國立台灣大學獸醫專業學院臨床動物醫學研究所

碩士論文

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台灣伴侶動物 A 型流行性感冒病毒之鑑定

Identification of Influenza A Virus in Companion Animals in Taiwan

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本論文係林慧亭（R01643010）在國立臺灣大學獸醫專業學院臨床動物醫學研究所完成之碩（博）士學位論文，於民國一百零三年六月二十七日承下列考試委員審查通過及口試及格，特此證明

口試委員：

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（指導教授）

劉朋誠　　王金和

系主任、所長　吳應寧　（簽名）

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將這份研究獻給 28 歲的自己，

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帶著完成這篇研究的毅力和勇氣，繼續走下去

中文摘要

A 型流行性感冒病毒在染伴侶動物犬和貓的感染報告非常少，然而近來許多 A 型流感病毒陸續從患有呼吸道疾病之犬貓被分離出來，部份亞型已經被證實可持續性在犬之間傳播。但在台灣的伴侶動物研究中，目前並無相關的 A 型流感病毒報告。本研究目的是以血清抗體檢測法及基因檢測法來調查臺灣地區伴侶動物 A 型流感病毒感染情形。以 Blocking ELISA 血清抗體檢測結果，台大動物醫院就診之家犬血清檢體，陽性率為 1.1% (3/281)；北中南等鄉村縣市，採集放養犬隻的血清檢體，陽性率則為 3.1% (6/193)。針對 H6 亞型 A 型流感病毒的血清凝集抑制試驗 (HI) 結果發現 9 隻 blocking ELISA 的陽性檢體中，有 3 隻測得為 HI H6 亞型陽性。One-step RT-PCR 檢測部份，犬 126 隻檢體中有 4 隻為陽性，陽性率為 2.1% (4/185)。PCR 陽性檢體中，成功從一隻有呼吸道症狀的病犬分離出 A 型流感病毒，命名為 A/canine/Taiwan/E01/2014 (H6N1)，此為世界首例犬 H6N1 自然感染之發現。此外，研究也分離出一起台灣雪貂自然感染 H1N1 新型流感之病例。研究結果顯示台灣伴侶動物確實存在 A 型流行性感冒病毒之感染，依據血清抗體和病毒基因檢測之陽性率，推測台灣犬流感病毒感染情形仍屬與散發性 (sporadic) 流行病，但未來仍有必要進一步對伴侶動 A 型流行性感冒持續的監控。

關鍵詞：禽流感、犬、雪貂、H6N1、A 型流行性感冒病毒、H1N1 新型流感

Abstract

Influenza was not considered an infectious disease in companion animals before. However, many subtypes of influenza A viruses have been isolated from dogs and cats around the world recently, such as H3N8, H3N2, H5N1, H5N2 and H1N1. Canine influenza virus (CIV) H3N8 and H3N2 subtypes were confirmed to be transmissible among dogs. The aim of this study was to investigate the prevalence of influenza A virus infection in companion animals in Taiwan using serological survey and one-step RT-PCR detection. Using blocking ELISA assay (bELISA), our results showed that the seroprevalence of pet dogs and rural free-ranging dogs was 1.1% (3/281) and 3.1% (6/193), respectively. The hemagglutination inhibition assay against avian H6 subtype also showed the presence of H6 antibody in three of nine bELISA positive serum samples. Furthermore, 2.1% (4/185) of positive RT-PCR detection in nasal swab samples were found in dogs. Among these four positive cases, an avian-origin influenza A virus was successfully isolated and designated as A/canine/Taiwan/E01/2014 (H6N1). It was the world's first report on dog naturally infected with an avian influenza H6N1 virus. Besides, we also identified a natural infection of influenza A(H1N1)pdm09 virus in a pet ferret in Taiwan. The results of this study indicated that the influenza A virus indeed exists in companion animals in Taiwan. According to the positive rate of influenza A antibody survey and nucleic acid detection, CIV infection in Taiwan belongs to sporadic cases, However, continual surveillance for influenza A virus infection in companion animals is still suggested.

Key words: Avian influenza virus, Canine, Ferret, H6N1, Influenza A virus, Influenza A(H1N1)pdm09 virus

Introduction

Influenza A virus belongs to family *Orthomyxoviridae* containing eight separate strand RNA segments and an envelope. Nowadays at least 18 HA and 11 NA subtypes have been identified based on hemagglutinin (HA) and neuraminidase (NA) on the surface of virus (Tong *et al.*, 2013). Shorebirds and waterfowl are considered reservoir of most influenza A virus subtypes, except H17, H18, N10 and N11 which were identified in bats (Wu *et al.*, 2014). Influenza A virus has wide host range and circulates in birds and some mammalian species. The genetic reassortment between animals and humans viruses may result in emerging new influenza subtypes in human population and cause an outbreak, such as influenza A(H1N1)pdm09. Avian influenza H5N1 and H7N9 are able to transmit to humans naturally (Claas *et al.*, 1998; Gao *et al.*, 2013). Influenza A virus is an important well-known zoonotic pathogen in the world (Lipatov *et al.*, 2004).

Previous studies of influenza A virus infection in mammals usually focused on human, swine, and horses. Companion animals were considered less susceptible with influenza A virus infection. However, the unusual fatal H5N1 virus infections in domestic dogs, cats and zoo felids were reported during the outbreak period of the avian influenza A (H5N1) virus in Asia during 2003-2004 (Keawcharoen *et al.*, 2004; Kuiken

et al., 2004; Songserm *et al.*, 2006a; Songserm *et al.*, 2006b). Meanwhile, canine influenza H3N8 subtype was first identified in the Unite State. Soon after, avian-origin H3N2 was also isolated from dogs in Korea (Song *et al.*, 2008). Hereafter, more and more studies reported the presence of antibodies against human or canine influenza viruses in dogs and cats (Dundon *et al.*, 2010; Kruth *et al.*, 2008; Lee *et al.*, 2009; McCullers *et al.*, 2011; Piccirillo *et al.*, 2010; Seiler *et al.*, 2010). These data indicated that dogs may play a role in the epidemiology of influenza A virus infection. Nevertheless, there is limited information about influenza A virus infection in companion animals in Taiwan.

Here we performed the serological and RT-PCR screening surveillance of influenza infection from 2012 to 2014 to address the possibility of influenza A virus infection in companion animals in Taiwan. Molecular analysis and the phylogenetic tree construction on the canine and ferret virus were also conducted. This study may provide further information of influenza A virus infection in companion animals and would help with the disease prevention in the future.

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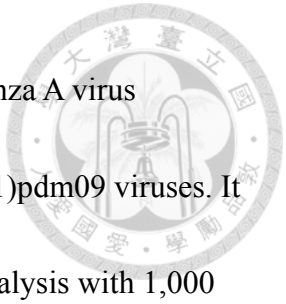


1. Literature reviews

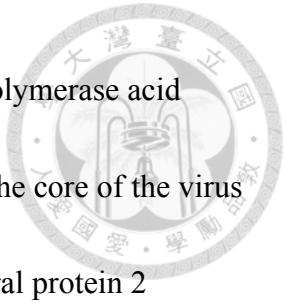
1.1 Influenza A virus

1.1.1 *Orthomyxoviridae*

Influenza viruses are enveloped viruses belonging to *Orthomyxoviridae* family *Orthomyxoviridae* family includes five different genera: influenza A, B and C; Thogotovirus; and Isavirus. Variation in influenza viruses is induced by the accumulation of nucleic acid point mutations (antigenic drift) during replication and genetic reassortment between two different influenza strains in the same cell. The new generated virus may have different host ranges comparing to the original strain (Cox and Subbarao, 2000; Lamb and Krug, 2001). Based on the nucleoprotein, influenza viruses can be classified as type A, B and C. Influenza A viruses have caused significant mobility and mortality of human from past to the present (Cox and Subbarao, 2000).

1.1.2 Genome structure of influenza A virus

The genome of influenza A virus contains eight negative, separate segmented RNAs which are translated into 11 proteins. The virus particle is about 80 to120 nanometers in diameter and usually spherical or filamentous in the shape. The hemaglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2) project from virus surface. Matrix protein 1 (M1) is laid under the envelope. The polymerase proteins



contains polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acid (PA). Other proteins include nucleoprotein (NP) which is located in the core of the virus particles and the non-structural proteins of nuclear export/nonstructural protein 2 (NEP/NS2) (Lamb and Krug, 2001).

1.1.3 Subtypes and nomenclature

Based on the hemaglutinin and neuraminidase which project on the envelope as spike, 18 different serotypes of HA and 11 different serotypes of NA have been identified (Tong *et al.*, 2013). Influenza A viruses are classified by these two surface glycoproteins, such as H1N1 and H5N1 subtypes. The nomenclature of a specific influenza strain is identified by virus type, host type (omission of isolation from human), geographical origin, strain number, year of isolation and HA/NA subtype. For example: A/California/04/2009 (H1N1) was influenza A virus isolated from human in California in 2009 with strain number of 04 (Wright and Webster, 2001).

1.1.4 Ecology of influenza A viruses

Wild waterfowl are considered the natural host of most known influenza A viruses (Webster *et al.*, 1992). Viruses replicate in the bird's intestinal mucosa and/or respiratory epithelium, and shed viruses in fecal or respiratory discharge (Webster *et al.*, 1978). Most of these infections are caused by low pathogenicity avian influenza (LPAI)

viruses. Mild respiratory distress, decreased egg production in chickens and turkeys may be observed, but it can be asymptomatic in ducks. However, H5 or H7 subtypes can mutate to become highly pathogenicity avian influenza (HPAI) viruses. HPAI viruses infection could cause severe clinical respiratory signs, organ hemorrhage and death (Beeler, 2009). Avian influenza A viruses had also been isolated from many animals, include seals, whales, horses, and pigs (Cox and Subbarao, 2000; Lagace-Wiens *et al.*, 2010).

1.1.5 The sialic acid receptor

Influenza A viral HA binds to the sialic acid receptor on the host tracheal epithelium. Avian influenza viruses bind to receptor glycolipids which possess sialyl-galactosyl residues with α 2, 3-gal linkages (SA α 2, 3-gal), whereas human influenza A viruses bind to SA α 2, 6-gal linkages (Suzuki, 2005). Therefore, it is limited for avian-adapted viruses to replicate in human host and cause sustained human outbreak without appropriate genetic alterations. Receptor specificity is considered to be an important species barrier (Cox and Subbarao, 2000). However, pigs which have α 2, 3-gal and α 2, 6-gal sialic acid receptors in the tracheal epithelium may be the intermediate hosts for reassortment of avian and mammalian species. This phenomenon causes the concern of pandemic potential risk of novel influenza A viruses (Cox and

Subbarao, 2000; Lagace-Wiens *et al.*, 2010).

1.1.6 Pandemic influenza

Swine and birds have been considered playing roles in generation of influenza pandemics. Avian and swine influenza are prominently involved in the history of influenza pandemics over four centuries (Lagace-Wiens *et al.*, 2010). In human history, there were three major pandemics in 20th century and one pandemic record in 2009: the 1918 to 1919 pandemic H1N1, the 1957 to 1958 pandemic H2N2, the 1968 pandemic H3N2, and the 2009 pandemic H1N1(Beeler, 2009; Cox and Subbarao, 2000; Lagace-Wiens *et al.*, 2010). These pandemic events had swine/avian origin and different epidemiologic patterns. For example, the 1918 pandemic H1N1 virus was thought to be a human/swine reassortment event which caused the most severe disease outbreaks in human history (dos Reis *et al.*, 2009). In 1974, influenza H2N2 pandemic began in Asia. This virus was originated from a single reassortment event between avian and human H1N1(Lagace-Wiens *et al.*, 2010). In 1968, a reassortment between avian H3 and human H2N2 generated a novel H3N2 viruses. It was also observed the extinction of H2N2 viruses after the outbreak of H3N2 in human populations (Cox and Subbarao, 2000). And in 2009, the novel H1N1 virus which was thought to be the triple-reassortment event with elements originating from Eurasia swine, north American

swine, avian and human led to the pandemic outbreaks in the world (Dawood *et al.*, 2009; Shinde *et al.*, 2009). In addition to these pandemic outbreaks, several influenza subtypes of avian origin could become the next pandemic strains. For example, there were several case reports about avian HPAI H5N1 and H7N9 infections in humans (Gao *et al.*, 2013; Peiris *et al.*, 2007; Uyeki and Cox, 2013). Concern for the emergence of a new pandemic is necessary even now there is no enough evidence of human to human transmission (Uyeki and Cox, 2013).

