wash buffer for discard other elements. 100 µl of Horseradish Peroxidase conjugate as secondary antibody against avian influenza antibody was added into each well and incubated at room temperature for 30 minutes. The plate was washed 5 times by using 1x wash buffer. The 100 µl of TMB substrate solution. The plate was incubated at room temperature for 15 minutes and 100 µl of stop solution was added. The OD was measured by ELISA reader and calculated by compared with the negative control. The positive sample was S/N ratio <0.6. The negative sample was S/N ratio > 0.7. The suspected sample was S/N ratio between 0.6 and 0.7 (Brown et al., 2009).

1.5.2 Hemagglutination Inhibition (HI) test

The positive and suspected serum sample from step 1.5.1 were tested for H5 subtype by HI test. The serum samples were treated by heat inactivation at 56 ºC for 30 minutes, 20% Kaolin for 30 minutes and 50% RBC for 60 minutes for remove non-specific inhibitor. The treated serum was 2-fold diluted with PBS in 96-well plate and 25 µl of 4HAU virus (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004, H5N1) was added in each well. The plate was incubated at room temperature for 60 minutes. The HI titer was interpreted from the last dilution that can inhibit the hemagglutination. The HI titer more than 40 was used as cut-off criteria for positive sample.

2. Phase 2: Genetic characterization and diversity analysis of influenza A viruses from free grazing ducks

2.1 Genetic characterization of Influenza A virus

The representative viruses from phase 1 were selected for genetic characterization based on subtype of the viruses and location of free-grazing duck flocks. The virus representing to its subtype in each free-grazing duck flock was selected and subjected to genetic characterization by whole genome sequencing of influenza A virus. In this study, 71 viruses were represented of 15 influenza A virus subtypes from 22 free-grazing duck flocks from 7 provinces. To perform whole genome sequencing, each gene segment (PB2, PB1, PA, HA, NP, NA, M and NS) was amplified by PCR using specific primer sets (Hoffmann et al., 2001) and newly designed primers. In brief, 30 µl of PCR mixture contained 1.5 µl of cDNA, 1.2 µM of each forward and reverse primer, 1× Top Taq Master Mix (QIAGEN), 1× loading dry, and 8.1 µl of distilled water. PCR conditions were initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing temperature depend on primers for 45 seconds, and extension at 72°C for 1 minute; and final extension at 72°C for 7 minutes. PCR products were then visualized by gel electrophoresis on a 1.2% of agarose gel in 0.5×Tris borate EDTA. The amplicons were purified using Nucleospin® PCR clean up kit. The purified PCR products were then sequenced using BigDye® Terminator v3.1 cycle sequencing kit (1st Base Laboratories, Kembangan, Malaysia). The nucleotide sequences of each gene were validated and assembled using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI, USA).

2.2 Phylogenetic and genetic analyses of Influenza A virus

The validated sequences of each gene segment were compared for nucleotide similarity with the reference influenza sequences from the GenBank database by using nucleotide BLAST tool. For pairwise comparison, nucleotide sequences and deduced amino acids of the viruses in this thesis were aligned with

those of reference IAV sequences from Thailand, IAV sequences of outbreak strains, IAV sequences from Eurasian lineage and North America lineage by using Muscle version 3.6, Molecular Evolutionary Genetics Analysis software version 7 (MEGA7) and MegAlign version 5.03 (DNASTARInc., Madison, WI, USA) software. Phylogenetic tree of 8 genes were generated with the reference sequences by using MEGA version 7 applying neighbor-joining method with 1,000 replicates of bootstrap. The phylogenetic tree analysis provided information of the molecular evolution and genetic diversity of Influenza A viruses.

3. Phase 3: Challenge of influenza A viruses from free-grazing ducks in animal models

In this thesis, we conducted the experiment under the Animal Use Protocol No. 1473008 of Institutional Animal Care and Use Committee (IACUC), the Faculty of Veterinary Science, Chulalongkorn University.

3.1 Influenza A virus selection criteria

In this thesis, 2 influenza A viruses from phase 2 were selected for animal challenge experiments based on the subtypes and genetic determinants for virulence potential of the viruses. The animal challenge experiments were conducted in 2 animal models, chicken and quails.

The representative viruses were

1) A/duck/Thailand/CU-11836C/2011 (H1N3) or Thai IAV-H1N3, collected from Kamphaeng Phet province on Dec 2011.
2) A/duck/Thailand/CU-12660T/2012 (H11N9) or Thai IAV-H11N9, collected from Ang Thong province on Oct 2012.

The Thai IAV-H1N3 and Thai IAV-H11N9 viruses have at least one of the following criteria:

a) Predominant subtype of avian influenza viruses circulating in Thai free-grazing ducks during 2010-2015
b) Avian influenza virus containing genetic determinants for virulence potential of the viruses.
c) Avian influenza viruses containing reassortant gene at least one gene segment from H5N1 or H7N9 viruses

3.2 Intravenous Pathogenicity Index (IVPI) test

Intravenous Pathogenicity Index (IVPI) test was conducted following OIE's IVPI protocol. For animal preparation, the experimental chickens (n=12) were housed at CU-LAC building in standard cages. The chickens were raised from 1 day-old until 6 weeks-old age. The chickens were sampled for blood, oropharyngeal and cloacal swabs on the 1st day of entry and one week before experiment to test for influenza antigens and antibodies. The chickens were free from influenza antigens and antibodies. The IVPI test was conducted when the chickens were age up to 6 weeks-old.

The IVPI protocol

In this thesis, we conducted IVPI test for 2 influenza viruses, Thai IAV-H1N3 and Thai IAV-H11N9 as following:

1) 0.1 ml of diluted 1/10 of $10^{6.5}$ EID_{50} influenza virus in sterile isotonic saline was inoculated intravenously (jugular vein) into each chicken (10 chickens/virus). 0.1ml of sterile isotonic saline was used for control chicken (2 chickens/control).

2) The clinical signs were observed at 24 hours interval for 10 days.

3) At each observation, each chicken was scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. ('sick' chickens showed one of the following signs and 'severely sick' showed more than one of the following signs: respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death.)

4) Chicken unable to eat or drink were euthanized and recorded as dead at the following day's observation time.

5) The intravenous pathogenicity index (IVPI) was calculated from the mean score per bird per observation over the 10-day period. An index of 3.00 means that all chickens died within 24 hours, an index of 0.00 means that no chicken showed any clinical sign during the 10-day observation period. Influenza A virus that has IVPI >1.2 is HPAI.

3.3 Challenge of influenza A virus in chickens and quails

To assess the pathogenicity of the two influenza viruses (Thai IAV-H1N3 and Thai IAV-H11N9) isolated from free-grazing ducks in chickens and quails, each virus were challenged in chickens (n=15/virus) and quails (n=15/virus).

For virus preparation, each virus was prepared and tested for the viral titer $10^{6.5}$ EID_{50}. For animal preparation (chickens and quails), the age of chickens and quails for challenge experiment were at 4 weeks old and 6 weeks old respectively. The animals were raised from 1 day-old birds until challenge experiment. The animals must be free from influenza A antigens and antibodies.

Influenza virus challenge experiments in animal models

1) The experimental animals (4 week-old chickens and 6 week old quails) were randomly divided into three groups;
   - Group 1: inoculated group (8 animals/virus)
     Group 1 was inoculated with $10^{6.5}$ EID_{50} influenza virus via intra-nasal route.
   - Group 2: contacted group (4 animals/virus)
     Group 2 was placed in the same cage with group 1 at 2 day-post-inoculation (dpi).
   - Group 3: control group (3 animals/virus)
     Group 3 was inoculated with diluent (PBS buffer) via intra-nasal route as control.
2) Oropharyngeal and cloacal swabs were collected from each animals at 0, 1, 3, 5, 7, 10 and 14 dpi for evaluating viral shedding. Blood samples (chicken: 1-3 ml and quail: 0.5-1 ml) were collected from each animals at 0, 7, 10 and 14 dpi. for evaluating antibody response.
3) Clinical signs (depression, diarrhea, cyanosis, edema, respiratory involvement and neurological sign) were observed and recorded daily. At 14 dpi all animals were terminated.
4) Gross lesions were observed and recorded during necropsy. Visceral organs (lung, trachea, liver, kidney and ileum) from death animals and euthanized animals were collected by aseptic technique and store in 10% formalin buffer for histopathology.

In this thesis, the swab samples were processed for influenza A virus detection and quantitation by rRT-PCR to evaluate viral shedding. The protocol for influenza A virus detection and quantitation was mentioned in phase 1.4.2. The serum samples were processed for influenza antibody detection by blocking ELISA to evaluate immune response. The protocol for influenza A virus antibody response was

mentioned in phase 1.5.1. The tissues from internal organs were processed for histopathological lesions by H&E straining.

Statistical analysis of significant differences of virus shedding titers was evaluated with an analysis of variance (ANOVA) by SPSS program and student *t-test* comparing between groups, p-values < 0.05 were considered to be statistically significant.

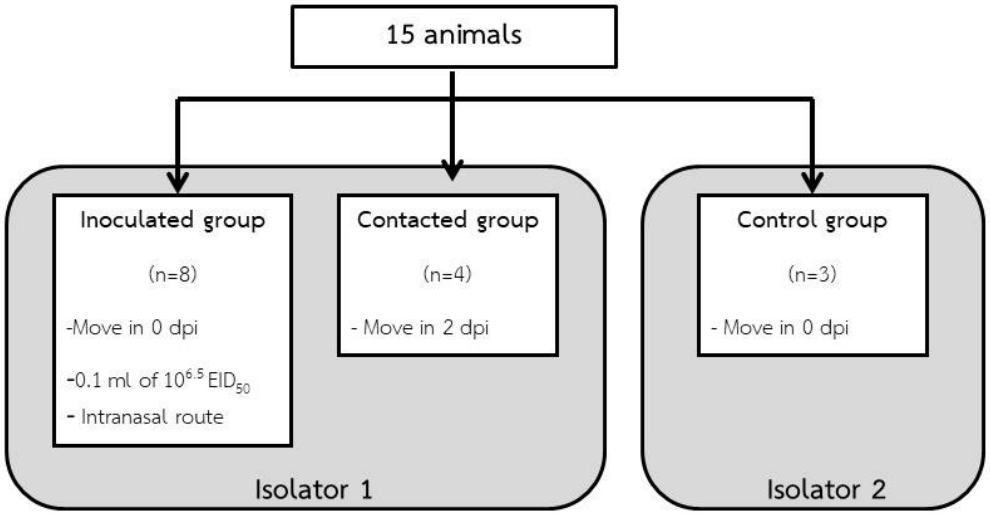

Figure 2 Group separation of the experimental animals (chickens and quails)

The experimental animals (chickens and quails) were divided in to 3 groups. Two days after inoculation, the contacted group were moved into inoculated group cage.

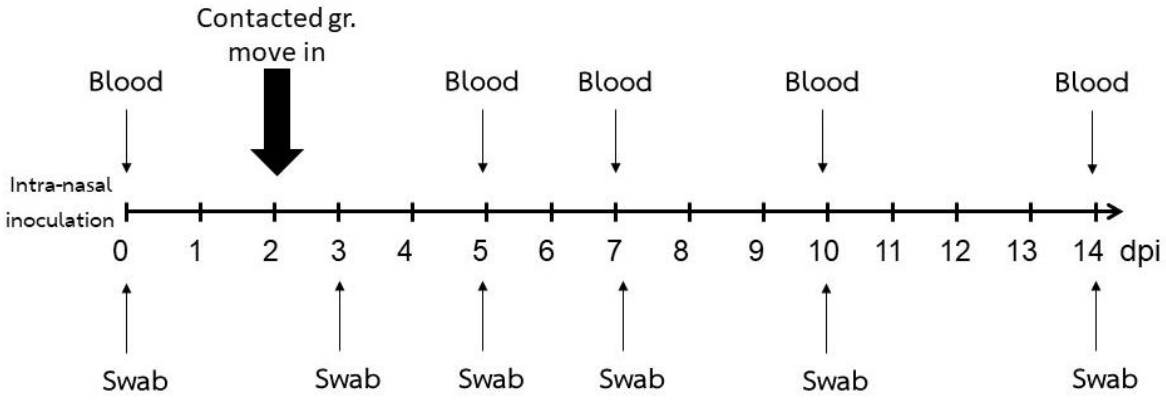

Figure 3 Sample collection (swabs and blood) timeline for influenza challenge experiment

CHAPTER 3

RESULTS

1. Phase 1: Influenza A surveillance in free-grazing ducks

In this thesis, influenza A surveillance in free-grazing ducks was retrospectively and prospectively performed during May 2010 to February 2015. The total number of the samples was 5,983 including oropharyngeal swabs (n=2,012), cloacal swabs (n=2,012) and serum samples (n=1,959). The samples were collected from 24 districts of 10 provinces in central and lower northern parts of Thailand. The provinces selected for sample collection in the study were Ang Thong, Ayutthaya, Kamphaeng Phet, Nakhon Pathom, Nakhon Sawan, Pathum Thani, Phichit, Phitsanulok, Sukhothai and Suphan Buri. One hundred and twenty six flocks of free-grazing ducks were included for sample collection. Size of the flocks of free-grazing ducks vary from 600 to 15,000 ducks per flock. Age of ducks was ranging from 20 days-old to 3 years. For sample collection, 10 to 30 ducks per flock were randomly selected for sample collection. In this study, ducks were classified into 2 groups based on age including young ducks (3 weeks old - 5 months old) and laying ducks (more than 5 months old).

For influenza A virus detection, rRT-PCR assay was used for the detection of Influenza A viruses from oropharyngeal and cloacal swab samples. Our result showed 1.77 % (71/4,024) of samples positive for influenza A virus. The influenza A viruses were detected in young-duck flocks (28.57%; 12/42) more than laying-duck flocks (11.90%; 10/84) (Table 1). And the viruses were detected in ducks from large flocks (≥ 6,000 ducks per flock) (35.29%; 6/17) more than ducks from small flocks (14.00%; 14/100) (Table 2). In this thesis, representative viruses (n=27) were selected for genetic characterization by whole genome sequencing. The viruses were selected based on the following criteria; 1) virus with high viral titer, 2) virus represent each subtype, 3) virus represent positive flock. The viruses were then subtyped into 15

subtypes. Fifteen subtypes of influenza A viruses were H1N3, H1N9, H3N8, H4N6, H4N9, H5N3, H5N8, H6N1, H7N4, H9N7, H10N6, H10N7, H11N6, H11N7 and H11N9. It is noted that the H3N8 and H4N6 subtypes were frequently found in almost every years and provinces suggesting predominant subtypes of influenza A viruses in free grazing ducks. Table 3 showed details of avian influenza viruses characterized in this thesis.

For serological test, antibody against influenza A virus and specific antibody against influenza A virus subtype H5 were tested by using blocking ELISA and H5-HI test, respectively. Of 1,959 serum samples, 70.24 % (1,376/1959) were positive for influenza A antibody by blocking ELISA. The ELISA positive samples were then tested for specific antibody for H5 virus by using H5-HI test. Of 1,376 serum samples tested, 6.40 % (88/1376) were positive by H5-HI test.

By age of ducks, free-grazing ducks can be classified into 2 groups based on age including young ducks (3 weeks old - 5 months old) and laying ducks (more than 5 months old). Of total 126 free grazing flocks, 17.46% (22/126) were influenza positive from swab samples. The young duck flocks were found positive (28.57%) more than laying duck flocks (11.90%). While, influenza A antibody could be detected with high positivity (92.86%) in laying duck flocks more than young duck flocks (59.52%). The influenza virus positivity was detected with highest % positivity (35.71%) in large flock size (6,000-10,000 ducks/flock) and no positivity in ≤1,000 flock size. Table 1 and 3.2 showed number of influenza positive in free grazing duck flocks by age of duck and flock size.

By year, during 2010-2015, influenza A virus and antibody against influenza A virus could be detected in free-grazing duck flocks sampled from each year. Average of influenza positive swab samples was 1.77%, highest in Year 2 (4.20%) and lowest

in Year 1 (0.77%). While, influenza A antibody could be detected with highest % positivity in Year 1 (97.48%) and lowest in Year 4 (42.73%). Moreover, the specific antibody against H5 subtype was detected with highest % positivity (7.85%) in Year 1 and no positivity in Year 5. Table 4 showed detail of sample collection, influenza A virus detection by rRT-PCR and serological detection by blocking ELISA and H5-HI test, by year during 2010-2015.

By location, in this thesis, the samples were collected from 10 provinces. In total 126 free grazing duck flocks in 24 districts of 10 provinces were included for sample collection. Of 10 provinces, influenza A virus could be detected from 7 provinces. The province with highest positive samples was Kamphaeng Phet (5.69%) (Table 5). Moreover, influenza antibody could be detected in free grazing ducks from 9 provinces. Of the 9 provinces, 5 provinces had high percentage positivity (≥80%), Phichit, Nakhon Sawan, Sukhothai and Ang Thong. Specific H5 antibody could be detected in 5 provinces and was highest in Nakhon Sawan province (7.95%). Table 5 show detail of sample collection influenza A virus detection by rRT-PCR and serological detection by blocking ELISA and H5-HI test, by province.

Table 1 Results of serology (blocking ELISA and H5-HI test) and virus detection (rRT-PCR) by age of duck

Age of duck	Number of flocks	Positive virus rRT-PCR	Positive antibody ELISA	Positive antibody HI (H5)
Young ducks (3 weeks old to < 5 months old)	42	12 (28.57%)	25 (59.52%)	3 (7.14%)
Laying ducks (≥5 months old)	84	10 (11.90%)	78 (92.86%)	28 (33.33%)

Table 2 Results of serology (blocking ELISA and H5-HI test) and virus detection (rRT-PCR) by flock size

Flock size	Number of flocks	Positive virus rRT-PCR	Positive antibody ELISA	Positive antibody HI (H5)
≤1,000	10	0 (0.00%)	8 (80.00%)	2 (20.00%)
1,001-3,000	77	12 (15.58%)	65 (84.42%)	19 (24.68%)
3,001-6,000	13	2 (15.38%)	11 (84.62%)	1 (7.69%)
6,001-10,000	14	5 (35.71%)	11 (78.57%)	2 (14.29%)
> 10,000	3	1 (33.33%)	0 (0.00%)	0 (0.00%)
Not available	9	2 (22.22%)	8 (88.88%)	7 (77.77%)

Table 3 List of Thai avian influenza viruses characterized in this thesis

No.	Collection date	No. of viruses	Strain name of represent virus	ID	Subtype	Flock no.	Province (district)
1	26 May 2010	2	A/duck/Thailand/CU-7518C/2010	7518C	H3N8	F1	Nakhon Sawan (Takhli)
2	17 Sep 2010	1	A/duck/Thailand/CU-8319T/2010	8319T	H9N7	F1	Nakhon Sawan (Mueang)
3	22 Dec 2010	2	A/duck/Thailand/CU-9744C/2010	9744C	H7N4	F3	Nakhon Sawan (Chum Saeng)
4	18 Jan 2011	14	A/duck/Thailand/CU-10507T/2011	10507T	H7N4	F4	Nakhon Sawan (Kao Liao)
5	20 Dec 2011	1	A/duck/Thailand/CU-11836C/2011	11836C	H1N3	F1	Kamphaeng Phet (Khlong Khlung)
6	20 Dec 2011	4	A/duck/Thailand/CU-11840T/2011	11840T	H4N6	F1	Kamphaeng Phet (Khlong Khlung)
7	20 Dec 2011	2	A/duck/Thailand/CU-11869C/2011	11869C	H1N9	F4	Kamphaeng Phet (Khlong Khlung)
8	8 Feb 2012	4	A/duck/Thailand/CU-12205C/2012	12205C	H5N8	F1	Phitsanulok (Bang Rakam)
9	19 Oct 2012	3	A/duck/Thailand/CU-12657T/2012	12657T	H11N6	F1	Ang Thong (Wiset Chai Chan)
10	19 Oct 2012	2	A/duck/Thailand/CU-12659T/2012	12659T	H4N6	F1	Ang Thong (Wiset Chai Chan)
11	19 Oct 2012	2	A/duck/Thailand/CU-12660T/2012	12660T	H11N9	F1	Ang Thong (Wiset Chai Chan)
12	19 Oct 2012	1	A/duck/Thailand/CU-12666C/2012	12666C	H4N6	F2	Ang Thong (Wiset Chai Chan)
13	19 Oct 2012	2	A/duck/Thailand/CU-12677T/2012	12677T	H11N6	F3	Ang Thong (Wiset Chai Chan)
14	19 Oct 2012	1	A/duck/Thailand/CU-12679C/2012	12679C	H4N6	F3	Ang Thong (Wiset Chai Chan)
15	27 Feb 2013	1	A/duck/Thailand/CU-13011C/2013	13011C	H6N1	F1	Kamphaeng Phet (Khanu Woralaksaburi)
16	8 Mar 2013	1	A/duck/Thailand/CU-13105C/2013	13105C	H4N6	F3	Phitsanulok (Bang Rakam)
17	30 Aug 2013	1	A/duck/Thailand/CU-13582C/2013	13582C	H4N9	F1	Suphan Buri (Song Phi Nong)
18	30 Aug 2013	2	A/duck/Thailand/CU-13600C/2013	13600C	H4N6	F3	Suphan Buri (Song Phi Nong)
19	30 Aug 2013	4	A/duck/Thailand/CU-13604T/2013	13604T	H3N8	F3	Suphan Buri (Song Phi Nong)
20	10 Sep 2013	3	A/duck/Thailand/CU-13738C/2013	13738C	H4N6	F3	Ayutthaya (Lat Bua Luang)
21	23 Dec 2013	2	A/duck/Thailand/CU-14201C/2013	14206C	H5N3	F1	Kamphaeng Phet (Khanu Woralaksaburi)
22	28 Jan 2014	1	A/duck/Thailand/CU-14284C/2014	14284C	H10N7	F2	Phitsanulok (Bang Krathum)
23	28 Jan 2014	3	A/duck/Thailand/CU-14288C/2014	14288C	H3N8	F3	Phitsanulok (Bang Krathum)
24	19 Feb 2014	1	A/duck/Thailand/CU-14421C/2014	14421C*	H3N8	F1	Sukhothai (Kong Krailat)
25	19 Feb 2014	1	A/duck/Thailand/CU-14442C/2014	14442C	H11N9	F3	Sukhothai (Kong Krailat)
26	12 Jan 2015	1	A/duck/Thailand/CU-15857T/2015	15857T	H10N6	F1	Suphan Buri (Si Prachan)
27	12 Feb 2015	3	A/duck/Thailand/CU-16340C/2015	16340C	H11N7	F3	Kamphaeng Phet (Khlong Khlung)

* This virus was sequenced only HA and NA gene

Table 4 Detail of the sample collection and results of serology (ELISA and H5-HI test) and virus detection (rRT-PCR) by year

Year	Collecting date	Number of visits	Number of samples			Positive virus	Positive antibody	
			OP	CS	B	rRT-PCR	ELISA	HI (H5)
1	May 2010 – Jan 2011	11	982	982	929	15 (0.77%)	737* (97.48%)	73 (7.85%)
2	Sep 2011 – Feb 2012	5	250	250	250	21 (4.20%)	162 (64.80%)	2 (0.89%)
3	Sep 2012 – Mar 2013	6	290	290	290	13 (2.24%)	213 (73.45%)	3 (1.03%)
4	Aug 2013 – Feb 2014	7	330	330	330	18 (2.73%)	141 (42.73%)	10 (3.03%)
5	Dec 2014 – Feb 2015	5	160	160	160	4 (1.25%)	123 (76.88%)	0 (0.00%)
Total		34	2,012	2,012	1,959	71 (1.77%)	1,376 (70.24%)	88 (6.40%)

OP=oropharyngeal swab, CS=cloacal swab, B=blood

*Tested samples = 756 samples

Table 5 Detail of sample collection and results of serology (ELISA and HI (H5) test) and virus detection (rRT-PCR) by province

No.	Province	Number of districts	Number of flocks	Number of samples			Positive virus	Positive antibody	
				OP	CS	B	rRT-PCR	ELISA	HI (H5)
1	Nakhon Sawan	5	33	914	914	893	15 (0.82%)	728* (91.57%)	71 (7.95%)
2	Phichit	4	16	226	226	205	0 (0.00%)	125** (96.15%)	4 (1.95%)
3	Kamphaeng Phet	3	19	202	202	191	23 (5.69%)	70 (36.65%)	0 (0.00%)
4	Suphan Buri	3	11	180	180	180	8 (2.20%)	128 (71.11%)	7 (3.89%)
5	Phitsanulok	2	15	150	150	150	9 (3.00%)	85 (56.67%)	0 (0.00%)
6	Ayutthaya	2	12	120	120	120	3 (1.25%)	69 (57.50%)	4 (3.33 %)
7	Sukhothai	1	10	100	100	100	2 (1.00%)	89 (89.00%)	2 (2.00%)
8	Nakhon Pathom	2	4	60	60	60	0 (0.00%)	19 (31.67%)	1 (1.67%)
9	Ang Thong	1	5	50	50	50	11 (11.00%)	32 (80.00%)	0 (0.00%)
10	Pathum Thani	1	1	10	10	10	0 (0.00%)	0 (0.00%)	0 (0.00%)
Total		24 districts	126 flocks	2,012	2,012	1,959	71 (1.77%)	1,376 (70.24%)	88 (6.40%)

*Tested samples = 795 samples, ** Tested samples = 130 samples

2. Phase 2: Genetic characterization and diversity analysis of influenza A viruses from free-grazing ducks

In this thesis, 27 representative influenza A viruses were selected from 71 positive samples. There were 15 influenza A virus subtypes among 27 isolates (Table 6). All 27 influenza A viruses were subjected to whole genome sequencing, except 1 virus (IAV-H3N8) was subjected to HA and NA gene sequencing due to low RNA quality. To analyze genetic diversity of influenza A viruses from free-grazing ducks, each gene of the viruses was compared with the reference viruses from the GenBank database. Phylogenetic trees of each gene were generated by using MEGA 7.0 program. From phylogenetic analysis, the origin of each gene of the viruses was identified and tabulated as genetic constellation of the influenza viruses.

2.1 Phylogenetic analysis of influenza A viruses from free-grazing ducks

2.1.1 Phylogenetic analysis of HA gene

HA1 gene

For IAV-H1 viruses, there were IAV-H1N3 (n=1) (CU-11836C) and IAV-H1N9 (n=1) (CU-11869C) viruses characterized in the study. From the phylogenetic tree of HA gene, two viruses (CU-11836C and CU-11869C) were closely related to H1N2 viruses isolated from ducks and chickens reported in China (Wu et al., 2012). Notably the Thai IAV-H1N3 and IAV-H1N9 viruses were grouped in separated cluster from a pdmH1N1-2009 group.

HA3 gene

For IAV-H3 viruses, the IAV-H3 viruses were grouped into 2 major groups, North American and Eurasian lineages. In this thesis, there were IAV-H3N8 viruses (n=4) designated as CU-7518C, CU-13604T, CU-14288C and CU-14421C. Phylogenetic

analysis showed that 4 representative IAV-H3 viruses were clustered into avian Eurasian lineage.

HA4 gene

For IAV-H4 viruses, the phylogenetic tree of the HA4 gene can be divided into the North American lineage and the Eurasian lineage. In this thesis, all of IAV-H4 viruses (IAV-H4N6 (n=7) and IAV-H4N9 (n=1)) were clustered in avian Eurasian lineage. However, one IAV-H4N6 virus (CU-12679C) was clustered away from other representative IAV-H4N6 viruses and shared only 95.6% to 95.9% nucleotide identity with the others.

