SURVEILLANCE OF INFLUENZA A VIRUSES IN A LIVE BIRD MARKET, YANGON, MYANMAR DURING 2017-2018

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การเฝ้าระวังเชื้อไวรัสไข้หวัดนกในตลาดค้าสัตว์ปีกมีชีวิต ในเมืองย่างกุ้ง สาธารณรัฐแห่งสหภาพพม่า ระหว่างปี ค.ศ. 2017-2018

น.ส.ขิ่น ทูเรน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ตลาดค้าสัตว์ปีกมีชีวิตมีความสำคัญต่อการแพร่กระจายของเชื้อไวรัสไข้หวัดนกชนิดรุนแรง (HPAI) ในประเทศพม่าการระบาดของโรคไข้หวัดนกชนิดรุนแรงสายพันธุ์เอชห้าเอ็นหนึ่ง (HPAI-H5N1) มีการรายงานเป็นครั้งแรกในปี ค.ศ. 2006 และมีรายงานการเฝ้าระวังเชื้อไวรัสไข้หวัดนกในตลาดค้าสัตว์ปีกมีชีวิตในประเทศพม่าตั้งแต่ปี ค.ศ. 2014 ภายใต้การดูแลของ Livestock Breeding and Veterinary Department และ FAO อย่างไรก็ตามยังขาดข้อมูลที่เกี่ยวกับลักษณะทางพันธุกรรมและวิวัฒนาการของเชื้อไวรัสไข้หวัดนกในประเทศพม่า ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้เพื่อตรวจหาเชื้อไวรัสไข้หวัดนกในสัตว์ปีกชนิดต่างๆในตลาดค้าสัตว์ปีกมีชีวิต มิงกาลา-ตวง-นยันต์ (Mingalar-Taung-Nyunt LBM) และเพื่อหาลักษณะและความหลากหลายทางพันธุกรรมของเชื้อไวรัสไข้หวัดนกชนิดเอในตลาดค้าสัตว์ปีกมีชีวิต ในช่วงระหว่างเดือนธันวาคมปี ค.ศ. 2017 ถึงเดือนธันวาคมปี ค.ศ. 2018 โดยเก็บตัวอย่างจำนวน 455 ตัวอย่าง จากไก่เนื้อ ไก่ไข่ ไก่หลังบ้าน และเป็ด จากตลาดค้าสัตว์ปีกมีชีวิต ผลการตรวจหาเชื้อไวรัสไข้หวัดนกชนิดเอ ในตัวอย่างแบบรวมจำนวน 91 ตัวอย่างด้วยวิธี realtime RT-PCR พบตัวอย่างที่ให้ผลบวกจำนวน 12 ตัวอย่าง และตรวจยืนยันว่าเป็นเชื้อไวรัส HPAI-H5N1 จำนวน 7 ตัวอย่าง ในการศึกษาครั้งนี้ได้ถอดรหัสพันธุกรรมทั้งหมดของเชื้อไวรัส (Whole-genome sequence) จำนวน 4 ตัวอย่าง ผลการศึกษา phylogenetic analyses แสดงให้เห็นว่าเชื้อไวรัส HPAI-H5N1 ในประเทศพม่าจัดอยู่ในกลุ่มเชื้อไวรัส HPAI-H5N1 กลุ่มย่อย 2.3.2.1c ซึ่งใกล้เคียงกับเชื้อไวรัสในประเทศจีนและเวียดนาม โดยสรุปจากการศึกษาครั้งนี้พบว่าตลาดค้าสัตว์ปีกมีชีวิตสามารถเป็นแหล่งแพร่กระจายเชื้อไวรัสไข้หวัดนกชนิดรุนแรงสายพันธุ์เอชห้าเอ็นหนึ่ง ทั้งในคนและสัตว์ปีกได้

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
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Khin Thurain : SURVEILLANCE OF INFLUENZA A VIRUSES IN A LIVE BIRD MARKET, YANGON, MYANMAR DURING 2017-2018. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Live bird markets play a significant role in highly pathogenic avian influenza (HPAI) virus spread and transmission. In Myanmar, the first HPAI-H5N1 outbreak was reported in 2006. Since 2014, routine influenza A virus surveillance in LBMs have been conducted in Myanmar by the Livestock Breeding and Veterinary Department in cooperation with FAO. However, there is limited information on virus characterization and evolution of influenza viruses in Myanmar. The objectives of this thesis were to detect influenza virus subtypes circulating in different poultry species from Mingalar-Taung-Nyunt live bird market (MTN-LBM) and to determine genetic characteristics and genetic diversity of influenza A viruses in the MTN-LBM. During December 2017 - December 2018, 455 swab samples were collected from broiler, layer, backyard chicken and ducks from the MTN-LBM. Screening of all 91 pooled samples for IAVs by real-time RT-PCR showed that 12 samples were positive for influenza A virus (M gene). In this study, 7 samples were identified as HPAI-H5N1. Among them, 4 IAVs were successfully isolated and characterized. Whole-genome sequence of 4 HPAI-H5N1 was revealed with highly pathogenic avian influenza characteristics. Phylogenetic analyses showed that the Myanmar HPAI-H5N1 viruses were clustered with HPAI-H5N1 clade 2.3.2.1c from China and Vietnam. In conclusion, this study suggests that MTN-LBM can contribute a significant risk of HPAI-H5N1 spread and transmission to poultry and human.

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

AI	Avian Influenza
bp	base pair
cDNA	Complementary deoxyribonucleic acid
CDC	Centers for Disease Control and Prevention
et al.	et alibi, and other
HA	Hemagglutinin
HPAI	Highly Pathogenic Avian Influenza
M	Matrix
NA	Neuraminidase
NP	Nucleoprotein
NS	Nonstructural protein
OIE	World Organization for Animal Health
PA	Polymerase acidic protein
PB1	Polymerase Basic Protein 1
PB2	Polymerase Basic protein 2
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
WHO	World Health Organization
µl	microliter

CHAPTER I

INTRODUCTION

Influenza caused by influenza viruses is an important infectious respiratory disease in both humans and animals. Based on the new classification system of influenza viruses, the virus has composed of four genera namely Alphainfluenzavirus (Influenza A), Betainfluenzavirus (Influenza B), Gammainfluenzavirus (Influenza C) and Deltainfluenzavirus (Influenza D) (ICTV, 2018). Influenza A virus can infect human and several animal species. Influenza B can infect human and cause seasonal human influenza endemics. Influenza C can cause disease in human, pig and it has been recently reported in cattle (Zhang et al., 2018). Influenza D can infect cattle and swine, but transmission to human has not been reported. Among them, influenza A virus is the most critical influenza of a public health concern due to its potential to cause the emergence of new and/or virulence influenza. Novel virulence influenza A virus variants may infect people and sustain human to human transmission leading to a pandemic outbreak.

Avian influenza virus causes respiratory disease in avian and mammalian species. Disease can range from asymptomatic infection to an acute and can lead to fatal. According to OIE terrestrial code, infection of poultry caused by any influenza A virus with an intravenous pathogenicity index (IVPI) greater than 1.2 or at least 75% mortality can define as a notifiable form of avian influenza. Avian influenza virus can be classified into two pathotypes according to its pathogenicity to poultry or birds, (1) Low pathogenic avian influenza (LPAI) causes mild or asymptomatic disease in poultry. LPAI is mostly maintaining in wild aquatic birds without severe clinical sings. LPAI infection in domestic poultry may show body weight loss or reduced egg

production in layer chicken (Spickler et al., 2008). (2) Highly pathogenic avian influenza (HPAI) causes severe clinical signs and can kill up to 90-100% of the flock. HPAI can spread rapidly and cause epidemic and/or pandemic outbreaks.

Highly pathogenic avian influenza subtype H5N1 (HPAI-H5N1) outbreaks have been reported worldwide. The first HPAI-H5N1 was isolated from a goose in Guangdong Province in China (Xu et al., 1999). In 1997, the first known case of H5N1 outbreak occurred in Hong-Kong causing the death and culling of million birds as well as human infection. In 2004, outbreaks of H5N1 spread across Asian countries and reported in many poultry and human deaths. Currently, HPAI-H5N1 has become enzootic in Asia and continues to cause many outbreaks in poultry and sporadic human infection. As of May 2019, WHO reported that 861 human HPAI-H5N1 infections with 455 deaths from 17 countries.

According to many previous studies, live bird markets (LBMs) play a significant role in HPAI spread and transmission in Southeast Asian countries. Thus, the HPAI transmission was threatening to human and animal health in those regions (Guan et al., 2002). Many countries such as China, Hong Kong, Indonesia, Vietnam and Thailand reported both HPAI and LPAI circulating in live bird markets. LBMs provide a suitable environment for influenza A viruses transmission and distribution. For example, a continuous supply of animals, animals from different sources and housing multiple avian species in close contact are favourable environments for influenza circulation, transmission and reassortment. Movement of human and poultry among farms and markets across different geographical areas may also contribute to the spread of avian influenza viruses. More recently, the emergence of novel viruses such as H10N8, H5N8 and H7N9 were reported with the concern of human infection through live bird markets (Pinsent et al., 2017). Due to its high densities of poultry, unhygienic

conditions, multiple subtypes of influenza A viruses circulating in the LBMs, thus the novel pathogenic re-assortment could be occurred.

Since 2014, routine surveillance of avian influenza viruses in LBMs in Myanmar have been conducted in Yangon, Mandalay and Northern Myanmar by the Livestock Breeding and Veterinary Department in cooperation with FAO. However, there are still limited scientific information on virus characterization, genetic characteristics and genetic relatedness of influenza viruses in Myanmar. In this thesis, we have characterized avian influenza viruses recovered from LBM in Yangon, Myanmar by next-generation sequencing. Phylogenetic analysis and genetic analysis of influenza viruses were performed. To fulfill the gap of information, our study objectives are as follow:

Objectives of this study

(1) To detect avian influenza virus subtypes circulating in different poultry in a live bird market in Yangon.

(2) To determine genetic characteristics and genetic diversity of avian influenza viruses recovered from the live bird market.

CHAPTER II

LITREATURES REVIEW

Avian influenza is a member of the genus influenza A virus or newly assigned Alphainfluenzavirus of the family Orthomyxoviridae. Influenza A virus can be classified into several subtypes according to its surface glycoproteins (HA and NA). There are 18 different HA subtypes (H1-H18), and 11 different NA subtypes (N1-N11) identified. Avian influenza virus can also be classified into two pathotypes, namely highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) based on its pathogenicity to chicken. According to the World Organization for Animal Health (OIE) terrestrial code, any avian influenza virus with intravenous pathogenicity index (IVPI) greater than 1.2 or at least 75% mortality in 4-8 week-old chicken can be defined as a highly pathogenic avian influenza (HPAI) (OIE, 2018). To date, influenza A subtypes, H5N1, H5N6, H7N4 and H7N7 cause highly pathogenic diseases.

The WHO, OIE and FAO have developed a nomenclature system to define clades of HPAI-H5 viruses (WHO, 2011). HPAI viruses become a global concern on both human and animal health as they can cause severe zoonotic disease with pandemic potential. On the other hand, LPAI viruses have been neglected due to their mild or asymptomatic infection in animals. Moreover, LPAI can increase the risk to both animal and human health through genetic mutation and reassortment.

Virology of influenza virus

Influenza virus is an enveloped, segmented RNA virus. The virus consists of 8 segments of negative-sense single-stranded RNA including hemagglutinin (HA), neuraminidase (NA), matrix (M), nucleoprotein (NP), non-structural (NS), polymerase (PA, PB1 and PB2). These genes encode at least 10 proteins, such as PB2, PB1, PA,

HA, NP, NA, M1, M2, NS1 and NS2. The hemagglutinin protein (HA) form spikes structure outside the viral envelope and the neuraminidase protein (NA) form mushrooms structure (Abdelwhab et al., 2010). The viral proteins and their functions in influenza virus are shown in Table 1.

Table 1. Gene segments and their protein functions in influenza virus

Segment	Nucleotides segment length	Encoded protein	Protein function	Reference
1	2341	PB2	Polymerase subunit mRNA cap recognition	(Bouvier and Palese, 2008)
2	2341	PB1	Polymerase subunit RNA elongation, endonuclease activity	(Steinhauer and Skehel, 2002)
		PB1-F2	Pro-apoptosis activity and Proinflammatory effect	(Chen et al., 2001)
		PB1-N40	Detrimental to virus replication	
3	2233	PA	Polymerase subunit Protease activity	(Bouvier and Palese, 2008)
4	1778	HA	Surface glycoprotein Major antigen, receptor binding & fusion activities	(Yoon et al., 2014)
5	1565	NP	RNA binding protein Nuclear import regulation	(Bouvier and Palese, 2008)
6	1413	NA	Surface glycoprotein Sialidase activity, virus release	(Steinhauer and Skehel, 2002)
7	1027	M1	Matrix protein vRNP interaction, RNA nuclear export regulation, viral budding	(Webster et al., 1992)
		M2	Ion channel Virus uncoating and assembly	
8	890	NS1	Non-structural protein Interferon antagonist protein Regulation of host gene expression	Bouvier and Palese, 2008)
		NEP/NS2	Non-structural protein Nucleocytoplasmic export of RNA	

The influenza viral replication cycle begins with attachment of HA protein to viral specific receptor binding site of host cell. Thus, HA plays a major antigen for receptor binding and fusion activities on specific host range of viruses. There are two types of receptor binding sites, avian specific receptor has sialic acid linked to galactose at alpha 2,3 (SA α2,3) and human specific receptor (SA α2,6). Life cycle of influenza virus can be divided into the following stages; (1) entry into the host cell, (2) entry of (ribonucleoproteins) vRNPs into the nucleus, (3) transcription and replication of viral genome, (4) export of vRNPs from the nucleus and (5) assembly and budding at the host cell plasma membrane (Samji, 2009).

Influenza virus has two major mechanisms for antigenic changes, namely antigenic shift and antigenic drift. Antigenic shift is a consequence of the segmented nature of the influenza virus genome. Antigenic shift is a result of the complete exchange of HA and /or NA genes of influenza virus. The gene segments can be exchanged among influenza viruses called “reassortment”. Antigenic shift is a major change of influenza A viruses and sometime is responsible for ‘new’ variants of influenza viruses. This new variant or novel virus can possibly cause pandemic influenza if there are no immunities in the human population. Another mechanism, antigenic drift is small changes or mutations of the gene during the RNA replication. This mechanism usually occurs at the antibody binding site of HA and NA. Antigenic drift of influenza virus may lead to mainly epidemic influenza (CDC, 2017).

Epidemiology of influenza

For pandemic influenza, in the 20th century, there were three major influenza pandemics caused by influenza A viruses. The first influenza pandemic “Spanish flu” occurred in 1918 caused by influenza A subtype H1N1 resulting in at least 50 million people deaths. In 1957, the influenza pandemic “Asian flu” caused by influenza A subtype H2N2 was reported with more than two million people deaths. In 1968, the

third pandemic “Hong Kong flu” caused by influenza A subtype H3N2, and approximately one million people died. In 1977, an endemic influenza outbreak caused by influenza A subtype H1N1, “Russian Flu” was also reported. In the 21st century, the first pandemic outbreak “Swine Flu” was occurred due to a new reassortment of influenza A subtype H1N1 (Smith et al., 2009).

For HPAI, in 2003-2004, at least eight countries including Vietnam, Thailand, Indonesia, Cambodia, Laos, South Korea, Japan and China reported HPAI-H5N1 outbreaks (Li et al., 2004). In May 2005, China reported more than 6,000 in wild birds death due to HPAI-H5N1 clade 2.2 (Chen et al., 2005). In 2007-2009, HPAI-H5N1 clade 2.3.4 was reported in live bird markets in southern China (Jiang et al., 2010). The HPAI-H5N1 clade 2.3.4 had also been reported in Laos, Malaysia and Thailand (Smith et al., 2006). In 2010, HPAI-H5N1 clade 2.3.2 has been reported in both poultry and wild birds in Asia and Europe (Abdelwhab et al., 2010). For the Republic of the Union of Myanmar, this country is a country in Southeast Asia, bordered by India, Bangladesh, Thailand, Laos and China. Myanmar has a tropical climate, and the average annual temperature is about 27.3 °C. Optimum environmental temperature (especially for 25 °C) is a significant risk factor for the transmission and distributions of HPAI (Zhang et al., 2014). Since 2006, several HPAI outbreaks have been reported in Myanmar. HPAI-H5N1 of clades 7, 2.3.2 and 2.3.4 have been identified. The epidemiological information of major HPAI-H5N1 outbreaks in Myanmar is as shown in Table 2.

Table 2. Epidemiological Information of major HPAI-H5N1 outbreaks in Myanmar

Wave	Location	Month/ year	AIV subtype	Clade	Affected species	Description	Reference
1st	Sagaing Mandalay	March/April 2006	H5N1	7	Layer, backyard	~ 650,000 poultry died and destroy	(Mon et al., 2012)
2nd	Yangon, Bago, Mon State	February, March 2007	H5N1	2.3.4	Layer, backyard	~100,000 poultry died and destroy	
3rd	Eastern Shan	November, December 2007	H5N1	2.3.4	Backyard	~35,000 birds were culled	
4th	Yangon, Sagaing	February, March 2011	H5N1	2.3.4 2.3.2	Layer, backyard	~25,00 birds were culled	
5th	Rakhine, Sagaing	January 2011	H5N1	N/A	Layer, Duck	~60,831 poultry were destroyed	(OIE, 2019)
6th	Sagaing, Bago	July 2012	H5N1	N/A	Layer, Bird	~28,30 poultry were culled	
7th	Sagaing	December 2015	H5N1	N/A	Layer chicken	~211,522 poultry were destroyed	
8th	Sagaing	April 2016	H5N1+ H9N2	N/A	Layer chicken	~86,338 poultry were destroyed	
9th	Taninthar yi	July 2017	H5N1	2.3.2.1 c	Layer chicken	~25,760 poultry were destroyed	

N/A: Not available

Epidemiology of influenza virus in live bird markets in Myanmar

During June 2006 to July 2008, the Livestock Breeding and Veterinary Department (LBVD) cooperated with Yangon City Development Council (YCDC) carried out an influenza serological surveillance program in three major live bird markets in Yangon. The surveillance result showed that 5.14% (38/738) from Mingalar-Taung-Nyunt LBM were seropositive for influenza viruses while two live bird markets (Hlaing-Tharyar LBM and Kyi-Myin-Dine LBM) showed seronegative. Of seropositive samples, HPAI-H5N1 viruses were also isolated from seropositive ducks (Kyaw, 2008). During 2014-2015, influenza A surveillance was conducted in LBMs located in Shan-State border of China-Myanmar. LPAI-H9N2 viruses were detected from healthy chicken in the LBMs, where sources of poultry were mostly from southern China. Whole genome sequencing of Myanmar LPAI-H9N2 viruses showed that the viruses were closely related to H9N2 virus of clade 4.2.5 from China. Genetic analysis showed that Myanmar LPAI-H9N2 viruses possessed low pathogenic characteristics and amantadine resistance (Lin et al., 2017). During 2014-2016, the Livestock Breeding and Veterinary Department (LBVD) cooperated with Food and Agriculture Organization of the United Nations (FAO) conducted active influenza surveillance in China-Myanmar border townships where act as entry points for poultry. From this surveillance, H5N6, H5N1 and H9N2 viruses were detected (Tun Win et al., 2017).