1.2 Influenza A virus in dogs

1.2.1 Equine origin canine influenza A virus (H3N8)

Canine influenza virus (CIV) H3N8 subtype was first isolated from the lung tissue of greyhounds in Florida in 2004. Eight gene segments were identified as an equine influenza virus origin with a unique lineage of H3N8 influenza virus (Crawford *et al.*, 2005). Direct contact, intake or inhalation of infectious droplets through nasal or oral mucosa was considered to be the route of transmission. Transmission of H3N8 from dogs to dogs was confirmed by virus isolation and serologic researches in 2005 (Brankston *et al.*, 2007; Payungporn *et al.*, 2008). Seroprevalence of CIV was below 5% in the Unite State and 49% in multi-dog households or shelters (Chambers *et al.*, 2013; Priestnall *et al.*, 2013). Nowadays, CIV H3N8 subtype has remained enzootic in

the USA (Chambers *et al.*, 2013; Priestnall *et al.*, 2013). CIV infection may result in ciliated damage in upper and lower respiratory tracts and the development of pneumonia. Clinical signs of anorexia, lethargy, respiratory discharge, coughing and fever are often noted. Besides, the environmental, host-associated factors and secondary bacteria pathogens are critical to the development of severe disease, such as fatal pneumonia (Beeler, 2009; Priestnall *et al.*, 2013). Necrotizing tracheitis/bronchitis could be observed by histopathological examination (Castleman *et al.*, 2010). So far case reports of CIV H3N8 infection have been published in both of the USA and Australia (Gibbs and Anderson, 2010; Kirkland *et al.*, 2010).

1.2.2 Avian origin canine influenza A virus (H3N2)

Canine influenza H3N2 subtype lineage was first reported in South Korea in 2007 (Song *et al.*, 2008). This subtype was isolated from dogs with severe respiratory disease. Genetic analysis showed that all eight gene segments have 95.5% to 98.9% homology to avian influenza viruses (Song *et al.*, 2008). Ingestion of poultry products or aerosol inhalation was the possible transmission route (Beeler, 2009; Gibbs and Anderson, 2010). Clinical signs and pathological examination results of the H3N2 infection were similar to those infected with H3N8 CIV (Song *et al.*, 2009). The ability of intraspecies transmission was demonstrated by experimental and natural infections

(Li *et al.*, 2010; Song *et al.*, 2008; Song *et al.*, 2009; Su *et al.*, 2013a). Sustained transmission in canine populations was verified by the serological evidence and subsequent infection cases of CIV H3N2 in South Korea, China and Thailand (Bunpapong *et al.*, 2014; Lee *et al.*, 2009; Li *et al.*, 2010; Song *et al.*, 2009; Su *et al.*, 2013a; Su *et al.*, 2013b; Sun *et al.*, 2014; Sun *et al.*, 2013; Wang *et al.*, 2013).

1.2.3 Highly pathogenicity avian influenza (H5N1)

In 2004, HPAI infection in a dog after ingestion infected poultry was documented in Thailand. Severe edema or congestion in pulmonary and extrapulmonary tissue, such as liver, kidney and spleen were observed (Songserm *et al.*, 2006b). Six hundred and twenty nine village dogs in Thailand were also found to have antibodies to H5N1(Butler, 2006). Mild respiratory signs, transient elevated body temperature or subclinical infection were recorded in experimental inoculation dogs. Virus shedding from nasal cavity and seroconvertion were also detected in some inoculated dogs. Howeve, transmission between infected dogs and dogs which closely contacted with was not established (Giese *et al.*, 2008; Maas *et al.*, 2007). Dogs are demonstrated to be susceptible to H5N1 through these experiment inoculation studies, but sustained transmission among dogs has not been reported so far.

1.2.4 Influenza A(H1N1)pdm09 virus

In 2009, there were sporadic clinical cases of A(H1N1)pdm09 infection in dogs following close contact with humans who had recently experienced influenza-like illness (ILI) in China and the USA (Dubovi, 2010; Lin *et al.*, 2012a). The further experimental and transmission studies were performed. However, the inoculated dogs only showed mild symptoms, and the capability of intraspecies transmission was limited (Lin *et al.*, 2012a). Serological evidence of A(H1N1)pdm09 virus infection in dogs was reported in many countries. There were 0.7% and 3.8% A(H1N1)pdm09 seroprevalence in dogs in Italy and in Japan, respectively (Dundon *et al.*, 2010; Horimoto *et al.*, 2014). So far no further reports on H1N1 transmission from dogs to humans are available.

1.2.5 H3N1 and H5N2 subtypes

A novel H3N1 subtype was also isolated from one dog during the surveillance program in South Korea in 2007 to 2010 (Song *et al.*, 2012). All gene segments except HA gene of this virus had highest nucleotide sequence similarity (99.1%- 99.9%) to A(H1N1)pdm09 viruses. Dogs infected H3N1 subtype didn't exhibit notable syndrome, but virus shedding from nasal cavity was detected (Song *et al.*, 2012). Besides, H5N2 subtype which was generated by complex reassortment of viral genomes was identified from a dog with classical flu signs in China in 2009 (Zhan *et al.*, 2012). Intraspecies transmission of H5N2 was demonstrated via experiment inoculation. These data

highlighted the role that dogs may play in transmission and spread of influenza (Song *et al.*, 2013).

1.3 Influenza in ferrets

Influenza induced rhinitis was reported in Ferret (*Mustela putorius furo*) in 1933. The high susceptibility of ferrets to influenza A virus made ferrets an attractive mammalian model for associated studies (Baker, 1998; Smith *et al.*, 1933). There were several reasons for ferrets to be a good model for influenza studies. First, α 2, 6-gal sialic acid receptor is predominant in upper respiratory tract of humans and ferrets (Belser *et al.*, 2011). Human and avian influenza viruses could replicate efficiently in ferrets without pre-adaptation (Belser *et al.*, 2011). And the long trachea structure is easy to compartmentalize the upper and lower respiratory tracts. It would help to analyze the infection effect of different viruses strains on the different respiratory tract locations. (Ball, 2002; Renegar, 1992). Second, numerous clinical signs of influenza infection in ferrets are similar to humans. Elevated body temperatures, nasal discharge and sneezing are so frequently observed in infected ferrets (Belser *et al.*, 2011). The infection severity depends on age, virus strains, immune status of host, environment and secondary pathogen infection. Sometimes clinical signs are very mild but it can also develop to pneumonia. Clinical assessment provides the information to evaluate the

status of virus replication and transmission effectiveness. Ferret have been thought as an ideal transmission model to explore the infection ability of influenza viruses (Herlocher *et al.*, 2001; Maines *et al.*, 2006; Yen *et al.*, 2007). Third, ferrets are large enough to monitor clinical parameters easily, such as temperatures, heart rate, and respiratory rate (Ball, 2002; Maher and DeStefano, 2004). In previous studies, HPAI H5N1 (Zitzow *et al.*, 2002), A(H1N1)pdm09 (Huang *et al.*, 2012; Munster *et al.*, 2009), seasonal H1N1 (Smith *et al.*, 2011), human origin H3N2 (Herlocher *et al.*, 2001) and canine H3N2 (Lee *et al.*, 2013) had used ferrets to perform pathogenesis and transmission researches. Nowadays, ferrets have become one kinds of popular companion animals. Several cases of human origin influenza infection in ferrets have been reported.(Campagnolo *et al.*, 2013; Patterson *et al.*, 2009; Yoon *et al.*, 2012). Therefore, it's essential to conduct the influenza A viruses surveillance on companion ferrets which have close contact with human begins .

1.4 Diagnosis of influenza A virus infection

RT-PCR targeting at M gene or NP gene is now the commonly used method to identify influenza A viruses infection (Dubovi, 2010). This assay had been validated to detect all HA subtypes of influenza A viruses (Fouchier *et al.*, 2000; Lee *et al.*, 2001). The influenza A virus subtype can be determined by full gene analysis using RT-PCR

and sequencing (Hoffmann *et al.*, 2001). Nasal swabs and respiratory discharge are the appropriate clinical specimens for RT-PCR (Dubovi, 2010). Virus isolation can be performed using embryonated chicken eggs, but the sensitivity is less than RT-PCR methods (Anderson *et al.*, 2012). For CIV infection, the maximum virus shedding is in two to four days after infection, and decline by day seven. The experiment inoculation showed that viruses shedding was difficult to be detected via RT-PCR by seven days post infection (Dubovi, 2010; Dubovi and Njaa, 2008). Therefore, RT-PCR and virus isolation could be false negative if sample collection was outside the virus shedding period (Deshpande *et al.*, 2009a). Hemagglutinin inhibition (HI) is the standard test to detect previous exposure to influenza A viruses. Antibody responses to CIV infection can be detected as early as seven days post infection (Deshpande *et al.*, 2009a). However, using 18 different HA subtype reference viruses to screen for all influenza A viruses infection seems impossible. Nowadays, blocking ELISA test coating NP as antigen to identify all influenza A subtypes in poultry or other animal species had been developed (Brown *et al.*, 2009). The convalescent serum specimens can be used to define influenza A virus infection (Dubovi, 2010).

1.5 Treatment and prevention of influenza A virus infection in companion animals

The treatment for influenza A virus infection in companion animals is not unusual. Supportive care such as fluid therapy, oxygen supply and bronchodilators are needed to relieve clinical signs. And broad-spectrum antibiotics are often used to control or prevent secondary bacterial infection (Dubovi, 2010). Antiviral drugs, such as adamantanes and oseltamivir are being discouraged in both human and animal medicine for several reasons (Beeler, 2009). First, there are no clinical oseltamivir evaluation studies in dogs and oseltamivir needs to be administered vey early before virus spreading over the body. Second, most of dogs can recover from adequent supportive care. Last, antiviral drugs shouldn't be overused due to the public health concern for the possibility of drug resistance (Beeler, 2009). CIV H3N8 subtype vaccine has been used in the USA (Deshpande *et al.*, 2009b). CIV H3N2 subtype vaccine is now also being developed in Korea (Lee *et al.*, 2010).

2. Identification of dog H6N1 infection through avian influenza survey in Taiwan

2.1 Abstract

Canine influenza A virus (CIV) has been reported around the world, but no CIV infection information in Taiwan is available. The aim of this study was to systematically investigate the influenza A virus prevalence in dogs from 2012 through 2014 in Taiwan using serological and virologic methods. The serological survey using blocking ELISA (bELISA) revealed that the influenza A positive rate was 1.1% (3/281) and 3.1 % (6/193) in household dogs and rural free-ranging dogs, respectively. Furthermore, the hemagglutination inhibition (HI) assay against avian H6 subtype also showed the presence of H6 antibody in three of nine bELISA positive serum samples. A/canine/Taiwan/E01/2014 (H6N1) was isolated from a dog nasal swab sample. Molecular analysis of eight gene segments of this virus revealed that it was closely related to avian influenza A viruses (H6N1) circulating in Taiwan. This virus harbored a single E627K substitution in the polymerase basic 2 protein (PB2) which might increase the replication ability in mammals. To our knowledge, this is the first report on H6N1 subtype natural infection in dog in the world. These data suggested that avian A(H6N1) infection in dogs may have existed in Taiwan. Our findings would spotlight the potential risks of avian-canine interspecies transmission of avian influenza viruses.

2.2 Introduction

Influenza A virus belongs to family *Orthomyxoviridae* and contains eight separate strand RNA segments with envelope. Nowadays at least 18 HA and 11 NA subtypes have been identified based on hemagglutinin (HA) and neuraminidase (NA) proteins on the surface of viruses (Tong *et al.*, 2013). Influenza A virus has wide host range and causes varied disease symptoms. It has become an important zoonotic pathogen in the world (Lipatov *et al.*, 2004). Influenza infection was not considered an infectious disease in dogs and cats before. However, interspecies transmission of an entire equine influenza A virus (H3N8) was first identified in an outbreak of respiratory disease occurred in greyhounds at racetrack in Florida in 2004 (Crawford *et al.*, 2005). In 2007, avian-origin H3N2 CIV was also discovered and confirmed in South Korea (Song *et al.*, 2008). Both H3N8 and H3N2 subtypes were able to cause sustained transmission among dogs (Payungporn *et al.*, 2008; Song *et al.*, 2009). Besides, sporadic influenza infection in dogs were successively reported in countries in Asia, such as H3N2, H3N1, H5N2 and H5N1 subtype in China, South Korea and Thailand (Bunpapong *et al.*, 2014; Li *et al.*, 2010; Lin *et al.*, 2012b; Song *et al.*, 2012; Song *et al.*, 2013; Songserm *et al.*, 2006b; Sun *et al.*, 2013; Zhan *et al.*, 2012). Nevertheless, no information about CIV infection in Taiwan, where is located in eastern Asia, neighboring with China, South Korea, and Thailand. Therefore, serological survey and

one-step RT-PCR screening were performed in this study to address the possibility of influenza A virus infection in dogs in Taiwan from November 2012 through February 2014. Phylogenetic analysis on the virus isolate, A/canine/Taiwan/E01/2014 (H6N1), was also conducted to compar its molecular characteristics with other closely related strains, especially the human-infecting H6N1 virus (A/Taiwan/2/2013). This study could provide information of influenza A virus infection in dogs in Taiwan and also aroused attention to the interspecies infection of local influenza A virus strains.