HA5 gene

For IAV-H5 viruses, the HA5 viruses can be classified into 2 major clusters, North American lineage and Eurasian lineage. Regularly, the HA5 gene can also be classified into sub-clusters or namely “clade”. In this thesis, the IAV-H5 viruses, IAV-H5N8 (n=1) (CU- 12205C) and IAV-H5N3 (CU-14201C) were clustered in Eurasian lineage but were in separated clades from HPAI-H5 viruses (clade 0; Gs/Ds/96).

HA6 gene

For IAV-H6 viruses, the HA6 gene of Influenza A virus was divided into 2 lineages; Eurasian lineage and North American lineage. In this thesis, the IAV-H6N1 virus (n=1) (CU-13011C) was clustered into Eurasian lineage and separated from group of zoonotic IAV-H6N1 virus (Taiwan-like) (Wei et al., 2013).

HA7 gene

For IAV-H7 viruses, the phylogenetic tree of the HA7 gene can also be divided into the North American lineage and the Eurasian lineage. In this thesis, 2 viruses of IAV-H7N4 (CU-9744C and CU-10507T) were clustered in avian Eurasian lineage. Both H7N4 viruses shared 98.6% nucleotide identity. However, the Thai H7N4 viruses were clustered in separated group from China H7N9 group.

HA9 gene

For IAV-H9 viruses, the phylogenetic tree of HA9 gene, the Eurasian lineage can be split into 3 sub-lineages, represented by A/chicken/Beijing/1/1994 (BJ94), A/quail/Hong Kong/G1/1997 (G1) and A/chicken/Hong Kong/Y439/1997 (Y439) (Guo et al, 2000). In this thesis, one H9N7 virus (CU-8319T) belonged to Y439 sub-lineage.

HA10 gene

For IAV-H10 viruses, the phylogenetic tree of the HA10 gene can be divided into the North American lineage and the Eurasian lineage. In this thesis, the HA10 gene of IAV-H10N7 (CU-14284C) and IAV-H10N6 (CU-15857T) viruses were clustered in avian Eurasian lineage. Both Thai IAV-H10N7 and IAV-H10N6 viruses were grouped and closely related to human IAV-H10N8 virus with nucleotide identity to human H10N8 virus as 96.5% and 97.6%, respectively.

HA11 gene

For IAV-H11 viruses, the phylogenetic tree of the HA11 gene can be divided into the North American lineage and the Eurasian lineage. In this thesis, there were 3 subtypes of IAV-H11 viruses, IAV-H11N6 (n=2), IAV-H11N9 (n=2) and IAV-H11N7 (n=1). Phylogenetic analysis showed that HA11 gene of Thai IAV-H11 viruses were clustered in avian Eurasian lineage. The viruses were grouped into 2 different sub-groups. The sub-group of Ang Thong province (CU-12657T (H11N6), CU-12660T (H11N9) and CU-12677T (H11N6)) were closely related with previous Thai IAV-H11N3 viruses. The sub-group of Sukhothai and Kamphaeng Phet provinces (CU-14442C (H11N9) and CU-16340C (H11N7)) were closely related with China H11N9 viruses.

2.1.2 Phylogenetic analysis of NA gene

NA1 gene

For IAV-N1 viruses, the NA1 gene of Influenza A virus can be grouped into 2 major clusters, North American lineage and Eurasian lineage. In this thesis, the IAV-N1 virus, H6N1 (n=1) (CU-13011C) was clustered in Eurasian lineage but was in separated sub-group from HPAI-H5N1 viruses.

NA3 gene

For IAV-N3 viruses, the phylogenetic tree of the NA3 gene can be divided into the North American lineage and the Eurasian lineage. In this thesis, 2 viruses of IAV-H1N3 (CU-11836C) and IAV-H5N3 (CU-14201C) were clustered in avian Eurasian lineage.

NA4 gene

For IAV-N4 viruses, the phylogenetic tree of the NA4 gene can be divided into the North American lineage and the Eurasian lineage. In this thesis, 2 viruses of IAV-H7N4 (CU-9744C and CU-10507T) were clustered in avian Eurasian lineage but in 2 separated sub-groups. Both IAV-H7N4 viruses shared 91.3% nucleotide identity.

NA6 gene

For IAV-N6 viruses, the phylogenetic tree of the NA6 gene can be divided into the North American lineage and the Eurasian lineage. In this thesis, the NA6 gene of the representative viruses (n=10) were clustered in avian Eurasian lineage. They were separated into 3 sub-groups.

NA7 gene

For IAV-N7 viruses, the IAV-N7 viruses were grouped into 2 major groups, North American and Eurasian lineages. In this thesis, three viruses were clustered in avian Eurasian lineage but the viruses were separated into 2 groups; 1) Group of (n=1)

CU-8319T (H9N7) and 2) Group of (n=2) CU-14284C (H10N7) and CU-16340C (H11N7). The group 1 and group 2 shared <92.9% nucleotide identity.

NA8 gene

For IAV-N8 viruses, phylogenetic analysis of the NA8 gene showed that representative viruses (n=4) (CU-7518C (H3N8), CU-13604 (H3N8), CU-14288C (H3N8) and CU-14421C (H3N8) were clustered into avian Eurasian lineage. Interestingly, one IAV-H5N8 virus (CU-12205C) was clustered in Asian group of North American lineage.

NA9 gene

For IAV-N9 viruses, the phylogenetic tree of the NA9 gene can also be grouped into the North American lineage and the Eurasian lineage. In this thesis, the representative viruses (n=4), CU-11869C (H1N9), CU-12660T (H11N9), CU13582C (H4N9) and CU-14442C (H11N9), were clustered in avian Eurasian lineage but in separated group from China H7N9.

2.1.3 Phylogenetic analysis of internal gene

To identify genetic constellation of 27 representative viruses, each internal gene of the viruses were analyzed by phylogenetic analysis. The origin of each gene of the viruses was identified and tabulated as genetic constellation of the viruses (Table 6 and Figure 4).

PB2 gene

Phylogenetic analysis of the PB2 gene showed that Thai viruses were grouped into 6 different clusters; Cluster 1 to Cluster 6. Among clusters, there were <95.3% nucleotide sequence identities among viruses. All clusters grouped in the avian Eurasian lineage but in separated group from zoonotic influenza viruses (H5N1, H7N9 and H9N2). Except one virus, CU-13105C (H4N6), the PB2 gene of CU-13105C was clustered with the group of H7N9 and H9N2 viruses.

PB1 gene

Phylogenetic analysis of the PB1 gene showed that Thai viruses formed 6 clusters in avian Eurasian lineage. The viruses shared nucleotide sequence identities of <95.9%.

PA gene

Phylogenetic analysis of the PA gene showed that Thai viruses formed 5 clusters. Notably, one virus CU-10507T (H7N4) was clustered in North American sub-lineage.

NP gene

Phylogenetic analysis of the NP gene showed that Thai viruses formed 8 clusters and shared nucleotide sequence identities <94.5%. It is noted that IAV-H11N9 virus (CU-12660T) and IAV-H4N6 virus (CU-13105C) of cluster 7 and IAV-H11N7 virus (CU-16340C) of cluster 8 were belonged to Asian group 1 and Asian group 2, respectively. The Asian group 1 and 2 were divided from main avian lineage before separate to North American and Eurasian lineage.

M gene

Phylogenetic analysis of the M gene showed that Thai viruses formed 6 clusters. Interestingly, one IAV-H11N7 virus (CU-16340C) of cluster 6 was clustered in avian North American lineage.

NS gene

Phylogenetic analysis of the NS gene showed 2 distinct separated alleles; Allele A and Allele B (Ludwig et al., 1991). Thai IAV-H11N9 virus (CU-12660T) was clustered in Allele B. While other Thai viruses were clustered in Allele A. Five groups of the representative viruses were formed and shared nucleotide sequence identities of <97.1%.

In this thesis, the results from phylogenetic analysis of 8 genes of Thai influenza viruses isolated from free-grazing ducks revealed new reassortant viruses (n=3) (IAV-H11N9, IAV-H4N6 and IAV-H11N7). While the origin of most Thai viruses was from Avian Eurasian lineage I, some genes of new reassortant viruses were originated from other lineages (Table 6). According to the origins of each gene, the gene constellation of 26 representative viruses were identified to 24 patterns (Figure 4).

Table 6 Origins of internal genes of representative Thai IAV viruses.

No.	ID	Subtype	PB2	PB1	PA	NP	M	NS
1	7518C	H3N8						
2	8319T	H9N7						
3	9744C	H7N4						
4	10507T	H7N4						
5	11836C	H1N3						
6	11840T	H4N6						
7	11869C	H1N9						
8	12205C	H5N8						
9	12657T	H11N6						
10	12659T	H4N6						
11	12660T	H11N9						
12	12666C	H4N6						
13	12677T	H11N6						
14	12679C	H4N6						
15	13011C	H6N1						
16	13105C	H4N6						
17	13582C	H4N9						
18	13600C	H4N6						
19	13604T	H3N8						
20	13738C	H4N6						
21	14201C	H5N3						
22	14284C	H10N7						
23	14288C	H3N8						
24	14442C	H11N9						
25	15857T	H10N6						
26	16340C	H11N7						

A

No.	ID	Subtype	PB2	PB1	PA	NP	M	NS
1	7518C	H3N8						
2	8319T	H9N7						
3	9744C	H7N4						
4	10507T	H7N4						
5	11836C	H1N3						
6	11840T	H4N6						
7	11869C	H1N9						
8	12205C	H5N8						
9	12657T	H11N6						
10	12659T	H4N6						
11	12660T	H11N9						
12	12666C	H4N6						
13	12677T	H11N6						
14	12679C	H4N6						
15	13011C	H6N1						
16	13105C	H4N6						
17	13582C	H4N9						
18	13600C	H4N6						
19	13604T	H3N8						
20	13738C	H4N6						
21	14201C	H5N3						
22	14284C	H10N7						
23	14288C	H3N8						
24	14442C	H11N9						
25	15857T	H10N6						
26	16340C	H11N7						

B

Group 1 Group 2 Group 3 Group 4 Group 5 Group 6 Group 7 Group 8

Figure 4 Genotypes of the six internal genes of the representative viruses (A) Genotypes were defined based on phylogenetic analysis (show in supplement figure) (B) Color designations for different groups based on phylogenetic analysis. Genes of the same group are shown in the same color.

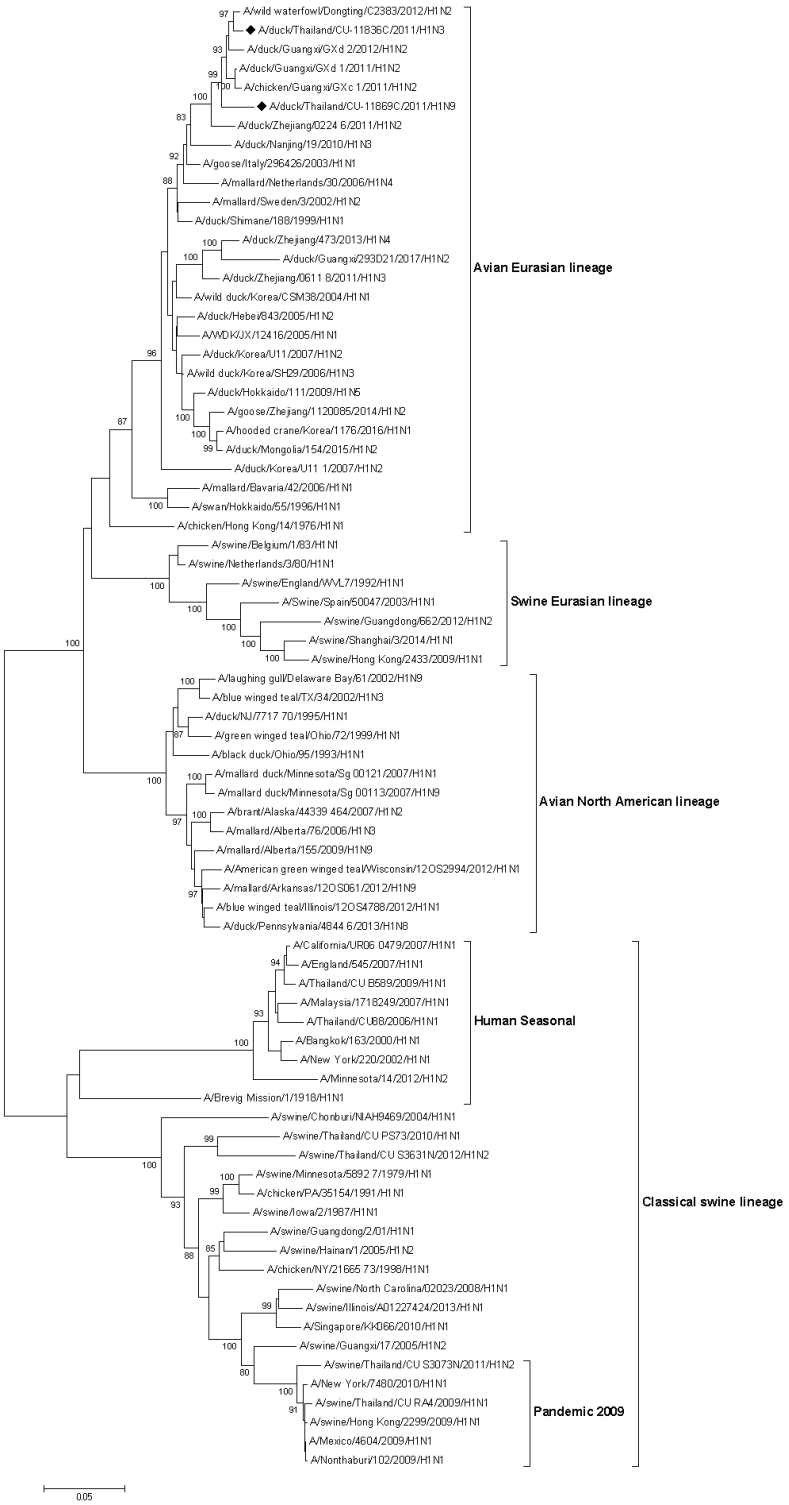

Figure 5 Phylogenetic analysis of HA1 gene

Phylogenetic analysis of HA1 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

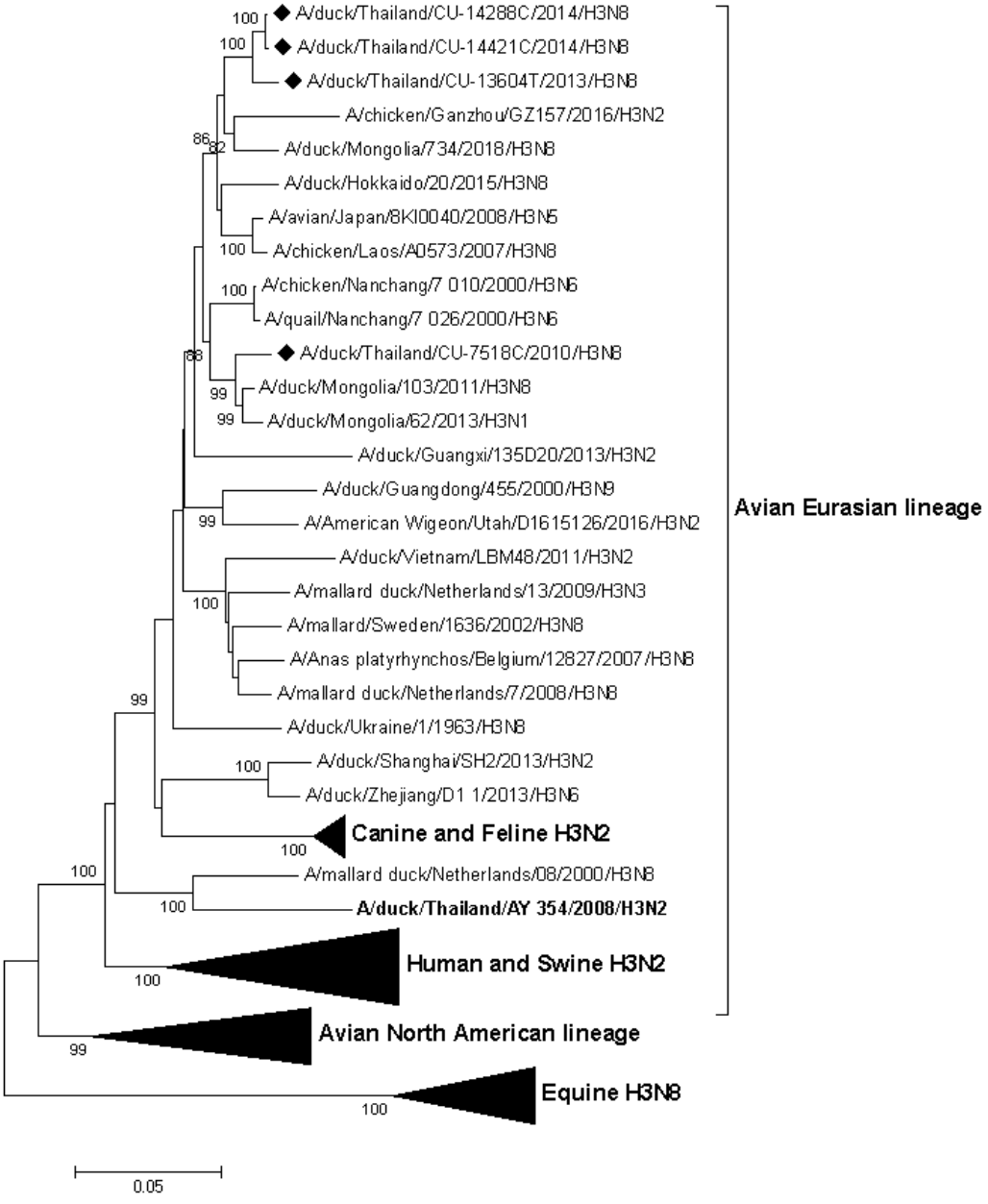

Figure 6 Phylogenetic analysis of HA3 gene

Phylogenetic analysis of HA3 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

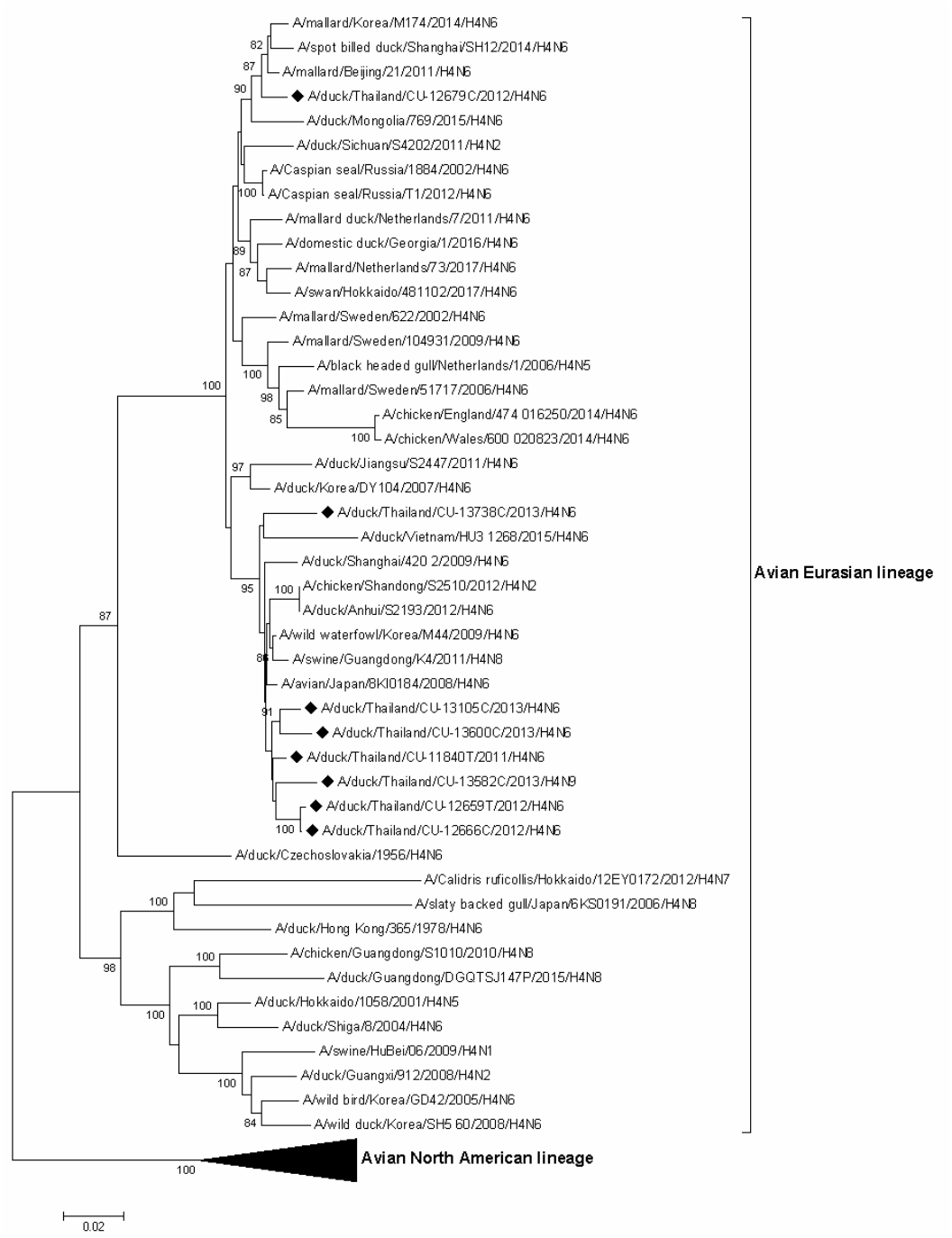

Figure 7 Phylogenetic analysis of HA4 gene

Phylogenetic analysis of HA4 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

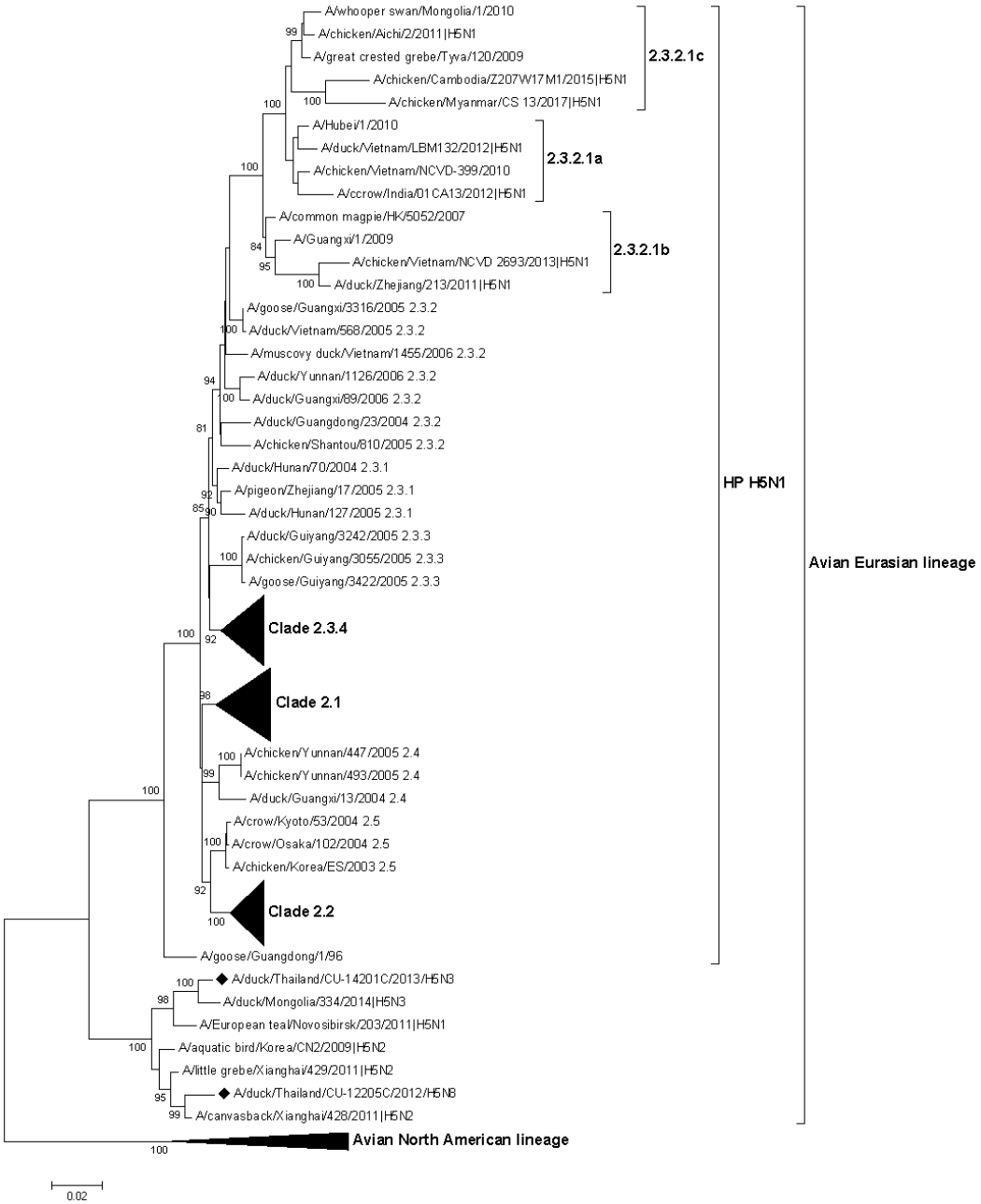

Figure 8 Phylogenetic analysis of HA5 gene

Phylogenetic analysis of HA5 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

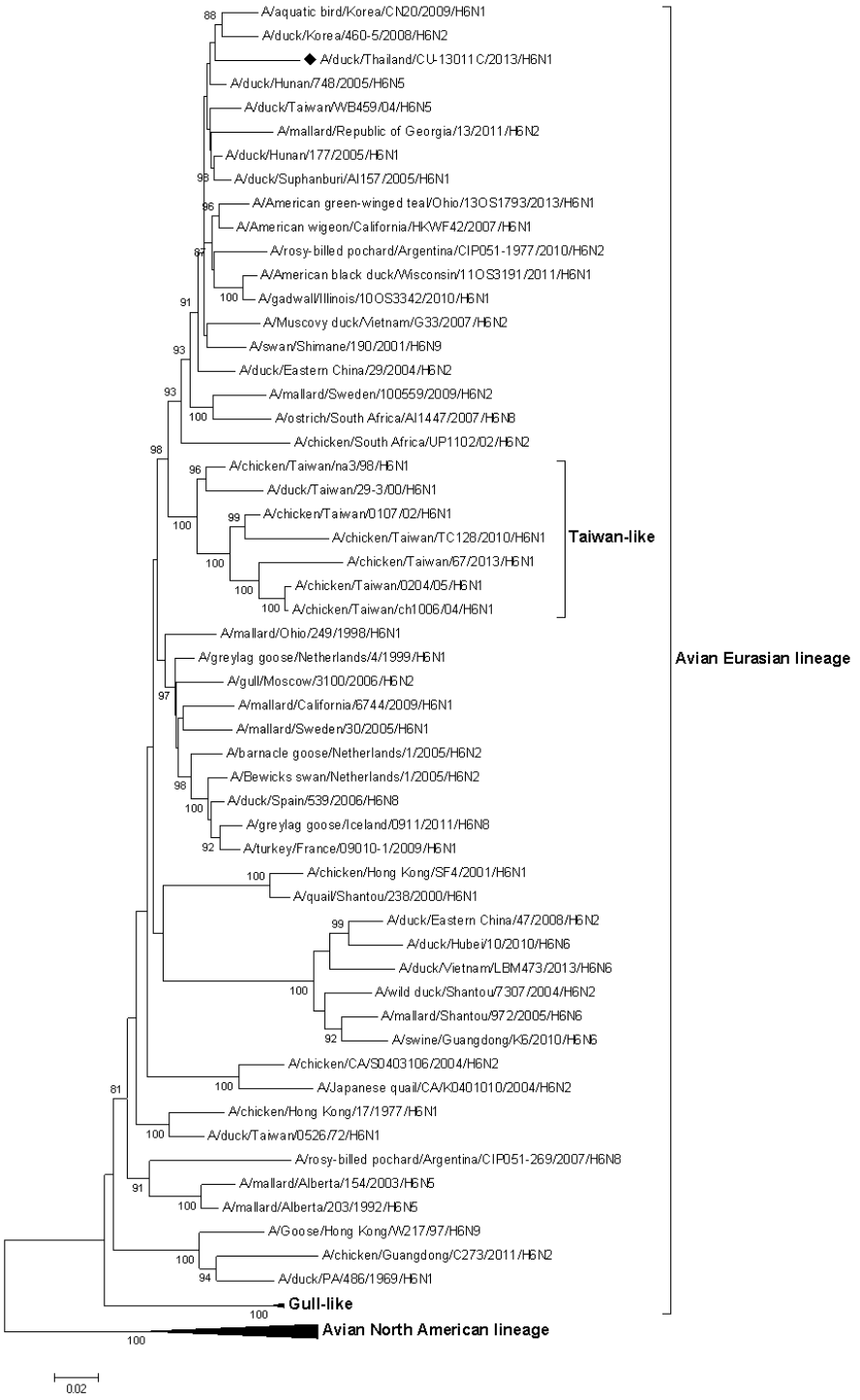

Figure 9 Phylogenetic analysis of HA6 gene

Phylogenetic analysis of HA6 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

