CHAPTER I

1.1 INTRODUCTION

Influenza is an acute contagious disease caused by influenza viruses. The viruses belong to the family *Orthomyxoviridae* which contains 5 genera; influenza A, B and C, thogotovirus and Isavirus (Webster et al., 1992; Krossoy et al., 1999). Influenza A, B and C are classified based on antigenic differences of their nucleopcapsid (NP) and matrix (M) proteins (Webster et al., 1992; Lee and Saif, 2009). All influenza pandemics in human are caused by genus influenza A. Moreover, influenza A viruses (IAV) have been reported to infect large number of animal species (Amonsin et al., 2006b; Amonsin et al., 2007; Taubenberger and Morens, 2010) and are well known as the zoonotic agent. Based on the antigenic properties of hemagglutinin (HA) and neuraminidase (NA) genes, IAV can be classified into 17 HA and 9 NA subtypes (Tong et al., 2012).

In the 20th century, three influenza pandemics were reported; 1) Spanish flu (H1N1) in 1918-1919, 2) Asian flu (H2N2) in 1957-1958 and 3) Hong Kong flu (H3N2) in 1968-1969. Spanish flu caused 20-40 million human deaths or might reach to 100 million, while each of Asian flu and Hong Kong flu caused 1-4 million deaths. These influenza pandemics affected not only the loss of life but also social and economic problems (Johnson and Mueller, 2002; Kilbourne, 2006). Although the origin of 1918 influenza virus is still unclear, some studies showed that hemagglutinin (HA) gene of the 1918 influenza virus may be directly associated with an avian source (Reid et al., 1999; Reid and Taubenberger, 2003; Garten et al., 2009). According to the genetic analysis of HA and neuraminidase (NA) gene, the 1957 and 1968 influenza viruses were derived from avian sources (Belshe, 2005).

The most recent influenza pandemic in human was occurred in 2009. A novel reassorted H1N1 was confirmed as a source of the pandemic. Genetic analysis of the virus revealed that it was derived from three different sources (avian, swine and human influenza) including HA gene from H1N2 classical swine of North American lineage, NA

and M genes from H1N1/H3N2 double reassortant of Eurasian swine lineage and triple reassortant internal gene cassette from H3N2 triple reassortant lineage. Later contains PB1 gene of human influenza virus, NS and NP genes of classical swine influenza virus, and PB2 and PA genes of avian influenza virus (Garten et al., 2009; Morens et al., 2009). The 2009 pandemic H1N1 has spread worldwide in more than 214 countries resulting in over 18,000 human deaths.

In 2011, novel reassortant swine influenza virus (SIV) H3N2 containing with internal genes of the 2009 pandemic H1N1 has been reported (Liu et al., 2011). In the same year, the Center for Disease Control and Prevention (CDC) reported two cases of children infected with swine origin-H3N2 influenza virus. This virus has been identified as "novel reassortant" because its genome contains seven gene segments of triple reassortant SIV H3N2 circulating in North America and matrix gene of the 2009 pandemic H1N1 (CDC, 2011b). In addition, the viruses has been reported of potential human to human transmission (CDC, 2011a).

Avian influenza is an infectious disease of birds caused by IAV. Highly pathogenic avian influenza viruses (HPAI) such as subtype H5 and H7 are responsible for massive economic losses in poultry industry. HPAI subtype H5 and H7 have been classified as list A diseases by the Office International des Epizooties (OIE). List A diseases have been referred to the transmissible diseases that have the potential for very serious and rapid spread and have a major impact on the international trade of animals and animal products. At the present, seven major waves of HPAI H5N1 outbreaks had been reported in Thailand. The first HPAI H5N1 outbreak in bird populations in Thailand was documented in 2004 (Viseshakul et al., 2004). In the same year, the first human case was also reported. It was associated with direct contact with sick chicken (Apisarnthanarak et al., 2004). As of April 2012, the total of 596 human cases infected with HPAI H5N1 have been reported in 15 countries worldwide and 349 among of them have been fatal (WHO, 2012) (Table 1.1).

Wild birds, especially wild waterfowls, are considered the main reservoir of low pathogenic avian influenza viruses (LPAI). Most subtypes of IAV (16 HA and 9 NA) and

some possible HA/NA combinations are known to infect and circulate in wild bird populations (Olsen et al., 2006a; Ip et al., 2008; Spackman, 2009). However, wild bird species may carry influenza viruses without any clinical signs. In this case, the viruses may evolve and become highly pathogenic to domestic poultry (Jourdain et al., 2010). Thus, wild birds can serve as a reservoir of influenza A viruses and a potential source of more pathogenic viruses (Rohani et al., 2009). Significance role of wild birds on avian influenza transmission, infection and evolution have been documented (Webster et al., 1992; Ludwig et al., 1995; Webster et al., 2007).

Quail is another important host for IAV. In 2008, Amonsin and his colleagues reported the presence of HPAI-H5N1 in live-bird markets in Thailand. The result showed that the prevalence of H5N1 virus was 1.4% (12/836). Interestingly, 5 of 12 HPAI-H5N1 were isolated from quails (41.4%, 5/12). Moreover, quails can serve as intermediate hosts or mixing vessel for IAVs due to their suitable for both avian and mammal influenza virus infections (Wan and Perez, 2006; Xu et al., 2007). While, most avian species usually carry one specific receptor for IAV termed sialic acid α2,3-galactose linked receptor (SA α2,3-gal). The respiratory and intestinal tracts of quails contain 2 types of receptors, SA α2,3-gal and SA α2,6-gal that are specific for avian and mammal influenza viruses, respectively.

Even the information of HPAI H5N1 in wild birds and quails has been previously reported in Thailand. However the information of LPAI in both avian populations is still limited. To fulfill and connect the gaps of the information, monitoring and genetic characterization of IAV in wild birds and quails were established. In this study, two high risk areas of Ayutthaya and Suphanburi provinces of central Thailand were selected and monitoring program were conducted in wild bird and quail populations. Sample collection, virus isolation, virus identification and genetic characterization were performed to determine the relationship of epidemiological data and molecular characteristics of IAV from each population. This study could provide the information on the dynamics of infection and genetic characterization of the viruses in wild bird and quail populations. The information gained from this study could help to promote an

awareness of IAV outbreaks and to develop disease prevention and control strategies in poultry in the future.

This dissertation is divided into 5 chapters. Chapter I is the introduction and literature review of IAVs in wild birds, quails and swine. Chapter II describes the diversity and genetic characteristic of IAVs in wild birds in high risk areas. Chapter III presents the diversity and genetic characteristic of IAVs in quails in two farms located in high risk areas. Chapter IV shows the development of high affinity DNA aptamers against SIV in pig. Chapter V is the conclusion of this dissertation.

Table 1.1 Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO (WHO, 2012).

Country	2003		2004		2005		2006		2007		2008		2009		2010		2011		2012		Total	
	case	death	case	death	case	death	case	death	Case	death	case	death	case	death	case	death	case	death	case	death	case	death
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	3	0	6	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	8	8	1	1	20	18
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	1	1	1	1	42	28
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	39	15	6	3	167	60
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	12	10	5	5	188	156
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	3	2
Laos	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	0	0	3	2	123	61
Total	**4**	**4**	**46**	**32**	**98**	**43**	**115**	**79**	**88**	**59**	**44**	**33**	**73**	**32**	**48**	**24**	**62**	**34**	**20**	**12**	**602**	**355**

Note; Total number of cases includes number of deaths. Data were updated on 12 April 2012.

1.2 OBJECTIVES

The objectives of this study are:

1. To describe the diversity and genetic characteristic of IAVs in wild birds.
2. To describe the diversity and genetic characteristic of IAVs in quails.
3. To develop high affinity DNA aptamers against SIV in pigs.

1.3 LITERATURE REVIEW

1.3.1 Morphology of influenza A viruses

Influenza A viruses (IAVs) are members of the family *Orthomyxoviridae*. This family contains 5 genera; influenza A, B and C, thogotovirus and Isavirus (Webster et al., 1992; Krossoy et al., 1999). Classification of the viruses in this family is based on the difference of their host ranges and pathogenicity. Influenza A, B and C are classified based on antigenic differences among their NP and M proteins (Webster et al., 1992; Lee and Saif, 2009). Most of influenza B virus was reported to infect human. Influenza B virus has been isolated from seals but it does not have a natural animal reservoir (Osterhaus et al., 2000). Few studies reported the evidence of influenza C virus in pigs and dogs (Guo et al., 1983; Webster et al., 1992). Only IAV has been reported to infect large numbers of warm-blooded animals including pigs, horses, dogs, cats, tiger, domestic birds and wild birds. Moreover, IAV is the only genus found in avian hosts and has the capability of interspecies transmission (Taubenberger and Morens, 2010).

IAV is an enveloped negative sense-single strand RNA virus with segmented genomes. The virus contains eight gene segments in its genomes encoding at least 12 recognized gene products (Figure 1.1, Table 1.2) (Lamb and Choppin, 1983; Webster et al., 1992; Sanz-Ezquerro et al., 1998; Chen et al., 2001; Steinhauer and Skehel, 2002; Robb et al., 2009; Wise et al., 2009). The virus particle consists of a lipid bilayer envelope derived from host. The lipid bilayer is embedded with three viral glycoproteins; 1) viral receptor HA protein, 2) NA receptor-destroying enzyme, 3) ion channel of M2

protein that inserted into the envelope. RNA segments of influenza A virus are encapsidated by nucleoproteins to form ribonucleotide nucleoprotein complexes associated with polymerase genes (PB1, PB2 and PA) (Webster et al., 1992; Brown, 2000a).

Subtyping of IAV is based on the antigenic properties of HA and NA surface proteins. At the present, there are 17 HA and 9 NA subtypes (Tong et al., 2012). Because IAVs have segmented genome, genetic reassortment is an important mechanism which can rapidly produce the genetic diversity of the virus. This mechanism occurs naturally among IAVs and is important in the emergence of influenza pandemics in human population e.g. the 2009 pandemic H1N1, the first influenza pandemic of the 21st century.

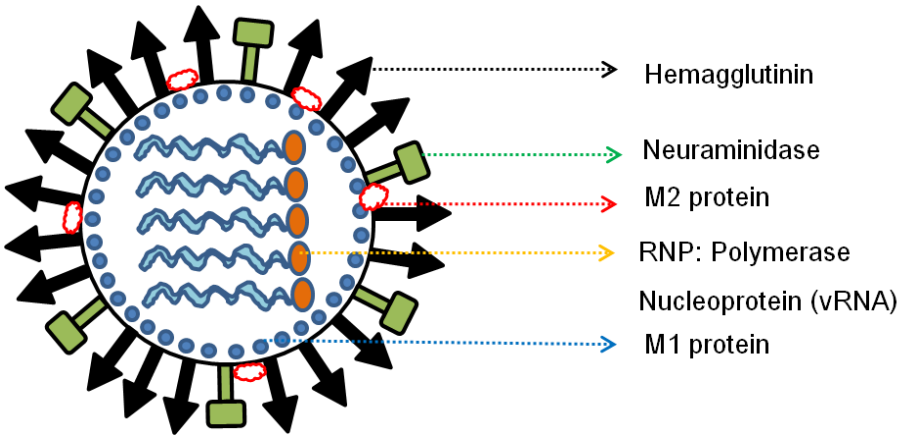

Figure 1.1. Schematic diagram of influenza A virus (modification from Webster et al., 1992)

Table 1.2 RNA segments and gene products of IAVs.

RNA segments	Gene products	Functions	References
1 PB2 (2,341)*	PB2 (759)**	- RNA transcriptase: host-cell RNA cap binding	Lamb and Choppin, 1983
2 PB1 (2,341)	PB1 (757)	- RNA transcriptase: elongation	Steinhauer and Skehel, 2002
	PB1-F2 (79 or 90)	- Proapoptotic virulence factor and proinflammatory effect	Chen et al., 2001
	PB1-N40 (718)	- Detrimental to virus replication - Function has not been fully identified yet	Wise et al., 2009
3 PA (2,233)	PA (716)	- RNA transcriptase, Protease activity - Phosphorylation	Steinhauer and Skehel, 2002 Sanz-Ezquerro et al., 1998
4 HA (1,778)	HA (566)	- Major surface glycoprotein - Binding to host cell surface SA receptors - Fusion between virion envelope and host cell	Lamb and Choppin, 1983 Webster et al., 1992
5 NP (1,565)	NP (498)	- Part of RNA transcriptase complex - RNA binding and nucleocytoplasmic transport of viral RNA - Target of host cytotoxic T-cell immune response	Steinhauer and Skehel, 2002 Webster et al., 1992
6 NA (1,413)	NA (454)	- Surface glycoprotein - Neuraminidase activity: release virus from host cell	Steinhauer and Skehel, 2002
7 M (1,027)	M1 (252)	- Matrix protein: major component of virion - Form a shell surrounding the virion nucleocapsids - Initiate progeny virus assembly	Steinhauer and Skehel, 2002 Webster et al., 1992
	M2 (97)	- Integral membrane protein - Proton channel : control pH of Golgi during HA synthesis - Acidification of interior of the virion during viral uncoating	Steinhauer and Skehel, 2002 Webster et al., 1992
8 NS (890)	NS1 (230)	- Non-structural protein - Regulation of mRNA splicing and translation - Anti-interferon protein: TNFα response	Steinhauer and Skehel, 2002
	NS2/NEP (121)	- Non-structural protein - Nucleocytoplasmic export of viral RNPs - Regulation of virus transcription and translation	Robb et al., 2009

*nucleotide length

**number of amino acid

1.3.2 Avian influenza virus

Avian influenza is an infectious disease of poultry caused by influenza A viruses (IAVs). Highly pathogenic avian influenza (HPAI) virus H5 and H7 are included as an Office International des Epizooties (OIE) List A disease. Since 2004 though 2008, seven major waves of HPAI H5N1 outbreaks were occurred in Thailand especially central part of the country. During each outbreak, many domesticated birds were culled and many poultry farms were closed as part of disease control and prevention. The term HPAI and LPAI have been explained by OIE relate to assessment of pathogenicity in poultry, not human (Table 1.3).

Table 1.3 Pathotypes of avian influenza viruses (WHO, 2009).

LPAI	HPAI
All subtypes of IAVs (H1-H16) including most of H5 and H7	H5 or H7 subtype
Low virulence but can serve as a progenitor for HPAI	Highly virulence
Respiratory disease and egg production drops	Severe systemic disease with very high mortality (except ducks and geese)
High morbidity (>50%), low mortality (<5%)	High mortality rate (~100%) of infected poultry flock
≤ 1.2 IVPI* in 4-to 8-week-old chicken	> 1.2 IVPI 4-to 8-week-old chicken
< 75% mortality rate in 4- to 8-week-old susceptible chickens within 10 days after intravenous inoculation	≥ 75% mortality rate in 4- to 8-week-old susceptible chickens within 10 days after intravenous inoculation
No multiple basic amino acid insertion at the HA cleavage site	Multiple basic amino acid insertion at the HA cleavage site

*IVPI = Intravenous pathogenicity index

1.3.3 Influenza A viruses in wild birds

Wild aquatic birds are considered the main reservoir of LPAI. They carry viruses without any clinical signs. LPAI may evolve in wild birds and become highly pathogenic for domestic poultry (Jourdain et al., 2010). Most subtypes of IAV (16 HA and 9 NA) and some possible HA/NA combinations are known to infect and circulate in several wild bird species (Olsen et al., 2006a; Ip et al., 2008; Spackman, 2009). Moreover, wild birds are also known as a potential source of novel human pathogenic strain (Rohani et al., 2009). It has been documented that wild birds in the order Anseriformes (e.g. duck, geese and swan) and Charadriiformes (e.g. snipe, gull) are the major natural reservoirs of LPAI (Webster et al., 1992). Wild birds in both orders have annual long-distance migratory pattern. This can allow LPAI and HPAI viruses transmission to other domestic birds or different wild bird species especially in feeding or resting areas (Olsen et al., 2006a).

Several studies have been discussed why wild birds are the important reservoir for influenza viruses. For example, first, gastrointestinal tract of birds has evolved to reduce volume and weight. Urine and intestinal contents are mixed at cloacae to form a thin mixture that can be easily to eject. Moreover, the passage time of food from gastrointestinal tract to cloacae of birds is quick because of short intestines. Second, birds have higher metabolic rate and body temperature (up to 43.5 ^{o}C) than humans. High body temperature is associated with the increase-rapid onset and progression of viral infection (Alexander, 1982). Third, because birds have no sweat gland, primary cooling mechanism of birds is a respiratory evaporative heat loss or panting. Since, bird lungs do not change air volume during the respiratory cycle immediately thus there is no flushing effect as in mammalian lungs. These characteristics can help promote the infectious cycle of avian influenza virus and also of other respiratory viruses e.g. infectious bronchitis virus (Alexander, 2000). Fourth, animal behaviors, wild birds prefer to form large flocks for feeding, resting and breeding. These behaviors can help spread of disease very quickly within the population (Kuiken et al., 2006).

At present, influenza surveillance in wild birds can provide the early warning signals to prepare the prevention and control strategies of introduction new IAVs to the areas (Olsen et al., 2006a). Recent study suggests that influenza surveillance programs should be included both cloacal and oropharyngeal swabs to gain an overall picture of the prevalence of IAVs in wild bird population (Jindal et al., 2010).

1.3.4 Influenza A viruses in quails

In 2008, the study on HPAI-H5N1 in Thailand reported the prevalence of virus in live-bird markets as 1.4% (12/836). Interestingly, 41.4% (5/12) of HPAI H5N1 viruses were isolated from quails (Amonsin et al., 2008). Moreover, many studies documented that several subtypes of IAVs could be found in quails in several countries. In Italy, 1999-2000, LPAI H7N1 has mutated to HPAI and then spread rapidly causing over 14 million bird deaths (Capua and Marangon, 2000; Alexander, 2003; Marangon et al., 2003). Quails were one of avian species affected with those outbreaks. In China and other Asian countries, IAV H6N1, H6N2 and H9N2 were well known as the important subtypes widely spread in poultry populations (Capua and Alexander, 2002; Cheung et al., 2007). In July and August 1998, five isolates of H9N2 viruses were identified from human in China. Afterwards, in March 1999, H9N2 viruses were isolated from two girls, age 4 years and 13 months, in Hong Kong (Peiris et al., 1999). Interestingly, these two viral isolates were similar to an IAV H9N2 isolated from quail in Hong Kong in 1997. However six internal genes of the H9N2 viruses from two girls were similar to H5N1 viruses. The similarity of the internal genes of quail virus to those of the H5N1 viruses suggested that virus from quail may be the internal gene donor. The results provided the evidence of reassortment of H9N2 and H5N1 subtypes circulating in chicken in live-bird markets in Hong Kong and consequent transmission to human population (Guan et al., 1999; Lin et al., 2000; Xu et al., 2007).

In general, AIV preferentially bind to sialic acid α2,3-galactose linked receptors (SA α2,3-gal), while human IAVs prefer to bind to sialic acid α2,6-galactose linked

receptors (SA α2,6-gal). It has been documented that SA α2,3-gal and SA α2,6-gal receptors are presented in both trachea and intestine of quails. As quails are susceptible to different subtypes of IAVs, they may pose the suitable characteristics of "an intermediate host" or "a mixing vessel" for virus reassortment and inter-species transmission (Wan and Perez, 2006; Xu et al., 2007). This characteristic is also observed in pigs which can be a potential source of human-avian IAV reassortment (Ito et al., 1998). For example, pig is the hosts responsible for generating influenza pandemics in 1957 and 1968 as well as the recent influenza pandemic in 2009 (Dawood et al., 2009; Garten et al., 2009; Guan et al., 2010).

1.3.5 Influenza A viruses in pigs

Swine influenza is an acute respiratory disease of pigs caused by IAVs. The importance of swine influenza not only causes significant loss in swine industry worldwide but also poses a risk for human public health. In the United State, influenza is considered one of the ten leading causes of death resulting over 50,000 life-year lost in 2010 (Murphy et al., 2012). Recent influenza pandemic in human known as the 2009 pandemic H1N1 was occurred in April 2009. Genetic analysis revealed that the viruses were originated by genetic reassortment among human, swine and avian influenza viruses. In 2011, the novel reassortant SIV H3N2 containing with internal genes of the 2009 pandemic H1N1 has been reported in pigs (Liu et al., 2011). After that zoonotic transmission of the virus was reported in two cases of infected children. Genetic analysis showed that its genome contains seven gene segments of triple reassortant SIV H3N2 circulating in North America since 1998 and matrix gene of the 2009 pandemic H1N1 (CDC, 2011b).

It is documented that pigs can serve as "mixing vessel" to generate novel viruses and transmit to human (Kida et al., 1994; Ito et al., 1998; Ma et al., 2009). Additionally, pigs have receptors for both avian and human influenza viruses that mean pigs are susceptible to infect with many subtypes of avian influenza viruses so far

identified (H1-H16) (Kida et al., 1994). However, pigs serve as major reservoirs of only three subtypes (H1N1, H1N2 and H3N2) which are endemic in swine farm throughout the world (Webster et al., 1992; Kida et al., 1994; Brown, 2000b).

In North America, SIV was first isolated from pigs in 1930 and classified as the classical swine H1N1 lineage. However, swine influenza was clinically recognized in pigs since 1918 (Shope, 1931; Brown, 2000b). In 1998, triple reassortant SIV H3N2 have been circulating in swine herds. The viruses contain PB1, HA and NA genes from human IAV, NP, M and NS genes from classical SIV, and PB2 and PA genes from avian influenza viruses (Webby et al., 2000; Zhou et al., 2000).

In Europe, respiratory diseases are the most important health disorders in finishing pigs. Even it can be caused by bacteria e.g. *Actinobacillus pleuroneumoniae*, IAVs have been identified as the most important causative agent (Loeffen et al., 1999). SIV H1N1 and H3N2 have co-circulated in swine farms since 1970s. Antigenic analysis of the initial SIV H3N2 revealed the virus was closely related to contemporary human influenza H3N2 (Ottis et al., 1982). In 1979, SIV H1N1 occurred naturally in swine herds in Belgium. The virus was antigenically closely related to IAV H1N1 isolated from ducks in North America and Germany (Pensaert et al., 1981). These evidences indicated that IAVs have crossed the species barrier to infect to new host species.

In Asia, particularly in China, classical SIV H1N1 is the predominantly subtype of IAVs isolated from pigs. The evidence of cross species transmission of IAV H1N1 from birds to pigs has been reported in southern China (Guan et al., 1996). Furthermore, sequence analysis of SIV H3N2 isolated in China indicated the introduction of IAV from avian species to pigs (Kida et al., 1988).

In Thailand, the first SIV H3N2 was detected since 1978. Serological and genetic analyses shown the virus was closely related to a contemporary human isolate (Nerome et al., 1981). In 1988, SIV H1N1 was the first isolated from pigs in Chonburi province (Kupradinun et al., 1991). Antigenic and genetic characteristics indicated that the virus was introduced through imported pigs from the United State (Kupradinun et al., 1991). As same in North America and Europe, three subtypes of IAVs (H1N1, H1N2

and H3N2) have been circulating in swine population in Thailand. However, H1N1 and H3N2 are the major subtypes recovered from pig population (Nakharuthai et al., 2008; Takemae et al., 2008; Takemae et al., 2011).

1.3.6 Standard methods for influenza A virus isolation and identification

The conventional techniques used for the diagnosis of IAVs are virus isolation and identification (OIE, 2009). For virus isolation, suspensions in viral transport medium (VTM) of oropharyngeal and cloacal swabs are inoculated into the allantoic cavity of 9-to 11-day-old specific pathogen-free (SPF) embryonated chicken eggs according to WHO/OIE recommendations. The eggs are then incubated at 37 ^{o}C (35-39 ^{o}C) for 3-7 days. The allantoic fluid of all eggs at the end of incubation period including any eggs containing dead or dying embryo are tested for the presence of hemagglutination activity by hemagglutination test (HA test). The allantoic fluids that give a negative reaction for HA test should be inoculated into at least one further passage. Samples that positive for HA test should be submitted to IAV subtype identification. The method recommended by WHO for subtyping of IAVs involves the use of highly specific antisera. Hemagglutination inhibition (HI) test has been used as the standard method for international trade of animals and also used commonly in veterinary diagnostic laboratories worldwide (Schild et al., 1980; OIE, 2009).

Even the conventional techniques are still used as the method of choice for at least the initial diagnosis of IAVs infection, however there tend to be costly, labour intensive, time consuming and requires maintenance of large stock of antisera (Alexander, 2008). Thus, modern diagnostic techniques for rapid detection of IAVs are developed over the past 10 years. Reverse transcription-polymerase chain reaction (RT-PCR) was developed for direct viral RNA detection and subtyping of IAVs using specific primers (Payungporn et al., 2004; Tsukamoto et al., 2008; Tsukamoto et al., 2009). Furthermore, one step-real time RT-PCR (rRT-PCR) was also developed for rapid detection using specific primers and probes (Spackman et al., 2002). However, direct viral RNA detection can be problematic due to the short time period for virus shedding.

Serological assays were also used to detect antibodies producing from infected animals are developed for IAVs surveillances e.g. enzyme-linked immunosorbent assays (ELISA), serum neutralization (SN) assay and HI test (Lee et al., 1993; Leuwerke et al., 2008; Marche and van den Berg, 2010). The optimal specificity and sensitivity of serological assays are still evaluated (Julkunen et al., 1985; Benne et al., 1994; Yoon et al., 2004).

1.3.7 Aptamers for influenza A viruses

Although antibodies have become the essential agents for wide range of application of diagnostic test, they also have some limitations. Antibodies are produced in animal system that difficult and expensive. Antibodies are sensitive and easy to denature with high temperature. They are also irreversible after denaturation. Thus, antibodies have a limited shelf life and not suitable to ship at ambient temperature. Moreover, the performance of the same antibody seems to have a batch-to-batch variation (Jayasena, 1999). Due to the limitations of antibody, a novel methodology called “Systematic Evolution of Ligands by Exponential enrichment; SELEX” was applied to use with IAVs (Jeon et al., 2004; Misono and Kumar, 2005; Gopinath et al., 2006; Cheng et al., 2008).

SELEX involves the selection of target molecules from a large pool of random oligonucleotides flanked with primer binding regions and then followed by PCR to yield the selected nucleic acid species for the next cycle. The cycles of selection and amplification are normally repeated 6-12 times, sometimes more, until the high affinity nucleic acid species called “aptamers” have evolved from the population called “aptamer” (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Schematic representation of SELEX is shown in figure 1.2.

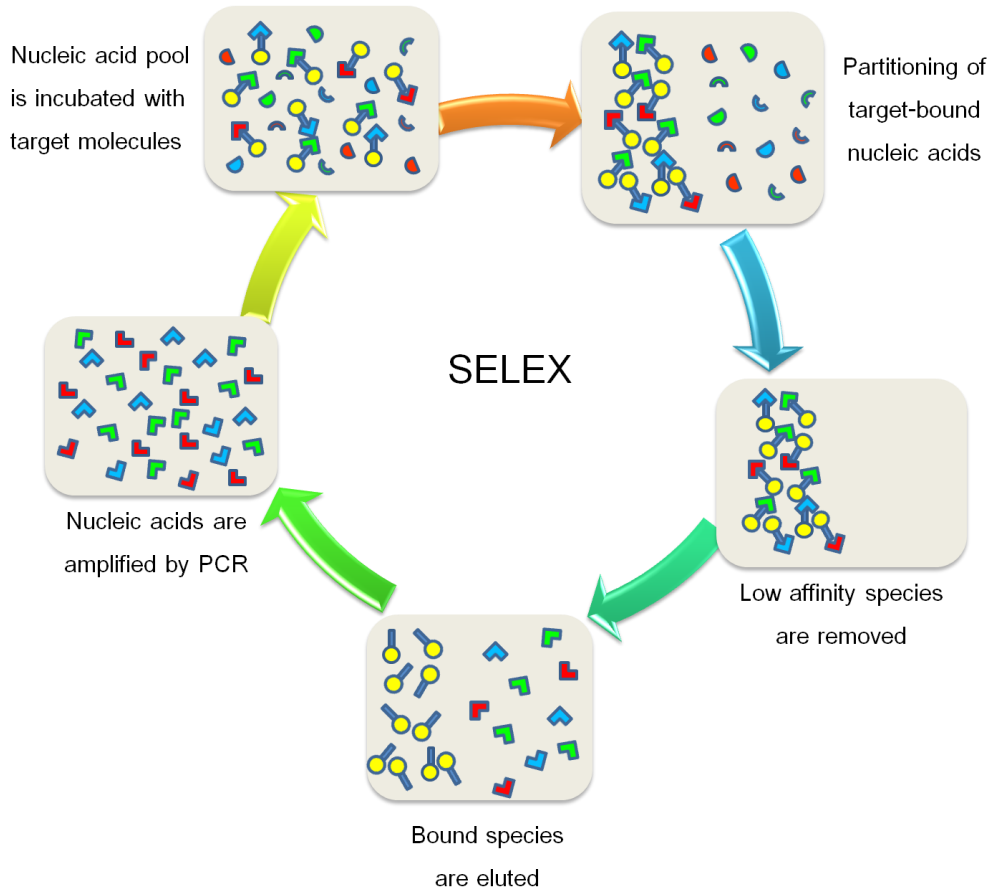

Figure 1.2 Schematic representation of a single SELEX round (modification from James, 2000). a) Nucleic acid libraries are incubated with target molecules, b) target-bound nucleic acid are partitioned, c) low binding affinity molecules are removed, d) the bound species are eluted, and e) the bound species are re-amplification by PCR for the next subsequent iterations.

Aptamers are short, unique, artificial nucleic acid ligands that can bind specifically to target molecule. They can completely be selected and characterized from complex synthetic libraries by *in vitro* process. Because aptamers can be chemically synthesized and all processes were done by *in vitro* techniques thus the physiological effect from animals are excluded (Luzi et al., 2003). They are stable to long-term storage and easily re-synthesized without batch-to-batch variation (Tuerk, 1997; Luzi et al., 2003). Furthermore, aptamers can be attached with reporter molecules such as

fluorescein, digoxigenin and biotin that increase the applicability for further applications (Syed and Pervaiz; Brody and Gold, 2000; Stojanovic et al., 2001; Bunka and Stockley, 2006; Javier et al., 2008; Yan et al., 2011). At present, aptamers are used in a wide range of applications (Ng et al., 2006; Keefe et al., 2010).

Table 1.4 Comparison between aptamers and antibodies (O'Sullivan, 2002)

Antibodies	Aptamers
Antibodies are glycoproteins (immunoglobulin)	Aptamers are oligonucleic acid (DNA or RNA) or peptide molecules
Requires animal system	Aptamers are identified completely through an *in vitro* process not requiring animal system
Limitations against target representing cellular components and toxins	Toxins as well as molecules that do not elicit good immune response can be used to generate high affinity aptamers
Antibodies are sensitive to temperature and easy to denature thus they have limited shelf life	Denatured aptamer can be regenerated, aptamers are stable during long term storage and can be transported at ambient temperature
Antibodies often suffer from batch-to-batch variation	Aptamers are produced by chemical synthesis with little or no batch-to-batch variation
Labeled antibodies might loss in binding affinity	Reporter molecules e.g. fluorescein, digoxigenin and biotin can be attached to aptamers in precise locations not involved in binding

In summary, the information of HPAI H5N1 in wild birds and quails in Thailand has been reported in several studies, but the information of LPAI is still limited. To fulfill and connect the gaps of the information of IAVs in wild bird and quail populations in Thailand, the monitoring programs on both HPAI and LPAI in wild birds and quails were established. Dynamics of infection and genetic characteristics of the viruses were reported in this study. The novel methodology; SELEX have been developed to generate the high affinity nucleic acid species for target molecules called “aptamer”, wide range of applications of aptamers have been documented including with IAVs detection.

CHAPTER II

Genetic characterization of influenza A virus subtype H12N1 isolated from a watercock and lesser whistling-ducks, Thailand

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2.1 Introduction

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae* and have been reported to cause infection in avian and mammalian species including humans. It has been known that wild bird species can serve as an important reservoir of avian influenza viruses. Several studies reported that wild waterfowl are considered the main reservoir of low pathogenic avian influenza (LPAI) viruses. Many of these wild bird species harbor the viruses without any clinical sign (Kida et al., 1980; Webster et al., 1992; Webster et al., 2007; Spackman, 2009). Since several subtypes of IAVs circulate in wild bird populations, the viruses may evolve or reassort and become more virulent for poultry or be potentially transmitted to domestic mammals and humans (Webster et al., 2007; Keawcharoen et al., 2008; Spackman, 2009). The importance of wild birds is they can migrate and distribute the viruses along their movement pathways (Webster et al., 1992; Olsen et al., 2006a; Krauss et al., 2007; Munster et al., 2007), thus surveillance of IAVs in wild bird population should be considered.

Since 2004, seven major waves of highly pathogenic avian influenza (HPAI) H5N1 virus outbreaks have been reported in Thailand. However, information on LPAI viruses circulating in the country is limited. Up to date, in the public databases, LPAI subtypes H3N1, H3N2, H4N6, H4N9, H8N4, H10N3 and H11N3 have been reported in avian species in Thailand (Wisedchanwet et al., 2011a; Wisedchanwet et al., 2011b). The H3N1 subtype is the only LPAI ever reported in wild bird species; A/mynah/Haneda-Thai/1976 (GenBank accession numbers M17070, available only NS gene). However,

IAV subtype H12N1 has never been reported in the country or even in Asia. To our knowledge, IAV subtype H12 was first reported in Canada in 1976 (Hinshaw and Webster, 1979), with the first H12N1 virus isolated from Mallard duck in Canada in 1983 (Hinshaw et al., 1985). Another H12N1 virus has been isolated from moustached warbler (*Acrocephalus melanopogon*) in Slovakia in 2006 (Gronesova et al., 2008). Throughout the past 30 years, based on public databases, numerous other H12 and N1 viruses have been isolated in Eurasia and North America indicating circulation of these subtypes in wild migratory birds. However, genetic information on the whole genome is available for only one H12N1 (A/mallard duck/Alberta/342/1983) in the public database (CY005344-CY005350).

To fulfill the information of IAVs both HPAI and LPAI circulating among wild bird species in Thailand, a 15-month monitoring program was conducted from February 2009 to April 2010. The monitoring program was conducted in 2 HPAI high-risk provinces of central Thailand, Ayutthaya and Suphanburi. In this study, the H12N1 subtype can only be identified and completed whole genome sequencing. Four H12N1 viruses were isolated from swab samples collected from 2 wild bird species, watercock and lesser whistling-duck. The viruses were also subjected to whole genome sequence analyses.

2.2 Materials and methods

2.2.1 Sample collection from wild birds

Two wild bird habitats in two provinces of central Thailand (Ayutthaya and Suphanburi provinces) were selected using the following criteria; 1) the provinces are high risk areas of HPAI-H5N1 outbreaks based on the previous records of HPAI outbreaks in Thailand, 2) participated villagers have expertise in wild bird handling and 3) wild bird species of specific interest were wild waterfowls, however, other species e.g. residential and terrestrial birds in the areas were also sampled.

In this study, we had worked with 2 participated villagers who could perform capture methods that minimize injury to birds. Most of wild birds were taken some

pictures for species identification. All of wild birds were released to their habitats immediately after sample collection. The capture method, species identification and sample collection were shown in figure 2.1.

2.2.1 Influenza A virus isolation

From February 2009 to April 2010, a 15-month monitoring program for IAVs among wild bird species was conducted in 2 provinces of central Thailand, Ayutthaya and Suphanburi, where HPAI-H5N1 outbreaks have previously been reported. Swab samples were collected from trapped wild birds at both locations monthly for 15 months. However, species of wild birds vary depending on seasonal and bird trap activities. Samples were collected from oropharynx and cloaca of individual bird using large- or small-sized sterile polyester tip swabs (depending on bird size) and placed individually in collecting tubes containing viral transport medium (VTM). The swab samples were maintained at 4 ^{o}C during collection and then submitted to the laboratory within 24 hours. Swab samples were stored at -80 ^{o}C until tested. A total of 2,994 swab samples were collected from 47 wild bird species of 25 families, 9 orders (Table 2.1). All swab samples were submitted to the Emerging and Re-emerging Infectious Diseases in Animals, Research Unit, Chulalongkorn University for virus isolation. The supernatant of VTM suspension was inoculated into 10-11 day-old specific pathogen-free (SPF) embryonated chicken eggs according to WHO/OIE recommendations at the Biosafety level 3 laboratory (BSL 3). To minimize false negative or missing some IAVs, in this study, all samples were inoculated at least 2 passages. The allantoic fluid of individual eggs of each passage was collected after 72-hour incubation and tested for the presence of hemagglutinating activity by hemagglutination test (HA test). The samples that positive for HA test at 4 HA unit/50 ul or greater were subsequently subjected to IAVs identification.

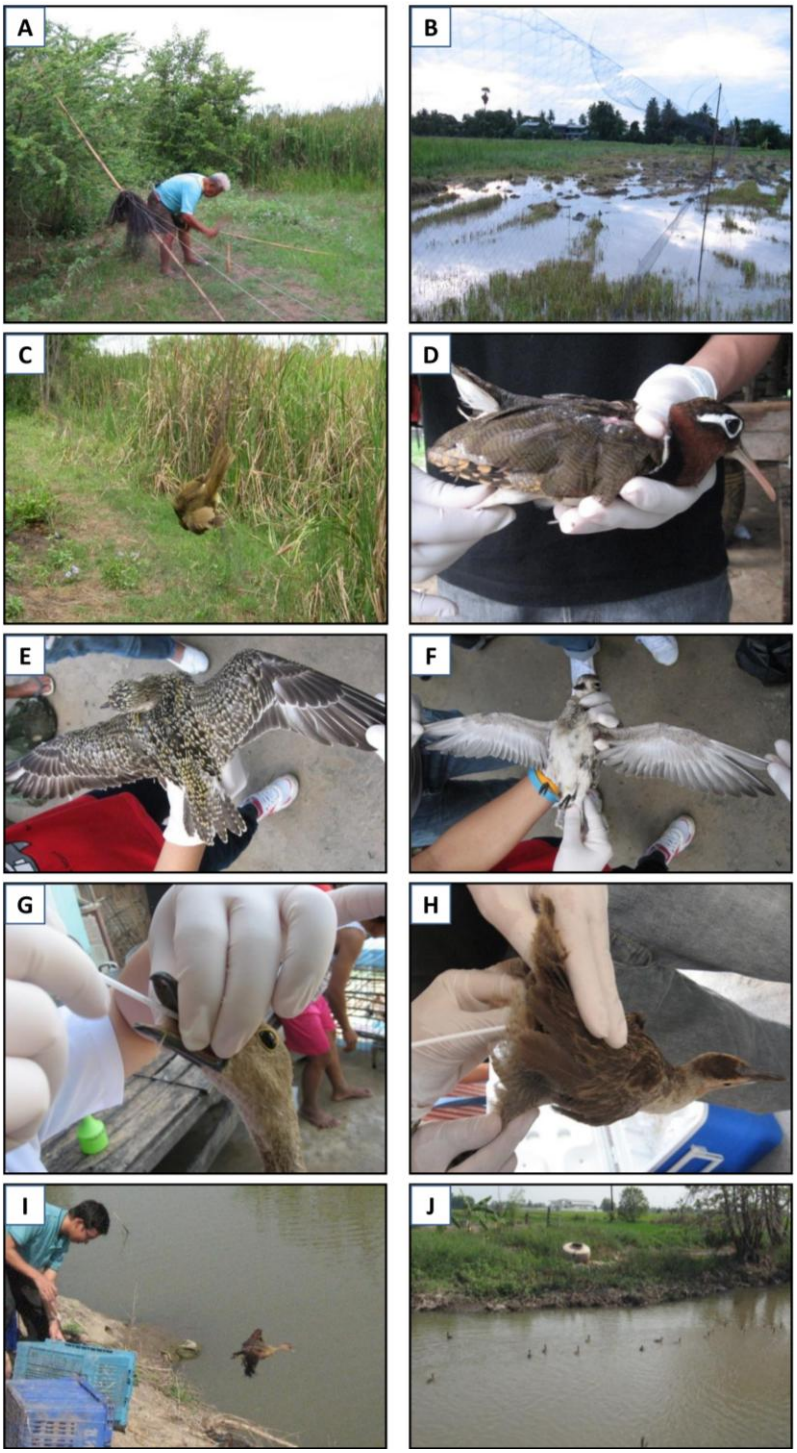

Figure 2.1 Wild birds capture method (A, B, C); species identification (D, E, F); sample collection from oropharynx (G) and cloaca (H); wild birds released after sample collection (I, J).