2.3 Materials methods

2.3.1 Serum specimen collection

From October 2012 through October 2013, 474 serum specimens were collected. Two dog groups were investigated in this serological survey. A total of 281 serum specimens were collected from the household dogs attending to the National Taiwan University Veterinary Hospital (NTUVH). And 193 serum specimens were obtained from rural free-ranging dogs enrolled in Rural Area Spay/Neuter Services (RASNS) program from counties of Taipei, Hsinchu, Miaoli, Yunlin, Chiayi. All the serum specimens were selected randomly from these two groups.

2.3.2 Influenza A virus antibody detection

All serum specimens were tested for antibodies against to influenza A virus

using a species-independent, blocking ELISA (bELISA) Influenza A Virus Antibody Test Kit (IDEXX, Westbrook, ME). The procedure was performed according to the manual and briefly introduced below. At first, 15 μl serum specimens was diluted in 135 μl of dilution buffer. Then 100 μl of each diluted serum sample, undiluted negative and positive control were dispensed into an antigen plate, respectively (negative and positive controls were dispensed into two wells). This plate would be incubated at 18-26 ^{o}C for 60 min, and then washed with wash solution. One hundred μl of anti-influenza A conjugate was added into each well and incubated for 30 min. The plate was washed again and dispensed with 100 μl of TMB substrate solution into each well. After 15 min, 100 μl of stop solution was added to stop all the reaction. Finally, the optical density (O.D.) of samples and controls were measured at 650 nm using an ELISA reader (TECAN, Seestrasse, Switzerland). Value of optical density of the serum sample divided by the mean O.D. of negative control was regarded as S/N ratio. If the S/N ratio is less than 0.60, the serum is interpreted influenza A antibody positive. Conversely, if S/N ratio is greater than or equal to 0.60, it is considered influenza A antibody negative.

2.3.3 Hemagglutination inhibition assay

All the serum samples with bELISA antibody positive to influenza A virus were further tested using hemaglutination inhibition (HI) assay. HI was performed using

A/chicken/Taiwan/2838V/2000 (H6N1). HI assay was operated according to the procedures which recommended by the World Organization for Animal Health (OIE). Briefly, 25 μl of 2-fold serial dilutions serum specimens were mixed 4 HA units of virus in a 96 well plate and incubated at 18-26 ºC for 30 min. Fifty μl of 1% chicken suspension RBC was added into each well and remained for 40 min at 18-26 ºC. The reciprocal of highest serum dilution was the HI titer. HI titer of 16 or more was considered evidence of previous exposure to influenza A H6 subtype.

2.3.4 Nasal swab specimen collection

Nasal swab specimens were collected from dogs which came to National Taiwan University Veterinary Hospital showing respiratory clinical signs, such as nasal discharge, sneezing and coughing. Specimen collections were from November 2012 through February 2014. Nasal swab was resuspended in 1mL viral transported medium (Creative, Taipei, Taiwan) and stored at -80ºC until processed.

2.3.5 RNA extraction

RNA was extracted with a commercial kit (Viral RNA Mini Kit, QIAGEN Inc., Hilden, Germany) according to manufacturer's instructions. Total RNA was eluted in a final volume of 50 μl.

2.3.6 Influenza A virus detection

One-step reverse transcription-PCR (RT-PCR) was performed on total RNA extraction from nasal swab specimens by using One-Step RT-PCR Kit (QIAGEN). Primer set (M52C/M253R) targeting on a highly conserved region of matrix (M) gene was used for influenza A nucleotide detection (Fouchier *et al.*, 2000). For each one-step RT-PCR, 5 μl RNA was mixed with a reaction mixture containing 5 μl of 5×QIAGEN One-Step RT-PCR Buffer, 1.0 μl of dNTPs (10 mM), 1.5 μl of forward and reverse primers (10 μM), 1 μl of QIAGEN One-Step RT-PCR Enzyme Mix, and distilled water in a final volume of 25 μl. One-step RT-PCR was performed using a thermocycler (Biometra, Goettingen, Germany). Reaction conditions were performed by reverse transcription step at 50 ºC for 30 min, initial denaturation step at 95 ºC for15 min, amplification for 40 cycles at 94 ºC for 30 s, 49 ºC for 30 s, 72 ºC for 30s, and followed by final extension at 72 ºC for 10 min. PCR product was analyzed by electrophoresis in 1.5% agarose gels (Amresco, Solon, OH) with ethidium bromide (0.2 mg/mL) staining.

The remaining nasal swab suspended solutions from RT-PCR positive cases were used for virus isolation.

2.3.7 Virus isolation

Nasal swab specimen suspended solutions were centrifuged at 1,500× g for 20 min in a refrigerated centrifuge (4 ºC) to remove tissue debris. The supernatant was filtered through a sterile 0.45 μm Millex syringe-driven Filter (Millipore, Billerica, MA) filter to be the innoculum, then inoculated into the allantoic sac of 10-day-old specific-pathogen-free chicken eggs (Animal health research institute, council of agriculture, executive yuan, Taipei, Taiwan) and incubated for 48 h at 35 ºC. Allantoic fluids were harvested for genetic analyses of influenza A virus. Allantoic fluids were harvested for influenza A virus genetic analysis after one to three passages.

2.3.8 Neucleotide sequencing and phylogenetic analysis

The full-length amplification of all eight gene segments was performed as described by using one-step RT-PCR with universal gene-specific primer sets (Hoffmann *et al.*, 2001). The amplified products of eight gene segments of influenza A virus were purified from agarose gels using QIAquick Gel Extraction Kits (QIAGEN), and cloned into the pGEM-T Easy vector (Promega Corp, Madison, WI), then transferred into Arrow 58sec DH5α competent cell (Arrowtec, Taipei, Taiwan).

Plasmids were sent for automated sequencing (Tri-l Biotech, Inc., Taipei, Taiwan)). The sequence identity of eight segmented genes was conducted with nucleotide Basic Local Alignment Search Tool (BLAST) in Global Initiative on Sharing All Influenza Data (GISAID) and in GenBank of National Center for Biotechnology Information (NCBI) .

The phylogenetic tree was constructed using complete sequences attained from sequence database. Multiple sequence alignments, and phylogenetic analyses were performed using Mega 6 software (Tamura *et al.*, 2013). Sequences were aligned using the ClustalW method. Phylogenic trees were built using maximum-likelihood method with1,000 replications of bootstrap analysis.

2.3.9 Statistical analysis

Fisher's exact test was used to evaluate the seroprevalence difference between household dogs and rural free-ranging dogs. Differences were considered significant at *p* value less than 0.05.

2.4 Results

2.4.1 Serological surveillance

A total 281 and 193 serum specimens were obtained from household dogs and rural free-ranging dogs, respectively. The serological positive rate was 1.1% (3/281) and 3.1% (6/193) using bELISA in household dogs and rural free-ranging dogs, respectively.

Based on the analysis of Fisher's exact test, there was no statistically significant difference (P = 0.168) in seroprevalence between these two groups. Furthermore, HI assay revealed that three of the nine bElISA positive serum samples showed antibodies against A/chicken/Taiwan/2838V/2000 (H6N1). The HI titers of these three samples were 16, 512 and >2048. All of the HI-positive serum specimens were collected from the countryside in Yunlin, Taiwan, which is a major poultry industry location in Taiwan.

2.4.2 Virus detection and isolation

Nasal swab specimens from four dogs were M gene positive detected using one-step RT-PCR. The positive rate was 2.1% (4/185). Further sequencing and sequence comparison proved that they were all influenza A virus. The remaining nasal swab suspended solutions from these four positive dogs were used to isolate the influenza A virus (5 eggs per specimen). Influenza A virus was successfully isolated from a case co-infected with canine distemper virus (CDV). At first passage, two embryos showing hemorrhage lesions died within 48 h (2/5) in which CDV and influenza A virus were both successfully detected using RT-PCR (Figure 2-7). At the second passage all five embryos showed hemorrhage lesions and died within 48 h, in which all showed positive in influenza A virus but no CDV could be detected. At the third passage, all five embryos appeared normal and were alive over 48 h. As with the

second passage, all eggs showed positive in influenza A virus but no CDV could be detected. The HA titer of allantoic fluid ranged from 256 to1024 in all of the influenza A virus positive eggs. This isolate was designated as A/canine/Taiwan/E01/2014 with the GenBank accession no. KM203337-KM203344.

2.4.3 Clinical findings of positive virus cases detected using RT-PCR

The most common syndrome was nasal discharge or coughing in the four one-step RT-PCR positive cases. Three of them were under six months of age, and adopted from animal shelter (n=1) or rescued from streets (n=2). Another was a 15 years old household pet. Canine herpesvirus co-infection was found in one of them, and CDV co-infection was found in another. The dog co-infected with CDV exhibited severe purulent nasal discharge, cough, and fever. PCR detection showed negative results for canine herpesvirus and canine adenovirus. Chest radiographs showed severe bilateral infiltration and air-bronchogram in lung field (Figure 2-9). The paired serum specimens (collected at day 1, 7, 14, and 19 after hospitalization) of this case were assessed with bELISA and HI assay against H6 subtype, but seroconversion was not observed. Persistent and large quantity of viral shedding from nasal discharge were detected using one-step RT-PCR every two days until the dogs died 19 days later.

2.4.4 Sequence analyses of A/canine/Taiwan/E01/2014

The sequence homology of A/canine/Taiwan/E01/2014 (H6N1) in eight gene segments was investigated in GISAID and GenBank (Table 2-1) . The HA and NA gene of this virus had the highest nucleotide sequence similarity (99%) to A/chicken/Taiwan/1843/2012 (H6N1) and A/chicken/Taiwan/2084/2012 (H6N1), respectively. The other genes, including polymerase basic protein 2 (PB2), polymerase basic 1 (PB1), NP and nonstructural protein (NS), were closely related to H6N1 avian influenza virus isolates from chicken in Taiwan at the similarity 97% to 99%. Whereas M gene and polymerase acidic protein (PA) gene also showed the highest nucleotide sequence similarity to A/chicken/Taiwan/2593/2012 (H5N2) at 99%. Based on the HA and NA sequence analysis result, A/canine/Taiwan/E01/2014 was identified as H6N1 subtype.

2.4.5 Molecular characterization of A/canine/Taiwan/E01/2014

Only one basic amino acid (PQIATR*G) was found at the HA cleavage site of A/canine/Taiwan/E01/2014. Besides, G228S substitution (H3 numbering) on receptor binding site of HA protein was also noted in this virus. In NA proteins, a-14 amino acid deletion in the NA stalk region was observed at the amino acid position 42 to 53 and 68 to 69. The amino acid H275Y substitution in NA, the marker of oseltamivir resistance,

was not found in this virus. In the M2 protein, A/canine/Taiwan/E01/2014 did carry a S31N substitution. The other important signatures associating with replication ability in mammalian hosts or pathogenecity, including E627K in the PB2 protein and the PDZ ligand domain (acronym of three proteins in the first letters: post synaptic density protein, drosophila disc large tumor suppressor, and zonula occludens-1 protein) at the c-terminal region of NS1 protein of this virus were also observed (Table 2-2).

2.4.6 Phylogenetic analysis

Phylogenetic analysis of HA and NA gene segments indicated that A/canine/Taiwan/E01/2014 belonged to an H6N1 lineage that had been circulating in chickens in Taiwan for more than 40 years (Figures 2-1; 2-2). Although, the lineage of internal gene segments (PB2, PB1, PA, NP, M and NS) also comprised H6N1 viruses isolated in Taiwan, some H5N2 isolates fell within a lineage of H6N1 were observed (Figures 2-3 to 2-5). Moreover, most gene segments of A/canine/Taiwan/E01/2014 were clustered together in Taiwan lineage viruses isolated during 2012-2013. Departed from other gene segments, the phylogenetic tree of PB1 gene indicated that A/chicken/Taiwan/A2837/2013 (H6N1) was closely related to A/canine/Taiwan/E01/2014 and clustered in the Taiwan lineage viruses which were isolated in 2009 (Figure 2-3).