Since influenza A viruses can circulate within live bird markets. Risk factors of human infection appear to be direct or indirect contact of live or dead poultry or contaminated environment at the LBMs. Handling live animals, slaughtering, processing and handling animal carcasses are significant risk factors for influenza infection. Thus, influenza surveillance in LBMs should be the routine activities for influenza prevention and control.

CHAPTER III

MATERIALS AND METHODS

This study consists of 4 phases. Phase 1; Sample collection from live bird market, Yangon, Myanmar. Phase 2; Screening and isolation of influenza A viruses. Phase 3; Identification and characterization of influenza A viruses. Phase 4; Phylogenetic and genetic analysis of influenza A viruses. In this study, Phase 1 and 2 were conducted at the Veterinary Diagnostic Laboratory, Yangon, Myanmar. Phase 3 and 4 were processed at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The conceptual framework of this study is shown in Figure 1.

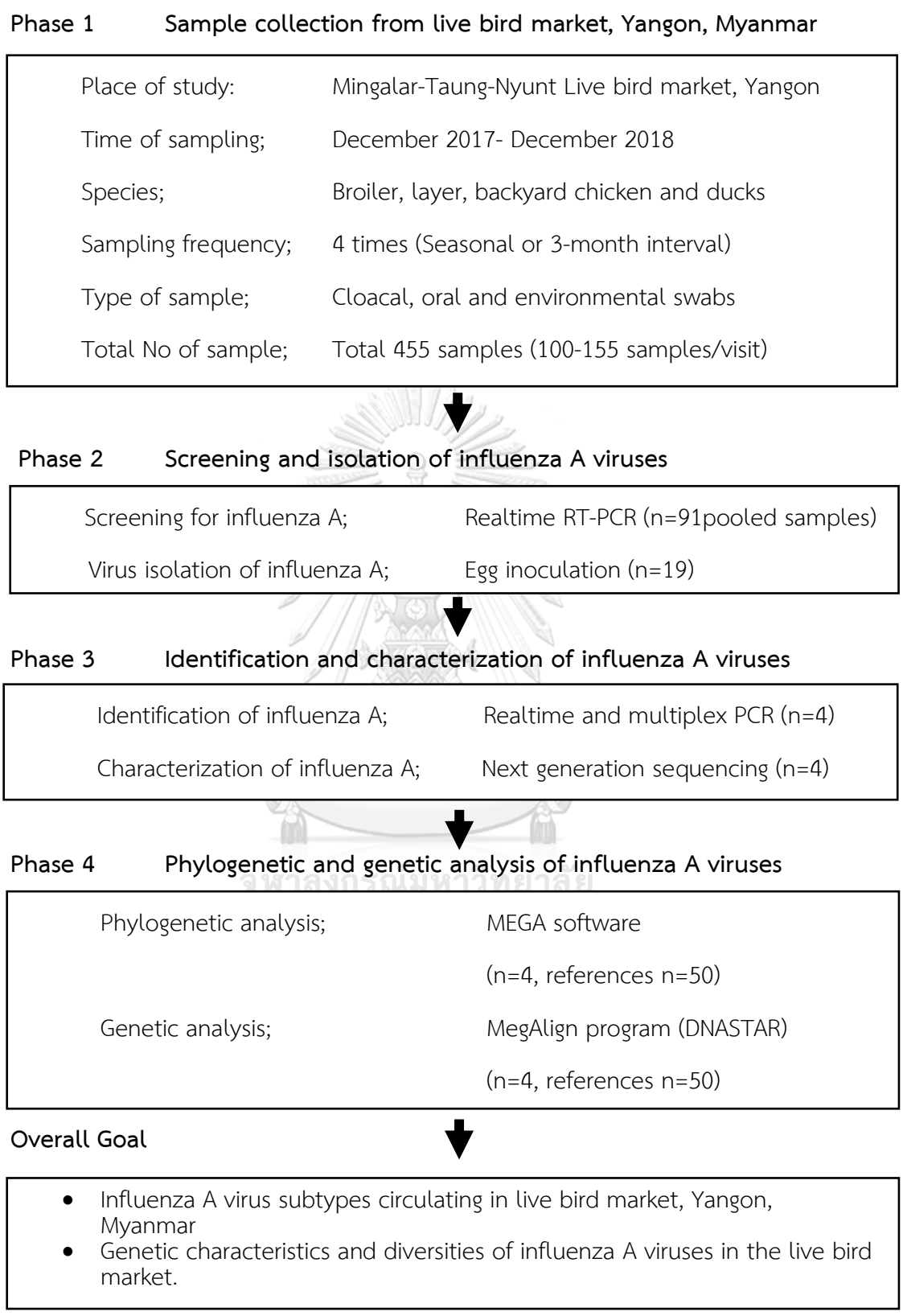

Figure 1. Conceptual frame work of the study

Phase 1 Sample collection from live bird market, Yangon, Myanmar

In this study, influenza surveillance was conducted at a live bird market (LBM) in Yangon, Myanmar. In Yangon, there are three significant LBMs including Mingalar-Taung-Nyunt LBM, Hlaing-Tharyar LBM and Kyi-Myin-Dine LBM. These LBMs are the places for many dealers and customers in Yangon to purchase live poultry for consumption or distribution to other local city markets. Among the three LBMs, Mingalar-Taung-Nyunt LBM is the largest and most functioning market in Yangon. It is located in Mingalar-Taung-Nyunt Township (Southeast of Yangon) (Figure 2). Furthermore, Mingalar-Taung-Nyunt LBM is situated by the river side of Pazundaung River, and workers use the water from this river for slaughtering and cleaning processes. Different avian species such as broiler chicken, layer chicken (spent hen), backyard chicken, Peking ducks and Muscovy ducks from different geographical regions including Ayeyarwaddy region, Bago Region and Yangon region are transporting daily to Mingalar-Taung-Nyunt LBM. Under the Authority of Yangon City Development Council (YCDC), there are approximately 60,000 birds are selling and buying in about 90 vendors shops within this market every day. Moreover, not only live animals are selling but also slaughtering and processing are done on-site in this LBM.

In this study, the samples were collected for four times, seasonally from December 2017- December 2018. In total, 455 samples from 16 shops were collected. In each visit, samples were collected from 4 vendors with different avian species (broiler chicken, layer chicken, backyard chicken and duck). The samples including cloacal swab, oral swab and environmental swabs (baskets, floor, scales, and water) were collected from different vendors. The collected samples were put into 2 ml viral transport media (VTM) and placed on ice for transportation to the

laboratory within 24 hours. Detail numbers and types of samples as well as date of sample collection were described in Table 3.

Phase 2 Screening and isolation of influenza A viruses

Screening and isolation of influenza A viruses were conducted at the BSL 2 laboratory, Livestock Breeding and Veterinary Department (LBVD), Yangon, Myanmar.

2.1 The cloacal, oral and environmental swab samples (baskets, floor, scales, and water) were pooled for 5:1 based on the same avian species, same location, and the same type of swabs. In detail, 200µl of each sample were pooled for 5:1 and final volume 1ml were obtained. In this study, 91 pooled samples were available and subjected to influenza A viruses screening. Detail of pooled samples were described in Table 3.

2.2 Viral RNA were extracted by using the QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany) to purify viral RNA according to assay protocol. In brief, first, the sample was lysed under highly denaturing conditions. Second, buffering conditions were then adjusted to provide optimum binding of RNA to QIAamp membrane. In order to wash away the contaminants efficiently, two different wash buffers were used in third and fourth steps. In last step, high-quality RNA was eluted in a special RNase-free buffer. Finally, purified RNA was obtained as free of protein, nuclease, other PCR inhibitors.

2.3 Screening of influenza A was carried out by using realtime RT-PCR specific primers and probe for the M gene. Realtime RT-PCR were conducted on a Rotor-Gene 3000 using (Corbett Research, Sydney, Australia) by utilizing the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen™: California, USA) with 0.4 µM final primers and probe

concentration (Spackman et al., 2002). In detail, 6.25 µl 2x reaction mixture, 0.5 µl forward primer (20pmol/ µl), 0.5 µl reverse primer (20 pmol/ µl), 0.5 µl probe (20pmol/ µl), 0.08 µl of 50M MgSO4, 0.25 µl SuperScript™ III, 4 µl RNA template and 0.42 µl RNase free water were contained in final volume 10 µl of each PCR mixture. The reactions were performed with the cycling conditions such as 15 min at 50 ºC, 2 min at 95 ºC and 40 cycles of 15 sec at 95 ºC and 30sec at 60 ºC. The result was analyzed by using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia) software. The samples with cycle threshold (Ct) of < 36 value were designated as influenza A positive, while those between 36 and 40 were considered as suspected and those > 40 ct value were identified as negative.

2.4 In this study, positive (n=12) and suspected (n=7) samples from realtime RT-PCR were subjected for virus isolation by egg inoculation. In detail, 150 µl of the supernatant of swab samples were inoculated to 9-11 days old specific pathogen free embryonated chicken eggs. The inoculated eggs were incubated at 37 ºC for 72 hours and monitored every day during incubation. After 72 hours incubation, the inoculated eggs were chilled at 4 ºC overnight. The allantoic fluid of each egg was collected and tested for hemagglutination test (HA test) by using 1% chicken red blood cells. The allantoic fluid samples with HA positive were further confirmed for influenza A virus antigen by realtime RT-PCR as previously described in 2.3.

2.5 The cDNA synthesis was carried out by using the influenza universal primer (Uni12) (Hoffmann et al., 2001) and the ImProm-II™ Reverse Transcription System. In detail, 5 µl of viral RNA were mixed with 5 µl of

Uni 12 primer and then amplified at 72 ºC for 15 minutes and 4 ºC for 5 minutes. The synthesized cDNAs were then transferred to the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand for further identification and characterization.

Phase 3 Identification and characterization of influenza A viruses

Identification and characterization of influenza A viruses were conducted at the BSL 3 at the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals (CU-EIDAS), Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

3.1 The cDNA samples were shipped to the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand for further identification and characterization.

3.2 Characterization of influenza A viruses were carried out by using next-generation sequencing (NGS). In detail, each gene segments (8 genes) of the positive cDNA samples were amplified by using one-step multiplex RT-PCR by using MBT 12 and MBT 13 primers (Xie et al., 2009). PCR products were confirmed by electrophoresis in 1.5% agarose gel. Then, PCR products were purified by using the QIAquick PCR Purification kit (Hilden, Germany). The purified PCR products were submitted to Novogen Company for illumine Highseq PE-150 sequencing. The DNA library generation were prepared by using NEB Next Multiplex OLIGOS for illumine (BioLabs ®Inc) that contains adaptors and primers suitable for multiplex sample preparation of next- generation sequencing on the illumina platform.

Phase 4 Phylogenetic and genetic analysis of influenza A viruses

Phylogenetic and genetic analysis of influenza A viruses were conducted at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

4.1 NGS data analysis was carried out by using CLC genomics workbench bio assembling software version 11.0.1 (CLC bio, 2005, Denmark). In detail, adapter list was created according to manual of NEB Next Multiplex OLIGOS for illumine (Index primer set 1) and imported our study viruses as illumina raw data. After that the nucleotide sequences were trimmed to remove adaptors and host nucleotide sequences. Nucleotide assembly were carried out by applying de novo assembly approach. The assembly contigs were then extracted and BLAST at NCBI. To be mapped read to reference, the influenza sequences were then downloaded from the GenBank database and imported as reference sequences. The result of mapping references was then extracted to consensus and exported nucleotide data as fasta format for further analysis. Finally, the whole genome nucleotide sequences (8 genes) of the viruses were submitted to the GenBank database.

4.2 For phylogenetic analysis, influenza viruses from this study and reference influenza viruses (n~50) were compared and evaluated. The reference viruses were obtained from Influenza Research Database and selected to represent clades of viruses found in Asian countries including clade 0, clade 1, clade 2.3.4 and clade 7, clade 2.3.2.1a, clade 2.3.2.1b and 2.3.2.1c. The phylogenetic trees were constructed by using MEGA software V7.0.26 using neighbor-joining method and 1000 bootstrap replications for the phylogeny tests. Maximum clade credibility (MCC) tree of all 8 gene

were generated by BEASTv1.10.4 with (BMCMC) algorithm. Uncorrelated relaxed clock model, coalescent constant size population with HKY with gamma 4 substitution and MCMC chain lengths 5000000 generations was used as parameters (Drummond and Rambaut, 2007). Representative clustering pattern was summarized by using tree annotator and tree were imaged by Fig Tree v1.4.3 software.

4.3 For genetic analysis, deduced amino acids of each gene of the viruses were aligned and analyzed by MegAlign program (DNASTAR) software. The amino acid determinant such as HA cleavage site, receptor binding site and anti-viral drug resistant determinants were evaluated.

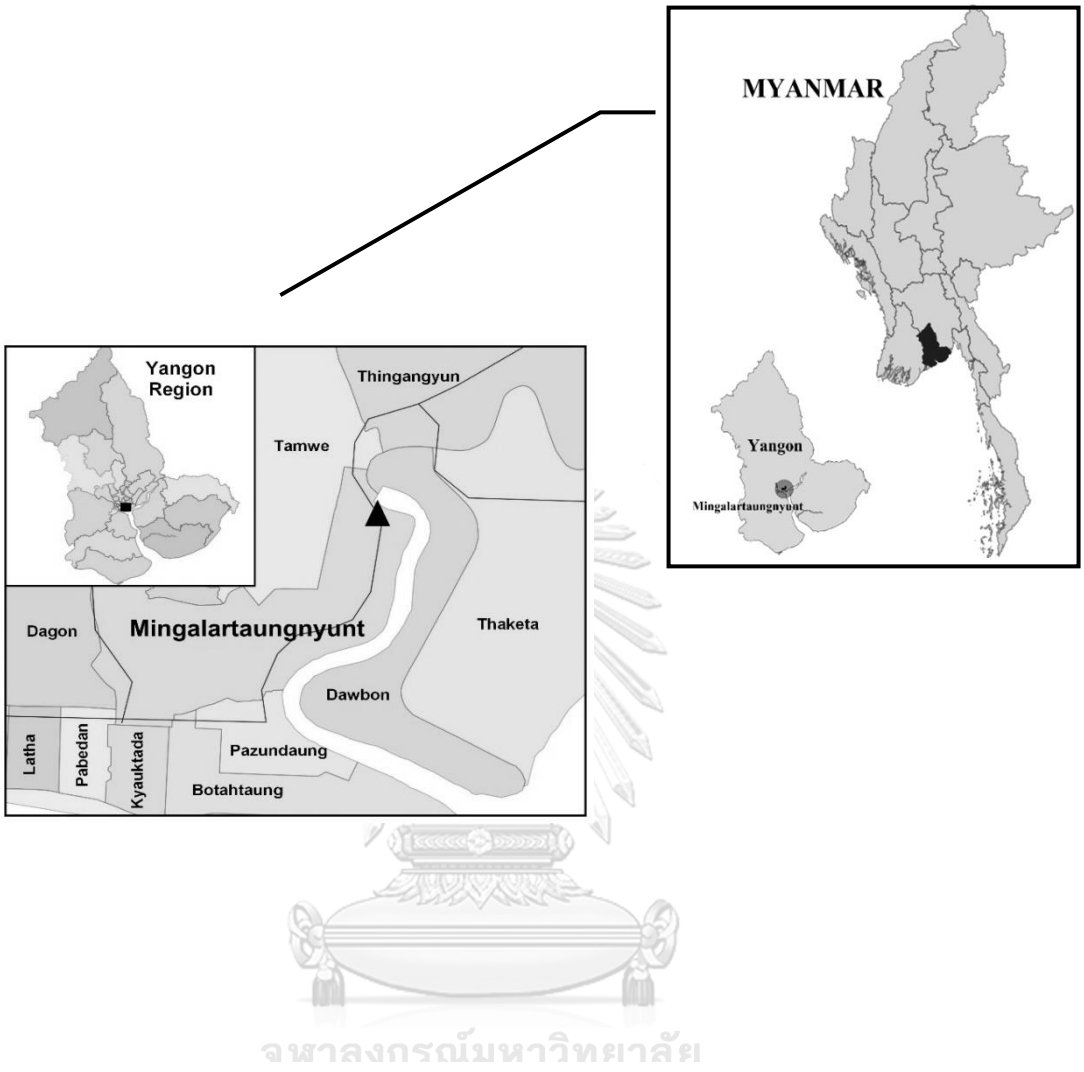

Figure 2. Location of Mingalar-Taung-Nyunt Live Bird Market, Yangon, Myanmar

CHAPTER IV

RESULTS

In this study, we conducted logtitudinal sample collection at Mingalar-Taung-Nyunt Live Bird Market, Yangon, Myanmar. There were 4 visits (3 months interval) for sample collection during 2017-2018. The samples were collected from broiler chicken (n=110), layer chicken (n=125), backyard chicken (n=110) and ducks (n=110). At the BSL2 laboratory, Yangon, the collected sample were pooled based on the same avian species, same location, and same type of swabs as shown in Table 3. Among 91 pooled samples, 12 samples were positive for influenza A (M gene) and 7 samples were considered as suspected for influenza A (Table 4). All of these positive and suspected samples were subjected for virus isolation by egg inoculation. The HA positive allantoic fluid were confirmed for influenza A virus by realtime RT-PCR. In detail, the oropharyngeal swab of layer chicken (n=3), the cloacal swab of layer chicken (n=2) and environmental swabs (n=2) were positive for influenza A virus by realtime RT-PCR (Figure 3 and Table 5). It is noted that all samples were examined for influenza A subtypes for H5, H7 and H9. Our result showed 7 samples could be identified as influenza A virus subtype H5N1. In this study, only 4 viruses were selected for characterization including 2 viruses from oropharyngeal swab of layer chicken (A/CK/Myanmar/MTN-LBM/OP11 and A/CK/Myanmar/MTN-LBM/OP12) and two viruses from cloacal swab of layer chicken (A/CK/Myanmar/MTN-LBM/CS13 and A/CK/Myanmar/MTN-LBM/CS14).