Table 2.1 Number of wild bird species and number of positive samples for IAVs identified by real time RT-PCR during February 2009 to April 2010.

Order	Family	Common name	Scientific name	Total no. of birds	real time RT-PCR	
					No.pos/ total sample tested	% pos
Anseriformes	Anatidae	Lesser Whistling-duck	*Dendrocygna javanica*	100	9/200	4.50
		Cotton Pygmy-goose	*Nettapus coromandelianus*	3	1/6	16.67
			Total	103	10/206	4.85
Charadriiformes	Scolopacidae	Pintail Snipe	*Gallinago stenura*	50	3/100	3.00
		Common Snipe	*Gallinago gallinago*	8	0/16	0
		Common Greenshank	*Tringa nebularia*	3	0/6	0
	Rostratulidae	Greater Painted-Snipe	*Rostratula benghalensis*	104	4/208	1.92
	Charadriidae	Red-wattled Lapwing	*Vanellus indicus*	26	1/52	1.92
		Pacific Golden Plover	*Pluvialis fulva*	2	0/4	0
		Grey-headed Lapwing	*Vanellus cinereus*	8	0/8	0
	Jacanidae	Bronze-winged Jacana	*Metopidius indicus*	3	1/6	16.67
		Pheasant-tailed Jacana	*Hydrophasianus chirurgus*	1	0/2	0
	Glareolidae	Oriental Pratincole	*Glareola maldivarum*	2	0/4	0

	Recurvirostridae	Black-winged Stilt	*Himantopus himantopus*	2	0/4	0
			Total	197	9/394	2.28
Columbiformes	Columbidae	Red Collared-Dove	*Streptopelia tranquebarica*	173	3/348	0.86
		Spotted Dove	*Stigmatopelia chinensis*	52	3/104	2.88
		Zebra Dove	*Geopelia striata*	119	4/238	1.68
			Total	344	10/688	1.45
Coraciiformes	Alcedinidae	White-throated Kingfisher	*Halcyon smyrnensis*	8	0/16	0
		Black-capped Kingfisher	*Halcyon pileata*	1	0/2	0
	Meropidae	Little Green Bee-eater	*Merops orientalis*	1	0/2	0
	Coraciidae	Indian Roller	*Coracias benghalensis*	1	0/2	0
			Total	11	0/22	0
Cuculiformes	Cuculidae	Asian Koel	*Eudynamys scolopaceus*	2	0/4	0
		Green-biled Malkoha	*Phaenicophaeus tristis*	1	0/2	0
			Total	3	0/6	0
Gruiformes	Rallidae	White-breasted Waterhen	*Amaurornis phoenicurus*	176	14/352	3.97
		Common Moorhen	*Gallinula chloropus*	10	0/20	0
		Watercock	*Gallicrex cinerea*	113	8/226	3.54
			Total	298	22/596	3.69

Passeriformes	Dicruridae	Black Drongo	*Dicrurus macrocercus*	28	1/56	1.78
	Passeridae	Plain-backed Sparrow	*Passer flaveolus*	13	0/26	0
		Eurasian Tree Sparrow	*Passer montanus*	6	0/12	0
	Hirundinidae	Barn Swallow	*Hirundo rustica*	2	0/4	0
	Estrildidae	Scaly-breasted Munia	*Lonchura punctulata*	9	0/18	0
	Sturnidae	White-vented Myna	*Acridotheres grandis*	50	2/100	2.00
		Asian Pied Starling	*Sturnus contra*	190	9/380	2.37
	Pycnonotidae	Streak-eared Bulbul	*Pycnonotus Blanfordi*	31	1/62	1.61
		Sooty-headed Bulbul	*Pycnonotus aurigaster*	5	0/10	0
	Ploceidae	Baya Weaver	*Ploceus philippinus*	96	1/192	0.52
		Streaked Weaver	*Ploceus manyar*	46	0/92	0
	Muscicapidae	Oriental Magpie-Robin	*Copsychus saularis*	4	0/8	0
	Laniidae	Brown Shrike	*Lanius cristatus*	2	0/2	0
			Total	483	14/966	1.45
Strigiformes	Tytonidae	Barn Owl	*Tyto alba*	7	0/14	0
	Strigidae	Collared Scops-Owl	*Otus bakkamoena*	1	0/2	0
			Total	8	0/16	0
Ciconiiformes	Ardeidae	Pond-Heron	*Ardeola spp*	19	1/38	2.63

		Cinnamon Bittern	*Ixobrychus cinnamomeus*	5	0/10	0
		Yellow Bittern	*Ixobrychus sinensis*	10	0/20	0
		Little Egret	*Egretta garzetta*	5	0/10	0
		Cattle egret	*Bubulcus ibis*	1	0/2	0
		Open-billed Stork	Anastomus oscitans	1	0/2	0
		Black-crowned Night-Heron	*Nycticorax nycticorax*	9	0/18	0
			Total	50	1/100	1.00
9 orders	25 families	47 species	**Total**	1,497	62/2,994	2.07

2.2.2 Influenza A virus identification and subtype determination

To identify IAVs, viral RNA was extracted from the allantoic fluid of HA-positive samples using the QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany) and confirmed as IAVs by real-time reverse transcription-PCR (rRT-PCR) using a TaqMan probe specific for the matrix (M) gene of IAVs (Spackman et al., 2002). To subtype IAV, cDNA synthesis was performed using the universal influenza oligonucleotide primer Uni12. The cDNA was used as template for RT-PCR with primers specific for the HA and NA genes (Tsukamoto et al., 2008; Tsukamoto et al., 2009).

2.2.3 Whole genome sequencing of influenza A virus

After confirming subtype, oligonucleotide primer sets were designed for whole genome sequencing by using Primer3 Input (v. 0.4.0). In addition, the primer sets for six internal genes were also obtained either from primer inventories or newly designed. The list of oligonucleotide sequences used in this study is shown in table 2.2. Whole genome sequencing of the viruses was conducted by amplifying each gene of the viruses. The resulting PCR products were then subjected to DNA sequencing using the Big Dye Terminator v.3.0 Cycle Sequencing Ready reaction (ABI, Foster city, CA). The nucleotide sequences of each gene were validated and assembled using SegMan software v.5.03 (DNASTAR Inc., Madison, WI, USA).

Table 2.2 Oligonucleotide primers used for whole genome sequencing of IAV subtype H12N1.

Genes	Forward primers	Reverse primers	Expected size [bp]
PB2	PB2F1 5'- AAT GTC ACA GTC CCG CAC TC -3'	PB2R1 5'- TCT GCA TGG CCA GGA TTT AT -3'	429
	PB2F2 5'- AGC RAA AGC AGG TCA AWT A -3'	PB2R2 5'- CCC ATT GCT GCY TTG CAT -3'	974
	PB2F3 5'- GGA TGG TRG ACA TYC TTA -3'	PB2R3 5'- GGT TCA AAY TCC ATC TTA TTG T -3'	859
	PB2F4 5'- CAA TGA TGT GGG ARA TCA A -3'	PB2R4 5'- TGG CCA TCA GTA GAA ACA A -3'	722
PB1	PB1F1 5'- TGA ATG GAT GTC AAY CCG ACT -3'	PB1R1 5'- GAT TTG CAT TCC GGG TGT T -3'	747
	PB1F2 5'- KCA CTG ACA CTG AAC ACA WTG -3'	PB1R2 5'- AAC ATG CCC ATC MTC ATT CC -3'	574
	PB1F3 5'- CAT GTT CGA AAG TAA GAG CAT GA -3'	PB1R3 5'- CCA TCY GAA ACC AMC AGT CCT -3'	717
	PB1F4 5'- TGG ATT TGT RGC CAA TTT CAG -3'	PB1R4 5'- TGA GYT CTT CAA TGG TGG AA -3'	764
PA	PAF1 5'-GCR AAA GCA GGT ACT GAT CC-3'	PAR1 5'-TGA GAA AGC TTG CCC TCA AT-3'	774
	PAF2 5'-ACC GAA CTT CTC CAG CCT TG-3'	PAR2 5'-TCA TAC TYG CAA TGT GCT CAA-3'	665
	PAF3 5'-TGA GAA YAT GGC ACC RGA GA-3'	PAR3 5'-CAA TYT GTT GAA GRG AYT GAA-3'	665
	PAF4 5'-ATG AAA TGG GGS ATG GAA AT-3'	PAR4 5'-TTT TTG GAC AGT ATG GAT AGC A-3'	450
HA	H12F1 5'- TTC TAA CAA CTG GTC TTG CTT ATG A -3'	H12R1 5'- AYC CTG GRT AGC AGA TTC CT -3'	303
	H12F2 5'- CCC AAA TGC GAT CTT TAT CTG -3'	H12R2 5'- TAA TCC ATC CTC CCT TGC TG -3'	468
	H12F3 5'- ATC ACC CAC CSA CTT CTG AC -3'	H12R3 5'- TTC TAC TTT CAA CCT CCG AAA A -3'	673
	H12F4 5'- AGC ACC CAG AAG GCA ATG GA -3'	H12R4 5'- CAA CGA ACA TTT CCA TTT TGA -3'	576

NP	NPF1 5'- CTC AAG GCA CCA AAC GAT CT -3'	NPR1 5'- GTT GCG TCC TCT CCA TTG TT -3'	468
	NPF2 5'- TGG GTG AGA GAG CTA ATT CTG -3'	NPR2 5'- CTT TGT GCT GCT GTT TGG AA -3'	560
	NPF3 5'- TTT CTG GAG AGG CGA AAA TG -3'	NPR3 5'- TCC TCT TGG GAC CAC TCT TG -3'	472
	NPF4 5'- GAG AAT CCA GCA CAT AAG AGT CAA -3'	NPR4 5'- GCA TTG TCT CCG AAC AAA TAA GA -3'	540
NA	N1F1 5'- AGC AAA AGC AGG AGT TTA AAA TG -3'	N1R1 5'- TGA TAG TGT CTG TTA TTA TGC C -3'	600
	N1F2 5'-TGT TGC TTG GTC AGC AAG TG-3'	N1R2 5'-TTG AGC CAT GCC AAT TAT CC-3'	386
	N1F3 5'-TCA CTA TGA GGA ATG CTC CTG-3'	N1R3 5'-CAC TGA AAA GCT ACT GTC CGT TT-3'	388
	N1F4 5'-CCA TGG GTA TCT TTC AAT CAA-3'	N1R4 5'-TCA ATG GTG AAT GGC AAC T-3'	500
M	MF1 5'- GGA GCR AAA GCA GGT AGA TR -3'	MR1 5'- TCT GCT CCA TAG CCT TWG CYG -3'	603
	MF2 5'- CCA GTG ARC GAG GAC TGC -3'	MR2 5'- TGT TGA CAA AAT GAC CAT CG -3'	765
NS	NSF1 5'- AGC RAA AGC AGG GTG ACA AA -3'	NSR1 5'- TCC ATT CAA RYC CTC CGA TG -3'	588
	NSF2 5'- CTT CGY CGA GAT CAG AAG TC -3'	NSR2 5'- TYA YTA AAT AAG CTG AAA CGA GAA -3'	737

2.2.4 Phylogenetic and genetic analyses of influenza A virus

Phylogenetic and genetic analyses were performed by comparing each gene segment of the viruses with those of IAVs from the GenBank database. Reference isolates were selected from different strain based on geographical origin. However, reference isolates from different subtypes were included to clarify the overall picture. Nucleotide sequences of each selected gene were aligned using Muscle v.3.6 (Edgar, 2004). Phylogenetic tree and molecular evolutionary analyses were conducted using the Bayesian Markov chain Monte Carlo (BMCMC) method (Drummond and Rambaut, 2007) and Molecular Evolutionary Genetics Analysis software, MEGA4 (Tamura et al., 2007). The nucleotide/deduced amino acid sequences of each gene and the amino acid sequence at the HA cleavage site of the viruses were aligned and compared for genetic analysis using MegAlign software v.5.03 (DNASTAR Inc.).

2.2.5 Nucleotide sequence accession numbers

The nucleotide sequences of the Thai H12N1 viruses were submitted to GenBank under accession numbers JN982500-JN982531.

2.3 Results

2.3.1 Influenza A virus subtype H12N1 from watercock and lesser whistling-ducks

Based on rRT-PCR, 67 out of 2,994 swab samples (2.07%) were positive to M gene of influenza viruses (Table 2.1). However, most of the samples cannot be identified the specific subtypes because of low virus titer. In this study, IAV subtype H12N1 can only be identified and completed whole genome sequencing. The H12N1 viruses were isolated from oropharyngeal swab of watercock (order *Gruiformes*, family *Rallidae*) (n=1) and cloacal swabs of lesser whistling-ducks (order *Anseriformes*, family *Anatidae*) (n=3) from one location of Ayutthaya province, central Thailand. The viruses were confirmed as IAVs by rRT-PCR using M gene specific PCR primers and hydrolysis probe (Spackman et al., 2002) and subsequently subtyped as H12N1 IAV by RT-PCR using subtype specific primers (Figure 2.1). A more detailed description of the H12N1 viruses

including collection date, host species, type of samples, location and GenBank accession numbers has been provided (Table 2.3).

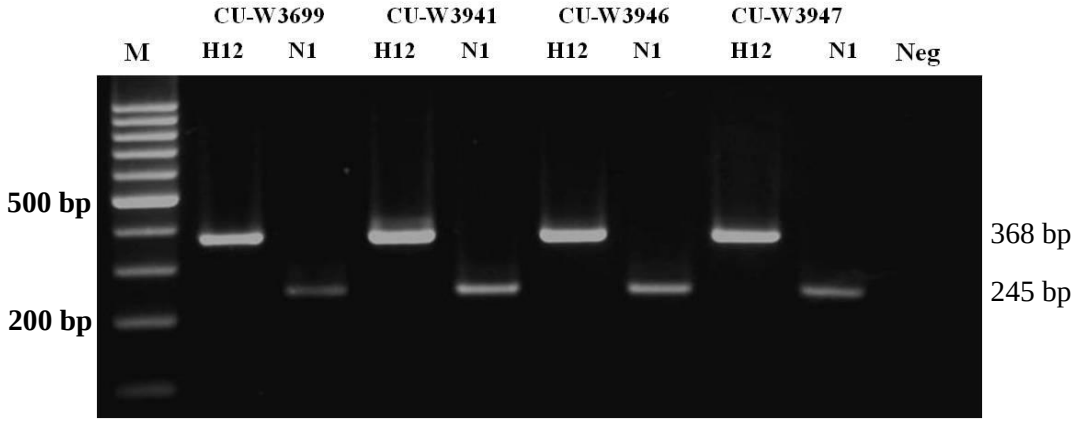

Figure 2.2 Subtype specific amplification by RT-PCR using a subtype specific primer set. Thai H12N1 viruses were positive for H12 and N1 subtypes. M; 100 bp marker, H12 (expected size 368 bp) and N1 (expected size 245 bp).

Table 2.3 Detailed description of Thai H12N1 viruses including collection date, host species, type of samples, location and GenBank accession numbers

Virus name	Collection date	Host common name	Host species	Type of sample*	Location	GenBank accession no.
CU-W3699	11 Dec 2009	Watercock	*Gallicrex cinerea*	OPS	Ayutthaya	JN982500-07
CU-W3941	26 Jan 2010	lesser whistling-duck	*Dendrocygna javanica*	CS	Ayutthaya	JN982508-14
CU-W3946	26 Jan 2010	lesser whistling-duck	*Dendrocygna javanica*	CS	Ayutthaya	JN982515-23
CU-W3947	26 Jan 2010	lesser whistling-duck	*Dendrocygna javanica*	CS	Ayutthaya	JN982524-31

*OPS= oropharyngeal swab; CS= cloacal swab

2.3.2 Whole genome sequences of Thai H12N1

In the present study, the viruses were designated as A/watercock/Thailand/ CU-W3699/2009; (CU-W3699), A/lesser whistling-duck/Thailand/CU-W3941/2010; (CU-W3941), A/lesser whistling-duck/Thailand/CU-W3946/2010; (CU-W3946) and A/lesser whistling-duck/Thailand/CU-W3947/2010; (CU-W3947).

To provide the information on the most closely related gene segments available in the GenBank database, nucleotide and deduced amino acid sequence similarities of each gene of the viruses were generated by using NCBI nucleotide BLAST program. Analysis of sequence identities showed that Thai H12N1 viruses display the most pronounced nucleotide and amino acid similarities to avian influenza viruses isolated in Eurasia. For example, the HA and NA genes of the H12N1 viruses display the highest nucleotide and amino acid similarities to A/mallard/Sweden/86/03 (H12N5) at 95.8% (amino acid identities, 98.2%) and A/aquatic bird/India/NIV-17095/2007(H11N1) at 97.7% (amino acid identities, 98.7%), respectively (Table 2.4).

Pairwise sequence comparison of Thai H12N1 viruses with the only one H12N1 isolate available in the database, A/Mallard Duck/Alberta/342/83 (H12N1) (Mallard/ALB/83) was also performed. The results showed that the nucleotide identities among Thai H12N1 viruses were ranging from 80.4-98.9% (amino acid identities, 75.8-100%) (Table 2.5). These results provide evidence that Thai H12N1 viruses are not closely related to another H12N1 virus isolated nearly 30 years prior and the closest nucleotide/amino acid identities of Thai H12N1 viruses are to gene segments from various viruses isolated from birds throughout Eurasia (Table 2.4 and 2.6).

Table 2.4 Nucleotide and amino acid identities (%) of the Thai H12N1 viruses compared with reference viruses in the GenBank database

Genes	Virus with the highest percentage of nucleotide identity	GenBank accession no.	Nucleotide and amino acid identities (%)			
			CU-W3699	CU-W3941	CU-W3946	CU- W3947
PB2	aquatic bird/Korea/w347/2008(H5N2)	GU361424	97.6 (98.0) [1-2257]	98.1 (98.1) [33-2280]	98.4 (98.5) [28-2280]	97.9 (98.7) [20-2260]
PB1	goose/Italy/296426/03(H1N1)	FJ432784	97.6 (99.5) [1-2260]	97.6 (99.2) [11-2301]	97.6 (99.2) [11-2301]	97.6 (99.5) [11-2258]
PB1-F2	mallard/Netherlands/28/2006(H3N1)	CY076911	96.3 (87.9) [95-367]	- -	- -	- -
	migratory duck/HK/MP5883/2005(H5N8)	EF597465	- -	98.5 (94.5) [95-367]	98.5 (94.5) [95-367]	98.5 (94.5) [95-367]
PA	duck/Jiang Xi/2374/2005(H3N6)	EF597408	98.6 (99.6) [1-2151]	98.7 (99.7) [1-2151]	98.6 (99.6) [1-2151]	98.5 (99.7) [1-2193]
HA	mallard/Sweden/86/03(H12N5)	CY060390	96.0 (98.4) [29-1679]	96.0 (98.4) [28-1695]	96.0 (98.4) [28-1666]	95.8 (98.2) [29-1680]
NP	spot-billed duck/Korea/546/08(H6N1)	GQ414896	98.1 (98.4) [8-1479]	98.8 (100.0) [1-1469]	98.6 (99.6) [1-1478]	98.4 (99.2) [7-1479]
NA	A/aquatic bird/India/NIV-17095/2007(H11N1)	CY055177	97.9 (98.9) [1-1406]	97.9 (98.7) [1-1403]	97.6 (98.9) [1-1403]	97.7 (98.7) [1-1401]

MP	mallard/Netherlands/20/2005(H12N8)	CY076969	98.0 (98.4)	-	-	-
			[1-967]	-	-	-
	env/Dongting Lake/Hunan/3-9/07(H10N8)	GQ325648	-	98.6 (99.1)	99.0 (99.4)	98.9 (99.1)
			-	[1-973]	[1-968]	[1-966]
MP1	aquatic bird/Korea/w120/2006(H5N2)	GU361272	97.1 (98.8)	-	-	-
			[1-759]	-	-	-
	aquatic bird/Korea/w193/07(H5N2)	GU361280	-	99.3 (100.0)	99.3 (100.0)	99.3 (100.0)
			-	[1-759]	[1-759]	[1-759]
MP2	A/chicken/Singapore/1994(H7N1)	EU014140	99.3 (98.9)	-	-	-
			[1-26,715-967]	-	-	-
	chicken/Singapore/93(H10N5)	EU014145	-	99.6 (100.0)	99.6 (100.0)	99.3 (100.0)
			-	[1-26,715-919]	[1-26,715-967]	[1-26,715-966]
NS	mallard/Sweden/65/2002(H10N9)	CY055179	98.4 (100.0)	98.4 (100.0)	98.4 (100.0)	98.4 (100.0)
			[1-831]	[1-831]	[1-831]	[1-831]
NS1	aquatic bird/India/NIV-17095/07(H11N1)	CY055179	98.8 (100.0)	98.8 (100.0)	98.8 (100.0)	98.8 (100.0)
			[1-693]	[1-693]	[1-693]	[1-693]
NS2	mallard/Sweden/65/02(H10N9)	CY060362	99.2 (100.0)	99.2 (100.0)	99.2 (100.0)	99.2 (100.0)
			[1-30,503-831]	[1-30,503-831]	[1-30,503-831]	[1-30,503-831]

(-) Amino acid identities; [-] Region of comparison

Table 2.5 Nucleotide and amino acid identities (%) of Thai H12N1 compared with Mallard/ALB/83

Mallard/ALB/83 (H12N1)													
Samples	Nucleotide and amino acid identities (%) compared with Mallard/ALB/83 (H12N1)												
	PB2	PB1	PB1-F2	PA	HA	NP	NA	MP	MP1	MP2	NS	NS1	NS2
CU-W3699	83.1 (98.5) [1-2257]	88.5 (99.2) [1-2260]	93.0 (81.3) [95-367]	92.1 (98.8) [1-2151]	80.7 (93.6) [29-1679]	89.7 (97.8) [8-1479]	86.6 (95.3) [1-1406]	92.0 (98.1) [1-967]	90.1 (99.2) [1-759]	98.6 (100) [1-26,715-967]	93.0 (94.9) [1-831]	93.5 (100) [1-693]	96.1 (100) [1-30,503-831]
CU-W3941	84.4 (98.7) [33-2280]	88.7 (98.9) [11-2301]	91.2 (75.8) [95-367]	92.1 (98.9) [1-2151]	80.4 (93.5) [28-1687]	90.1 (99.4) [1-1469]	86.5 (95.1) [1-1403]	92.5 (98.5) [1-973]	91.3 (99.2) [1-759]	98.9 (100) [1-26,715-971]	93.0 (94.9) [1-831]	93.5 (100) [1-693]	96.1 (100) [1-30,503-831]
CU-W3946	84.4 (99.1) [28-2280]	88.7 (98.9) [11-2301]	91.2 (75.8) [95-367]	92.1 (98.7) [1-2151]	80.5 (93.4) [28-1666]	90.1 (99.0) [1-1478]	86.4 (95.3) [1-1403]	93.0 (98.8) [1-968]	91.3 (99.2) [1-759]	98.9 (100) [1-26,715-967]	93.0 (94.9) [1-831]	93.5 (100) [1-693]	96.1 (100) [1-30,503-831]
CU-W3947	83.6 (99.2) [20-2260]	88.3 (99.2) [11-2258]	91.2 (75.8) [95-367]	92.1 (98.7) [1-2151]	80.6 (93.3) [29-1680]	90.0 (98.6) [7-1479]	86.4 (95.1) [1-1401]	92.9 (98.4) [1-966]	91.3 (99.2) [1-759]	98.6 (100) [1-26,715-966]	92.9 (93.5) [1-831]	93.5 (100) [1-693]	95.8 (100) [1-30,503-831]

Table 2.6 Deduced amino acid sequences at the HA cleavage site of the Thai H12N1 viruses compared with H12 subtype viruses accessible at GenBank

Viruses	Subtypes	Strain	Amino acid sequence at HA cleavage site	GenBank accession no.
CU-W3699	H12N1	Eurasian	PQAQDR/G	This study
CU-W3941	H12N1	Eurasian	PQAQDR/G	This study
CU-W3946	H12N1	Eurasian	PQAQDR/G	This study
CU-W3947	H12N1	Eurasian	PQAQDR/G	This study
Mallard duck/Alberta/342/83*	H12N1	North American	PQVQNR/G	CY006006
Duck/Alberta/60/76**	H12N5	North American	PQVQDR/G	AB288334
Mallard/Switzerland/WV4060166/06	H12N2	Eurasian	PQAQDR/G	GQ415321
Teal/Norway/10_1836/06	H12N2	Eurasian	PQAQDR/G	FM179754
Bar head goose/Mongolia/143/05	H12N3	Eurasian	PQVQDR/G	GQ907286
Duck/Hokkaido/66/01	H12N5	Eurasian	PQVQDR/G	AB288843
Chicken/THA/CUK2/04	HPAI H5N1	Eurasian	PQRERRRKKR/G	AY590568
Chicken/Rostock/8/34	HPAI H7N1	Eurasian	PEPSKKRKKR/G	M24457

*The only isolate of H12N1 viruses accessible at GenBank

**The first H12 subtype.

2.3.3 Phylogenetic and genetic analysis of Thai H12N1

Genetic relatedness of the Thai H12N1 was analyzed by comparing each gene of the H12N1 viruses with those of reference IAVs in the database. Phylogenetic tree of H12 gene was conducted including with all H12 reference isolates in Eurasia and some representatives of H12 reference isolates in North America. Phylogenetic tree of N1 gene was conducted including with some representatives of N1 reference isolates of each subtype in Eurasia and North America and some reference HPAI H5N1 isolates in Thailand. Phylogenetic trees of each gene were generated using the Bayesian Markov chain Monte Carlo (BMCMC) method (Drummond and Rambaut, 2007) with 40,000 generations and an average standard deviation of split frequencies < 0.05 and Molecular Evolutionary Genetics Analysis software, MEGA4 (Tamura et al., 2007) with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities. Phylogenetic analysis of the H12 genes revealed that the Thai H12N1 viruses were grouped into the Eurasian strain of avian influenza viruses (Figure 2.2). A similar result was also shown by phylogenetic analysis of the N1 gene in that N1 of the Thai H12N1 viruses was closely related to the viruses in the Eurasian strain (Figure 2.3). Phylogenetic analyses of six additional internal genes of the Thai H12N1 viruses also showed similar results to the HA and NA genes in that they were grouped into the Eurasian strain (Figure 2.4).

Genetic analysis of the HA gene showed that the Thai H12N1viruses did not contain any multiple amino acid insertions at the HA cleavage site indicating low pathogenic characteristics. On the other hand, the Thai HPAI H5N1 (A/chicken/THA/CUK2/04) and pathogenic H7N1 (chicken/Rostock/8/34) contained multiple basic amino acids (Table 2.6). The HA cleavage site of the H12N1 viruses displayed a -P-Q-A-Q-D-R/G- motif which differ from those of the North American viruses, Mallard/ALB/83 (H12N1) and Duck/ALB/76 (H12N5). However, this amino acid motif was similar to several viruses isolated from the European strain e.g. mallard/Sweden/86/03 (H12N5), mallard/Switzerland/4060166/06 (H12N2) and tern/Norway/10/1836/06 (H12N2) (Table

2.6). The result indicated that amino acid motifs at the HA cleavage site of the Thai H12N1 were similar to those of H12N2 of Eurasian strain viruses.

Figure 2.3 Phylogenetic analysis of the H12 gene of IAVs. All H12 isolates in Eurasia and some representatives of H12 isolates in North America were chosen to include in the analysis. The tree was constructed with two different software applications using MEGA v.4 with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities and BMCMC with 40,000 generations and an average standard deviation of split frequencies < 0.05 (bootstrap percentage, posterior probabilities). Thai H12N1 viruses are indicated by triangles and the only H12N1 virus in the GenBank database is indicated by a circle.

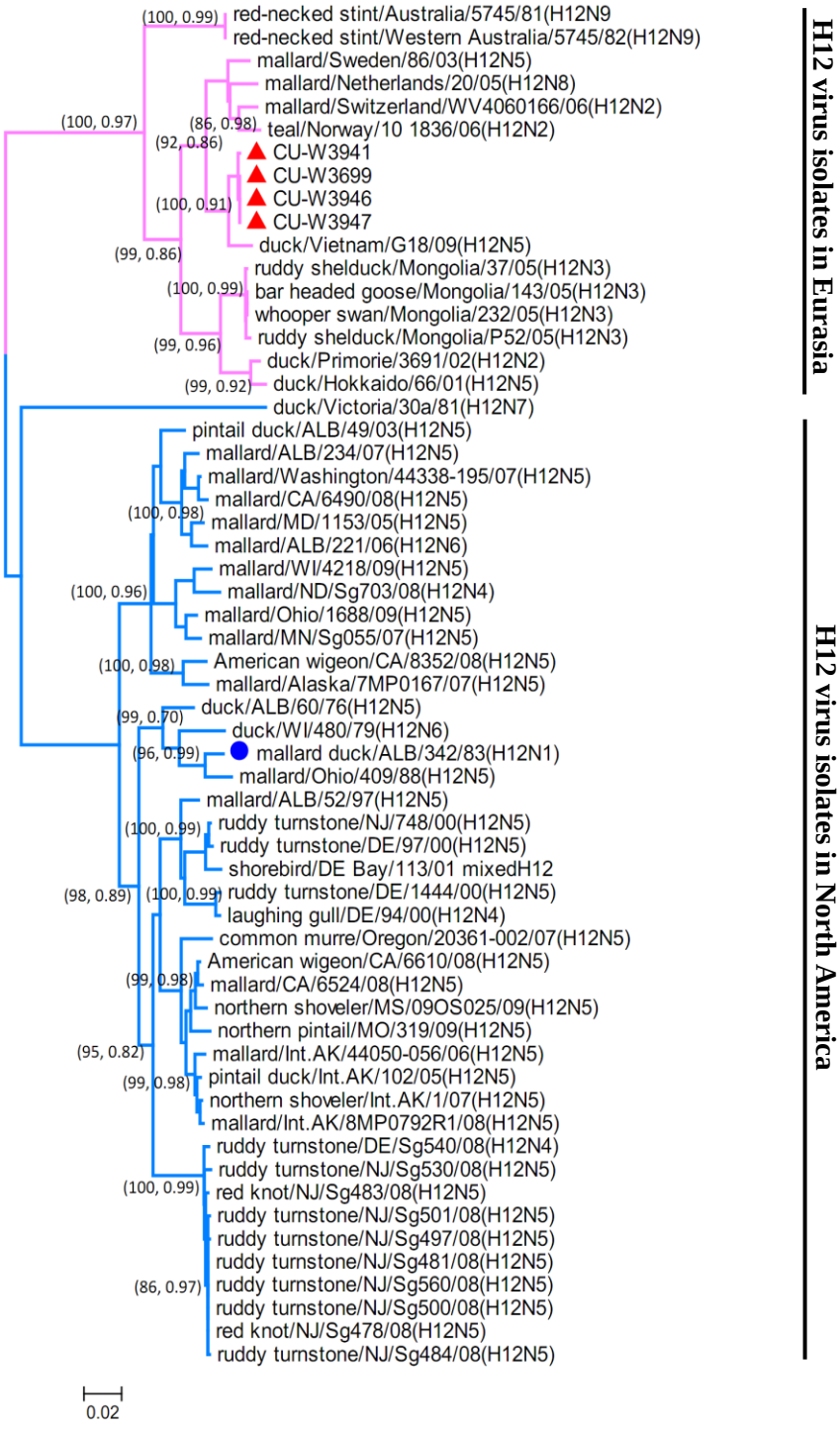

Figure 2.3 Phylogenetic analysis of the H12 gene of IAVs

Figure 2.4 Phylogenetic analysis of the N1 gene of IAVs. Some representatives of N1 isolates of each HA subtype in Eurasia and North America were chosen to include in the analysis. The tree was constructed with two different software applications using MEGA v.4 with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities and BMCMC with 40,000 generations and an average standard deviation of split frequencies < 0.05 (bootstrap percentage, posterior probabilities). Thai H12N1 viruses are indicated by triangles and the only H12N1 virus in the GenBank database is indicated by a circle.

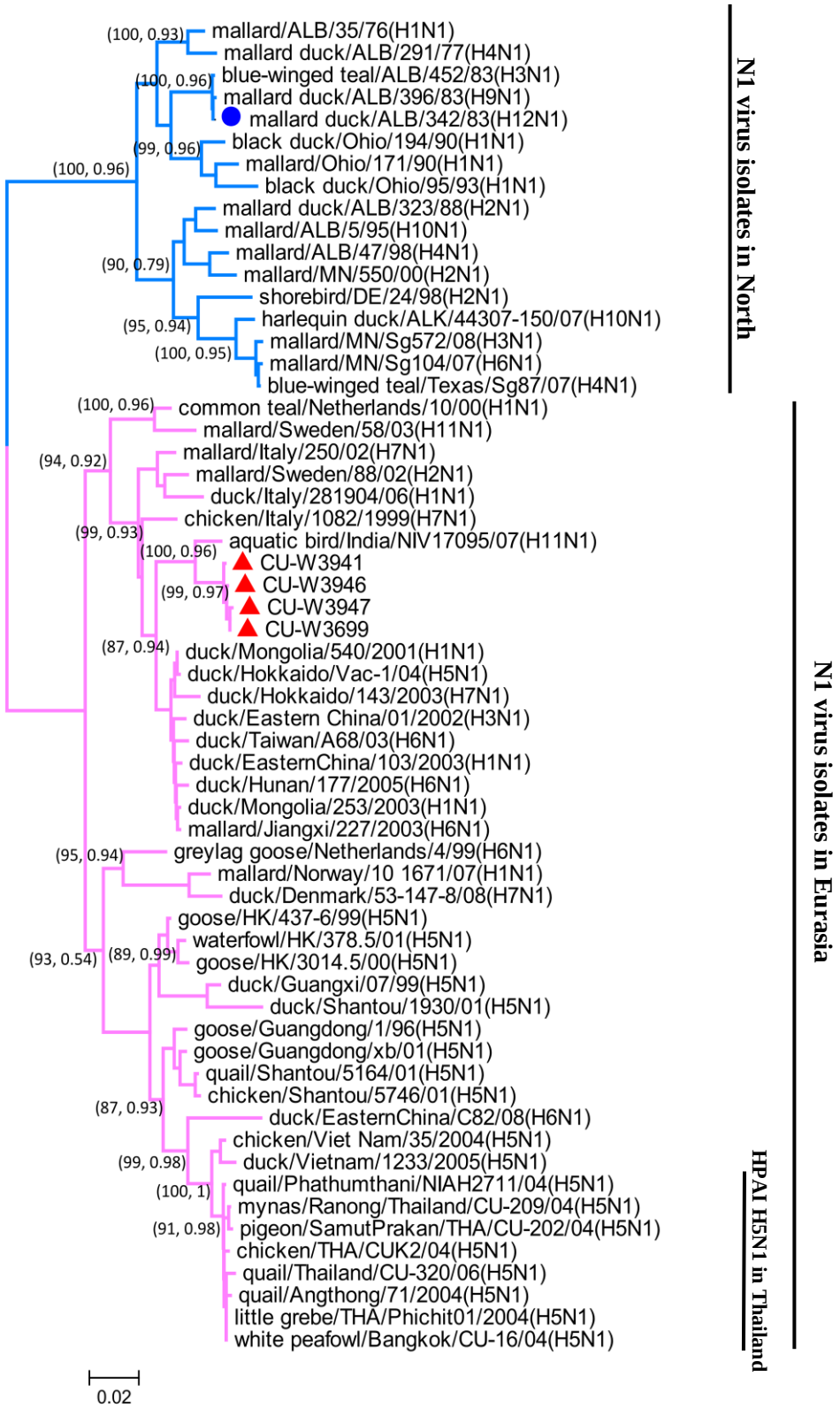

Figure 2.4 Phylogenetic tree of the N1 gene of IAVs

Figure 2.5 Phylogenetic analysis of six internal genes of influenza A viruses. All six internal genes of the Thai H12N1 viruses were grouped into the Eurasian strains based on geographical origin. Phylogenetic trees of each gene were generated using the Molecular Evolutionary Genetics Analysis software, MEGA4 (Tamura et al., 2007) with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities. The Thai H12N1 viruses are indicated by triangles and the only H12N1 virus in the GenBank database is indicated by a circle.

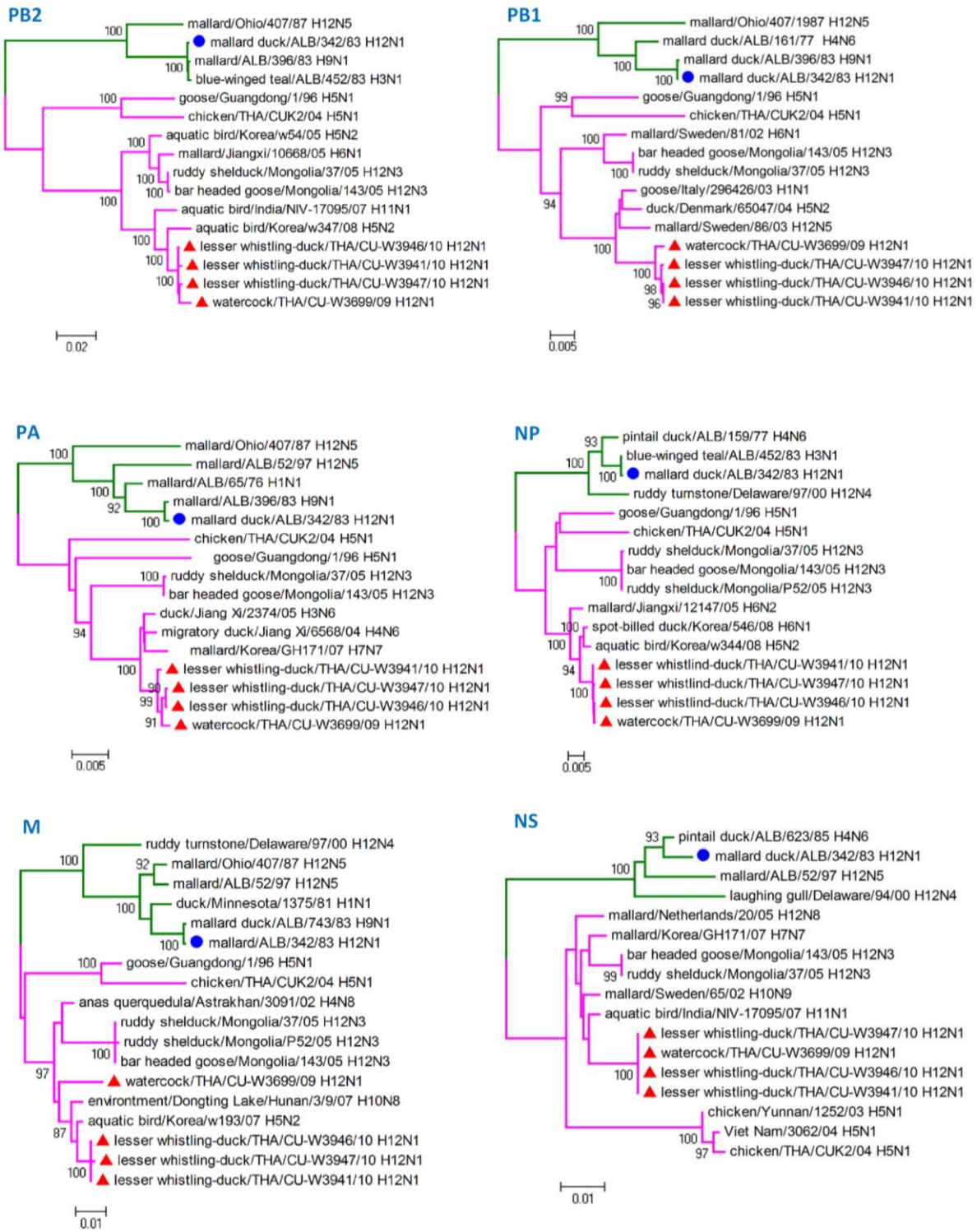

Figure 2.5 Phylogenetic analysis of six internal genes of IAVs.