2.5 Discussion

Transmission of avian influenza viruses to dogs have been reported in various countries (Song *et al.*, 2008; Songserm *et al.*, 2006b). For example, a fatal H5N1 infection in a dog after ingestion of an H5N1-infected duck was reported in Thailand in 2004 (Songserm *et al.*, 2006b). Avian-originated H3N2 CIV was identified from dogs with severe respiratory disease in 2007 in South Korea (Song *et al.*, 2008). All eight segmented genes of these two virus isolates described above had highly homology (96-99%) with avian influenza viruses. In contrast, the information about dog influenza infection and its relevance to local avian-originated viruses in Taiwan was absent. Avian influenza H6N1 viruses have been widespread in chicken in Taiwan since 1972 (Lee *et al.*, 2005; Lee *et al.*, 2006; Lu *et al.*, 1985). H6N1 viruses in Taiwan have established a unique lineage which differs from those circulating in Hong Kong and in Southeastern China. Previous study indicated that it would cause respiratory distress, decreased egg production or increased flock mortality in poultry (Lee *et al.*, 2006). Unlike avian species, H6 subtype infection in mammals was rare.

In order to investigate the potential risk of avian H6N1 infection in dogs, the HI test against avian H6 subtypes was performed. Among nine bELISA-positive serum specimens, there were three serum specimens showed positive results with HI titers of

32, 512 and 2048, respectively. Four dogs exhibited RT-PCR positive results (2.1%) among total 185 tested dogs from swab samples. These four dogs had similar history background, including young age tendency, complicated living environment (shelter or stray dogs) or concurrent infection with other pathogens. A/canine/Taiwan/E01/2014 was isolated from a dog which co-infected with canine distemper virus.

Based on the A/canine/Taiwan/E01/2014 molecular analysis results, HA, NA, PB1, PB2, NP and NS genes showed high homology (>97% nucleotide identity) with avian H6N1 isolates which are now concurrently prevalent in Taiwan. PA and M gene of A/canine/Taiwan/E01/2014 showed 99% nucleotide identity with A/chicken/Taiwan/2593/2013 (H5N2).

All eight-segmented genes were highly originated from avian sources, it was speculated that the whole avian influenza genome had transmitted to this dog. According to the phylogenetic analysis, eight segmented genes comprised all avian H6N1 viruses isolated in Taiwan. However, some avian H5N2 viruses which fell within the H6N1 lineage of six internal genes were also observed. According to previous study, avian H6N1 viruses had circulated in poultry in Taiwan more than 41 years (Lee *et al.*, 2006), and were found to co-circulate with H5N2 viruses at low levels in 2012-2013 (Lee *et al.*, 2014). Further phylogenetic analysis indicated that internal genes of H5N2

viruses have undergone multiple reassortment events with contemporary enzootic H6N1 viruses for the last two decades (Lee *et al.*, 2014). Since we didn't observe independent lineage of H5N2 viruses in our phylogenetic trees. A/canine/Taiwan/E01/2014 was considered a H6N1 subtype virus originated from avian H6N1 and was a reassortant from different avian H6N1 viruses circulating in Taiwan. To our knowledge, it was the world's first avian H6N1 infected canine case.

Avian H6 viruses had the ability to replicate in mice without pre-adaptation based on the study of Lee et al (Lee *et al.*, 2006). Beare and Webster also reported that humans inoculated with avian-origin H6N1 virus shed virus and exhibited mild clinical symptoms but wouldn't produce a detectable antibody response (Beare and Webster, 1991). On 20 May 2013, the world's first human H6N1 bird flu infected case was demonstrated in Taiwan (Shi *et al.*, 2013; Wei *et al.*, 2013; Yuan *et al.*, 2013). We therefore compared A/canine/Taiwan/E01/2014 with the human-infected H6N1 virus (A/Taiwan/2/2013) to further identify the molecular traits regarding host range and virulence (Table 2-2; 2-3; 2-4). Similar to A/Taiwan/2/2013, amino-acid G228S substitution in the HA of A/canine/Taiwan/E01/2014 was found at the receptor-binding domain (RBD). The G228S amino-acid substitution in HA was considered acquiring higher receptor affinity for α 2-6 sialic acid receptors which exist in human (Schrauwen

and Fouchier, 2014; Stevens *et al.*, 2006). Avian H6N1 viruses which harbor G228S substitution in HA have become an predominant strains in poultry in Taiwan since 2005 (Lee *et al.*, 2005; Wei *et al.*, 2013). Therefore, the impacts of G228S substitution in HA of A/canine/Taiwan/E01/2014 need further research. Besides, a 14-aminoacid deletion in the stalk region of NA was also noted in this canine isolate. Deletions in the NA stalk region was considered to be associated with the adaptation hallmark of waterfowl viruses to terrestrial poultry (Munier *et al.*, 2010; Schrauwen and Fouchier, 2014). Avian H6N1viruses with the 14-residue deletion in NA stalk have become the major strains in Taiwan since 2001 (Lee *et al.*, 2006). The C-terminus of NS1 in this canine isolate also contained a PDZ ligand domain (EPEZ sequence), which was considered a potential virulent strain in a mouse model (Jackson *et al.*, 2008; Obenauer *et al.*, 2006). Moreover, previous studies revealed that E627K and D701N substitutions in PB2 protein were important mammalian signatures (de Wit *et al.*, 2010; Hatta *et al.*, 2001; Mehle and Doudna, 2009; Subbarao *et al.*, 1993). PB2 E627K was observed to have high prevalence (70%) in the H7N9 human isolates, but not in H7N9 avian isolates. This substitution in H7N9 human isolates had been demonstrated to enhance the replication in mice. E627K was also associated with the virulence of HPAI H5N1 infection in human cases. E627K substitution was found in PB2 of A/canine/Taiwan/E01/2014, but not in that of A/Taiwan/2/2013. This suggests that

A/canine/Taiwan/E01/2014 might replicate more efficiently than A/Taiwan/2/2013 in mammalian host.

To address the susceptibility of dogs to avian A(H6N1)virus, the intranasal inoculation experiment in beagles with A/Mallard/San-Jiang/275/2007 (H6N1) was conducted in China in 2013. Seroconversion and virus shedding in nasal and fecal route were noted. However, there were many differences in molecular characterization between A canine/Taiwan/2014 and A/Mallard/San-Jiang/275/2007 (Table 2-2). Therefore, the experiment inoculation findings may not be able to correlate with the natural infection in dogs with contemporary avian A(H6N1) viruses in Taiwan.

The H6N1 infecting dog developed severe pneumonia and only survived for 19 days after hospitalization.. During this period, persistent and massive influenza A viral shedding from nasal discharge was detected but neither bELISA nor HI test observed detectable antibody. It was presumed that CDV co-infection may cause immunosuppression (Carvalho *et al*., 2012) and inhibit antibodies production against influenza A viruses (Hong *et al.*, 2013). The epidemiological information about this isolate was unclear due to the lack of serological data from 11 closely contacted dogs.

Except the one which was successfully isolated, there were three partial M gene RT-PCR positive cases that were failed to isolate viruses from remaining nasal swab

suspended solutions. Based on the sequence data of partial M gene segments (199bp, the 45 nucleotides at the primer M52/M253R binding site were excluded), all these cases showed five nucleotide differences compared to A/canine/Taiwan/E01/2014 (Figure 2-6). Although these partial M gene segments (199 bp) were too short to indicate the correct influenza A subtype, they didn't seem to have correlation with A/canine/Taiwan/E01/2014 (H6N1). Moreover, there were still six bELISA serum positive samples which exhibited HI test negative results against avian H6 subtypes among total nine samples. Based on the above observations, the possibility of other influenza A subtypes infection in dogs may exist in Taiwan. Further surveillance on influenza A virus infection in dogs was therefore necessary.

Table 2-1 . Homology of nucleotide sequence of A/canine/Taiwan/E01/2014 (H6N1) isolated in Taiwan compared with related sequence in GISAID.

Gene segment	Virus with the highest identity	Source	Identity %	Accession no.
PB2	A/chicken/Taiwan/1843/2012 (H6N1)	Avian	98%	EPI510830
PB1	A/chicken/Taiwan/A2837/2013 (H6N1)	Avian	97%	EPI459872
PA	A/chicken/Taiwan/2593/2012 (H5N2)	Avian	99%	EPI510622
HA	A/chicken/Taiwan/1843/2012 (H6N1)	Avian	99%	EPI519832
NP	A/chicken/Taiwan/67/2013 (H6N1)	Avian	98%	EPI510875
NA	A/chicken/Taiwan/2084/2012 (H6N1)	Avian	99%	EPI510837
M	A/chicken/Taiwan/2593/2012 (H5N2)	Avian	99%	EPI510660
NS	A/chicken/Taiwan/67/2013 (H6N1)	Avian	97%	EPI510878

Table 2-2. Molecular characterization comparison between A/canine/Taiwan/E01/2014, A/Taiwan/2/2013 and A/Mallard/San-Jiang/275/2007

Amino acid substitution	A/canine/Taiwan/E01/2014	A/Taiwan/2/2013	A/Mallard/San-Jiang/275/2007	Function (26, 29)
PB2				
E627K	K	E	E	Replication ability in mammalian host
D701Q	D	D	D	Nuclear import
PB1-F2				
N66S	N	Truncated form	S	Induction of apoptosis
HA				
Cleavage site	Single (PQIATR*G)	Single (PQIATR*G)	Single (PQIETR*G)	HA cleavage
Q226L	Q	Q	Q	Increased virus binding ability of α2-6 sialic acid receptor.
G228S	S	S	G	
NA				
H275Y	H	H	H	Oseltamivir resistance
41-52 and 68-69	Deleted	Deleted	Complete	Unknown
M2				
S31N	N	N	S	Adamantane resistance
NS1				
D92E	D	D	D	Unknown
EPEV sequence (C-terminus)	EPEV	EPEV	ESEV	PDZ ligand domain

* HA cleavage site

Table 2-3. Sequence alignment of the different sites of the PB2 gene. Amino acid sites differ from the 2012/2013 H6N1 viruses.

	117	184	318	355	434	575	627	640	659	671	679	683	709
A/canine/Taiwan/E01/2014 (H6N1)	T	T	G	R	L	M	K	I	S	N	P	A	G
A/Taiwan/2/2013 (H6N1)	.	.	R	.	.	.	E	V	.	D	.	T	S
A/chicken/Taiwan/692/2012 (H5N2)	.	.	R	.	.	.	E	V	.	D	.	T	S
A/chicken/Taiwan/689/2012 (H5N2)	.	.	R	.	.	.	E	V	.	D	.	T	S
A/chicken/Taiwan/2267/2012 (H6N1)	.	.	R	.	.	.	E	V	.	D	.	T	S
A/chicken/Taiwan/2331/2012 (H6N1)	.	.	R	.	.	.	E	V	.	D	.	T	S
A/chicken/Taiwan/1843/2012 (H6N1)	.	.	R	.	.	.	E	V	.	D	Q	T	S
A/chicken/Taiwan/2084/2012 (H6N1)	.	A	R	.	J	I	E	V	N	D	.	T	S
A/chicken/Taiwan/A2837/2013(H6N1)	A	.	R	Q	.	.	E	V	.	D	.	T	S
A/chicken/Taiwan/67/2013(H6N1)	.	.	R	Q	.	I	E	V	.	D	.	T	S

Table 2-4. Amino acid sequence alignment of the different sites of the HA gene.