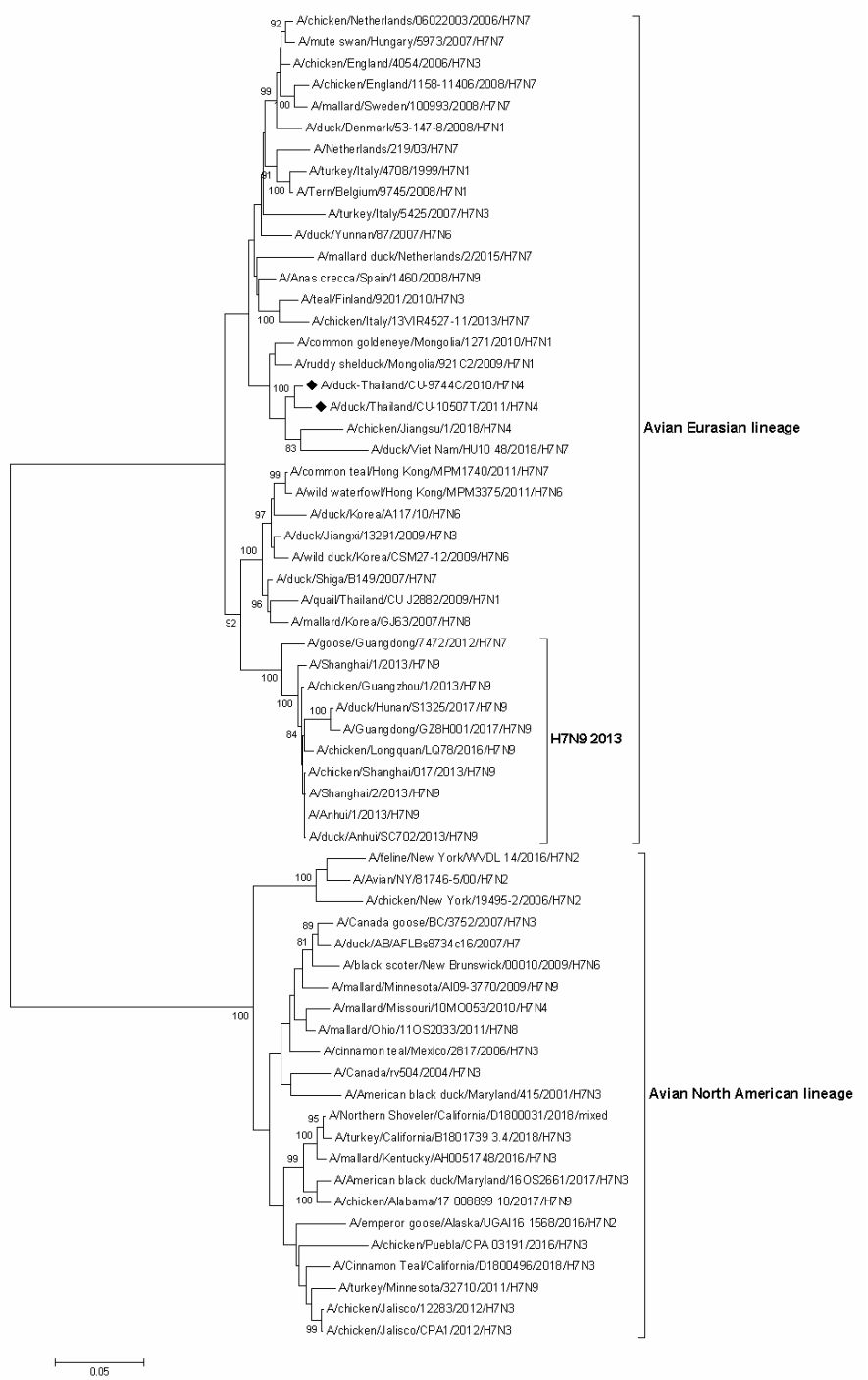

Figure 10 Phylogenetic analysis of HA7 gene

Phylogenetic analysis of HA7 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

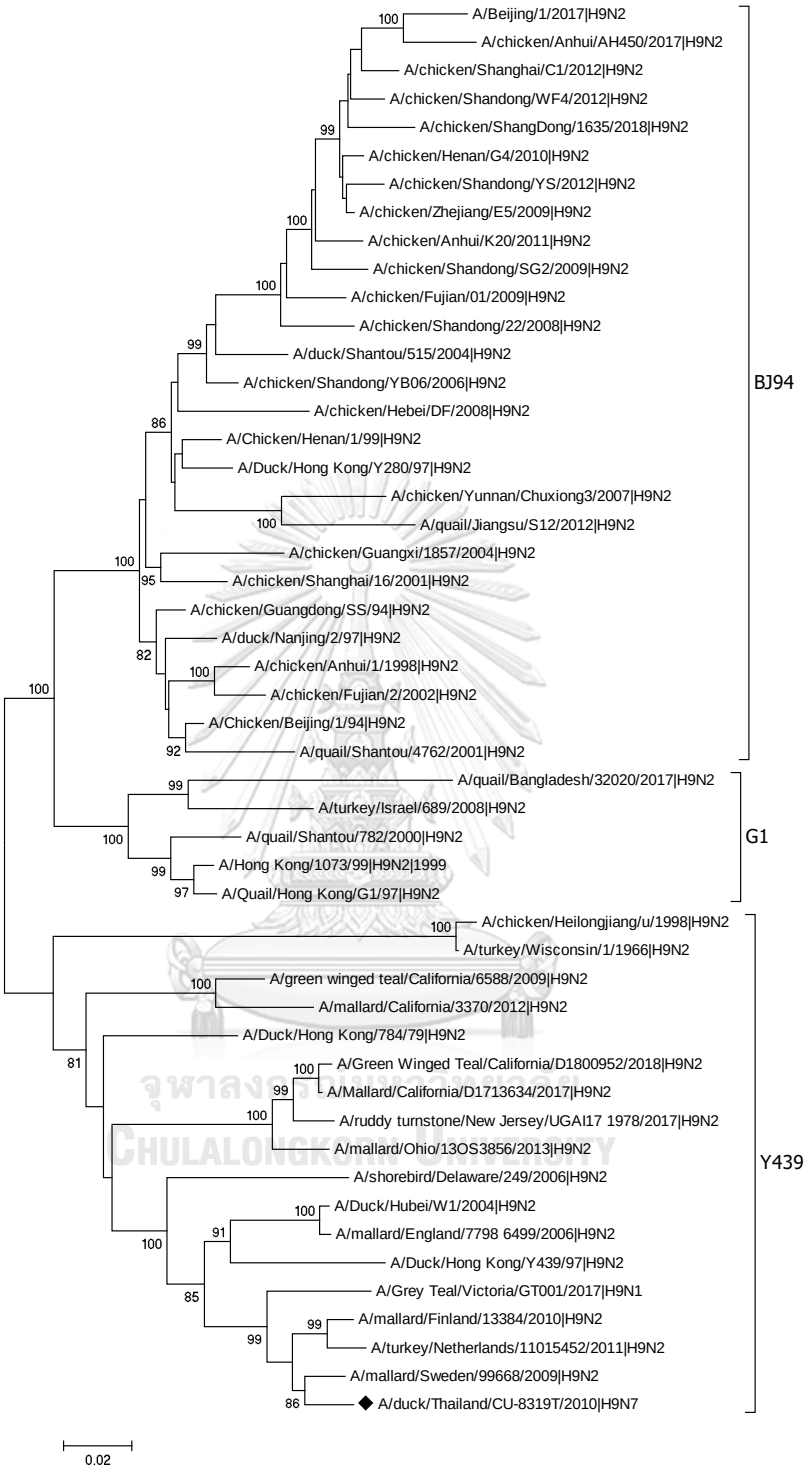

Figure 11 Phylogenetic analysis of HA9 gene

Phylogenetic analysis of HA9 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

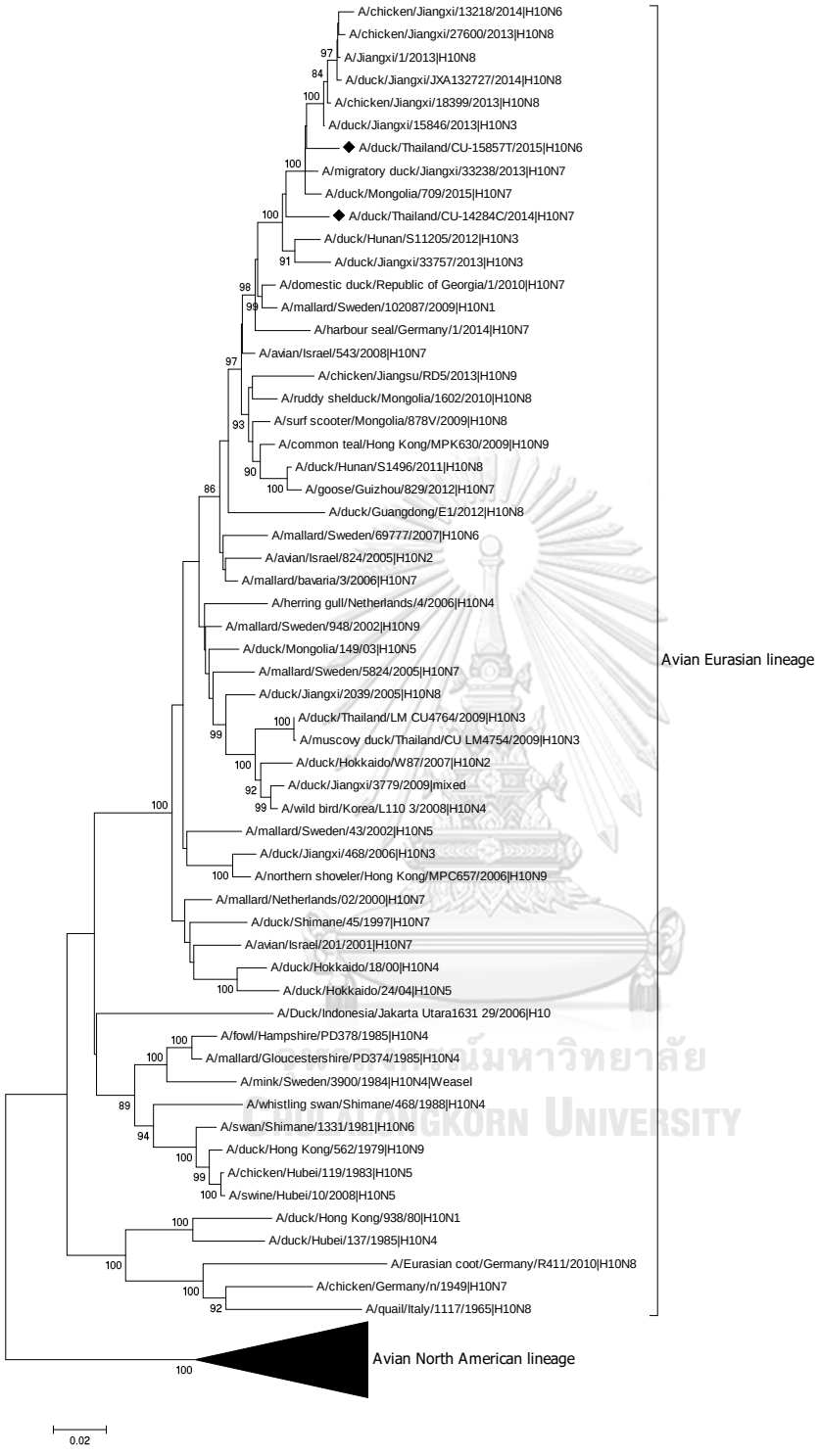

Figure 12 Phylogenetic analysis of HA10 gene

Phylogenetic analysis of HA10 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

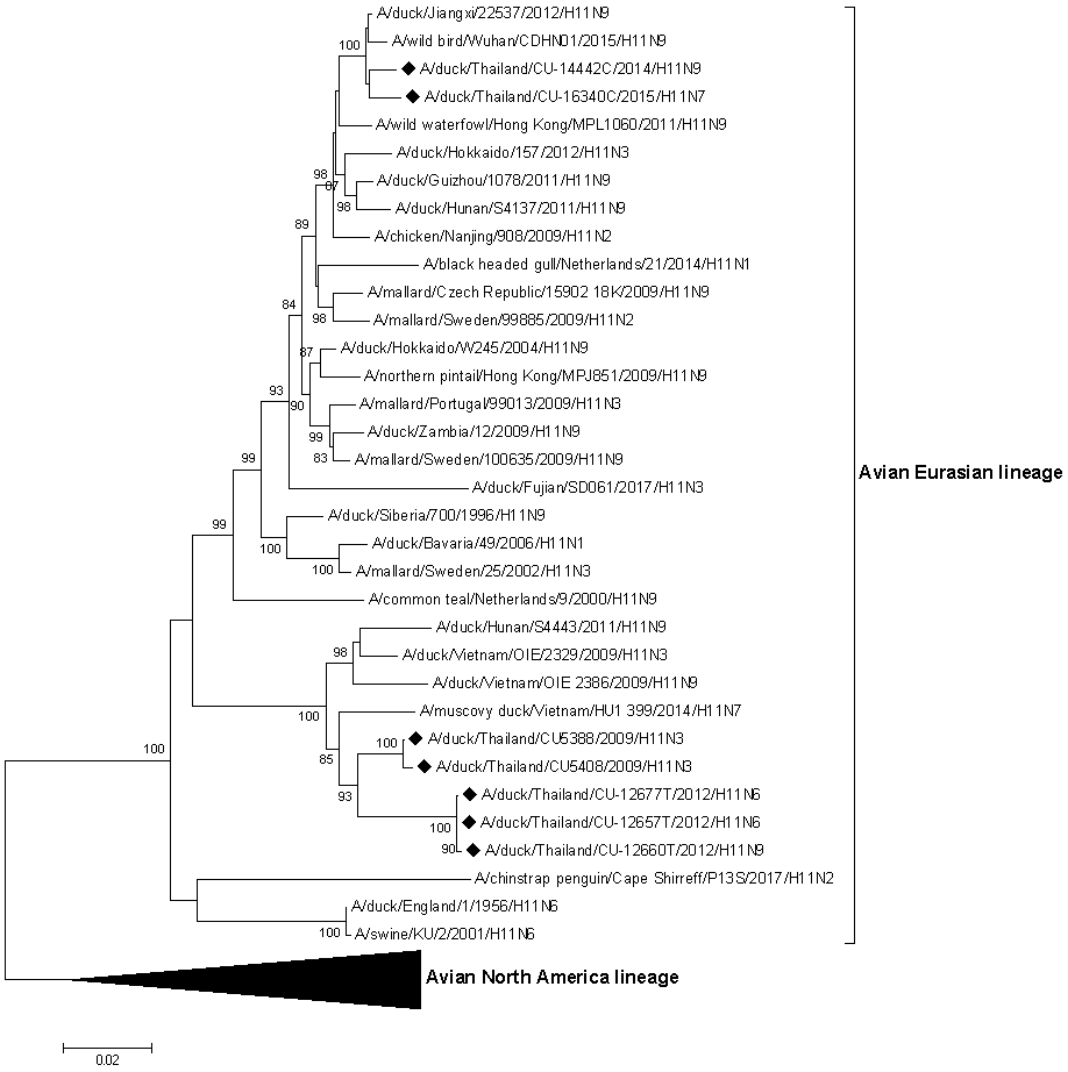

Figure 13 Phylogenetic analysis of HA11 gene

Phylogenetic analysis of HA11 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

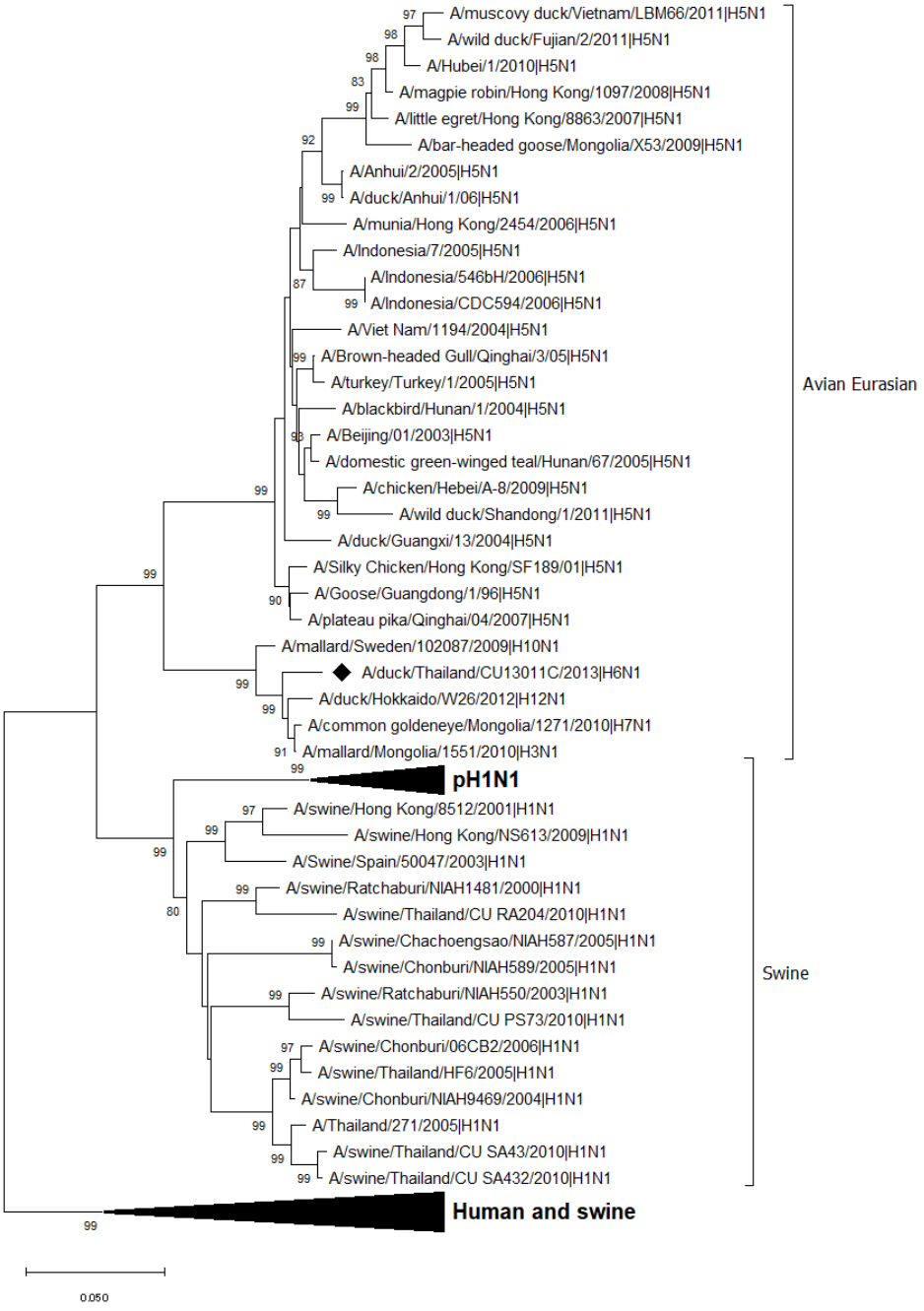

Figure 14 Phylogenetic analysis of NA1 gene

Phylogenetic analysis of NA1 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

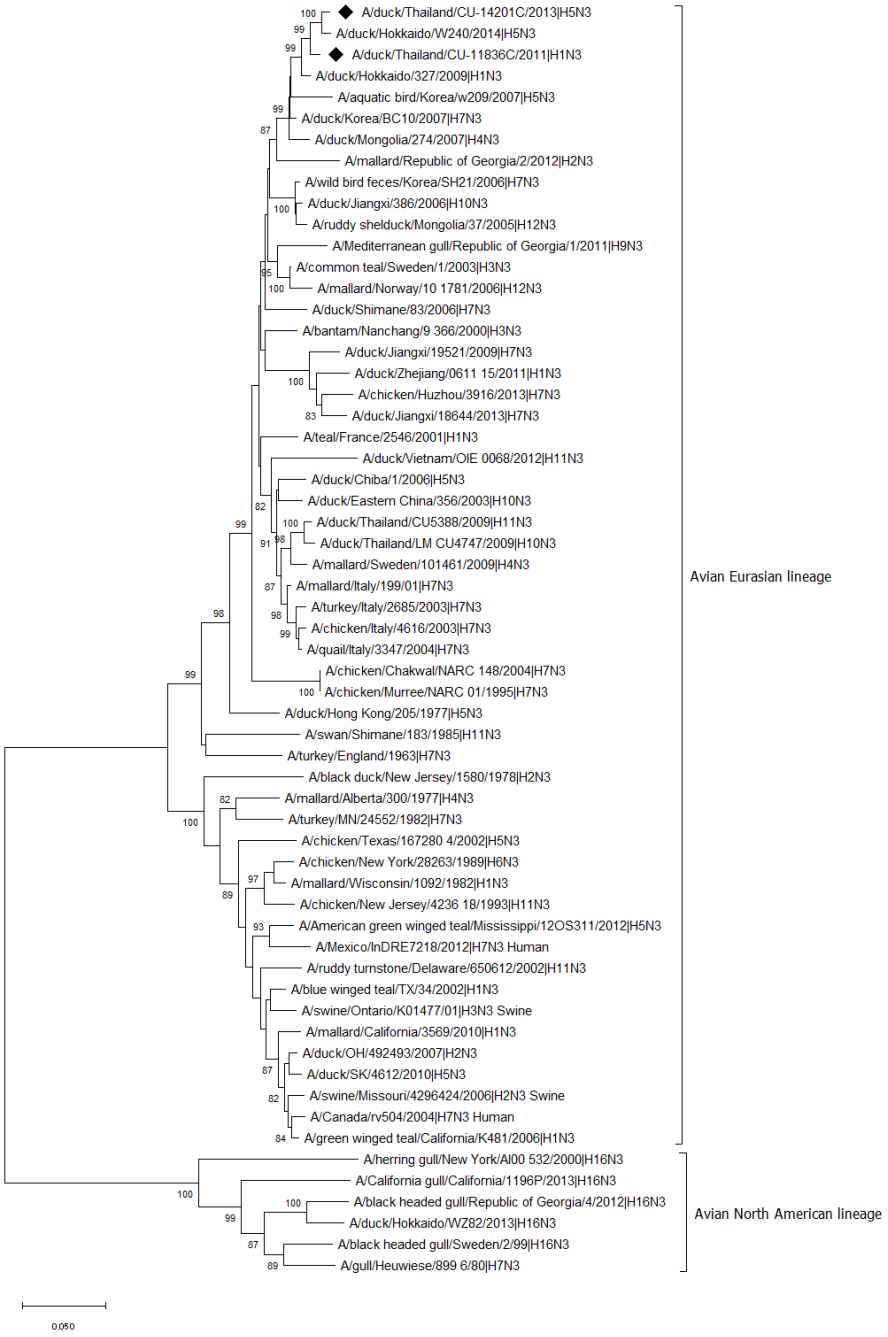

Figure 15 Phylogenetic analysis of NA3 gene

Phylogenetic analysis of NA3 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

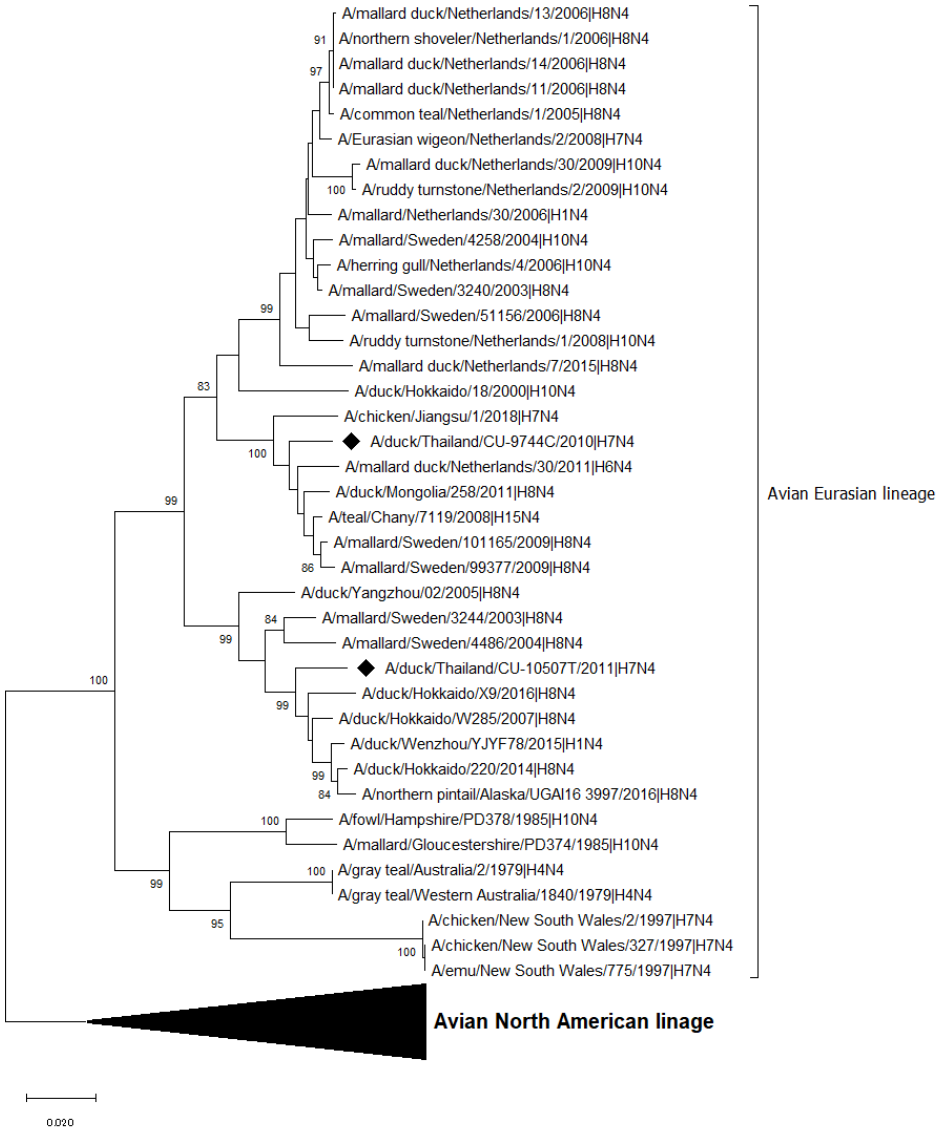

Figure 16 Phylogenetic analysis of NA4 gene

Phylogenetic analysis of NA3 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