2.4 Discussion and conclusion

From February 2009 through April 2010, a 15-month monitoring program of IAVs among wild bird species was conducted in 2 provinces of central Thailand, Ayutthaya and Suphanburi. In this study, we have collected and tested more than 2,000 swab samples from 47 wild bird species in 25 families, 9 orders. Four IAV subtype H12N1 were isolated from 2 bird species (watercock; *Gallicrex cinerea* and lesser whistling-duck; *Dendrocygna javanica*) at the same location but different time of sampling (December (n=1) and January (n=3)). As of November 2011, only one isolate of IAV H12N1 has ever been reported and made available to the GenBank database; Mallard/ALB/83. The virus was recovered from a mallard duck in Canada in 1983 (Hinshaw et al., 1985). The significance of this finding is that the Thai H12N1 viruses of this study represent the first Eurasian strain of H12N1 viruses ever reported based on geographical origin. It should be noted that the viruses were isolated during the winter season in Thailand when the temperature decreases to between 15 ^{o}C and 25 ^{o}C. It has been documented that the frequencies of H5N1 outbreaks in wild birds were significantly higher during winter season (from November to February) in Thailand (Siengsanan et al., 2009). The evidence that avian influenza viruses can survive and remain infective for extended durations during winter season might be due to the temperature dependent (Stallknecht et al., 1990; Shortridge et al., 1998). However, in this study, the occurrences of IAVs in wild bird were high during June to August (Figure 2.6). Based on our observation, during that period, many wild birds and free-grazing ducks have been observed. To our knowledge, farmers in the areas start growing rice in May and harvesting in August. This may lead to the high occurrence of IAVs.

Watercock (*Gallicrex cinerea)* belongs to the order *Gruiformes*, family *Rallidae*. Lesser whistling-duck (*Dendrocygna javanica)* belongs to the order *Anseriformes*, family *Anatidae*. Both species are native in most areas of South Asia, East Asia and Southeast Asia including Thailand (BirdLife International, 2009, 2011). To our knowledge about these 2 birds in Thailand, watercock is a common resident and wet-season visitor. Habitats of watercock are commonly in dense vegetation of marshes and paddy-field

margins. Lesser whistling-duck is also a common resident and may be a winter visitor to Thailand. Habitats of lesser whistling-duck are in flooded fields, marshy areas and lakes (Lekagul and Round, 2005). It should be noted that each year from September to December, several wild bird species migrate from neighboring countries to Thailand for resting, feeding and breeding and return to their habitats between April and May. In contrast, some bird species are local and move for a short distance between feeding areas. Based on our observation, watercock is a free-flying local habitant in a paddy field and moves between paddy fields, while the lesser whistling-duck is not local to these areas but a long distance free-flying bird from northern Thailand or neighboring countries especially during the winter season. It has been known that wild birds of the order Anseriformes e.g. Mallard, Teal, Pintail and Geese are considered main reservoirs of influenza virus (Webster et al., 1992; Hanson et al., 2003). Similarly, watercock of the order Gruiformes has been identified as an HPAI H5N1 reservoir by a previous study in Thailand (Amonsin et al., 2008). In this study, Thai H12N1 viruses were found in two wild bird species (watercock and lesser whistling-duck). Although both wild bird species differ by order and family, they share the same habitats that help promote cross transmission of influenza viruses among wild bird species and potential spread to domestic poultry populations such as free grazing ducks and backyard chicken (Matrosovich et al., 1999; Alexander, 2000; Cappelle et al., 2011).

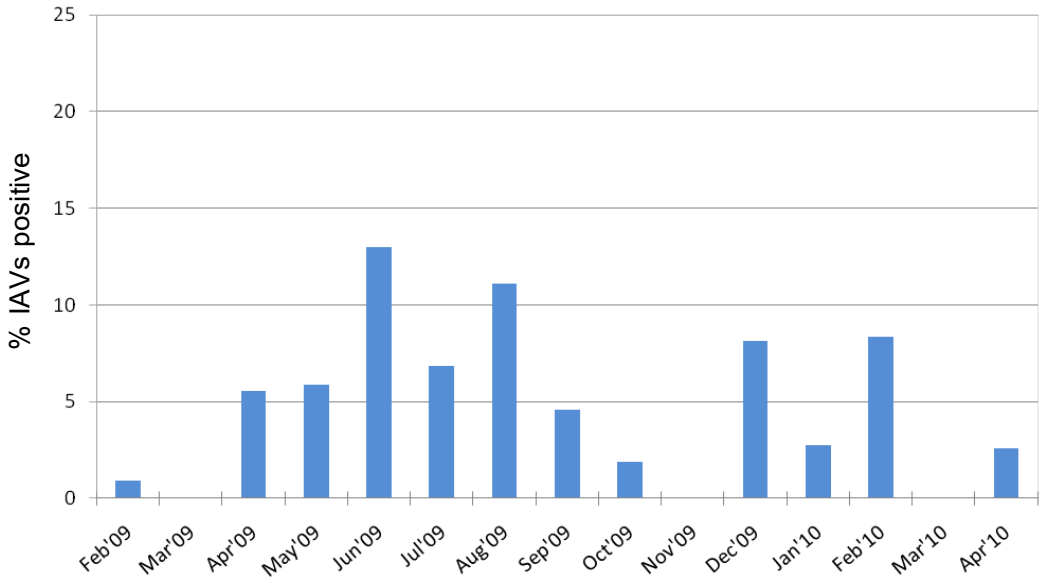

Figure 2.6 The occurrence of IAVs isolated in wild birds during February 2009 to April 2010

Phylogenetic and genetic analyses have shown that Thai H12N1 viruses were closely related to avian influenza viruses of the Eurasian strain (Table 2.4 and 2.6, Figure 2.3, 2.4 and 2.5). None of the eight genes of Thai H12N1viruses clustered with the only H12N1 (Mallard/ALB/83) available in the database, which belongs to the North American strain. These results provided additional information on the genetic diversity of H12N1 IAV. In addition, genetic analysis of the HA cleavage site of the viruses suggested that Thai H12N1 were of low pathogenicity for avian species. However, an animal model test such as the intravenous pathogenicity index test (IVPI) should be performed in order to further confirm the pathogenicity of the H12N1 virus (OIE, 2004). It should be noted that numerous other H12 and N1 influenza viruses have been isolated in North America and Eurasia throughout the past 30 years and all four H12N1 viruses in this study were closely related and shared a common ancestor of all 8 genes. This could indicate that IAV subtype H12N1 is one of the rarely detected subtype. Moreover, it may imply that the Thai H12N1 virus is probably a recent (and perhaps transient) reassortant circulating in the wild bird populations in Thailand.

Based on our investigation, monitoring programs in wild bird populations should be continuously conducted to further our understanding of IAVs circulating in wild birds in Thailand. In summary, this study has presented the first report on the new subtype H12N1 IAV in Thailand and the first genetic characterization of H12N1 in Eurasia. Moreover, the genetic information on H12N1 obtained by this study has provided a new Eurasian strain of H12N1 to the GenBank and Influenza Research Database.

CHAPTER III

Genetic characterization of influenza A virus subtype H7N1 isolated from quails, Thailand

3.1 Introduction

Avian influenza is an infectious disease of poultry caused by influenza A viruses (IAVs). Highly Pathogenic Avian Influenza (HPAI) viruses of the subtypes H5 and H7 are classified as Office International des Epizooties (OIE) List A diseases. Since 2004 though 2008, seven major waves of HPAI-H5N1 outbreaks were occurred in Thailand especially in central part of the country. During each outbreak, large numbers of domesticated birds were culled as part of disease control and prevention.

In 2008, the study on HPAI-H5N1 in Thailand reported the prevalence of the virus in live-bird markets as 1.4% (12/836). Interestingly, 41.4% (5/12) of HPAI-H5N1 viruses were isolated from quails (Amonsin et al., 2008). Moreover, many studies documented that several subtypes of IAVs could be isolated from quails in several countries e.g. H7N1 and H7N2 in Italy (Capua et al., 2002), H6N1 and H6N2 in China (Cheung et al., 2007) and H9N2 in China and Hong Kong (Guo et al., 2000; Capua and Alexander, 2002). In Italy, 1999-2000, the LPAI-H7N1 virus mutated to HPAI virus and then spread rapidly causing over 14 million bird deaths (Capua and Marangon, 2000; Alexander, 2003; Marangon et al., 2003). Quails were the one affected by those outbreaks.

In Thailand, even the information of HPAI-H5N1 has been reported in quails but the information of LPAI in quails is still limited. To fulfill the gap of the information of IAVs in quails, a 12-month monitoring program (May 2009 through April 2010) of IAVs in quails was conducted in two provinces in central Thailand, where presumably located in HPAI high-risk areas: Ayutthaya and Suphanburi provinces. In this study, three IAVs could be identified as subtype H7N1. Phylogenetic and genetic analyses were performed on one H7N1 virus. This study highlights the first report of H7N1 avian influenza virus in Thailand.

3.2 Material and Method

3.2.1 Influenza A virus isolation

From May 2009 through April 2010, a 12-month cross-sectional monitoring program of IAVs in quails was conducted in two provinces of central Thailand. Samples were collected from two quail farms where presumably located in HPAI high-risk areas of: Ayutthaya (n=1) and Suphanburi (n=1). The swab samples were collected from oropharynx and cloacae of individual quail. All of swab samples were deposited in viral transport medium (VTM). Samples were maintained at 4 $^{\circ}$C during transportation and submitted to the laboratory within 24 hours. A total of 2,040 swab samples were collected from 1,020 quails (oropharyngeal swab, OPS; n = 1,020, cloacal swab, CS; n = 1,020). Approximately 40 quails of each farm were sampled monthly. The virus isolation was conducted by using embryonated chicken eggs inoculation according to WHO/OIE recommendations (WHO, 2002). After 72-hour incubation, the allantoic fluid of individual eggs were collected and tested for the presence of hemagglutinin activity using hemagglutination test (HA test). The samples testing positive for HA test at 4 HA unit/50 ul or greater were subsequently subjected to IAV identification.

3.2.2 Influenza A virus identification and subtype determination

To identify IAVs, viral RNA was extracted from the allantoic fluid of HA positive samples by using the QIAamp Viral RNA Mini Kit (QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany). RNA samples were subjected for one-step real time RT-PCR (rRT-PCR) using TaqMan probe to detect matrix (M) gene of IAVs (Spackman et al., 2002). To subtype IAVs, cDNA was synthesized from the rRT-PCR positive samples using the influenza universal primer Uni12. The cDNA was then used as template for subtype identification using specific primer for HA and NA genes (Tsukamoto et al., 2008; Tsukamoto et al., 2009).

Due to the low virus titer, only one virus was selected and then subjected for whole genome sequencing. Nested PCR was performed to increase specificity of the test by using universal primer sets for IAVs to amplify the full-length of each gene

(Hoffmann et al., 2001). Each gene of IAV was amplified by using oligonucleotide primer sets either from our primer inventories or newly designed primer sets by using Primer3 (v.0.4.0) (Table 3.1). PCR products were submitted for DNA sequencing by using the Big Dye Terminator v.3.0 Cycle Sequencing Ready reaction (ABI, Foster city, CA) at the Molecular Informatics Laboratory Limited, Hong Kong. The nucleotide sequences of each gene were then validated and assembled using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI, USA).

Table 3.1 Oligonucleotide primers used for HA and NA genes of IAV subtype H7N1

Primer name	Primers for H7 HA gene	Length (bp)	Tm (°C)	Product size (bp)
H7F1	5'- CAA AAG CAG GGG ATA CAA A -3'	19	58.6	491
H7R1	5'- CTC TGC ATA GAA TGA AGA TCC -3'	21	55.9	
H7F2	5'- CAA TGG GAT TCA CAT ACA GC -3'	20	55.3	463
H7R2	5'- GCA TCA ACC TGT ACT CCA CT -3'	20	57.3	
H7F3	5'- GAC CAA RCT MTA YGG RAG TG -3'	20	49.2	816
H7R3	5'- CCA TCT TCT TCA GCA TTC TC -3'	20	55.3	
H7F4	5'- GAG AAT GCT GAA GAA GAT GG -3'	20	55.3	259
H7R4	5'- ATA GTG CAC YGC ATG TTT -3'	28	47.9	
	Primers for N1 NA gene			
N1F1	5'- CAG GAG TTC AAA ATG AAT CCA A -3'	22	51.2	600
N1R1	5'- AAA GCA AGA GCC ATT TAC ACA -3'	21	52.8	
N1F2	5'- TGT TGC TTG GTC AGC AAG TG -3'	20	61.05	386
N1R2	5'- TTG AGC CAT GCC AAT TAT CC -3'	20	60.81	
N1F3	5'- TCA CTA TGA GGA ATG CTC CTG -3'	21	57.91	388
N1R3	5'- CAC TGA AAA GCT ACT GTC CGT TT -3'	23	59.87	
N1F4	5'- CCA TGG GTA TCT TTC AAT CAA -3'	21	57.41	500
N1R4	5'- TCA ATG GTG AAT GGC AAC T -3'	19	57.42	

3.2.3 Phylogenetic and genetic analyses of influenza A viruses

Phylogenetic and genetic analyses were performed by comparing each gene segment of the viruses with reference IAVs available in the GenBank database. Reference nucleotide sequences of IAVs were retrieved to include all geographical origins (Eurasia and North America) and all of different subtypes; H1-H16 (n=48) and N1-N9 (n=54). Nucleotide sequences of each genes were aligned by using Muscle v.3.6 (Edgar, 2004). Phylogenetic tree and molecular evolutionary analyses were

conducted using the Bayesian Markov chain Monte Carlo (BMCMC) method (Drummond and Rambaut, 2007) and Molecular Evolutionary Genetics Analysis software, MEGA4 (Tamura et al., 2007). The nucleotide sequences and deduced amino acids of each gene of the viruses were aligned and compared for genetic analysis using MegAlign software v.5.03 (DNASTAR Inc., Madison, WI, USA).

3.3 Results

3.3.1 The occurrence of influenza A viruses in quails

In this study, a 12-month monitoring program of IAVs was conducted in two quail farms in central Thailand from May 2009 through April 2010. The result showed that the occurrence of IAVs in quails was 1.18% (24/2,040). Among the positive samples, 15 samples were from OPS (15/24, 62.50%) and 9 samples were from CS (9/24, 37.50%). The occurrence of IAVs was high in May and September 2009 (table 3.2 and figure 3.1).

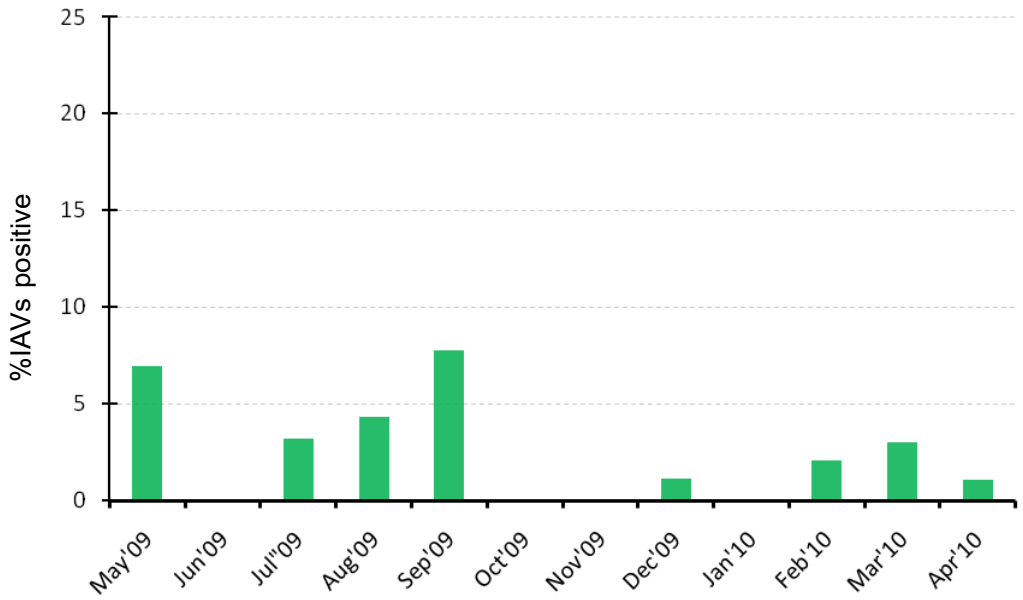

Figure 3.1 The occurrence of IAVs isolated in quails during May 2009 to April 2010.

Table 3.2 Number of collected samples, HA positive and IAVs isolated from quails

Months	Suphanburi					Ayutthaya					Total				
	Quails (n)	No.HA positive,(%)		No.IAV positive[a],(%)		Quails (n)	No.HA positive,(%)		No.IAV positive[a],(%)		Quails (n)	No.HA positive,(%)		No.IAV positive[a],(%)	
		OPS	CS	OPS	CS		OPS	CS	OPS	CS		OPS	CS	OPS	CS
May'09	43	0	4 (9.30)	0	3 (6.97)	-			-	-	43	0	4 (9.30)	0	3 (6.97)
Jun'09	-			-	-	50	0	0	0	0	50	0	0	0	0
Jul'09	42	0	0	0	0	51	3 (5.88)	0	3 (5.88)	0	93	3 (3.22)	0	3 (3.22)	0
Aug'09	41	1 (2.44)	0	1 (2.44)	0	52	2 (3.85)	4 (7.69)	0	3 (5.77)	93	3 (3.22)	4 (4.30)	1 (1.07)	3 (3.22)
Sep'09	41	1 (2.44)	1 (2.44)	1 (2.44)	0	49	7 (14.28)	3 (6.12)	5 (10.20)	1(2.04)[b]	90	8 (8.89)	4 (4.44)	6 (6.67)	1 (1.11)
Oct'09	40	0	0	0	0	50	0	0	0	0	90	0	0	0	0
Nov'09	43	10 (23.25)	9 (20.93)	0	0	47	0	0	0	0	90	10 (11.11)	9 (10.0)	0	0
Dec'09	40	10 (25.0)	0	0	0	50	14 (28.0)	16 (32.0)	1(2.0)[b]	0	90	24 (26.67)	16 (17.78)	1 (1.11)	0
Jan'10	41	0	7 (17.07)	0	0	50	6 (12.0)	2 (4.0)	0	0	91	6 (6.59)	9 (9.89)	0	0
Feb'10	45	7 (15.56)	4 (8.89)	1 (2.22)	1 (2.22)	51	1 (1.96)	3 (5.88)	0	0	96	8 (8.33)	7 (7.29)	1 (1.04)	1 (1.04)
Mar'10	50	0	4 (8.0)	0	0	50	7 (14.0)	17 (34.0)	2 (4.0)	1 (2.0)	100	7 (7.0)	21 (21.0)	2 (2.0)	1 (1.0)
Apr'10	43	0	6 (13.95)	0	0	51	2 (3.92)	0	1(1.96)[b]	0	94	2 (2.13)	6 (6.38)	1 (1.06)	0
Total	469	29 (6.18)	35 (7.46)	3 (0.64)	4 (0.85)	551	42 (7.62)	45 (8.17)	12 (2.18)	5 (0.91)	1,020	71 (6.96)	80 (7.84)	15 (1.47)	9 (0.88)

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

[b]Samples that could be identified as IAVs subtype H7N1.

3.3.2 Influenza A virus subtype H7N1 in quails

Due to the low virus titer, among 24 IAVs positive, only 3 samples could be identified as subtype H7N1. The result of one-step rRT-PCR of three IAVs subtype H7N1 isolated from this study was shown in figure 3.2. The viruses were designed as A/quail/THA/CU-J1835/2009 (CU-J1835); A/quail/THA/CU-J2882/2009 (CU-J2882) and A/quail/THA/CU-J3293/2010 (CU-J3293). All three H7N1 were isolated from 45-days-old quails from a farm in Ayutthaya province. Detail description of the three H7N1 viruses was shown in table 3.3. The H7N1 viruses were isolated from a cloacal swab (n=1, CU-J1835) and oropharyngeal swab (n=2, CU-J2882 and CU-J3293). Based on the information that all three H7N1 viruses were isolated from the same farm, nucleotide and amino acid identities (%) of HA and NA genes among the viruses are similar (table 3.4). Thus, only one H7N1 virus (CU-J2882) was selected as the representative and subjected for whole genome sequencing.

Table 3.3 Detailed description of IAV subtype H7N1 isolated from quails

Viruses	Subtype	Collection date	Host Name	Age (days)	Sample type	Location
CU-J1835	H7N1	Sep'09	Japanese quail	45	CS	Ayutthaya
CU-J2882	H7N1	Dec'09	Japanese quail	45	OPS	Ayutthaya
CU-J3293	H7N1	April'10	Japanese quail	45	OPS	Ayutthaya

Note; CS= cloacal swab; OPS= oropharyngeal swab

Table 3.4 Nucleotide and amino acid comparison (%) of HA and NA genes among three H7N1 viruses isolated in this study

CU-J2882 (H7N1)		
Samples	Nucleotide and amino acid identities (%) compared with CU-J2882	
	HA	NA
CU-J1835	100.0 (100.0) [1399-1671, 271 bp]	97.3 (94.7) [227-839, 612bp]
CU-J3293	100.0 (100.0) [1399-1671, 271 bp]	100.0 (100.0) [43-517, 473 bp]

Note: (-) Amino acid identity; [-] Region compared, nucleotide length (base pairs)

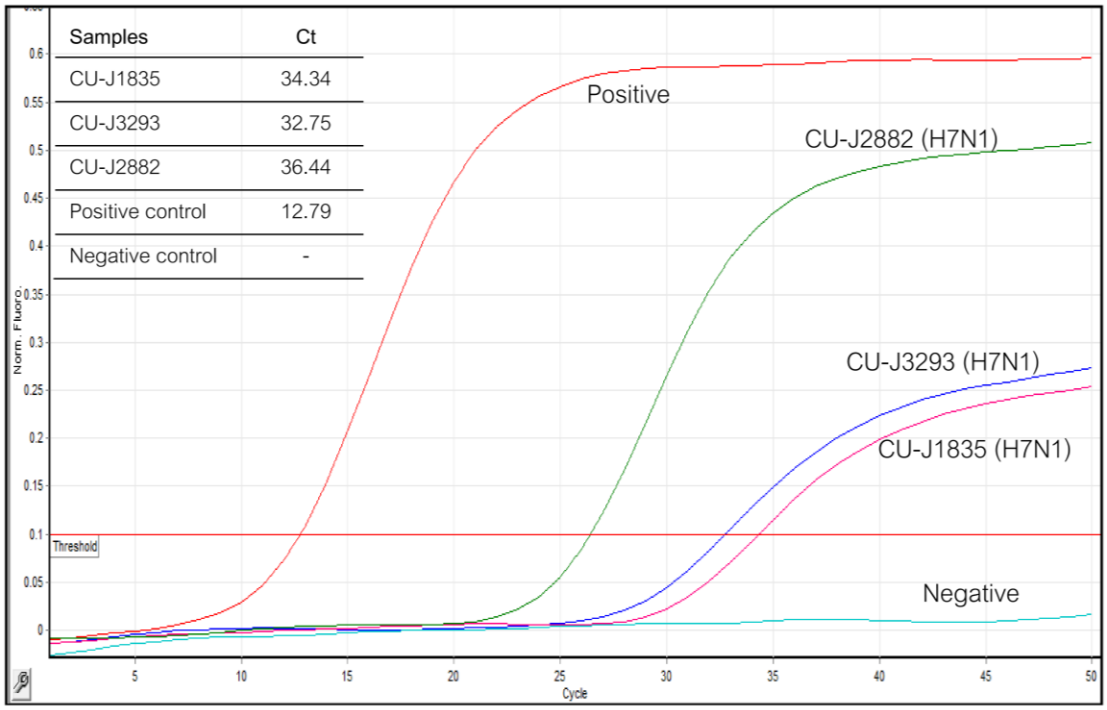

Figure 3.2 The result of one-step rRT-PCR of IAVs subtype H7N1 isolated from this study. Threshold cycle (CT value) was calculated from the point at which the fluorescence crosses the threshold (the threshold in this study was set up at the fluorescence intensity = 0.1). CU-J2882 was chosen and subjected for whole genome sequencing.

3.3.3 Genetic and phylogenetic analyses of LPAI H7N1 (CU-J2882)

To analyze the genetic relatedness of CU-J2882 with reference IAVs available in the GenBank database, nucleotide sequence similarity of each gene of the virus were investigated by using NCBI nucleotide BLAST program and MegAlign software (DNASTAR). Our results showed that HA gene of CU-J2882 was closely related to IAV from Japan, A/duck/Shiga/B149/07(H7N7), with 98.1% nucleotide identity. It is noted that most of the gene segments of CU-J2882 were closely related to LPAI virus of Eurasian strain. Interestingly, NA gene of the virus displays the highest nucleotide and amino acid similarities to HPAI-H5N1 in Thailand; A/quail/Phathumthani/ NIAH2711/04 (H5N1) at 97.1% (amino acid identity, 98.4%) (table 3.5).

Phylogenetic analysis of HA gene showed that CU-J2882 was grouped into Eurasian lineage of H7 avian influenza viruses (figure 3.3). As similar to HA gene, phylogenetic analysis of the internal genes (PB1, PA, NP, M and NS) showed that the virus was grouped into LPAI of Eurasian strain (figure 3.5). In addition, NA gene of the virus was grouped into HPAI-H5N1 of Eurasian lineage (clade 1 H5N1 viruses) (figure 3.4).

Genetic analysis of HA gene of CU-J2882 showed that the virus does not contain any multiple basic amino acid insertions at the connecting peptide sequence comparing with both HPAI H7 and LPAI H7. This evidence indicates low pathogenic characteristic of the virus. In this study, amino acids at the connecting peptide sequence of the Thai H7N1 poses P-E-I-P-K-G-R-/G. Amino acid at receptor-binding residues (HA142, 143, 175, 182, 185; numbering system is based on consensus H7 amino acid sequence) and amino acid at right edge and left edge of receptor-binding pockets were also analyzed (table 3.6). HA1 domain of CU-J2882 has no amino acid deletion at position 215-220 comparing with reference LPAI H7N2 viruses isolated in live-bird market in North America; A/avian/NY/70411-12/2000 and A/guinea fowl/PA/7777/1996) (Suarez et al., 1999). In addition, the 20-amino acid deletion in the NA stalk region has been found in the virus.

Table 3.5 Sequence comparison of CU-J2882 compared with reference IAVs available in the GenBank database

Genes	Nucleotide sequence compared	Virus with the highest percentage nucleotide identities	Nucleotide and amino acid identities (%)	GenBank accession no.
PB2*	1,618 - 2,264 [647]	A/pintail/Aomori/1130/08(H1N3)	98.8 (99.1)	AB546177.1
PB1*	1,081 - 2,254 [1,174]	lesser whistling-duck/CU-W3941/10(H12N1)	97.2 (100.0)	JN982509.1
PA	1– 2,148 [2,148]	A/duck/Guizhou/888/2006(H6N5)	99.0 (99.7)	CY109281.1
HA	1 - 1,671 [1,671]	A/duck/Shiga/B149/07(H7N7)	98.0 (97.8)	AB558257.1
NP	7 - 1,487 [1,481]	A/spot-billed duck/Korea/546/08(H6N1)	97.0 (99.2)	GQ414896.1
NA	1 – 1,343 [1,343]	A/open-billed stork/NS/BBD0404F/04(H5N1)	99.0 (99.8)	DQ989971.1
M	1 - 961 [961]	A/duck/Thailand/CU5408/09(H11N3)	99.7 (100.0)	CY062599.1
M1	1 - 759 [759]	A/muscovy duck/THA/CU-LM4775/09(H10N3)	100.0 (100.0)	CY062599.1
M2	1 - 26, 715 - 961[273]	A/chicken/Singapore/94(H7N1)	100.0 (100.0)	EU014140.1
NS	1 - 828 [828]	A/aquatic bird/India/NIV-17095/07(H11N1)	98.7 (99,6)	CY055179.1
NS1	1 - 693 [693]	A/aquatic bird/India/NIV-17095/07(H11N1)	98.8 (99.6)	CY055179.1
NS2	1 - 30, 503 - 828[357]	A/mallard/Sweden/65/02(H10N9)	99.2 (100.0)	CY060362.1

*Gene segments that could not be completely amplified.

(-) Amino acid identities; [-] length of nucleotides compared (base pairs).

Figure 3.3 Phylogenetic analysis of the H7 gene of IAVs. Some representatives of H7 isolates in Eurasia and North America were chosen to include in the analysis. The tree was constructed with two different software applications using MEGA v.4 with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities and BMCMC with 40,000 generations and an average standard deviation of split frequencies < 0.05 (bootstrap percentage, posterior probabilities). Thai H7N1 virus (CU-J2882) is indicated by a triangle.

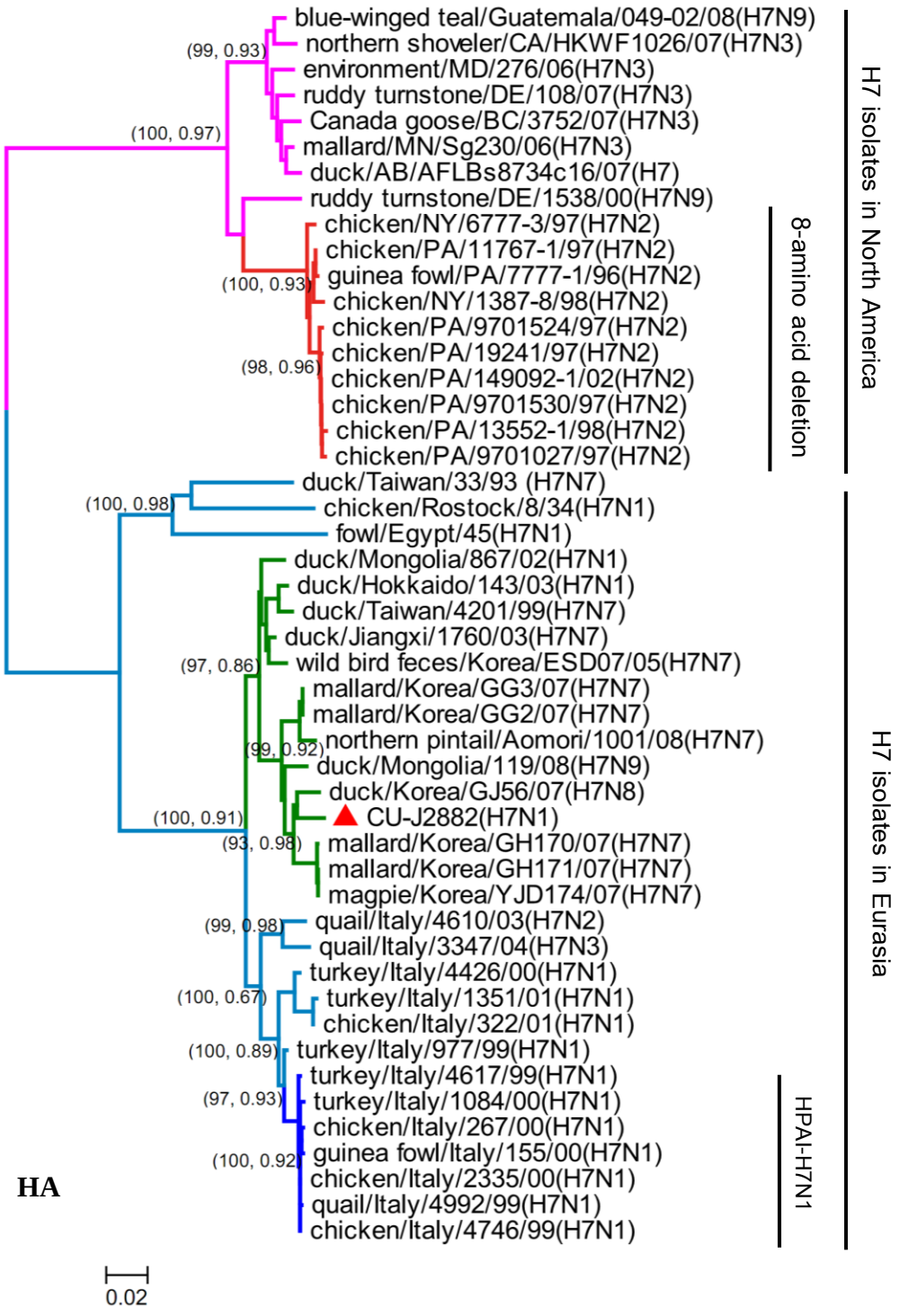

Figure 3.3 Phylogenetic analysis of H7 gene of IAVs

Figure 3.3 Phylogenetic analysis of the N1 gene of IAVs. Some representatives of N1 isolates of each HA subtype in Eurasia and North America were chosen to include in the analysis. The tree was constructed with two different software applications using MEGA v.4 with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities and BMCMC with 40,000 generations and an average standard deviation of split frequencies < 0.05 (bootstrap percentage, posterior probabilities). Thai H7N1 virus (CU-J2882) is indicated by a triangle.

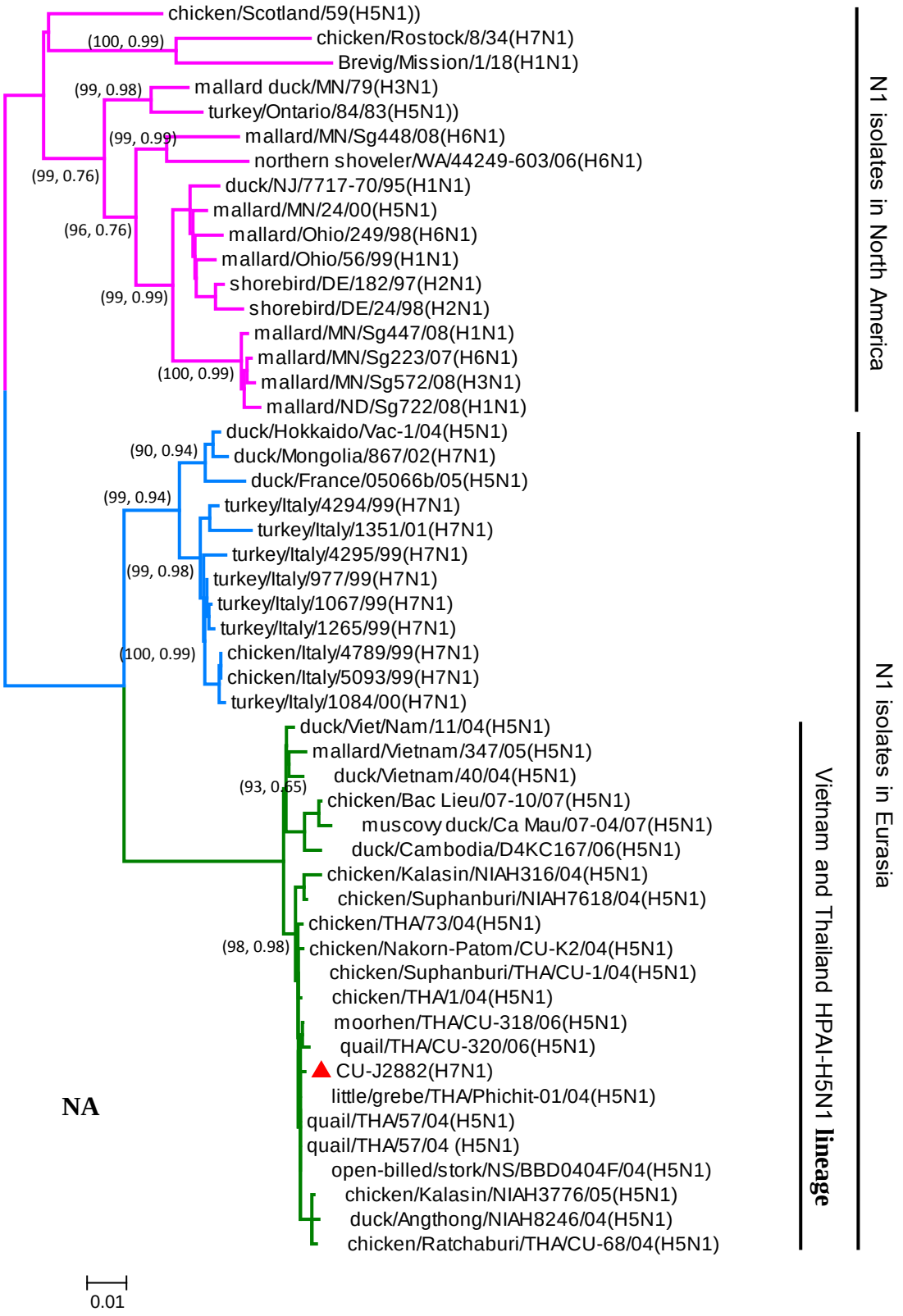

Figure 3.4 Phylogenetic analysis of N1 gene of IAVs

Figure 3.5 Phylogenetic analysis of the internal genes of CU-J2882 (H7N1). Five internal genes of the Thai H7N1 virus (PB1, PA, NP, M and NS) were grouped into the Eurasian strains based on geographical origin. Phylogenetic trees of each gene were generated using the Molecular Evolutionary Genetics Analysis software, MEGA4 (Tamura et al., 2007) with the neighbor-joining algorithm and bootstrap analysis with 1,000 nucleotide identities. Thai H7N1 virus is indicated by a triangle and the Thai H12N1 viruses isolated from wild birds described in Chapter II are included to analyse and indicated by circles. Only 1,174 nucleotides of PB1 gene were used for phylogenetic analysis. In this study, PB2 gene of the virus was not included for phylogenetic analysis.