	32	37	41	45	48	86	96	98	100	106	108	130	135	142	143	157	160	186	192	199	200
A/canine/Taiwan/E01/2014	V	N	K	K	N	N	V	K	V	F	G	N	N	T	D	D	T	P	D	G	K
A/Taiwan/2/2013	.	.	.	.	.	.	A	N	L	.	.	S	.	I	.	.	.	L	.	D	.
A/chicken/Taiwan/2437/2012	.	.	.	.	.	.	.	N	L	.	.	S	.	I	.	.	.	.	.	D	.
A/chicken/Taiwan/A2837/2013	.	.	.	.	.	.	.	N	L	.	.	S	.	I	.	.	.	.	.	D	.
A/chicken/Taiwan/67/2013	.	.	.	.	.	.	A	N	L	.	.	S	.	I	.	.	.	.	.	D	.
A/chicken/Taiwan/1843/2012	.	.	.	.	.	.	.	.	.	.	E	.	.	I	.	.	.	.	.	D	.
A/chicken/Taiwan/2084/2012	.	.	.	N	K	.	.	.	.	.	.	.	.	I	.	.	.	.	N	D	.
A/chicken/Taiwan/0706/2003	I	.	.	.	.	T	.	N	.	.	.	S	K	I	.	E	A	.	.	D	R
A/chicken/Taiwan/0204/2005	I	.	.	.	.	.	.	N	.	L	.	S	.	I	.	E	A	.	.	D	R
A/chicken/Taiwan/TC147/2010	I	S	N	N	.	T	.	N	.	L	.	S	.	I	.	E	A	.	.	D	R
A/chicken/Taiwan/A342/2005	I	S	.	.	.	T	.	N	.	L	.	S	.	I	N	E	A	.	.	D	R

	226	228	238	244	263	273	280	287	289	302	306	314	324	328	329	377	388	480	481	495	503
A/canine/Taiwan/E01/2014	Q	S	K	H	T	D	D	A	A	L	K	E	N	I	A	D	S	M	E	K	G
A/Taiwan/2/2013	.	.	R	N	.	.	.	T	V	.	E	.	.	.	.	.	N	.	.	.	.
A/chicken/Taiwan/2437/2012	.	.	R	N	.	.	.	.	V	.	E	.	.	.	.	.	N	.	.	.	.
A/chicken/Taiwan/A2837/2013	.	.	R	N	.	.	.	.	V	.	E	.	.	.	.	.	N	.	.	.	.
A/chicken/Taiwan/67/2013	.	.	R	N	K	.	.	.	V	.	E	.	.	.	.	.	N	.	.	.	.
A/chicken/Taiwan/1843/2012	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	S	.	.	E	.
A/chicken/Taiwan/2084/2012	.	.	.	N	.	.	.	.	V	Q	E	.	.	.	.	.	N	.	M	.	.
A/chicken/Taiwan/0706/03	.	.	.	N	.	N	Y	.	V	.	E	.	D	.	E	.	N	I	.	D	.
A/chicken/Taiwan/0204/05	.	.	.	N	.	.	.	.	V	.	E	.	.	.	.	.	N	I	.	E	.
A/chicken/Taiwan/TC147/2010	.	.	.	N	.	N	.	.	V	.	E	K	.	V	E	N	T	I	.	D	R
A/chicken/Taiwan/A342/2005	.	.	.	N	.	N	.	.	V	.	E	.	.	.	E	.	N	I	.	D	.

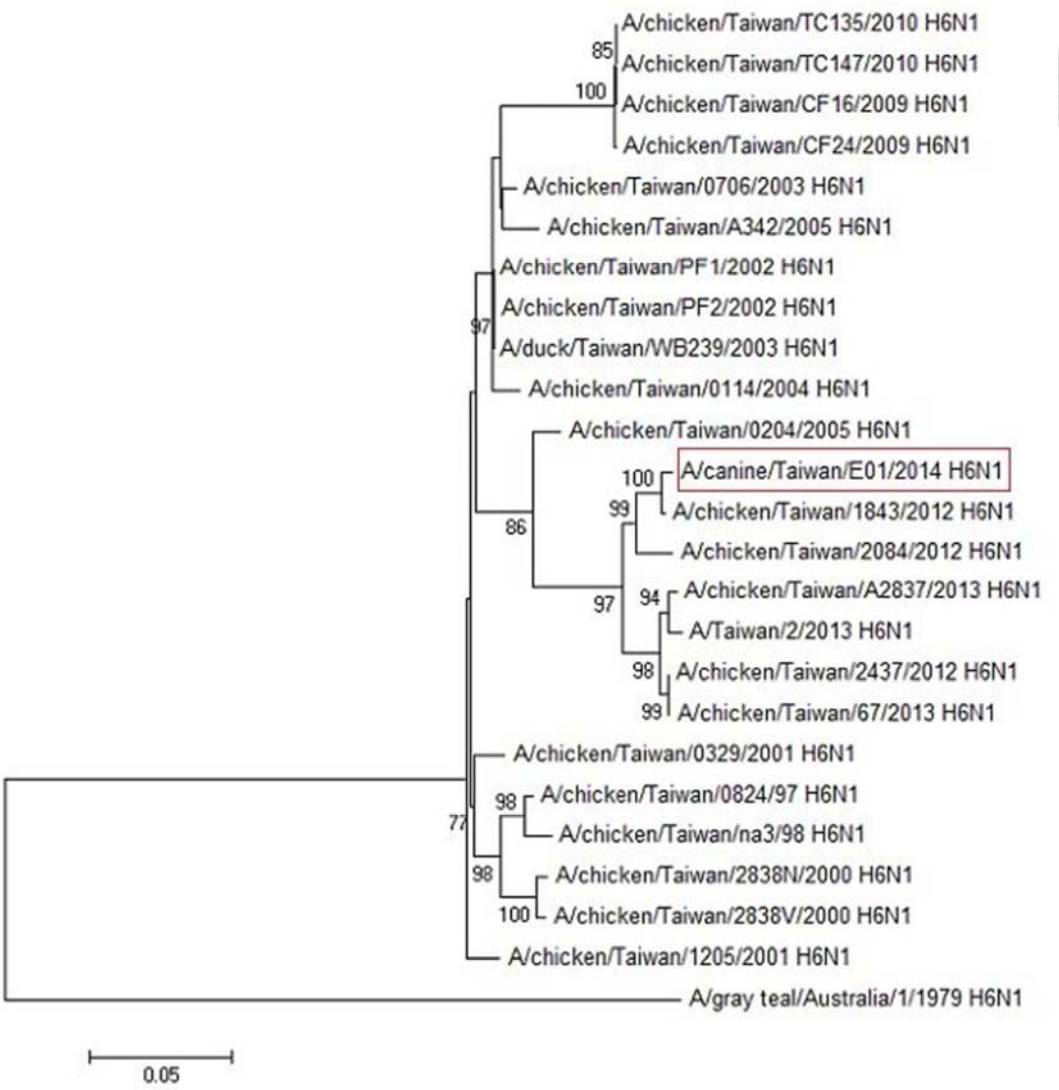


Figure 2-1. Phylogenetic relationship between the complete HA genes of A/canine/Taiwan/E01/2014 and other avian influenza virus lineages. HA gene of A/canine/Taiwan/E01/2014 was clustered with H6N1 strains isolated from 2012 to 2013 in Taiwan. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree. Scale bar indicates nucleotide substitutions per site.

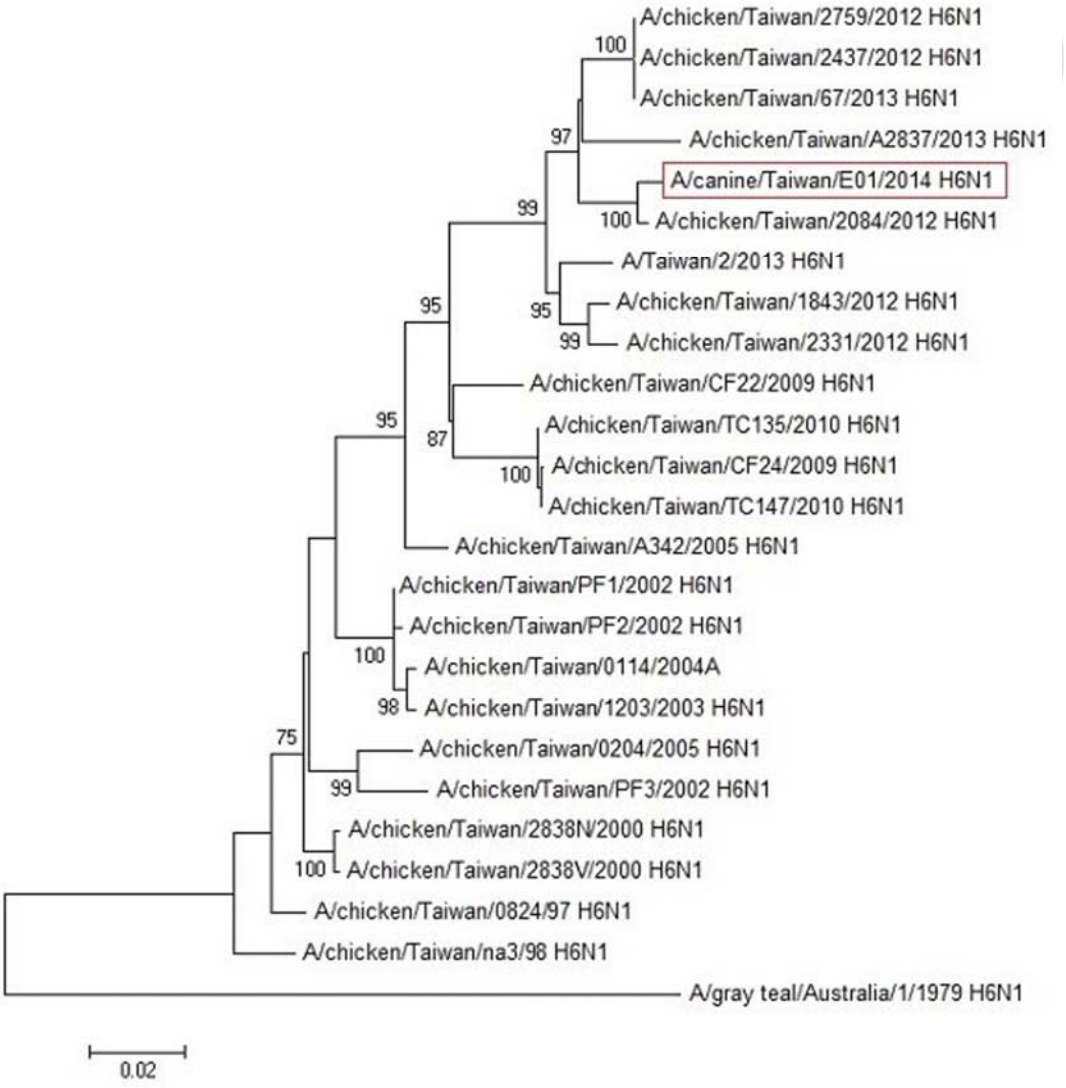


Figure 2-2. Phylogenetic relationship between the complete NA genes of A/canine/Taiwan/E01/2014 and other avian influenza virus lineages. NA gene of A/canine/Taiwan/E01/2014 was clustered with H6N1 strains isolated from 2012 to 2013 in Taiwan. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree. Scale bar indicates nucleotide substitutions per site.