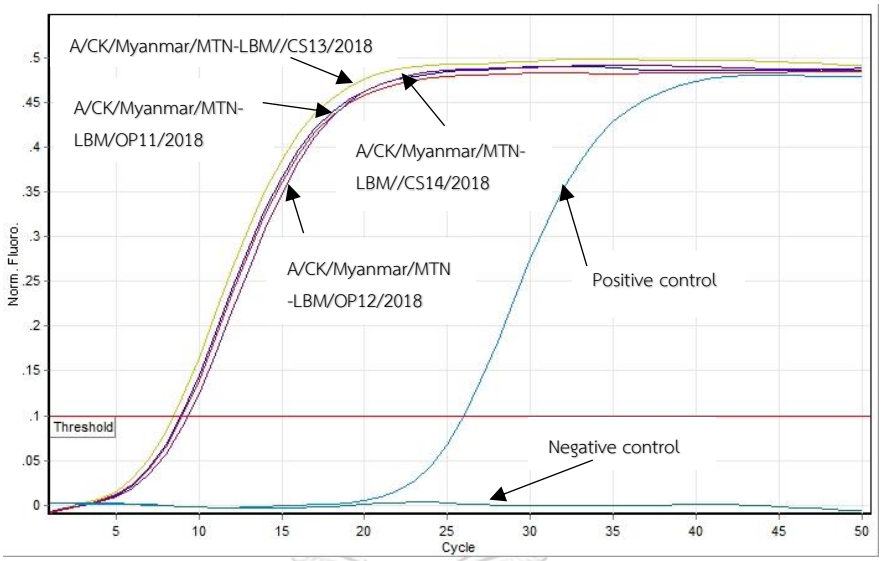

Figure 3. Identification of Influenza A virus (M gene) by realtime RT-PCR. The Ct value lower than 36 indicates influenza A virus positive and Ct value between 36-40 indicate suspected

Table 3. Description of date of sample collection, host species, numbers of collected samples and numbers of pooled samples from MTN-LBM in this study

Visit	Month/ year	Species	Number of samples collected			Number of sample tested (pooled sample)
			OP*	CS*	EV*	
1	Dec/17	Broiler	10	10	5	25 (5)
		Layer	10	10	5	25 (5)
		Backyard chicken	10	10	5	25 (5)
		Duck	10	10	5	25 (5)
2	Apr/18	Broiler	10	10	5	25 (5)
		Layer	10	10	5	25 (5)
		Backyard chicken	10	10	5	25 (5)
		Duck	10	10	5	25 (5)
3	Jul/18	Broiler	10	10	5	25 (5)
		Layer	10	10	5	25 (5)
		Backyard chicken	10	10	5	25 (5)
		Duck	10	10	5	25 (5)
4	Dec/18	Broiler	15	15	5	35 (7)
		Layer	20	20	10	50 (10)
		Backyard chicken	15	15	5	35 (7)
		Duck	15	15	5	35 (7)
			185	185	85	455 (91)

*OP= Oropharyngeal swab, *CS= cloacal swabs and *EV= environmental swabs

Identification and isolation of influenza A virus

Identification and isolation of influenza A viruses were conducted at the BSL 2 laboratory, Livestock Breeding and Veterinary Department (LBVD), Yangon, Myanmar. In this study, 91 pooled samples were subjected to RNA extraction and screening of influenza A virus. These samples were subjected to realtime RT-PCR by using specific primers and probe for M gene. Detail information of virus identification and virus isolation were as shown in Table 4 and 5. In detail, 12 out of 91 pooled samples that showing Ct value of <36 were considered as positive for influenza virus. In addition, 7 pooled samples with Ct value of >36 and <40 were considered as suspected for influenza A virus. All positive and suspected samples were subjected for virus isolation to 9-11 days old specific pathogen free embryonated chicken eggs. After 72 hours incubation, the allantoic fluid of each egg were collected and tested for hemagglutination test (HA test) by using 1% chicken red blood cells. In this study, 4 allantoic fluid samples were showed HA positive and other samples showed HA negative. All 4 HA positive samples and remaining 8 realtime positive samples were subjected to cDNA synthesis by using the influenza universal primer (Uni12) and the ImProm-II^{TM} Reverse Transcription System. The cDNA of 12 samples were shipped to the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

Table 4. Description of influenza A virus identification by realtime RT-PCR

Sample collection time	Screening of IAV by realtime PCR (M gene)		
	Positive Ct < 36	Suspected Ct between 36 and 40	Negative Ct >40
Dec/2017	6	3	11
April/2018	4	0	16
July/2018	1	1	18
Dec/2018	1	3	27
Total	12	7	72

Table 5. Description of samples by virus isolation, subtyping and whole genome sequencing

Time	Sample ID	Species	Type of sample	Virus isolation	Sub-type	Sequencing
Dec/ 2017	A/CK/Myanmar/ MTN-LBM/OP11	Layer	Oral swab	Positive	H5N1	Whole genome sequencing
	A/CK/Myanmar/ MTN-LBM/OP12	Layer	Oral swab	Positive	H5N1	Whole genome sequencing
	A/CK/Myanmar/ MTN-LBM/CS13	Layer	Cloacal swab	Positive	H5N1	Whole genome sequencing
	A/CK/Myanmar/ MTN-LBM/CS14	Layer	Cloacal swab	Positive	H5N1	Whole genome sequencing
	A/CK/Myanmar/ MTN-LBM/EV15	Layer	Environ mental swab	Negative	H5N1	-
	A/Du/Myanmar/ MTN-LBM/OP16	Duck	Oral swab	Negative	-	-
April/ 2018	A/CK/Myanmar/ MTN-LBM/OP26	Layer	Oral swab	Negative	H5N1	-
	A/CK/Myanmar/ MTN-LBM/OP27	Layer	Oral swab	Negative	-	-
	A/CK/Myanmar/ MTN-LBM/EV30	Layer	Environ mental swab	Negative	H5N1	-
	A/CK/Myanmar/ MTN-LBM/OP36	Back yard	Oral swab	Negative	-	-
July/ 2018	A/CK/Myanmar/ MTN-LBM/EV50	Layer	Environ mental swab	Negative	-	-
Dec/ 2018	A/CK/Myanmar/ MTN-LBM/OP61	Broiler	Oral swab	Negative	-	-
Total	12			12	12 (7)	12 (4)

To reconfirm influenza A virus, positive cDNA samples were then amplified by multiplex RT-PCR reactions using two primers (MBT 12 and MBT 13). In this study, 4 viruses (MTN-LBM/OP11, MTN-LBM/OP12, MTN-LBM/CS13 and MTN-LBM/CS14) could be amplified and reconfirmed as influenza A virus (Figure 4). PCR products were then purified and checked for DNA concentration for whole genome sequencing. The DNA concentration of 4 viruses were as shown in Table 6.

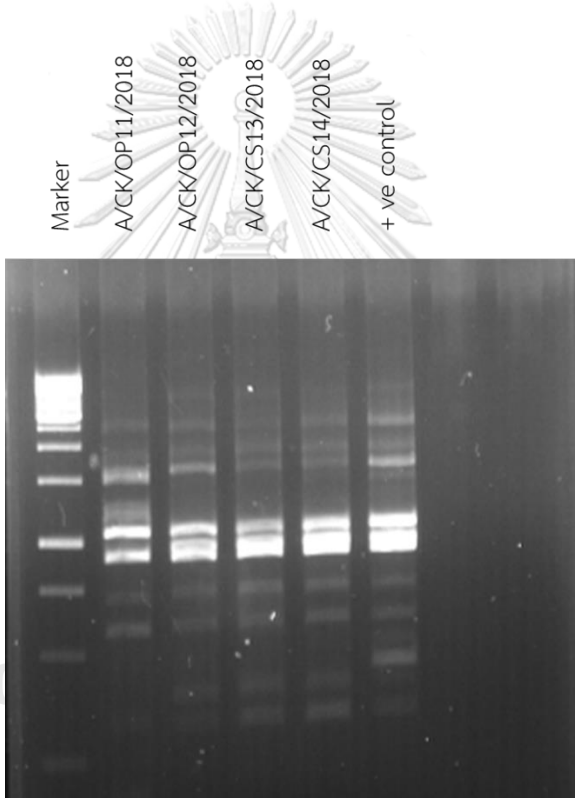

Figure 4. Amplification of 8 genes of influenza A virus by multiplex RT-PCR

Table 6. Description of DNA concentration of MTN-LBM H5N1 viruses by spectro-photometer

Isolated sample	Host	Type of sample	Nanodrop result		
			Concentration >60ng/µl	260/280 (1.8)	260/230 (2.0-2.2)
A/CK/Myanmar/ MTN-LBM/OP11	Layer chicken	Oropharyngeal swab	212.1	1.9	2.07
A/CK/Myanmar/ MTN-LBM/OP12	Layer chicken	Oropharyngeal swab	174.8	1.91	1.76
A/CK/Myanmar/ MTN-LBM/CS13	Layer chicken	Cloacal swab	197	1.88	1.78
A/CK/Myanmar/ MTN-LBM/CS14	Layer chicken	Cloacal swab	180	1.91	2.15

Characterization of influenza A viruses

For whole genome sequencing, the purified PCR products of 4 influenza viruses were submitted to Novogen Company for illumine Highseq PE-150 by next-generation sequencing. The DNA library generation were prepared by using NEB Next Multiplex OLIGOS for illumine (BioLabs ®Inc) kit. In detail, the kit components of 960 µl of NEB Next adaptor for illumine (15µM), 288 µl of USER enzymes and 10µl per well of NEB Next Index/ Universal primer mix (5µM) are transferred to PCR plate containing the virus of interest.

For NGS data analysis, based on de novo assembly result, approximately 2-4 million high quality reads were obtained for analysis. The results of nucleotide identities, closet related viruses and their accession numbers were obtained and provided in Table 10. The map reads for each gene of the MTN-LBM viruses were ranging from 302,053-3,499,339 reads and the sequence coverage were ranging from 18,154-563,492 coverages, respectively. The detail of nucleotide sequences with the length, reads and the average coverage were described in Table 9. In summary, the

whole genome sequences of the 4 viruses obtained from this study were submitted to the GenBank database under the accession number of MN046331-MN046362 (Table 7).

In this study, the remaining 8 realtime RT-PCR positive cDNA samples were re-tested to reconfirm the subtypes by using subtype specific primers for H5, H7 and H9. Our result showed that 3 out of 8 samples (MTN-LBM/EV15, MTN-LBM/EV30 and MTN-LBM/OP26) were positive and subtyped as influenza H5N1 as shown in Figure 5. The other 5 samples could not be subtyped due to high Ct value (low viral RNA).

Table 7. Description of influenza A viruses characterized in this study, GenBank accession numbers and clade of the viruses

No	Virus	Accession no	Species	Clade	Location
1	A/CK/Myanmar/MTN-LBM/OP11/2018	MN046331-MN046338	Layer	Clade 2.3.2.1c	MTN-LBM Yangon
2	A/CK/Myanmar/MTN-LBM/OP12/2018	MN046355-MN046362	Layer	Clade 2.3.2.1c	MTN-LBM Yangon
3	A/CK/Myanmar/MTN-LBM/CS13/2018	MN046339-MN046346	Layer	Clade 2.3.2.1c	MTN-LBM Yangon
4	A/CK/Myanmar/MTN-LBM/CS14/2018	MN046347-MN046354	Layer	Clade 2.3.2.1c	MTN-LBM Yangon

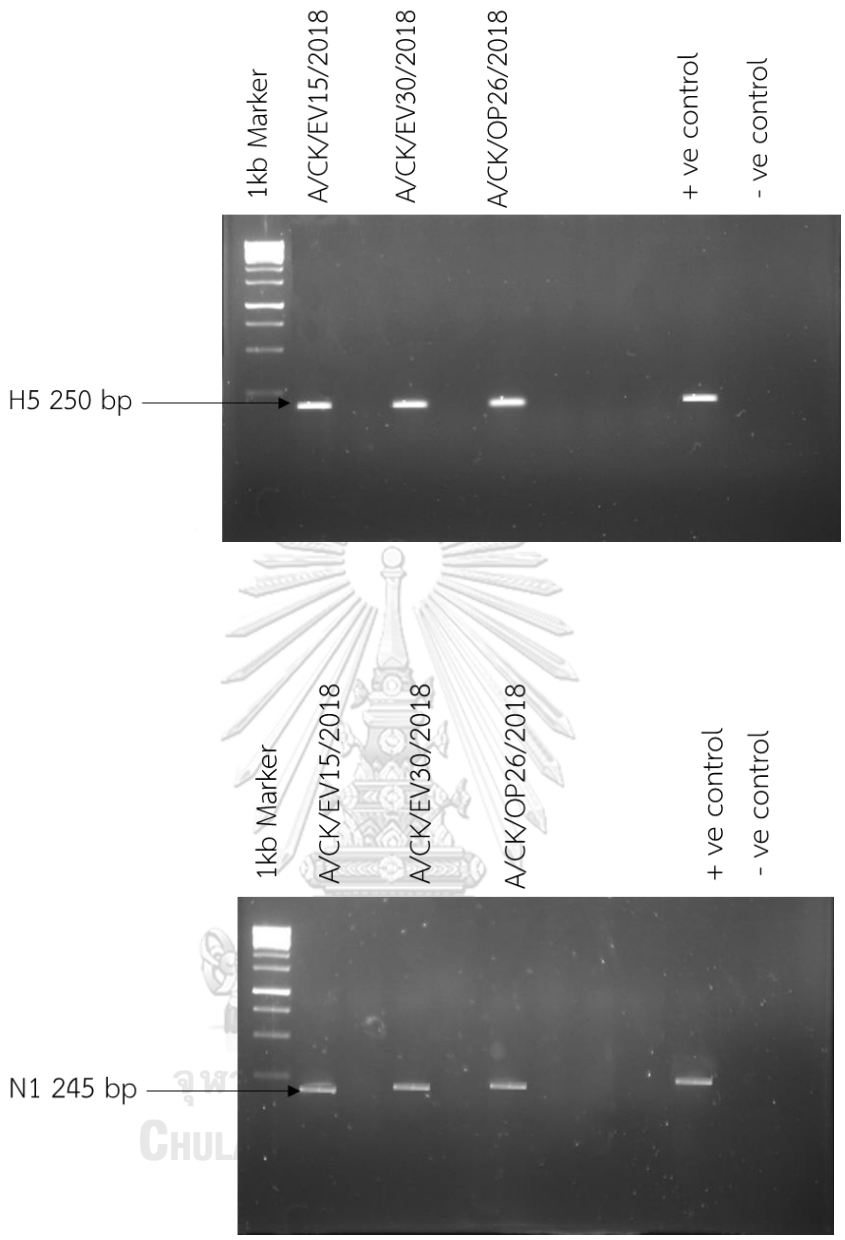

Figure 5. Subtyping of influenza, A viruses and identification of influenza A subtype H5N1 virus

Table 8. Influenza A virus subtyping of remaining realtime RT-PCR positive samples (n=8)

Sample ID	Sample collection Time line	Host	Type of sample	Subtyping result					
				H5	H7	H9	N1	N2	N7
A/CK/Myanmar/ MTN-LBM/EV15*	December 2017	Layer	**EV swab	+	-	-	+	-	-
A/Duck/Myanmar /MTN-LBM/OP16	December 2017	Duck	**OP swab	-	-	-	-	-	-
A/CK/Myanmar/ MTN-LBM/OP26*	April 2018	Layer chicken	OP swab	+	-	-	+	-	-
A/CK/Myanmar/ MTN-LBM/OP27	April 2018	Layer chicken	OP swab	-	-	-	-	-	-
A/CK/Myanmar/ MTN-LBM/EV30*	April 2018	Layer	EV swab	+	-	-	+	-	-
A/CK/Myanmar/ MTN-LBM/OP36	April 2018	Backyard chicken	OP swab	-	-	-	-	-	-
A/CK/Myanmar/ MTN-LBM/EV50	July 2018	Layer	EV swab	-	-	-	-	-	-
A/CK/Myanmar/ MTN-LBM/OP61	December 2018	Broiler	OP swab	-	-	-	-	-	-
Total			8	3	0	0	3	0	0

* H5N1 positive sample

**OP= Oropharyngeal swab and **EV= environmental swabs

Table 9. Alignment metrics from Illumina Highseq sequencing of Myanmar HPAI-H5N1

A/Chicken/Myanmar/MTN-LBM/OP11/2018			
Gene	Length	Mapped reads	Average coverage
PB2	2,280	441,121	25,999
PB1	2,274	2,352,134	140,025
PA	2,119	671,379	44,330
HA	1,704	609,332	49,469
NP	1,565	950,859	89,149
NA	1,357	445,601	45,677
M	1,027	2,866,840	405,126
NS	829	2,860,475	474,751
A/Chicken/Myanmar/MTN-LBM/OP12/2018			
Gene	Length	Mapped reads	Average coverage
PB2	2,341	302,053	18,154
PB1	2,341	2,173,245	133,750
PA	2,162	575,871	35,043
HA	1,776	711,195	58,075
NP	1,565	349,103	32,541
NA	1,398	404,734	41,502
M	1,027	2,192,894	307,436
NS	875	2,668,431	443,652
A/Chicken/Myanmar/MTN-LBM/CS13/2018			
Gene	Length	Mapped reads	Average coverage
PB2	2,280	308,623	19,063
PB1	2,274	2,792,554	169,863
PA	2,162	521,368	33,064
HA	1,704	728,682	57,926
NP	1,529	458,066	42,520
NA	1,357	567,493	58,197
M	1,027	3,130,004	439,533
NS	875	3,499,339	563,492
A/Chicken/Myanmar/MTN-LBM/CS14/2018			
Gene	Length	Mapped reads	Average coverage
PB2	2,280	463,790	28,206
PB1	2,274	1,807,472	105,886
PA	2,162	503,105	33,097
HA	1,704	532,699	43,229
NP	1,529	791,448	74,317
NA	1,357	429,662	44,391
M	1,027	2,401,984	339,153
NS	840	2,393,534	396,649

Table 10. Nucleotide identities of Myanmar HPAI-H5N1 viruses against their closest relatives

A/Chicken/Myanmar/MTN-LBM/OP11/2018			
Gene	**Closest viruses**	**Accession no**	**% identity**
PB2	A/quail/Vietnam/NCVD-3794/2013(H5N1)	KY170964	98.2
PB1	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171157	98.2
PA	A/chicken/DongNai/0182/2013(H5N1)	KU311521	98.4
HA	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171159	97.7
NP	A/muscovy duck/Vietnam/LBM228/2012(H5N1)	AB786685	98.2
NA	A/environment/Changsha/213/2014(H5N1)	KX247934	98.2
M	A/quail/Vietnam/CVVI-50/2014(H5N1)	KP872898	98.7
NS	A/duck/Vietnam/NCVD14-A447/2014(H5N1)	KY171131	98.3
A/Chicken/Myanmar/MTN-LBM/OP12/2018			
Gene	**Closest viruses**	**Accession no**	**% identity**
PB2	A/quail/Vietnam/NCVD-3794/2013(H5N1)	KY170964	98.2
PB1	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171157	98.1
PA	A/chicken/DongNai/0182/2013(H5N1)	KU311521	98.3
HA	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171159	97.6
NP	A/muscovy duck/Vietnam/LBM228/2012(H5N1)	AB786685	98.1
NA	A/environment/Changsha/213/2014(H5N1)	KX247934	98.1
M	A/quail/Vietnam/CVVI-50/2014(H5N1)	KP872898	98.7
NS	A/muscovy duck/Long An/43/2013(H5N1)	AB972743	98.2
A/Chicken/Myanmar/MTN-LBM/CS13/2018			
Gene	**Closest viruses**	**Accession no**	**% identity**
PB2	A/quail/Vietnam/NCVD-3794/2013(H5N1)	KY170964	98.2
PB1	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171157	98.1
PA	A/chicken/DongNai/0182/2013(H5N1)	KU311521	98.3
HA	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171159	97.6
NP	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171160	98.4
NA	A/environment/Changsha/213/2014(H5N1)	KX247934	98.1
M	A/quail/Vietnam/CVVI-50/2014(H5N1)	KP872898	98.7
NS	A/muscovy duck/Long An/43/2013(H5N1)	AB972743	98.2
A/Chicken/Myanmar/MTN-LBM/CS14/2018			
Gene	**Closest viruses**	**Accession no**	**% identity**
PB2	A/quail/Vietnam/NCVD-3794/2013(H5N1)	KY170964	98.2
PB1	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171157	98.1
PA	A/chicken/DongNai/0182/2013(H5N1)	KU311521	98.3
HA	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171159	97.7
NP	A/chicken/Vietnam/NCVD14-A461/2014(H5N1)	KY171160	98.4
NA	A/environment/Changsha/213/2014(H5N1)	KX247934	98.1
M	A/quail/Vietnam/CVVI-50/2014(H5N1)	KP872898	98.7
NS	A/duck/Vietnam/NCVD14-A447/2014(H5N1)	KY171131	98.3

Characteristics of Hemagglutinin gene (HA)

Phylogenetic analysis of HA gene

Four representative H5N1 viruses were analyzed by comparing the nucleotide sequences with 47 reference H5N1 viruses from various locations especially from Southeast Asia. In this study, five different clades mostly found in Asia were included in the analysis (clade 0, clade 1, clade 7, clade 2.3.4, clade 2.3.2.1a, 2.3.2.1b, clade 2.3.2.1c). From phylogenetic tree, based on the HA gene, four H5N1 viruses isolated from Mingalar-Taung-Nyunt live bird market (MTN-LBM) were grouped within the H5N1 viruses of clade 2.3.2.1c and closely related with the H5N1 viruses from China (A/ environment/ Changsha/ 213/ 2014) and Vietnam (A/ chicken/ Vietnam/ NCVD14-A461/ 2014) with high nucleotide identities ranging 99-100%. The H5N1 viruses from this study were clustered separately from the previous Myanmar H5N1 viruses of clade 7 and clade 2.3.4 (Figure 6).