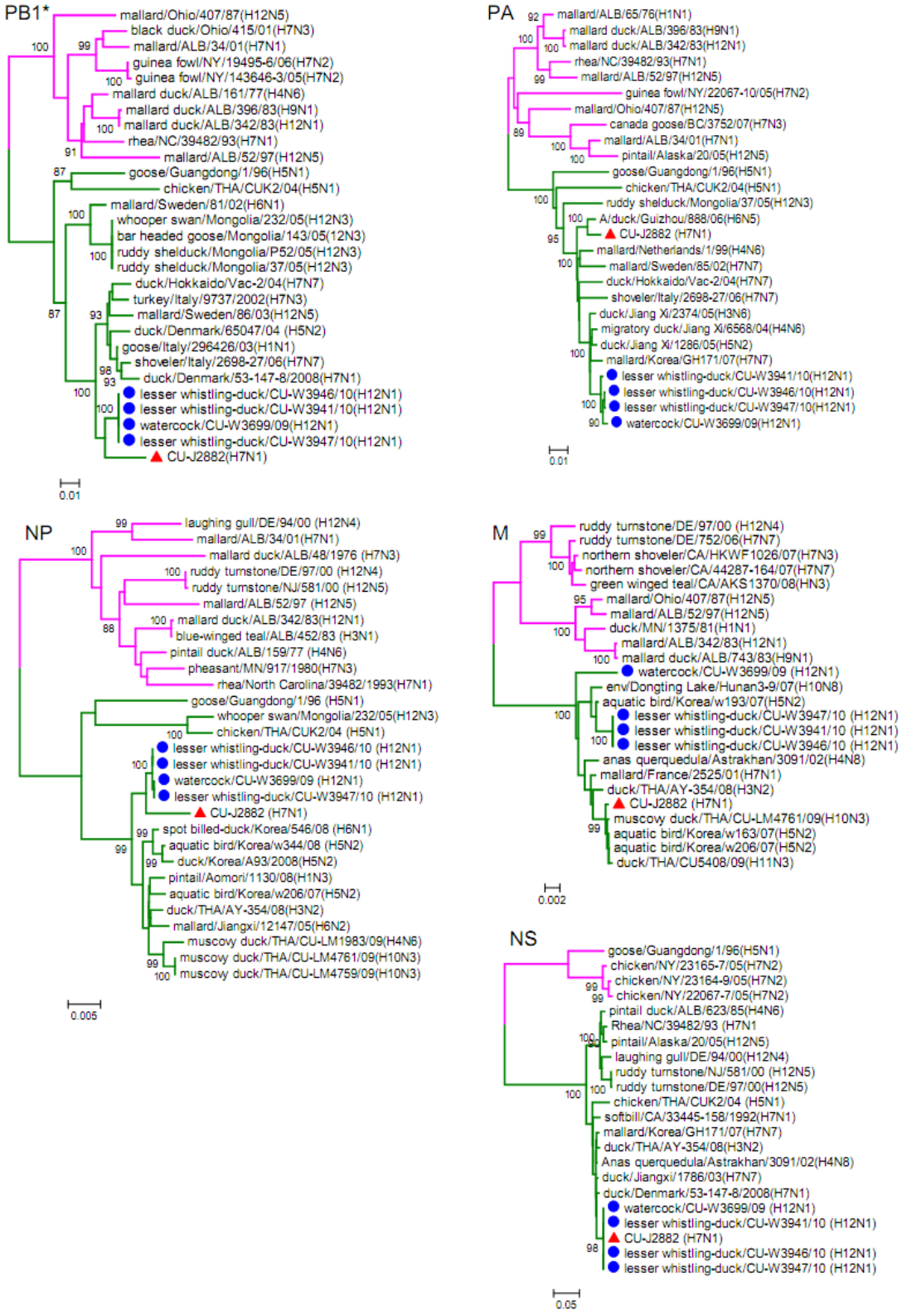

Figure 3.5 Phylogenetic analysis of the internal genes of Thai H7N1 virus.

Table 3.6 Amino acid comparison of HA gene of CU-J2882 with reference IAV subtype H7 available in the Genbank database

Viruses	Subtype	Pathotype	[a]Amino acid position of HA gene										[b]Deletion in HA1	GenBank accession no.
			N-glycan		Right edge of RBS	Receptor-binding residues					Left edge of RBS	HA cleavage site		
			123	149	124-128	142	143	175	182	185	215-220	315-325		
CU-J2882	H7N1	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	No	This study
Turkey/Italy/977/99	H7N1	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	No	CY024754
Duck/Korea/GJ56/07	H7N8	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-I-P-K-G-R/G	No	FJ750853.1
Rhea/NC/39482/93	H7N1	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-N-P-K-T-R/G	No	EF470586.1
Guinea fowl/PA/7777/96	H7N2	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	----------R	P-E-N-P-K-P-R/G	Yes	AF072394.2
Avian/NY/70411-12/00	H7N2	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	----------R	P-E-K-P-K-P-R/G	Yes	AY240878.1
NY/107/03	H7N2	LPAI	N	N	G-A-T-S-A	W	L	H	Q	L	----------R	P-E-K-P-K-P-R/G	Yes	EU587368
Chicken/Rostock/8/34	H7N1	HPAI	N	N	G-T-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-P-S-K-K-R-K-K-R/G	No	M24457
Turkey/Italy/4617/99	H7N1	HPAI	N	N	G-A-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-I-P-K-G-S-R-V-R-R/G	No	CY025141
Chicken/Netherland/1/03	H7N1	HPAI	N	N	G-A-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-I-P-K-R-R-R-R/G	No	AY338458
Netherland/219/03	H7N7	HPAI	N	N	G-T-T-S-A	W	L	H	Q	L	N-G-Q-S-G-R	P-E-I-P-K-R-R-R-R/G	No	AY338459

[a] Eight-amino acid deletion within the putative receptor binding site in HA1 domain. [b] Amino acid numbering system is based on the consensus H7 amino acid sequence.

Abbreviation; NC= North Carolina, PA= Pennsylvania, NY= New York.

3.4 Discussion and conclusion

Since 2004, seven major waves of the HPAI-H5N1 outbreaks were reported in Thailand. Quail farms were also affected by the outbreaks due to animal culling and movement restriction as well as farm closing (Amonsin et al., 2006a; Amonsin et al., 2008; Uchida et al., 2008). In this study, a 12-month monitoring program of IAVs in quails was conducted from May 2009 through April 2010. The study-quail farms were located in the HPAI-high risk provinces. However, the pronounced difference between two quail farms was the biosecurity systems. In Suphanburi, quail farm management with high biosecurity had been conducted as per the Thai Department of Livestock Development (Thai DLD) recommendations for diseases control and prevention. For example, the farm had human and animal restriction movements, disinfections and semi-close housing. On the other hand, in Ayutthaya, a quail farm had low biosecurity or backyard system. Pets, terrestrial birds and wild birds can access inside the farm. It is noted that the owner of a quail farm in Suphanburi allowed to collect samples from non-egg production female quails (8-9 month old). While the owner of a quail farm in Ayutthaya allowed to collect sample from the 45-days-old quails. Therefore, in this study, 2 major factors affecting the occurrence of IAVs have to be concerned; (i) biosecurity system, (ii) age of quails.

Out of 2,040 samples, 24 samples were identified as IAVs by rRT-PCR (1.18%). The occurrence of positive samples was high in May and September 2009. As shown in the previous study of IAVs in wild birds in Thailand, the occurrence of IAVs were high in June to August (Wongphatcharachai et al., 2012). However, the relationship of IAVs between wild bird and quail populations does not established in this study. Most of positive samples were from the quail farm in Ayutthaya (17/24, 70.83%), on the other hand, 7 samples (7/24, 29.17%) were from the quail farm in Suphanburi. It should be noted that no clinical sign relating to IAVs infection in the farms were observed in this study.

Due to the low virus titer, three rRT-PCR positive samples can only be further identified as IAV subtype H7N1. The virus CU-J2882 with highest virus titer (CT value <

30) was selected and subjected for genetic characterization (figure 3.2). It should be noted that the virus could not be completely amplified the entire genes for PB2 and PB1. Since we used nested PCR and amplified with universal primer sets for full-length amplification, thus it may not be completed prior further steps.

Phylogenetic analysis showed that most genes (PB2, PB1, PA, HA, NP, M and NS) were closely related to LPAI virus of Eurasian strain. Genetic analysis at the HA connecting peptide shown the LPAI characteristic. However, an animal model test such as the intravenous pathogenicity index test (IVPI) should be performed to confirm the pathogenicity of Thai H7N1 virus. Concerning with N1 NA gene, the NA gene of Thai H7N1shown more closely related to HPAI-H5N1 in Vietnam and Thailand lineage. This evidence leads to the speculation that there is a possibility of genetic reassortment of H7Nx and HxN1 viruses circulating in poultry populations in Thailand. However, further experiment such as plaque assay is needed to confirm this observation (Sugiura et al., 1972; Tobita et al., 1975). In this study, HA1 domain of Thai H7N1 has no amino acid deletion comparing with those references of H7N2 viruses isolated in live-bird market in North America (Table 3).

In 1996, new genotype of H7N2 has been emerged in North America and become predominantly in avian species in live bird markets. The viruses contain 8-amino acid deletion including 5 amino acids of left edge of RBS in HA1 domain (Suarez et al., 1999; Spackman et al., 2003; Belser et al., 2008). The effect of this deletion has not been fully identified yet. It is documented that right edge and left edge of RBS are required for the formation of the active site cleft of HA (Weis et al., 1988; Gamblin et al., 2004). Seven-amino acid deletion in HA gene of IAV H3N2 generated in laboratory was reported a slightly increased affinity of RBS for sialic acid α2,3-galactose linked receptor (Daniels et al., 1987). The mutations in RBS of HA gene were responsible for adaptation of IAVs to be the pandemic strains (Matrosovich et al., 2000; Nobusawa et al., 2000; Stevens et al., 2006; Stevens et al., 2008; Yang et al., 2010). Thus, this mutation might lead the viruses to be well adapted to infect new hosts.

In conclusion, this study highlights the first IAV subtype H7N1 ever reported in Thailand. Monitoring and genetic characterization of LPAI H7 should be continue conducted because it may become highly pathogenic as reported in Italy (Capua and Marangon, 2000). Monitoring of IAVs in quails could help promote an awareness of LPAI influenza infections in animals in the future.

CHAPTER IV

High affinity DNA aptamers against swine influenza H3N2 viruses

This study has been done at the University of Minnesota as part of research collaboration in aboard of the Chulalongkorn University Dutsadi Phiphat scholarship. The study described in this chapter is related to influenza A virus detection and would be useful for influenza prevention and control in the future.

4.1 Introduction

Influenza is an acute contagious disease caused by influenza viruses. Viruses belong to the family *Orthomyxoviridae* contain 5 genera; influenza A, B and C, thogotovirus and Isavirus (Webster et al., 1992; Krossoy et al., 1999). All influenza pandemics in human have been caused by genus influenza A. Moreover, influenza A viruses (IAVs) have been reported to infect a wide range of animal species including humans (Lvov et al., 1978; Webster et al., 1992; Amonsin et al., 2006b; Amonsin et al., 2007; Taubenberger and Morens, 2010). Based on the antigenic properties of hemagglutinin (HA) and neuraminidase (NA) genes, IAVs can be divided into 17 HA and 9 NA subtypes (Tong et al., 2012).

Swine influenza virus (SIV) was first isolated from pigs in 1930 and identified as H1N1. Swine influenza is not only an important cause of respiratory disease in swine industry throughout the world but also poses an important for human public health concern. Since human, avian and swine IAVs can replicate in pigs thus pigs have been implicated as “mixing vessels” that generate novel viruses with wide host range (Kida et al., 1994; Ito et al., 1998; Ma et al., 2009). Even pigs can be infected with many subtypes of IAVs, they serve as major reservoirs of only three subtypes (H1N1, H1N2 and H3N2) which are endemic in swine population worldwide (Webster et al., 1992; Kida et al., 1994; Brown, 2000b).

Since 1998, triple reassortant SIV H3N2 have been circulating in swine herds worldwide. The viruses contain PB1, HA and NA genes from human IAV, NP, M and NS genes from classical SIV, and PB2 and PA genes from avian influenza viruses (AIV) (Webby et al., 2000; Zhou et al., 2000). Recently, swine H3 viruses in the phylogenetic cluster IV that emerged as vaccinal escape variants of Cluster III have now become dominant in pig populations in North America (Olsen et al., 2006b; Gramer et al., 2007; Vincent et al., 2008).

In 2011, the novel reassortant SIV H3N2 containing internal genes of the 2009 pandemic H1N1 have been reported (Liu et al., 2011). In the same year, the Center for Disease Control and Prevention (CDC) reported two pediatric cases with swine origin-H3N2 influenza virus. This virus has been identified as "novel reassortant" because their genomes contain seven gene segments of triple reassortant SIV H3N2 circulating in North America since 1998 and matrix gene of the 2009 pandemic H1N1 (CDC, 2011b).

Current diagnostic tools rely on isolation of the viruses followed by hemagglutination or genome sequencing that are expensive and time consuming. Thus novel subtype specific ligands and methods are desperately needed for rapid testing and subtyping of IAVs in the field.

Aptamers are short, unique, single stranded nucleic acid ligands that can bind specifically to target molecule. They can completely be selected and characterized from complex synthetic libraries by *in vitro* process called "systematic evolution of ligands by exponential enrichment; SELEX" (Ellington and Szostak, 1990; Tuerk and Gold, 1990). This process starts with random oligonucleotides flanked with primer binding regions for PCR amplification.

An example of aptamer for therapeutic agent in humans is pegaptanib sodium (Macugen®). It is the first aptamer product to be successfully developed as therapeutic agent. It has been approved by the US FDA in 2004 for the treatment of all types of neovascular age-related macular degeneration (AMD). The function of this aptamer is to inhibit a vascular endothelial growth factor (VEGF), a major regulator of unusual and excessive blood vessel growth and permeability in the eyes (Ng et al., 2006). The major

advantage of aptamers is that they can be completely selected and characterized through *in vitro* process by SELEX method. Therefore, it can potentially reduce or even replace the use of animals.

Based on our experience, we found that most of researchers failed with SELEX before getting the aptamers. The importance for aptamer selection and characterization includes;

1) Target molecules;

   The most highly conserved target molecules (proteins) are better than less conserved. Therefore, aptamers for IAVs are very difficult since these viruses have been classified to be quasispecies due to their extremely high mutation rate and exhibit significant genetic diversity. However, DNA aptamers developed from this study have already been proven that they can now be used in rapid detection and typing protocols for field applications.

2) Primers and PCR conditions;

   We found that these two factors are very important since inappropriate conditions can potentially produce undesired by-products. In this current study, we have used several conditions to reduce unspecific reactions as much as possible e.g. touch-down PCR, asymmetric PCR, new designed primers, number of PCR cycles.

3) Blocking reagents for aptamer dot-blot assay;

   We found that the importance step for the aptamer dot-blot assay is blocking step. At the beginning, we failed with this assay by using bovine serum albumin (BSA) as blocking reagent. However, after modification by changing the blocking reagent from BSA to fish gelatin combining with sheared salmon sperm DNA, we have succeeded to use our DNA aptamers to identify IAVs by dot-blot assay.

In the current study, high affinity DNA aptamers against recombinant HA protein from SIV H3 cluster IV were selected and characterized. This is the first report of DNA aptamers for IAVs demonstrating homologous and heterologous viral subtype specificity. Furthermore, our study shows that selected candidates bind directly to virus suggesting that these ligands can be translated into a rapid testing device for field use.

4.2 Materials and Methods

4.2.1 Randomized single-stranded DNA library and primers

Single-stranded DNA library (WAP40) consisting of randomized 40-mer DNA sequence flanked by constant primer binding regions, primers (sense strand; WP18 and antisense strand; WP20) and selected aptamers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Sequences of primers and DNA library are shown in table 4.1.

Table 4.1 Sequences of primers and DNA library used in this study

Name	Sequence
WAP40	5'-GTGCAGTCAAAGACGTCC-N(40)-GGCAATCGCACTTCATGGTC-3'
WP18	5'-GTGCAGTCAAAGACGTCC-3'
WP20	5'-GGCAATCGCACTTCATGGTC-3'
Bio-WP18	Biotin 5'-GTGCAGTCAAAGACGTCC-3'
Bio-WP20	Biotin 5'-GGCAATCGCACTTCATGGTC-3'
M13F	5'-GTTTTCCCAGTCACGAC-3'
M13R	5'-CAGGAAACAGCTATGAC-3'

4.2.2 Recombinant 6xHis-tagged H3 hemagglutinin protein

Recombinant 6xHis-tagged H3 hemagglutinin protein (recombinant swH3 protein) from SIV H3N2; A/swine/Minnesota/SG-00235/2007, was cloned and expressed in a baculovirus system in our laboratory. The recombinant swH3 protein was used as

template for selection and characterization of DNA aptamers. The reference recombinant HA proteins from Biodefense and Emerging Infections Research (BEI) Resources were used to test the specificity of aptamers. Detail descriptions of the recombinant HA proteins used in this study are shown in table 4.2.

Table 4.2 Detailed descriptions of the recombinant HA proteins used in this study

Proteins	Influenza virus origins	Sources of protein
H3 (template)	A/swine/Minnesota/SG-00235/07(H3N2)	St. Jude Children's Research: used for SELEX
H1	A/Solomon Islands/3/06 (H1N1)	BEI resource: NR-15170
H2	A/Singapore/1/57 (H2N2)	BEI resource: NR-2668
H3	A/Uruguay/716/07 (H3N2)	BEI resource: NR-15168
H5	A/Hong Kong/156/97 (H5N1)	BEI resource: NR-652
H5	A/bar-headed goose/ Qinghai/1A/05 (H5N1)	BEI resource: NR-9666
H6	A/teal/Hong Kong/W312/97 (H6N1)	BEI resource: NR-653
H7	A/Netherlands/219/03 (H7N7)	BEI resource: NR-2633
H7	A/Canada/rv444/04 (H7N3)	BEI resource: NR-12073
H9	A/Hong Kong/1073/99 (H9N2)	BEI resource: NR-654
6xHis-tagged NP	A/swine/Minnesota/07002083/07 (H1N1)	Assoc. Prof. Srinand Sreevatsan

4.2.3 Preparation of recombinant swH3 protein and Ni-NTA magnetic agarose bead matrix

Approximately 100 µL of Ni-NTA magnetic agarose bead (Qiagen, Hilden, Germany) were separated from solution using magnetic apparatus. Beads were washed three times with 500 µL of 50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, 0.005%Tween20, pH 8.0 (washing buffer) and then conjugated with recombinant swH3 protein in 500 µL of 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0 (binding buffer) at 4 ^{o}C with gentle shaking. After 48 hours incubation, protein-bead matrix was washed three times with 500 µL of washing buffer. After washing, the protein-bead matrix was stored at 4 ^{o}C and used within a day. In subsequent iterations of SELEX, the recombinant swH3 protein was gradually reduced from 30 µg to 0.4 µg (table 4.3).

4.2.4 Negative selection

Negative selection was performed in every iteration of SELEX to remove any non-specific candidates that bind to the Ni-NTA magnetic agarose beads. The aptamer library was denatured at 95 ^{o}C for 10 min and then incubated at room temperature for 10 min prior use. Three sets of approximately 100 µL of Ni-NTA beads were prepared by separating from solution and wash three times with 500 µL of washing buffer. The denatured aptamer library was added to Ni-NTA beads in 500 µL of 50 mM NaH_2PO_4, 50 mM NaCl, 20 mM imidazole, pH 8.0 (interaction buffer) and incubated at room temperature for 30 min by using rotisserie shaker. After the third negative selection, unbound library was then subjected to positive SELEX.

Table 4.3 Amount of recombinant swH3 protein used for each round of SELEX

SELEX rounds	Protein (ug)	Note
R1	30.00	N
R2	30.00	N
R3	25.00	N
R4	25.00	N
R5	20.00	N
R6	20.00	N
R7	15.00	N
R8	15.00	N
R9	7.50	N
R10	3.75	N
R11	1.88	N
R12	1.50	N
R13	0.75	N
R14	0.40	N and C
R15	0.40	N and C

N, Negative selection; C, Counter SELEX

4.2.5 SELEX

After negative selection, the spent aptamer library was submitted to positive SELEX against recombinant swH3 protein. The aptamer library was conjugated with protein-bead matrix in 500 µL of interaction buffer and incubated at room temperature using rotisserie shaker. After 1 hour incubation, unbound library or poor binders were removed by 11 wash steps with 1x PBS containing 0.05% Tween20 (1x PBST). Protein-bound aptamers were recovered with 100 µL of nuclease-free water and then subjected to polymerase chain reaction (PCR). PCR was performed to amplify all protein-bound aptamer candidates. Briefly, 3 µL of protein-bound aptamers were mixed with 5 pmol of forward primer (WP18) and biotinylated reverse primer (Bio-WP20), 2x HotStarTaq DNA

polymerase (Qiagen, Hilden, Germany) and nuclease-free water (final volume 50 µL). PCR was performed using to the following conditions: 95 °C for 15 min, 15 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s, and 72 °C for 7 min. Amplicons from PCR were then used as template for asymmetric-touchdown PCR to enrich for the sense strand to be applied back in the next round of SELEX. Briefly, 2 µL of amplicons were mixed with 30 pmol WP18 and 1.2 pmol Bio-WP20 (ratio of forward and reverse primers was 25:1). Touchdown PCR conditions used were 95 °C 15 min, 9 cycles of 95 °C 15 s, 72 °C 15 s (gradually decrease 1 °C each cycle), and 72 °C 15 s, 11 cycles of 95 °C 15 s, 63 °C 15 s and 72 °C 15 s, and final extension at 72 °C 3 min. Approximately 400 µL of amplicons from asymmetric-touchdown PCR was purified by MiniElute® PCR purification kit (Qiagen, Hilden, Germany) and then eluted with 60 µL of nuclease-free water. Subsequent iterations of SELEX used the sense strand aptamer thus purified PCR amplicons were mixed with Dynal M280 Streptavidin super paramagnetic beads (Invitrogen™, Dynal AS, Oslo, Norway) and then snap cooling for 15 min to remove the antisense strand. This process was repeated for a total of 15 iterations of SELEX. Binding affinity and specificity of aptamer candidates were investigated by chemiluminescent electromobility shift analysis (LightShift®, Chemiluminescent EMSA Kit, Pierce) before cloning and sequencing.

4.2.6 Counter SELEX to remove any His-tag binding candidates

At the last 2 rounds of SELEX (14th and 15th), 6xHis-tagged recombinant nucleoprotein (rNP protein) from SIV H1N1; A/swine/Minnesota/07002083/2007, was used for counter SELEX to remove any non-specific candidates that bind to 6xHis-tag portion of the protein. Protein-bead matrix was prepared by following the same procedures as used for recombinant swH3 protein. Aptamer pool was incubated with rNP protein at room temperature for 1 hour and then collected the supernatant using magnetic apparatus. The supernatant containing aptamer candidates was then submitted to positive SELEX.

4.2.7 Cloning and sequencing of the aptamer candidates

PCR amplicons containing aptamer pool of 8th and 15th round of SELEX were purified by MiniElute® PCR purification kit (Qiagen, Hilden, Germany). Purified amplicons were cloned into TA vector (pCR®2.1-TOPO®) and then transformed into chemically competent TOP10 *E.coli* cell by heat shock at 42 ^{o}C as manufacturer's recommendation (TOPO TA Cloning® Kit, Invitrogen™, Carlsbad, CA, USA). Transformed white colonies of *E.coli* cells were chosen and amplified by PCR with M13 primers. PCR amplicons were then submitted to the Biomedical Genomics Center at the University of Minnesota (BMGC, St. Paul, MN) for sequencing using the Sanger sequencing method. DNA sequences from enriched pool of the 15th round of SELEX were used to identify redundancy in selected sequences motifs by conducting a Clustal W alignment using MEGA4 (Tamura et al., 2007). Selected aptamer sequences were further analyzed for secondary structure prediction using mfold web server (Zuker, 2003).

4.2.8 Electrophoretic mobility shift assay (EMSA)

Selected aptamer sequences were synthesized and labeled with biotin (5') while amplicons containing enriched aptamer pool were amplified by PCR with Bio-WP18. Aptamer-protein binding assay was analyzed using LightShift® chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) as per manufacturer's instruction with a few modifications. Briefly, selected aptamers (20 fmol) were added to recombinant swH3 protein in 1x binding buffer and nuclease-free water (final volume 20 µL) and then incubated at room temperature for 30 min. Samples were loaded onto the 8% native polyacrylamide gel (Bio-Rad, Hercules, CA, USA) in 0.5x TBE buffer and then electrotransferred to a positively charged nylon membrane (Biodyne® B 0.45 µm, Pensacola, FL, USA). The membrane was processed and developed with Chemiluminescent Nucleic Acid Detection Module (Pierce, Rockford, IL, USA) as per manufacturer's instructions. Reactions on the membrane was then visualized and imaged with LabWorks™ 4.5 imaging system (UVP products, Upland, CA, USA)

4.2.9 Hemagglutination inhibition test

Hemagglutination inhibition (HI) test was performed to prove that the selected aptamers recognized the active site of the HA protein of SIV H3N2 and inhibit the viral infectivity. Turkey red blood cells (RBCs) were diluted to 0.5% in PBS. HA test was performed in microtiter 96-well plate with 50 µL of samples containing virus and 50 µL of 0.5%RBC. Samples were incubated at room temperature for 30-45 min and agglutination of RBC was inspected. For hemagglutination inhibition test, 500 pmol of each aptamer was added to the virus before addition of RBC and incubated at room temperature for 30 min.

4.2.10 DNase I footprinting assay

DNase I footprinting assay was performed to identify binding site(s) on the selected aptamer(s) (Zianni et al., 2006; Wang et al., 2011). The recombinant swH3 protein (4-6 ug) was mixed with 1 pmol of 5'FAM-HA68 in 1x binding buffer (Lightshift®, Pierce), nuclease-free water was added to a final volume 50 µL and then incubated at room temperature for 1 hour. After incubation, samples were treated with 0.2 U of DNase I, amplification grade (Invitrogen™ , Carlbad, CA, USA) and incubated at 37 °C for 5 min. To inactivate DNase I, 2 mM EDTA was added to each sample and incubated at 70 °C for 10 min. Recombinant protein from human influenza virus (recombinant hH1 protein); A/Solomon Islands/3/06 (H1N1) was used as negat ive control and treated under identical conditions. A negative control (without protein) was applied using nuclease-free water in place of protein. After reaction, samples were purified by MiniElute PCR purification kit (Qiagen, Hilden, Germany) and eluted with 14 µL of nuclease-free water. Approximately 12 µL of purified samples were submitted to the BMGC for fragment analysis on a 3130XL Genetic Analyzer. The binding region of aptamer was analyzed by Peak Scanner software v.1.0 (Applied Biosystems, Foster City, CA, USA) with Liz500 as internal size standard.

4.2.11 Specificity of each aptamer against recombinant HA proteins in different subtypes of influenza A viruses

Aptamer dot-blot assay was modified and performed to test the specificity of each aptamer by using recombinant HA proteins in different subtypes of IAVs from BEI resource; H1, H2, H3, H5, H6, H7 and H9 (table 4.2). Each protein was diluted with nuclease free-water to a concentration of 50 ng/μL and 2 μL and blotted onto nitrocellulose membrane (Protran®, Whatman™, Pierce). Membrane was blocked with 5% fish gelatin (Sigma®, St.Louis, MO) in 1x PBST at 4 °C. After 12 hours incubation, the membrane was incubated with 5'DIG-aptamers at the final concentration 100 nM in 1% fish gelatin at room temperature with gentle shaking for 1 hour and then washed 5 times with 1x PBST in 5 min increments. The membrane was incubated with peroxidase-conjugated IgG fraction monoclonal mouse anti-digoxigenin (Jackson Immuno Research Laboratories Inc., PA, USA) in 1:12,500 dilutions in 1% fish gelatin in 1x PBST at room temperature for 30 min. Membrane was then washed once with 1x PBS and developed with luminal-enhancer and stable peroxide solution in 1:1 dilution (Lightshift®, Thermo) at room temperature for 5 min. The membrane was visualized and imaged by LabWorks 4.5 software (UVP products, Upland, CA, USA).

4.2.12 Aptamers binding affinity

The recombinant swH3 protein was prepared at a concentration 25 ng/μL by diluting with 50mM Tris, 250mM NaCl, pH8.2 (protein buffer). Nitrocellulose membranes (Protan®, Whatman™, Pierce) were blotted with 2 μL of the protein (50 ng) in triplicate and dried at room temperature for 1 hour. Membranes were blocked with 5% fish gelatin in 1x PBST at 4 °C. After 12 hours incubation, the membranes were incubated with different concentration of 5' Bio-aptamer in two-fold dilution (1-1,024 nM) in 1% fish gelatin in 1x PBST at room temperature for 1 hour with gentle shaking. The membranes were washed with 1x PBST for 5 min with gentle shaking. After 5 times of washing, the membranes were incubated with NeutrAvidin Protein, Horseradish Peroxidase Conjugated (Thermo®) in 1:12,500 dilutions in 1% fish gelatin in PBST at room

temperature for 30 min. Membranes were washed once with 1x PBS and developed with luminol-enhancer and stable peroxide solution in 1:1 dilution (Lightshift®, Thermo) at room temperature for 5 min. Membrane were visualized and imaged by LabWorks 4.5 software. Chemiluminescence intensity was calculated by ImagJ 1.45s software. K_D was calculated based on nonlinear regression.

4.2.13 Influenza live virus detection by aptamer dot-blot assay

All processes working with live viruses were performed in a class II biosafety cabinet. SIV isolates of North America belonging to phylogenetic lineages of H3 were obtained from the Minnesota Veterinary Diagnostic Laboratory (MVDL) as blind samples in minimal essential medium (MEM). AIV H3N2 in allantoic fluid was used as the representative for H3 cluster I; A/mallard/South Dakota/SG128/2007 (H3N2) (Ramakrishnan et al., 2010). Phylogenetic clusters of the reference viruses used in this study were shown in figure 4.1. Samples containing live viruses were centrifuged at full-speed (13,000 rpm) for 5 min prior use. Then 2 µL of each culture (supernatant) was blotted onto the nitrocellulose membranes in triplicate (Protan®, Whatman™). The membranes were dried at room temperature for 1 hour and blocked with 5% fish gelatin (Sigma®, St.Louis, MO) in 1x PBST at 4 °C. After 12 hours incubation, denatured sheared salmon sperm DNA (Ambion®, Austin, TX) was added to the membranes with blocking buffer at the concentration of 10 ng/µL and then incubated at room temperature for 20 min. Membrane was incubated with 5'DIG-aptamers (100 nM) in 1% fish gelatin at room temperature for 1 hour and then washed five times for 5 min each with 1x PBST. The washing and developing steps were done as described above.

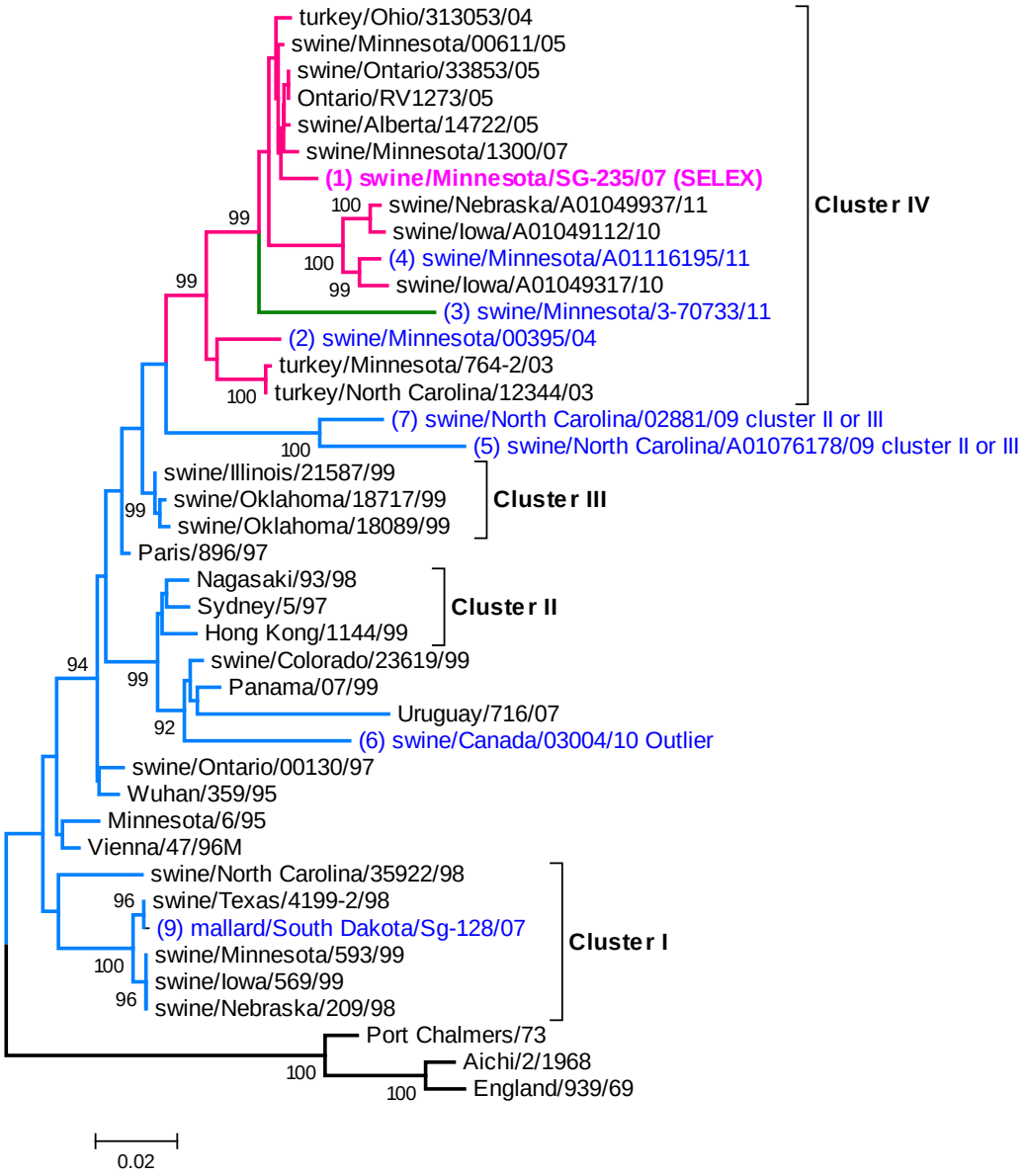

Figure 4.1 Phylogenetic cluster of IAVs subtype H3. (1) SIV used for SELEX, (2) to (9) the reference SIVs obtained from the Minnesota Veterinary Diagnostic Laboratory (MVDL). All numbers are linked to detailed description of reference influenza live viruses used in this study in Table 4.5.

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4.3 Results

4.3.1 Selection of DNA aptamers against recombinant swH3 protein from SIV H3N2

Enriched aptamer pool from the 8th round was cloned into *E.coli* and 80 colonies were sequenced. A tendency toward sequence saturation and thus aptamer candidate enrichment was identified and seven additional iterations of SELEX were performed. After 15 rounds of SELEX, enriched aptamer pool showed high affinity against recombinant swH3 protein by gel shift assay (data not shown). Aptamer pool was cloned into *E.coli* and 95 white colonies were submitted for DNA sequencing. The sequence similarity was compared and five sequences were chosen for further characterizations (figure 4.2). By aptamer dot-blot assay, four aptamer candidates were shown to be specific to recombinant swH3 protein and one (HA1c) not bind to any proteins.

4.3.2. Specificity of four aptamer candidates

Aptamer dot-blot assay was performed to test the specificity of the selected four aptamers. Results show that all aptamers are highly specific to H3 subtype compared with other 10 different recombinant proteins representing 7 different subtypes (figure 4.3).

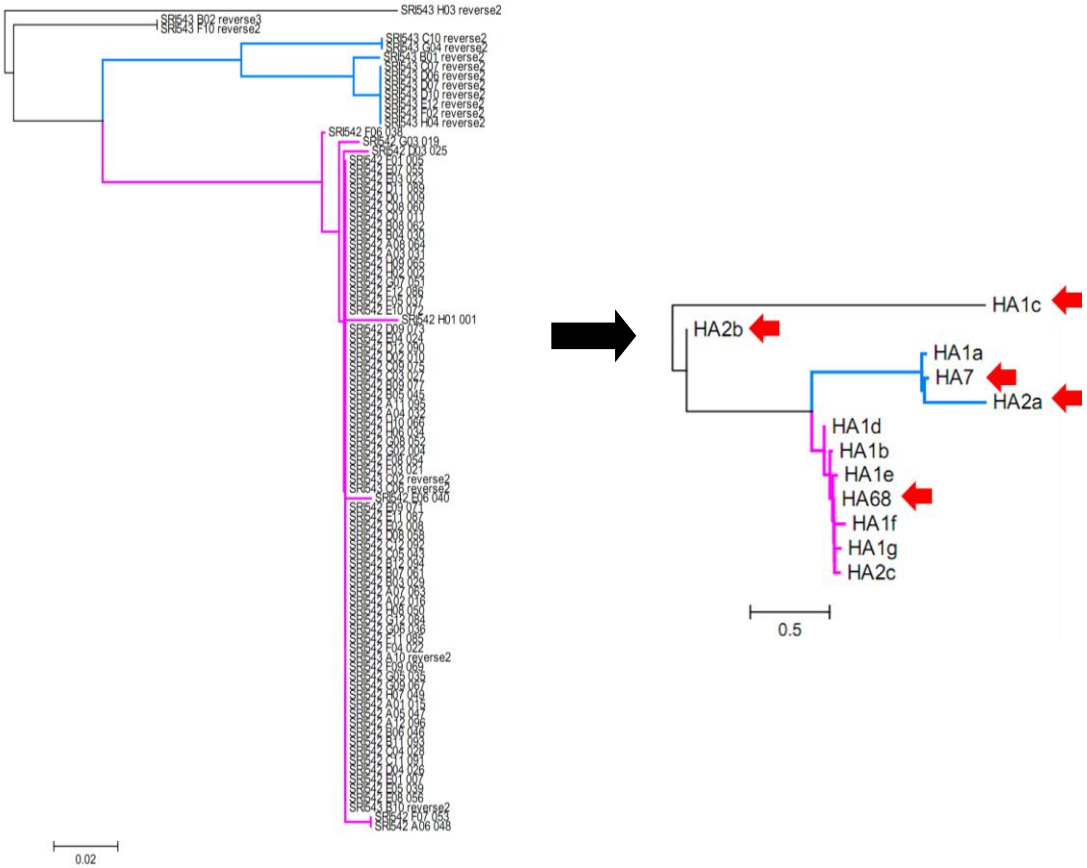


Figure 4.2 DNA sequences from enriched aptamer pool of the 15th round of SELEX were used to identify redundancy in selected sequences motifs by conducting a Clustal W alignment. The sequence similarity was compared and five sequences were chosen for further characterizations; HA1c, HA2b, HA7, HA2a and HA68.

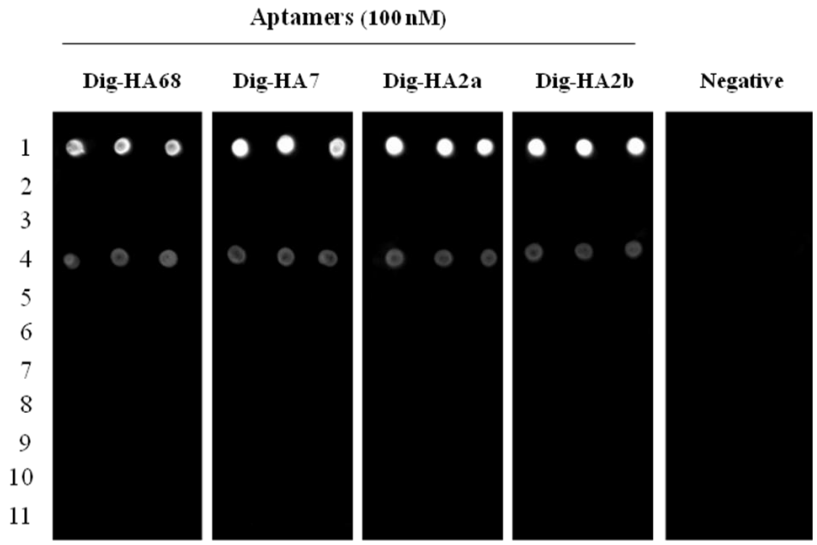


Figure 4.3 Subtype specificity of each aptamer against different recombinant HA proteins by aptamer-dot blot assay.