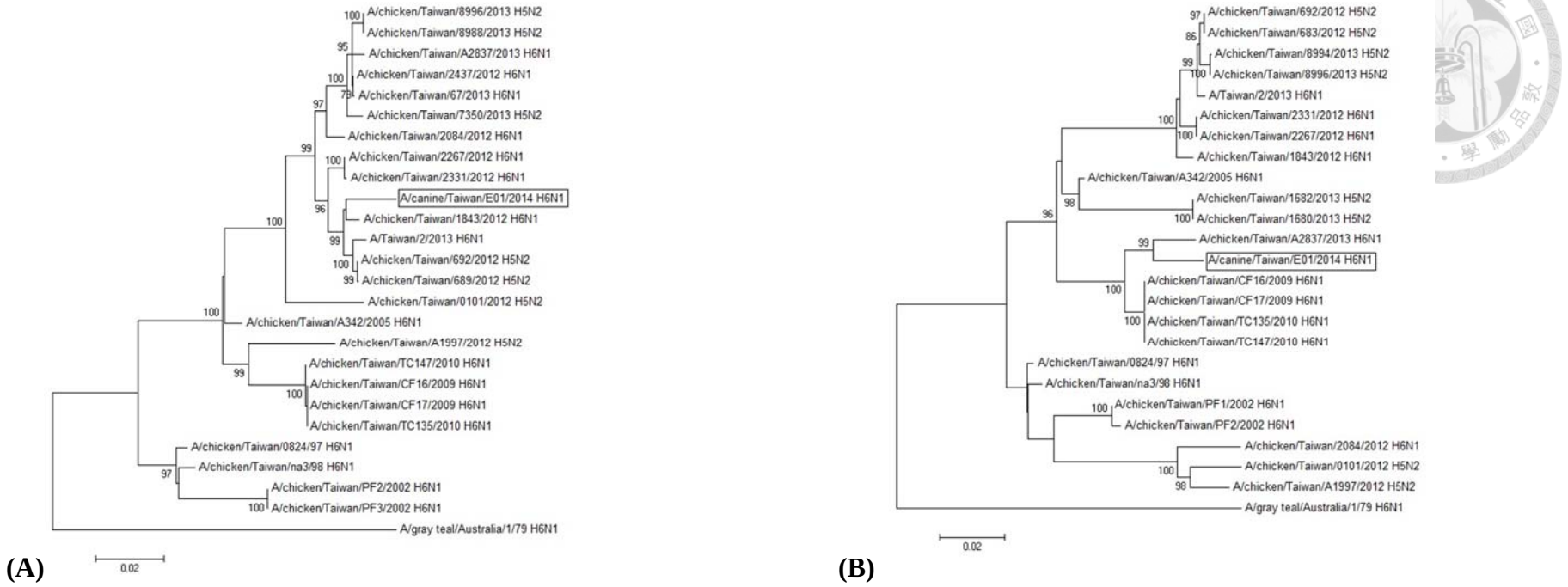
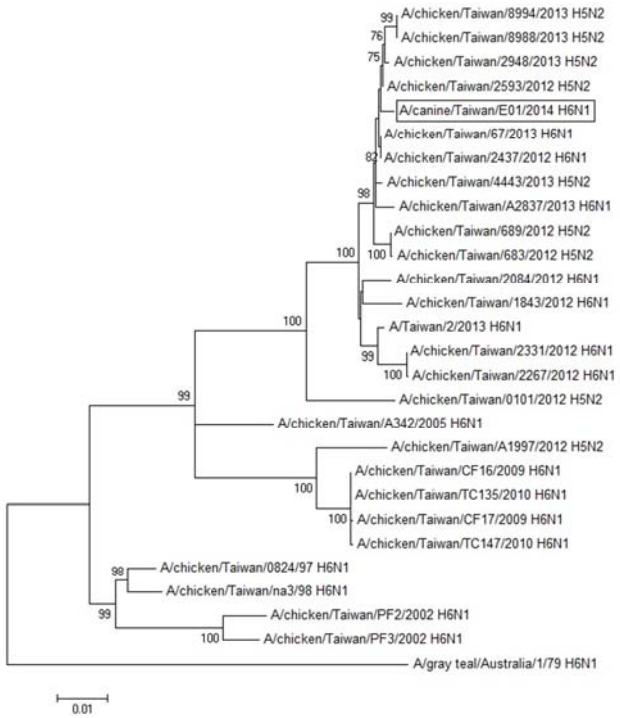
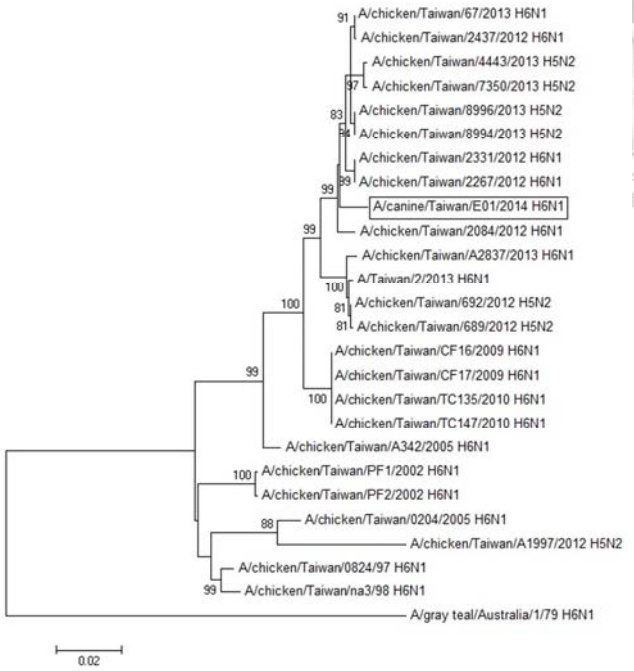


Figure 2-3. (A) Phylogenetic relationship between the complete PB2 genes of A/canine/Taiwan/E01/2014 and other avian influenza virus lineages. (B) Phylogenetic relationship between the complete PB1 genes of A/canine/Taiwan/E01/2014 and other avian influenza A virus lineages. PB2 and PB1 gene of A/canine/Taiwan/E01/2014 are clustered with H6N1 strains isolated from 2012 to 2013 in Taiwan. Some avian H5N2 viruses fall within the H6N1 lineage. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree. Scale bar indicates nucleotide substitutions per site.

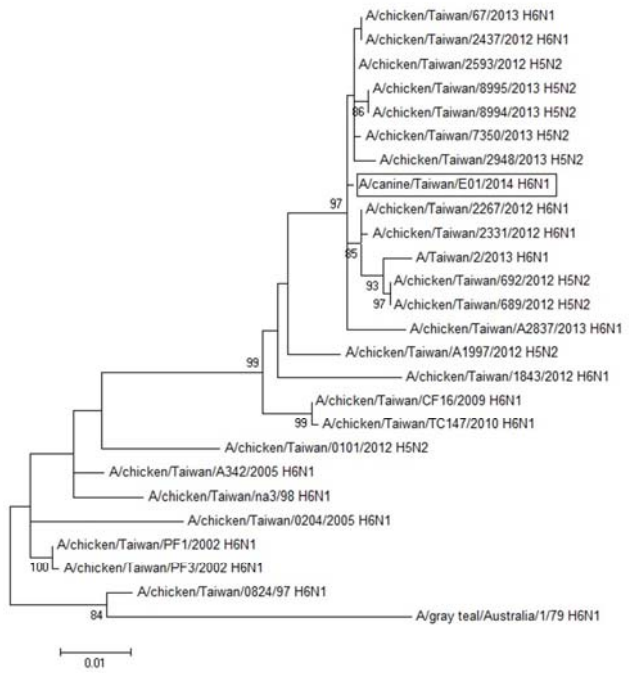


(C)



(D)

Figure 2-4. (C) Phylogenetic relationship between the complete PA genes of A/canine/Taiwan/E01/2014 and other avian influenza A virus lineages. (D) Phylogenetic relationship between the complete NP genes of A/canine/Taiwan/E01/2014 and other avian influenza A virus lineages. PA and NP gene of A/canine/Taiwan/E01/2014 are clustered with H6N1 strains isolated from 2012 to 2013 in Taiwan. Some avian H5N2 viruses fall within the H6N1 lineage. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree. Scale bar indicates nucleotide substitutions per site.



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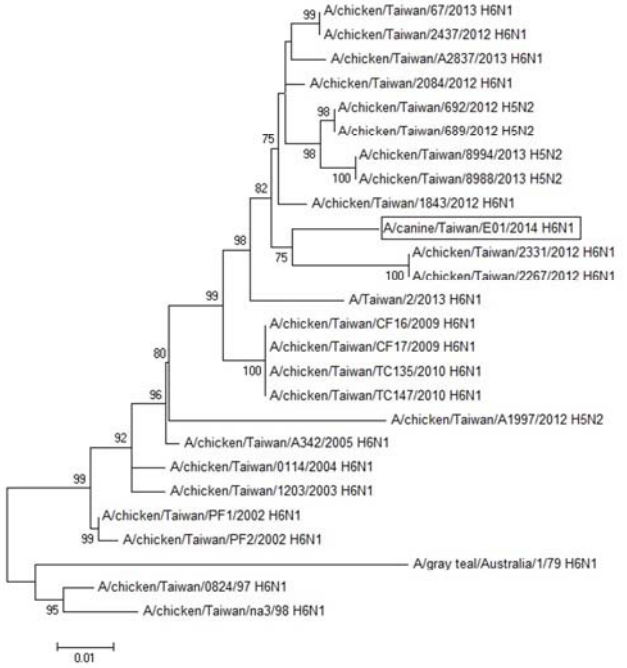


(F)

Figure 2-5. (E) Phylogenetic relationship between the complete M genes of A/canine/Taiwan/E01/2014 and other avian influenza A virus lineages. (F) Phylogenetic relationship between the complete NS genes of A/canine/Taiwan/E01/2014 and other avian influenza A virus lineages. M and NS gene of A/canine/Taiwan/E01/2014 are clustered with H6N1 strains isolated from 2012 to 2013 in Taiwan. Some avian H5N2 viruses fall within the H6N1 lineage. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree. Scale bar indicates nucleotide substitutions per site.

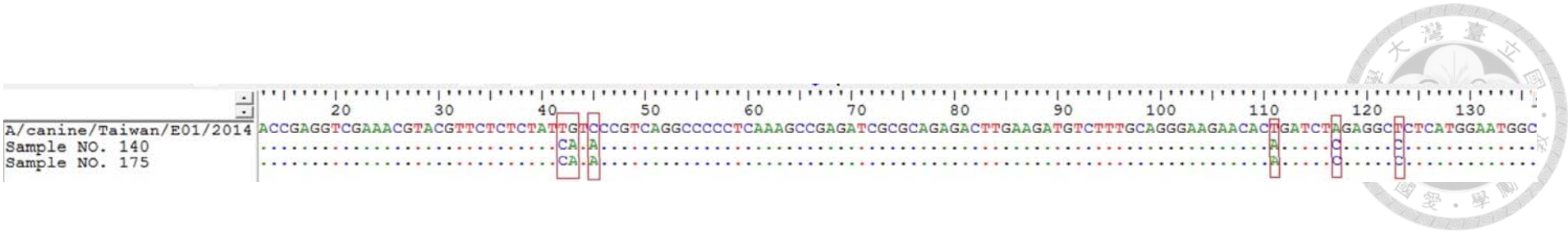


Figure 2-6. Sequence alignment of amplified DNA using primers M52C/M253R. Sample number 140 and 175 were two of three partial M gene sequences (199bp, the 45 nucleotides at the primer M52/M253R binding sites were excluded) that were failed to isolate viruses from remaining nasal swab suspended solutions. Alignment results showed five nucleotides differences, compared to A/canine/Taiwan/E01/2014.

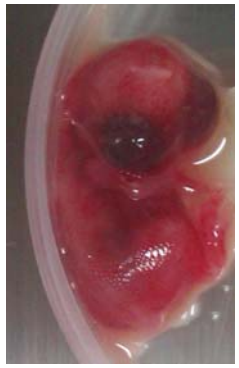
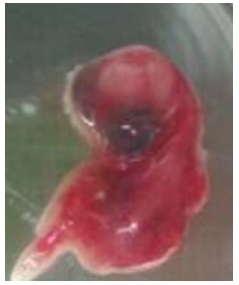
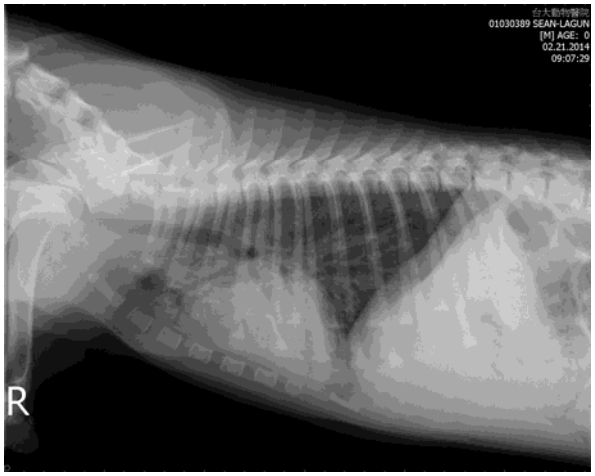
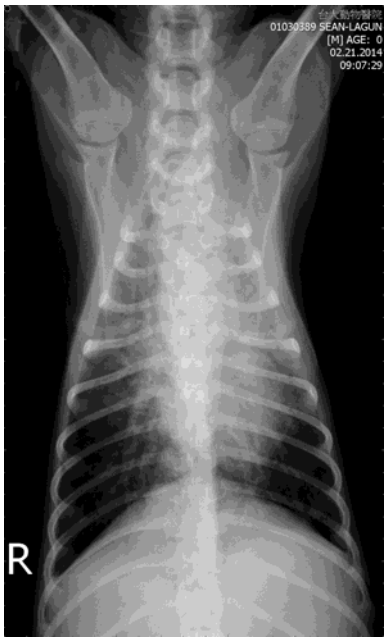
(A)  (B)  (C)  (D)  (E)  (F) 

Figure 2-7. (A) One of the A/canine/Taiwan/E01/2014 infected embryos in the first passage died with hemorrhage lesion in 48h. (B)-(F) Hemorrhage lesions were noted at five A/canine/Taiwan/E01/2014 infected embryos which died within 48h at the second passage.

Figure 2-8. The appearances of five embryos infected with A/canine/Taiwan/E01/2014 were normal and all alive after 48h at the third passage. The right last embryo with congestion appearance was resulted in artificial mechanical damage to this embryo.



(A)



(B)

Figure 2-9. Chest radiographs of the dog with confirmed A/canine/Taiwan/E01/2014 infection. (A) Right lateral view. (B) Dorsal ventral view. Chest radiographs showed severe bilateral infiltration and air-bronchogram in lung field.

3. Natural infection case of influenza A(H1N1)pdm09 virus in a pet ferret in Taiwan

3.1 Abstract

Ferrets have been demonstrated to have high susceptibility to influenza virus infection and possess the ability to transmit virus among individuals. In this study, a natural influenza A(H1N1)pdm09 virus (A(H1N1)pdm09) infection in a pet ferret (*Mustela putorius furo*) in Taiwan in 2013 was identified. The ferret was closely contacted with family members who had recently experienced influenza-like illness. Nasal swab was submitted to the laboratory for influenza A viruses molecular screening and virus isolation. Both of the nasal swab and virus isolation showed positive results using one-step RT-PCR targeting at matrix gene (M). Phylogenetic analysis indicated that all of the eight segmented genes were closely related to the lineage of A(H1N1)pdm09 viruses isolated in Taiwan. This study may provided a perspective view on the relationship of natural influenza A virus transmission between pet ferrets and the local human population.