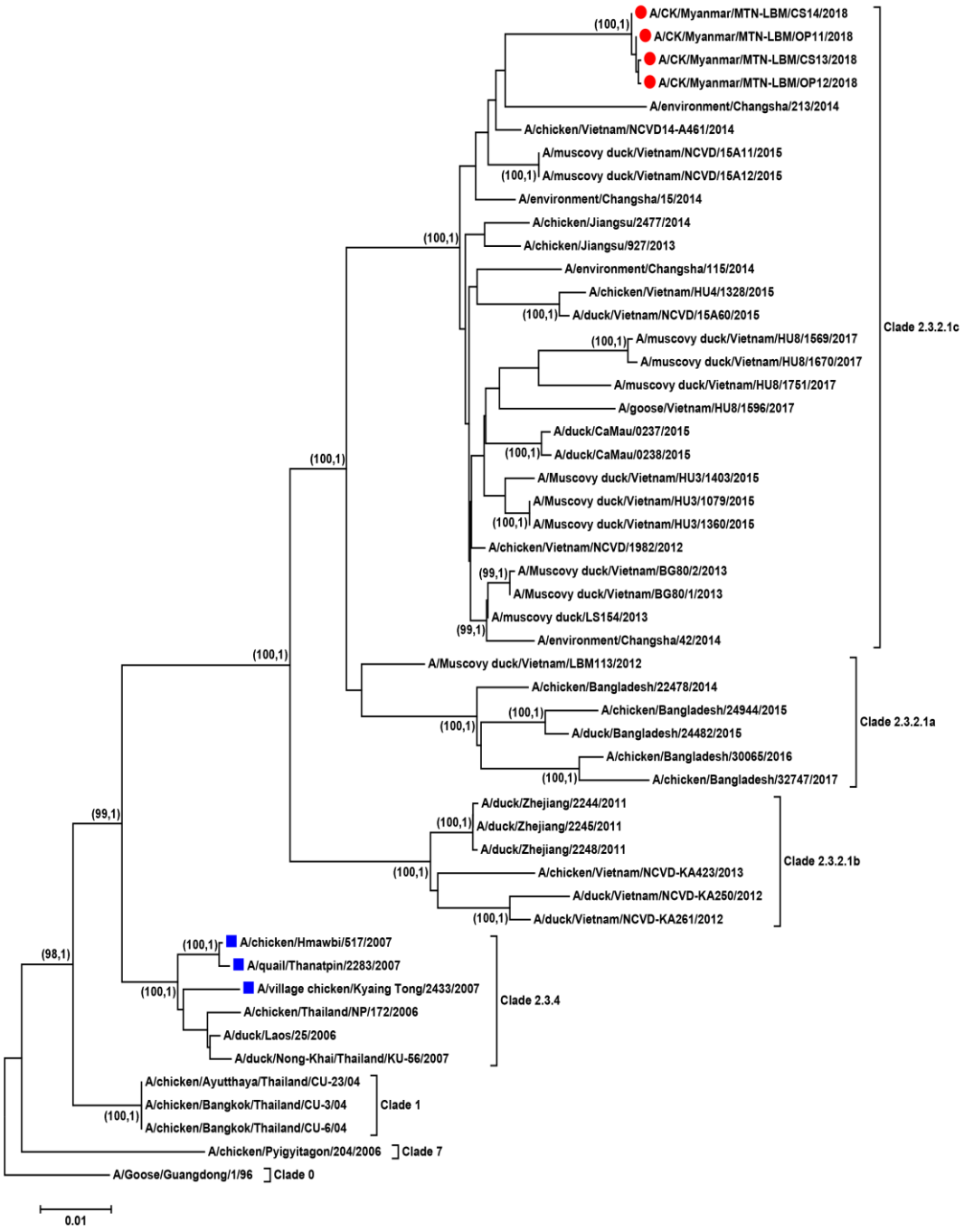

Figure 6. Phylogenetic tree of the HA gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar viruses

Genetic analysis of HA gene

The deduced amino acid of HA gene at key determinants such as HA cleavage site, receptor binding site and N-linked glycosylation sites of 4 H5N1 viruses are as shown in Figure 7. HA cleavage site motifs of 4 H5N1 viruses at amino acid position 321-330 have multiple basic amino acid sequences (PQRERRRR/G) indicating the characteristic of highly pathogenic avian influenza virus (OFFLU and Network, 2014). In this study, the polymorphisms of HA cleavage sites were observed between H5N1 viruses from this study and previous Myanmar H5N1 viruses such as A/CK/Hmawbi/517 (PLREKRRKR/G), A/quail/Thanatpin/2283/2007(PLREKRRKR/G), A/ village-chicken/ KyaingTong/2433/2007 (PLREKRRKR/G) and A/chicken/Pyigyitagon/ 204/2006 (PQREGRRKKR/G) while A/goose/Guangdong/1/1996 possesses (PQRERRRKKR/G). The amino acid receptor binding site (RBS) of the HA protein of 4 H5N1 viruses in this study have Leucine at position 129 (129L) and 175 (175L), Glutamine (Q) and Glycine (G) at position 222 and 224 (222Q and 224G) indicating that preferentially binding to α-2,3 linkages (α-2,3-SA) of avian host cell surface (Ha et al., 2001). On the other hand, Guangdong and Thailand H5N1 viruses possess Serine (S) at position 129 (129S) which is different from the H5N1 viruses from this study. Potential N-linked glycosylation sites (PGS) of HA gene of all H5N1 viruses possess (N-S) at position 10-12 and 286-288, (N-T) at position 11-13, 23-25, 165-167 and 193-195. All H5N1 viruses possessed (G-A) at position 154-156 (154G and 156A) suggesting low virulence of the virus (Table 11).

Table 11. Genetic analysis of HA gene: Deduced amino acid at key determinants at the HA cleavage sites, receptor binding sites and glycosylation sites

	HA gene				
	HA cleavage	Receptor binding site			
Virus	321-330	129[a]	175[a]	222[b]	224[b]
A/Gs/Guangdong/1/1996	PQRERRRKKR/G	S	L	Q	G
A/CK/Ayutthaya/Thailand/CU 23/04	PQRERRRKKR/G	L	L	Q	G
A/CK/Pyigyitagon/204/2006	PQREGRRKKR/G	L	L	Q	G
A/CK/Thailand/NP 172/2006	PLRERRRK/G	S	L	Q	G
A/Du/Nong Khai/Thailand/KU 56/2007	PLRERRRK/G	S	L	Q	G
A/CK/Hmawbi/517/2007	PLREKRRKR/G	L	L	Q	G
A/Qu/Thanatpin/2283/2007	PLREKRRKR/G	L	L	Q	G
A/V-CK/KyaingTong/2433/2007	PLRERRRKR/G	L	L	Q	G
A/CK/Bangladesh/30065/2015	PQRERRRKR/G	L	L	Q	G
A/CK/Bangladesh/32747/2015	PQRERRRKR/G	L	L	Q	G
A/CK/Jiangsu/927/2013	PQRERRRKR/G	L	L	Q	G
A/Du/Zhejiang/2244/2011	PQIERRRRKR/G	L	L	Q	G
A/Du/Vietnam/NCVD KA250/2012	PQIERRRRKR/G	L	L	Q	G
A/CK/Vietnam/NCVD 1982/2012	PQRERRRKR/G	L	L	Q	G
A/DU/LS 154/2013	PQRERRRKR/G	L	L	Q	G
A/CK/Vietnam/NCVD14/A461/2014	PQRERRRKR/G	L	L	Q	G
A/CK/Cambodia/Z850W49M1/2015	PLREKRRKR/G	L	L	Q	G
A/CK/Cambodia/Z3W7M/2015	PLREKRRKR/G	L	L	Q	G
A/CK/Kediri/04160512/2016	PLRERRRKR/G	L	L	Q	G
A/CK/Pati/04160433/2016	PLRERRRKR/G	L	L	Q	G
A/CK/Myanmar/MTN-LBM/OR11/2018*	**PQRERRRR/G**	**L**	**L**	**Q**	**G**
A/CK/Myanmar/MTN-LBM/OR12/2018*	**PQRERRRR/G**	**L**	**L**	**Q**	**G**
A/CK/Myanmar/MTN-LBM/CS13/2018*	**PQRERRRR/G**	**L**	**L**	**Q**	**G**
A/CK/Myanmar/MTN-LBM/CS14/2018*	**PQRERRRR/G**	**L**	**L**	**Q**	**G**

[a] At amino acid position 129 and 175: Leucine (L) and Serine (S).

[b] At amino acid position 222 and 224: Glutamine (Q) and Glycine (G) respectively.

Virus	HA gene						
	Glycosylation sites						
	10-12[a]	11-13	23-25	154-156[b]	165-167[c]	193-195[d]	286-288
A/Gs/Guangdong/1/1996	N-S	N-T	N-T	N-A	N-T	N-T	N-S
A/CK/Ayutthaya/Thailand/CU 23/04	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/CK/Pyigyitagon/204/2006	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/CK/Thailand/NP 172/2006	N-S	N-T	N-T	N-T	N-T	R-T	N-S
A/Du/Nong Khai/Thailand/KU 56/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/CK/Hmawbi/517/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/Qu/Thanatpin/2283/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/V-CK/KyaingTong/2433/2007	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/CK/Bangladesh/30065/2015	N-S	N-T	N-T	D-T	N-T	N-T	N-S
A/CK/Bangladesh/32747/2015	N-S	N-T	N-T	D-T	N-T	N-T	N-S
A/CK/Jiangsu/927/2013	N-S	N-T	N-T	D-A	N-T	N-T	N-S
A/Du/Zhejiang/2244/2011	N-S	N-T	N-T	D-A	K-T	N-T	N-S
A/Du/Vietnam/NCVD KA250/2012	N-S	N-T	N-T	D-A	N-T	N-T	N-S
A/CK/Vietnam/NCVD 1982/2012	N-S	N-T	N-T	G-A	N-T	N-T	N-S
A/DU/LS 154/2013	N-S	N-T	N-T	D-A	N-T	N-T	N-S
A/CK/Vietnam/NCVD14/A461/2014	N-S	N-T	N-T	D-A	N-T	N-T	N-S
A/CK/Cambodia/Z850W49M1/2015	N-S	N-T	N-T	N-T	**N-I**	**N-D**	N-S
A/CK/Cambodia/Z3W7M/2015	N-S	N-T	N-T	N-T	N-T	N-T	N-S
A/CK/Kediri/04160512/2016	N-S	N-T	N-T	N-A	N-T	N-T	N-S
A/CK/Pati/04160433/2016	N-S	N-T	N-T	N-A	N-T	N-T	N-S
A/CK/Myanmar/MTN-LBM/OR11/2018*	**N-S**	**N-T**	**N-T**	**G-A**	**N-T**	**N-T**	**N-T**
A/CK/Myanmar/MTN-LBM/OR12/2018*	**N-S**	**N-T**	**N-T**	**G-A**	**N-T**	**N-T**	**N-T**
A/CK/Myanmar/MTN-LBM/CS13/2018*	**N-S**	**N-T**	**N-T**	**G-A**	**N-T**	**N-T**	**N-T**
A/CK/Myanmar/MTN-LBM/CS14/2018*	**N-S**	**N-T**	**N-T**	**G-A**	**N-T**	**N-T**	**N-T**

[a] Amino acid at glycosylation site of HA gene: Asparagine (N), Serine (S), Threonine (T)

[b] Aspartic acid (D), Glycine (G) and Alanine (A)

[c] Lysine (K)

[d] Arginine (R) respectively

HA analysis

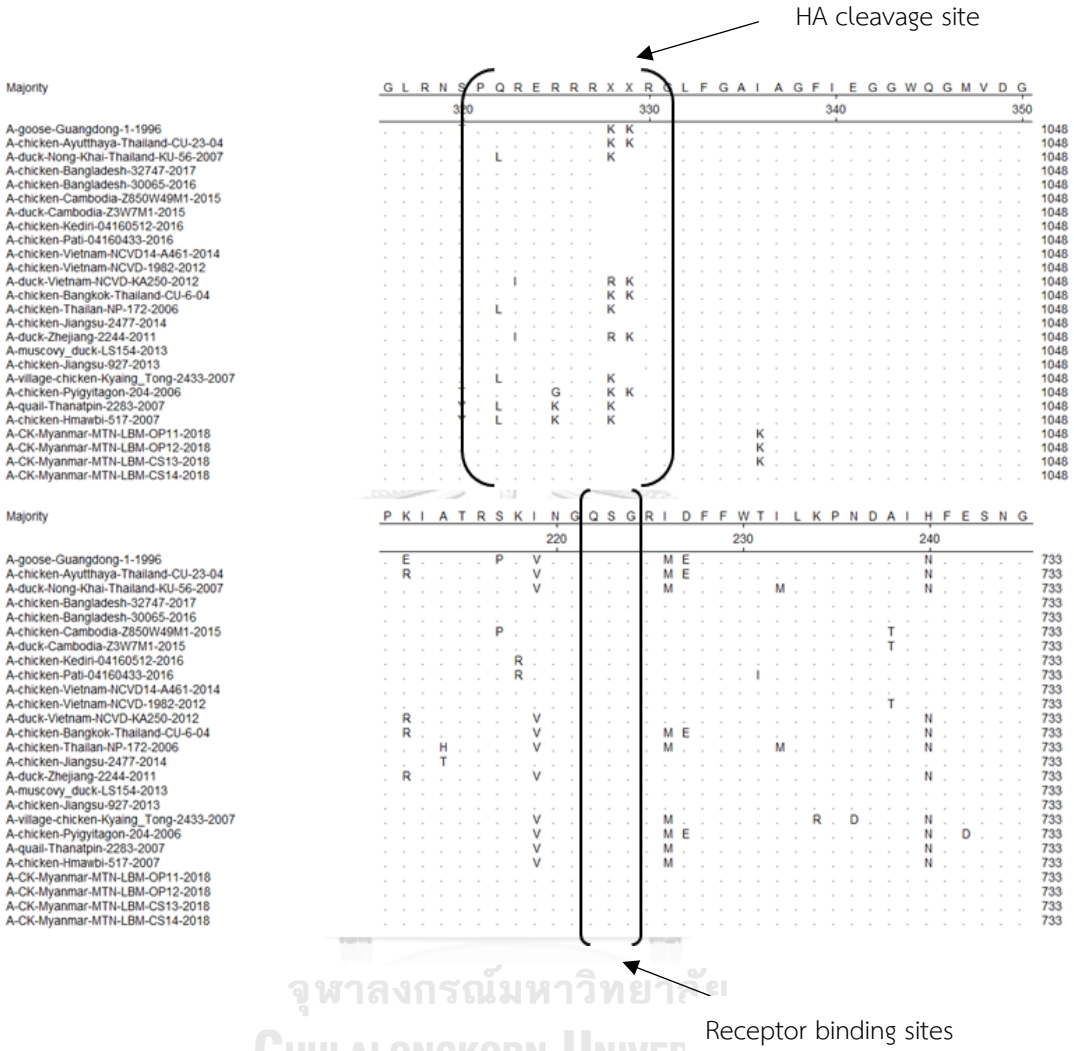

Figure 7. Comparison of deduced amino acid of HA cleavage site at positions 321-330, receptor binding sites at position 222 & 224 and glycosylation sites at 154-156. The last 4 nucleotide sequences are H5N1 viruses in this study

Characteristics of Neuraminidase gene (NA gene)

Phylogenetic analysis of NA gene

The genetic relatedness of the NA gene of H5N1 viruses is shown in Figure 8. Similar to HA gene, NA gene of 4 H5N1 viruses were clustered with the H5N1 viruses of clade 2.3.2.1c including the viruses from China (A/environment/ Changsha/213/2014, A/environment/Changsha/15/2014) and Vietnam (A/chicken/ Vietnam/NCVD14-A461/2014, A/Muscovy duck/Vietnam/NCVD/15A11/2015 and A/Muscovy duck/Vietnam/NCVD/15A12/ 2015).