Table 4.4 Detailed description of recombinant HA proteins for aptamer-dot blot assay

Sample no.	Influenza virus origins	Subtypes
1	A/swine/Minnesota/SG235/07	H3N2
2	A/Solomon Islands/3/06	H1N1
3	A/Singapore/1/57	H2N2
4	A/Uruguay/716/07	H3N2
5	A/Hong Kong/156/97	H5N1
6	A/bar-headed goose/ Qinghai/1A/05	H5N1
7	A/teal/Hong Kong/W312/97	H6N1
8	A/Canada/rv444/04	H7N3
9	A/Netherlands/219/03	H7N7
10	A/Hong Kong/1073/99	H9N2
11	A/swine/MN/07002083/07 (NP*)	H1N1

*6xHis-tagged recombinant NP protein

4.3.3 Aptamer binding to recombinant swH3 protein

Aptamer HA68 and HA7 were chosen to test with EMSA to show that the aptamer specificity to recombinant swH3 protein and not to an extraneous 6xHis-tagged recombinant protein (NP protein). Results show that the aptamers specifically recognized recombinant swH3 protein in a dose-dependent fashion (figure 4.4).

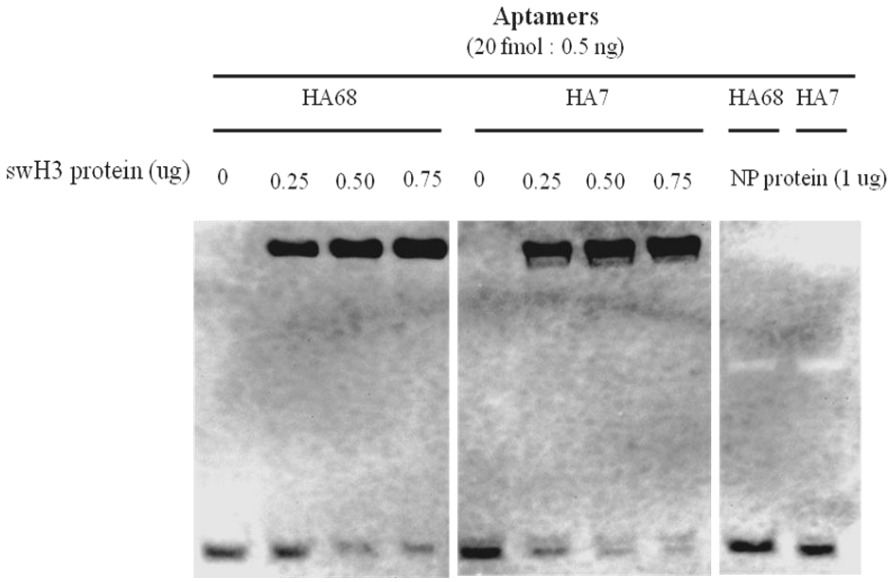


Figure 4.4 Two selected aptamer candidates, HA68 and HA7, were screened using electromobility shift assay (EMSA). Aptamers were electrophoresed with 6xHis-tagged-recombinant swH3 protein and 6xHis-tagged recombinant NP protein. Both aptamers specifically recognized to recombinant swH3 protein in a dose-dependent fashion.

4.3.4 Hemagglutination inhibition test (HI test)

Since the aptamers were enriched and selected by using recombinant protein, we want to show that these aptamers can bind to live virus. A/swine/Minnesota/SG-00235/2007 (H3N2) was used in a HI test for this purpose. All four aptamers at the concentration of 2.5 μM, HA68, HA7, HA2a and HA2b were able to completely inhibit the agglutination of RBC at 16, 8, 16 and 4 HA unit/50 μL, respectively (figure 4.5). In addition, we show none of selected aptamers bind to a heterologous virus;

A/swine/Minnesota/SG-00239/2007 (H1N2). The result indicates the aptamers bind specifically to HA protein likely at or around the receptor binding site(Hirst, 1941; Weis et al., 1988) of SIV H3N2 and do not bind to SIV H1N2.

4.3.5 Determination of the dissociation constant (K_D) of each aptamer

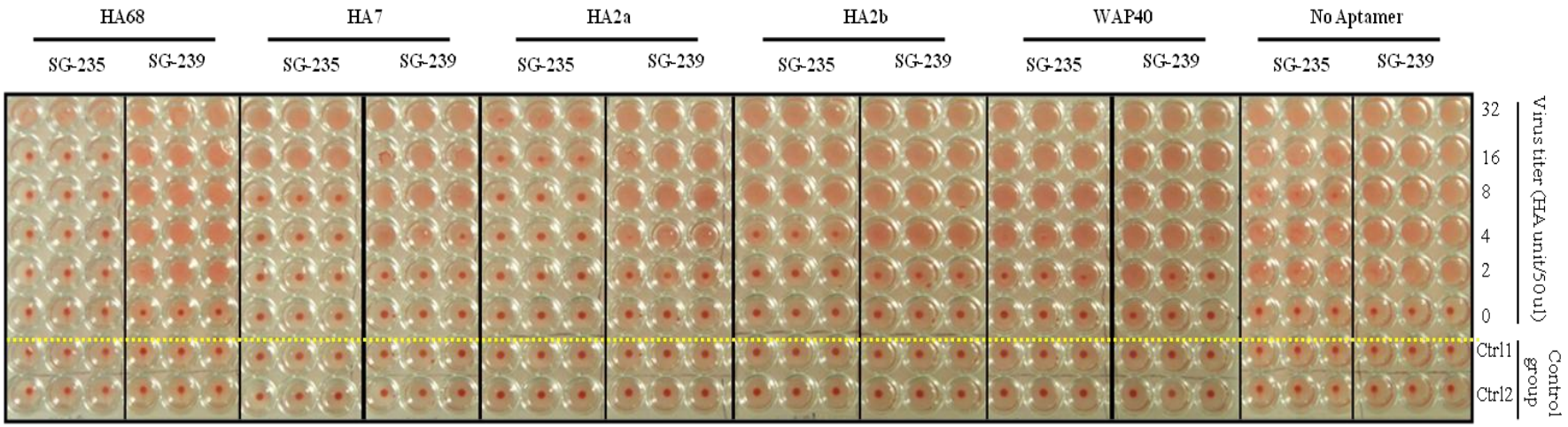
To describe the strength of binding affinity between aptamer and recombinant swH3 protein, dissociation constants (K_D) of each aptamer were calculated from dot-blot chemiluminescence intensity based on nonlinear regression. The results of binding curve were fit to the equation;

$$CI = \frac{CI_{max} \cdot [aptamer]}{K_D + [aptamer]}$$

In the equation above, CI = measure signal of chemiluminescence intensity, CI max = the maximum signal of chemiluminescence intensity, [aptamer] = concentration of aptamer, K_D = the dissociation constant. Binding affinity of HA68, HA7, HA2a and HA2b were 7.1, 22.3, 16.0 and 3.7 nM, respectively (figure 4.6).

4.3.6 Binding region of aptamer HA68 to recombinant H3 protein

DNase I footprinting assay was performed to identify binding region of aptamer to recombinant protein. In this study, HA68 was chosen for this purpose. The binding region of HA68 with recombinant swH3 protein was identified to be between nucleotide residues 8 and 40 (figure 4.7). Identical result was obtained using recombinant hH3 protein derived from A/Uruguay/716/2007 (H3N2). In addition, HA68 did not bind to recombinant hH1 protein; A/Solomon Islands/2/2006(H1N1) (figure 4.8).



Note: Final concentration of aptamer = 2.5 uM, SG-235; A/swine/Minnesota/SG-00235/2007(H3N2), SG-239; A/swine/Minnesota/SG-00239/2007(H1N2),
Control 1 = no virus control (this group contains aptamer + RBC + PBS – no virus),
Control 2 = no aptamer and virus control (this group contains RBC + PBS – no aptamer – no virus)

Figure 4.5 Hemagglutination inhibition test was performed to show that the selected aptamers can bind specifically to HA protein of SIV H3N2; A/swine/Minnesota/SG-00235/2007 (H3N2). All four aptamers at the concentration of 2.5 μM, HA68, HA2a, HA7 and HA2b were able to completely inhibit the agglutination of turkey red blood cell at 16, 16, 8, and 4 HA unit/50 μL, respectively, compared with no inhibition of unselected library (WAP40). Additionally, all four selected aptamers did not bind to a heterologous virus; A/swine/Minnesota/SG-00239/2007 (H1N2), indicating the selected aptamers bind specifically to HA protein likely at or around binding site of SIV H3N2 and do not bind to SIV H1N2.

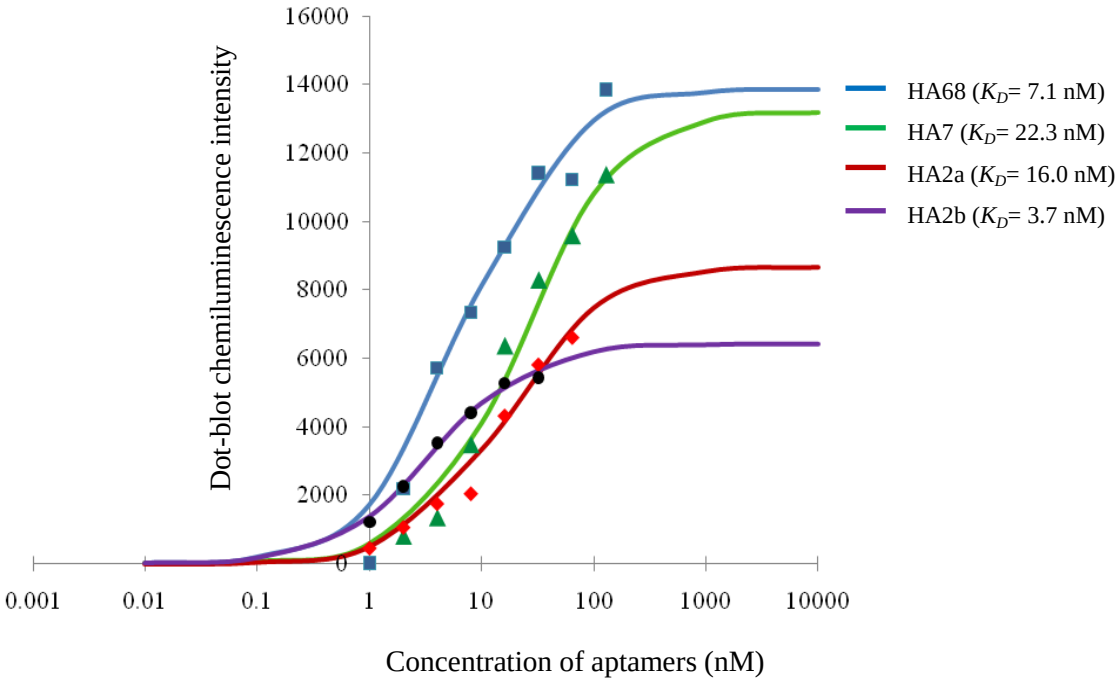


Figure 4.6 The dissociation constant (K_D) of each DNA aptamer was used to describe the strength of binding affinity between aptamer and recombinant swH3 protein. K_D was calculated from dot-blot chemiluminescence intensity based on nonlinear regression. Binding affinity of HA2b, HA68, HA2a, and HA7 were 3.7, 7.1, 16.0 and 22.3 nM, respectively.

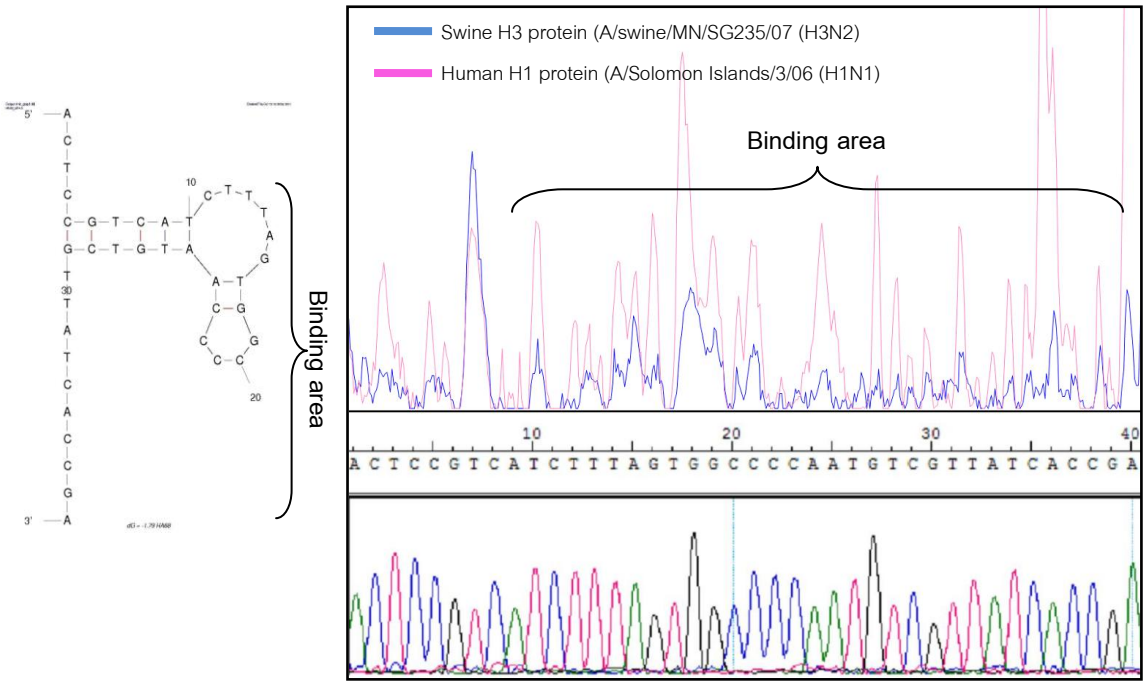


Figure 4.7 Aptamer binding site of HA68 was identified by DNase I footprinting assay. (Upper pannel) Fluorescein-labeled aptamer was reacted with recombinant swH3 protein (blue peak) or recombinant hH1 protein (pink peak) and then digested with DNase. Fragments protected by their binding to recombinant swH3 protein were identified using a 3130XL Genetic Analyzer. The binding site of aptamer was analyzed using PeakScanner software v.1.0 (ABI, Foster City, CA, USA). Aptamer residues 8-40 were protected from DNase, indicating that this region contains the recombinant swH3 protein binding site. (Lower panel) Nucleotide sequence chromatogram is shown. (Left panel) Secondary structure prediction of aptamer HA68 was analyzed using mfold web server and the putative binding sites are shown.

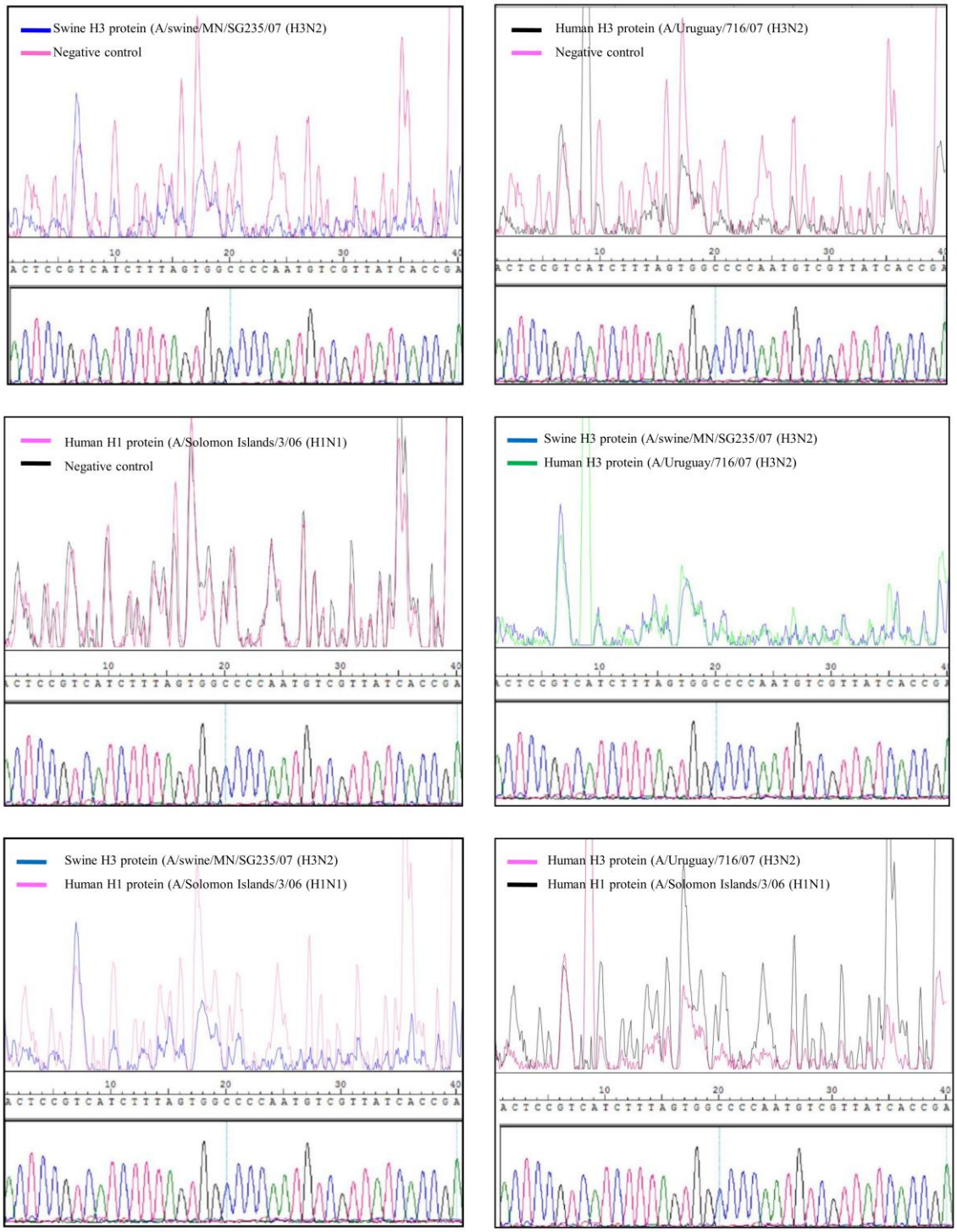


Figure 4.8 DNase I footprinting of aptamer HA68 against different recombinant proteins; swine H3N2, human H3N2 and human H1N1

4.3.7 Application of aptamers to detect influenza live viruses

Dot-blot assay was performed to prove the aptamer could be applied to detect live viruses. The reference SIVs were provided by the MVDL and used as the representative of each H3 cluster. The results showed that HA68 was highly specific to all four lineages of the H3 subtype. Further, the other three aptamers specifically identified H3 live viruses belonging to the phylogenetic clusters I and IV alone (figure 4.8 and table 4.3). It is likely that this application was affected by virus titer in the cultured samples (figure 4.9).

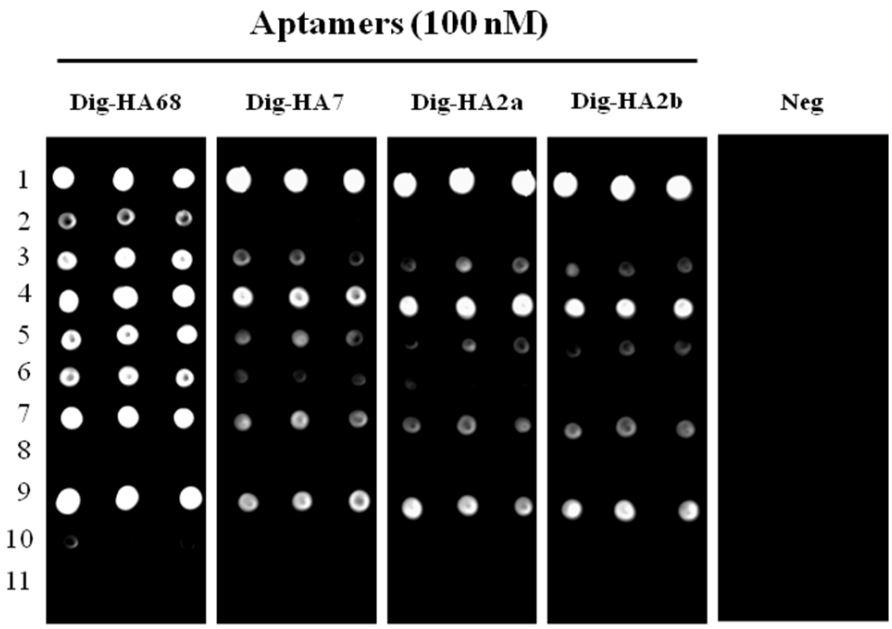


Figure 4.9 Aptamer dot-blot assay was performed to detect reference influenza live viruses in difference phylogenetic lineages of H3 (sample number 1-7 and 9, detail descriptions of each sample are shown in Table 3). SIV H1N2 (8), AIV H4N8 (10) and 10%FBS in MEM (11) were used as negative controls. 2 µl of each sample containing live viruses was blotted onto nitrocellulose membrane in triplicate and then detected with digoxigenin-labeled aptamers. Aptamer H68 was highly specific to all four lineage of H3 subtype. While, other three aptamer specifically identified H3 live viruses in clusters I and IV. However, it is likely that this application was affected by virus titer in the cultured samples.

Table 4.5 Detailed description of the reference influenza live viruses used in this study and the results of each aptamers detected by aptamer-dot blot assay

Samples*	Subtypes	HA titer**	H3 clusters	Results of each aptamer			
				HA68	HA7	HA2a	HA2b
1. A/swine/MN/SG235/07	H3N2	64	IV	+	+	+	+
2. A/swine/MN/00395/04	H3N2	2	IV	+	-	-	-
3. A/swine/MN/3-70733/11	H3N2	6	IV	+	+	+	+
4. A/swine/MN/A01116195/11	H3N2	16	IV	+	+	+	+
5. A/swine/NC/A01076178/1	H3N2	32	II or III	+	+/-	+/-	-
6. A/swine/Canada/03004/10	H3N2	16	Outlier	+	+/-	-	-
7. A/swine/NC/02881/09	H3N2	16	II or III	+	+	+	+
8. A/swine/MN/SG239/07	H1N2	16	-	-	-	-	-
9. A/mallard/SD /Sg-00128/07	H3N2	4	I	+	+	+	+
10. A/blue-winged teal/LO/Sg-00163/07	H4N8	16	-	-	-	-	-
11. MEM+10%FBS	Negative	-	-	-	-	-	-

*Reference SIVs H3N2 (Sample number 2-7) were provided by the Minnesota Veterinary Diagnostic Laboratory (MVDL).

**HA titer has been reported as HA unit/50 ul

SD; South Dakota, MN; Minnesota, NC; North Carolina, LA; Louisiana.

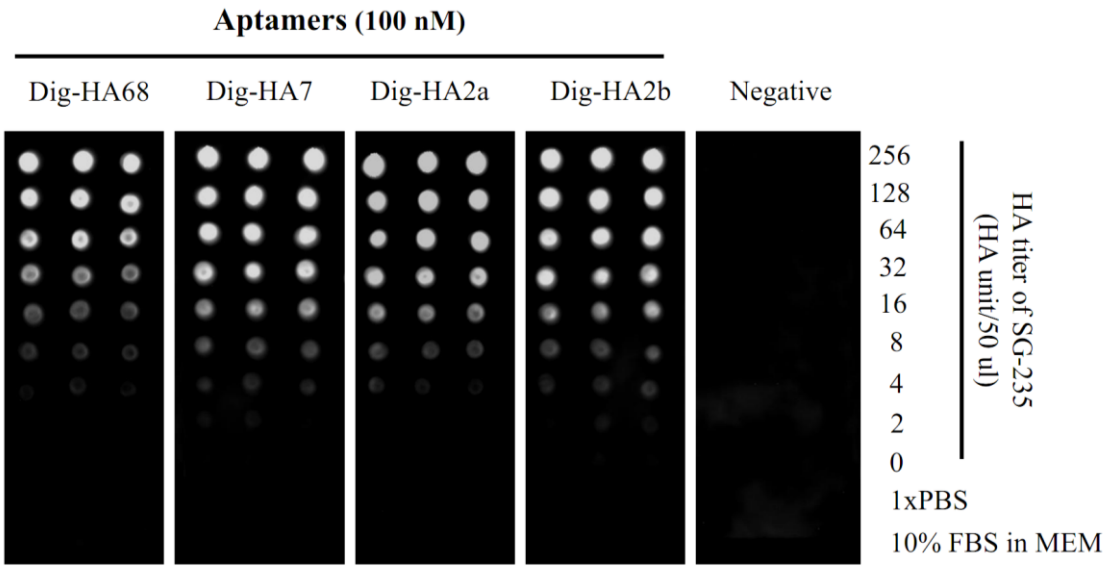


Figure 4.10 Chemiluminescent intensity of aptamer dot-blot assay was affected by virus titer (sample containing influenza live virus, SG-235, was diluted with 1x PBS in two-fold dilution).

4.4 Discussion and conclusion

Since 1998, SIV H3N2 initially isolated in North America, has become endemic in swine herds. Although pigs can be infected with many subtypes of IAVs but H1N1, H3N2 are most important and frequently isolated from pigs worldwide (Brown, 2000b). Currently, most diagnostic tools rely on HI test and virus isolation, both of which are time consuming and require extensive laboratory resources including RBCs or embryos from specific pathogen free chicken and cell culturing facilities. Although antibodies have been made for a wide range of applications, they are also associated with limitations. The process of antibody production starts in animals or cell cultures that are difficult and expensive. Frozen stocks of antibodies might lose their activities. The performance of the same antibody seems to have a batch-to-batch variation. Moreover, antibodies are sensitive to temperature and irreversible after denaturation thus they have a limited shelf life and not suitable to transport at ambient temperature (Jayasena, 1999).

On the other hand, aptamers show promise of being ideal candidates for molecular targeting applications. Aptamers can be chemically synthesized and all processes were done by *in vitro* techniques. Aptamers can be applied on a wide range of matrices primary clinical specimen. This addresses the main advantage of aptamer over antibody since it can reduce the physiological variations from animals and replace the animal systems (Luzi et al., 2003). Moreover, aptamers can be easily and inexpensively synthesized without batch-to-batch variation (Tuerk, 1997; Luzi et al., 2003). They are also stable to long-term storage and easy to transport at ambient temperature. Similar to antibodies, aptamers can also be labeled with reporter molecules such as fluorescein, biotin and digoxigenin that increase the applicability for further applications (Syed and Pervaiz; Brody and Gold, 2000; Stojanovic et al., 2001; Bunka and Stockley, 2006; Javier et al., 2008; Yan et al., 2011). DNA and RNA aptamers have shown similar function and performance in term of affinity and specificity (Gold et al., 1995). RNA molecules are susceptible to enzymatic degradation. In addition, DNA aptamers are easier to prepare, stable, and can be amplified in one step by PCR and manipulated for selection process. Current applications are primarily DNA based (Breaker, 1997).

IAVs attach to host cells via sialic-acid (SA) receptor. This receptor is also found on RBCs of several species of animals. Turkey, guinea pigs, type O-human blood and chicken RBCs are traditionally used for HI test (Ito et al., 1997; Louisirirotchanakul et al., 2007; Kayali et al., 2008). Due to the presentation of SA receptor on RBCs, influenza viruses can agglutinate RBCs (Hirst, 1941). Since the aptamers in this study have been developed based on recombinant swH3 protein thus HI test using turkey RBC was performed to prove the aptamers can bind to viral HA. The result of HI test showed that all four aptamers showed an inhibitory effect of *in vitro* viral infectivity by HI test suggesting both diagnostic and therapeutic potential for the selected aptamers (figure 4.5).

IAVs have been identified in mixed infections due to the genomic heterogeneity identified by next generation sequencing and exhibit significant genetic diversity

(Ramakrishnan et al., 2009; Lauring and Andino, 2010). HA protein is the part of virus that recognized by host immune system. Therefore, incremental variations largely result in antigenic drift (Bean et al., 1992; Zhang et al., 2007; Lauring and Andino, 2010). Developing aptamers to identify subtype and its application to use with other IAVs within subtype is challenging.

In this study, high affinity DNA aptamers against SIV H3N2 were selected and characterized. The results of aptamer-dot blot assay showed that aptamers in this study can be used to detect and differentiate influenza subtypes. Aptamer HA68 has shown high sensitivity and specificity to all four SIV H3 clusters. Other three aptamers (HA7, HA2a and HA2b) have shown high sensitivity and specificity to SIV H3 cluster I and IV (table 4.3 and figure 4.8). For future applications, HA68 can be used for detection and subtyping of SIV and other aptamers can be applied to specify the phylogenetic cluster.

CHAPTER V

CONCLUSION

This dissertation was conducted for 3 main objectives. The first objective was to describe the diversity and genetic characteristic of IAVs in wild birds. The second objective was to describe the diversity and genetic characteristic of IAVs in quails in HPAI-H5N1 high risk areas. The third objective was to develop high affinity DNA aptamers against HA protein of SIV H3N2. For the first objective, we provided the occurrence of IAVs in wild bird population. In this study, four H12N1 were recovered from two wild bird species; watercock (n=1) and lesser whistling-ducks (n=3). Based on the information from public database, Thai H12N1 viruses are the first H12N1 of Eurasian strain. For the second objective, we provided the occurrence of IAVs in quails. This study showed the first IAV subtype H7N1 ever reported in Thailand. Genetic characteristic of one IAV H7N1 was also elucidated. For the third objective, we have developed high affinity DNA aptamers against SIV H3N2. We already proved that the aptamers developed in this study can also be used for rapid detection and typing protocols for field applications.

The diversity and genetic characteristics of IAVs circulating in wild birds was described in Chapter II. Up to date, except HPAI-H5N1, LPAI subtype H3N1 is the only one strain reported in wild bird species in Thailand; A/mynah/Haneda-Thai/1976 (available only NS gene, GenBank accession number M17070). Due to the information of IAVs in wild bird population in Thailand is still limited, in this study a 15-month monitoring program was conducted from February 2009 to April 2010 in two HPAI-H5N1 high-risk areas of central Thailand, Ayutthaya and Suphanburi provinces. Wild birds were caught by local villagers and immediately released after sample collection. During 15 months, we collected and tested 2,994 swab samples from 47 wild bird species in 25 families, 9 orders. Based on the result of rRT-PCR, the occurrence of IAVs in wild birds was 2.07% (67/2,994). It has previously been reported that the frequencies of IAVs outbreak in wild birds were significantly higher during winter season in Thailand

(November to February) (Siengsanan et al., 2009). In addition, the occurrence of IAVs in wild birds in this current study was high during June to August 2009. Based on our observation, during that time, wild birds had a lot of food in rice fields since farmers have just finished harvesting.

Due to the low virus titer, most of the samples could not be identified the specific subtypes. In this study, four IAVs subtype H12N1 can only be identified and completed for whole genome sequencing. Genetic characteristics showed that all eight genes of Thai H12N1 viruses were closely related to IAVs of the Eurasian strain and classified to be low pathogenic. Thai H12N1 viruses were isolated from two wild bird species (watercock and lesser whistling-duck) in the same location (Ayutthaya province). During sample collection, we also found that wild birds have shared the same feeding areas with free-grazing ducks. This helps promote cross transmission from wild bird species to domestic poultry populations. We suggest that monitoring of IAVs in wild bird species should be continuously conducted to further understanding of IAVs circulating in wild bird population.

Chapter III described the diversity and genetic characteristic of IAVs in quails. As the previous study of HPAI-H5N1 in Thailand, the prevalence of the virus in live-bird markets was 1.4% (12/836). Interestingly, 41.4% (5/12) of HPAI-H5N1 viruses was isolated from quails (Amonsin et al., 2008). In this study, a 12-month monitoring program (May 2009 through April 2010) of IAVs in quails was conducted in two provinces in central Thailand where presumably located in HPAI-H5N1 high-risk areas: Ayutthaya and Suphanburi. A total of 2,040 swab samples from quails was collected and tested. Based on rRT-PCR, 1.18% of swab samples (24/2,040) was positive to IAVs and the occurrence was high in May and September 2009. Three samples can be identified as IAVs subtype H7N1 and only one sample was chosen and subjected for whole genome sequencing. Due to low virus titer, nested PCR was performed to increase specificity of the test and H7 HA and N1 NA genes were successfully completed. Genetic analysis at HA cleavage site showed that the virus was classified to be low pathogenic. Phylogenetic analysis showed that most genes (PB2, PB1, PA, HA, NP, M and NS) were

closely related to LPAI of Eurasian strain. It should be noted that NA gene of Thai H7N1shown more closely related to HPAI-H5N1 in Vietnam and Thailand lineage. This evidence leads to the speculation that there is a possibility of genetic reassortment of H7Nx and HxN1 viruses circulating in poultry populations in Thailand. We strongly suggest routine monitoring IAVs in quails for further understanding of IAVs circulating in quail population and how they may impact on public health.

High affinity DNA aptamers against HA protein of SIV H3N2 were developed and the methods for selection and characterization were described in Chapter IV. In this study, DNA aptamers against HA protein of SIV H3 cluster IV were developed through the SELEX method. To our knowledge, this technology was first developed from 3 independent research groups during 1988 to 1989 and published their results in 1990 (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990). Currently, most diagnostic tools rely on HI test and virus isolation, both of which are time consuming and require extensive laboratory resources including RBCs or embryos from specific pathogen free chicken and cell culturing facilities. Although antibodies have been made for a wide range of applications, they also have some limitations. Due to the advantage of aptamers over antibodies, aptamers are shown the ideal candidates for molecular targeting applications. In this study, four candidate aptamers were identified and characterized. The results of aptamer dot-blot assay showed that selected DNA aptamers can be used to detect and differentiate influenza subtypes. Furthermore, all four aptamers showed an inhibitory effect of *in vitro* viral infectivity by HI test suggesting both diagnostic and therapeutic potential for the selected aptamers.

In conclusion, this dissertation reported the novel subtypes of IAVs circulating in Thailand; H12N1 in wild bird species and H7N1 in quails. Monitoring and genetic characterization of IAVs in wild bird and quail populations should be continuously conducted to promote an awareness of IAVs infection in animals and humans in the future. Subtype specific aptamers against SIVs H3N2 developed from this study can also be used in rapid detection and typing protocols for field applications.

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APPENDICES

APPENDIX A

Criteria of sample collection from wild birds and quails

1. **Collection of samples from wild birds**
    - Two wild bird habitats in two provinces of central Thailand (Ayutthaya and Suphanburi provinces) will be selected using the following criteria:
        - The provinces are high risk areas of HPAI H5N1 outbreaks based on the previous records of HPAI outbreaks in Thailand.
        - Participated villagers have expertise in wild bird handling. They could perform capture methods that minimize injury to birds.
    - Wild bird species of specific interest will be wild waterfowls, however, other species e.g. residential and terrestrial birds in the areas will also be sampled
    - Each wild bird will be swabbed from the oropharynx and cloacae (tracheal exudates, cloacal contents and feces) using sterile cotton swab.
    - Swab samples will be stored immediately in viral transport media (VTM) at 4 ^{o}C and shipped to the laboratory at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University within 24 hours.

2. **Collection of samples from quails**
    - Two quail farms from two provinces of central part of Thailand (Ayutthaya and Suphanburi provinces) will be selected using the criteria:
        - HPAI H5N1 outbreaks have previously been reported in the areas and farms have been also affected by the outbreaks.
        - There are high densities of quail farms in the high risk areas.

- Wild bird habitats are located around the farms.

- Each quail will be swabbed from the oropharynx and cloacae (tracheal exudates, cloacal contents and feces) using sterile cotton swab.
- Swab samples will be stored immediately in viral transport media (VTM) at 4 ^{o}C and shipped to the laboratory within 24 hours.