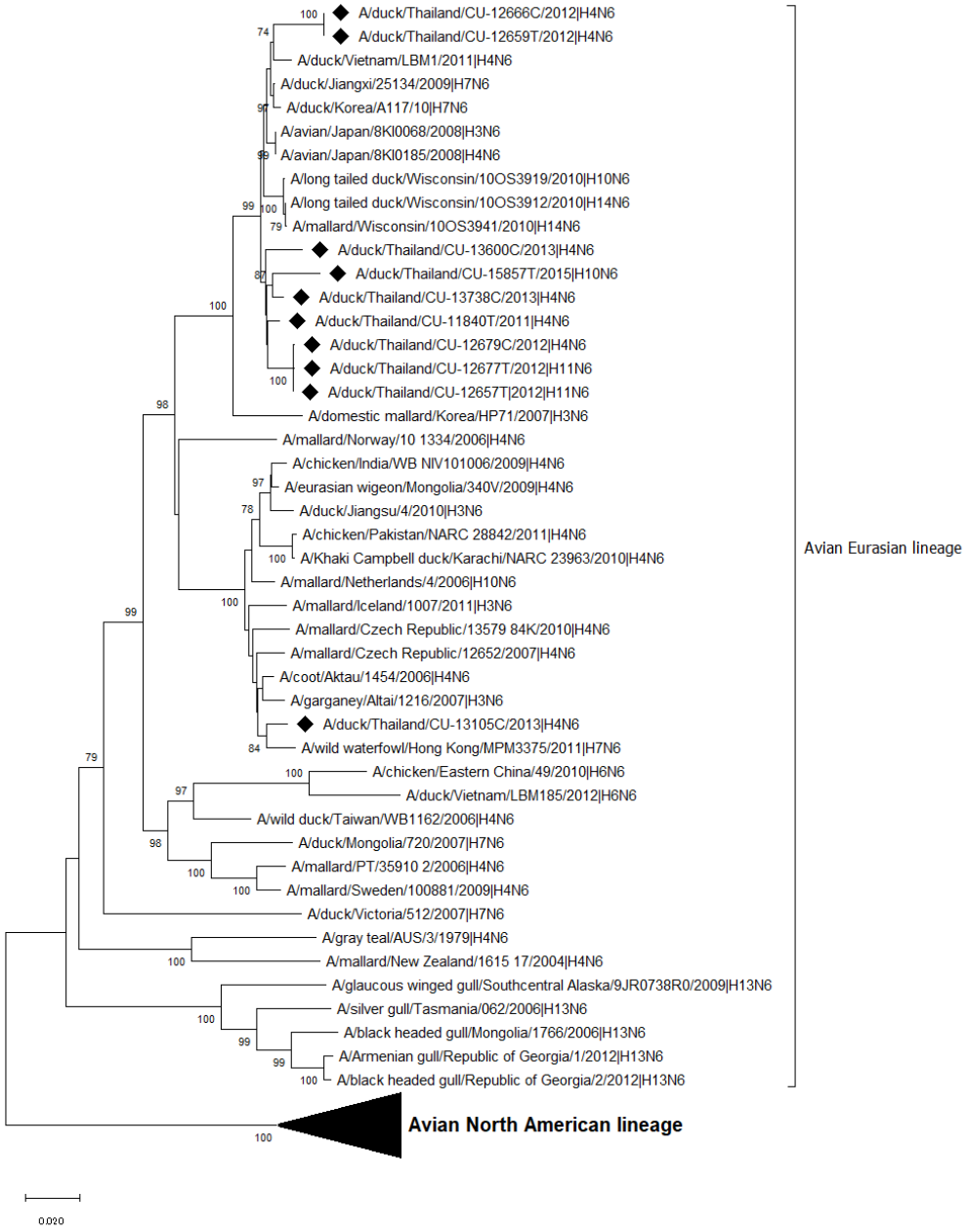

Figure 17 Phylogenetic analysis of NA6 gene

Phylogenetic analysis of NA6 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

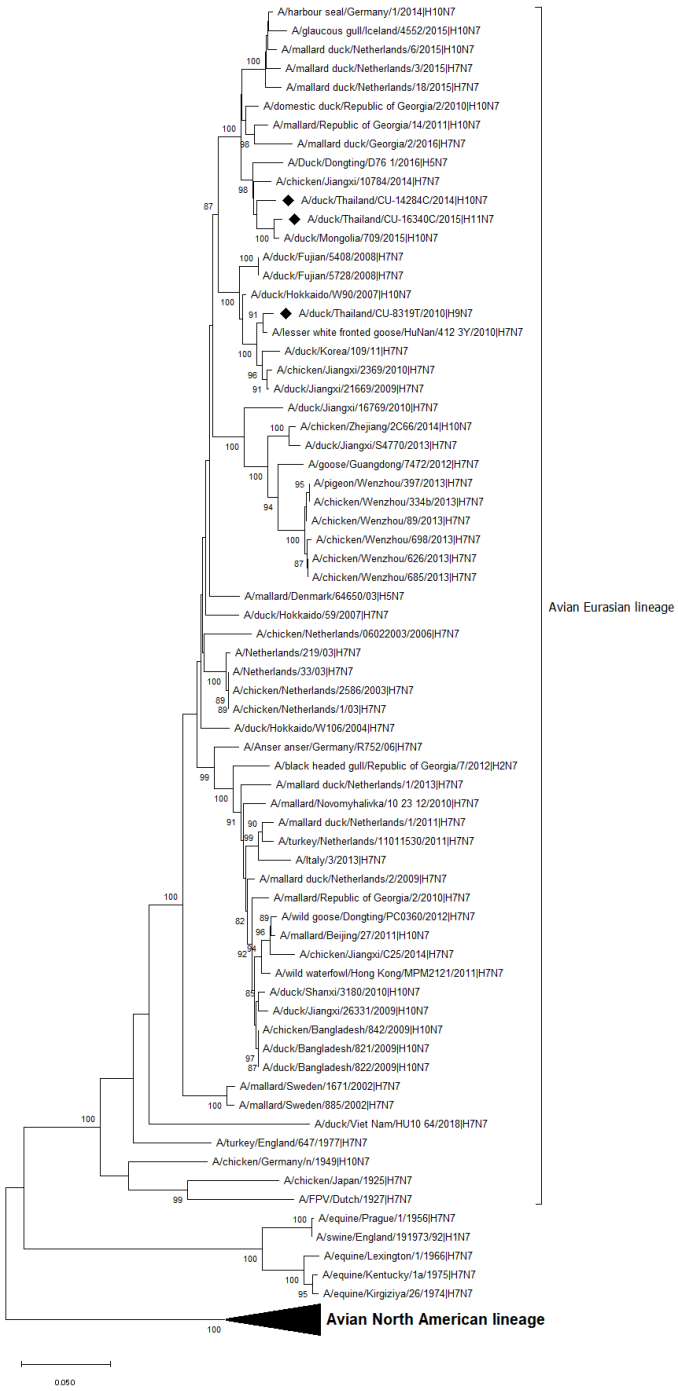

Figure 18 Phylogenetic analysis of NA7 gene

Phylogenetic analysis of NA7 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

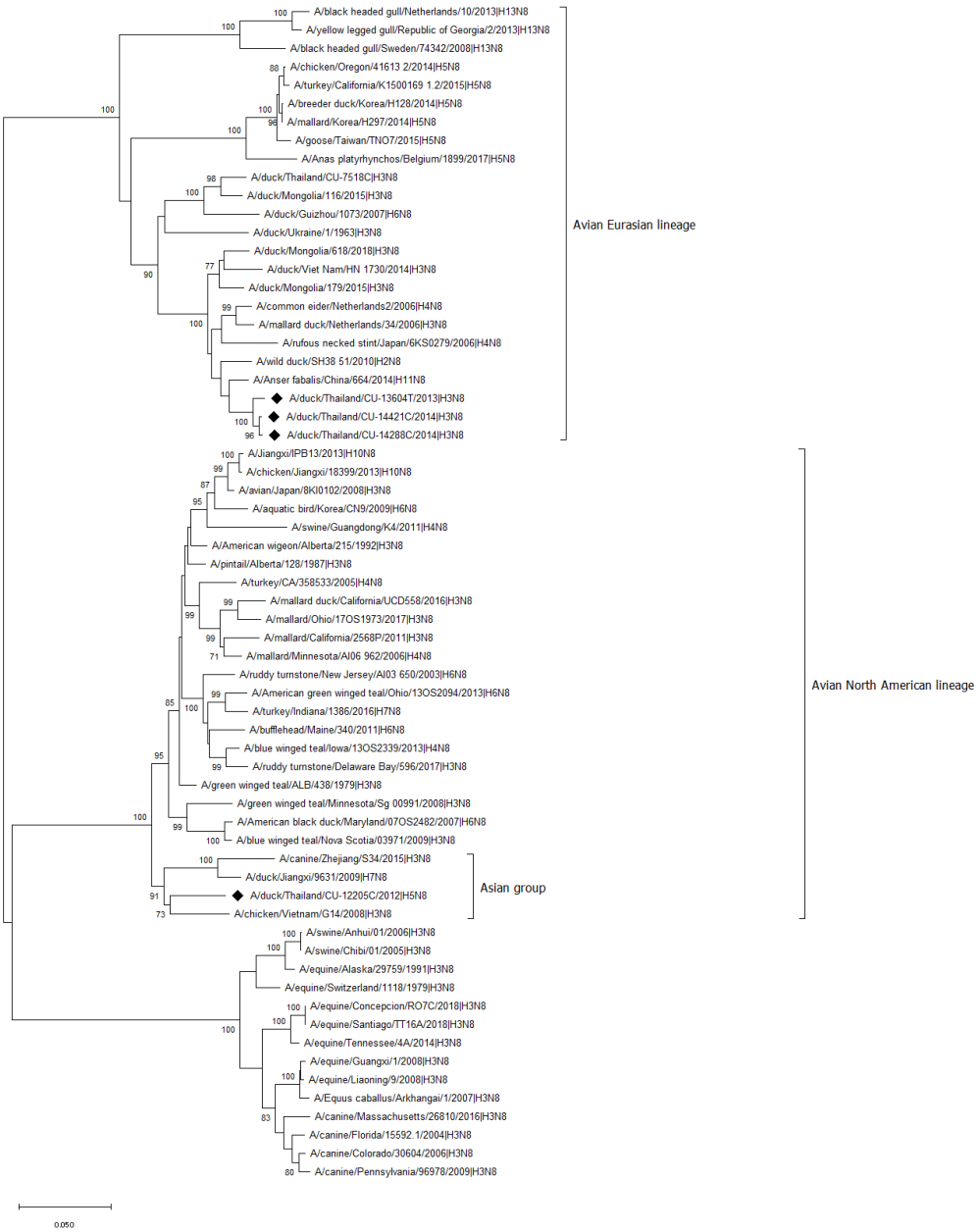

Figure 19 Phylogenetic analysis of NA8 gene

Phylogenetic analysis of NA8 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

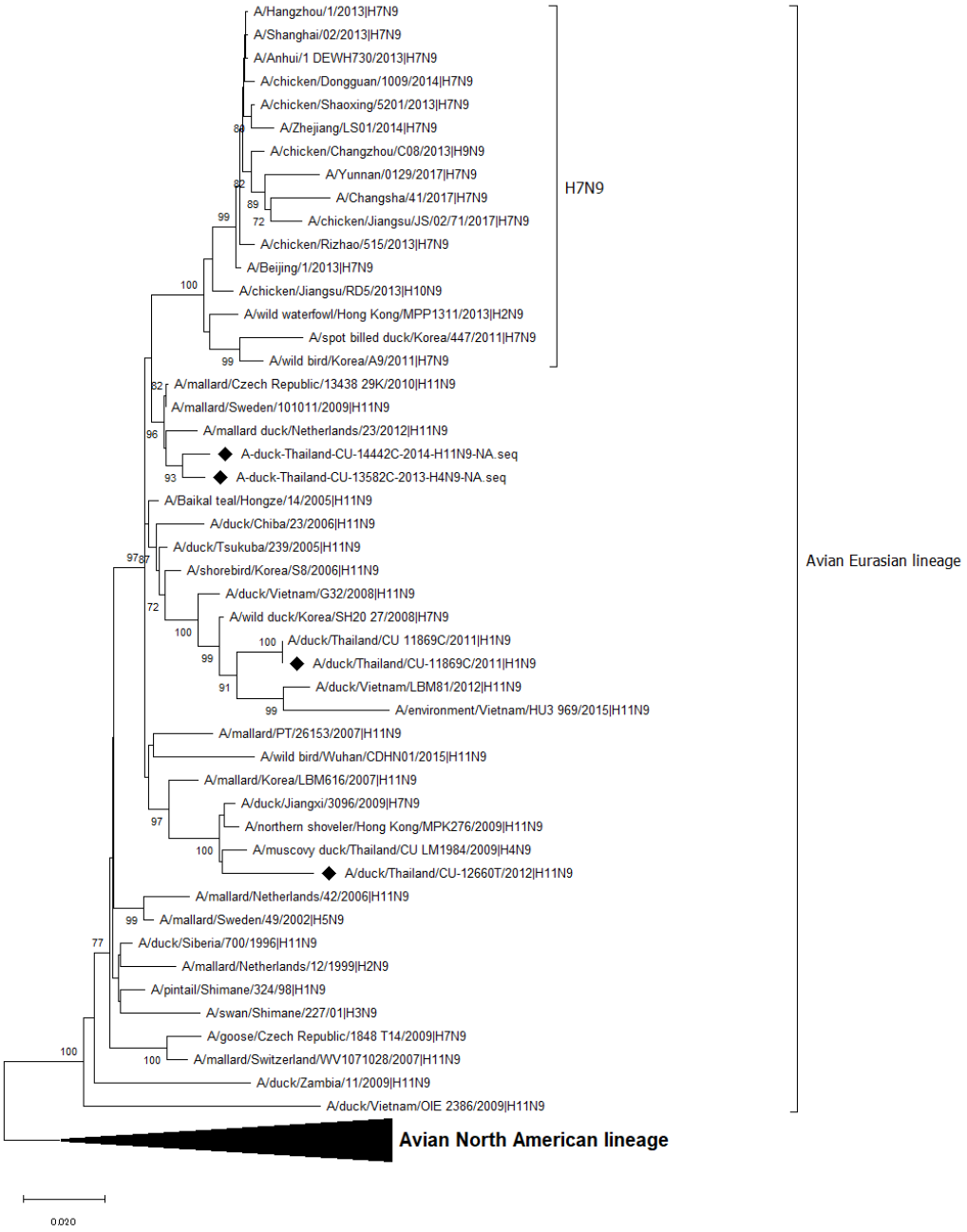

Figure 20 Phylogenetic analysis of NA9 gene

Phylogenetic analysis of NA9 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are presented by diamond. The scale bar represents the distance unit between sequence pairs.

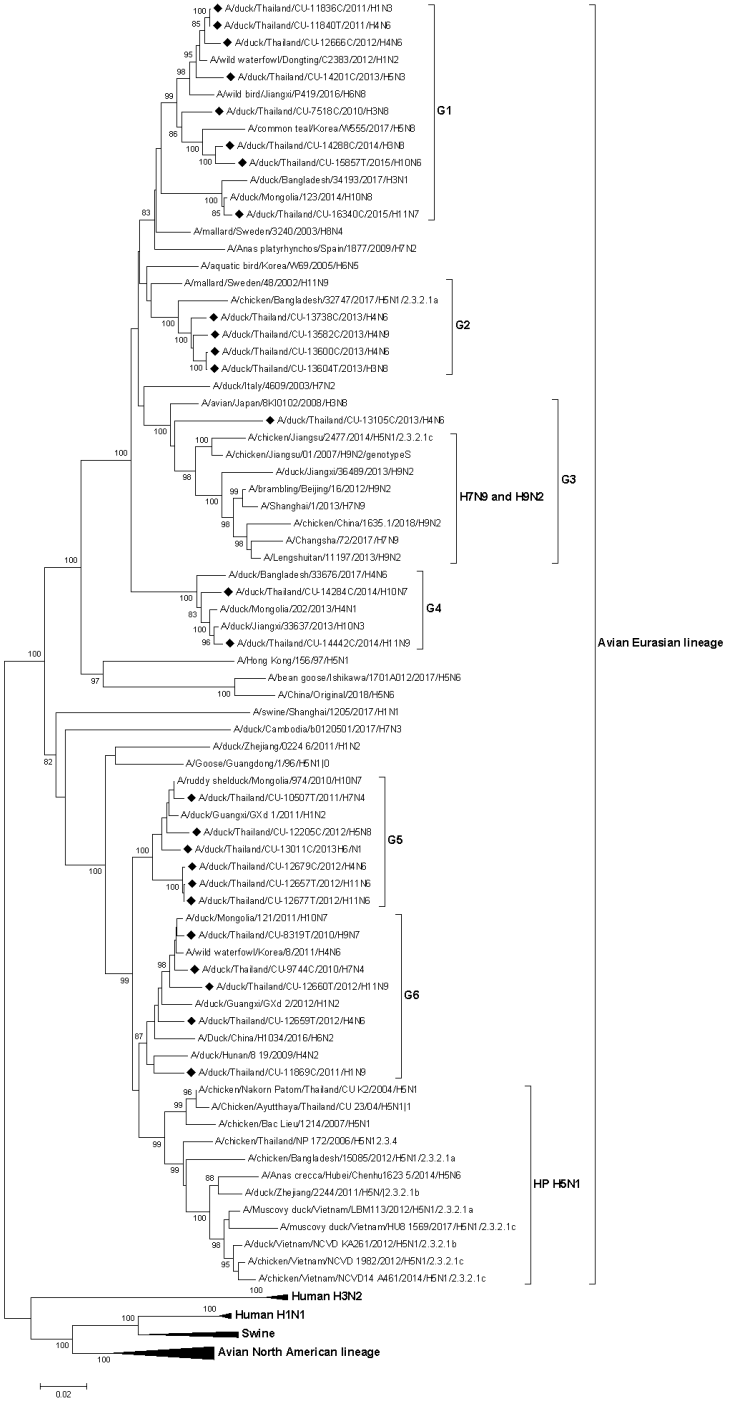

Figure 21 Phylogenetic analysis of PB2 gene

Phylogenetic analysis of PB2 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are highlight by a diamond. The scale bar represents the distance unit between sequence pairs.

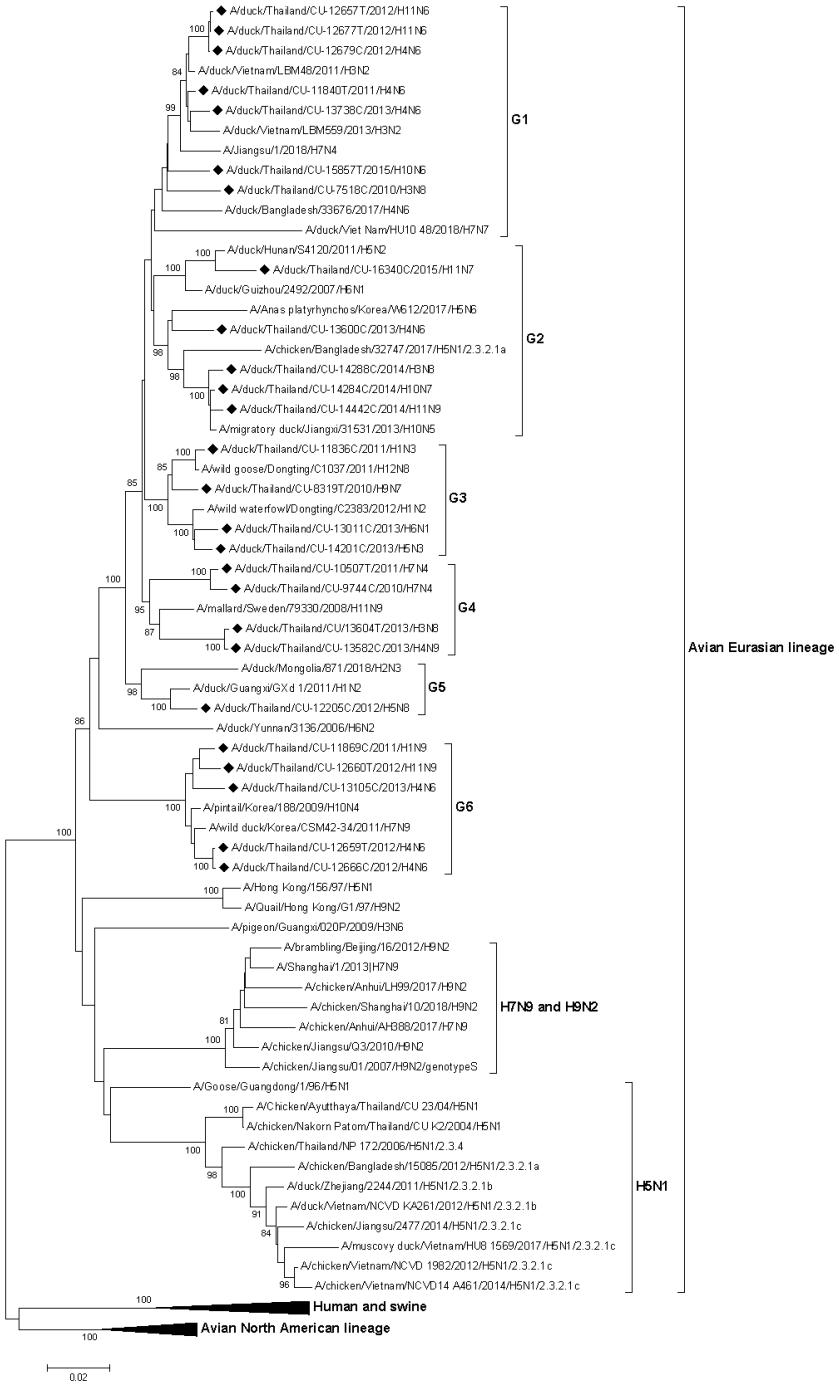

Figure 22 Phylogenetic analysis of PB1 gene

Phylogenetic analysis of PB1 gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are highlight by a diamond. The scale bar represents the distance unit between sequence pairs.

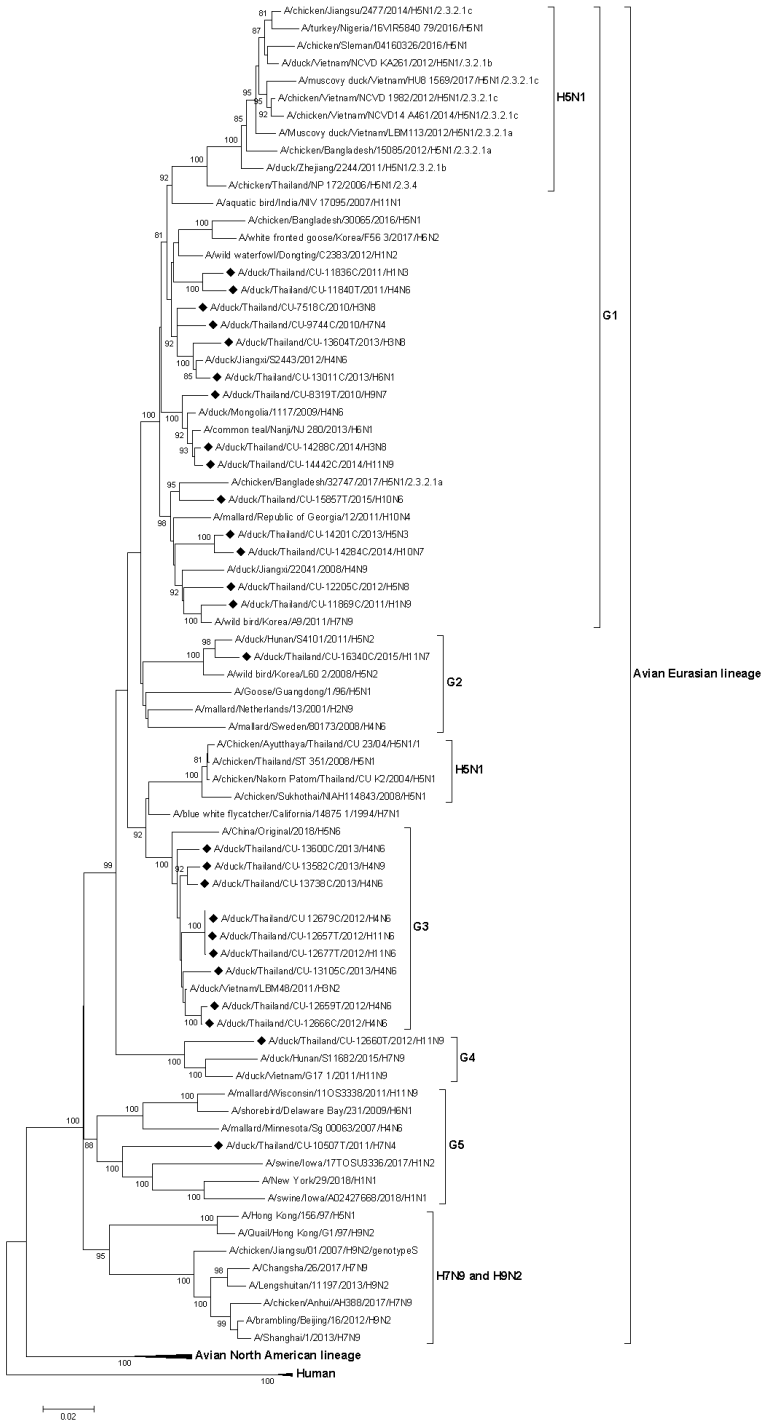

Figure 23 Phylogenetic analysis of PA gene

Phylogenetic analysis of PA gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are highlight by a diamond. The scale bar represents the distance unit between sequence pairs.

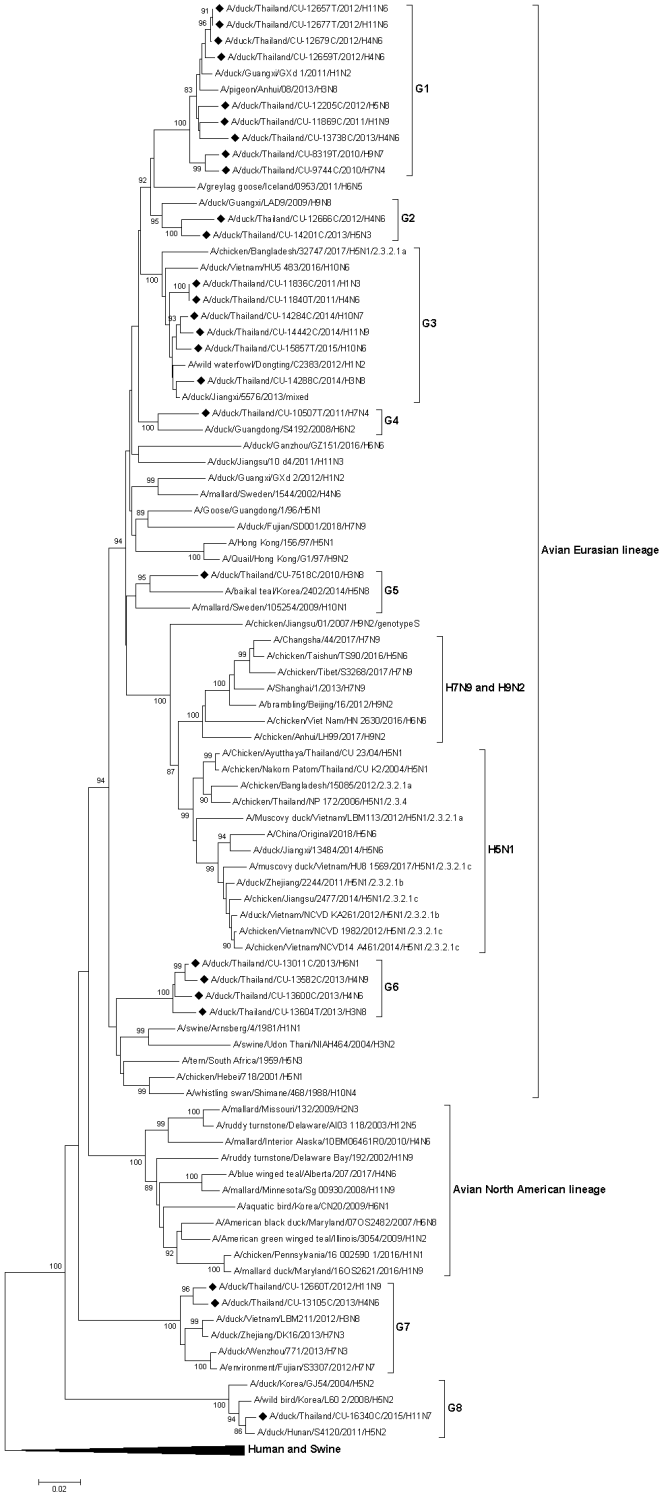

Figure 24 Phylogenetic analysis of NP gene

Phylogenetic analysis of NP gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are highlight by a diamond. The scale bar represents the distance unit between sequence pairs.