3.2 Case introduction

Influenza-induced rhinitis in ferret was first reported in 1933. They were demonstrated to have high susceptibility to type A and B of human influenza viruses,

and obtain the ability to transmit virus mutually (Baker, 1998; Smith *et al.*, 1933). Nowadays, ferrets have been considered an ideal mammalian model for influenza A virus studies (Maher and DeStefano, 2004). According to the previous inoculation experiments, moderate focal bronchopneumonia in a ferret inoculated with 10^2 $TCID_{50}$ dose of A/California/4/2009 was observed at 7 days post inoculation (Smith *et al*., 2011). Necrotizing rhinitis, trachetitis, bronchitis and bronchiolitis were also revealed in ferrets in inoculated with 10^6 $TCID_{50}$ dose of A/Netherlands/602/2009. A(H1N1)pdm09 virus-inoculated animals exhibited clinical symptoms of lethargy, sneezing, nasal discharge, anorexia and weight loss (Munster *at al*., 2009). In this article, we identified a natural influenza A virus infection case in a pet ferret that had been in close contact with persons who had recently experienced influenza-like illness (ILI). It was an eight-year-old neutered female ferret attended to National Taiwan University Veterinary Hospital (NTUVH) in July 2013 showing panting, coughing, open-mouth breathing, inappetence, poor activity and low body temperature (36.6 ^oC). Past history showed there was adenocarcinoma in left adrenal gland and unilateral adrenalectomy was performed at four years old. Multiple lymph nodes swellings have been noted since November 2012. Lymphoma was suspected due to the monoclonal gammopathy of serum protein electrophoresis (SPEP) test. However, further biopsy checking was declined by the owner.

Two family members of the household who had close contact with this ferret appeared ILI symptoms within one week before the ferret's visit at NYUVH. The ILI signs of the owners included fever, coughing and nasal discharge. The ferret became sick three days later after the onset of owner's clinical sings. Chest radiographs showed a moderate to severe diffusive increase in bronchial markings, and mesenchymal tissue opacity in both left and right lungs (Figure 3-1). Bacterial culture of pleural effusion was negative. The ferret was treated with subcutaneous fluid, antibiotics, and airway nebulization. After two days of therapy, nasal discharge was eliminated, but the ferret still exhibited lethargy and dyspnea (oxygen saturation < 80%). Euthanasia was executed on the third day of hospitalization. Necropsy was not performed because of owner's disagreement. PCR detection of the ferret nasal swab sample showed negative results for canine distemper virus and feline coronavirus but positive result for influenza A virus. The nasal swab sample was then submitted to virus isolation.

Complete molecular analysis for this ferret isolate was performed. Phylogenetic tree construction and comparison with other viruses, including A(H1N1)pdm09 viruses which were isolated in Taiwan, were made. This study results would highlight the zoonotic role of influenza A virus played in both pet ferrets and human living in the same household.

3.3 Materials and methods

The ferret's nasal swab sample was dissolved in 1mL of viral transported medium (Creative, Taipei, Taiwan). Influenza A virus M gene was detected using primer set M52C/M253R (Fouchier *et al.*, 2000) with one step RT-PCR kit (QIAGEN, Hiloden, Germany). One aliquot of nasal swab sample solution was inoculated into the allantoic sac of 10-day-old specific-pathogen-free chicken eggs (Animal health research institute, council of agriculture, executive yuan, Taipei, Taiwan) which were incubated for 48 hours at 35 ^{o}C. Allantoic fluid was harvested to conduct influenza A virus genotyping. The entire eight segmented genes of influenza A virus were amplified using the universal primer set described by Hoffmann *et al* (Hoffmann *et al.*, 2001). The amplified products were purified from agarose gels (Amresco, solon, OH) using QIAquick® Gel Extraction Kits (QIAGEN), cloned into the pGEM-T Easy vector (Promega Corp, Madison, WI), and then transferred into Arrow 58sec DH5αcompetent ell (Arrowtec, Taipei, Taiwan). Plasmids were sent for automated sequencing (Tri-l Biotech, Inc., Taipei, Taiwan).

The sequence identity of eight segmented genes was conducted with nucleotide Basic Local Alignment Search Tool (BLAST) in GenBank of National Center for Biotechnology Information (NCBI). Phylogenetic analysis was conducted using virus

genes which were representative of relevant major gene lineages from different hosts. Complete reference virus sequences were downloaded from GenBank. Multiple sequence alignments, and phylogenetic analyses were performed using Mega 6 (Tamura et al., 2031) soft ware. Sequences were aligned with the Clustal W method. Phylogenic trees were conducted using maximum likelihood with bootstrap analysis with 1,000 replications.

3.4 Results

3.4.1 Phylogenetic analysis

Both of the original nasal swab sample and virus isolate were positive in M gene detection using one-step RT-PCR. This virus was designated as A/ferret/Taiwan/E01/2013. The eight segmented gene sequences of A/ferret/Taiwan/E01/2013 were submitted to GenBank under the accession numbers of KJ702009-KJ702016. All gene segments of A/ferret/Taiwan/E01/2013 had the highest nucleotide sequence similarity to H1N1 viruses. Eight segmented genes fell into the same cluster as A(H1N1)pdm09 viruses which were initially identified in April 2009 in the United States. A/ferret/Taiwan/E01/2013 was most closely related to the lineage of A(H1N1)pdm09 viruses which were isolated in Taiwan (Figures 3-2 to 3-9). Its hemagglutinin (HA) and M genes were most closely to the A/Taiwan/80205/2013 strain.

3.4.2 Molecular characterizations of A/ferret/Taiwan/E01/2013

Moreover, a unique mutation of HA2 (E374K) was found in A/ferret/Taiwan/E01/2013. The amino acid H275Y substitution in neuraminidase (NA), the marker of oseltamivir resistant, was not found in A/ferret/Taiwan/2013. Two other mutations (C154T and G238A) of M gene in A/ferret/Taiwan/E01/201were also observed.

3.5 Discussion

In the previous studies, ferrets were demonstrated to have high susceptibility to influenza virus infection and have the ability to transmit mutually (Baker, 1998; Smith *et al.*, 1933). The animal has become a suitable model for human influenza virus studies nowadays (Belser *et al.*, 2011). Ferrets infected with A(H1N1)pdm09 virus were also reported in other countries (Patterson *et al.*, 2009; Swenson *et al.*, 2010). As regards the epidemic situation of A(H1N1)pdm09 viruses in Taiwan, there was a prevalence peak in November 2009 but intensively declined in January 2010 after a mass vaccination program. During the 2009-2010 influenza season, the mortality was 1.9 deaths per million in Taiwan. However, A(H1N1)pdm09 still recurred during Taiwan's 2010-11 influenza season after WHO announced that the world shifted into the post-pandemic period on August 10, 2010. The mortality rate in 2010-2011 influenza season was 5.5

deaths per million population (Chuang *et al.*, 2012; Ho *et al.*, 2010; Lan *et al.*, 2013). Based on the previous studies, A(H1N1)pdm09 viruses have begun to evolve and diversify into at least 7 clades (clade 1 to 7) with various spatial and geographic patterns (Nelson *et al.*, 2009). Clade 7 virus strain was the predominantly imported strain in August 2009 in Taiwan. The viruses which reemerged in 2012-2013 were classified into three novel clades 11.2, 12.1 and 12.2 according to HA phylogenetic investigations (Nelson *et al.*, 2009; Yang *et al.*, 2011; Yang *et al.*, 2014). In this study, the phylogenetic analysis of HA gene of A/ferret/Taiwan/E01/2013 revealed that it belonged to clade 11.1, which was branched from the previous predominant clade 11 that circulated in 2010 to 2011 in Taiwan. A/ferret/Taiwan/E01/2013 held the HA amino acid characteristics of clade 11 (D114N, S202T, S468N) and the additional substitutions features of clade 11.1(V251I, K300E) (Yang *et al.*, 2014). It indicated that A/ferret/Taiwan/E01/2014 possessed the features specific to A(H1N1)pdm09 viruses circulated in Taiwan.

The major human influenza virus strains circulating in Taiwan from 2009 to 2010 were found to have a unique mutation in HA2, E374K, numbering without signal peptide (17 amino acids) (Kao *et al.*, 2012). The same feature was also noted in A/ferret/Taiwan/E01/2014. However, E374K mutation had no significant differences in cross-microneutralization test and hemagglutination inhibition serologic reaction (Kao

et al., 2012).

Mutation (H275Y N1 numbering) in A(H1N1)pdm09 was discovered in June 2009 and found to become oseltamivir-resistant (Baz *et al.*, 2009; WHO, 2010). In Taiwan, the first oseltamivir-resistant A(H1N1)pdm09 virus strain was isolated from a 20-year-old man in October 2009 after four-days of treatment with standard dose of oseltamivir (Sy *et al.*, 2010). The oseltamivir-resistant viruses were detected during May 2009 to April 2011 and the results showed 1.1 % (15/1335) patients of A(H1N1)pdm09-positive cases had H275Y-mutation in Taiwan (Yang et al., 2013). A/ferret/Taiwan/E01/2013 was not found to have histidine to tyrosine substitution at amino acid 275, indicating that it was still sensitive to oseltamivir.

A/ferret/Taiwan/E01/2013 was also found to have C154T and G238A mutations on M gene which would result in mismatches at primer binding sites for real-time RT-PCR assay developed by Ward et al (Ward *et al.*, 2004). These two mutations were also noted in other A(H1N1)pdm09 viruses isolated in 2012-2013 in Taiwan (Yang *et al.*, 2014). These two novel mutations were found to decrease the sensitivity of commonly employed real-time RT-PCR assays since the mismatches located at primer binding sites. Therefore, it was recommended that simultaneous use of real-time RT-PCR assay targeting different genes will improve the accuracy of virus identification (Ward *et al.*,

2004; Yang *et al.*, 2014).

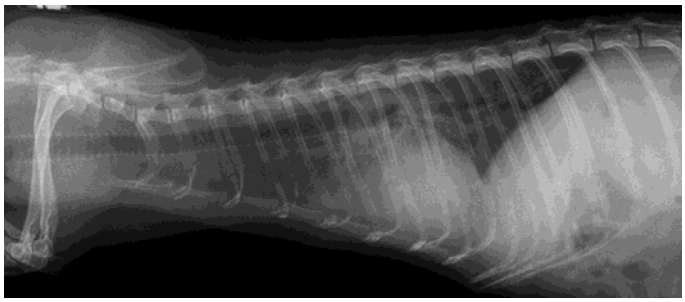
Because of severe lethargy and dyspnea, euthanasia was finally executed on the third day of hospitalization. Due to lack of necropsy, it was impossible to confirm the neoplasia diagnosis and explore the relationship between A(H1N1)pdm09 infection and neoplasia. According to the previous studies, most cases of A(H1N1)pdm09 infection were mild, but patients with underlying disease, immunosuppression, pregnancy, cancer or chronic diseases may have greater incidence to acquire influenza infections or aggravate the severity of infection due to the disruption of immune system. Serious clinical presentations, such as acute respiratory distress syndrome and death may develop (Gill *et al*., 2010; Hajjar *et al.*, 2010; Thirumala *et al.*, 2010). The suspected neoplasia and old age could be negative impacts on immunity of this ferret, and aggravated the disease severity.

It could not be directly proven that the family members were the influenza A virus sources for the ferret. However, based on the RT-PCR, virus isolation and phylogenetic analysis results, this ferret did have A(H1N1)pdm09 virus infection. Moreover, the molecular analysis results revealed that A/ferret/Taiwan/E01/2013 possessd a number of unique features of localized A(H1N1)pdm09 viruses in Taiwan.

Transmission of 2009 pandemic H1N1 virus from human to ferret had been

reported before (Patterson *et al.*, 2009; Swenson *et al.*, 2010). However, A/ferret/Taiwan/E01/2014 (H1N1) was the first isolate identified from a pet ferret bearing the specific characteristics of human influenza viruses circulated in Taiwan. It indicated that this isolate may co-circulate with other human influenza viruses in Taiwan and increased the zoonotic risk. Nevertheless, influenza A virus infection in pet ferrets was not routinely diagnosed by veterinary practitioners in Taiwan. Therefore, further surveillance on influenza A virus infection in pet ferrets is necessary.