APPENDIX B

Nucleotide sequences

A/watercock/THA/CU-W3699/2009(H12N1)

Polymerase basic protein 2 (PB2) gene 2,257 nucleotide; GenBank: JN982500

CTAACAACTGGTCTTGCTTATGACAAGATTTGCATCGGCTATCAGACGAATAACTCAACCGATACGGTAAACACACTAATTGAAC
AGAATGTCCCAGTCACTCAGGTGGAAGAGCTCGTGCATGGTCAAGTCAATCCGATCCTATGCAGTACAGAACTGGGGTCACCC
CTAGTGCTTGACGATTGCTCTCTGGAGGGCTTGATCCTAGGCAATCCCAAATGCGATCTCTATCTGAATGGTAGAGAATGGTCAT
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CTTCAATCAAGAAATATGAAAGAGTGAAAATGTTCGACTTCACCAAATGGAATGTCACTTACACTGGAACGAGCAAAGCTTGTAAT
AACACCTCAAACCAAGGATCATTCTACAGGAGCATGAGATGGCTCACTTTGAAGTCAGGACAATTCCCAGTGCAAACCGATGAG
TACAAAAACACTAGGGATTCGGACATTGTATTCACATGGGCTATTCATCACCCACCCACTTCTGACGAGCAGGTGAAGCTCTACA
AGAACTCCGACACTCTCTCGTCAGTCACAACGGATGAAATTAACAGGAGTTTCAGGCCTAACATAGGGCCAAGACCCCTGGTTA
GGGGACAACAAGGGAGGATGGATTATTATTGGGCTGTTCTAAAGCCAGGGCAGACAGTGAAGATACAGACTAATGGAAATCTAA
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CCGAATGTCAGTTAAATGAAGGAGTAATGAACACCAGCAAACCGTTTCAGAATACCAGCAAGCATTACATTGGGAAATGCCCTA
GATATATACCATCGAACAGTTTGAAACTGGCGATAGGGCTCAGGAATGTTCCCCAGGCCCAAGACCGAGGGCTCTTTGGAGCA
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CATTGCAGCAGACAGGGACAGCACCCAGAAGGCAATGGACAACATGCAGAACAAACTGAACAATGTCATTGACAAAATGAACA
AGCAATTCGAAGTGGTGAACCACGAATTTTCGGAGGTTGAAAGTAGAATAAATATGATCAATTCAAAAATTGACGATCAAATAACA
GACATATGGGCATATAATGCGGAACTGCTAGTTTTGCTGGAGAATCAGAAGACACTAGACGAGCACGATGCGAATGTTAGGAAT
CTCCATGACCGAGTCAGAAGGGTATTAAAAGAAAATGCGATAGATACTGAGGATGGGTGTTTTGAAATACTACACAAATGTGATG
ATGAATGCATGGATACAATCAAGAACGGGACTTACAACCACCAGAAATACGAAGAAGAGAGCAAGCTTGAGAGACAAAGGATT
AATGGAGTGAAACTTGAAGAGAATTCTACATATAAAATCTTGAGCATCTACAGCAGTGTTGCCTCAAGCTTAGTATTACTGCTCAT
GATTATTGGGGGTTTCATTTTCGGATGTCAAAATGGAAATGTTCGTTG

Polymerase basic protein 1 (PB1) gene 2,259 nucleotides; GenBank: JN982501

ATGGATGTCAATCCGACTTTACTTTTCTTAAAGGTGCCAGCGCAAAATGCTATAAGTACTACATTCCCTTACACTGGAGATCCTCC
ATACAGCCATGGAACAGGAACAGGATACACCATGGACACAGTCAACAGAACACATCAGTATTCAGAAAAGGGAAAGTGGACAA
CAAACACAGAGACCGGAGCACCCCAACTCAACCCGATTGATGGACCCTTACCTGAGGATAATGAGCCCAGTGGATATGCACAA
ACAGATTGTGTATTGGAAGCAATGGCTTTCCTTGAAGAATCCCACCCAGGGATCTTTGAAAACTCTTGTCTTGAAACGATGGAAG
TTGTTCAGCAAACAAGAGTGGACAAACTAACCCAAGGTCGCCAGACTTATGACTGGACATTGAATAGAAACCAACCGGCTGCAA
CTGCTTTGGCCAACACTATAGAGGTTTTCAGATCGAACGGTCTGACGGCCAACGAATCAGGGAGACTAATGGATTTTCTCAAGG
ATGTGATGGAATCAATGGATAAAGAAGAAATGGAAATAACAACACATTTCCAAAGAAAGAGAAGAGTAAGGGACAACATGACCA
AGAAGATGGTCACACAAAGAACAATAGGGAAGAAGAAGCAGAGGCTGAACAAGAGGAGCTACTTAATAAGAGCACTGACGCTG
AACACAATGACAAAGGATGCAGAGAGAGGCAAATTGAAGAGGCGGGCAATTGCAACACCCGGGATGCAGATTAGAGGATTCG
TGTACTTTGTCGAAACACTGGCGAGGAGCATCTGTGAGAAACTTGAGCAATCTGGACTCCCCGTTGGAGGGAATGAGAAGAAG

GCCAAATTGGCAAATGTCGTGAGAAAAATGATGACTAATTCACAAGATACAGAGCTCTCCTTTACAATTACTGGAGACAACACCA
AATGGAATGAGAATCAAAATCCTCGGATGTTCCTGGCAATGATAACATACATCACAAGAAACCAACCTGAATGGTTTAGAAATGT
CTTGAGCATTGCCCCTATAATGTTCTCAAACAAAATGGCGAGATTAGGAAAAGGGTACATGTTTGAAAGTAAGAGCATGAAACTA
CGGACACAAATACCGGCAGAAATGCTTGCAAACATTGACTTGAAATACTTCAACGAGTCAACGAGAAAGAAAATCGAGAAAATA
AGACCTCTGCTAATTGATGGCACAGCCTCATTGAGTCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACAGTATTGGGA
GTCTCAATCCTGAATCTTGGGCAAAAGAGGTACACCAAAACCACATACTGGTGGGATGGACTCCAATCCTCTGATGATTTCGCTC
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TGATAAACAACGACCTTGGACCAGCAACAGCTCAGATGGCTCTTCAGCTATTCATCAAGGACTATAGGTACACATACCGATGCC
ACAGGGGTGATACACAAATTCAAACGAGGAGATCATTCGAGCTGAGGAAGCTGTGGGAGCAGACCCGTTCAAAGGCAGGGCT
GTTGGTATCAGACGGAGGACCAAATCTATACAACATTCGGAATCTCCACATCCCAGAGGTCTGCTTGAAATGGGAACTGATGGA
TGAAGATTACCAAGGCAGGCTGTGTAATCCTCTGAATCCGTTTGTCAGTCATAAGGAAATTGAGTCTGTAAACAATGCTGTAGTAA
TGCCAGCCCACGGCCCAGCCAAGAGCATGGAATATGATGCTGTTGCAACTACACACTCATGGATCCCTAAGAGGAACCGTTCT
ATTCTCAATACCAGCCAAAGGGGAATTCTTGAGGATGAGCAAATGTACCAGAAGTGTTGCAATCTATTCGAGAAATTCTTCCCCA
GTAGTTCATACAGGAGGCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGATGCACGCATTGATT
TCGAATCTGGAAGGATTAAGAAAGAAGAGTTCGCTGAGATCATGAAGATCTGTTCCACCATTGAAGAACTC

Polymerase acidic protein (PA) gene 2,217 nucleotides; GenBank: JN982502

AGCAAAAGCAGGTACTGATCCGAAATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATTGTCGAGCTTGCGGAAAAAGCA
ATGAAAGAATACGGGGAAGATCCGAAAATCGAAACGAACAAATTTGCTGCAATATGCACACACTTAGAAGTCTGTTTCATGTATT
CGGATTTCCACTTTATTGATGAACGAGGCGAATCAATAATTGTAGAATCTGGCGATCCGAATGCATTATTGAAACACCGATTTGAG
ATAATTGAAGGGAGAGACCGAACAATGGCCTGGACAGTGGTAAATAGTATCTGCAACACCACAGGAGTCGATAAGCCTAAATTC
CTCCCAGATTTGTATGACTACAAAGAGAACCGATTCATCGAAATTGGAGTGACACGAAGGGAAGTTCACATATACTATCTAGAAA
AAGCCAACAAGATAAAATCAGAGAAGACACACATTCACATATTCTCATTCACTGGAGAGGAAATGGCCACTAAAGCGGACTATA
CCCTTGATGAGGAGAGCAGAGCAAGAATCAAAACCAGGCTGTTCACCATAAGGCAAGAAATGGCCAGTAGGGGTCTATGGGAT
TCCTTTCGTCAGTCCGAGAGAGGCGAAGAGACAATTGAAGAAAGATTTGAAATCACAGGAACCATGCGCAGGCTTGCCGACCA
AAGTCTCCCACCGAACTTCTCCAGCCTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGGGCAA
GCTTTCTCAAATGTCAAAAGAAGTGAACGCCAGGATTGAGCCATTTCTGAAAACAACACCACGCCCTCTCAGATTACCTGATGG
GCCTCCCTGCTCTCAGCGGTCGAAGTTCTTACTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAAGGGGAAGG
TATACCACTATATGATGCAATCAAATGCATGAAGACATTTTTCGGTTGGAAAGAGCCCAACATCGTAAAACCACATGAAAAAGGC
ATAAACCCCAATTACCTCCTGGCTTGGAAGCAGGTGCTGGCAGAACTCCAAGACATTGAGAATGAGGAGAAAATTCCAAAAACA
AAGAACATGAAGAAAACAAGCCAATTGAAGTGGGCACTTGGTGAGAACATGGCACCAGAGAAAGTAGACTTTGAGGACTGCAA
AGATGTTAGTGATCTAAGACAGTATGACAGTGATGAACCAGAGTCTAGATCGCTAGCAAGCTGGATCCAGAGCGAATTCAACAA
AGCATGCGAATTGACAGATTCAAGTTGGATTGAACTTGATGAAATAGGGGAAGATGTTGCTCCAATTGAGCACATTGCGAGTATG
AGAAGAAACTATTTCACAGCGGAAGTATCCCATTGCAGGGCTACTGAATACATAATGAAGGGGGTGTACATAAACACAGCCCTG
TTGAATGCATCCTGTGCAGCCATGGATGACTTCCAACTGATTCCAATGATAAGCAAGTGCAGAACCAAAGAAGGAAGACGGAAG
ACAAATCTGTATGGATTCATTATAAAAGGGAGATCCCATTTGAGGAATGACACCGATGTGGTAAACTTTGTGAGCATGGAATTCTC
TCTTACTGACCCGAGGCTGGAACCACACAAGTGGGAAAAGTACTGTATTCTCGAGATAGGAGACATGCTCCTACGGACTGCAAT
AGGCCAAGTGTCAAGGCCCATGTTCCTGTATGTGAGAACCAATGGGACTTCCAAGATCAAGATGAAATGGGGCATGGAAATGA
GGCGATGCCTTCTTCAATCCCTTCAACAAATTGAGAGCATGATTGAGGCCGAGTCTTCTGTTAAAGAGAAAGACATGACCAAAGA

ATTCTTTGAGAACAAATCAGAAACATGGCCAATTGGGGAATCACCTAAAGGGGTAGAGGAAGGCTCCATTGGGAAGGTGTGCA
GAACATTACTAGCAAAATCTGTATTCAACAGCCTATATGCATCTCCACAACTCGAGGGGTTTTCAGCTGAATCAAGAAAATTGCTT
CTCATTGTTCAGGCACTTAGGGACAACCTGGAACCTGGGACCTTCGATCTTGGAGGGCTATATGAAGCAATTGAGGAGTGCCTG
ATTAACGATCCCTGGGTTTTGCTTAATGCGTCTTGGTTCAACTCCTTCCTCACACATGCACTGAAATAGTTGTGGCAATGCTACTAT
TTGCTATCCATACTGTCCAAAAAA

Hemagglutination (HA) gene 1,469 nucleotides; GenBank: JN982503

CTAACAACTGGTCTTGCTTATGACAAGATTTGCATCGGCTATCAGACGAATAACTCAACCGATACGGTAAACACACTAATTGAAC
AGAATGTCCCAGTCACTCAGGTGGAAGAGCTCGTGCATGGTCAAGTCAATCCGATCCTATGCAGTACAGAACTGGGGTCACCC
CTAGTGCTTGACGATTGCTCTCTGGAGGGCTTGATCCTAGGCAATCCCAAATGCGATCTCTATCTGAATGGTAGAGAATGGTCAT
ACATTGTGGAGAGGCCCAAGGAGATGGAAGGAATCTGCTATCCAGGATCAATAGAGAACCAAGAAGAGTTGAGATCATTGTTCT
CTTCAATCAAGAAATATGAAAGAGTGAAAATGTTCGACTTCACCAAATGGAATGTCACTTACACTGGAACGAGCAAAGCTTGTAAT
AACACCTCAAACCAAGGATCATTCTACAGGAGCATGAGATGGCTCACTTTGAAGTCAGGACAATTCCCAGTGCAAACCGATGAG
TACAAAAACACTAGGGATTCGGACATTGTATTCACATGGGCTATTCATCACCCACCCACTTCTGACGAGCAGGTGAAGCTCTACA
AGAACTCCGACACTCTCTCGTCAGTCACAACGGATGAAATTAACAGGAGTTTCAGGCCTAACATAGGGCCAAGACCCCTGGTTA
GGGGACAACAAGGGAGGATGGATTATTATTGGGCTGTTCTAAAGCCAGGGCAGACAGTGAAGATACAGACTAATGGAAATCTAA
TTGCACCTGAATATGGCCATCTAATCACTGGGAAATCACACGGGAGAATACTTAAAAACAACCTGCCGATCGGCCAATGCACGA
CCGAATGTCAGTTAAATGAAGGAGTAATGAACACCAGCAAACCGTTTCAGAATACCAGCAAGCATTACATTGGGAAATGCCCTA
GATATATACCATCGAACAGTTTGAAACTGGCGATAGGGCTCAGGAATGTTCCCCAGGCCCAAGACCGAGGGCTCTTTGGAGCA
ATCGCTGGATTCATAGAAGGAGGGTGGCCCGGATTGGTGGCTGGTTGGTATGGGTTCCAACATCAAAACTCTGAGGGAACAGG
CATTGCAGCAGACAGGGACAGCACCCAGAAGGCAATGGACAACATGCAGAACAAACTGAACAATGTCATTGACAAAATGAACA
AGCAATTCGAAGTGGTGAACCACGAATTTTCGGAGGTTGAAAGTAGAATAAATATGATCAATTCAAAAATTGACGATCAAATAACA
GACATATGGGCATATAATGCGGAACTGCTAGTTTTGCTGGAGAATCAGAAGACACTAGACGAGCACGATGCGAATGTTAGGAAT
CTCCATGACCGAGTCAGAAGGGTATTAAAAGAAAATGCGATAGATACTGAGGATGGGTGTTTTGAAATACTACACAAATGTGATG
ATGAATGCATGGATACAATCAAGAACGGGACTTACAACCACCAGAAATACGAAGAAGAGAGCAAGCTTGAGAGACAAAGGATT
AATGGAGTGAAACTTGAAGAGAATTCTACATATAAAATCTTGAGCATCTACAGCAGTGTTGCCTCAAGCTTAGTATTACTGCTCAT
GATTATTGGGGGTTTCATTTTCGGATGTCAAAATGGAAATGTTCGTTG

Nucleoprotein (NP) gene 1,525 nucleotides; GenBank: JN982504

TAGCAAAAGGCAGGTAGATAATCACTCACTGAGTGACATCAATATCATGGCGTCTCAAGGCACCAAACGATCTTATGAACAGAT
GGAGACCGGTGGAGAACGCCAGAATGCCACTGAGATCAGAGCATCTGTTGGAAGGATGGTTGGTGGAATTGGGAGGTTCTACA
TACAGATGTGCACTGAACTCAAACTCAGTGACTATGAAGGAAGGCTGATCCAAAACAGCATAACAATCGAGAGAATGGTTCTCT
CTGCATTTGATGAAAGGAGAAACAAATACCTGGAAGAACATCCCAGTGCGGGGAAAGACCCGAAGAAGACTGGAGGTCCAATT
TATCGAAGGAGAGATGGGAAATGGGTGAGAGAaCTGATTCTGTATGACAAAGAGGAGATCAGGAGAATCTGGCGTCAAGCGAA
CAATGGAGAAGACGCAACTGCTGGTCTCACTCATCTGATGATCTGGCATTCCAACCTGAATGATGCCACATACCAGAGAACAAG
AGCGCTCGTGCGTACTGGAATGGACCCCCGGATGTGCTCCCTCATGCAAGGATCAACTCTCCCGAGGAGATCTGGAGCTGCTG
GTGCAGCGGTAAAGGGAGTCGGGACGATGGTGATGGAGCTAATTCGAATGATAAAACGAGGGATTAATGATCGGAATTTCTGG
AGAGGCGAAAATGGACGAAGGACAAGGATTGCATATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACA
AAGAGCAATGATGGACCAGGTGCGAGAAAGCAGGAATCCTGGAAATGCTGAAATTGAGGATCTCATCTTCCTGGCACGGTCTG
CACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCAGTAGCCAGTGGATACGACT

TTGAGAGAGAAGGATACTCTCTAGTTGGAATAGATCCTTTCCGTTTGCTTCAAAACAGCCAGGTCTTCAGTCTCATTAGACCCAAT
GAGAACCCAGCACACAAGAGTCAATTGGTGTGGATGGCATGCCATTCTGCAGCATTCGAAGACCTGAGAGTCTCAAGCTTCATC
AGAGGAACAAGAGTAGTCCCAAGAGGACAACTATCCACCAGAGGAGTTCAAATTGCTTCAAACGAGAATATGGAAACAATGGA
CTCTAGCACTCTTGAACTGAGGAGCAGATATTGGGCTATAAGAACCAGAAGTGGAGGAAACACCAACCAACAGAGAGCATCTG
CAGGACAAATCAGTGTACAGCCCACTTTCTCAGTACAGAGAAATCTTCCCTTCGAAAGAGCGACCATTATGGCAGCGTTCACAG
GGAATACTGAAGGTAGAACATCAGACATGAGGACTGAAATCATAAGAATGATGGAAAGTGCCAGACCAGAAGATGTGTCTTTCC
AGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCT
TATTTCTTCGAAAACAATGCA

Neuraminidase (NA) gene 1,427 nucleotides; GenBank: JN982505

TAGCAAAAGCAGGAGTTTAAAATGAATCCAAATCAAAAGATAATAACCATTGGATCAATCTGCATGGTAATTGGAATAGCCAGCTT
GATGCTACAAATTGGGAACATAATCTCTATATGGGTTAGCCATTCAATTCAGACAGGGAACCAATATCAGCCTGAACCATGCAAT
CAAAGCATCATTACTTATGAAAACAACACCTGGGTAAATCAGACGTATGTCAACATTAGCAATACCAATTTTCTTGCTGAACAGGC
TGTCACTTCGGTGGCATTAGCGGGCAATTCATCTCTTTGCCCTATTAGTGGGTGGGCTATATACAGTAAAGATAATGGTATAAGAA
TTGGTTCCAAGGGGGATGTGTTTGTTATAAGAGAGCCATTCATCTCATGCTCCCACTTAGAATGCAGAACCTTCTTTTTGACCCAG
GGGGCTTTGCTGAATGACAAGCATTCTAATGGGACCGTCAAAGATAGGAGCCCCTACAGAACTTTGATGAGTTGTCCCGTGGGT
GAGGCTCCTTCCCCGTACAATTCAAGATTTGAGTCTGTTGCTTGGTCGGCAAGTGCTTGTCATGATGGTATCAGTTGGTTGACAAT
CGGAATTTCTGGTCCAGACAATGGGGCTGTGGCTGTATTGAAGTACAACGGCATAATAACGGACACTATCAAGAGTTGGAGGAA
TAACATTTTGAGAACTCAAGAGTCTGAGTGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGATGGACCAAGTAATGGGC
AGGCCTCATACAAAATCTTCAAGATAGAAAAAGGGAAAGTAGTCAAGTCAGTTGAATTGAATGCCCCTAATTACCACTACGAGGA
ATGCTCCTGTTATCCTGATGCGGGCGAAATTATGTGTGTGTGCAGGGACAACTGGCATGGCTCAAATCGGCCGTGGGTATCTTT
CAATCAAAATCTGGAGTATCAAATAGGATATATATGCAGTGGGCTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGCAG
TTGCGGTCCAATGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTTTGGATTGGAAGGACA
AAAAGTACTAGTTCCAGAAGCGGCTTTGAGATGATTTGGGATCCGAATGGATGGACTGAAACGGACAGTAGCTTTTCAGTGAAG
CAAGACATCGTAGCAATAACTGACTGGTCAGGGTATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGATTAGATTGCATG
AGGCCTTGTTTCTGGGTTGAGCTAATCAGAGGGCGGCCCAAAGAGAACACAATTTGGACTAGTGGGAGCAGCATATCCTTTTGT
GGTGTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCACCATTGACAA

Matrix protein (M) gene 982 nucleotides; GenBank: JN982506

GGTAGATATTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCGTCCCATCAGGCCCCCTCAAAGCCGAG
ATCGCGCAGAGACTTGAAGATGTCTTTGCAGGGAAGAACGCCGATCTCGAGGCTCTCATGGAATGGCTAAAGACAAGACCAAT
CCTGTCACCTCTAACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTT
TGTCCAAAATGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAGGGCAGTCAAACTGTACAGGAAATTGAAAAGAGAGA
TAACATTCCATGGGGCTAAAGAAGTTGCACTCAGCTATTCAACCGGTGCACTTGCCAGTTGTATGGGTCTCATATACAACAGGAT
GGGAACAGTGACCACAGAAGTAGCTTTTGGCCTAGTGTGTGCCACCTGTGAGCAGATTGCTGATTCACAGCATCGGTCCCACA
GGCAAATGGTAACTACCACCAACCCGCTAATCAGGCATGAAAACAGAATGGTACTGGCCAGCATTACAGCTAAGGCTATGGAG
CAGATGGCTGGGTCAAGTGAGCAGGCAGCAGAAGCCATGGAGGTTGCTAGTCAGGCTAGGCAGATGGTGCAGGCGATGAGG
ACAATTGGGACTCACCCTAGCTCCAGTGCCGGTCTGAAAGATGATCTTCTTGAAAATTTGCAGGCCTACCAGAAACGGATGGGA
GTGCAAATGCAGCGATTCAAGTGATCCTCTCATTATTGCCGCAAGTATTATTGGGATCTTGCACTTGATATTGTGGATTCTTGATC

GTCTTTTCTTCAAATGTATTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTTCTACGGAAGGAGTGCCTGAGTCTATGAGG
GAAGAGTATCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGTCATTTTGTCAAC

Nonstructural protein (NS) gene 858 nucleotides; GenBank; JN982507

AGCAAAAGGCAGGGTGACAAAAACATAATGGACTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGC
AAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGGGGAAGAGG
CAGCACTCTTGGTCTGGACATCGAGACGGCTACCCGTGCGGGAAAGCAGATAGTGGAGCGGATTCTAGAAGAAGAATCTGATG
AGGCACTTAAAATGACTATTGCTTCAGTGCCGGCTTCACGCTACCTAACTGACATGACTCTTGAAGAGATGTCAAGGGACTGGTT
CATGCTCATGCCTAAACAGAAAGTAGCAGGTTCCCTTTGCATCAGAATGGACCAGGCAATAATGGATAAAAACATCATATTGAAA
GCAAACTTCAGTGTGATTTTTGACCGACTGGAGACCCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGGGAGAAA
TCTCACCGTTACCTTCTCTTCCAGGACATACTGATGAGGATGTCAAAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAA
TGATAACACAGTTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGGATGGGAGACCTCCACTCCCTCC
AAAGCAGAAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAATAAGATGGCTGATTGAAGAAGTGCGACATAGAT
TGAAAATCACAGAAAACAGCTTCGAACAGATAACGTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAGAGATAAGAAC
TTTCTCGTTTCAGCT

A/lesser whistling-duck/THA/CU-W3941/2010(H12N1)

Polymerase basic protein 2 (PB2) gene 2,284 nucleotide; GenBank: JN982508

GTCGCATTCTCGCACTCGCGAGATACTGACAAAACCCACTGTGGACCATATGGCCATAATCAAGAAATATACGTCAGGAAGACA
GGAGAAGAATCCTGCACTTAGGATGAAATGGATGATGGCAATGAAATATCCGATTACAGCAGACAGAAGGATAATGGAGATGAT
CCCTGAAAGAAATGAGCAAGGTCAGACTCTTTGGAGCAAGACAAATGATGCTGGATCAGACAGAGTGATGGTGTCACCTTTGGC
TGTGACGTGGTGGAaTAGAAATGGACCAACGACAAGTACAGTCCATTATCCAAAGGTATATAAAACCTACTTTGAAAAGGTTGAA
AGGTTAAAGCATGGAACCTTCGGCCCCGTTCATTTTCGAAATCAGGTTAAGATACGCCGCAGGGTTGACATAAACCCGGGCCAT
GCAGATCTCAGTGCTAAAGAAGCACAAGACGTCATCATGGAGGTCGTTTTCCCAAACGAAGTCGGAGCCAGGATATTGACATCA
GAGTCACAGTTAACAATAACAAAGGAAAAGAAGGAGGAGCTTCAGGACTGTAAGATTGCCCCTTTAATGGTGGCATaCATGTTG
GAgAGAGAACTGGTTCGTAAAACCAGATTCCTGCCAGTAGCTGGCGGGACAAGCAGCGTGTATATCGAGGTGTTGCACTTGACT
CAAGGGACCTGCTGGGAACAAATGTACACGCCGGGAGGAGAAGTGAGAAATGATGACGTTGATCAGAGTTTAATTATTGCTGCT
AGAAATATCGTTAGGAGAGCAACAGTATCAGCAGACCCATTGGTTTcGCTCTTGGAGATGTGCCATAGTACACAAATCGGCGGG
ATAAGGATGGTAGACATCCTTAGACAGAATCCAACAGAAGAGCAAGCCGTGGACATATGCAAAGCAGCAATGGGTCTAAGAAT
CAGTTCATCTTTCAGCTTTGGAGGTTTCACTTTCAAAAGGACAAGTGGGTCATCTGTCAAAAGAGAAGAGGAAGTGCTCACCGGC
AACCTCCAAACATTGAAAATAAGAGTGCATGAAGGGTATGAGGAATTCACCATGGTTGGGCGAAGAGCAACAGCCATTCTAAGG
AAAGCAACCAGAAGGCTGATCCAATTGATAGTAAGTGGAAGAGACGAGCAGTCAATCGCCGAAGCGATCATAGTGGCAATGGT
GTTCTCCCAAGAGGATTGCATGATAAAAGCAGTACGAGGTGATTTGAATTTTGTCAATAGAGCGAATCAGCGGCTCAATCCTATG
CATCAGCTCCTGAGGCATTTCCAAAAGGATGCAAAAGTACTGTTCCAAAACTGGGGAATTGAACCCATTGACAATGTCATGGGG
ATGATAGGAATATTGCCTGATATGACCCCCAGCACAGAGATGTCATTAAGAGGAGTGAGAGTCAGTAAGATGGGAGTGGATGAA
TATTCCAGTACTGAGAGGGTGGTCGTGAGTATTGATCGTTTCTTGAGGGTCCGAGACCAGAGAGGAAACGTGCTCTTGTCCCCT
GAAGAGGTTAGTGAAACACAGGGAACAGAGAAGCTGACGATAACATATTCATCGTCCATGATGTGGGAAATCAATGGCCCGGA
ATCAGTGTTAGTTAACACATATCAATGGATCATTAGAAACTGGGAAACTGTGAAGATTCAGTGGTCCCAAGACCCTACAATGCTAT

ACAACAAGATGGAGTTTGAGCCCTTTCAGTCCTTGGTGCCTAAGGCTGCCAGAGGCCAGTATAGTGGATTTGTGAGGACGCTAT
TCCAGCAGATGCGTGATGTGCTGGGGACCTTTGACACTGTCCAGATAATAAAGCTACTTCCATTTGCAGCAGCCCCACCGGAAC
AGAGTAGGATGCAGTTCTCTTCTCTAACTGTAAACGTAAGGGGTTCAGGAATGAGAATACTTGTGAGAGGCAACTCCCCTGTGTT
CAACTATAACAAGGCAACCAAGAGGCTTACAGTCCTTGGAAAGGATGCAGGTGCATTGACAGAAGACCCAGATGAGGGGACAG
CAGGAGTGGAGTCTGCGGTATTAAGAGGATTCCTAATTCTGGGCAAAGAAGACAAAAGATATGGACCAGCATTGAGCATCAATG
AATTGAGCAATCTTGCGAAAGGGGAGAAGGCTAATGTGTTGATAGGGCAAGGAGACGTGGTGTTGGTGATGAAACGGAAACGG
GACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTCGAATTGTTTAAAAACGAC
CTTGTTTCTACGG

Polymerase basic protein 1 (PB1) gene 2,271 nucleotides; GenBank: JN982509

ATGGATGTCAATCCGACTTTACTTTTCTTAAAGGTGCCAGCGCAAAATGCTATAAGTACTACATTCCCTTACACTGGAGATCCCCC
ATACAGCCATGGAACAGGAACAGGGTACACCATGGACACAGTCAACAGAACACATCAGTACTCAGAAAAGGGAAAGTGGACAA
CAAACACAGAGACCGGAGCACCCCAACTCAACCCAATTGATGGACCCTTACCTGAGGACAACGAGCCAAGCGGATATGCACA
AACAGATTGCGTGTTGGAAGCAATGGCTTTCCTTGAAGAATCCCACCCAGGAATCTTTGAAAACTCTTGTCTTGAAACGATGGAA
GTTGTTCAGCAAACAAGAGTGGACAAACTAACCCAAGGTCGCCAGACTTATGACTGGACATTGAATAGAAACCAACCAGCTGCA
ACTGCTTTGGCCAACACTATAGAGGTTTTCAGATCGAACGGTCTGACGGCCAACGAATCAGGGAGACTAATGGATTTTCTCAAG
GATGTGATGGAATCAATGGATAAAGAAGAAATGGAAATAACAACACATTTCCAAAGAAAGAGAAGAGTAAGGGACAACATGACC
AAGAAGATGGTCACACAAAGAACAATAGGGAAGAAGAAGCAGAGGCTGAACAAGAGGAGCTACTTAATAAGAGCACTGACGCT
GAACACAATGACAAAGGATGCAGAGAGAGGCAAATTGAAGAGGCGGGCAATTGCAACACCCGGGATGCAGATTAGAGGATTC
GTGTACTTTGTCGAAACACTGGCGAGGAGCATCTGTGAGAAACTTGAGCAATCTGGACTCCCCGTTGGAGGGAATGAGAAGAA
GGCCAAATTGGCAAATGTCGTGAGAAAAATGATGACTAATTCACAAGATACAGAGCTCTCCTTTACAATTACTGGAGACAACACC
AAATGGAATGAGAATCAAAATCCTCGGATGTTCCTGGCAATGATAACATACATCACAAGAAACCAACCTGAATGGTTTAGAAATG
TCTTGAGCATTGCCCCTATAATGTTCTCAAACAAAATGGCGAGATTAGGAAAAGGGTACATGTTTGAAAGTAAGAGCATGAAACT
ACGGACACAAATACCGGCAGAAATGCTTGCAAACATTGACTTGAAATACTTCAACGAGTCAACGAGAAAGAAAATCGAGAAAAT
AAGACCTCTGCTAATTGATGGCACAGCCTCATTGAGTCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACAGTATTGGGA
GTCTCAATCCTGAATCTTGGGCAAAAGAGGTACACCAAAACCACATACTGGTGGGATGGACTCCAATCCTCTGATGATTTCGCTC
TCATAGTGAATGCACCGAATCATGAGGGGATACAAGCAGGGGTGGATAGGTTCTATAGGACCTGCAAACTGGTTGGCATCAACA
TGAGCAAAAAGAAGTCTTACATAAACCGGACAGGAACTTTTGAGTTCACAAGCTTTTTCTACCGCTATGGGTTTGTGGCCAACTTC
AGTATGGAGTTACCCAGCTTTGGAGTGTCTGGAATCAATGAATCGGCTGACATGAGCATTGGAGTTACAGTGATAAAGAACAATA
TGATAAACAACGACCTTGGACCAGCAACAGCTCAGATGGCTCTTCAGCTATTCATCAAGGACTATAGGTACACATACCGATGCC
ACAGGGGTGATACACAAATTCAAACGAGGAGATCATTCGAGCTGAGGAAGCTGTGGGAGCAGACCCGTTCAAAGGCAGGGCT
GTTGGTATCAGACGGAGGACCAAATCTATACAACATTCGGAATCTCCACATCCCAGAGGTCTGCTTGAAATGGGAACTGATGGA
TGAAGATTACCAAGGCAGGCTGTGTAATCCTCTGAATCCGTTTGTCAGTCATAAGGAAATTGAGTCTGTAAACAATGCTGTAGTAA
TGCCAGCCCACGGCCCAGCCAAGAGCATGGAATATGATGCTGTTGCAACTACACACTCATGGATCCCTAAGAGGAACCGTTCT
ATTCTCAATACCAGCCAAAGGGGAATTCTTGAGGATGAGCAAATGTACCAGAAGTGTTGCAATCTATTCGAGAAATTCTTCCCCA
GTAGTTCATACAGGAGGCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGATGCACGCATTGATT
TCGAATCTGGAAGGATTAAGAAAGAAGAGTTCGCTGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGCAAAAG

Polymerase acidic protein (PA) gene 2,148 nucleotides; GenBank: JN982510

ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATTGTCGAGCTTGCGGAAAAAGCAATGAAAGAATACGGGGAAGATCCG
AAAATCGAAACGAACAAATTTGCTGCAATATGCACACACTTAGAAGTCTGTTTCATGTATTCGGATTTCCACTTTATTGATGAACGA
GGCGAATCAATAATTGTAGAATCTGGCGATCCGAATGCATTATTGAAACACCGATTTGAGATAATTGAAGGGAGAGACCGAACAA
TGGCCTGGACAGTGGTAAATAGTATCTGCAACACCACAGGAGTCGATAAGCCTAAATTCCTCCCAGATTTGTATGACTACAAAGA
GAACCGATTCATCGAAATTGGAGTGACACGAAGGGAAGTTCACATATACTATCTAGAAAAAGCCAACAAGATAAAATCAGAGAA
GACACACATTCACATATTCTCATTCACTGGAGAGGAAATGGCCACTAAAGCGGACTATACCCTTGATGAGGAGAGCAGAGCAAG
AATCAAAACCAGGCTGTTCACCATAAGGCAAGAAATGGCCAGTAGGGGTCTATGGGATTCCTTTCGTCAGTCCGAGAGAGGCG
AAGAGACAATTGAAGAAAGATTTGAAATCACAGGAACCATGCGCAGGCTTGCCGACCAAAGTCTCCCACCGAACTTCTCCAGC
CTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGGGCAAGCTTTCTCAAATGTCAAAAGAAGTGA
ACGCCAGGATTGAGCCATTTCTGAAAACAACACCACGCCCTCTCAGATTACCTGATGGGCCTCCCTGCTCTCAGCGGTCGAAGT
TCTTACTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAAGGGGAAGGTATACCACTATATGATGCAATCAAATG
CATGAAGACATTTTTCGGCTGGAAAGAGCCCAACATCGTAAAACCACATGAAAAAGGCATAAACCCCAATTACCTCCTGGCTTG
GAAGCAGGTGCTGGCAGAACTCCAAGACATTGAGAATGAGGAGAAAATTCCAAAAACAAAGAACATGAAGAAAACAAGCCAAT
TGAAGTGGGCACTTGGTGAGAACATGGCACCAGAGAAAGTAGACTTTGAGGACTGCAAAGATGTTAGTGATCTAAGACAGTATG
ACAGTGATGAACCAGAGTCTAGATCGCTAGCAAGCTGGATCCAGAGCGAATTCAACAAAGCATGCGAATTGACAGATTCAAGTT
GGATTGAACTTGATGAAATAGGGGAAGATGTTGCTCCAATTGAGCACATTGCGAGTATGAGAAGAAACTATTTCACAGCGGAAGT
ATCCCATTGCAGGGCTACTGAATACATAATGAAGGGGGTGTACATAAACACAGCCCTGTTGAATGCATCCTGTGCAGCCATGGA
TGACTTCCAACTGATTCCAATGATAAGCAAGTGCAGAACCAAAGAAGGAAGACGGAAGACAAATCTGTATGGATTCATTATAAAA
GGGAGATCCCATTTGAGGAATGACACCGATGTGGTAAACTTTGTGAGCATGGAATTCTCTCTTACTGACCCGAGGCTGGAACCA
CACAAGTGGGAAAAGTACTGTGTTCTCGAGATAGGAGACATGCTCCTACGGACTGCAATAGGCCAAGTGTCAAGGCCCATGTTC
CTGTATGTGAGAACCAATGGGACTTCCAAGATCAAGATGAAATGGGGCATGGAGATGAGGCGATGCCTTCTTCAATCCCTTCAA
CAAATTGAGAGCATGATTGAGGCCGAGTCTTCTGTCAAAGAGAAAGACATGACCAAAGAATTCTTTGAGAACAAATCAGAAACAT
GGCCAATTGGAGAATCACCTAAAGGGGTGGAGGAAGGCTCCATTGGGAAGGTGTGCAGAACATTACTAGCAAAATCTGTATTCA
ACAGCCTATATGCATCTCCACAACTCGAGGGATTTTCAGCTGAATCAAGAAAATTGCTTCTCATTGTTCAGGCACTTAGGGACAA
CCTGGAACCTGGGACTTTCGATCTTGGGGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGTTTTGCTTAAT
GCGTCTTGGTTCAACTCCTTCCTCACACATGCACTGAAA

Hemagglutination (HA) gene, 1,661 nucleotides; GenBank: JN982511

AATTCTAACAACTGGTCTTGCTTATGACAAGATTTGCATCGGCTATCAGACGAATAACTCAACCGATACGGTAAACACACTAATTG
AACAGAATGTCCCAGTCACTCAGGTGGAAGAGCTCGTGCATGGTCAAGTCAATCCGATCCTATGCAGTACAGAACTGGGGTCA
CCCCTAGTGCTTGACGATTGCTCTCTGGAGGGCTTGATCCTAGGCAATCCCAAATGCGATCTCTATCTGAATGGTAGAGAATGGT
CATACATTGTGGAGAGGCCCAAGGAGATGGAAGGAATCTGCTATCCAGGATCAATAGAGAACCAAGAAGAGTTGAGATCATTGT
TCTCTTCAATCAAGAAATATGAAAGAGTGAAAATGTTCGACTTCACCAAATGGAATGTCACTTACACTGGAACGAGCAAAGCTTGT
AATAACACCTCAAACCAAGGATCATTCTACAGGAGCATGAGATGGCTCACTTTGAAGTCAGGACAATTCCCAGTGCAAACCGAT
GAGTACAAAAACACTAGGGATTCGGACATTGTATTCACATGGGCTATTCATCACCCACCCACTTCTGACGAGCAGGTGAAGCTC
TACAAGAACTCCGACACTCTCTCGTCAGTCACAACGGATGAAATTAACAGGAGTTTCAGGCCTAACATAGGGCCAAGACCCCTG
GTTAGGGGACAACAAGGGAGGATGGATTATTATTGGGCTGTTCTAAAGCCAGGGCAGACAGTGAAGATACAGACTAATGGAAAT
CTAATTGCACCTGAATATGGCCATCTAATCACTGGGAAATCACACGGGAGAATACTTAAAAACAACCTGCCGATCGGCCAATGC
ACGACCGAATGTCAGTTAAATGAAGGAGTAATGAACACCAGCAAACCGTTTCAGAATACCAGCAAGCATTACATTGGGAAATGC

CCTAAATATATACCATCGAACAGTTTGAAACTGGCGATAGGGCTCAGGAATGTTCCCCAGGCCCAAGACCGAGGGCTCTTTGGA
GCAATCGCTGGATTCATAGAAGGAGGGTGGCCCGGATTGGTGGCTGGTTGGTATGGGTTCCAACATCAAAACTCTGAGGGAAC
AGGCATTGCAGCAGACAAGGACAGCACCCAGAAGGCAATGGACAACATGCAGAACAAACTGAACAATGTCATTGACAAAATGA
ACAAGCAATTCGAAGTGGTGAACCACGAATTTTCGGAGGTTGAAAGTAGAATAAATATGATCAATTCAAAAATTGACGATCAAATA
ACAGACATATGGGCATATAATGCGGAACTGCTAGTTTTGCTGGAGAATCAGAAGACACTAGACGAGCACGATGCGAATGTTAGG
AATCTCCATGACCGAGTCAGAAGGGTATTAAAAGAAAATGCGATAGATACTGAGGATGGGTGTTTTGAAATACTACACAAATGTG
ATGATGAATGCATGGATACAATCAAGAACGGGACTTACAACCACCAGAAATACGAAGAAGAGAGCAAGCTTGAGAGACAAAGG
ATTAATGGAGTGAAACTTGAAGAGAATTCTACATATAAAATCTTGAGCATCTACAGCAGTGTTGCCTCAAGCTTAGTATTACTGCTC
ATGATTATTGGGGGTTTCATTTTCGGATGTCAAAATGGAAATGTTCGTTGTACTTTCT

Nucleoprotein (NP) gene 1,514 nucleotides; GenBank: JN982512

AGCAAAAAGCAGGTAGATAATCACTCACTGAGTGACATCAATATCATGGCGTCTCAAGGCACCAAACGATCTTATGAACAGATG
GAGACCGGTGGAGAACGCCAGAATGCCACTGAGATCAGAGCATCTGTTGGAAGGATGGTTGGTGGAATTGGGAGGTTCTACAT
ACAGATGTGCACTGAACTCAAACTCAGTGACTATGAAGGAAGGCTGATCCAAAACAGCATAACAATCGAGAGAATGGTTCTCTC
TGCATTTGATGAAAGGAGAAACAAATACCTGGAAGAACATCCCAGTGCGGGGAAAGACCCGAAGAAGACTGGAGGTCCAATTT
ATCGAAGGAGAGATGGGAAATGGGTGAGAGAACTGATTCTGTATGACAAAGAGGAGATCAGGAGAATCTGGCGTCAAGCGAAC
AATGGAGAAGACGCAACTGCTGGTCTCACTCATCTGATGATCTGGCATTCCAACCTGAATGATGCCACATACCAGAGAACAAGA
GCGCTCGTGCGTACTGGAATGGACCCCCGGATGTGCTCCCTCATGCAAGGATCAACTCTCCCGAGGAGATCTGGAGCTGCTG
GTGCAGCGGTGAAGGGAGTCGGGACGATGGTGATGGAGCTAATTCGAATGATAAAACGAGGGATTAATGATCGGAATTTCTGG
AGAGGCGAAAATGGACGAAGGACAAGGATTGCATATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACA
AAGAGCAATGATGGACCAGGTGCGAGAAAGCAGGAATCCTGGAAATGCTGAAATTGAGGATCTCATCTTCCTGGCACGGTCTG
CACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCAGTAGCCAGTGGATACGACT
TTGAGAGAGAAGGATACTCTCTAGTTGGAATAGATCCTTTCCGTTTGCTTCAAAACAGCCAGGTCTTCAGTCTCATTAGACCCAAT
GAGAACCCAGCACACAAGAGTCAATTGGTGTGGATGGCATGCCATTCTGCAGCATTCGAAGACCTGAGAGTCTCAAGCTTCATC
AGAGGAACAAGAGTAGTCCCAAGAGGACAACTATCCACCAGAGGAGTTCAAATTGCTTCAAACGAGAATATGGAAACAATGGA
CTCTAGCACTCTTGAACTGAGGAGCAGATATTGGGCTATAAGAACCAGAAGTGGAGGAAACACCAACCAACAGAGAGCATCTG
CAGGACAAATCAGTGTACAGCCCACTTTCTCAGTACAGAGAAATCTTCCCTTCGAAAGAGCGACCATTATGGCAGCGTTCACAG
GGAATACTGAAGGTAGAACATCAGACATGAGGACTGAAATCATAAGAATGATGGAAAGTGCCAGACCAGAAGATGTGTCTTTCC
AGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCT
TATTTCTTCGG