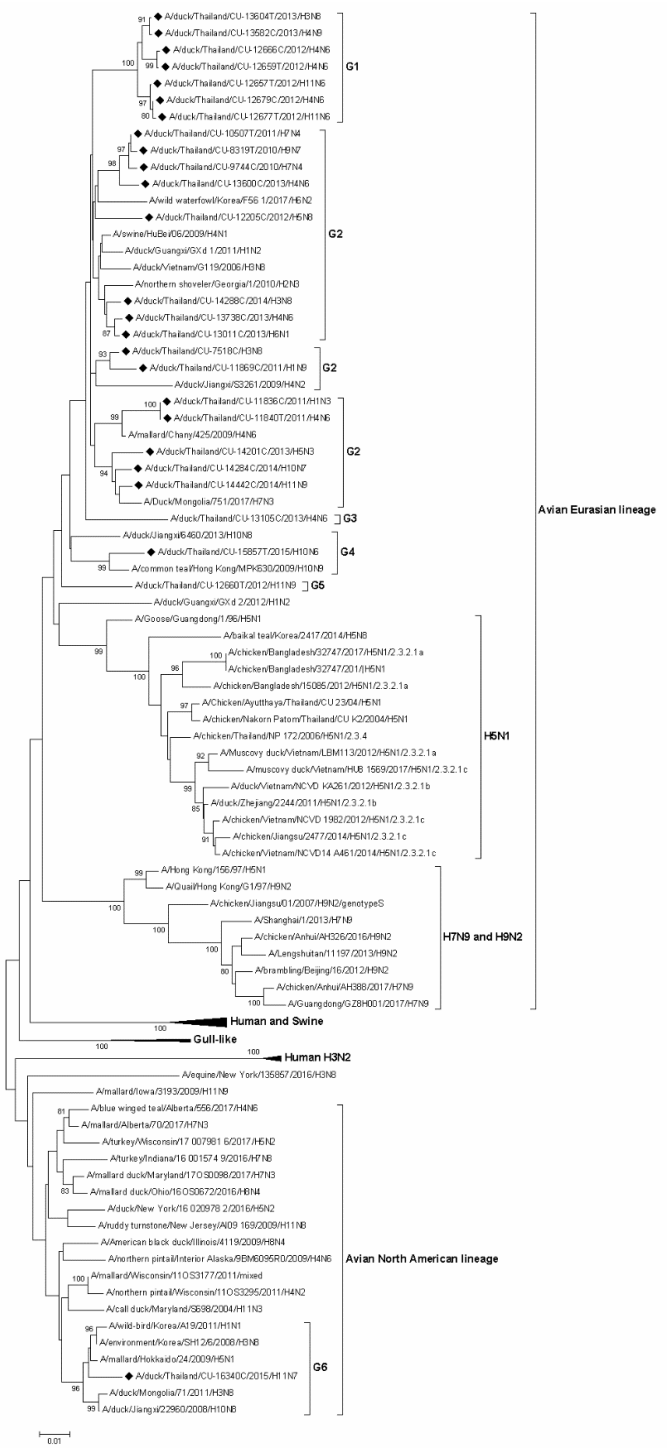

Figure 25 Phylogenetic analysis of M gene

Phylogenetic analysis of M gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are highlight by a diamond. The scale bar represents the distance unit between sequence pairs.

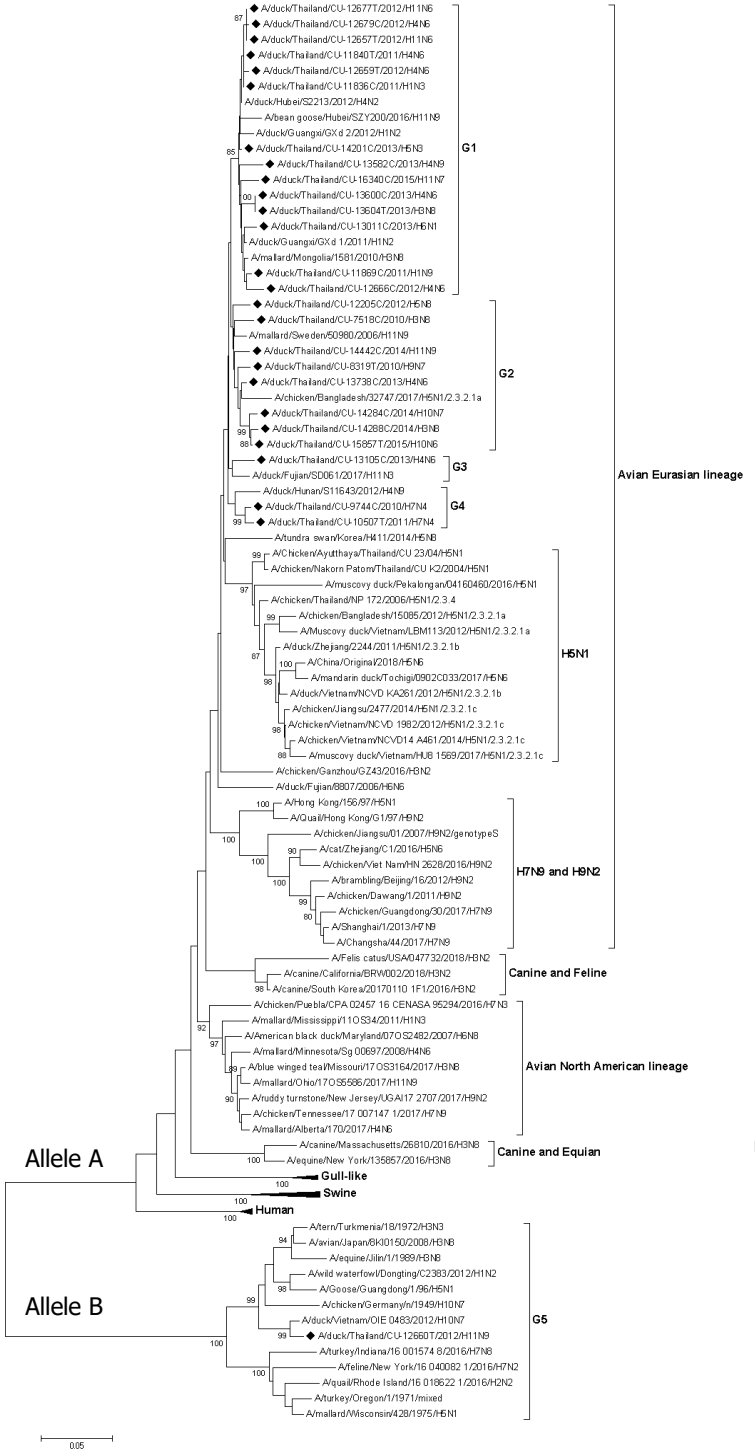

Figure 26 Phylogenetic analysis of NS gene

Phylogenetic analysis of NS gene of the representative viruses in this thesis and other influenza A viruses. The representative viruses are highlight by a diamond. The scale bar represents the distance unit between sequence pairs.

3. Phase 3: Challenge of influenza A viruses from free-grazing ducks in animal models

From phase 1 and 2, we selected influenza A viruses subtype H1N3 (n=1) and H11N9 (n=1) for animal challenge study. The viruses were designated as A/duck/Thailand/CU-11836C/2011 (H1N3) (CU-11836C) and A/duck/Thailand/CU-12660T/2012 (H11N9) (CU-12660T). The rationale for the selection of these 2 influenza A viruses are as following:

- The Thai IAV-H1N3 virus is the first avian H1 subtype ever reported in Thailand. The H1 subtype can infect and cause endemic influenza in humans and pigs. However, the H1 virus circulates in avian species as reservoir host. Genetic analysis of Thai IAV-H1N3 virus (CU-11836C) suggesting that Thai IAV-H1N3 virus is low pathogenic avian influenza virus (LPAI). But, the human-like amino acid at position M2-11 suggested virulence of the virus and required further investigation.
- The Thai IAV-H11N9 virus (CU-12660T) is a novel reassortant virus with unique internal genes (NP and NS gene). Moreover, the unique virulence determinants were observed at PB2-627 (PB2-627G) and PA-55 (PA-55N). Thus CU-12660T virus was selected for further investigation.

3.1 Determination of pathogenicity and virulence of Thai IAV-H1N3 (CU-11836C) virus

3.1.1 Intravenous Pathogenicity Index (IVPI) test

For IVPI test, the Thai IAV-H1N3 virus (CU-11836C) was propagated to $10^{6.5}$ EID_{50} and was diluted as 10 fold-dilutions in sterile isotonic saline. The viral suspension (0.1 ml) was used for intravenous injection at jugular vein in 6-week-old chickens (n=10) (free from influenza antibodies) (Figure 27). The challenged chickens were monitored for clinical signs at 24-hour intervals for 10 days. At each observation, each chicken is scored 0 (normal; no sign), 1 (sick; 1 clinical sign), 2 (severely sick; ≥2 clinical signs) and 3 (dead). The clinical signs are respiratory sign,

depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs. The index is calculated as the mean score per bird per observation. The IVPI is the mean score per bird per observation over the 10-days period. In this thesis, during experimental period, one chicken was sudden death after virus infection while other were normal. In this study, total score is 12 and thus the IVPI index of Thai H1N3 virus is 0.12 indicating LPAI characteristic of Thai H1N3 (CU-11836C) (Table 7). Noted that the IVPI index more than 1.2 is considered as HPAI.

Table 7 Detail of Intravenous Pathogenicity Index (IVPI) test of Thai IAV-H1N3 (CU-11836C)

Clinical signs	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	Total	Score
Normal	10	10	10	10	10	10	9	9	9	9	96×0	0
Sick	0	0	0	0	0	0	0	0	0	0	0×1	0
Severe Sick	0	0	0	0	0	0	0	0	0	0	0×2	0
Dead	0	0	0	0	0	0	1	1	1	1	4×3	12
											Total	12

Clinical signs (respiratory sign, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs) were scored.

Normal: No clinical signs

Sick: Animal showed 1 clinical sign

Sever sick: Animal showed ≥2 clinical signs

The IVPI index of this virus = 12/(10x10) = 0.12

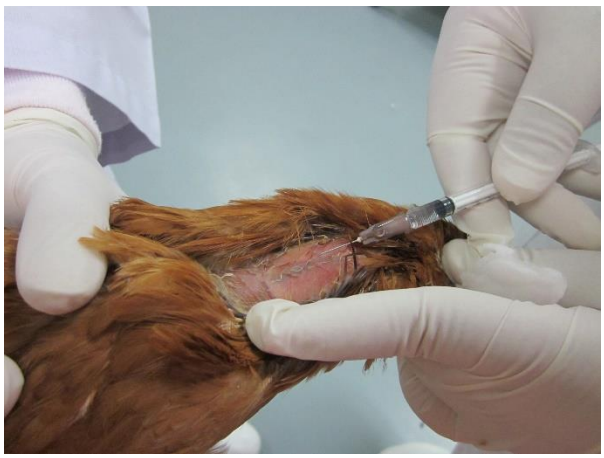

Figure 27 IVPI test by injection of $10^{6.5}$ EID_{50} virus at jugular vein

3.1.2 Challenge of Thai IAV-H1N3 (CU-11836C) virus in experimental chickens

The Thai IAV-H1N3 virus (CU-11836C) was challenged in chickens (n=15) to determine clinical presentations, pathological changes, viral shedding and viral transmission. The challenge experiment was conducted in separated ABSL2 room. The challenge experiment composed three experiment groups. Group 1; Inoculated group (n=8) was inoculated with 0.1 ml of the virus via intranasal route. Group 2; Contacted group (n=4) was moved to animal cage of inoculated birds at 2 dpi. Group 3; Control group (n=3) was placed in separate room from group 1 and 2 and inoculated with 0.1 ml PBS (Figure 28). Oropharyngeal and cloacal swab samples were collected from all the animals at 0, 1, 3, 5, 7, 10 and 14 dpi. The swab samples were processed for influenza virus detection and quantitation by rRT-PCR. Blood samples were collected from all birds at 0, 7, 10 and 14 dpi. The serum samples were detected for influenza antibody by NP ELISA. After experiment was terminated, all chickens were euthanized, necropsied and collected of the visceral organs (lung, trachea, liver, kidney and ileum). The organs were examined for pathological changes.

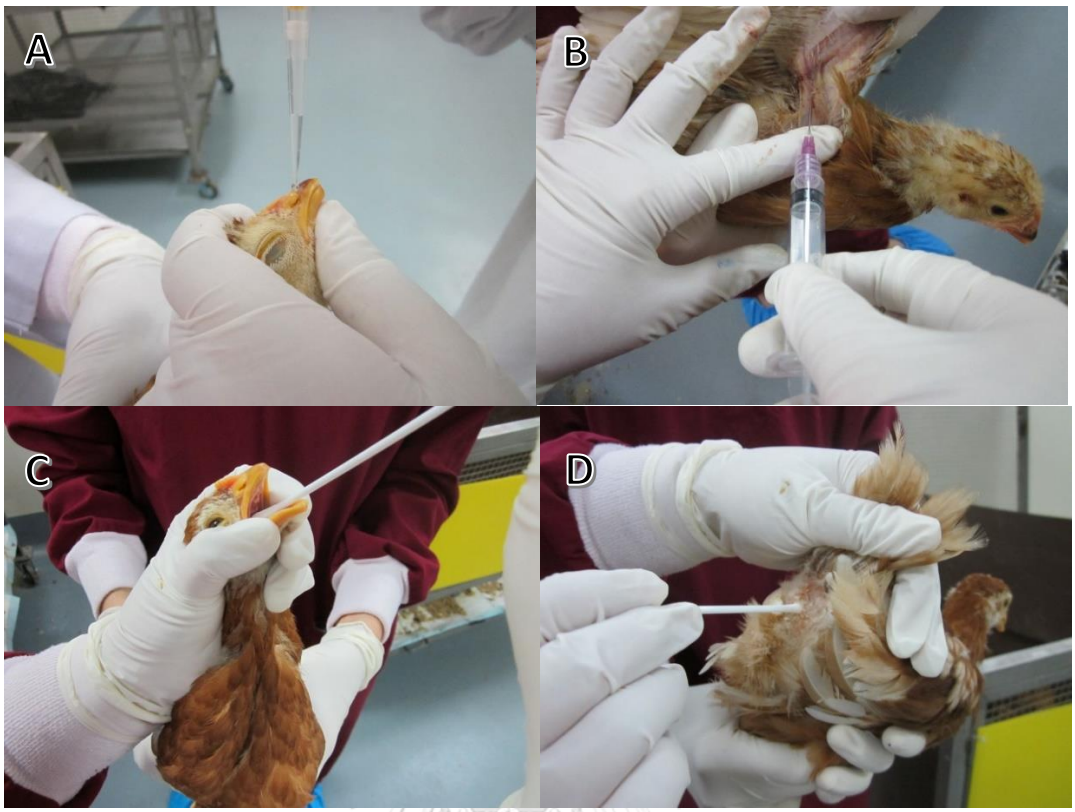

Figure 28 Viral inoculation via intranasal of experimental chickens
(A) The experimental chickens were inoculated with the virus via intranasal. (B) blood collection from wing vein, (C) oropharyngeal swab and (D) cloacal swab

The result of Thai IAV-H1N3 virus (CU-11836C) infection in experimental chickens showed that all chickens did not show any clinical signs during 14 dpi. The chickens of inoculated group (group 1) shed the virus from respiratory tract but not in the intestinal tract. The chicken shed the virus in respiratory tract at highest 62.5% (5/8 birds) at 3 dpi and last until 10 dpi. While the chickens of contacted group and control group did not shed the virus from the respiratory and intestinal tract (Table 8). In this study, viral titer from infected chickens were examined and the result showed that the viral shedding period was vary from 3 to 10 days. The highest viral titer was observed at 1 dpi (5.64±2.70), followed by 3 dpi (5.96±2.68) (Table 9). No statistic significant of viral titer between inoculated group and contacted group as well as between day post inoculation was observed (P-value <0.05) (Table 21).

Table 8 Viral shedding determination of Thai IAV-H1N3 (CU11836C) descripted by number of positive chickens.

		Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Inoculated gr.	OP	2/8 (25%)	5/8 (62.5%)	4/8 (50%)	3/8 (37.5%)	1/8 (12.5%)	0/8
	CS	0/8	0/8	0/8	0/8	0/8	0/8
Contacted gr.	OP	0/4	0/4	0/4	0/4	0/4	0/4
	CS	0/4	0/4	0/4	0/4	0/4	0/4
Control gr.	OP	0/3	0/3	0/3	0/3	0/3	0/3
	CS	0/3	0/3	0/3	0/3	0/3	0/3

* Number of positive chicken/number of chickens in each group

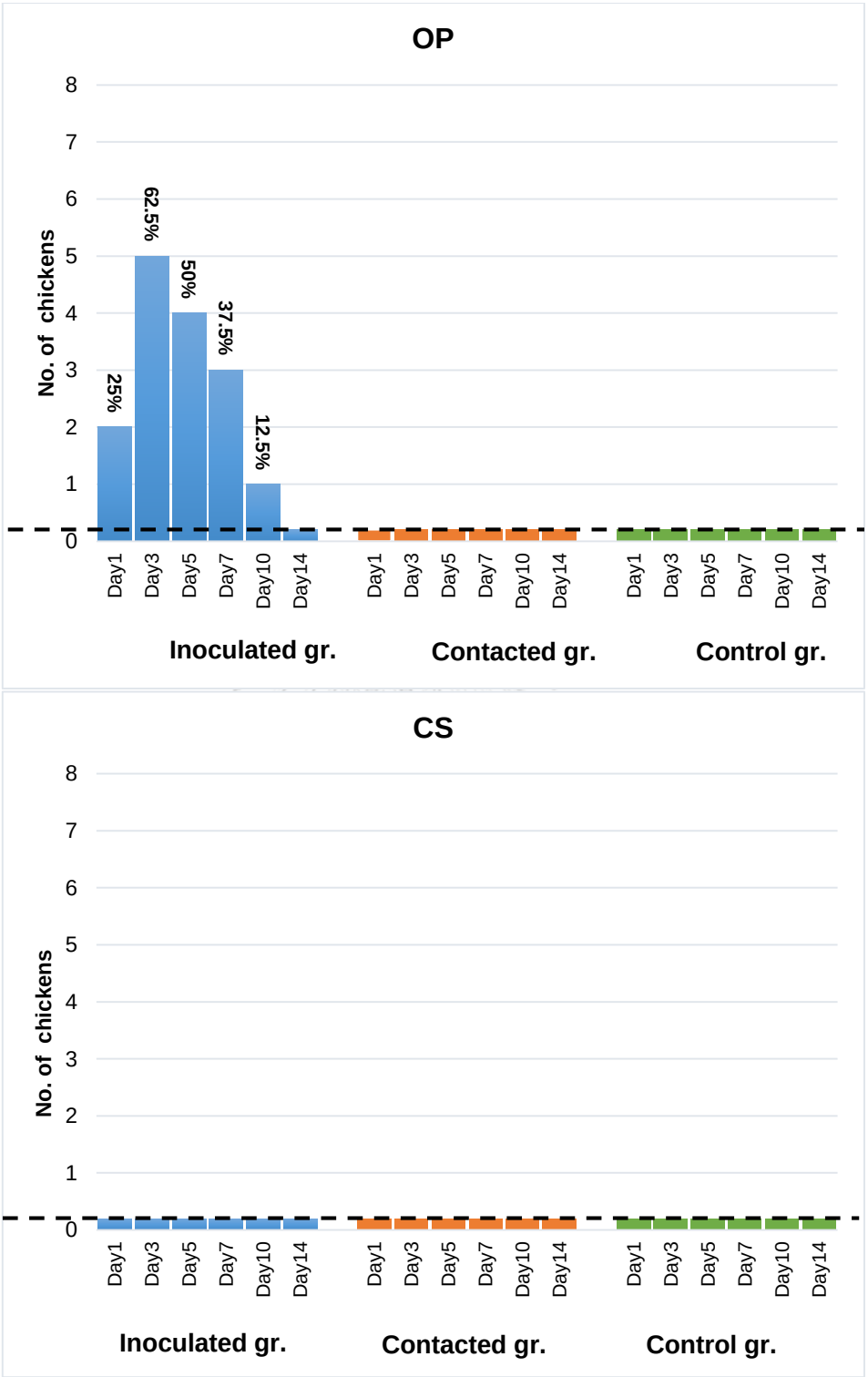

Figure 29 Viral shedding of Thai IAV-H1N3 (CU-11836C) descripted by number of positive chickens; A) Oropharyngeal swab B) Cloacal swab

Table 9 Viral shedding determination of Thai IAV-H1N3 (CU11836C) descripted by virus titers (copy number)

Inoculated gr.	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Chicken#1	6.32	5.71	-	-	-	-
Chicken#2	6.16	6.82	5.13	3.81	-	-
Chicken#3	-	-	-	-	-	-
Chicken#4	-	4.80	4.41	4.71	-	-
Chicken#5	-	-	-	-	-	-
Chicken#6	-	-	-	-	-	-
Chicken#7	-	4.50	5.40	3.84	4.37	-
Chicken#8	-	5.03	5.36	-	-	-
Mean ±SD	5.64±2.71	5.96±2.68	4.90±2.55	3.91±2.01	3.46±1.44	-

Number in gray boxes; number of viral copy number [log10 (copies/µl)].

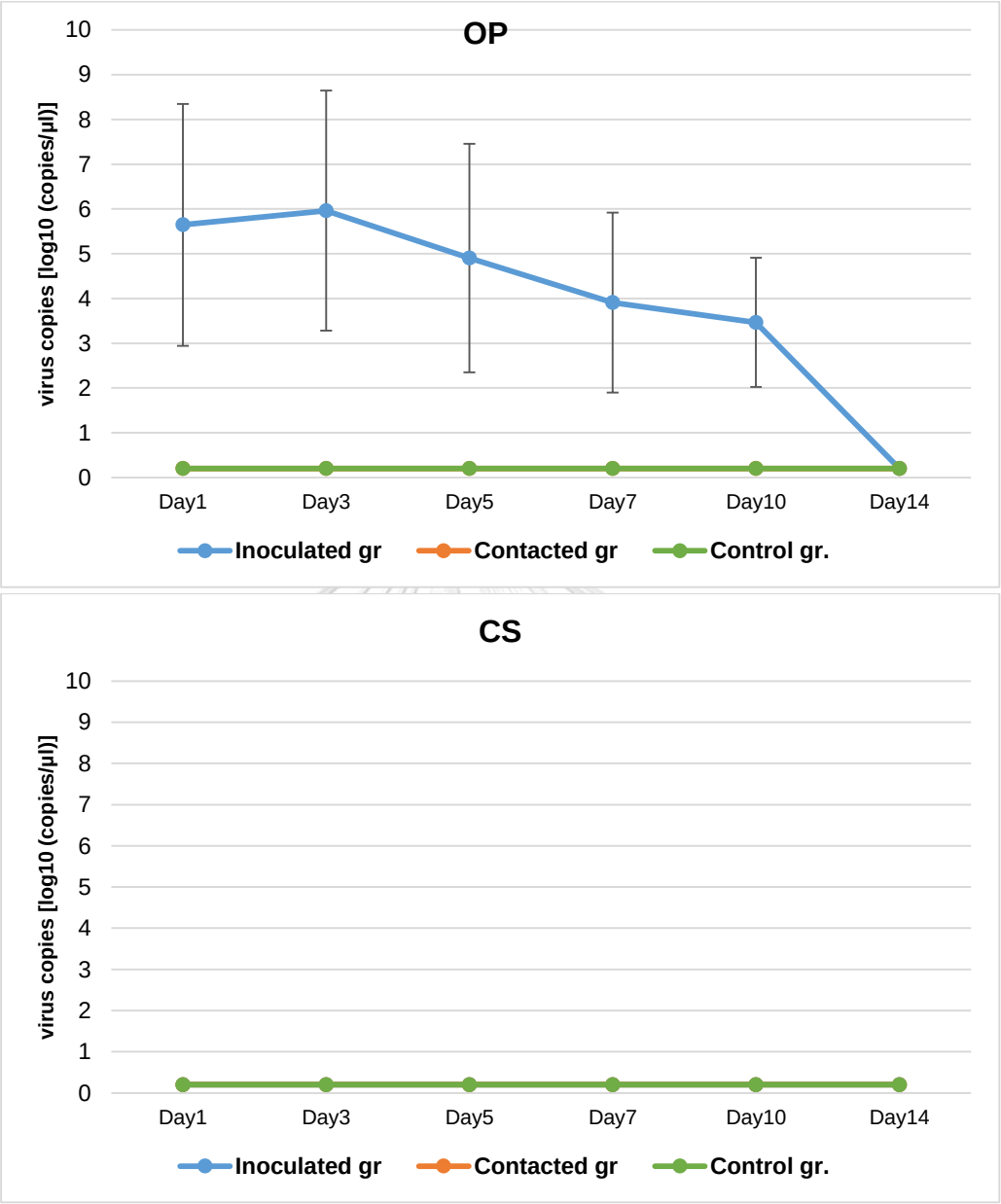

Figure 30 Viral shedding of Thai IAV-H1N3 (CU-11836C) in chickens descripted by virus titers (copy number), A) Oropharyngeal swab B) Cloacal swab

For serological response to Thai IAV-H1N3 virus (CU-11836C) infection in experimental chickens, 87.50% (7/8) of chickens in inoculated group (group 1) showed positive for influenza antibody. The chickens showed antibody response since 7 dpi (n=6). In contacted group (group 2), 25% (1/4) showed positive antibody at 14 dpi (Table 10).

Table 10 Serological response determination of Thai IAV-H1N3 (CU11836C) descripted by number of positive influenza antibody chickens by blocking ELISA.