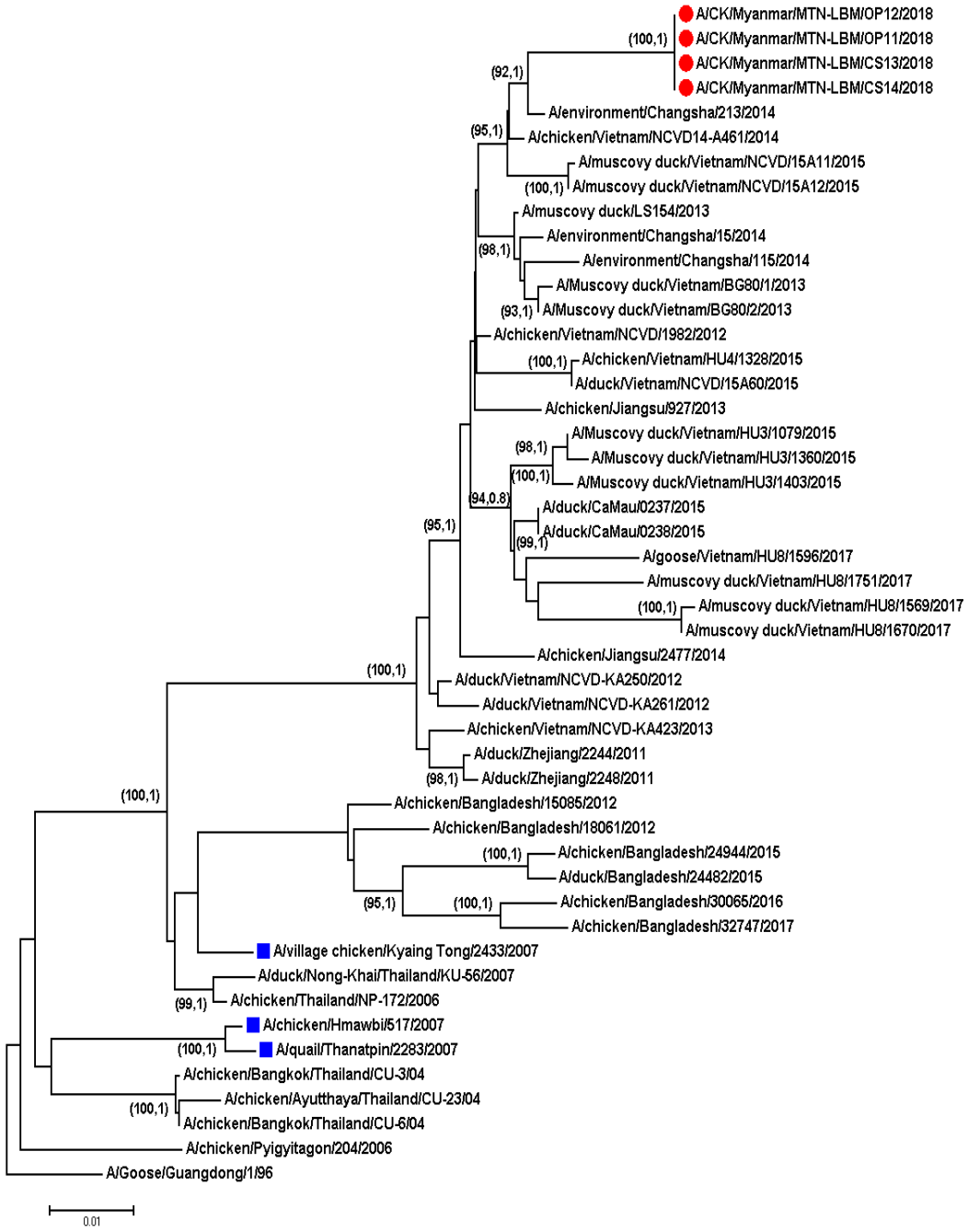

Figure 8. Phylogenetic tree of the NA gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicat the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar H5N1 viruse

Genetic analysis of NA gene

NA gene of all H5N1 viruses contained 20 amino acids deletion at the NA stalk region (position 49-68), while these 20 aa deletions was not observed in the H5N1 viruses of clade 0 (A/goose/Guangdong/1/1996). For Oseltamivir resistance determinants at position 119, 275, 293, and 295, all H5N1 viruses contained Glutamic acid (119E), Histidine (275H), Arginine (293R) and Asparagine (295N). The amino acid substitutions at the positions E119V, H275Y, R293K and N295S can result in Oseltamivir resistance. Our result indicated that all H5N1 viruses in this study are still sensitive to Oseltamivir similar to the previous Myanmar H5N1 viruses. Genetic analyses of NA gene at NA stalk region and Oseltamivir resistant determinants are shown in Table 12 and Figure 9.

Table 12. Genetic analyses of NA gene at NA stalk region and Oseltamivir resistant determinants

Virus	NA gene				
	NA stalk region	Oseltamivir resistance			
	49-68[a]	119[b]	275[c]	293[d]	295[e]
A/Gs/Guangdong/1/1996	No deletion	E	H	R	N
A/CK/Ayutthaya/Thailand/CU 23/04	20-aa deletion	E	H	R	N
A/CK/Pyigyitagon/204/2006	20-aa deletion	E	H	R	N
A/CK/Thailand/NP 172/2006	20-aa deletion	E	H	R	N
A/Du/Nong Khai/Thailand/KU 56/2007	20-aa deletion	E	H	R	N
A/CK/Hmawbi/517/2007	20-aa deletion	E	H	R	N
A/Qu/Thanatpin/2283/2007	20-aa deletion	E	H	R	N
A/V-CK/KyaingTong/2433/2007	20-aa deletion	E	H	R	N
A/CK/Bangladesh/30065/2015	20-aa deletion	E	H	R	N
A/CK/Bangladesh/32747/2015	20-aa deletion	E	H	R	N
A/CK/Jiangsu/927/2013	20-aa deletion	E	H	R	N
A/Du/Zhejiang/2244/2011	20-aa deletion	E	H	R	N
A/Du/Vietnam/NCVD KA250/2012	20-aa deletion	E	H	R	N
A/CK/Vietnam/NCVD 1982/2012	20-aa deletion	E	H	R	N
A/DU/LS 154/2013	20-aa deletion	E	H	R	N
A/CK/Vietnam/NCVD14/A461/2014	20-aa deletion	E	H	R	N
A/CK/Cambodia/Z850W49M1/2015	20-aa deletion	E	H	R	N
A/CK/Cambodia/Z3W7M/2015	20-aa deletion	E	H	R	N
A/CK/Kediri/04160512/2016	20-aa deletion	E	H	R	N
A/CK/Pati/04160433/2016	20-aa deletion	E	H	R	N
A/CK/Myanmar/MTN-LBM/OR11/2018*	**20-aa deletion**	**E**	**H**	**R**	**N**
A/CK/Myanmar/MTN-LBM/OR12/2018*	**20-aa deletion**	**E**	**H**	**R**	**N**
A/CK/Myanmar/MTN-LBM/CS13/2018*	**20-aa deletion**	**E**	**H**	**R**	**N**
A/CK/Myanmar/MTN-LBM/CS14/2018*	**20-aa deletion**	**E**	**H**	**R**	**N**

[a] Amino acid position 49-68 shows 20 amino acid deletion

[b] Amino acid position 119 contains E119V mutation indicates Oseltamivir resistance

[c] Amino acid position 275 contains H275Y mutation indicates Oseltamivir resistance

[d] Amino acid position 293 contains R293K mutation indicates Oseltamivir resistance

[e] Amino acid position 295 contains N295S mutation indicates Oseltamivir resistance

NA analysis

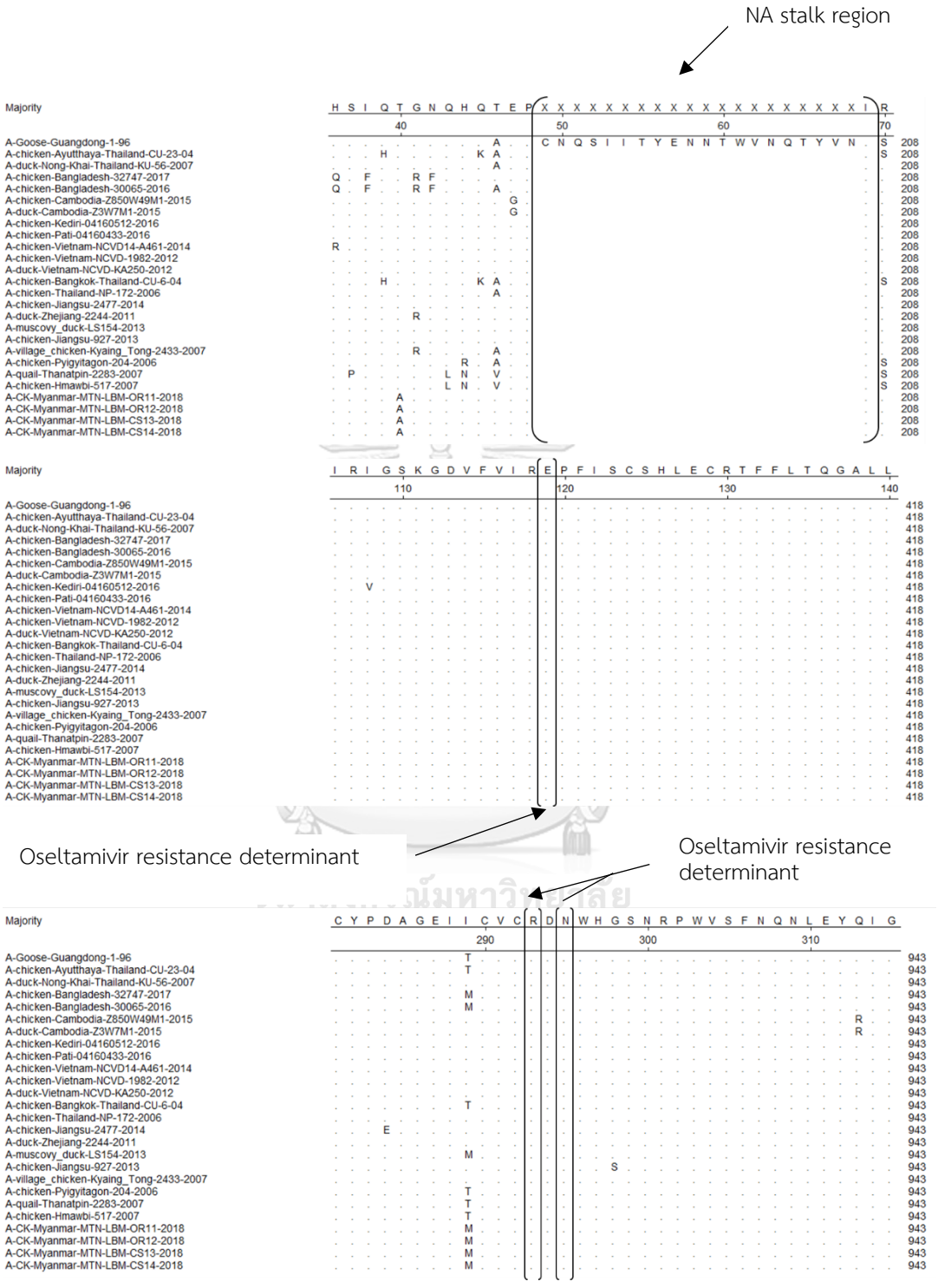

Figure 9. Comparison of deduced amino acids at NA stalk region at position 49-68 and some Oseltamivir resistant determinants at position 119, 293 and 295

Characteristics of Matrix Gene (M gene)

Phylogenetic analysis of M gene

Based on phylogenetic analysis of the M gene, all 4 H5N1 viruses from this study were clustered with the H5N1 viruses of clade 2.3.2.1c, same as HA and NA genes. However, unlike the HA and NA genes, the H5N1 viruses were clustered with the Vietnam H5N1 viruses from 2015 and 2017 (A/chicken/Vietnam/HU4/1328/2015, A/duck/Vietnam/NCVD/15A60/2015 and A/goose/Vietnam/HU8/1596/2017) (Figure 10).

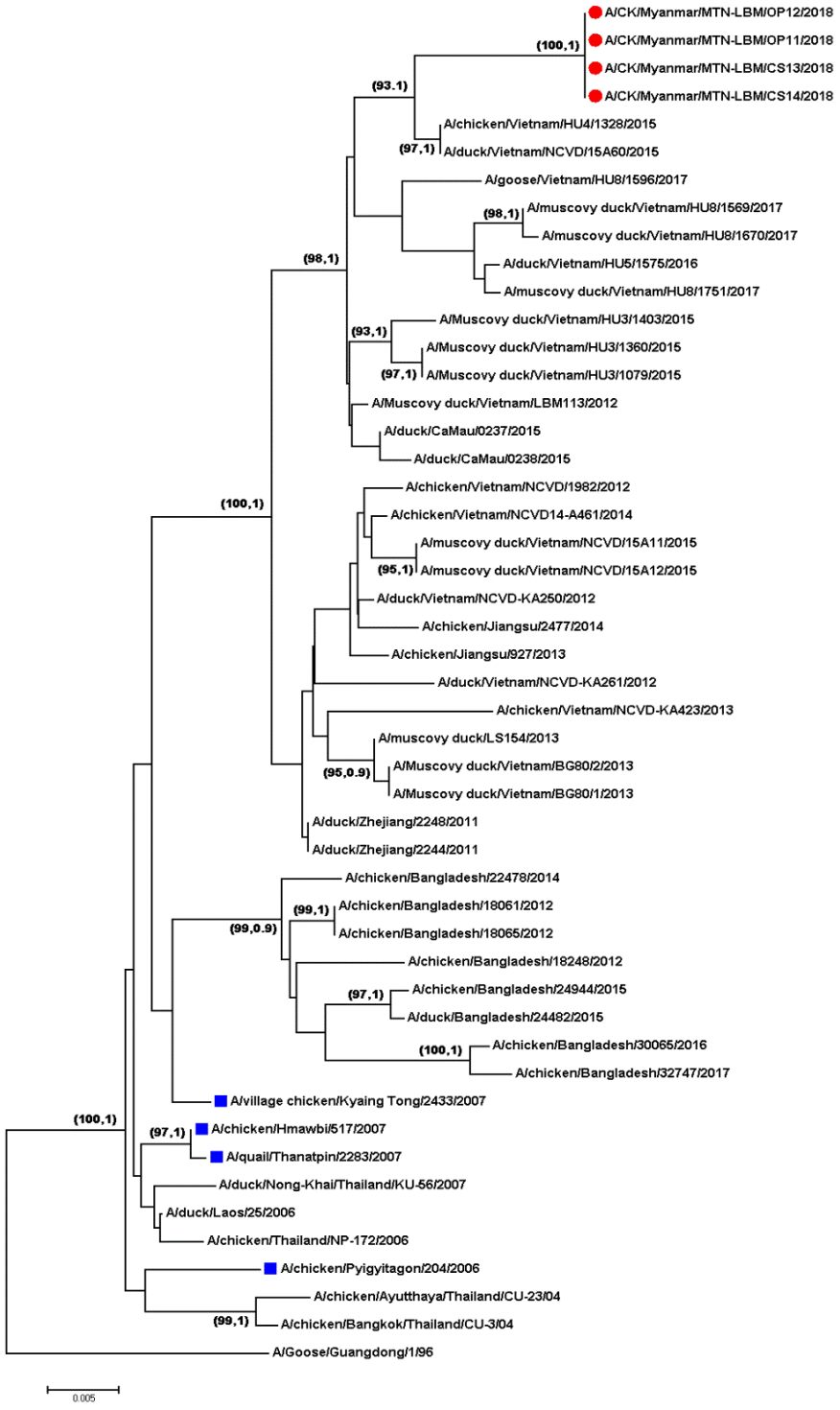

Figure 10. Phylogenetic tree of the M gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar H5N1 viruse

Genetic analysis of M gene

The analysis of Amantadine resistance at M gene of 4 H5N1 viruses from this study showed Leucine (L) at position 26 (26L), Valine (V) at position 27 (27V), Alanine (A) at position 30 (30A) and Serine (S) at position 31 (31S) indicating Amantadine sensitive viruses. Amantadine resistance can occur when amino acid substitutions as L26F, V27A, A30T, A30V, S31N. The amino acids related to human-like characteristics such as Valine (V) at position 28 (28V) was observed in all H5N1 viruses. While, the amino acids related to avian-like characteristics such as Glutamic acid (E) at position 16 (16E) and Leucine (L) at position 55 (55L) of M2 protein were found in all H5N1 viruses. The characteristics of all H5N1 viruses in this study were still agreed with Myanmar H5N1 viruses from previous study. Key determinants of matrix gene such as amantadine resistant amino acids and amino acids showing avian-like and human-like amino acids were shown in Table 13 and Figure 11.

Table 13. Genetic analysis of M2 protein at Amantadine resistant determinants and avian and human-like amino acids

Virus	M gene								
	Amantadine resistance amino acid					Human like amino acid		Avian like amino acid	
	26[a]	27[a]	30[a]	31[a]	64[a]	66[a]	28[b]	16[c]	55[c]
A/Gs/Guangdong/1/1996	L	V	A	S	S	E	V	E	L
A/CK/Ayutthaya/Thailand/CU 23/04	I	V	A	N	A	A	V	E	L
A/CK/Pyigyitagon/204/2006	L	V	A	S	S	A	V	E	L
A/CK/Thailand/NP 172/2006	L	V	A	S	S	E	V	E	L
A/Du/Nong Khai/Thailand/KU 56/2007	L	V	A	S	S	E	V	E	L
A/CK/Hmawbi/517/2007	L	V	A	S	S	E	V	E	L
A/Qu/Thanatpin/2283/2007	L	V	A	S	S	E	V	E	L
A/V-CK/KyaingTong/2433/2007	L	V	A	S	S	E	V	E	L
A/CK/Bangladesh/30065/2015	L	V	A	S	S	E	V	E	L
A/CK/Bangladesh/32747/2015	L	V	A	S	S	E	V	E	L
A/CK/Jiangsu/927/2013	L	I	A	S	S	E	I	E	L
A/Du/Zhejiang/2244/2011	L	V	A	S	S	E	V	E	L
A/Du/Vietnam/NCVD KA250/2012	L	T	A	S	S	E	T	E	L
A/CK/Vietnam/NCVD 1982/2012	L	I	A	S	S	E	I	E	L
A/DU/LS 154/2013	L	V	A	S	S	E	V	E	L
A/CK/Vietnam/NCVD14/A461/2014	L	I	A	S	S	E	I	E	L
A/CK/Cambodia/Z850W49M1/2015	L	V	A	N	S	E	V	V	F
A/CK/Cambodia/Z3W7M/2015	L	V	A	S	S	E	V	E	L
A/CK/Kediri/04160512/2016	L	I	A	S	S	E	F	E	L
A/CK/Pati/04160433/2016	L	I	A	S	S	E	F	E	L
A/CK/Myanmar/MTNLBM/OR11/2018*	**L**	**V**	**A**	**S**	**S**	**E**	**V**	**E**	**L**
A/CK/Myanmar/MTNLBM/OR12/2018*	**L**	**V**	**A**	**S**	**S**	**E**	**V**	**E**	**L**
A/CK/Myanmar/MTNLBM/CS13/2018*	**L**	**V**	**A**	**S**	**S**	**E**	**V**	**E**	**L**
A/CK/Myanmar/MTNLBM/CS14/2018*	**L**	**V**	**A**	**S**	**S**	**E**	**V**	**E**	**L**

[a] Amantadine resistance can result when mutation occur at position L26F, V27A, A30T, A30V, S31N

[b] Characteristics of human-like amino acid at position 16 E (glutamic acid) and 55 L (Leucine)

[c] Characteristics of avian-like amino acid at position 28 V (Valine)