Neuraminidase (NA) gene 1,416 nucleotides; GenBank: JN982513

GCAGAGTTTAAAATGAATCCAAATCAAAAGATAATAACCATTGGATCAATCTGCATGGTAATTGGAATAGCCAGCTTGATGCTACA
AATTGGGAACATAATCTCTATATGGGTTAGCCATTCAATTCAGACAGGGAACCAATATCAGCCTGAACCATGCAATCAAAGCATC
ATTACTTATGAAAACAACACCTGGGTAAATCAGACGTATGTCAACATTAGCAATACCAATTTTCTTGCTGAACAGGCTGTCACTTC
GGTGGCATTAGCGGGCAATTCATCTCTTTGCCCTATTAGTGGGTGGGCTATATACAGTAAAGATAATGGTATAAGAATTGGTTCCA
AGGGGGATGTGTTTGTTATAAGAGAGCCATTCATCTCATGCTCCCACTTAGAATGCAGAACCTTCTTTTTGACCCAGGGGGCTTT
GCTGAATGACAAGCATTCTAATGGGACCGTCAAAGATAGGAGCCCCTACAGAACTTTGATGAGTTGTCCCGTGGGTGAGGCTCC
TTCCCCGTACAATTCAAGATTTGAGTCTGTTGCTTGGTCGGCAAGTGCTTGTCATGATGGTATCAGTTGGTTGACAATCGGAATTT

CTGGTCCAGACAATGGGGCTGTGGCTGTATTGAAGTACAACGGCATAATAACGGACACTATCAAGAGTTGGAGGAATAACATTT
TGAGAACTCAAGAGTCTGAGTGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGATGGACCAAGTAATGGGCAGGCCTC
ATACAAAATCTTCAAGATAGAAAAAGGGAAAGTAGTCAAGTCAGTTGAATTGAATGCCCCTAATTACCACTACGAGGAGTGCTCC
TGTTATCCTGATGCGGGCGAAATTATGTGTGTGTGCAGGGACAACTGGCATGGCTCAAATCGGCCGTGGGTATCTTTCAATCAA
AATCTGGAGTATCAAATAGGATATATATGCAGTGGGCTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGCAGTTGCGGT
CCAATGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTTTGGATTGGAAGGACAAAAAGTA
CTAGTTCCAGAAGCGGCTTTGAGATGATTTGGGATCCGAATGGATGGACTGAGACGGACAGTAGTTTCTCAGTGAAGCAAGACA
TCGTAGCAATAACTGACTGGTCAGGGTATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGATTAGATTGCATGAGGCCTT
GTTTCTGGGTTGAGCTAATCAGAGGGCGGCCCAAAGAGAACACAATTTGGACTAGTGGGAGCAGCATATCCTTTTGTGGTGTAA
ATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCACCATTGAA

Matrix protein (M) gene 998 nucleotides; GenBank: JN982514

GCAAAAAACAGGTAGATATTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCGTCCCATCAGGCCCCCTC
AAAGCCGAGATCGCGCAGAGACTTGAAGATGTCTTTGCAGGGAAGAACGCCGATCTCGAGGCTCTCATGGAATGGCTAAAGAC
AAGACCAATCCTGTCACCTCTAACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCG
TAGACGCTTTGTCCAAAATGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAGAGCAGTCAAACTGTACAGGAAGTTGAA
AAGAGAGATAACATTCCATGGGGCTAAAGAAGTTGCACTCAGTTATTCAACCGGTGCACTTGCCAGTTGCATGGGTCTCATATAC
AACAGGATGGGAACGGTGACCACAGAAGTGGCTTTTGGCCTAGTGTGTGCCACCTGTGAGCAGATTGCTGATTCGCAGCATCG
GTCCCACAGGCAGATGGTAACTACCACCAACCCACTAATCAGGCATGAAAACAGAATGGTGCTGGCTAGCACCACGGCTAAGG
CTATGGAGCAGATGGCTGGGTCAAGTGAGCAGGCAGCGGAAGCCATGGAAGTTGCTAGTCAGGCCAGGCAGATGGTGCAGG
CAATGAGGACAATTGGAACTCACCCTAGCTCCAGTGCCGGTCTGAAAGATGATCTTCTTGAAAATTTGCAGGCCTACCAGAAAC
GGATGGGAGTGCAAATGCAGCGATTCAAGTGATCCTCTCGTTATTGCCGCAAGTATCATTGGGATCTTGCACTTGATATTGTGGA
TTCTTGATCGTCTTTTCTTCAAATGTATTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTTCTACGGAAGGAGTGCCTGAG
TCTATGAGGGAAGAATATCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGTCATTTTGTCAACATCCAC

Nonstructural protein (NS) gene 858 nucleotides; GenBank; JN982515

AGCAAAAGGCATGGTGACAAAAACATAATGGACTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCA
AACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGGGGAAGAGGC
AGCACTCTTGGTCTGGACATCGAGACGGCTACCCGTGCGGGAAAGCAGATAGTGGAGCGGATTCTAGAAGAAGAATCTGATGA
GGCACTTAAAATGACTATTGCTTCAGTGCCGGCTTCACGCTACCTAACTGACATGACTCTTGAAGAGATGTCAAGGGACTGGTTC
ATGCTCATGCCTAAACAGAAAGTAGCAGGTTCCCTTTGCATCAGAATGGACCAGGCAATAATGGATAAAAACATCATATTGAAAG
CAAACTTCAGTGTGATTTTTGACCGACTGGAGACCCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGGGAGAAAT
CTCACCGTTACCTTCTCTTCCAGGACATACTGATGAGGATGTCAAAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAAT
GATAACACAGTTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGGATGGGAGACCTCCACTCCCTCCA
AAGCAGAAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAATAAGATGGCTGATTGAAGAAGTGCGACATAGATT
GAAAATCACAGAAAACAGCTTCGAACAGATAACGTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAGAGATAAGAACT
TTCTCGTTTCAGCT

A/lesser whistling-duck/THA/CU-W3946/2010 (H12N1)

Polymerase basic protein 2 (PB2) gene 2,284 nucleotide; GenBank: JN982516

TTGATGTCGCAGTCTCGCACTCGCGAGATACTGACAAAAACCACTGTGGACCATATGGCCATAATCAAGAAATATACGTCAGGA
AGACAGGAGAAGAATCCTGCACTTAGGATGAAATGGATGATGGCAATGAAATATCCGATTACAGCAGACAGAAGGATAATGGAG
ATGATCCCTGAAAGAAATGAGCAAGGTCAGACTCTTTGGAGCAAGACAAATGATGCTGGATCAGACAGAGTGATGGTGTCACCT
TTGGCTGTGACGTGGTGGAATAGAAATGGACCAACGACAAGTACAGTCCATTATCCAAAGGTATATAAAACCTACTTTGAAAAGG
TTGAAAGGTTAAAGCATGGAACCTTCGGCCCCGTTCATTTTCGAAATCAGGTTAAGATACGCCGCAGGGTTGACATAAACCCGG
GCCATGCAGATCTCAGTGCTAAAGAAGCACAAGACGTCATCATGGAGGTCGTTTTCCCAAACGAAGTCGGAGCCAGGATATTGA
CATCAGAGTCACAGTTAACAATAACAAAGGAAAAGAAGGAGGAGCTTCAGGACTGTAAGATTGCCCCTTTAATGGTGGCATACA
TGTTGGAGAGAGAACTGGTTCGTAAAACCAGATTCCTGCCAGTAGCTGGCGGGACAAGCAGCGTGTATATCGAGGTGTTGCAC
TTGACTCAAGGGACCTGCTGGGAACAAATGTACACGCCGGGAGGAGAAGTGAGAAATGATGACGTTGATCAGAGTTTAATTATT
GCTGCTAGAAATATCGTTAGGAGAGCAACAGTATCAGCAGACCCATTGGCTTCGCTCCTGGAGATGTGCCATAGTACACAAATC
GGCGGGATAAGGATGGTAGACATCCTTAGACAGAATCCAACAGAAGAGCAAGCCGTGGACATATGCAAAGCAGCAATGGGTCT
AAGAATCAGTTCATCTTTCAGCTTTGGAGGTTTCACTTTCAAAAGGACAAGTGGGTCATCTGTCAAAAGAGAAGAGGAAGTGCTC
ACCGGCAACCTCCAAACATTGAAAATAAGAGTGCATGAAGGGTATGAGGAATTCACCATGGTTGGGCGAAGAGCAACAGCCAT
TCTAAGGAAAGCAACCAGAAGGCTGATCCAATTGATAGTAAGTGGAAGAGACGAGCAGTCAATCGCCGAAGCGATCATAGTGG
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TCCTATGCATCAGCTCCTGAGGCATTTCCAAAAGGATGCAAAAGTACTGTTCCAAAACTGGGGAATTGAACCCATTGACAATGTC
ATGGGGATGATAGGAATATTGCCTGATATGACCCCCAGCACAGAGATGTCATTAAGAGGAGTGAGAGTCAGTAAGATGGGAGT
GGATGAATATTCCAGTACTGAGAGGGTGGTCGTGAGTATTGATCGTTTCTTGAGGGTCCGAGACCAGAGAGGAAACGTGCTCTT
GTCCCCTGAAGAGGTTAGTGAAACACAGGGAACAGAGAAGCTGACGATAACATATTCATCGTCCATGATGTGGGAAATCAATGG
CCCGGAATCAGTGTTAGTTAACACATATCAATGGATCATTAGAAACTGGGAAACTGTGAAGATTCAGTGGTCCCAAGACCCTACA
ATGCTATACAACAAGATGGAGTTTGAGCCCTTTCAGTCCTTGGTGCCTAAGGCTGCCAGAGGCCAGTATAGTGGATTTGTGAGG
ACGCTATTCCAGCAGATGCGTGATGTGCTGGGGACCTTTGACACTGTCCAGATAATAAAGCTACTTCCATTTGCAGCAGCCCCA
CCGGAACAGAGTAGGATGCAGTTCTCTTCTCTAACTGTAAACGTAAGGGGTTCAGGAATGAGAATACTTGTGAGAGGCAACTCC
CCTGTGTTCAACTATAACAAGGCAACCAAGAGGCTTACAGTCCTTGGAAAGGATGCAGGTGCATTGACAGAAGACCCAGATGA
GGGGACAGCAGGAGTGGAGTCTGCGGTATTAAGAGGATTCCTAATTCTGGGCAAAGAAGACAAAAGATATGGACCAGCATTGA
GCATCAATGAATTGAGCAATCTTGCGAAAGGGGAGAAGGCTAATGTGTTGATAGGGCAAGGAGACGTGGTGTTGGTGATGAAA
CGGAAACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTCGAATTGTT
TAAAAACGACCTTGTTTCTACG

Polymerase basic protein 1 (PB1) gene 2,271 nucleotides; GenBank: JN982517

ATCCGACTTTACTTTTCTTAAGGGTGCCAGCGCAAAATGCTATAAGTACTACATTCCCTTACACTGGAGATCCCCCATACAGCCAT
GGAACAGGAACAGGGTACACCATGGACACAGTCAACAGAACACATCAGTACTCAGAAAAGGGAAAGTGGACAACAAACACAG
AGACCGGAGCACCCCAACTCAACCCAATTGATGGACCCTTACCTGAGGACAACGAGCCAAGCGGATATGCACAAACAGATTGC
GTGTTGGAAGCAATGGCTTTCCTTGAAGAATCCCACCCAGGAATCTTTGAAAACTCTTGTCTTGAAACGATGGAAGTTGTTCAGC
AAACAAGAGTGGACAAACTAACCCAAGGTCGCCAGACTTATGACTGGACATTGAATAGAAACCAACCAGCTGCAACTGCTTTGG
CCAACACTATAGAGGTTTTCAGATCGAACGGTCTGACGGCCAACGAATCAGGGAGACTAATGGATTTTCTCAAGGATGTGATGG
AATCAATGGATAAAGAAGAAATGGAAATAACAACACATTTCCAAAGAAAGAGAAGAGTAAGGGACAACATGACCAAGAAGATGG

TCACACAAAGAACAATAGGGAAGAAGAAGCAGAGGCTGAACAAGAGGAGCTACTTAATAAGAGCACTGACGCTGAACACAATG
ACAAAGGATGCAGAGAGAGGCAAATTGAAGAGGCGGGCAATTGCAACACCCGGGATGCAGATTAGAGGATTCGTGTACTTTGT
CGAAACACTGGCGAGGAGCATCTGTGAGAAACTTGAGCAATCTGGACTCCCCGTTGGAGGGAATGAGAAGAAGGCCAAATTG
GCAAATGTCGTGAGAAAAATGATGACTAATTCACAAGATACAGAGCTCTCCTTTACAATTACTGGAGACAACACCAAATGGAATG
AGAATCAAAATCCTCGGATGTTCCTGGCAATGATAACATACATCACAAGAAACCAACCTGAATGGTTTAGAAATGTCTTGAGCATT
GCCCCTATAATGTTCTCAAACAAAATGGCGAGATTAGGAAAAGGGTACATGTTTGAAAGTAAGAGCATGAAACTACGGACACAA
ATACCGGCAGAAATGCTTGCAAACATTGACTTGAAATACTTCAACGAGTCAACGAGAAAGAAAATCGAGAAAATAAGACCTCTGC
TAATTGATGGCACAGCCTCATTGAGTCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACAGTATTGGGAGTCTCAATCCT
GAATCTTGGGCAAAAGAGGTACACCAAAACCACATACTGGTGGGATGGACTCCAATCCTCTGATGATTTCGCTCTCATAGTGAAT
GCACCGAATCATGAGGGGATACAAGCAGGGGTGGATAGGTTCTATAGGACCTGCAAACTGGTTGGCATCAACATGAGCAAAAA
GAAGTCTTACATAAACCGGACAGGAACTTTTGAGTTCACAAGCTTTTTCTACCGCTATGGGTTTGTGGCCAACTTCAGTATGGAGT
TACCCAGCTTTGGAGTGTCTGGAATCAATGAATCGGCTGACATGAGCATTGGAGTTACAGTGATAAAGAACAATATGATAAACAA
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TACACAAATTCAAACGAGGAGATCATTCGAGCTGAGGAAGCTGTGGGAGCAGACCCGTTCAAAGGCAGGGCTGTTGGTATCAG
ACGGAGGACCAAATCTATACAACATTCGGAATCTCCACATCCCAGAGGTCTGCTTGAAATGGGAACTGATGGATGAAGATTACC
AAGGCAGGCTGTGTAATCCTCTGAATCCGTTTGTCAGTCATAAGGAAATTGAGTCTGTAAACAATGCTGTAGTAATGCCAGCCCA
CGGCCCAGCCAAGAGCATGGAATATGATGCTGTTGCAACTACACACTCATGGATCCCTAAGAGGAACCGTTCTATTCTCAATAC
CAGCCAAAGGGGAATTCTTGAGGATGAGCAAATGTACCAGAAGTGTTGCAATCTATTCGAGAAATTCTTCCCCAGTAGTTCATAC
AGGAGGCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGATGCACGCATTGATTTCGAATCTGG
AAGGATTAAGAAAGAAGAGTTCGCTGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGCAAAAGTAGTGAATTTA
GCTTGTCCTTCATGAAAAA

Polymerase acidic protein (PA) gene 2,148 nucleotides; GenBank: JN982518

ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATTGTCGAGCTTGCGGAAAAAGCAATGAAAGAATACGGGGAAGATCCG
AAAATCGAAACGAACAAATTTGCTGCAATATGCACACACTTAGAAGTCTGTTTCATGTATTCGGATTTCCACTTTATTGATGAACGA
GGCGAATCAATAATTGTAGAATCTGGCGATCCGAATGCATTATTGAAACACCGATTTGAGATAATTGAAGGGAGAGACCGAACAA
TGGCCTGGACAGTGGTAAATAGTATCTGCAACACCACAGGAGTCGATAAGCCTAAATTCCTCCCAGATTTGTATGACTACAAAGA
GAACCGATTCATCGAAATTGGAGTGACACGAAGGGAAGTTCACATATACTATCTAGAAAAAGCCAACAAGATAAAATCAGAGAA
GACACACATTCACATATTCTCATTCACTGGAGAGGAAATGGCCACTAAAGCGGACTATACCCTTGATGAGGAGAGCAGAGCAAG
AATCAAAACCAGGCTGTTCACCATAAGGCAAGAAATGGCCAGTAGGGGTCTATGGGATTCCTTTCGTCAGTCCGAGAGAGGCG
AAGAGACAATTGAAGAAAGATTTGAAATCACAGGAACCATGCGCAGGCTTGCCGACCAAAGTCTCCCACCGAACTTCTCCAGC
CTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGGGCAAGCTTTCTCAAATGTCAAAAGAAGTGA
ACGCCAGGATTGAGCCATTTCTGAAAACAACACCACGCCCTCTCAGATTACCTGATGGGCCTCCCTGCTCTCAGCGGTCGAAGT
TCTTACTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAAGGGGAAGGTATACCACTATATGATGCAATCAAATG
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AAGCAGGTGCTGGCAGAACTCCAAGACATTGAGAATGAGGAGAAAATTCCAAAAACAAAGAACATGAAGAAAACAAGCCAATTA
AGTGGGCACTTGGTGAGAACATGGCACCAGAGAAAGTAGACTTTGAGGACTGCAAAGATGTTAGTGATCTAAGACAGTATGACA
GTGATGAACCAGAGTCTAGATCGCTAGCAAGCTGGATCCAGAGCGAATTCAACAAAGCATGCGAATTGACAGATTCAAGTTGGA
TTGAACTTGATGAAATAGGGGAAGATGTTGCTCCAATTGAGCACATTGCGAGTATGAGAAGAAACTATTTCACAGCGGAAGTATC
CCATTGCAGGGCTACTGAATACATAATGAAGGGGGTGTACATAAACACAGCCCTGTTGAATGCATCCTGTGCAGCCATGGATGA

CTTCCAACTGATTCCAATGATAAGCAAGTGCAGAACCAAAGAAGGAAGACGGAAGACAAATCTGTATGGATTCATTATAAAAGG
GAGATCCCATTTGAGGAATGACACCGATGTGGTAAACTTTGTGAGCATGGAATTCTCTCTTACTGACCCGAGGCTGGAACCACA
CAAGTGGGAAAAGTACTGTATTCTCGAGATAGGAGACATGCTCCTACGGACTGCAATAGGCCAAGTGTCAAGGCCCATGTTCCT
GTATGTGAGAACCAATGGGACTTCCAAGATCAAGATGAAATGGGGCATGGAGATGAGGCGATGCCTTCTTCAATCCCTTCAACA
AATTGAGAGCATGATTGAGGCCGAGTCTTCTGTCAAAGAGAAAGACATGACCAAAGAATTCTTTGAGAACAAATCAGAAACATGG
CCAATTGGAGAATCACCCAAAGGAGTGGAGGAAGGCTCCATTGGGAAGGTGTGCCGAACATTACTAGCAAAATCTGTATTCAAC
AGCCTATATGCATCTCCACAACTCGAGGGGTTTTCAGCTGAATCAAGAAAATTGCTTCTCATTGTTCAGGCACTTAGGGACAACC
TGGAACCTGGGACCTTCGATCTTGGGGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGTTTTGCTTAATG
CGTCTTGGTTCAACTCCTTCCTCACACATGCACTGAAA

Hemagglutination (HA) gene, 1,661 nucleotides; GenBank: JN982519

ATTCTAACAACTGGTCTTGCTTATGACAAGATTTGCATCGGCTATCAGACGAATAACTCAACCGATACGGTAAACACACTAATTGA
ACAGAATGTCCCAGTCACTCAGGTGGAAGAGCTCGTGCATGGTCAAGTCAATCCGATCCTATGCAGTACAGAACTGGGGTCAC
CCCTAGTGCTTGACGATTGCTCTCTGGAGGGCTTGATCCTAGGCAATCCCAAATGCGATCTCTATCTGAATGGTAGAGAATGGTC
ATACATTGTGGAGAGGCCCAAGGAGATGGAAGGAATCTGCTATCCAGGATCAATAGAGAACCAAGAAGAGTTGAGATCATTGTT
CTCTTCAATCAAGAAATATGAAAGAGTGAAAATGTTCGACTTCACCAAATGGAATGTCACTTACACTGGAACGAGCAAAGCTTGT
AATAACACCTCAAACCAAGGATCATTCTACAGGAGCATGAGATGGCTCACTTTGAAGTCAGGACAATTCCCAGTGCAAACCGAT
GAGTACAAAAACACTAGGGATTCGGACATTGTATTCACATGGGCTATTCATCACCCACCCACTTCTGACGAGCAGGTGAAGCTC
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GTTAGGGGACAACAAGGGAGGATGGATTATTATTGGGCTGTTCTAAAGCCAGGGCAGACAGTGAAGATACAGACTAATGGAAAT
CTAATTGCACCTGAATATGGCCATCTAATCACTGGGAAATCACACGGGAGAATACTTAAAAACAACCTGCCGATCGGCCAATGC
ACGACCGAATGTCAGTTAAATGAAGGAGTAATGAACACCAGCAAACCGTTTCAGAATACCAGCAAGCATTACATTGGGAAATGC
CCTAGATATATACCATCGAACAGTTTGAAACTGGCGATAGGGCTCAGGAATGTTCCCCAGGCCCAAGACCGAGGGCTCTTTGGA
GCAATCGCTGGATTCATAGAAGGAGGGTGGCCCGGATTGGTGGCTGGTTGGTATGGGTTCCAACATCAAAACTCTGAGGGAAC
AGGCATTGCAGCAGACAGGGACAGCACCCAGAAGGCAATGGACAACATGCAGAACAAACTGAACAATGTCATTGACAAAATGA
ACAAGCAATTCGAAGTGGTGAACCACGAATTTTCGGAGGTTGAAAGTAGAATAAATATGATCAATTCAAAAATTGACGATCAAATA
ACAGACATATGGGCATATAATGCGGAACTGCTAGTTTTGCTGGAGAATCAGAAGACACTAGACGAGCACGATGCGAATGTTAGG
AATCTCCATGACCGAGTCAGAAGGGTATTAAAAGAAAATGCGATAGATACTGAGGATGGGTGTTTTGAAATACTACACAAATGTG
ATGATGAATGCATGGATACAATCAAGAACGGGACTTACAACCACCAGAAATACGAAGAAGAGAGCAAGCTTGAGAGACAAAGG
ATTAATGGAGTGAAACTTGAAGAGAATTCTACATATAAAATCTTGAGCATCTACAGCAGTGTTGCCTCAAGCTTAGTATTACTGCTC
ATGATTATTGGGGGTTTCATTTTCGGATGTCAAAATG

Nucleoprotein (NP) gene 1,514 nucleotides; GenBank: JN982520

TTAGCAAAAAGCAGGTAGATAATCACTCACTGAGTGACATCAATATCATGGCGTCTCAAGGCACCAAACGATCTTATGAACAGAT
GGAGACCGGTGGAGAACGCCAGAATGCCACTGAGATCAGAGCATCTGTTGGAAGGATGGTTGGTGGAATTGGGAGGTTCTACA
TACAGATGTGCACTGAACTCAAACTCAGTGACTATGAAGGAAGGCTGATCCAAAACAGCATAACAATCGAGAGAATGGTTCTCT
CTGCATTTGATGAAAGGAGAAACAAATACCTGGAAGAACATCCCAGTGCGGGGAAAGACCCGAAGAAGACTGGAGGTCCAATT
TATCGAAGGAGAGATGGGAAATGGGTGAGAGAACTGATTCTGTATGACAAAGAGGAGATCAGGAGAATCTGGCGTCAAGCGAA
CAATGGAGAAGACGCAACTGCTGGTCTCACTCATCTGATGATCTGGCATTCCAACCTGAATGATGCCACATACCAGAGAACAAG

AGCGCTCGTGCGTACTGGAATGGACCCCCGGATGTGCTCCCTCATGCAAGGATCAACTCTCCCGAGGAGATCTGGAGCTGCTG
GTGCAGCGGTGAAGGGAGTCGGGACGATGGTGATGGAGCTAATTCGAATGATAAAACGAGGGATTAATGATCGGAATTTCTGG
AGAGGCGAAAATGGACGAAGGACAAGGATTGCATATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACA
AAGAGCAATGATGGACCAGGTGCGAGAAAGCAGGAATCCTGGAAATGCTGAAATTGAGGATCTCATCTTCCTGGCACGGTCTG
CACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCAGTAGCCAGTGGATACGACT
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GAGAACCCAGCACACAAGAGTCAATTGGTGTGGATGGCATGCCATTCTGCAGCATTCGAAGACCTGAGAGTCTCAAGCTTCATC
AGAGGAACAAGAGTAGTCCCAAGAGGACAACTATCCACCAGAGGAGTTCAAATTGCTTCAAACGAGAATATGGAAACAATGGA
CTCTAGCACTCTTGAACTGAGGAGCAGATATTGGGCTATAAGAACCAGAAGTGGAGGAAACACCAACCAACAGAGAGCATCTG
CAGGACAAATCAGTGTACAGCCCACTTTCTCAGTACAGAGAAATCTTCCCTTCGAAAGAGCGACCATTATGGCAGCGTTCACAG
GGAATACTGAAGGTAGAACATCAGACATGAGGACTGAAATCATAAGAATGATGGAAAGTGCCAGACCAGAAGATGTGTCTTTCC
AGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCT
TATTTCTTCGAAAACAATGCA

Neuraminidase (NA) gene 1,416 nucleotides; GenBank: JN982521

ATGAATCCAAATCAAAAGATAATAACCATTGGATCAATCTGCATGGTAATTGGAATAGCCAGCTTGATGCTACAAATTGGGAACAT
AATCTCTATATGGGTTAGCCATTCAATTCAGACAGGGAACCAATATCAGCCTGAACCATGCAATCAAAGCATCATTACTTATGAAA
ACAACACCTGGGTAAATCAGACGTATGTCAACATTAGCAATACCAATTTTCTTGCTGAACAGGCTGTCACTTCGGTGGCATTAGC
GGGCAATTCATCTCTTTGCCCTATTAGTGGGTGGGCTATATACAGTAAAGATAATGGTATAAGAATTGGTTCCAAGGGGGATGTG
TTTGTTATAAGAGAGCCATTCATCTCATGCTCCCACTTAGAATGCAGAACCTTCTTTTTGACCCAGGGGGCTTTGCTGAATGACAA
GCATTCTAATGGGACCGTCAAAGATAGGAGCCCCTACAGAACTTTGATGAGTTGTCCCGTGGGTGAGGCTCCTTCCCCGTACAA
TTCAAGATTTGAGTCTGTTGCTTGGTCGGCAAGTGCTTGTCATGATGGTATCAGTTGGTTGACAATCGGAATTTCTGGTCCAGACA
ATGGGGCTGTGGCTGTATTGAAGTACAACGGCATAATAACGGACACTATCAAGAGTTGGAGGAATAACATTTTGAGAACTCAAG
AGTCTGAGTGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGATGGACCAAGTAATGGGCAGGCCTCATACAAAATCTT
CAAGATAGAAAAAGGGAAAGTAGTCAAGTCAGTTGAATTGAATGCCCCTAATTACCACTACGAGGAGTGCTCCTGTTATCCTGAT
GCGGGCGAAATTATGTGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCCGTGGGTATCTTTCAATCAAAATCTGGAGTAT
CAAATAGGATATATATGCAGTGGGCTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGCAGTTGCGGTCCAATGTCCTCT
AACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTTTGGATTGGAAGGACAAAAAGTACTAGTTCCAGAA
GCGGCTTTGAGATGATTTGGGATCCGAATGGATGGACTGAGACGGATAATAGTTTCTCAGTGAAGCAAGACATCGTAGCAATAA
CTGACTGGTCAGGGTATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGATTAGATTGCATGAGGCCTTGTTTCTGGGTTG
AGCTAATCAGAGGGCGGCCCAAAGAGAACACAATTTGGACTAGTGGGAGCAGCATATCCTTTTGTGGTGTAAATAGTGACACTG
TGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCACCATTGA

Matrix protein (M) gene 998 nucleotides; GenBank: JN982522

AAAAACAGGTAGATATTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCGTCCCATCAGGCCCCCTCAAA
GCCGAGATCGCGCAGAGACTTGAAGATGTCTTTGCAGGGAAGAACGCCGATCTCGAGGCTCTCATGGAATGGCTAAAGACAAG
ACCAATCCTGTCACCTCTAACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAG
ACGCTTTGTCCAAAATGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAGAGCAGTCAAACTGTACAGGAAGTTGAAAAG
AGAGATAACATTCCATGGGGCTAAAGAAGTTGCACTCAGTTATTCAACCGGTGCACTTGCCAGTTGCATGGGTCTCATATACAAC
AGGATGGGAACGGTGACCACAGAAGTGGCTTTTGGCCTAGTGTGTGCCACCTGTGAGCAGATTGCTGATTCGCAGCATCGGTC

CCACAGGCAGATGGTAACTACCACCAACCCACTAATCAGGCATGAAAACAGAATGGTGCTGGCTAGCACCACGGCTAAGGCTA
TGGAGCAGATGGCTGGGTCAAGTGAGCAGGCAGCGGAAGCCATGGAAGTTGCTAGTCAGGCCAGGCAGATGGTGCAGGCAA
TGAGGACAATTGGAACTCACCCTAGCTCCAGTGCCGGTCTGAAAGATGATCTTCTTGAAAATTTGCAGGCCTACCAGAAACGGA
TGGGAGTGCAAATGCAGCGATTCAAGTGATCCTCTCGTTATTGCCGCAAGTATCATTGGGATCTTGCACTTGATATTGTGGATTCT
TGATCGTCTTTTCTTCAAATGTATTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTTCTACGGAAGGAGTGCCTGAGTCTA
TGAGGGAAGAATATCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGTCATTTTGTCAACA

Nonstructural protein (NS) gene 858 nucleotides; GenBank; JN982523

AAAAGGCAGGGTGACAAAAACATAATGGACTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAAC
GATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGGGGAAGAGGCAG
CACTCTTGGTCTGGACATCGAGACGGCTACCCGTGCGGGAAAGCAGATAGTGGAGCGGATTCTAGAAGAAGAATCTGATGAGG
CACTTAAAATGACTATTGCTTCAGTGCCGGCTTCACGCTACCTAACTGACATGACTCTTGAAGAGATGTCAAGGGACTGGTTCAT
GCTCATGCCTAAACAGAAAGTAGCAGGTTCCCTTTGCATCAGAATGGACCAGGCAATAATGGATAAAAACATCATATTGAAAGCA
AACTTCAGTGTGATTTTTGACCGACTGGAGACCCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGGGAGAAATCT
CACCGTTACCTTCTCTTCCAGGACATACTGATGAGGATGTCAAAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAATGA
TAACACAGTTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGGATGGGAGACCTCCACTCCCTCCAAA
GCAGAAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAATAAGATGGCTGATTGAAGAAGTGCGACATAGATTGA
AAATCACAGAAAACAGCTTCGAACAGATAACGTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAGAGATAAGAACTTT
CTCGTTTCAGCT

A/lesser whistling-duck/THA/CU-W3947/2010(H12N1)

Polymerase basic protein 2 (PB2) gene 2,284 nucleotide; GenBank: JN982524

TAAGAGATTTAATGTCACAGTCCCGCACTCGCGAGATACTGACAAAAACCACTGTGGACCATATGGCCATAATCAAGAAATATAC
GTCAGGAAGACAGGAGAAGAATCCTGCACTTAGGATGAAATGGATGATGGCAATGAAATATCCGATTACAGCAGACAGAAGGA
TAATGGAGATGATCCCTGAAAGAAATGAGCAAGGTCAGACTCTTTGGAGCAAGACAAATGATGCTGGATCAGACAGAGTGATGG
TGTCACCTTTGGCTGTGACGTGGTGGAATAGAAATGGACCAACGACAAGTACAGTCCATTATCCAAAGGTATATAAAACCTACTT
TGAAAAGGTTGAAAGGTTAAAGCATGGAACCTTCGGCCCCGTTCATTTTCGAAATCAGGTTAAGATACGCCGCAGGGTTGACAT
AAACCCGGGCCATGCAGATCTCAGTGCTAAAGAAGCACAAGACGTCATCATGGAGGTCGTTTTCCCAAACGAAGTCGGAGCCA
GGATATTGACATCAGAGTCACAGTTAACAATAACAAAGGAAAAGAAGGAGGAGCTTCAGGACTGTAAGATTGCCCCTTTAATGGT
GGCATACATGTTGGAGAGAGAACTGGTTCGTAAAACCAGATTCCTGCCAGTAGCTGGCGGGACAAGCAGCGTGTATATCGAGG
TGTTGCACTTGACTCAAGGGACCTGCTGGGAACAAATGTACACGCCGGGAGGAGAAGTGAGAAATGATGACGTTGATCAGAGT
TTAATTATTGCTGCTAGAAATATCGTTAGGAGAGCAACAGTATCAGCAGACCCATTGGCTTCGCTCTTGGAGATGTGCCATAGTA
CACAAATCGGCGGGATAAGGATGGTAGACATCCTTAGACAGAATCCAACAGAAGAGCAAGCCGTGGACATATGCAAAGCAGCA
ATGGGTCTAAGAATCAGTTCATCTTTCAGCTTTGGAGGTTTCACTTTCAAAAGGACAAGTGGGTCATCTGTCAAAAGAGAAGAGG
AAGTGCTCACCGGCAACCTCCAAACATTGAAAATAAGAGTGCATGAAGGGTATGAGGAATTCACCATGGTTGGGCGAAGAGCA
ACAGCCATTCTAAGGAAAGCAACCAGAAGGCTGATCCAATTGATAGTAAGTGGAAGAGACGAGCAGTCAATCGCCGAAGCGAT

CATAGTGGCAATGGTGTTCTCCCAAGAGGATTGCATGATAAAAGCAGTACGAGGTGATTTGAATTTTGTCAATAGAGCGAATCAG
CGGCTCAATCCTATGCATCAGCTCCTGAGGCATTTCCAAAAGGATGCAAAAGTACTGTTCCAAAACTGGGGAATTGAACCCATTG
ACAATGTCATGGGGATGATAGGAATATTGCCTGATATGACCCCCAGCACAGAGATGTCATTAAGAGGAGTGAGAGTCAGTAAGA
TGGGAGTGGATGAATATTCCAGTACTGAGAGGGTGGTCGTGAGTATTGATCGTTTCTTGAGGGTCCGAGACCAGAGAGGAAAC
GTGCTCTTGTCCCCTGAAGAGGTTAGTGAAACACAGGGAACAGAGAAGCTGACGATAACATATTCATCGTCAATGATGTGGGAA
ATCAATGGTCCGGAATCAGTGTTAGTTAACACATATCAATGGATCATTAGAAACTGGGAAACTGTGAAGATTCAGTGGTCCCAAG
ACCCTACAATGCTATACAACAAGATGGAGTTTGAGCCCTTTCAGTCCTTGGTGCCTAAGGCTGCCAGAGGCCAGTATAGTGGAT
TTGTGAGGACGCTATTCCAGCAGATGCGTGATGTGCTGGGGACCTTTGACACTGTCCAGATAATAAAGCTACTTCCATTTGCAGC
AGCCCCACCGGAACAGAGTAGGATGCAGTTCTCTTCTCTAACTGTAAACGTAAGGGGTTCAGGAATGAGAATACTTGTGAGAGG
CAACTCCCCTGTGTTCAACTATAACAAGGCAACCAAGAGGCTTACAGTCCTTGGAAAGGATGCAGGTGCATTGACAGAAGACCC
AGATGAGGGGACAGCAGGAGTGGAGTCTGCGGTATTAAGAGGATTCCTAATTCTGGGCAAAGAAGACAAAAGATATGGACCAG
CATTGAGCATCAATGAATTGAGCAATCTTGCGAAAGGGGAGAAGGCTAATGTGTTGATAGGGCAAGGAGACGTGGTGTTGGTGA
TGAAACGGAAACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGGA

Polymerase basic protein 1 (PB1) gene 2,271 nucleotides; GenBank: JN982525

CCGACTTTACTTTTCTTAAAGGTGCCAGCGCAAAATGCTATAAGTACTACATTCCCTTACACTGGAGATCCCCCATACAGCCATG
GAACAGGAACAGGGTACACCATGGACACAGTCAACAGAACACATCAGTACTCAGAAAAGGGAAAGTGGACAACAAACACAGA
GACCGGAGCACCCCAACTCAACCCAATTGATGGACCCTTACCTGAGGACAACGAGCCAAGCGGATATGCACAAACAGATTGC
GTGTTGGAAGCAATGGCTTTCCTTGAAGAATCCCACCCAGGAATCTTTGAAAACTCTTGTCTTGAAACGATGGAAGTTGTTCAGC
AAACAAGAGTGGACAAACTAACCCAAGGTCGCCAGACTTATGACTGGACATTGAATAGAAACCAACCAGCTGCAACTGCTTTGG
CCAACACTATAGAGGTTTTCAGATCGAACGGTCTGACGGCCAACGAATCAGGGAGACTAATGGATTTTCTCAAGGATGTGATGG
AATCAATGGATAAAGAAGAAATGGAAATAACAACACATTTCCAAAGAAAGAGAAGAGTAAGGGACAACATGACCAAGAAGATGG
TCACACAAAGAACAATAGGGAAGAAGAAGCAGAGGCTGAACAAGAGGAGCTACTTAATAAGAGCACTGACGCTGAACACAATG
ACAAAGGATGCAGAGAGAGGCAAATTGAAGAGGCGGGCAATTGCAACACCCGGGATGCAGATTAGAGGATTCGTGTACTTTGT
CGAAACACTGGCGAGGAGCATCTGTGAGAAACTTGAGCAATCTGGACTCCCCGTTGGAGGGAATGAGAAGAAGGCCAAATTG
GCAAATGTCGTGAGAAAAATGATGACTAATTCACAAGATACAGAGCTCTCCTTTACAATTACTGGAGACAACACCAAATGGAATG
AGAATCAAAATCCTCGGATGTTCCTGGCAATGATAACATACATCACAAGAAACCAACCTGAATGGTTTAGAAATGTCTTGAGCATT
GCCCCTATAATGTTCTCAAACAAAATGGCGAGATTAGGAAAAGGGTACATGTTTGAAAGTAAGAGCATGAAACTACGGACACAA
ATACCGGCAGAAATGCTTGCAAACATTGACTTGAAATACTTCAACGAGTCAACGAGAAAGAAAATCGAGAAAATAAGACCTCTGC
TAATTGATGGCACAGCCTCATTGAGTCCTGGAATGATGATGGGCATGTTCAATATGCTGAGCACAGTATTGGGAGTCTCAATCCT
GAATCTTGGGCAAAAGAGGTACACCAAAACCACATACTGGTGGGATGGACTCCAATCCTCTGATGATTTCGCTCTCATAGTGAAT
GCACCGAATCATGAGGGGATACAAGCAGGGGTGGATAGGTTCTATAGGACCTGCAAACTGGTTGGCATCAACATGAGCAAAAA
GAAGTCTTACATAAACCGGACAGGAACTTTTGAGTTCACAAGCTTTTTCTACCGCTATGGGTTTGTGGCCAACTTCAGTATGGAGT
TACCCAGCTTTGGAGTGTCTGGAATCAATGAATCGGCTGACATGAGCATTGGAGTTACAGTGATAAAGAACAATATGATAAACAA
CGACCTTGGACCAGCAACAGCTCAGATGGCTCTTCAGCTATTCATCAAGGACTATAGGTACACATACCGATGCCACAGGGGTGA
TACACAAATTCAAACGAGGAGATCATTCGAGCTGAGGAAGCTGTGGGAGCAGACCCGTTCAAAGGCAGGGCTGTTGGTATCAG
ACGGAGGACCAAATCTATACAACATTCGGAATCTCCACATCCCAGAGGTCTGCTTGAAATGGGAACTGATGGATGAAGATTACC
AAGGCAGGCTGTGTAATCCTCTGAATCCGTTTGTCAGTCATAAGGAAATTGAGTCTGTAAACAATGCTGTAGTAATGCCAGCCCA
CGGCCCAGCCAAGAGCATGGAATATGATGCTGTTGCAACTACACACTCATGGATCCCTAAGAGGAACCGTTCTATTCTCAATAC
CAGCCAAAGGGGAATTCTTGAGGATGAGCAAATGTACCAGAAGTGTTGCAATCTATTCGAGAAATTCTTCCCCAGTAGTTCATAC

AGGAGGCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGATGCACGCATTGATTTCGAATCTGG
AAGGATTAAGAAAGAAGAGTTCGCTGAGATCATGAAGATCTGTTCCACCATTGAAGAA

Polymerase acidic protein (PA) gene 2,148 nucleotides; GenBank: JN982526

ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATTGTCGAGCTTGCGGAAAAAGCAATGAAAGAATACGGGGAAGATCCG
AAAATCGAAACGAACAAATTTGCTGCAATATGCACACACTTAGAAGTCTGTTTCATGTATTCGGATTTCCACTTTATTGATGAACGA
GGCGAATCAATAATTGTAGAATCTGGCGATCCGAATGCATTATTGAAACACCGATTTGAGATAATTGAAGGGAGAGACCGAACAA
TGGCCTGGACAGTGGTAAATAGTATCTGCAACACCACAGGAGTCGATAAGCCTAAATTCCTCCCAGATTTGTATGACTACAAAGA
GAACCGATTCATCGAAATTGGAGTGACACGAAGGGAAGTTCACATATACTATCTAGAAAAAGCCAACAAGATAAAATCAGAGAA
GACACACATTCACATATTCTCATTCACTGGAGAGGAAATGGCCACTAAAGCGGACTATACCCTTGATGAGGAGAGCAGAGCAAG
AATCAAAACCAGGCTGTTCACCATAAGGCAAGAAATGGCCAGTAGGGGTCTATGGGATTCCTTTCGTCAGTCCGAGAGAGGCG
AAGAGACAATTGAAGAAAGATTTGAAATCACAGGAACCATGCGCAGGCTTGCCGACCAAAGTCTCCCACCGAACTTCTCCAGC
CTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGGGCAAGCTTTCTCAAATGTCAAAAGAAGTGA
ACGCCAGGATTGAGCCATTTCTGAAAACAACACCACGCCCTCTCAGATTACCTGATGGGCCTCCCTGCTCTCAGCGGTCGAAGT
TCTTACTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAAGGGGAAGGTATACCACTATATGATGCAATCAAATG
CATGAAGACATTTTTCGGTTGGAAAGAGCCCAACATCGTAAAACCACATGAAAAAGGCATAAACCCCAATTACCTCCTGGCTTGG
AAGCAGGTGCTGGCAGAACTCCAAGACATTGAGAATGAGGAGAAAATTCCAAAAACAAAGAACATGAAGAAAACAAGCCAATT
GAAGTGGGCACTTGGTGAGAACATGGCACCAGAGAAAGTAGACTTTGAGGACTGCAAAGATGTTAGTGATCTAAGACAGTATGA
CAGTGATGAACCAGAGTCTAGATCGCTAGCAAGCTGGATCCAGAGCGAATTCAACAAAGCATGCGAATTGACAGATTCAAGTTG
GATTGAACTTGATGAAATAGGGGAAGATGTTGCTCCAATTGAGCACATTGCGAGTATGAGAAGAAACTATTTCACAGCGGAAGTA
TCCCATTGCAGGGCTACTGAATACATAATGAAGGGGGTGTACATAAACACAGCCCTGTTGAATGCATCCTGTGCAGCCATGGAT
GACTTCCAACTGATTCCAATGATAAGCAAGTGCAGAACCAAAGAAGGAAGACGGAAGACAAATCTGTATGGATTCATTATAAAAG
GGAGATCCCATTTGAGGAATGACACCGATGTGGTAAACTTTGTGAGCATGGAATTCTCTCTTACTGACCCGAGGCTGGAACCAC
ACAAGTGGGAAAAGTACTGTATTCTCGAGATAGGAGACATGCTCCTACGGACTGCAATAGGCCAAGTGTCAAGGCCCATGTTCC
TGTATGTGAGAACCAATGGGACTTCCAAGATCAAGATGAAATGGGGCATGGAAATGAGGCGATGCCTTCTTCAATCCCTTCAAC
AAATTGAGAGCATGATTGAGGCCGAGTCTTCTGTCAAAGAGAAAGACATGACCAAAGAATTCTTTGAGAACAAATCAGAAACATG
GCCAATTGGAGAATCACCCAAAGGAGTGGAGGAAGGCTCCATTGGGAAGGTGTGCCGAACATTACTAGCAAAATCTGTATTCAA
CAGCCTATATGCATCTCCACAACTCGAGGGGTTTTCAGCTGAATCAAGAAAATTGCTTCTCATTGTTCAGGCACTTAGGGACAAC
CTGGAACCTGGGACCTTCGATCTTGGGGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGTTTTGCTTAAT
GCGTCTTGGTTCAACTCCTTCCTCACACATGCACTGAAA

Hemagglutination (HA) gene, 1,661 nucleotides; GenBank: JN982527

CTAACAACTGGTCTTGCTTATGACAAGATTTGCATCGGCTATCAGACGAATAACTCAACCGATACGGTAAACACACTAATTGAAC
AGAATGTCCCAGTCACTCAGGTGGAAGAGCTCGTGCATGGTCAAGTCAATCCGATCCTATGCAGTACAGAACTGGGGTCACCC
CTAGTGCTTGACGATTGCTCTCTGGAGGGCTTGATCCTAGGCAATCCCAAATGCGATCTCTATCTGAATGGTAGAGAATGGTCAT
ACATTGTGGAGAGGCCCAAGGAGATGGAAGGAATCTGCTATCCAGGATCAATAGAGAACCAAGAAGAGTTGAGATCATTGTTCT
CTTCAATCAAGAAATATGAAAGAGTGAAAATGTTCGACTTCACCAAATGGAATGTCACTTACACTGGAACGAGCAAAGCTTGTAAT
AACACCTCAAACCAAGGATCATTCTACAGGAGCATGAGATGGCTCACTTTGAAGTCAGGACAATTCCCAGTGCAAACCGATGAG
TACAAAAACACTAGGGATTCGGACATTGTATTCACATGGGCTATTCATCACCCACCCACTTCTGACGAGCAGGTGAAGCTCTACA

AGAACTCCGACACTCTCTCGTCAGTCACAACGGATGAAATTAACAGGAGTTTCAGGCCTAACATAGGGCCAAGACCCCTGGTTA
GGGGACAACAAGGGAGGATGGATTATTATTGGGCTGTTCTAAAGCCAGGGCAGACAGTGAAGATACAGACTAATGGAAATCTAA
TTGCACCTGAATATGGCCATCTAATCACTGGGAAATCACACGGGAGAATACTTAAAAACAACCTGCCGATCGGCCAATGCACGA
CCGAATGTCAGTTAAATGAAGGAGTAATGAACACCAGCAAACCGTTTCAGAATACCAGCAAGCATTACATTGGGAAATGCCCTA
GATATATACCATCGAACAGTTTGAAACTGGCGATAGGGCTCAGGAATGTTCCCCAGGCCCAAGACCGAGGGCTCTTTGGAGCA
ATCGCTGGATTCATAGAAGGAGGGTGGCCCGGATTGGTGGCTGGTTGGTATGGGTTCCAACATCAAAACTCTGAGGGAACAGG
CATTGCAGCAGACAGGGACAGCACCCAGAAGGCAATGGACAACATGCAGAACAAACTGAACAATGTCATTGACAAAATGAACA
AGCAATTCGAAGTGGTGAACCACGAATTTTCGGAGGTTGAAAGTAGAATAAATATGATCAATTCAAAAATTGACGATCAAATAACA
GACATATGGGCATATAATGCGGAACTGCTAGTTTTGCTGGAGAATCAGAAGACACTAGACGAGCACGATGCGAATGTTAGGAAT
CTCCATGACCGAGTCAGAAGGGTATTAAAAGAAAATGCGATAGATACTGAGGATGGGTGTTTTGAAATACTACACAAATGTGATG
ATGAATGCATGGATACAATCAAGAACGGGACTTACAACCACCAGAAATACGAAGAAGAGAGCAAGCTTGAGAGACAAAGGATT
AATGGAGTGAAACTTGAAGAGAATTCTACATATAAAATCTTGAGCATCTACAGCAGTGTTGCCTCAAGCTTAGTATTACTGCTCAT
GATTATTGGGGGTTTCATTTTCGGATGTCAAAATGGAATGGTTCGTTG

Nucleoprotein (NP) gene 1,514 nucleotides; GenBank: JN982528

AGCAAAAGGCGGGTAGATAATCACTCACTGAGTGACATCAATATCATGGCGTCTCAAGGCACCAAACGATCTTATGAACAGATG
GAGACCGGTGGAGAACGCCAGAATGCCACTGAGATCAGAGCATCTGTTGGAAGGATGGTTGGTGGAATTGGGAGGTTCTACAT
ACAGATGTGCACTGAACTCAAACTCAGTGACTATGAAGGAAGGCTGATCCAAAACAGCATAACAATCGAGAGAATGGTTCTCTC
TGCATTTGATGAAAGGAGAAACAAATACCTGGAAGAACATCCCAGTGCGGGGAAAGACCCGAAGAAGACTGGAGGTCCAATTT
ATCGAAGGAGAGATGGGAAATGGGTGAGAGAACTGATTCTGTATGACAAAGAGGAGATCAGGAGAATCTGGCGTCAAGCGAAC
AATGGAGAAGACGCAACTGCTGGTCTCACTCATCTGATGATCTGGCATTCCAACCTGAATGATGCCACATACCAGAGAACAAGA
GCGCTCGTGCGTACTGGAATGGACCCCCGGATGTGCTCCCTCATGCAAGGATCAACTCTCCCGAGGAGATCTGGAGCTGCTG
GTGCAGCGGTAAAGGGAGTCGGGACGATGGTGATGGAGCTAATTCGAATGATAAAACGAGGGATTAATGATCGGAATTTCTGG
AGAGGCGAAAATGGACGAAGGACAAGGATTGCATATGAGAGAATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACA
AAGAGCAATGATGGACCAGGTGCGAGAAAGCAGGAATCCTGGAAATGCTGAAATTGAGGATCTCATCTTCCTGGCACGGTCTG
CACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCAGTAGCCAGTGGATACGACT
TTGAGAGAGAAGGATACTCTCTAGTTGGAATAGATCCTTTCCGTTTGCTTCAAAACAGCCAGGTCTTCAGTCTCATTAGACCCAAT
GAGAACCCAGCACACAAGAGTCAATTGGTGTGGATGGCATGCCATTCTGCAGCATTCGAAGACCTGAGAGTCTCAAGCTTCATC
AGAGGAACAAGAGTAGTCCCAAGAGGACAACTATCCACCAGAGGAGTTCAAATTGCTTCAAACGAGAATATGGAAACAATGGA
CTCTAGCACTCTTGAACTGAGGAGCAGATATTGGGCTATAAGAACCAGAAGTGGAGGAAACACCAACCAACAGAGAGCATCTG
CAGGACAAATCAGTGTACAGCCCACTTTCTCAGTACAGAGAAATCTTCCCTTCGAAAGAGCGACCATTATGGCAGCGTTCACAG
GGAATACTGAAGGTAGAACATCAGACATGAGGACTGAAATCATAAGAATGATGGAAAGTGCCAGACCAGAAGATGTGTCTTTCC
AGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCT
TATTTCTTCGGAGACAATGCA

Neuraminidase (NA) gene 1,416 nucleotides; GenBank: JN982529

ATGAATCCAAATCAAAAGATAATAACCATTGGATCAATCTGCATGGTAATTGGAATAGCCAGCTTGATGCTACAAATTGGGAACAT
AATCTCTATATGGGTTAGCCATTCAATTCAGACAGGGAACCAATATCAGCCTGAACCATGCAATCAAAGCATCATTACTTATGAAA
ACAACACCTGGGTAAATCAGACGTATGTCAACATTAGCAATACCAATTTTCTTGCTGAACAGGCTGTCACTTCGGTGGCATTAGC
GGGCAATTCATCTCTTTGCCCTATTAGTGGGTGGGCTATATACAGTAAAGATAATGGTATAAGAATTGGTTCCAAGGGGGATGTG

TTTGTTATAAGAGAGCCATTCATCTCATGCTCCCACTTAGAATGCAGAACCTTCTTTTTGACCCAGGGGGCTTTGCTGAATGACAA
GCATTCTAATGGGACCGTCAAAGATAGGAGCCCCTACAGAACTTTGATGAGTTGTCCCGTGGGTGAGGCTCCTTCCCCGTACAA
TTCAAGATTTGAGTCTGTTGCTTGGTCGGCAAGTGCTTGTCATGATGGTATCAGTTGGTTGACAATCGGAATTTCTGGTCCAGACA
ATGGGGCTGTGGCTGTATTGAAGTACAACGGCATAATAACGGACACTATCAAGAGTTGGAGGAATAACATTTTGAGAACTCAAG
AGTCTGAGTGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGATGGACCAAGTAATGGGCAGGCCTCATGCAAAATCTT
CAAGATAGAAAAAGGGAAAGTAGTCAAGTCAGTTGAATTGAATGCCCCTAATTACCACTACGAGGAGTGCTCCTGTTATCCTGAT
GCGGGCGAAATTATGTGTGTGTGCAGGGACAACTGGCATGGCTCAAATCGGCCGTGGGTATCTTTCAATCAAAATCTGGAGTAT
CAAATAGGATATATATGCAGTGGGCTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGCAGTTGCGGTCCAATGTCCTCT
AACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTTTGGATTGGAAGGACAAAAAGTACTAGTTCCAGAA
GCGGCTTTGAGATGATTTGGGATCCGAATGGATGGACTGAGACGGATAATAGTTTCTCAGTGAAGCAAGACATCGTAGCAATAA
CTGACTGGTCAGGGTATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGATTAGATTGCATGAGGCCTTGTTTCTGGGTTG
AGCTAATCAGAGGGCGGCCCAAAGAGAACACAATTTGGACTAGTGGGAGCAGCATATCCTTTTGTGGTGTAAATAGTGACACTG
TGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCACC

Matrix protein (M) gene 998 nucleotides; GenBank: JN982530

TTGGAGCAAAAGCAGGTAGATGTTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCGTCCCATCAGGCC
CCCTCAAAGCCGAGATCGCGCAGAGACTTGAAGATGTCTTTGCAGGGAAGAACGCCGATCTCGAGGCTCTCATGGAATGGCTA
AAGACAAGACCAATCCTGTCACCTCTAACTAAGGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTG
CAGCGTAGACGCTTTGTCCAAAATGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAGAGCAGTCAAACTGTACAGGAA
GTTGAAAAGAGAGATAACATTCCATGGGGCTAAAGAAGTTGCACTCAGTTATTCAACCGGTGCACTTGCCAGTTGCATGGGTCTC
ATATACAACAGGATGGGAACGGTGACCACAGAAGTGGCTTTTGGCCTAGTGTGTGCCACCTGTGAGCAGATTGCTGATTCGCAG
CATCGGTCCCACAGGCAGATGGTAACTACCACCAACCCACTAATCAGGCATGAAAACAGAATGGTGCTGGCTAGCACCACGGC
TAAGGCTATGGAGCAGATGGCTGGGTCAAGTGAGCAGGCAGCGGAAGCCATGGAAGTTGCTAGTCAGGCCAGGCAGATGGTG
CAGGCAATGAGGACAATTGGAACTCACCCTAGCTCCAGTGCCGGTCTGAAAGATGATCTTCTTGAAAATTTGCAGGCCTACCAG
AAACGGATGGGAGTGCAAATGCAGCGATTCAAGTGATCCTCTCGTTATTGCCGCAAGTATCATTGGGATCTTGCACTTGATATTG
TGGATTCTTGATCGTCTTTTCTTCAAATGTATTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTTCTACGGAAGGAGTGCC
TGAGTCTATGAGGGAAGAATATCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGTCATTTTTTCAA

Nonstructural protein (NS) gene 858 nucleotides; GenBank; JN982531

AAAAGGCAGGGTGACAAAAACATAATGGACTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAAC
GATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGGGGAAGAGGCAG
CACTCTTGGTCTGGACATCGAGACGGCTACCCGTGCGGGAAAGCAGATAGTGGAGCGGATTCTAGAAGAAGAATCTGATGAGG
CACTTAAAATGACTATTGCTTCAGTGCCGGCTTCACGCTACCTAACTGACATGACTCTTGAAGAGATGTCAAGGGACTGGTTCAT
GCTCATGCCTAAACAGAAAGTAGCAGGTTCCCTTTGCATCAGAATGGACCAGGCAATAATGGATAAAAACATCATATTGAAAGCA
AACTTCAGTGTGATTTTTGACCGACTGGAGACCCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGGGAGAAATCT
CACCGTTACCTTCTCTTCCAGGACATACTGATGAGGATGTCAAAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAATGA
TAACACAGTTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGGATGGGAGACCTCCACTCCCTCCAAA
GCAGAAACGGAAAATGGCGAGAACAATTGAGTCAGAAGTTTGAAGAAATAAGATGGCTGATTGAAGAAGTGCGACATAGATTGA
AAATCACAGAAAACAGCTTCGAACAGATAACGTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAGAGATAAGAACTTT
CTCGTTTCAGCT

A/quail/THA/CU-J2882/2009 (H7N1)

Polymerase basic protein 2 (PB2) gene 647 nucleotides

TACGGTCCTGAGTCAGTGCTTGTTAACACTTATCAATGGATCATCAGAAATTGGGAGACTGTGAAGATTCAATGGTCTCAAGACC
CTACGATGTTGTATAATAAGATGGAGTTTGAACCGTTCCAATCCTTGGTACCTAAAGCTGCTAGAGGTCAATACAGTGGATTTGTG
AGAACACTATTCCAACAAATGCGTGACGTACTGGGGACATTTGATACAGTTCAGATAATAAAGCTGCTACCATTTGCAGCAGCCC
CACCGGAGCAGAGCAGGATGCAGTTTTCTTCTCTGACTGTGAATGTGAGAGGCTCAGGAATGAGAATACTCGTAAGGGGCAAC
TCCCCTGTATTCAACTACAATAAGGCAACCAAAAGGCTTACTGTTCTTGGAAAGGATGCAGGTGCATTAACGGAGGATCCAGAT
GAGGGAACAGCCGGAGTGGAATCTGCAGTACTGAGGGGATTCTTAATTCTAGGCAAGGAGGACAAAAGATACGGACCAGCATT
GAGCATCAATGAACTGAGCAATCTTGCGAAAGGGGAGAAAGCTAATGTGCTGATAGGGCAAGGAGACGTGGTGTTGGTGATGA
AACGGAAACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCG

Polymerase basic protein 1 (PB1) gene 1,174 nucleotides

AGCATGAAGCTGCGGACGCAAATACCAGCAGAAATGCTTGCAAACATTGACCTGAAATACTTCAACGAATCAACAAGGAAGAAA
ATCGAGAAAATAAGGCCTCTTCTAATAGATGGCACAGCCTCACTGAGCCCTGGAATGATGATGGGCATGTTCAACATGCTGAGT
ACAGTCTTAGGAGTCTCAATTCTGAATCTTGGGCAGAAGAGGTACACCAAAACCACATACTGGTGGGACGGACTCCAGTCCTCT
GATGATTTCGCTCTCATAGTGAATGCACCGAATCATGAGGGAATACAAGCAGGAGTGGATAGGTTCTACAGGACCTGCAAACTG
GTTGGGATCAATATGAGCAAAAAGAAGTCTTACATAAATCGGACAGGAACATTTGAGTTCACAAGCTTTTTCTACCGTTATGGATT
TGTGGCCAATTTCAGTATGGAGTTACCCAGCTTTGGAGTGTCTGGAATCAATGAATCGGCTGACATGAGCATTGGAGTTACAGTG
ATAAAGAACAATATGATAAACAACGACCTTGGACCAGCAACAGCTCAAATGGCTCTTCAGCTATTCATCAAGGACTATAGGTACA
CATACCGATGCCACAGGGGTGATACACAAATTCAAACGAGGAGATCATTCGAGCTGAGGAAGCTGTGGGAGCAGACCCGTTCA
AAGGCAGGGCTGTTGGTATCAGACGGAGGACCAAATCTATACAACATTCGGAATCTCCACATCCCAGAGGTCTGCTTGAAATGG
GAACTGATGGATGAAGATTACCAAGGCAGGCTGTGTAATCCTCTGAATCCGTTTGTCAGTCATAAGGAAATTGAGTCTGTAAACA
ATGCTGTAGTAATGCCAGCCCACGGCCCAGCCAAGAGCATGGAATATGATGCTGTTGCAACTACACACTCATGGATCCCTAAGA
GGAACCGTTCTATTCTCAATACCAGCCAAAGGGGAATTCTTGAGGATGAGCAAATGTACCAGAAGTGTTGCAATCTATTCGAGAA
ATTCTTCCCCAGTAGTTCATACAGGAGGCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGACGC
ACGCATTGATTTCGAATCTGGAAGGATTAAGAAAGAAGAGTTCGCTGAGATCATGAAGATCTGTTCCACCATTGAAG

Polymerase acidic protein (PA) gene 2,193 nucleotides

ATGGAAGATTTTGTGCGACAATGCTTCAATCCAATGATTGTCGAGCTTGCGGAAAAGGCAATGAAAGAATATGGGGAAGATCCA
AAAATCGAAACGAACAAATTTGCCGCAATATGCACACACTTAGAAGTCTGTTTCATGTATTCGGATTTCCACTTTATTGATGAACGA
GGCGAATCAATAATTGTAGAATCTGGCGATCCAAATGCATTACTGAAGCACCGATTTGAGATAATTGAAGGGAGAGACCGAACA
ATGGCCTGGACAGTGGTGAATAGTATCTGCAACACCACAGGAGTCGATAAGCCTAAATTCCTCCCAGATTTGTATGACTACAAAG
AGAACCGATTCATTGAAATTGGAGTGACACGAAGGGAAGTTCACATATACTATCTAGAAAAAGCCAACAAGATAAAATCAGAGAA
GACACACATTCACATATTCTCATTCACTGGAGAGGAAATGGCCACCAAAGCGGACTATACCCTTGATGAAGAGAGCAGAGCAAG
AATCAAGACCAGGCTTTTCACTATAAGACAAGAAATGGCCAGCAGGGGTCTATGGGATTCCTTTCGTCAGTCCGAAAGAGGCGA
AGAGACAGTTGAAGAAAGATTTGAAATCACAGGGACCATGCGCAGGCTCGCCGACCAAAGTCTCCCACCGAACTTCTCCAGCC

TTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGGGCAAGCTTTCTCAAATGTCAAAAGAAGTGAA
CGCCAGAATTGAGCCATTTCTGAAGACAACACCACGCCCTCTCAGATTACCTGATGGGCCTCCCTGCTCTCAGCGGTCGAAGTT
CTTGCTGATGGATGCCCTTAAATTAAGCATCGAAGACCCGAGTCATGAGGGGGAAGGTATACCGCTATACGATGCAATCAAATG
CATGAAGACATTTTTCGGCTGGAAAGAGCCCAACATCGTAAAACCACATGAAAAAGGCATCAACCCCAATTACCTCCTGGCTTG
GAAGCAGGTGCTGGCAGAACTCCAAGATATTGAAAATGAGGAGAAAATCCCAAAAACAAAAAACATGAAGAAAACAAGCCAATT
GAAGTGGGCACTTGGTGAGAACACGGCACCAGAGAAAGTAGACTTTGAGGACTGCAAAGATGTTAGCGATCTAAGACAGTATG
ACAGTGATGAACCAGAGTCTAGATCGCTAGCAaGCTGGATCCAAAGTGAATTCAACAAGGCATGCGAATTGACAGATTCAAGTT
GGATTGAACTTGATGAAATAGGGGAAGACGTTGCTCCAATTGAGCACATTGCGAGTATGAGAAGAAACTATTTCACAGCAGAAG
TATCCCATTGTAGGGCCACTGAATACATAATGAAGGGAGTGTACATAAACACAGCCCTGTTGAATGCATCCTGTGCAGCCATGG
ATGACTTCCAATTGATTCCAATGATAAGCAAGTGCAGAACCAAAGAAGGAAGACGGAAAACTAATTTGTATGGATTCATTATAAAA
GGAAGATCCCATTTGAGAAATGACACCGATGTGGTAAACTTTGTGAGCATGGAATTCTCTCTCACTGACCCGAGGCTGGAGCCA
CACAAGTGGGAAAAGTACTGTGTTCTCGAGATAGGAGACATGCTCCTCCGGACTGCAATAGGCCAAGTGTCAAGGCCCATGTT
CCTGTATGTGAGAACCAATGGGACTTCTAAGATCAAGATGAAATGGGGCATGGAAATGAGGCGATGCCTTCTTCAATCCCTTCAA
CAAATTGAGAGCATGATTGAGGCCGAGTCTTCTGTCAAAGAGAAAGACATGACCAAAGAATTCTTTGAGAACAAATCAGAAACAT
GGCCAATTGGAGAATCACCCAAAGGAGTGGAGGAAGGCTCCATTGGGAAGGTGTGCCGAACATTACTAGCAAAATCTGTATTC
AACAGCCTATATGCATCTCCACAACTCGAGGGGTTTTCAGCTGAATCAAGAAAATTGCTTCTCATTGTTCAGGCACTTAGGGACA
ACCTGGAACCTGGGACCTTCGATCTTGGGGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAACGATCCCTGGGTTTTGCTTA
ATGCGTCTTGGTTCAACTCCTTCCTCACACATGCACTGAAATAGTTGTGGCAATGCTACTATTTGCTATCCATACTGTCCAAAAAA

Hemagglutinin (HA) gene 1,671 nucleotides

ATGAACAcTCAAATcCTGGTATTCGCTCTGGTTGCAATCATTCCGACAAATGCAGACAAAATATGCCTCGGACATCATGCTGTGTC
AAACGGAACTAAAGTAAACACATTAACTGAGAGAGGAGTGGAAGTTGTTAATGCGACTGAAACAGTGGAACGGACAAACATCCC
CAGGATCTGCTCAAAAGGAAAAAGGACAGTTGACCTAGGTCAATGTGGACTCCTGGGGACAGTCACTGGGCCACCCCAATGTG
ACCAATTCCTAGAATTTTCAGCCGATTTAATTATTGAGAGGCGAGAAGGAAGTGATGTCTGTTATCCTGGGAAGTTCGTGGATGA
AGAAGCTCTGAGGCAAATTCTCAGAGAATCAGGCGGAATTGACAAGGAAACAATGGGATTCACATACAGCGGAATAAGAACTAA
TGGAGCAACCAGTGCATGTAGGAGATCAGGATTTTCATTCTATGCAGAAATGAAATGGCTCCTGTCAAACACAGATAATGCTGCT
TTCCCGCAAACGACTAAATCATACAAAAATACAAGGAAAGACCCAGCTCTAATAATATGGGGGATTCATCATTCTGGATCAACTG
CAGAACAGACCAAGCTATATGGGAGTGGAAACAAACTAATAACAGTTGGGAGTTCTAATTATCAACAGTCTTTTGTACCGAGTCC
AGGAGCGAGACCACAAGTTAATGGCCAATCTGGAAGGATTGATTTTCATTGGCTGATGCTAAACCCCAATGATACAGTCACTTTC
AGTTTCAATGGGGCCTTCATAGCTCCAGACCGTGCAAGCTTCCTGAGAGGAAAATCTATGGGGATTCAGAGTGGAGTACAGGTT
GATGCCAATTGTGAAGGGGATTGCTATCACAATGGAGGAACAATAATAAGTAATTTGCCCTTTCAGAACATAAATAGCAGGGCAG
TAGGGAAATGTCCGAGATATGTTAAGCAAGAGAGTCTGCTGCTAGCAACAGGGATGAAGAATGTTCCTGAGATTCCAAAGGGAA
GAGGTCTATTTGGTGCTATAGCAGGTTTCATTGAAAATGGATGGGAAGGTCTAATTGATGGGTGGTATGGTTTCAAGCATCAAAAT
GCACAGGGAGAGGGGATTGTTGCAGATTACAAAAGCACTCAATCGGCAATTGATCAAATAACAGGGAAATTAAACCGGCTCATA
GAAAGAACCAACCAACAATTTGAGTTGATAGACAATGAGTTCACTGAGGTTGAAAAGCAAATTGGTAATGTGATAAATTGGACCA
GAGACTCCATGACGGAAGTGTGGTCCTACAATGCTGAACTCTTAGTAGCGATGGAGAACCAGCACACAATTGATCTGGCTGACT
CAGAAATGAATAAACTATACGAACGAGTGAAGAGACAGCTGAGAGAGAATGCTGAAGAAGATGGCACTGGTTGCTTTGAGATAT
TTCACAAATGTGATGATGATTGTATGGCCAGTATTAGAAACAACACCTATGATCACAGCAAGTACAGGGAAGAGGCAATGCAAAA
TAGAATACAGATTGATCCAGTCAAAcTAAGCAGTGGCTACAAAGATGTGATACTTTGGTTTAGCTTCGGGGCATCATGTTTCATAC
TTCTGGCCATTGCAATGGGCCTTGTCTTCATATGTGTGAAGAATGGAAACATGCGGTGCACT

Nucleoprotein (NP) gene 1,481 nucleotides

TCTCAAGGCACCAAACGATCTTATGAACAGATGGAAACTGGTGGGGAACGCCAGAATGCTACTGAGATCAGGGCATCTGTTGG
AAGAATGGTCAGTGGCATTGGGAGGTTCTACATACAGATGTGCACAGAACTCAAACTCAGTGACTATGAAGGGAGGCTGATCCA
GAACAGCATAACAATAGAGAGAATGGTACTCTCTGCATTTGATGAAAGAAGGAACAGATGCCTGGAAGAACACCCCAGTGCGG
GAAAGGACCCGAAGAAGACTGGAGGTCCAATTTATCGGAGGAGAGACGGGAAATGGGTGAGAGAGCTAATTCTGTATGACAAA
GAGGAGATCAGGAGAATCTGGCGTCAAGCGAACAATGGAGAAGACGCAACTGCTGGTCTCACTCATCTGATGATCTGGCATTC
CAACCTGAATGATGCCACATACCAGAGAACAAGAGCGCTCGTGCGTACTGGAATGGACCCCCGGATGTGCTCCCTCACGCAA
GGATCAACTCTCCCGAGGAGATCTGGAGCTGCTGGTGCAGCGGTGAAGGGAGTCGGGACGATGGTGATGGAGCTAATTCGAA
TGATAAAACGAGGGATTAATGATCGGAATTTCTGGAGAGGCGAAAATGGACGAAGGACAAGGATTGCATATGAGAGAATGTGCA
ACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGAGCAATGATGGACCAGGTGCGAGAAAGCAGGAATCCTGGAAATGCT
GAAATTGAGGATCTCATCTTCCTGGCACGGTCTGCACTCATCCTGAGAGGATCAGTGGCCCATAAGTCCTGCTTGCCTGCTTGT
GTGTACGGACTTGCAGTAGCCAGTGGATACGACTTTGAGAGAGAAGGATACTCTCTAGTTGGAATAGATCCTTTCCGTTTGCTTC
AAAACAGCCAGGTCTTCAGTCTCATTAGACCCAATGAGAACCCAGCACACAAGAGTCAATTGGTGTGGATGGCATGCCATTCTG
CAGCATTCGAAGACCTGAGAGTCTCAAGCTTCATCAGAGGAACAAGAGTGGTCCCAAGAGGACAACTATCCACCAGAGGAGTT
CAAATTGCTTCAAACGAGAATATGGAAACAATGGACTCTAGCACTCTTGAACTGAGGAGCAGATATTGGGCTATAAGAACCAGAA
GTGGAGGAAACACCAACCAACAGAGAGCATCTGCAGGACAAATCAGTGTACAGCCCACTTTCTCAGTACAGAGAAATCTTCCCT
TCGAAAGAGCGACCATTATGGCAGCGTTCACAGGGAATACTGAAGGTAGAACATCAGACATGAGGACTGAAATCATAAGAATGA
TGGAAAGTGCCAGACCAGAAGATGTGTCTTTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGAT
CGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAATGCAGAGGAGTA

Neuraminidase (NA) gene 1,355 nucleotides

CAGGAGTTCAAAATGAATCCAAATAAGAAGATAATAACCATCGGATCAATCTGTATGGTAACTGGAATGGTTAGCTTAATGTTACA
AATTGGGAACTTGATCTCAATATGGGTCAGTCATTCAATTCACACAGGGAATCAACACAAAGCTGAACCAATCAGCAATACTAATT
TTCTTATTGAGAAAGCTGTGGCTTCAGTAAAATTAGCAGGCAATTCATCTCTTTGCCCCATTAATGGATGGGCTGTATACAGTAAG
GACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGAGCCATTCATCTCATGCTCCCACTTGGAATGCAGAA
CTTTCTTTTTGACTCAGGGAGCCTTGCTGAATGACAAGCACTCCAATGGGACTGTCAAAGACAGAAGCCCTCACAGAACATTAAT
GAGTTGTCCTGTGGGTGAGGCTCCCTCCCCATATAACTCAAGGTTTGAGTcTGTTGCTTGGTCAGCAAGTGCTTGCCATGATGGC
ACCAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAGTGGGGCTGTGGCTGTATTGAAATACAATGGCATAATAACAGACACTA
TCAAGAGTTGGAGGAATAACATACTGAGAACTCAAGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGAC
GGACCAAGTAATGGTCAGGCATCACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATCAGTCGAATTGGATGCTCCTA
ATTATCACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGAAATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCG
GCCATGGGTGTCCTTCAATCAAAATCTGGAGTATCAAATAGGATATATATGCAGTGGGCTTTTCGGAGACAATCCACGCCCCAAT
GATGGAACAGGCAGTTGCGGTCCAATGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTTT
GGATTGGAAGGACAAAAAGTACTAGTTCCAGAAGCGGCTTTGAGATGATTTGGGATCCGAATGGATGGACTGAGACGGATAATA
GTTTCTCAGTGAAGCAAGACATCGTAGCAATAACTGACTGGTCAGGGTATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAG
GATTAGATTGCATGAGGCCTTGTTTCTGGGTTGAGCTAATCAGAGGGCGGCCCAAAGAGAACACAATTTGGACTAGTGGGAGCA
GCATATCCTTTTGTGGTGTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCACCATTGA

Matrix protein (M) gene 961 nucleotides

ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCGTCCCGTCAGGCCCCCTCAAAGCCGAGATCGCGCAGAGACTT
GAAGATGTCTTTGCAGGGAAGAACACCGATCTTGAGGCTCTCATGGAATGGCTAAAGACAAGACCAATCCTGTCACCTCTGACT
AAGGGGATTTTAGGGTTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTA
AATGGGAATGGAGACCCAAACAACATGGACAGAGCAGTCAAACTGTACAGGAAGTTGAAAAGAGAGATAACATTCCATGGGGC
TAAGGAAGTTGCACTCAGCTACTCAACCGGTGCACTTGCCAGTTGTATGGGTCTCATATACAACAGGATGGGAACAGTGACCAC
AGAAGTGGCTTTTGGCCTAGTGTGTGCCACCTGTGAGCAGATTGCTGATTCACAGCATCGGTCCCACAGGCAGATGATAACTAC
CACCAACCCACTAATCAGGCATGAAAACAGGATGGTGCTAGCCAGCACTACGGCTAAGGCTATGGAGCAGATGGCTGGGTCG
AGTGAGCAGGCAGCGGAAGCCATGGAGGTTGCTAGTCAGGCTAGGCAGATGGTGCAGGCGATGAGGACAATTGGAACTCACC
CTAGCTCCAGTGCCGGTCTGAAAGATGATCTTCTTGAAAATTTGCAGGCCTACCAGAAACGGATGGGAGTGCAAATGCAGCGAT
TCAAGTGATCCTCTCGTTATTGCCGCAAGTATCATTGGGATCTTGCACTTGATATTGTGGATTCTTGATCGTCTTTTCTTCAAATGTA
TTTATCGTCGCCTTAAATACGGTTTGAAAAGAGGGCCTTCTACGGAAGGAGTGCCTGAGTCTATGAGGGAAGAGTATCGGCAGg
AACAGCAGAGTGCTGTGGATGTTGACGATGGTCATTTT

Nonstructural protein (NS) gene 829 nucleotides

ATGGACTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAACGATTTGCAGACCAAGAACTGGGTG
ATGCCCCATTCCTTGACCGGCTTCGCCGAGATCAGAAGTCCCTAAGGGGAAGAGGCAGCACTCTTGGTCTGGACATCGAGACG
GCTACCCGTGCGGGAAAGCAGATAGTGGAGCGGATTCTAGAAGAAGAATCTGATGAGGCACTTAAAATGACTATTGCTTCAGTG
CCGGCTTCACGCTACCTAACTGACATGACTCTTGAAGAGATGTCAAGGGACTGGTTCATGCTCATGCCTAAACAGAAAGTAGCA
GGTTCCCTTTGCATCAGAATGGACCAGGCAATAATGGATAAAAACATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACCGACT
GGAGACCCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGGGAGAAATCTCACCGTTACCTTCTCTTCCAGGACAT
ACTGATGAGGATGTCAAAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAATGATAACACAGTTCGAGTCTCTGAAACTC
TACAGAGATTCGCTTGGAGAAGCAGTAATGAGGATGGGAGACCTCCACTCCCTCCAAAGCAGAAACGGAAAATGGCGAGAACA
ATTGAGTCAGAAGTTTGAAGAAATAAGATGGCTGATTGAAGAAGTGCGACATAGATTGAAAATCACAGAAAACAGCTTCGAACAG
ATAACGTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAGAGATAAGAACTTTCTCGTTTCAC

APPENDIX C

Single letter code for amino acids

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

BIOGRAPHY

Dr. Manoosak Wongphatcharachai was born on January 31, 1983 in Nakhon-sawan, Thailand. He is the first son of Mr. Montri and Mrs. Sirawadee Wongphat-charachai. In 2002, he enrolled in the Degree of Doctor of Veterinary Medicine in the Faculty of Veterinary Science, Chulalongkorn University, Thailand. After graduation with second class honours, he enrolled in the Degree of Doctor of Philosophy Program in Veterinary Public Health, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2008. During study in the PhD program, he received the financial support from the Chulalongkorn University Dutsadi Phiphat scholarship.

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