	Day 7	Day 10	Day 14
Inoculated gr.	6/8 (75%)	7/8 (87.5%)	7/8 (87.5%)
Contacted gr.	0/4	0/4	1/4 (25%)
Control gr.	0/3	0/3	0/3

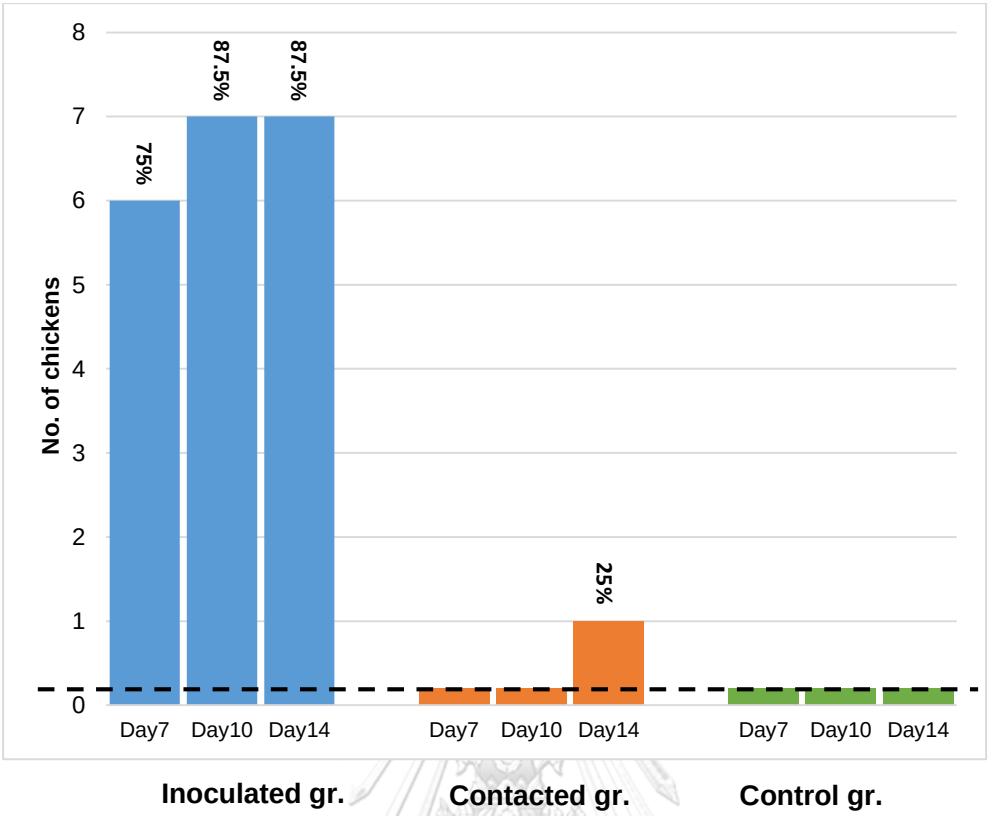

Figure 31 Serological response of Thai IAV-H1N3 (CU11836C) descripted by number of positive Influenza antibody chickens by blocking ELISA.

For pathological changes to Thai IAV-H1N3 virus (CU-11836C) infection in experimental chickens, our result showed that histopathological lesions of infected chickens in inoculated group (group 1) and contacted group (group 2) (one seropositive chicken) showed lesions mostly restricted to the lung and spleen. The common histopathological finding was lymphocytic proliferation. Chronic catarrhal bronchitis with bronchial associated lymphoid proliferation, lymphoid aggregation or new lymphoid follicle in spleen were also observed. Significant lesions in the organs did not observe in uninfected chickens (viral shedding negative birds).

3.1.3 Challenge of Thai IAV-H1N3 (CU-11836C) virus in experimental quails

The experimental protocol was the same as challenge of Thai IAV-H1N3 virus in experimental chickens. Our result showed that all quails did not show any clinical signs throughout the experiment. The quails from inoculated group (group 1) shed the virus from both respiratory (6/8) and intestinal tract (2/8). One quail from contacted group (group 2) shed the virus at 1 dpi to 3 dpi. The quails shed the virus at highest 87.5% (7/8) at 1 dpi and 3 dpi and last until 7 dpi. (Table 11). The viral shedding from infected quails was observed from 1 to 7 days. The highest viral titer was observed at 1 dpi (6.21±2.59), follow by 3 dpi (6.08±2.58) and 5 dpi (4.11±2.01) (Table 12). The viral titer from respiratory tract at 1 and 3 dpi were statistic significant higher than viral titer at 5 dpi, P-value <0.05. (Table 21). But, no statistic significant of viral titer between inoculated group and contacted group was observed (P-value <0.05) (Table 23).

Table 11 Viral shedding determination of Thai IAV-H1N3 (CU11836C) virus descripted by number positive quails

		Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Inoculated gr.	OP	6/8* (75%)	6/8 (75%)	5/8 (62.5%)	0/8	0/8	0/8
	CS	1/8 (12.5%)	2/8 (25%)	1/8 (12.5%)	1/8 (12.5%)	0/8	0/8
Contacted gr.	OP	1/4 (25%)	1/4 (25%)	1/4 (25%)	0/4	0/4	0/4
	CS	0/4	0/4	0/4	0/4	0/4	0/4
Control gr.	OP	0/3	0/3	0/3	0/3	0/3	0/3
	CS	0/3	0/3	0/3	0/3	0/3	0/3

* Number of positive quail/number of quails in each group

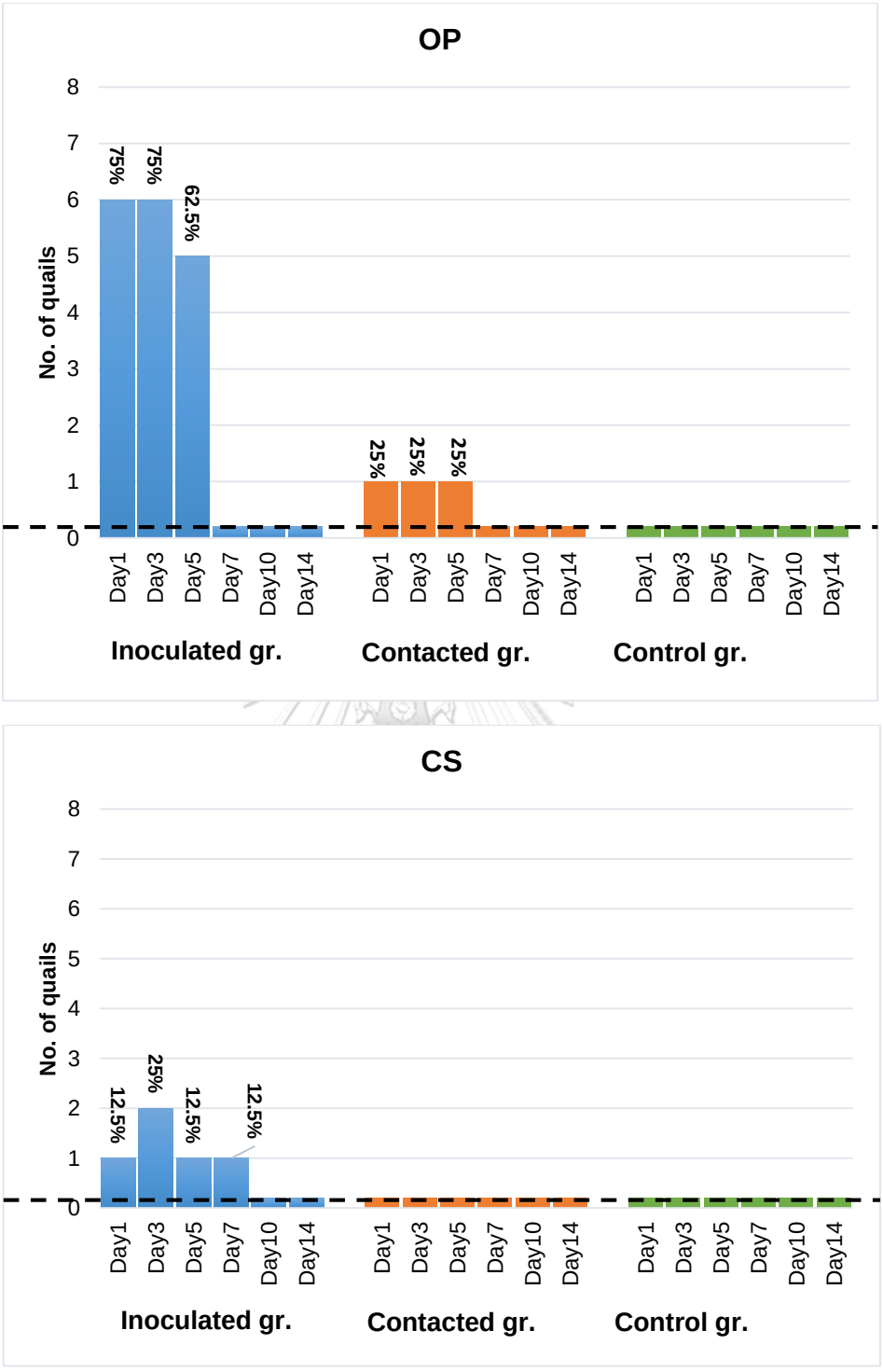

Figure 32 Viral shedding of Thai IAV-H1N3 (CU11836C) descripted by number of H1N3 virus positive quails

Table 12 Viral shedding determination of Thai H1N3 (CU11836C) descripted by virus titers (copy number)

Inoculated gr.		Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Quail#1	OP	4.77	5.74	3.77	-	-	-
	CS	-	-	-	-	-	-
Quail#2	OP	5.50	5.48	3.48	-	-	-
	CS	-	-	-	-	-	-
Quail#3	OP	4.88	6.62	4.66	-	-	-
	CS	-	4.42	4.65	4.45	-	-
Quail#4	OP	-	-	-	-	-	-
	CS	5.59	6.44	-	-	-	-
Quail#5	OP	5.96	5.62	4.60	-	-	-
	CS	-	-	-	-	-	-
Quail#6	OP	6.89	6.63	-	-	-	-
	CS	-	-	-	-	-	-
Quail#7	OP	6.60	4.76	3.95	-	-	-
	CS	-	-	-	-	-	-
Quail#8	OP	-	-	-	-	-	-
	CS	-	-	-	-	-	-
Mean ±SD	OP	6.21±2.59	6.08±2.58	4.11±2.01	-	-	-
	CS	4.68±1.84	5.54±2.40	3.74±1.62	3.54±1.47	-	-
Contacted gr.		Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Quail#1	OP	-	-	-	-	-	-
	CS	-	-	-	-	-	-
Quail#2	OP	4.67	6.02	5.76	-	-	-
	CS	-	-	-	-	-	-
Quail#3	OP	-	-	-	-	-	-
	CS	-	-	-	-	-	-
Quail#4	OP	-	-	-	-	-	-
	CS	-	-	-	-	-	-
Mean ±SD	OP	4.06±2.02	5.41±2.60	5.15±2.49	-	-	-
	CS	-	-	-	-	-	-

Number in gray boxes; number of viral copy number [log10 (copies/µl)].

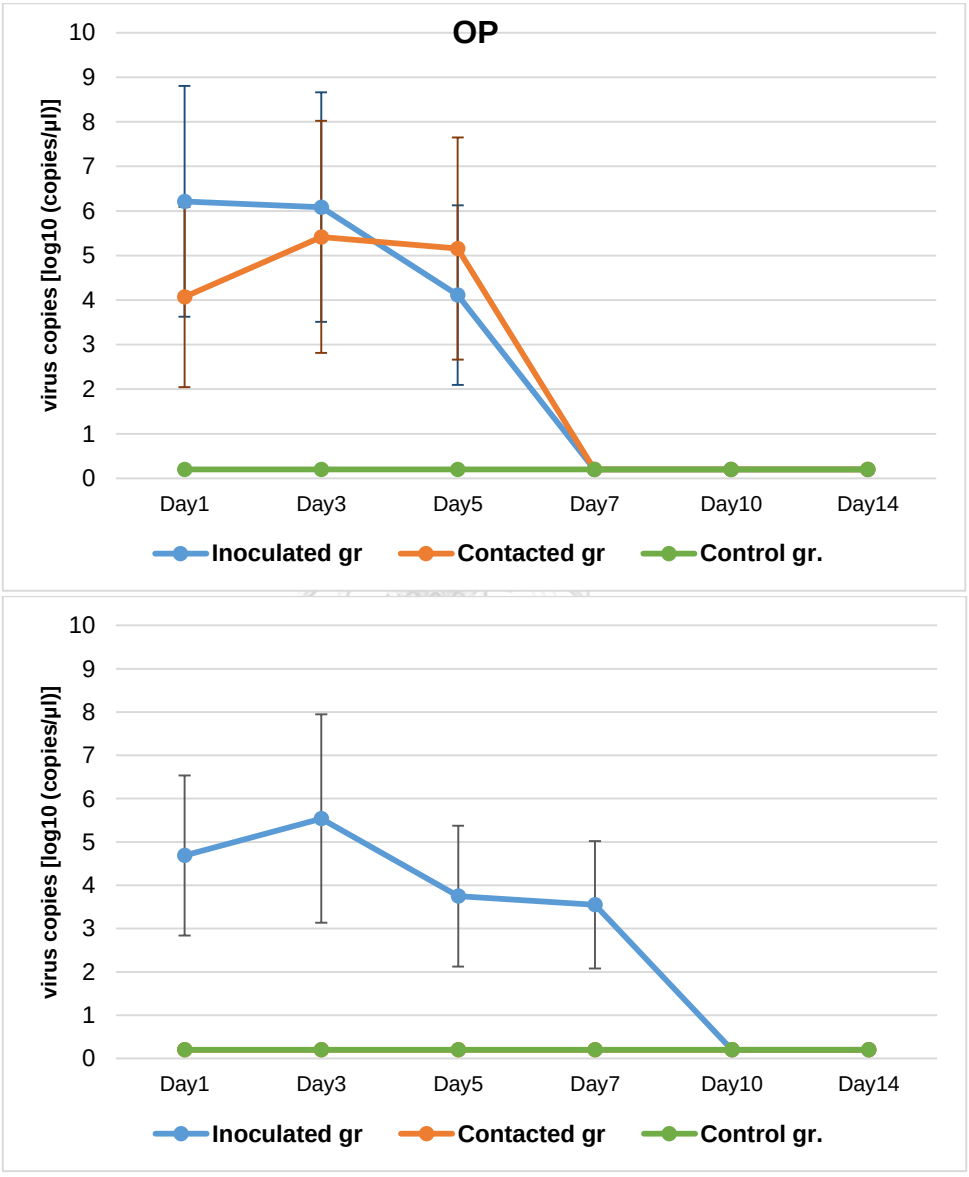

Figure 33 Viral shedding of Thai IAV-H1N3 (CU11836C) in quails descripted by virus titers (copy number). A). Oropharyngeal swab, B) Cloacal swab

For serological response to Thai H1N3 virus (CU-11836C) infection in experimental quails, influenza antibodies were detected in 7 out of 8 quails in inoculated group (group 1) at 7, 10 and 14 dpi. One out of 4 quails in contact group (group 2) showed antibody response at 7 dpi to 14 dpi (Table 13).

Table 13 Serological response determination of Thai IAV-H1N3 (CU11836C) descripted by number of positive influenza antibody quails by blocking ELISA

	Day 7	Day 10	Day 14
Inoculated gr.	7/8 (87.5%)	7/8 (87.5%)	7/8 (87.5%)
Contacted gr.	1/4 (25%)	1/4 (25%)	1/4 (25%)
Control gr.	0/3	0/3	0/3

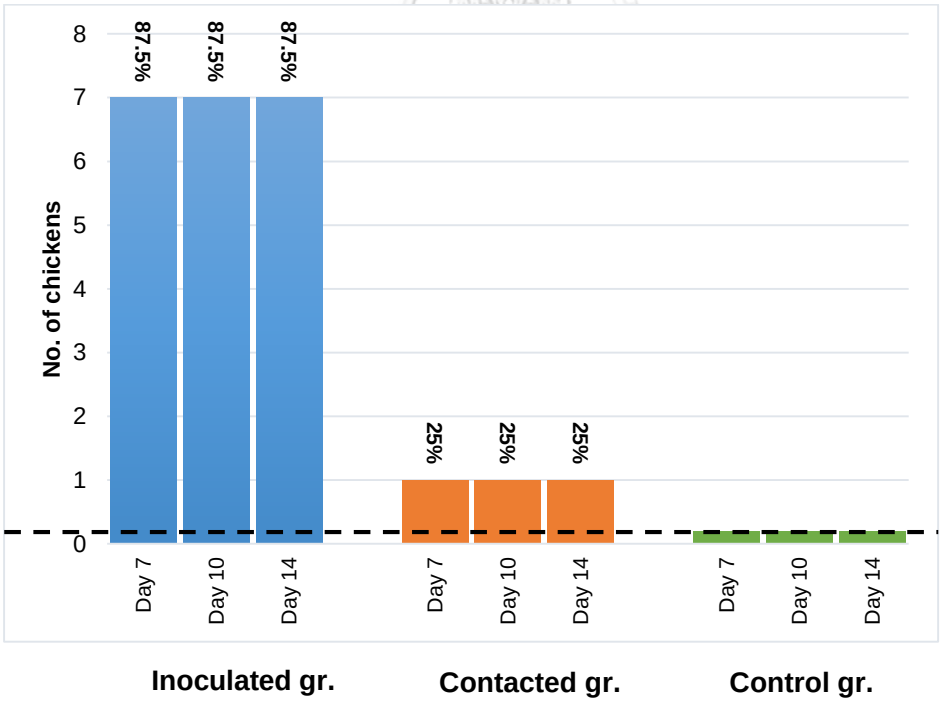

Figure 34 Serological response of Thai IAV-H1N3 (CU11836C) descripted by number of positive Influenza antibody quails by blocking ELISA

For pathological changes to Thai IAV-H1N3 virus (CU-11836C) infection in experimental quails, no significant microscopic lesions were observed in trachea, lung, intestine and kidney of all quails. Notably, 2 infected quails in inoculated group (group 1) showed mild focal lymphoid proliferation in liver (n=1) and spleen (n=1).

3.2 Determination of pathogenicity and virulence of Thai IAV-H11N9 (CU-12660T) virus

3.2.1 Intravenous Pathogenicity Index (IVPI) test

The Intravenous Pathogenicity Index (IVPI) test of Thai IAV-H11N9 (CU-12660T) was conducted following OIE's IVPI protocol. At day 4, the chickens (n=2) showed clinical signs including depress (2/10) and gasped for breathing (1/10). At day 6 and day 7, all chickens (n=10) showed both clinical signs. The chicken gradually recovered at the end of experiment at day 10. In this study, total score is 94 and thus the IVPI index of Thai H11N9 is 0.94 indicating LPAI characteristic of Thai IAV-H11N9 (CU-12660T) (Table 14).

Table 14 Detail of Intravenous Pathogenicity Index (IVPI) test of Thai IAV-H11N9 (CU-12660T)

Clinical signs*	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	Total	Score
Normal	10	10	10	7	0	0	0	0	2	6	45×0	0
Sick	0	0	0	2	8	0	0	2	3	1	16×1	16
Severe Sick	0	0	0	1	2	10	10	8	5	3	39×2	78
Dead	0	0	0	0	0	0	0	0	0	0	0×3	0
											Total	**94**

*Clinical signs (respiratory sign, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs) were scored.

Normal: No clinical signs

Sick: Animal showed 1 clinical sign
(The chickens showed depression or gasp for breathing)

Sever sick: Animal showed ≥2 clinical signs
(The chickens showed depression and gasp for breathing)

The IVPI index of this virus = 94/(10x10) = 0.94

3.2.2 Challenge of Thai IAV-H11N9 (CU-12660T) virus in experimental chickens

The experimental protocol of Thai IAV-H11N9 virus (CU-12660T) in experimental chickens was the same as those of Thai IAV-H1N3 virus. All chickens did not show any clinical signs during the experiment. For virus shedding, all chickens (8/8) of inoculated group (group 1) shed the virus from respiratory tract at 3 dpi but not in the intestinal tract. The virus shedding was last until 5 dpi (3/8). However the chickens of the contacted group (group 2) and control group (group 3) did not shed the virus from both respiratory and intestinal tracts (Table 15). In this study, highest viral titer from infected chickens was observed at 3 dpi (4.32±1.47) (Table 16). The viral titer at 3 dpi were statistic significant higher than viral titer at 5 dpi, P-value <0.05 (Table 22). The viral titer of inoculated group at 1 and 3 dpi were statistic significant higher than viral titer of contacted group (P-value <0.05) (Table 24).

Table 15 Viral shedding determination of Thai IAV-H11N9 (CU-12660T) descripted by number of positive chickens

		Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Inoculated gr.	OP	7/8* (87.5%)	8/8 (100%)	3/8 (37.5%)	0/8	0/8	0/8
	CS	0/8	0/8	0/8	0/8	0/8	0/8
Contacted gr.	OP	0/4	0/4	0/4	0/4	0/4	0/4
	CS	0/4	0/4	0/4	0/4	0/4	0/4
Control gr.	OP	0/3	0/3	0/3	0/3	0/3	0/3
	CS	0/3	0/3	0/3	0/3	0/3	0/3

* Number of positive chicken/number of chickens in each group

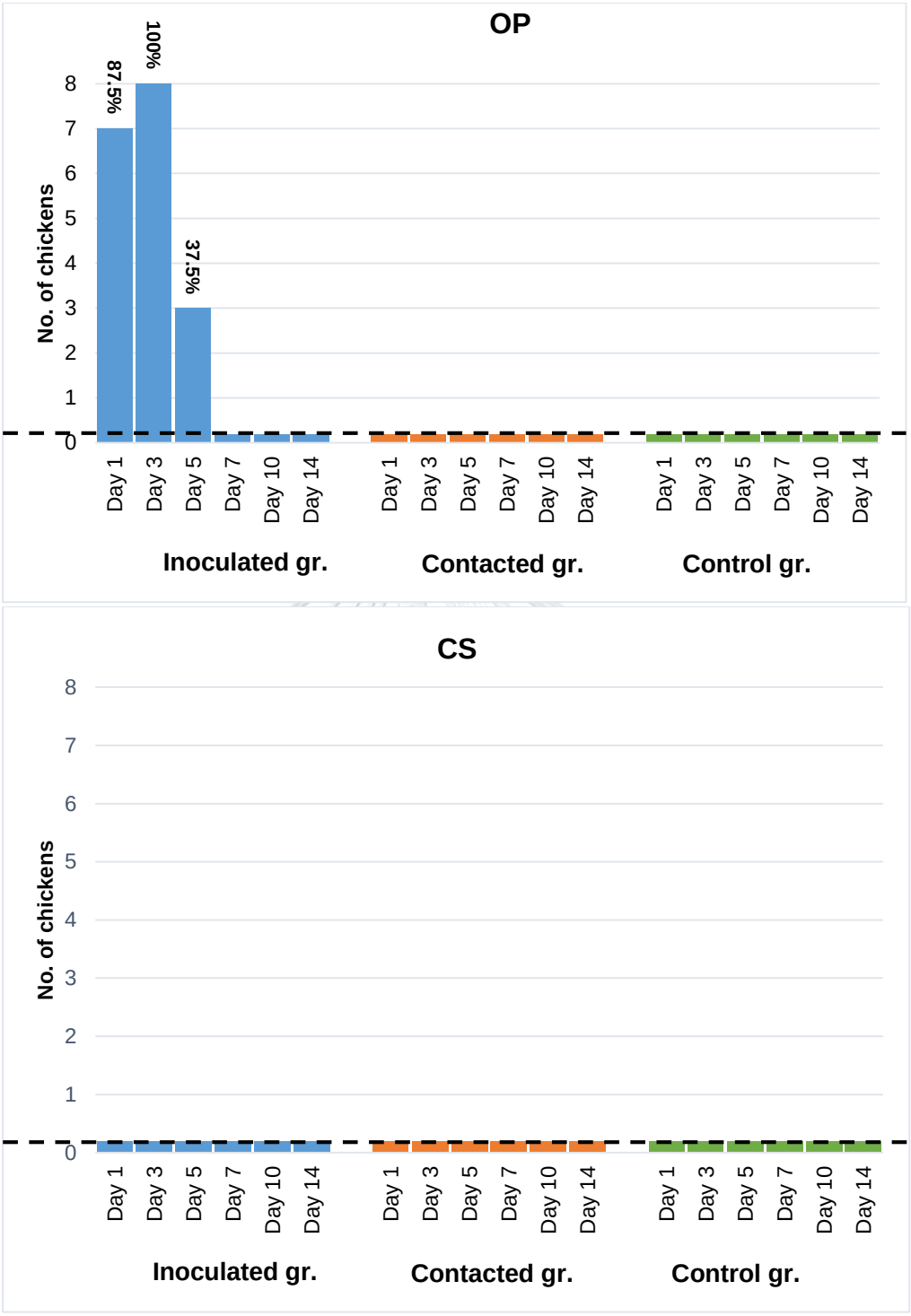

Figure 35 Viral shedding of Thai IAV-H11N9 (CU-12660T) descripted by number of positive chickens, A) Oropharyngeal swab B) Cloacal swab

Table 16 Viral shedding determination of Thai IAV-H11N9 (CU-12660T) in chickens descripted by virus titers (copy number)

Inoculated gr.	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Chicken#1	3.34	3.50	-	-	-	-
Chicken#2	3.27	2.85	-	-	-	-
Chicken#3	3.07	4.21	3.38	-	-	-
Chicken#4	4.35	4.09	3.70	-	-	-
Chicken#5	-	2.30	-	-	-	-
Chicken#6	4.47	5.08	4.87	-	-	-
Chicken#7	2.52	3.46	-	-	-	-
Chicken#8	3.37	4.03	-	-	-	-
Mean ±SD	3.87±1.63	4.32±1.47	4.01±1.97	-	-	-

Number in gray boxes: number of viral copy number [log10 (copies/µl)].

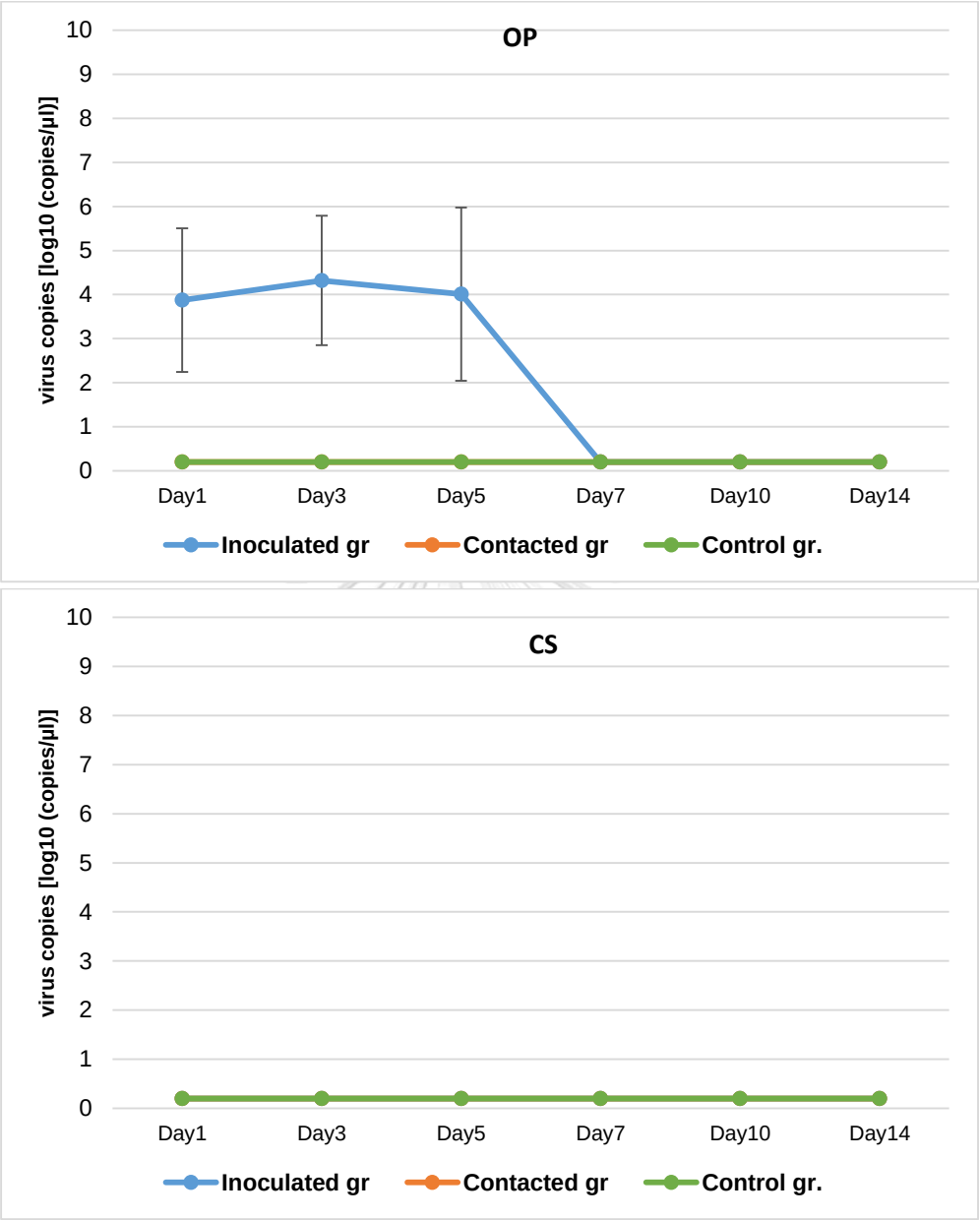

Figure 36 Viral shedding determination of Thai IAV-H11N9 (CU-12660T) in chickens descripted by virus titers (copy number), A) Oropharyngeal swab B) Cloacal swab

For serological response to Thai IAV-H11N9 virus (CU-12660T) infection in experimental chickens, 6 of 8 chickens (75.00%) of inoculated group (group 1) showed positive for influenza antibody. The chickens showed influenza antibody at 7 dpi. (n=4), 10 dpi (n=5), and 14 dpi (n=6). Influenza antibody was not detected in all chickens from contact group (group 2) and control group (group 3) (Table 17).