M2 analysis

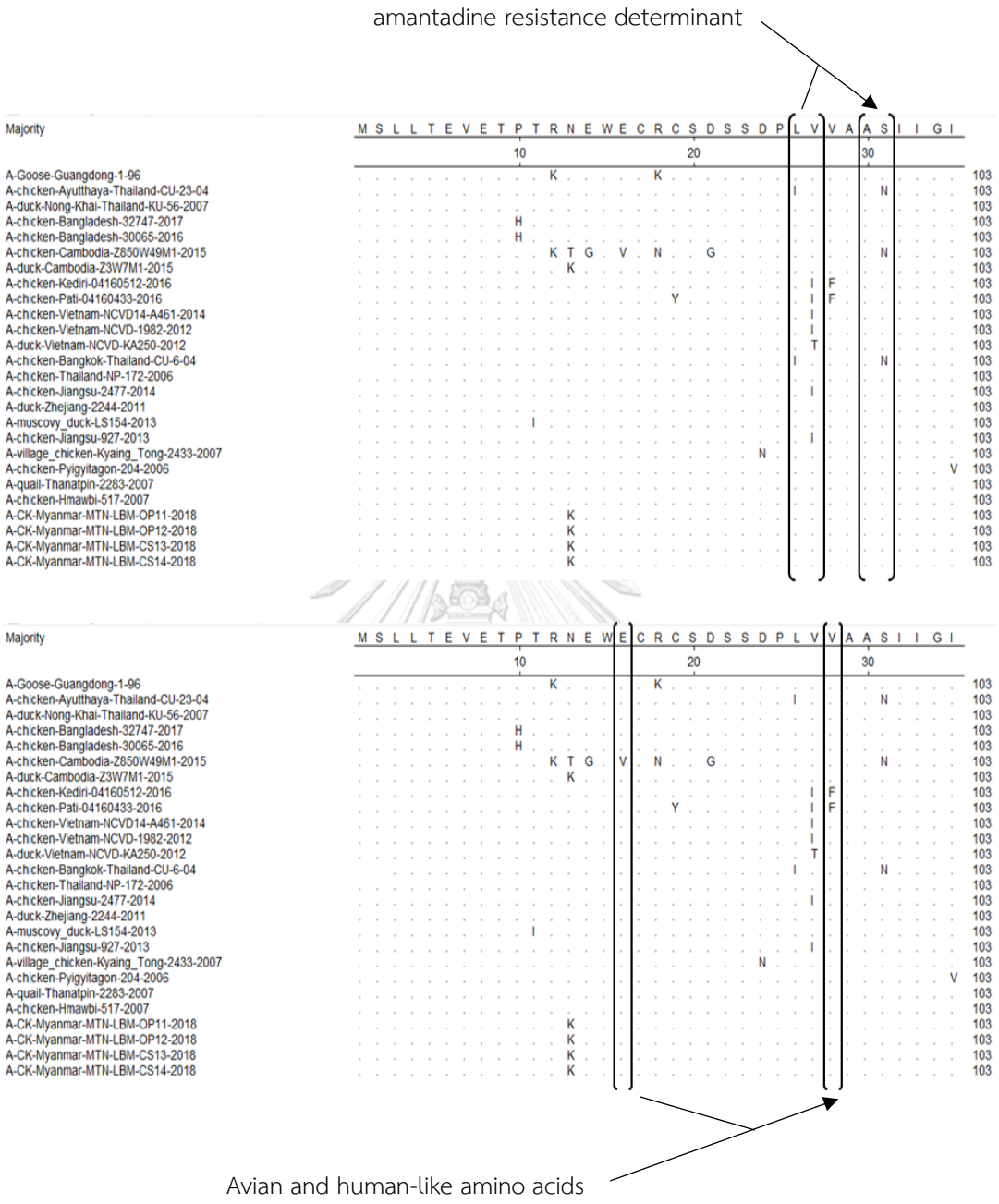

Figure 11. Comparison of deduced amino acid of M2 protein at Amantadine resistant determinants at positions 26, 27, 30 and 31 and avian and human-like characteristics at position 16 and 28

Characteristics of Polymerase genes (PB2, PB1, PA)

Phylogenetic analysis of polymerase gene (PB2, PB1, PA)

The genetic relatedness of PB2, PB1 and PA genes of H5N1 viruses comparing with those of H5N1 viruses from different countries are shown in Figures 12, 14 and 16. The polymerase genes of 4 H5N1 viruses were clustered with the H5N1 viruses of clade 2.3.2.1c similar to the previously described HA, NA and M genes. The PB2 gene of H5N1 viruses were grouped together with the Vietnam virus of clade 2.3.2.1c (A/chicken/Vietnam/HU4-1328/2015). Similar to PB2 gene, the phylogenetic tree of PB1 gene of MTN-LBM H5N1 viruses showed that 4 H5N1 viruses clustered with the viruses of clade 2.3.2.1c and closely related to A/chicken/Vietnam/NCVD14-A461/2014. Also, PA gene of 4 H5N1 viruses were grouped together with the viruses of clade 2.3.2.1c A/chicken/Vietnam/HU4-1328/2015 and A/duck/Vietnam/NCVD-15A60/2015.

Genetic analysis of polymerase genes (PB2, PB1 and PA)

Genetic analysis of PB2 gene of all H5N1 viruses showed Glutamic acid (E) at position 627 (627E) and Arginine (R) at position 355 (335R) indicating low virulence in mammals. The amino acids Alanine (A) at position 119 (119A), Threonine (T) at position 661 (661T), Glycine (G) at position 667 (667G) and 702 (702G) were also observed (Figure 13). Genetic analysis of PB1 gene showed that H5N1 viruses possessed Lysine (K) at position 198 (198K) indicating low virulence in mammals (Figure 15). Genetic analysis of PA gene showed that amino acid Serine (S) at position 409 (409S) suggesting avian-like characteristic (Figure 17). Detail of genetic analysis of polymerase genes of H5N1 viruses are shown in Table 14.

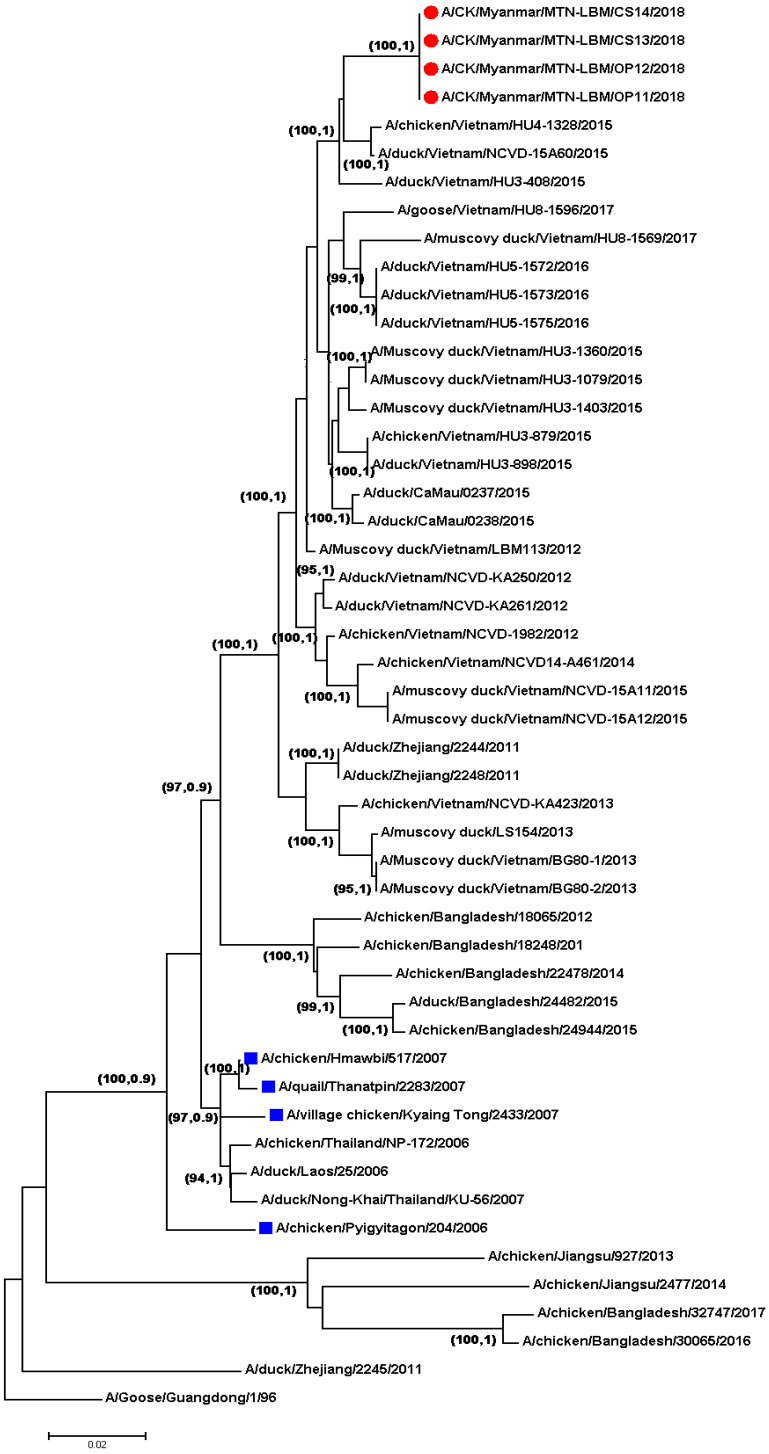

Figure 12. Phylogenetic tree of the PB2 gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar H5N1 viruses

Table 14. Genetic analysis of polymerase genes (PB2, PB1 and PA) at the human/avian-like and virulence determinants

Virus	PB2 gene						PB1	PA
	H/A like[a]				Virulence[b]		Virule-nce[c]	H/A like[d]
	199	661	667	702	627	355	198	409
A/Gs/Guangdong/1/1996	A	A	V	K	E	K	K	S
A/CK/Ayutthaya/Thailand/CU	A	A	V	K	E	R	K	S
A/CK/Pyigyitagon/204/2006	A	A	V	K	E	R	K	S
A/CK/Thailand/NP 172/2006	A	A	I	K	E	R	K	S
A/Du/Nong Khai/Thailand/KU	A	A	I	K	E	R	K	S
A/CK/Hmawbi/517/2007	A	A	V	K	E	R	K	S
A/Qu/Thanatpin/2283/2007	A	A	V	T	E	R	K	S
A/V-CK/KyaingTong/2433/2007	A	A	I	K	E	R	K	S
A/CK/Bangladesh/30065/2015	A	A	V	K	E	R	K	S
A/CK/Bangladesh/32747/2015	A	A	V	K	E	R	K	S
A/CK/Jiangsu/927/2013	A	A	V	K	E	R	K	S
A/Du/Zhejiang/2244/2011	A	A	V	K	E	R	K	S
A/Du/Vietnam/NCVD KA250/2012	A	A	V	K	E	R	K	S
A/CK/Vietnam/NCVD 1982/2012	A	A	V	K	E	R	K	S
A/DU/LS 154/2013	A	A	V	K	E	K	K	S
A/CK/Vietnam/NCVD14/A461/201	A	A	V	K	E	R	K	S
A/CK/Cambodia/Z850W49M1/201	A	A	V	K	E	R	K	S
A/CK/Cambodia/Z3W7M/2015	A	A	V	K	E	R	K	S
A/CK/Kediri/04160512/2016	S	A	V	K	E	R	K	S
A/CK/Pati/04160433/2016	A	A	V	K	E	K	K	S
A/CK/Myanmar/MTNLBM/OR11/	**A**	**A**	**V**	**K**	**E**	**R**	**K**	**S**
A/CK/Myanmar/MTNLBM/OR12/	**A**	**A**	**V**	**K**	**E**	**R**	**K**	**S**
A/CK/Myanmar/MTNLBM/CS13/	**A**	**A**	**V**	**K**	**E**	**R**	**K**	**S**
A/CK/Myanmar/MTNLBM/CS14/	**A**	**A**	**V**	**K**	**E**	**R**	**K**	**S**

[a] Human like amino acid of PB2 gene at position 199A, 661T, 667V and 702K

[b] Virulence determinant amino acid of PB2 gene at position E627K, K355R

[c] Virulence determinant amino acid of PB1 gene at position 198 K

[d] Human like amino acid of PA gene at position 409 S

PB2 analysis

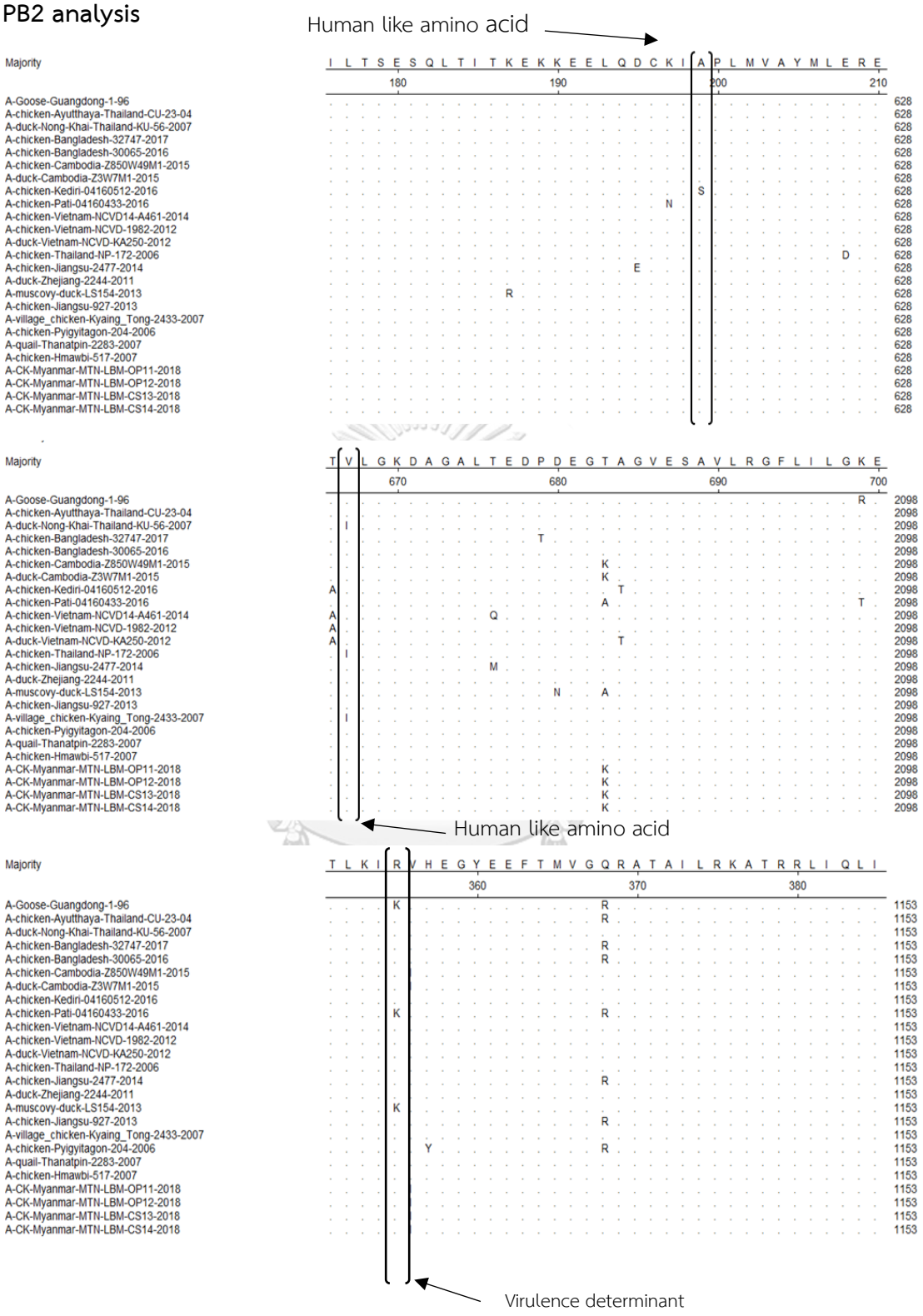

Figure 13. Comparison of deduced amino acids of PB2 at the human/avian-like amino acids at position 199 and 667 and virulence determinants at position 355.

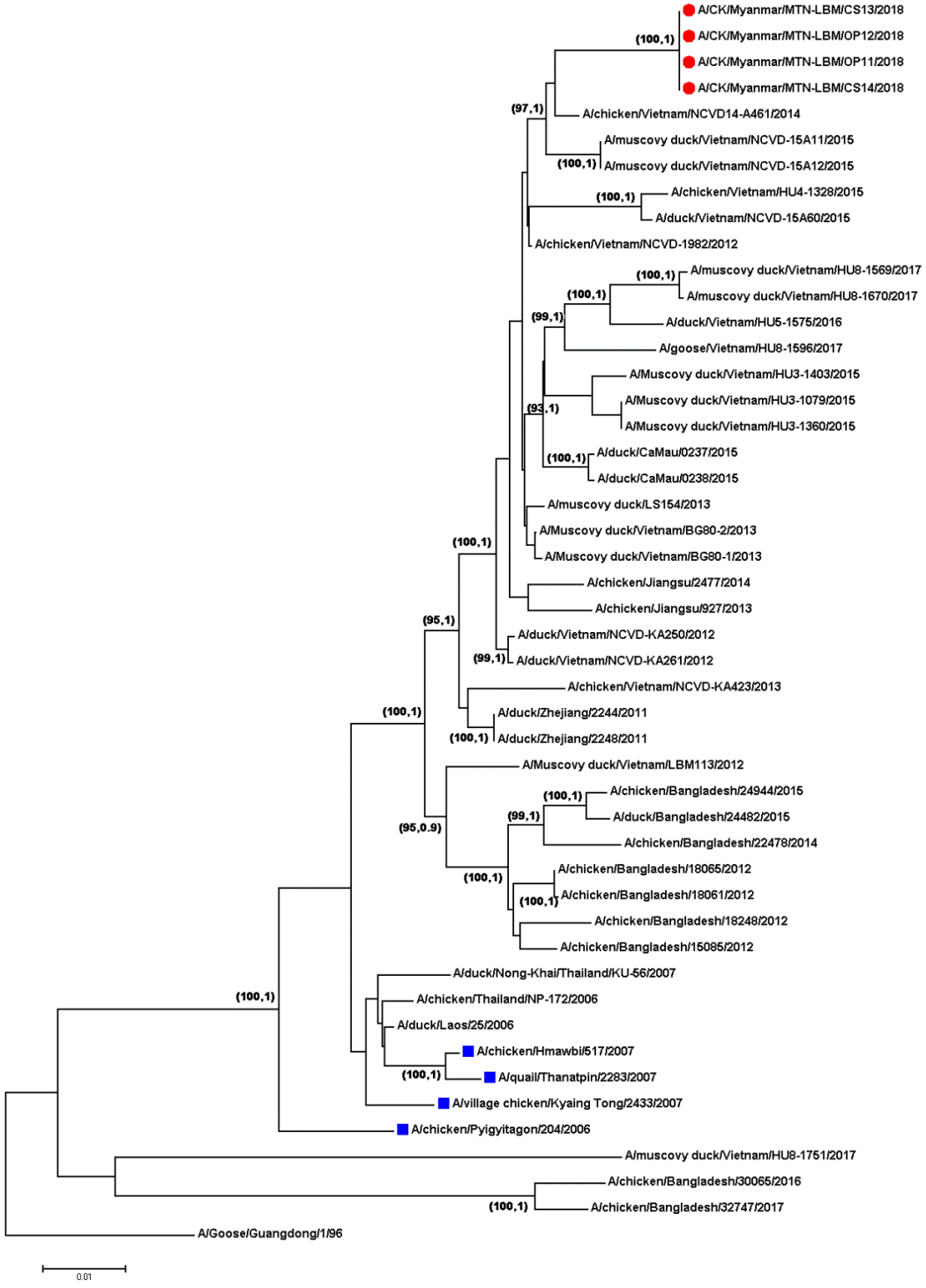

Figure 14. Phylogenetic tree of the PB1 gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar H5N1 viruses.

PB1 analysis

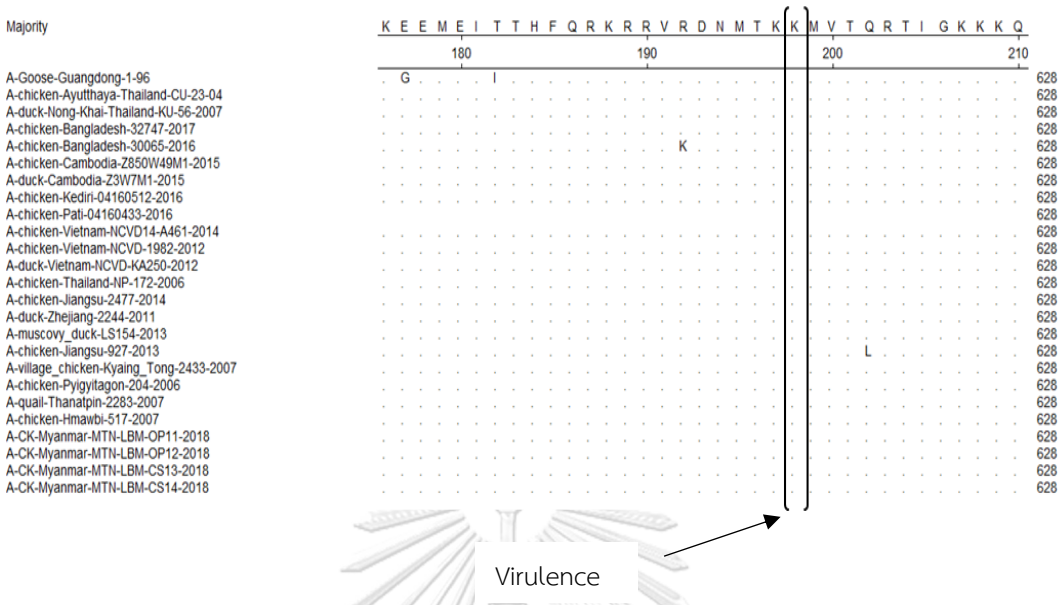

Figure 15. Comparison of deduced amino acids of PB1 protein at virulence determinant (position 198)

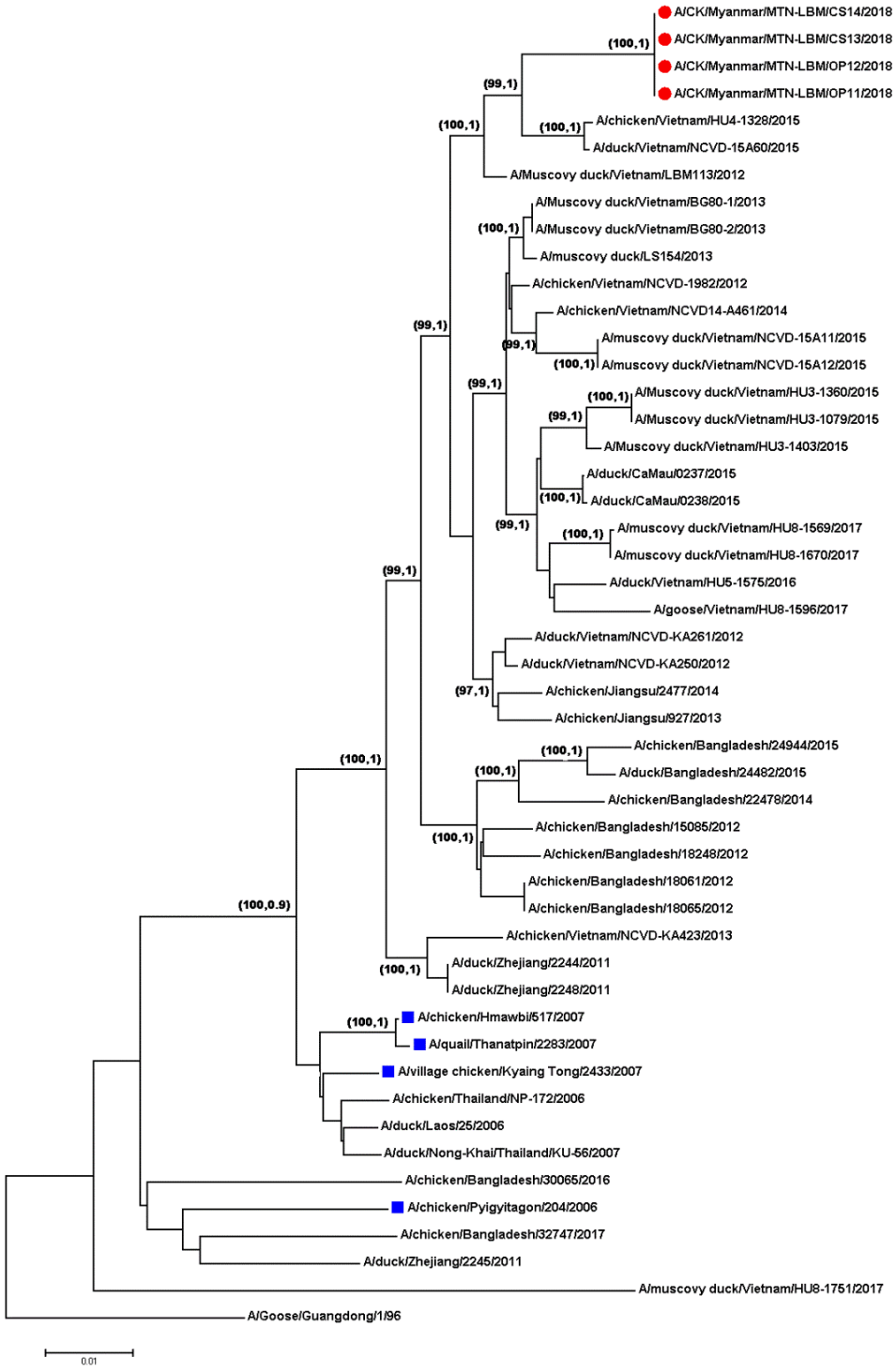

Figure 16. Phylogenetic tree of the PA gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the Myanmar H5N1 viruses

PA analysis

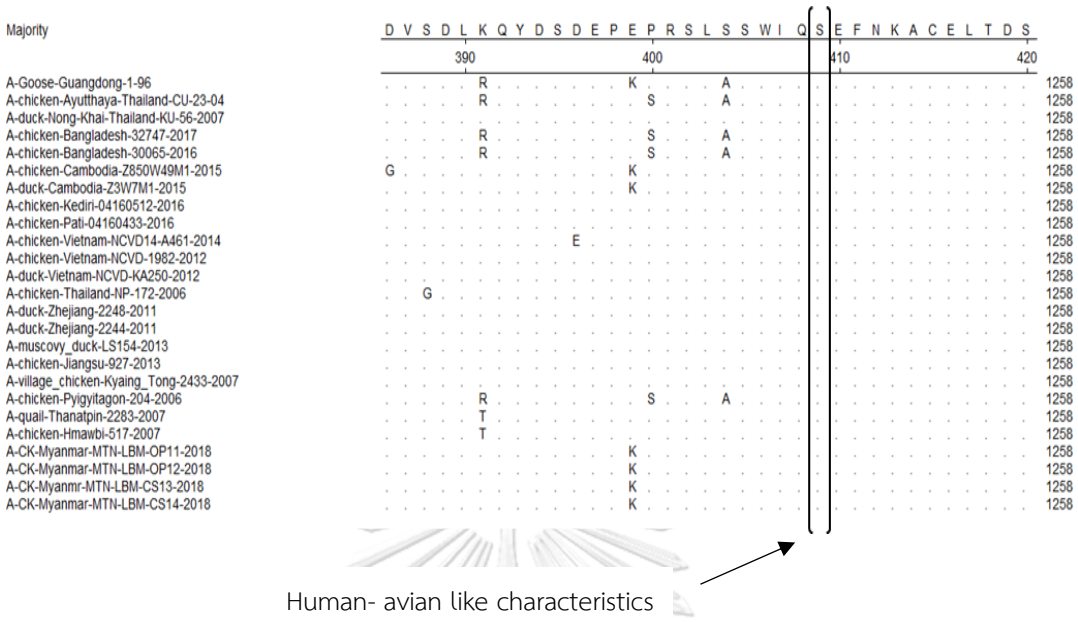

Figure 17. Comparison of deduced amino acid of PA protein at human/avian-like determinants (position 409)

Characteristics of nonstructural protein (NS gene)

Phylogenetic analysis of NS gene

Figure 18 shows the genetic relatedness of NS gene of H5N1 viruses in this study. Phylogenetic analysis of 4 H5N1 viruses revealed that Myanmar viruses clustered with the H5N1 viruses of clade 2.3.2.1c, similar to the HA, NA, M, PB2, PB1 and PA. All H5N1 viruses grouped together with the chicken and duck H5N1 viruses from Vietnam in 2015.

Genetic analysis of NS gene

Five amino acid deletions at position 80-84 were observed in 3 H5N1 viruses (MTN-LBM/OP11/2018, MTN-LBM/OP12 /2018, MTN-LBM/CS14/ 2018). However, one virus (MTN-LBM/CS13/2018) do not contain amino acid deletion, which is similar to ancestor virus (A/goose/Guangdong/1/1996). It had been known that the 5 amino acid deletions in NS gene is related to the increasing virulence in avian and mammalian species. Moreover, C-terminal (Carboxy terminal) motif (ESEV) was observed in all H5N1 viruses. Virulence determinants in NS gene were also analyzed all H5N1 viruses showed substitution from aspartic acid (D) to glutamic acid (E) at position 92 (D92E). Genetic analysis of NS protein is shown in Table 15 and Figure 19.

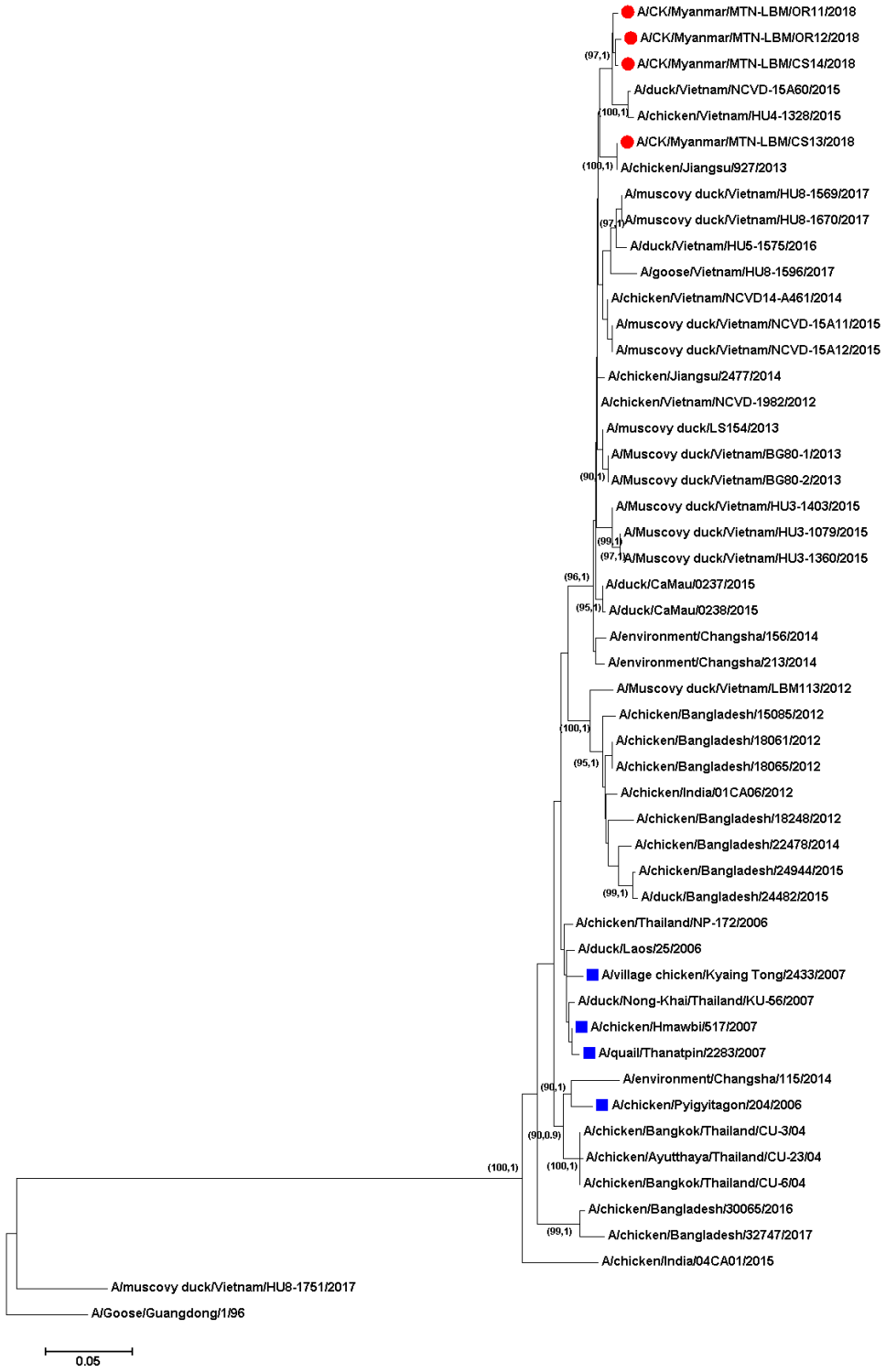

Figure 18. Phylogenetic tree of the NS gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar H5N1 viruses

NS analysis

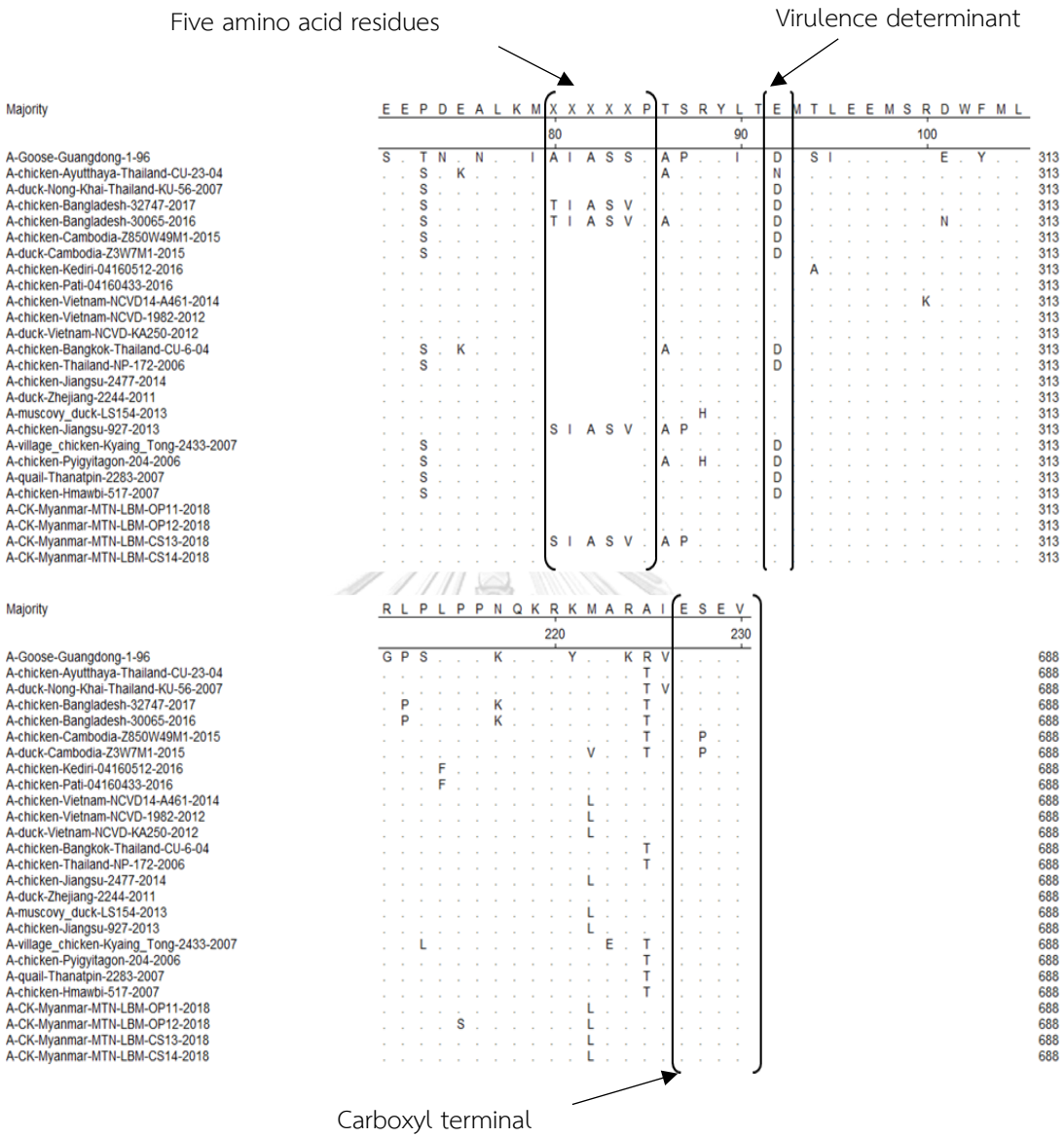

Figure 19. Comparison of deduced amino acids of NS1 protein at virulence determinants (position of 80-85) and C-terminal motif (position 227-230)

Table 15. Genetic analysis of NS1protein and NP protein at the amino acid deletion, virulence determinants and human-avian like amino acids

Virus	NS1 gene			NP gene
	5 amino acid		Virulence	H/A like
	80-84[a]	92[b]	Carboxyl	136[d]
A/Gs/Guangdong/1/1996	No	D	ESEV	M
A/CK/Ayutthaya/Thailand/CU 23/04	Yes	N	ESEV	L
A/CK/Pyigyitagon/204/2006	Yes	D	ESEV	L
A/CK/Thailand/NP 172/2006	Yes	D	ESEV	L
A/Du/Nong Khai/Thailand/KU 56/2007	Yes	D	ESEV	L
A/CK/Hmawbi/517/2007	Yes	D	ESEV	L
A/Qu/Thanatpin/2283/2007	Yes	D	ESEV	L
A/V-CK/KyaingTong/2433/2007	Yes	D	ESEV	L
A/CK/Bangladesh/30065/2015	No	D	ESEV	L
A/CK/Bangladesh/32747/2015	No	D	ESEV	L
A/CK/Jiangsu/927/2013	No	E	ESEV	L
A/Du/Zhejiang/2244/2011	Yes	E	ESEV	L
A/Du/Vietnam/NCVD KA250/2012	Yes	E	ESEV	L
A/CK/Vietnam/NCVD 1982/2012	Yes	E	ESEV	L
A/DU/LS 154/2013	Yes	E	ESEV	L
A/CK/Vietnam/NCVD14/A461/2014	Yes	E	ESEV	L
A/CK/Cambodia/Z850W49M1/2015	Yes	D	EPEV	L
A/CK/Cambodia/Z3W7M/2015	Yes	D	EPEV	**L**
A/CK/Kediri/04160512/2016	Yes	E	ESEV	**L**
A/CK/Pati/04160433/2016	Yes	E	ESEV	**L**
A/CK/Myanmar/MTN-LBM/OR11/2018*	**Yes**	**E**	**ESEV**	**L**
A/CK/Myanmar/MTN-LBM/OR12/2018*	**Yes**	**E**	**ESEV**	**L**
A/CK/Myanmar/MTN-LBM/CS13/2018*	**No**	**E**	**ESEV**	**L**
A/CK/Myanmar/MTN-LBM/CS14/2018*	**Yes**	**E**	**ESEV**	**L**

[a] 5 amino acid deletion in NS1 gene at position 80-84

[b] Virulence determinant amino acids of NS1 gene at position D92E

[c] Carboxy terminal of NS1 protein: ESEV (Glutamic acid, Serine, Glutamic acid, and Valine)

[d] Human-avian like amino acid of NP gene at position 136 (Leucine) and (Methionine)

Characteristics of nucleoprotein gene (NP gene)

Phylogenetic analysis of NP gene

The genetic relatedness of NP gene of H5N1 viruses is shown in Figure 20. All H5N1 viruses clustered with the H5N1 viruses of clade 2.3.2.1c and closely related with A/chicken/Vietnam/NCVD14-A461/2014.