Table 17 Serological response determination of Thai IAV-H11N9 (CU-12660T) descripted by number of positive Influenza antibody chickens by blocking ELISA

	Day 7	Day 10	Day 14
Inoculated gr.	4/8 (50%)	5/8 (62.5%)	6/8 (75%)
Contacted gr.	0/4	0/4	0/4
Control gr.	0/3	0/3	0/3

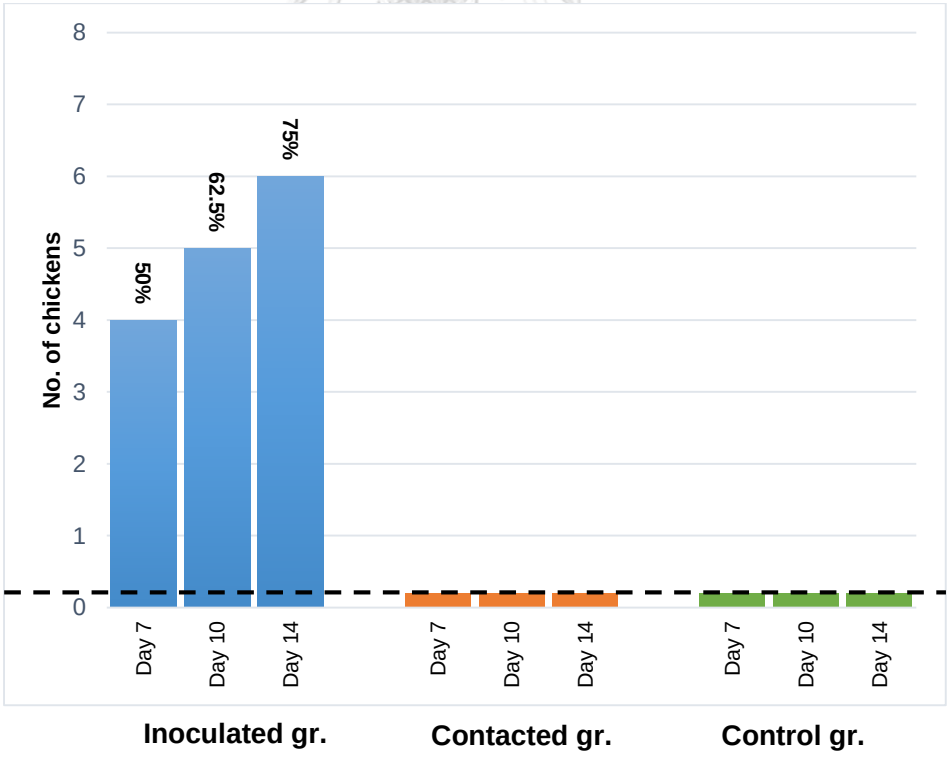

Figure 37 Serological response of Thai IAV-H11N9 (CU-12660T) descripted by number of positive Influenza antibody chickens by blocking ELISA.

For pathological changes to Thai IAV-H11N9 virus infection in experimental chickens, our result showed that histopathological lesions of infected chickens in inoculated group (group 1) were observed in lung and spleen. The histopathological findings were mild to moderate chronic bronchitis with lymphoid proliferation and focal new lymphoid follicle in spleen. No significant lesions observed in trachea, intestine, liver and kidney.

3.2.3 Challenge of Thai IAV-H11N9 (CU-12660T) virus in experimental quails

The experimental protocol of Thai IAV-H11N9 virus (CU-12660T) was the same as challenge in experimental chickens. All quails did not show any clinical signs during the challenge. Our result showed that the quails from inoculated group (group 1) shed the virus from respiratory tract at 1 and 3 dpi (3/8) and at 5, 7, 10 dpi (1/8). The quails of contacted group (group 2) and control group (group 3) did not shed the virus from both respiratory and intestinal tract (Table 18). The viral shedding from infected quails was observed from 1 to 10 days. The highest viral titer was observed at 3 dpi (5.10±2.57) follow by 1 dpi (4.93±2.57) (Table 19). No statistic significant of viral titer between inoculated group and contacted group as well as viral titer between day post inoculation was observed (P-value <0.05) (Table 22-24).

Table 18 Viral shedding determination of Thai IAV-H11N9 (CU-12660T) descripted by number of positive quails.

		Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Inoculated gr.	OP	3/8 (37.5%)	3/8 (37.5%)	1/8 (12.5%)	1/8 (12.5%)	1/8(12.5%)	0/8
	CS	0/8	0/8	0/8	0/8	0/8	0/8
Contacted gr.	OP	0/4	0/4	0/4	0/4	0/4	0/4
	CS	0/4	0/4	0/4	0/4	0/4	0/4
Control gr.	OP	0/3	0/3	0/3	0/3	0/3	0/3
	CS	0/3	0/3	0/3	0/3	0/3	0/3

* Number of positive quails/number of quails in each group

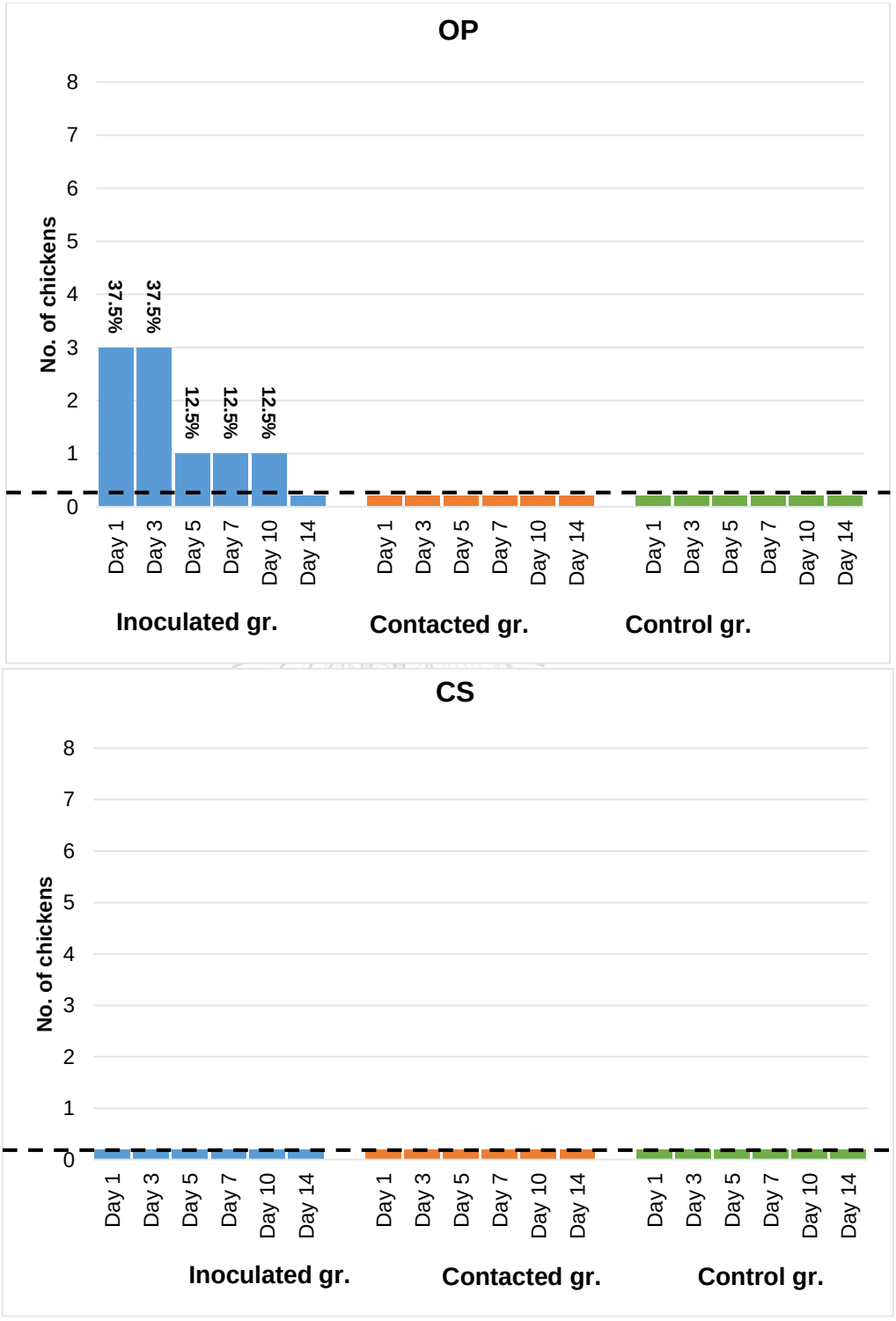

Figure 38 Viral shedding of Thai IAV-H11N9 (CU-12660T) descripted by number of H11N9 virus positive quails, A) Oropharyngeal swab B) Cloacal swab

Table 19 Viral shedding determination of Thai IAV-H11N9 (CU-12660T) in quails descripted by virus titers (copy number)

Inoculated gr.	Day 1	Day 3	Day 5	Day 7	Day 10	Day14
Quail#1	-	-	-	-	-	-
Quail#2	-	-	-	-	-	-
Quail#3	5.40	5.91	-	-	-	-
Quail#4	5.05	5.11	-	-	-	-
Quail#5	5.51	4.86	5.23	4.62	4.66	-
Quail#6	-	-	-	-	-	-
Quail#7	-	-	-	-	-	-
Quail#8	-	-	-	-	-	-
Mean ±SD	4.93±2.57	5.10±2.57	4.32±1.73	3.72±1.53	3.76±1.54	-

Number in gray boxes; number of viral copy number [log10 (copies/µl)].

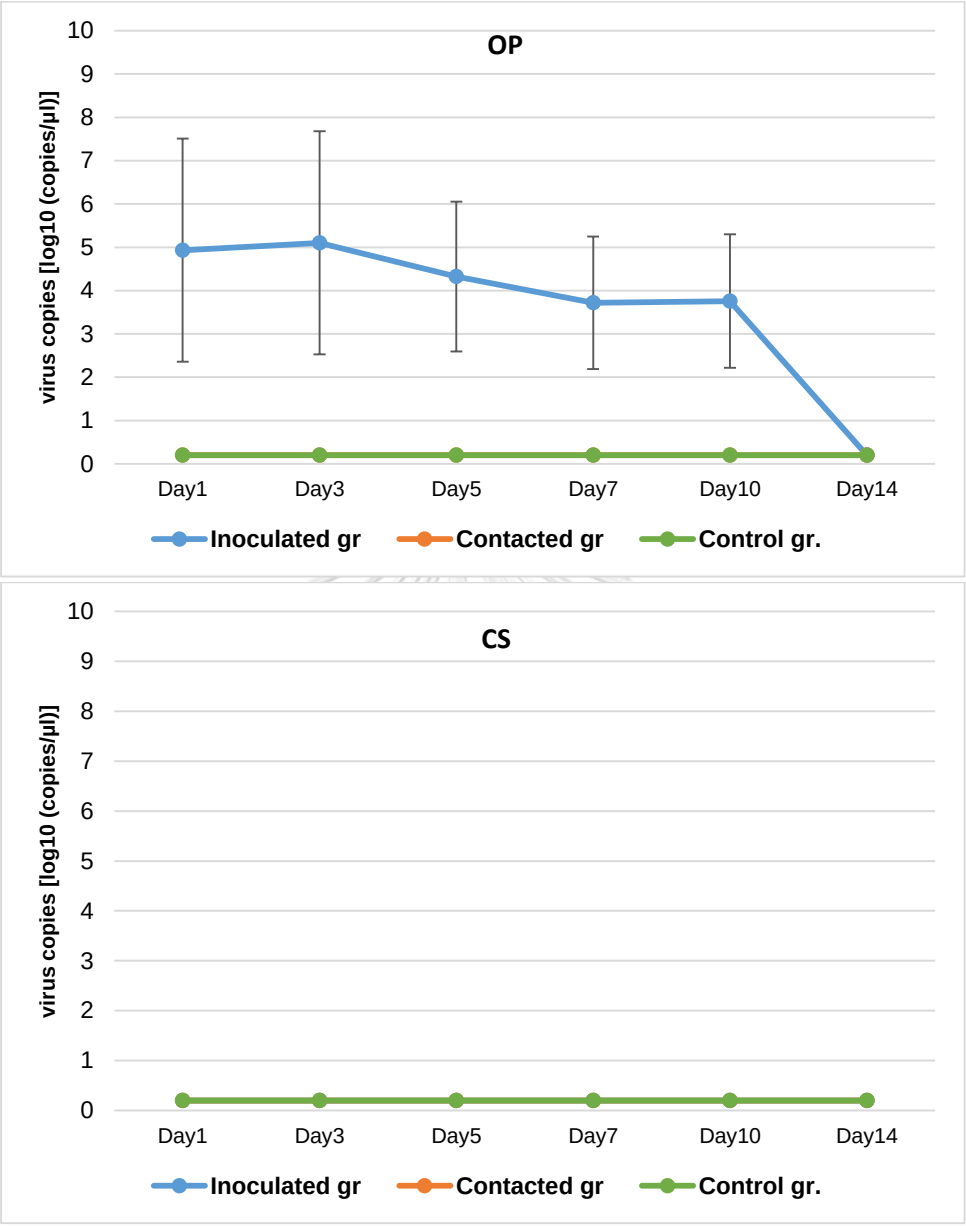

Figure 39 Viral shedding of Thai IAV-H11N9 (CU-12660T) in quails descripted by virus titers (copy number)

For serological response to Thai IAV-H11N9 virus (CU-12660T) infection in experimental quails. Influenza antibodies were detected in quails in inoculated group (group 1) at 7 dpi (2/8), 10 dpi (3/8), 14 dpi (1/8). Quails in contact group (group 2) and control group (group 3) did not show any influenza antibody response (Table 20).

Table 20 Serological response determination of Thai IAV-H11N9 (CU-12660T) descripted by number of positive Influenza antibody quails by blocking ELISA

	Day 7	Day 10	Day 14
Inoculated gr.	2/8 (25%)	3/8 (37.5%)	1/8 (12.5%)
Contacted gr.	0/4	0/4	0/4
Control gr.	0/3	0/3	0/3

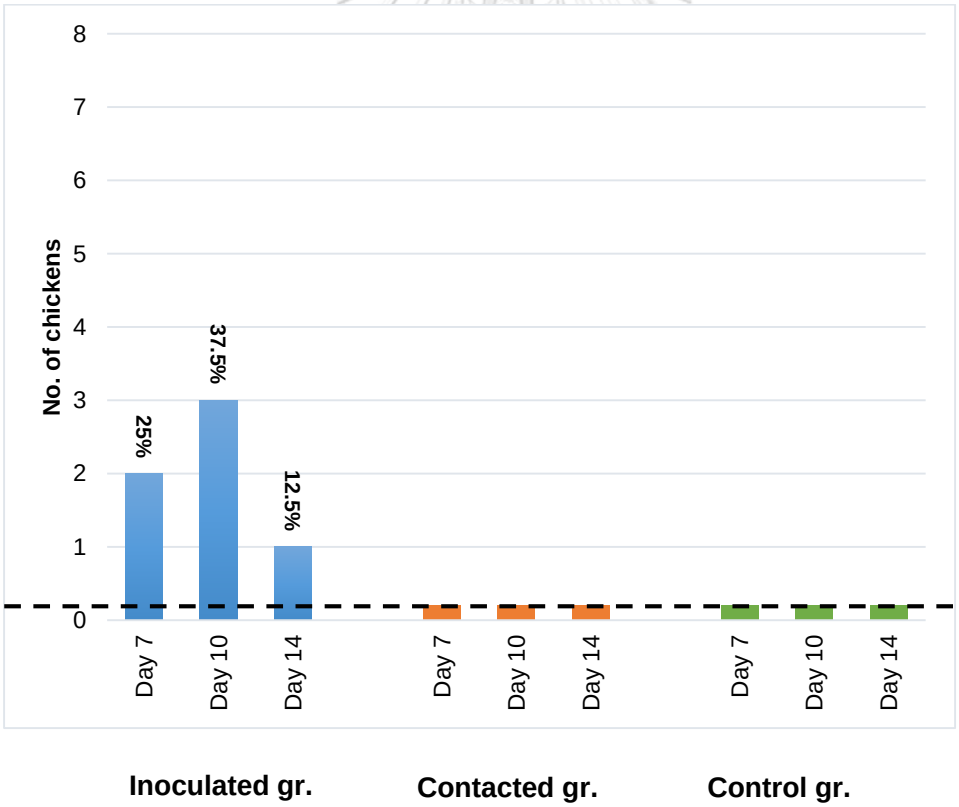

Figure 40 Serological response of Thai IAV-H11N9 (CU-12660T) descripted by number of positive Influenza antibody quails by blocking ELISA

For pathological changes to Thai IAV-H11N9 virus infection in experimental quails, no significant microscopic lesions was observed in all organs. Only mild degree of lymphoid aggregation was found in the liver of infected quail (n=1).

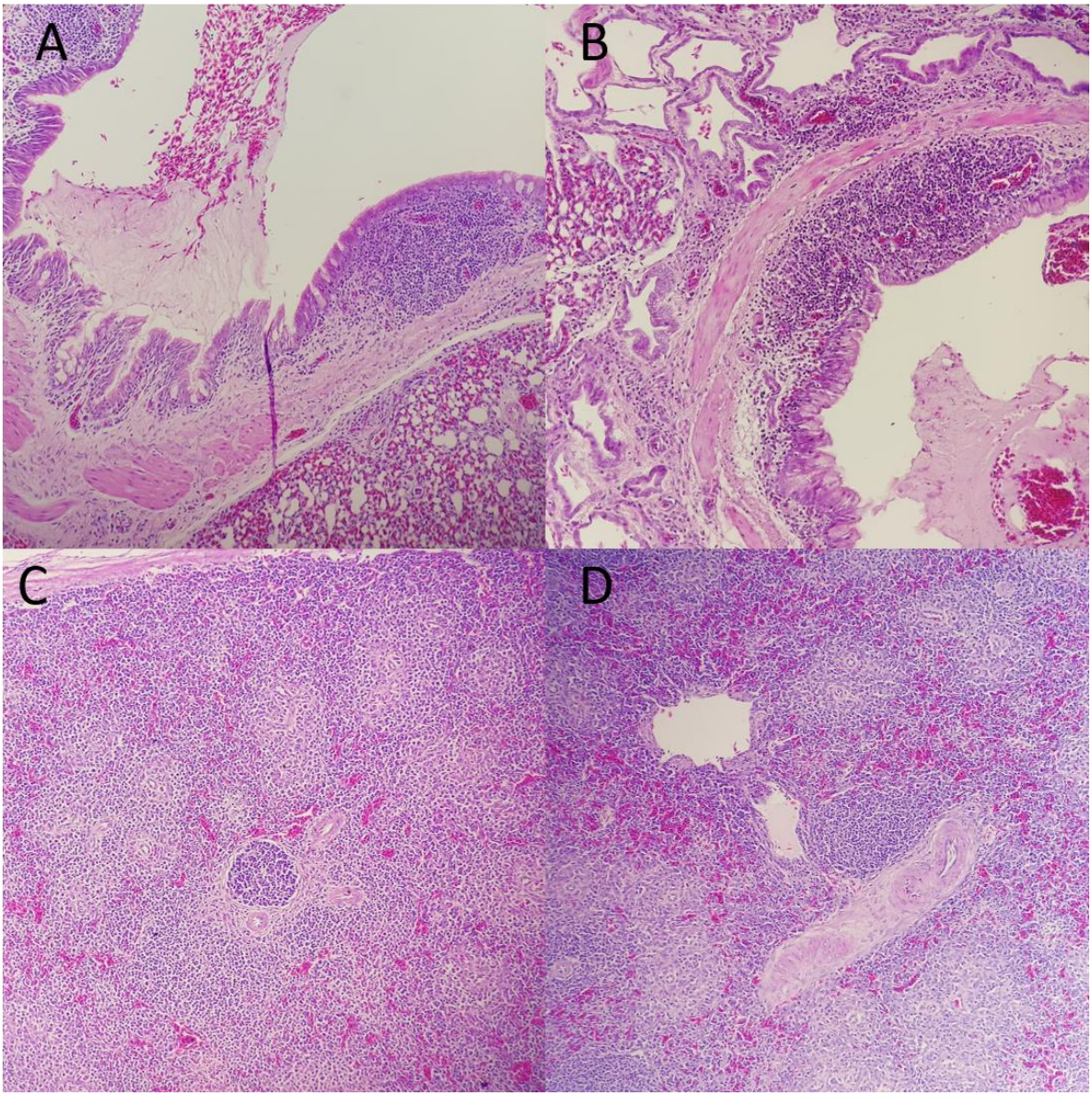

Figure 41 Histopathological findings of chickens inoculated with Thai IAV-H1N3 (CU11836C) and Thai IAV-H11N9 (CU-12660T).
(A and B) Sections from chicken lung exhibited mild to moderate chronic bronchitis with lymphoid proliferation. (C) Focal new lymphoid follicle in chicken spleen. (D) Lymphoid aggregation in chicken spleen.

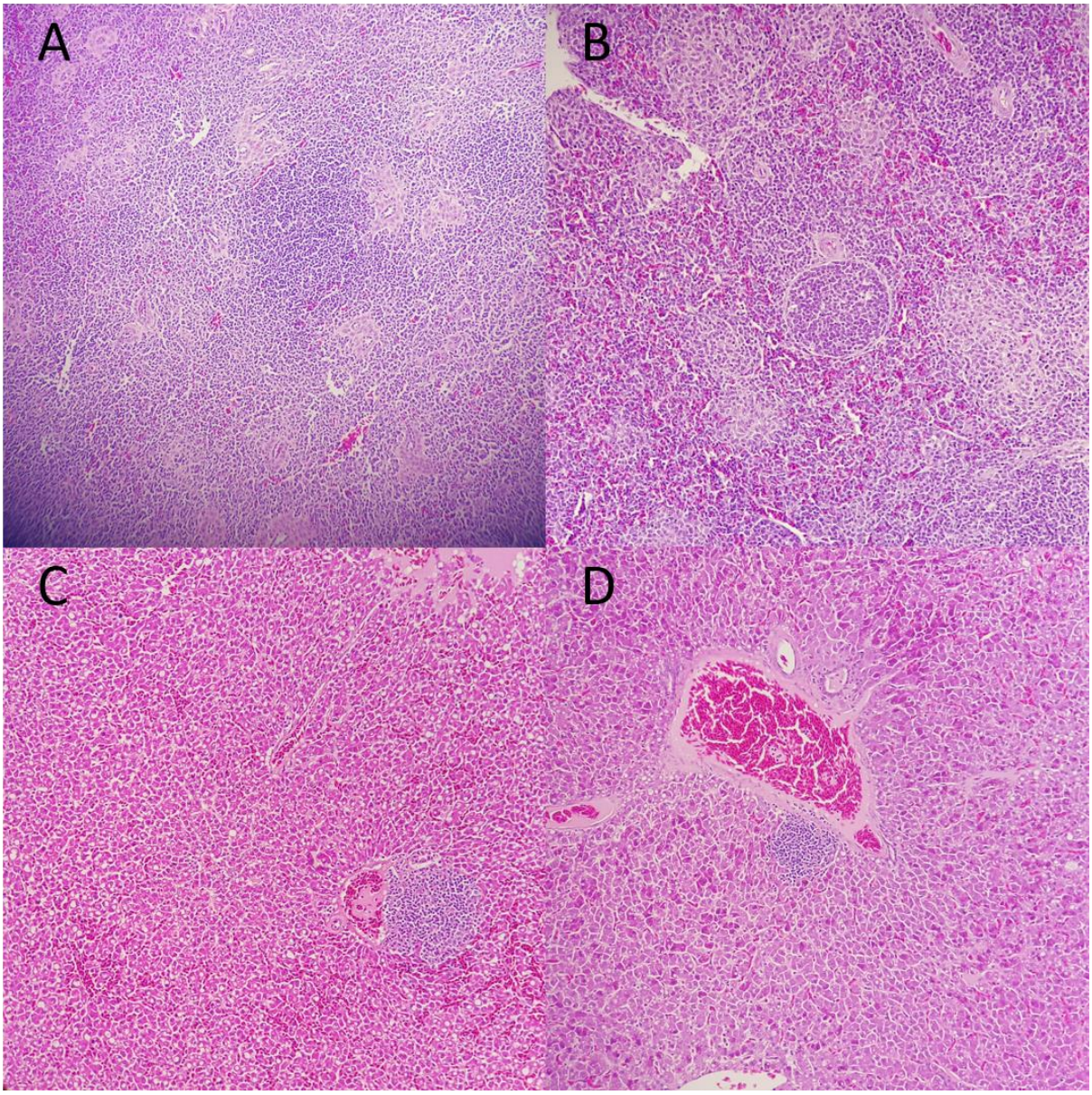

Figure 42 Histopathological findings of quails inoculated with Thai IAV-H1N3 (CU11836C) and Thai IAV-H11N9 (CU-12660T).

(A and B) Sections from quail spleen. (C and D) Sections from quail liver. (A) Lymphoid aggregation in quail spleen. (B) New lymphoid follicle in quail spleen. (C and D) Lymphoid aggregation in quail liver.