Genetic analysis of NP gene

Genetic analysis of NP protein is shown in Table 14. The genetic characteristics such as avian-like and human-like amino acids at key determinant sites were analyzed. The avian-like characteristics of amino acid residue (Leucine) at position 136 (136L) was found in all H5N1 viruses as shown in Figure 21.

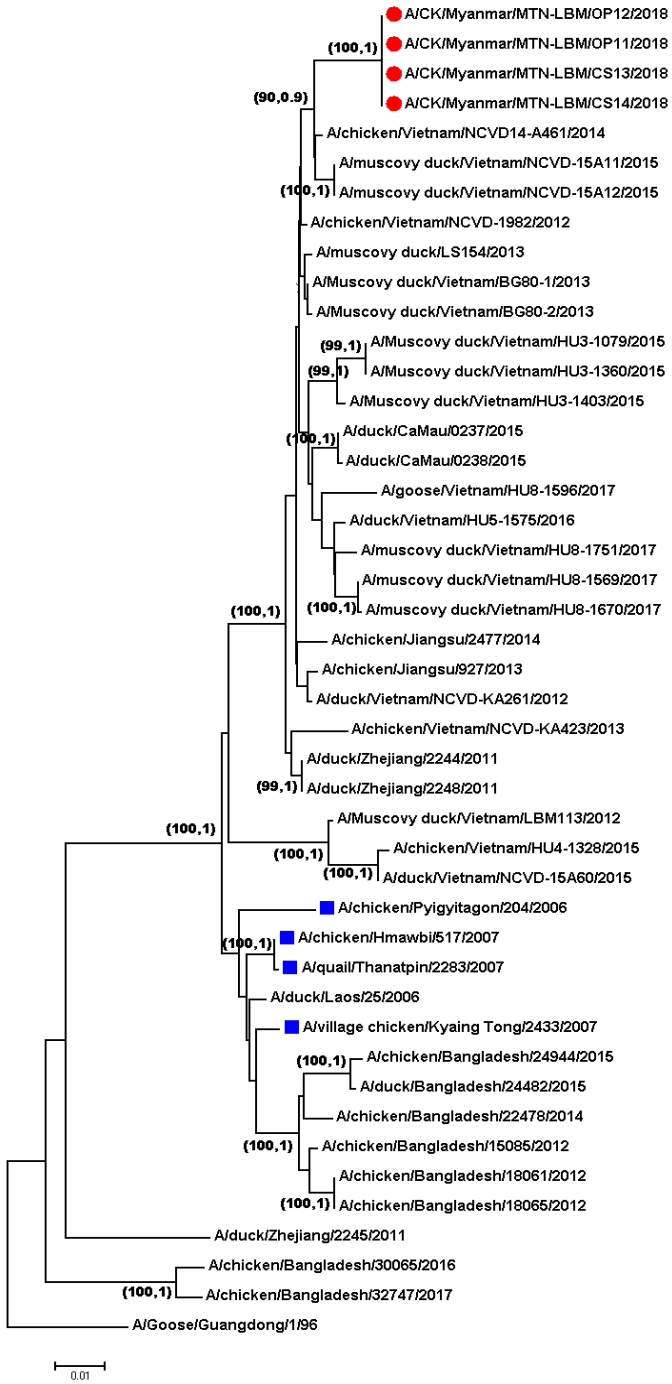

Figure 20. Phylogenetic tree of the NP gene. The phylogenetic tree was generated by using the neighbor-joining algorithm with the Kimura 2-parameter model with bootstrap values of 1,000. The red circles indicate the H5N1 viruses of this study and the blue square boxes indicate the previous Myanmar H5N1 viruses

NP analysis

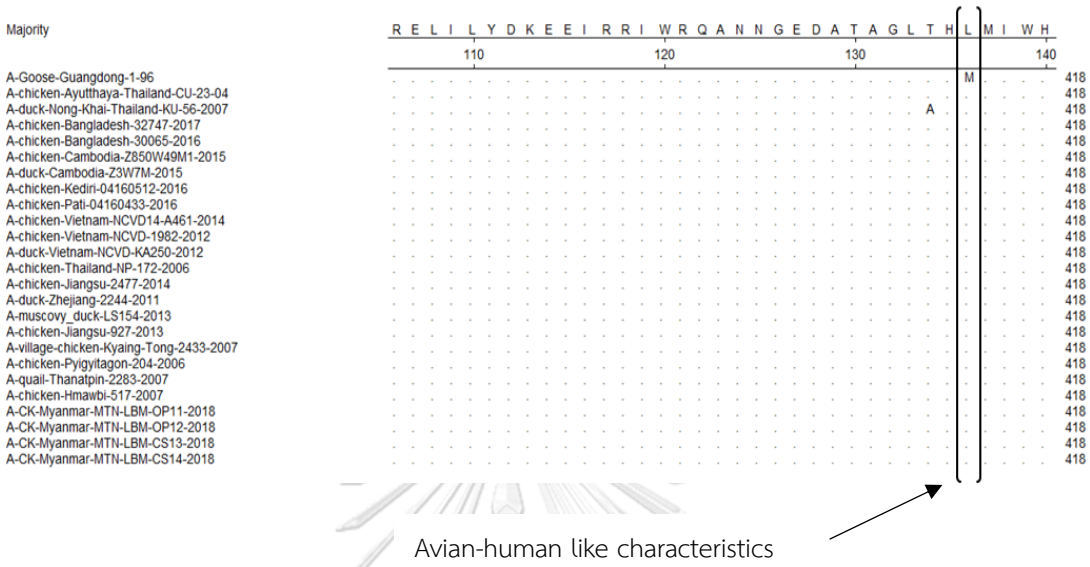

Figure 21. Comparison of deduced amino acids of NP protein at avian and human-like characteristics (position 136)

CHAPTER V

DISCUSSION

In Myanmar, the first avian influenza outbreak was reported in chicken in 2006. Since then, influenza surveillance has been conducting in backyard and commercial poultry as well as in live bird market. Previous study reported that 5.1% (38/738) of avian species from a LBM in Yangon was seropositive for influenza A viruses (Kyaw, 2008). Moreover, HPAI-H5N1, HPAI-H5N6 and LPAI-H9N2 subtypes have been documented in LBMs in Myanmar (Tun Win et al., 2017). However, genetic information of H5N1 viruses from LBM in Myanmar is still limited. In this study, we conducted influenza surveillance in MTN-LBM in Yangon region during December 2017 to December 2018. In total, 455 samples including oropharyngeal swabs (n=185), cloacal swab (n=185) and environmental swabs (n=85) from broiler, layer, backyard and duck species were collected in this study. This thesis also conducted phylogenetic and genetic analyses of the HPAI-H5N1 virus recovered from this LBM. It is noted that H5N1 viruses isolated from LBM in this study were collected from healthy chicken. Thus, farmers, vendors and workers should concern about the poultry carrying HPAI-H5N1 viruses when handling, slaughtering and trading. These may pose a risk to the human who interface with poultry and environment at all level of the LBM.

In this study, 12 out of 91 pooled samples were positive and 7 samples were suspected for influenza A viruses by realtime RT-PCR with Ct values ranging from 20.1-38.9. In detail, 9.8% (9/91) was collected from layer chicken and 1.1% were collected from broiler (1/91), backyard (1/91) and duck (1/91). Based on our findings, it could be concluded that out of four poultry species in LBM, layer chicken and their environment have higher prevalence of influenza A virus. This observation was

consistence with the previous report from Myanmar that most of the HPAI-H5N1 outbreaks were originated from commercial layer farms (Mon et al., 2012). This might be due to longer production time of layer chicken and longer contact with other bird species such as the sparrow, pigeon, crow. Otherwise, the illegal used of influenza vaccine might be another possible explanation. Moreover, domestic poultry and mammals in the farms such as duck, backyard and dogs in medium and small-scale commercial farms may posed the risk of influenza virus transmission.

In this study, 4 HPAI-H5N1 viruses could be successfully isolated from both cloacal swabs (CS) and oropharyngeal swabs (OP) of layer chicken. The realtime RT-PCR showed Ct values of 20.1-26.5. On the other hand, some environmental samples showed realtime RT-PCR positive for influenza A with high Ct values but the virus isolation for these environmental samples were not successful. However, subtyping of these 2 environmental samples were successful and the results showed H5N1 positive. Our result indicated that environment from the LBM including floor, basket, scales and water could be contaminated with H5N1 viruses. Therefore, LBM and their environment play an important role for maintaining and persistence of influenza viruses especially HPAI-H5N1 viruses throughout the year. Our results also suggested that influenza A viruses could be contaminated and sustained in the market chain through the avian species to the environment (Khan et al., 2018). Many previous studies reported multiple influenza A virus subtypes could be simultaneously circulated in the LBMs (Amonsin et al., 2008). In this study, the 4 HPAI-H5N1 viruses could be traced back to the layer farms located in Hmawbi Township, Yangon. Thus, the circulation of influenza viruses in poultry production chain could be continuously entered into the LBMs through infected poultry. Moreover, live bird market closure is not practiced in LBMs in Yangon, Myanmar. This situation may favor the maintenance

of circulation of IAVs in the LBMs and also increased the risk of poultry to poultry and poultry to human transmission.

In this study, the whole genome sequences of 4 HPAI-H5N1 viruses from Myanmar LBM were elucidated. Phylogenetic trees were constructed by comparing 8 genes of 50 HPAI-H5N1 viruses including previous Myanmar H5N1 viruses and H5N1 viruses from China, Thailand, Vietnam and Bangladesh. The selection criteria for the reference viruses were based on the availability of all 8 gene segments, geographically related and trading with Myanmar. The phylogenetic trees of all of 8 genes showed that Myanmar H5N1 viruses were grouped into clade 2.3.2.1c. It has been documented that clade 2.3.2.1 H5N1 viruses were identified for the first time in China in 2004. It was reported that the H5N1 viruses clade 2.3.2.1 was circulating in wild birds during 2009 (Hu et al., 2011). After 2009 the H5N1 viruses clade 2.3.2.1 had evolved into sub-clade of 2.3.2.1a, 2.3.2.1b and 2.3.2.1c (Bi et al., 2015). Among them, clade 2.3.2.1c have caused severe outbreaks in both wild birds and domestic birds along Central Asian flyway (China and Russia) in 2014-2015. It has been known that there are four major migratory flyways which two of them covering of Myanmar (Central Asian Flyway and the East Asian-Australasian Flyway). Based on the previous reports, not only transportation and trade promoted influenza virus among poultry (Magalhaes et al., 2012), but also the migration of wild birds promoted viral spread among regions. Thus, migratory birds could be one of possible factors for the introduction of this HPAI-H5N1 virus into Myanmar. To this fact, monitoring of migratory sites in the country should be routinely conducted during the migratory season in order to reduce the risk to poultry and humans (Meng et al., 2019).

Genetic analyses of each genes of HPAI-H5N1 viruses were reported in this study. The nucleotide sequences of the H5N1 viruses were compared with those of clade 0 (GS/Gd), the previous H5N1 viruses from Myanmar, Vietnam, China, Thailand

and Bangladesh. Based on genetic analysis of HA gene, HPAI-H5N1 viruses possessed multiple basic amino acids (PQRERRRR/G) at HA cleavage site indicating highly pathogenic avian influenza viruses. Amino acid differences were found in this cleavage site between HPAI-H5N1 viruses from this study and Yangon (Hmawbi) H5N1 virus (PLREKRRKR/G). The receptor binding sites of HA1 protein possessed 222Q-224G. This result demonstrated that HPAI-H5N1 viruses remained the same characteristics with preferential binding to avian host cell surface. The glycosylation sites at position 154-156 of HA gene of HPAI-H5N1 viruses showed 154G-156A indicating loss of glycosylation sites resulting in lower virulence viruses. For NA gene analysis, HPAI-H5N1 viruses possessed 20-aa deletion at NA stalk region representing viral adaptation from wild aquatic birds to poultry species (Li et al., 2014). Myanmar HPAI-H5N1 viruses showed both Oseltamivir and Amantadine susceptible. For NS gene, 3 of HPAI-H5N1 viruses (MTN-LBM/OP11/2018, MTN-LBM/OP12/2018, MTN-LBM/CS14/2018) possessed 5 aa deletion suggesting increased virulence in avian and mammalian species. While, 1 of Myanmar HPAI-H5N1 virus (MTN-LBM/CS13/2018) do not contain this deletion suggesting lower virulence. It is noted that Myanmar HPAI-H5N1 viruses contained human and avian-like characteristics at position of 16E, 28V, 55L of M gene, 409S of PA gene and 136L of NP gene.

In Myanmar, HPAI-H5N1 viruses of clade 7, clade 2.3.4, clade 2.3.2 and clade 2.3.2.1c have been reported from 2006 until now. The latest H5N1 outbreak was reported on July, 2017 in Southern Myanmar and HPAI-H5N1 viruses of clade 2.3.2.1c had been identified. Therefore, it is well noted that our finding had confirmed that the recent HPAI-H5N1 viruses circulating in Myanmar was predominated by HPAI-H5N1 viruses of clade 2.3.2.1c. In Myanmar, there is limited genetic information of HPAI-H5N1 clade 2.3.2.1c HPAI H5N1 viruses. Therefore, this study could fulfill the

gap of knowledge of genetic characteristics of Myanmar HPAI-H5N1 of clade 2.3.2.1c isolated from LBM.

Based on the data of World Animal Health Organization (OIE), the H5 subtypes including H5N1, H5N2, H5N6 and H5N8 from more than 20 countries were reported during 2019. These data indicated the important role of H5 viruses in both poultry production and public health significant. Therefore, monitoring of influenza viruses in poultry especially in LBM setting is essential to identify the risk in susceptible avian species and their environment. Early detection of the influenza virus, conducting timely surveillance programs in poultry should be performed. It is also important to timely report the surveillance findings of influenza viruses for the preparedness of public health (Khan et al., 2018). In Yangon, Mingalar-Taung-Nyunt Live Bird Market (MTN-LBM) is the largest and most functioning in Myanmar with approximately 60,000 birds entering daily. Different bird species from different regions such as Hmawbi Livestock zone, Bago Region, Ayeyarwaddy Region are transported to MTN-LBM. Therefore, birds from different biosecurity level are housing together in the vendor shops. Moreover, workers and vendors in LBM are lack of awareness of personal hygiene and biosafety. In Myanmar, agriculture and livestock zones are establishing in order to mitigate and control animal disease outbreaks. However. the system of LBM closure and management pattern are still posing difficulty. The importation of infected raw poultry products, legal or illegal importation of infected live domestic poultry and illegal use of H5N1 vaccine, weak biosecurity and biosafety could be possible factors for influenza virus circulation in Myanmar. It has been reported that Livestock Breeding and Veterinary Department (LBVD) implemented various strategies for prevention and control of HPAI as well as LAPI outbreaks in Myanmar.

Conclusion and recommendations

This surveillance study was conducted in Mingalar-Taung-Nyunt live bird market, Yangon, Myanmar during 2017-2018. In this study, 91 pooled samples were available and tested for influenza A viruses. Among 91 pooled samples, 12 samples were positive for influenza A (M gene). 7 samples were identified as H5N1 subtype and 4 HPAI-H5N1 viruses were successfully isolated. These 4 viruses were subjected to whole genome sequencing by next-generation sequencing and complete genome of those viruses were elucidated.

Phylogenetic analyses showed that Myanmar HPA- H5N1 viruses were clustered into clade 2.3.2.1c and grouped with China and Vietnam viruses. Genetic analysis of HPAI-H5N1 viruses were analyzed and compared with 50 viruses of different countries. Key determinants such as HA cleavage site of HPAI-H5N1 viruses were different from previous Myanmar H5N1 viruses. It is noted that NA stalk region, antiviral drug resistance, virulence determinants and avian and human-like characteristics were still remaining the same. Based on our findings, LBMs could be the reservoirs of influenza virus transmission and threaten to both human and animal health. Thus, subsequently surveillance programs should be carried out not only in livestock sector but also in human sector. Moreover, the result from this study, whole genome sequences of 4 Myanmar HPAI-H5N1 viruses were submitted to the GenBank Database for the scientific community. Monitoring of the circulation, diversity and relatedness of influenza viruses are beneficial for prevention and control of influenza viruses in the LBMs in Myanmar.

To reduce the risk of influenza viruses in MTN-LBM, the suggestions and recommendations are as following;

1. OIE recommendations should be followed such as knowledge about significance of infectious agents and biosecurity practices at all levels of operations in LBM including drivers, owners, handlers and processors should be strengthened.
2. Awareness activities on biosecurity and self-hygiene should be frequently encouraged for public and stakeholders at different levels and different sectors.
3. Birds from suspected and/or infected flocks should not be delivered to LBM.
4. LBM should be frequently emptied, cleaned and disinfected.
5. Surveillance programs should be carried out in LBMs to detect infectious diseases of poultry.
6. Tracing of birds entering and leaving the markets should be accomplished.

The results from this study have provided benefits such as,

1. Whole genome sequencing, phylogenetic characteristics and genetic analysis of HPAI- H5N1 viruses in Myanmar were available. This information present genetic diversity of Myanmar H5N1 from this study and previous Myanmar influenza viruses recovered from LBMs and outbreaks.
2. Advanced molecular characterization technique of influenza viruses such as next generation sequencing, data analysis by CLC software, genetic analysis and phylogenetic analysis software tool can be applicable for further influenza surveillance programs in Veterinary Diagnostic Laboratories in Myanmar.
3. Genetic information of HPAI-H5N1 virus from MTN-LBM from this study were submitted and published in the GenBank database (http://www.ncbi.nlm.nih.gov) for scientific communities.

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