Table 21 Statistical analysis (P-value) of viral titer between days post inoculation (dpi) of inoculated group of Thai IAV-H1N3 (CU-11836C) by student t-test

Challenge of Thai H1N3 virus in chickens	OP	CS
1 dpi and 3 dpi	0.382	1
1 dpi and 5 dpi	0.721	1
1 dpi and 7 dpi	0.959	1
1 dpi and 10 dpi	0.645	1
1 dpi and 14 dpi	0.442	1
3 dpi and 5 dpi	0.645	1
3 dpi and 7 dpi	0.130	1
3 dpi and 10 dpi	0.500	1
3 dpi and 14 dpi	0.038	1
5 dpi and 7 dpi	0.382	1
5 dpi and 10 dpi	0.161	1
5 dpi and 14 dpi	0.105	1
7 dpi and 10 dpi	0.442	1
7 dpi and 14 dpi	0.234	1
10 dpi and 14 dpi	0.721	1
Challenge of Thai H1N3 virus in quails	OP	CS
1 dpi and 3 dpi	1	0.721
1 dpi and 5 dpi	0.050	0.959
1 dpi and 7 dpi	0.010	0.959
1 dpi and 10 dpi	0.010	0.721
1 dpi and 14 dpi	0.010	0.721
3 dpi and 5 dpi	0.050	0.721
3 dpi and 7 dpi	0.010	0.721
3 dpi and 10 dpi	0.010	0.442
3 dpi and 14 dpi	0.010	0.442
5 dpi and 7 dpi	0.038	0.959
5 dpi and 10 dpi	0.038	0.721
5 dpi and 14 dpi	0.038	0.721
7 dpi and 10 dpi	1	0.721
7 dpi and 14 dpi	1	0.721
10 dpi and 14 dpi	1	1

Numbers in gray boxes are P-value ≤0.05

Table 22 Statistical analysis (P-value) of viral titer between days post inoculation (dpi) of inoculated group of Thai IAV-H11N9 (CU-12260T) by student t-test

Challenge of Thai IAV-H11N9 virus in chickens	OP	CS
1 dpi and 3 dpi	0.382*	1
1 dpi and 5 dpi	0.382	1
1 dpi and 7 dpi	0.002	1
1 dpi and 10 dpi	0.002	1
1 dpi and 14 dpi	0.002	1
3 dpi and 5 dpi	0.050	1
3 dpi and 7 dpi	0	1
3 dpi and 10 dpi	0	1
3 dpi and 14 dpi	0	1
5 dpi and 7 dpi	0.234	1
5 dpi and 10 dpi	0.234	1
5 dpi and 14 dpi	0.234	1
7 dpi and 10 dpi	1	1
7 dpi and 14 dpi	1	1
10 dpi and 14 dpi	1	1
Challenge of Thai IAV-H11N9 virus in quails	OP	CS
1 dpi and 3 dpi	0.959	1
1 dpi and 5 dpi	0.328	1
1 dpi and 7 dpi	0.328	1
1 dpi and 10 dpi	0.328	1
1 dpi and 14 dpi	0.234	1
3 dpi and 5 dpi	0.442	1
3 dpi and 7 dpi	0.328	1
3 dpi and 10 dpi	0.328	1
3 dpi and 14 dpi	0.234	1
5 dpi and 7 dpi	0.959	1
5 dpi and 10 dpi	0.959	1
5 dpi and 14 dpi	0.721	1
7 dpi and 10 dpi	0.959	1
7 dpi and 14 dpi	0.721	1
10 dpi and 14 dpi	0.721	1

Numbers in gray boxes are P-value ≤0.05

Table 23 Statistical analysis (P-value) of viral titer between inoculated group and contacted group of Thai IAV-H1N3 (CU-11836C) by student t-test

Challenge of Thai IAV-H1N3 virus in chickens	OP	CS
1 dpi	0.570	1
3 dpi	0.109	1
5 dpi	0.214	1
7 dpi	0.368	1
10 dpi	0.808	1
14 dpi	1	1
Challenge of Thai IAV-H1N3 virus in quails	OP	CS
1 dpi	0.073	0.808
3 dpi	0.283	0.570
5 dpi	0.570	0.808
7 dpi	1	0.808
10 dpi	1	1
14 dpi	1	1

Numbers in gray boxes are P-value ≤0.05

Table 24 Statistical analysis (P-value) of viral titer between inoculated group and contacted group of Thai IAV-H11N9 (CU-12260C) by student t-test

Challenge of Thai IAV-H11N9 virus in chickens	OP	CS
1 dpi	0.016	1
3 dpi	0.004	1
5 dpi	0.368	1
7 dpi	1	1
10 dpi	1	1
14 dpi	1	1
Challenge of Thai IAV-H11N9 virus in quails	OP	CS
1 dpi	0.368	1
3 dpi	0.368	1
5 dpi	0.808	1
7 dpi	0.808	1
10 dpi	0.808	1
14 dpi	1	1

Numbers in gray boxes are P-value ≤0.05

CHAPTER 4

DISCUSSION

1. Phase 1: Influenza A surveillance in free-grazing ducks

Free-grazing duck is one of the reservoir hosts for influenza virus. Free-grazing ducks can be infected with influenza virus without clinical signs. Thus, free-grazing ducks can receive and/or spread the virus to and/or from wild birds, domestic birds, domestic animals and humans (Huang et al., 2010; Kim et al., 2010). In Thailand, during HPAI-H5N1 outbreaks, it has been reported that free-grazing ducks were considered as important risk factor of the HPAI-H5N1 outbreaks (Gilbert et al., 2006; Tiensin et al., 2005). The HPAI-H5N1 infected ducks showed no clinical signs with low mortality and morbidity. Due to the free-grazing ducks can carry HPAI-H5N1 viruses without clinical appearance as well as their nature of frequent movement between rice fields, these factors contributed to increasing risk of HPAI-H5N1 virus to widely spread in the country (Hulse-Post et al., 2005; Songserm et al., 2006). Since 2008, The HPAI-H5N1 outbreak has not been reported in Thailand, however, routine surveillance of influenza A virus in free-grazing ducks has been continuously conducted to determine and monitor the status of HPAI and other LPAI viruses in the country.

In this thesis, a surveillance of influenza A viruses in free-grazing ducks was performed during 2010-2015 in 24 districts of 10 provinces in central and lower northern parts of Thailand. Our result found 1.77% of influenza A virus antigen by rRT-PCR and 70.24% of influenza A antibody by blocking ELISA and 6.40% of specific H5 antibody. Influenza A virus can be detected from the young-duck flocks (3 weeks-old - 5 months-old) with 28.57% positivity, which higher than the laying-duck flocks (>5 months-old) (11.90%). While influenza antibody can be detected from the laying-duck flocks (92.86%) more than the young-duck flocks (59.52%). In the thesis, 26 influenza A viruses were selected for genetic characterization by whole genome

sequencing. Fifteen subtypes of influenza A viruses including H1N3, H1N9, H3N8, H4N6, H4N9, H5N3, H5N8, H6N1, H7N4, H9N7, H10N6, H10N7, H11N6, H11N7 and H11N9 could be identified. For serological result, influenza antibody can be detected in every years of the surveillance with the highest positivity in Year 1 (May 2010 – Jan 2011) (97.48% for influenza A virus antibody and 7.85% for specific H5 antibody).

The result from serological surveillance for influenza antibody in free-grazing ducks in Thailand suggested that free-grazing ducks in this study (2010 to 2015) had influenza A antibody and specific H5 antibody lower than previous study in Thailand in 2010. A previous study was conducted in Suphanburi province in July and August of 2010 and found 85% positivity for influenza A antibody and 12% positivity for specific H5 antibody (Beaudoin et al., 2014). In this thesis, the laying duck flocks were positive for influenza antibody (ELISA; 92.86% and HI-H5; 33.33%) more than the young duck flocks (ELISA; 59.52%% and HI-H5; 7.14%). Similar to previous study, older ducks (mean age; 9.1 months) found influenza seropositive more than younger ducks (mean age; 5.1 months). High level of seropositivity in laying duck flocks suggested that the older ducks have opportunity and duration of exposure to the viruses from grazing in the rice fields more than the younger ducks. In contrast, the result from surveillance for influenza antigen in free-grazing ducks in Thailand suggested that the young duck flocks shed influenza virus (28.57%) more than the laying duck flocks (11.90%). In previous study, surveillance of influenza A virus in free-grazing ducks in Thailand in 2011 reported the highest of virus detection from the ducks at 3.2 to 3.7 months-old (Boonyapisitsopa et al., 2016). Another study in Thailand during the H5N1 outbreaks, the HPAI-H5N1 viruses were found in 1.4 to 3.1 months-old ducks (Songserm et al., 2006). In this thesis, only 1.77% of samples showed influenza virus positive which is lower than the report from previous studies. For examples, 14% of ducks from live-poultry markets and duck farms in southern China from Jan 2006 to Dec 2007 were positive for influenza viruses (Huang et al.,

2012). The virus isolation rate in domestic ducks was 3.5% in China, 2011-2012 (Deng et al., 2013). Influenza surveillance in Vietnam in 2010-2012 found 5% influenza viruses from domestic ducks, Muscovy ducks, and chickens (Okamatsu et al., 2013).

In this thesis, 15 subtypes of influenza A viruses were identified in free-grazing ducks. Influenza A virus subtypes H3N8 and H4N6 were predominant subtypes and constantly circulated in free-grazing ducks in Thailand. Both H3N8 and H4N6 subtypes were found almost every years for example H3N8 viruses were found in 2010 (n=2), 2013 (n=4) and 2014 (n=3) as well as H4N6 viruses were found in 2010 (n=4), 2012 (n=2) and 2013 (n=6). Our result agreed with previous report that the H3N8 and H4N6 viruses had been detected in free-grazing ducks in 2010-2011 in Thailand (Boonyapisitsopa et al., 2016). In SEA countries, the H3N8 viruses was reported in ducks during H5N1 outbreak in Laos (Boltz et al., 2010). Furthermore, H3N8 and H4N6 viruses isolated from ducks and mallards in Vietnam were published in the GenBank database. The reports from other countries also indicated that H3 virus is the major virus subtype in domestic ducks, live poultry markets, and wild birds in Korea (Choi et al., 2012). Both H3N8 and H4N6 subtypes were predominant subtypes in mallards in northwestern Minnesota from 2007-2016 (Hollander et al., 2018). The influenza viruses subtype H4N6, H4N9, H7N6 and H10N3 had also been reported in Muscovy ducks housing in live bird markets (Jairak et al., 2016; Wisedchanwet et al., 2011). This observation was in agreement with our findings that H4N6 and H4N9 had been found in free-grazing ducks. In Thailand, other LPAI viruses e.g. H12N1, were isolated from watercock and lesser-whistling ducks (Wongphatcharachai et al., 2012). There are many reports of influenza A viruses circulating in ducks in Asia such as H5N6 in Muscovy ducks in Vietnam, H7N4 in ducks in Cambodia, H5N3 in waterfowl and domestic ducks in China (Tsunekuni et al., 2019; Vijaykrishna et al., 2019). It should be noted that some subtypes of influenza virus in this thesis such as H1N3, H1N9,

H5N3, H5N8, H7N4, H10N6, H10N7, H11N6, H11N7 and H11N9 viruses have never been reported in Thailand.

From our observations in this surveillance, the free-grazing ducks shared the habitat (rice fields) with wild birds and other domestic animals when the ducks housing in the backyard pens. Many wild bird species were found in the rice fields, for example little egret, Asian open-bill stork, swallows, drongo and pigeon. Sharing habitat between wild and domestic ducks increased the risk of influenza virus transmission between both populations (Cappelle et al., 2014). For example, there is an evidence in China that identical LPAI viruses could be isolated from both domestic ducks and wild birds (Duan et al., 2011).

In summary, in phase 1 of this thesis, influenza A virus antigen (1.77%) and influenza A antibody (70.24%) and specific H5 antibody (6.40%) were detected in free-grazing ducks in Thailand during 2010-2015. Free-grazing ducks of younger age had higher risk for influenza shedding. Fifteen subtypes of influenza A viruses were identified from 27 influenza viruses characterized by whole genome sequencing. The predominant subtypes of influenza A viruses in free-grazing ducks were H3N8 and H4N6 viruses. While, some subtypes of influenza A viruses have never been reported in Thailand. Genetic characterization and diversity analysis of 27 representative influenza A viruses from free-grazing ducks in phase 1 were carried out in phase 2.

2. Phase 2: Genetic characterization and diversity analysis of influenza A viruses from free-grazing ducks

In phase 2, 27 representative influenza A viruses were selected from 71 positive samples for genetic characterization by whole genome sequencing. Of 27 representative viruses, 15 subtypes of influenza A viruses were identified in free-grazing ducks. Whole genome sequences of 26 representative viruses were elucidated, while one H3N8 virus only HA and NA gene sequences were available. To analyze genetic diversity of the viruses, each gene of the representative viruses were compared with the reference viruses from the GenBank database. Phylogenetic trees of each gene were generated and analyzed to identify the origin of each gene of the viruses. Genetic constellation of the 26 representative influenza viruses in this phase was provided.

The results of phylogenetic analysis of Thai influenza viruses isolated from free-grazing ducks revealed new reassortant viruses including IAV-H11N9, IAV-H4N6 and IAV-H11N7 viruses. It has been known that the origin of most Thai influenza viruses was from Avian Eurasian lineage, but some genes of new reassortant viruses (IAV-H11N9, IAV-H4N6 and IAV-H11N7 viruses) were originated from other lineages. For example, the NP gene of 3 viruses were clustered in avian Eurasian lineage II (Asian group 1) and avian Eurasian lineage III (Asian group 2). Both groups were divided from main avian lineage before separate to North American and Eurasian lineage. The Thai IAV-H11N9 virus (CU-12660T) and Thai IAV-H4N6 virus (CU-13105C) were clustered in Asian group 1 which in the same group with H3N8 virus from Vietnam and H7N3 and H7N7 viruses from China (Cui et al., 2016). The Thai IAV-H11N7 virus (CU-16340C) was clustered in avian Eurasian lineage III (Asian group 2). In this group, Asian group 2, had only 6 viruses, 4 viruses (H5N2 and H11N9) from China (Kim et al., 2010) and 2 viruses (H5N2) from South Korea (Deng et al., 2013). The M gene of Thai IAV-H11N7 virus (CU-16340C) was grouped into North American lineage. The NS gene of Thai IAV-H11N9

virus (CU-12660T) was grouped into Allele B while other Thai influenza viruses were grouped into Allele A.

At least 24 patterns of genetic constellation of 26 representative viruses were identified in this study. The genetic diversity of influenza A viruses observed in this study suggested that influenza A viruses originate from several sources were circulating in free-grazing ducks in Thailand. Thus, reassortant influenza viruses (IAV-H11N9, IAV-H4N6 and IAV-H11N7 viruses) could be observed in this study. There are many evidences of reassortant viruses from several origin of viruses from wild ducks, domestic ducks and virulence viruses. Example, HPAI-H7N9 virus from human case originated genes from waterfowl (Yang et al., 2019), novel recombinant H1N2 influenza virus between domestic ducks and wild waterfowl isolated from wild waterfowl in China in 2012 (Zhu et al., 2014), New reassortant H5N8 HPAI viruses of wild duck origin were isolated from waterfowl in China (Song et al., 2015).

In this thesis, influenza subtype H1N3 and H1N9 viruses are the first to report of avian H1 subtype in Thailand. Notably, H1 subtype influenza virus can infect wide host range (human, swine and avian) and cause of pandemic influenza (pdmH1N1-2009). Origins of pdmH1N1-2009 virus were from human, swine and avian viruses (Smith et al., 2009). Some previous study suggested that the circulation of H1 subtype in reservoir hosts, e.g. wild birds, ducks poses a continuous threat for future influenza pandemics in humans (Kocer et al., 2015). From our analysis, the Thai IAV-H1N3 virus (CU-11836C) was closely related with H1N2 virus isolated from chicken in China (nucleotide identity; 98.1%).

It has been known that H11 subtype influenza virus may has a zoonotic potential. Although, most H11N9 viruses found in *Anseriformes* order and few H11N9 viruses found in *Charadriiformes* orders, and rarely in *Galliformes* order (Li et al., 2008). The free-grazing ducks are probably infected with H11N9 viruses. However, there are evidences of H11 infection in humans e.g. seropositive for H11 antibody of chicken growers, duck hunters and wildlife professionals (Kayali et al., 2011). It has been know that an important genetic determinant of host adaptation and increase virulence of influenza A virus is at the position PB2-627 (Schat et al., 2012). At PB2-627, the PB2-627 mutation (E627K) can improve viral replication and more virulence of H5N1 virus in mice (Hatta et al., 2001; Shinya et al., 2004). Interestingly, the Thai IAV-H11N9 virus (CU-12660T) contain glycine (G) at PB2-627 which is very rare in avian influenza viruses and never been reported in mammalian influenza viruses. There is no report of the function of glycine at PB2-627.

In summary, in phase 2 of this thesis, 27 representative influenza A viruses were analyzed by phylogenetic analysis to identify the origin of each gene of the viruses. At least, 24 patterns of genetic constellation of the viruses could be identified. Three viruses, IAV-H11N9, IAV-H4N6 and IAV-H11N7 viruses, are new reassortant viruses. The origin of NP gene was originated from Asian group 1, same as Vietnamese and Chinese viruses. One IAV-H11N9 virus (CU-12260T), has unique amino acid substitution at virulence determinant (PB2-627) of influenza A virus. In the next phase, the viruses, IAV-11N9 (CU-12260T) and IAV-H1N3 (CU-11836C), were selected and used for animal challenge study to determine pathogenicity of the viruses in experimental chickens and quails.

3. Phase 3: Challenge of influenza A viruses from free-grazing ducks in animal models

In phase 3, Thai IAV-H1N3 virus (CU-11836C) and IAV-H11N9 virus (CU-12260T) were selected for the challenge in animal models (chickens and quails). The IAV-H1N3 and IAV-H11N9 viruses were selected based on their unique subtypes and potential virulence of the viruses (based on the virulent determinants). For H1N3 virus, the H1 subtype influenza A virus from avian species has never been reported in Thailand, thus IAV-H1N3 virus was selected for the challenge in animal model. For H11N9 virus, the Thai IAV-H11N9 virus is a novel reassortant virus and poses unique virulent determinants at PB2-627 (PB2-627G), thus the IAV-H11N9 virus was also selected for the challenge. Both Thai IAV-H1N3 virus (CU-11836C) and IAV-H11N9 virus (CU-12260T) were characterized for their virulence potential by IVPI test in chickens and challenging in animal models, chickens and quails.

The Thai IAV-H1N3 and IAV-H11N9 viruses were assigned as LPAI viruses based on the genetic analysis at the HA cleavage site (no multiple-basic amino acids). The pathogenicitiy of IAV-H1N3 and IAV-H11N9 viruses were also confirmed by standard method for classifying avian influenza virus, intravenous pathogenicity index (IVPI). The IVPI index of Thai IAV-H1N3 was 0.12 and of Thai H11N9 was 0.94, indicating low pathogenicity avian influenza viruses.

From animal challenge study, both IAV-H1N3 and IAv-H11N9 viruses can infect chickens and quails. However, infected chickens with Thai IAV-H1N3 and IAV-H11N9 viruses did not show clinical signs. Our results showed some evidences that chickens and quails can shed the viruses and develop antibody response during the experiment period. In detail, the IAV-H1N3 virus can replicate in respiratory tract of inoculated chickens (50%) but limit to transmit to contacted chickens. However one

chicken of contacted group developed influenza antibody at 14 dpi suggesting IAV-H1N3 infection in contacted group (even though no viral shedding from swabs). In quails, the IAV-H1N3 virus can replicate both in respiratory tract (75%) and intestinal tract (25%) of inoculated quails. The IAV-H1N3 virus can also be detected in the respiratory of quails (25%) of contacted group. The quails also developed antibody response to IAV-H1N3 virus since 7 dpi.

For H11N9 virus, the IAV-H11N9 virus can infected and replicated in respiratory tract of chickens and quails. The inoculated chickens (100%) can shed the H11N9 virus more than inoculated quails (37.5%). However, none of chickens and quails of contacted group can shed the virus or develop antibody response. For pathological changes to Thai IAV-H1N3 and IAV-H11N9 virus infection in chickens, the lesions could be observed in lung and spleen. The histopathological findings were chronic bronchitis with lymphoid proliferation and lymphoid proliferation in spleen. But in quail, the lesion, lymphoid aggregation could be observed in liver and spleen.

In this study, the ThaiIAV-H1N3 virus was shed from respiratory tract of experimental chickens and quails. In contrast, in previous study, H1 avian influenza viruses can replicated in experimental chickens and can be detected from both oropharyngeal and cloacal swabs with high titer (Kang et al., 2014). For H11 subtype virus, Thai IAV-H11N9 virus was shed from respiratory tract and transmitted to contacted birds. While the study in Colombia reported that the H11N2 virus can replicate and transmit effectively in chickens (Jimenez-Bluhm et al., 2016). The H11 avian influenza virus can cross infect between chickens and humans (Kayali et al., 2011). Other studies also showed that LPAI virus replication was restricted mainly to respiratory tracts (Campitelli et al., 2002; Choi et al., 2005).

The viral replication of Thai IAV-H1N3 virus in quails was observed in both respiratory and intestinal tracts. It has been reported that receptors for influenza virus in quails distribute in respiratory and intestinal tracts of avian (SAα2,3-gal) and mammalian (SAα2,6-gal) species. Thus quails can act as a mixing vessel for generating new reassortant viruses. On the other hand, majority of the receptors for influenza virus in chicken in both trachea and intestine is SAα2,3-gal or avian receptor. It has been documented that chicken may express minority of SAα2,6-gal or mammalian receptor in trachea but not in intestine (Wan and Perez, 2006; Yu et al., 2011). In previous study, experimental quails infected with duck H3N2, pH1N1-2009 and swine viruses can shed the viruses and new reassortant viruses from both respiratory and intestinal tracts (Thontiravong et al., 2012a). Similar to this thesis, the challenged quails with Thai IAV-H1N3 can shed the virus from both respiratory and intestinal tracts.

The histopathological findings including lymphocytic tracheitis and bronchitis and pneumonia were commonly found in LPAI infection in poultry. Moreover, lymphocytic depletion in cloacal bursa, thymus, spleen and other area with lymphocytic accumulation were found in LPAI infection in poultry (Pantin-Jackwood and Swayne, 2009). Microscopic lesions in quails were heterophilic-to-lymphocytic tracheitis, bronchitis, peribronchiolar cuffing with heterophilic and lymphocytic infiltration (Thontiravong et al., 2012b). In our experiment, lymphoid aggregation restricted in lung (infected chickens) and spleen (infected chickens and quails) were observed.

In summary, in phase 3 of this thesis, Thai IAV-H1N3 virus (CU-11836C) and IAV-H11N9 virus (CU-12260T) are classified as LPAI viruses by IVPI test. Both IAV-H1N3 and IAV-H11N9 viruses can infect chickens and quails. The IAV-H11N9 virus replicated

only in respiratory tract of chickens. But, the IAV-H1N3 virus can replicate in both respiratory and intestinal tract of quails. The IAV-H1N3 virus was shed from respiratory tract and transmit to contacted birds (both chickens and quails). The histopathological findings were chronic bronchitis in lung and lymphoid aggregation in lung and spleen which commonly found in LPAI infection in poultry.

CHAPTER 5

CONCLUSIONS

Influenza A virus (IAV) is an important zoonotic pathogen. The virus can infect several animal species including humans and can transmit interspecies. Free-grazing ducks are important reservoir hosts for influenza virus. In this thesis, we conducted the multi-years influenza A surveillance in free-grazing ducks, identified IAV subtypes and determined potential virulence of the IAVs isolated from free-grazing ducks in Thailand.

In phase 1, the multi-years influenza A surveillance in free-grazing ducks was conducted in 24 districts of 10 provinces in central and lower northern part of Thailand during 2010-2015. The findings from this phase of the thesis are as following:

1.1 The total number of the samples (n=5,983) including oropharyngeal swabs (n=2,012), cloacal swabs (n=2,012) and serum (n=1,959) were examined for influenza A virus and influenza A antibody.

1.2 Our results showed that 1.77% of influenza A virus antigen by rRT-PCR and 70.24% of influenza A antibody by blocking ELISA and 6.40% of specific H5 antibody could be found in free-grazing ducks.

1.3 Our results showed that 15 subtypes of influenza A viruses including H1N3, H1N9, H3N8, H4N6, H4N9, H5N3, H5N8, H6N1, H7N4, H9N7, H10N6, H10N7, H11N6, H11N7 and H11N9 could be identified. The predominant subtypes of influenza A viruses in free-grazing ducks were H3N8 and H4N6. Some subtypes of influenza A viruses have never been reported in Thailand.

In phase 2, whole genome sequences of 26 representative viruses were elucidated, while one H3N8 virus only HA and NA gene sequences were available. Phylogenetic trees of each gene were generated and analyzed to identify the origin of each gene of the viruses. Genetic constellation of the 26 representative influenza viruses in this phase was elucidated. The findings from this phase of the thesis are as following:

2.1 At least 24 patterns of genetic constellation of 26 representative viruses were identified. This findings suggested that influenza A viruses originated from several sources were circulating in free-grazing ducks in Thailand.

2.2 Influenza A viruses from free-grazing ducks contained genes from Eurasian lineage. But, the reassortant viruses (IAV-H11N9, IAV-H4N6 and IAV-H11N7) contained NP, M and NS genes originated from other lineages.

2.3 A unique of amino acid substitution at PB2-627 (PB2-627G) was observed in a Thai IAV-H11N9 virus.

2.4 Circulation of several subtypes and diversity of influenza A viruses in free-grazing ducks indicated that the ducks could infect with the viruses from several sources and could probably generate novel reassortant influenza A virus.

In phase 3, influenza A virus subtype H1N3 (n=1) and H11N9 (n=1) were selected and used for animal challenge study in chickens and quails. The viruses were designated as A/duck/Thailand/CU-11836C/2011 (H1N3) (CU-11836C) and A/duck/Thailand/CU-12660T/2012 (H11N9) (CU-12660T). The findings from this phase of the thesis are as following:

3.1 Thai IAV-H1N3 virus is the first H1 subtype isolated from avian in Thailand. While Thai IAV-H11N9 virus is a novel reassortant virus and present the unique genetic determinants at PB2-627 (PB2-627G).

3.2 Thai IAV-H1N3 and IAV-H11N9 viruses were confirmed as low pathogenicity avian influenza (LPAI) viruses by IVPI test.

3.3 Thai IAV-H1N3 and IAV-H11N9 viruses can infect in chickens and quails in experimental setting. The viruses mainly replicated in respiratory tract and limit to transmission to contacted birds. Except Thai IAV-H1N3 virus can replicated in both respiratory and intestinal tracts and can transmission to contacted quails.

In conclusion the results in this thesis provided significant findings which will benefit the strategic planning for influenza prevention and control measures in Thailand. The significant findings are

1. There are several subtypes of influenza A viruses circulating in free-grazing ducks. At least 15 subtypes of influenza A viruses including H1N3, H1N9, H3N8, H4N6, H4N9, H5N3, H5N8, H6N1, H7N4, H9N7, H10N6, H10N7, H11N6, H11N7 and H11N9 circulating in free-grazing ducks in Thailand during 2010-2015. In addition, some influenza A subtypes have never been reported in Thailand.

2. At least 24 patterns of genetic constellation were observed indicating genetic diversity of Thai influenza A viruses isolated from free-grazing ducks. New reassortant viruses (IAV-H11N9, IAV-H4N6 and IAV-H11N7) and virus with unique amino acid substitution at PB2-627 (PB2-627G) (H11N9) were observed in free-grazing ducks in Thailand.

3. The IAV-H1N3 and IAV-H11N9 viruses were examined for their virulence and pathogenicity. The IAV-H1N3 and IAV-H11N9 viruses were classified as low pathogenicity avian influenza (LPAI) viruses. In animal experiment, both IAV-H1N3 and IAV-H11N9 viruses can infect chickens and quails. The IAV-H1N3 virus can also transmit among quails in direct contact.

Our findings confirmed that free-grazing ducks are important reservoir hosts for influenza A virus in Thailand. Several subtypes and new reassortant viruses are circulating in the free-grazing ducks and some viruses able to infect other animals especially chickens and quails. Based on the results of this thesis, the future recommendations for influenza prevention and control measures in free-grazing ducks including

1. Restriction or zoning of grazing areas for free-grazing ducks can help decrease opportunity of sharing or transmitting influenza A virus from the free-grazing ducks
2. Routine and continue monitoring of influenza A virus in free-grazing ducks should be conducted to determine the status and dynamic of the viruses in Thailand.

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