(A)



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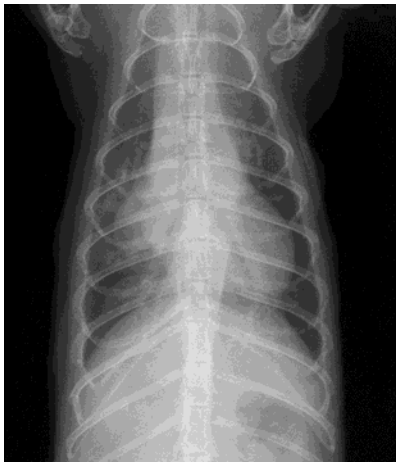


Figure 3-1. Chest radiographs of the ferret with confirmed A/ferret/Taiwan/E01/2013 infection. (A) Right lateral view. (B) Dorsal ventral view. The radiographs show visible alveolar pattern with a moderate to severe diffusive increase in bronchial markings. Increased mesenchymal tissue opacity is observed in both left and right lung lobes at dorsal ventral view.

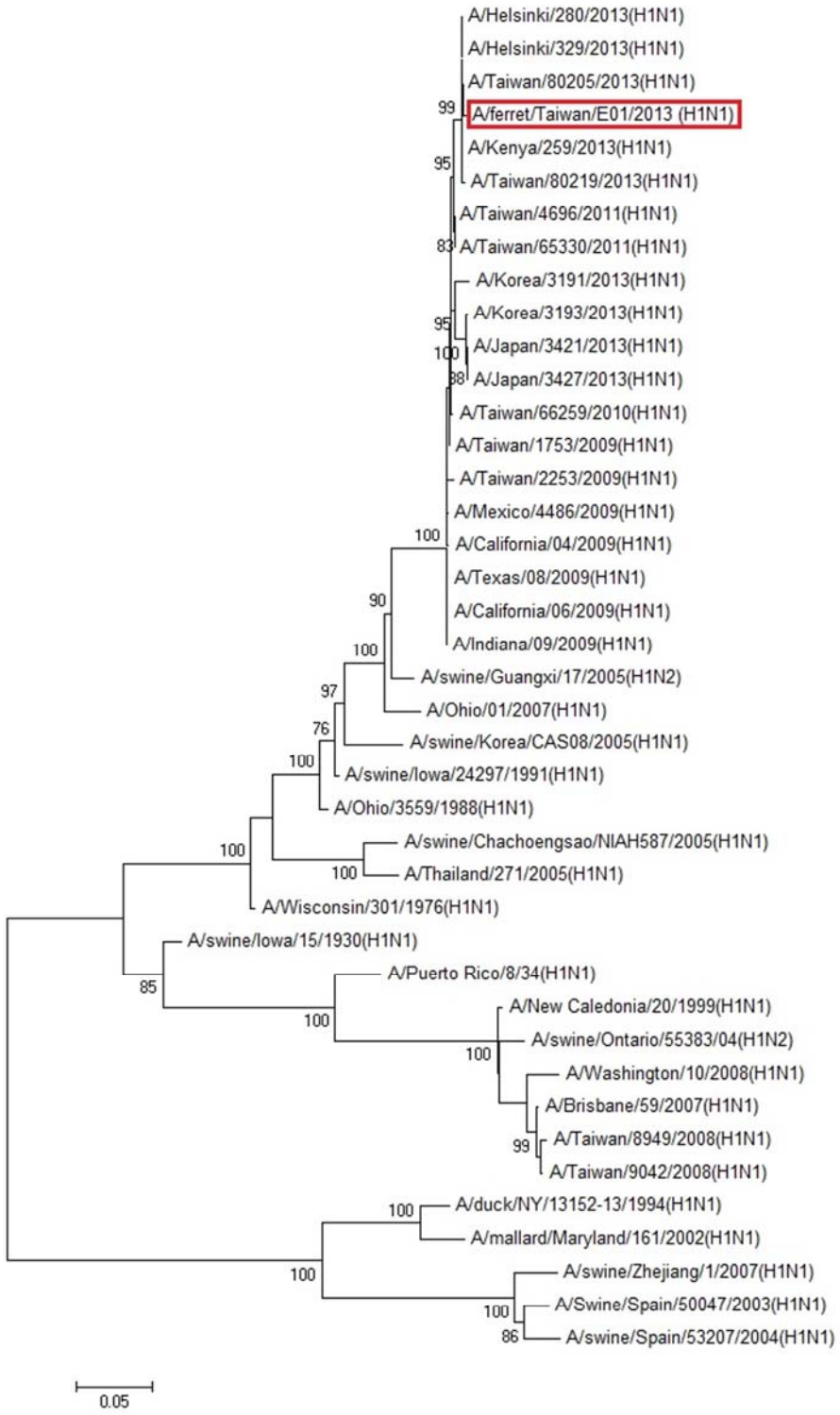


Figure 3-2. Phylogenetic tree of complete HA gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. A/ferret/Taiwan/E01/2013 is grouped with A(H1N1)pdm09 viruses. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree.

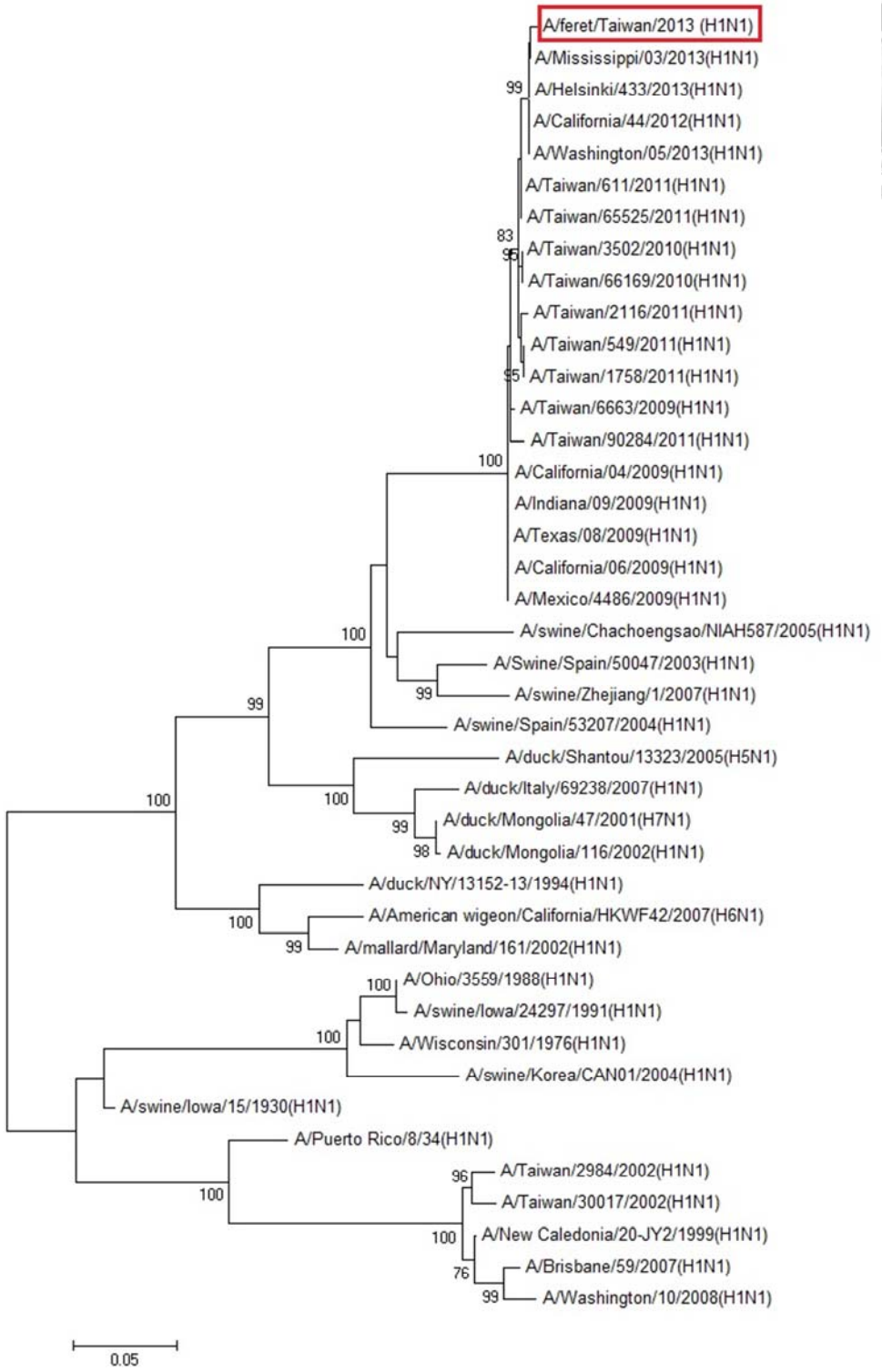


Figure 3-3. Phylogenetic tree of complete NA gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. A/ferret/Taiwan/E01/2013 is grouped with A(H1N1)pdm09 viruses. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree.

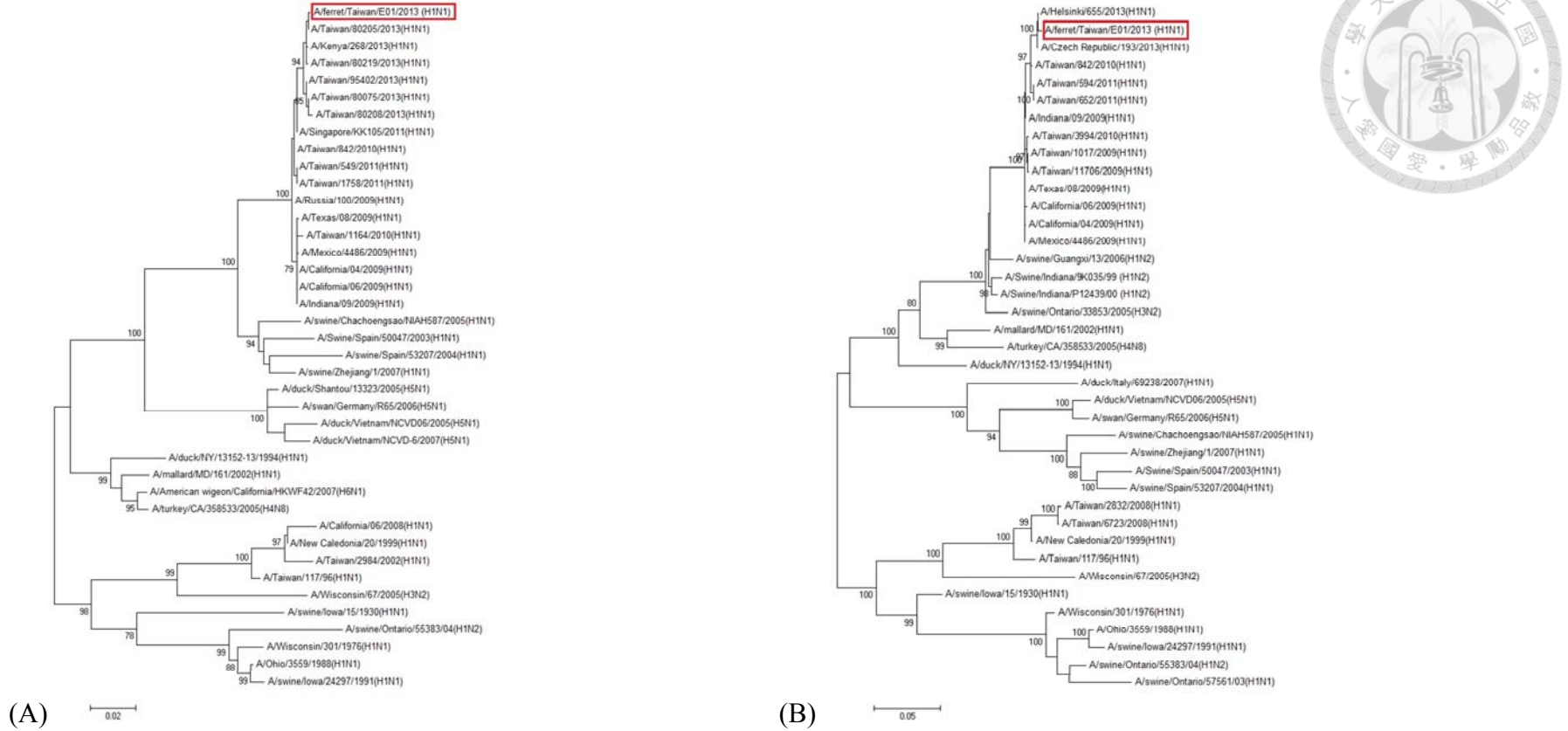


Figure 3-4. (A) Phylogenetic tree of complete M gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. (B) Phylogenetic tree of complete PB2 gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. A/ferret/Taiwan/E01/2013 is grouped with A(H1N1)pdm09 viruses. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree.

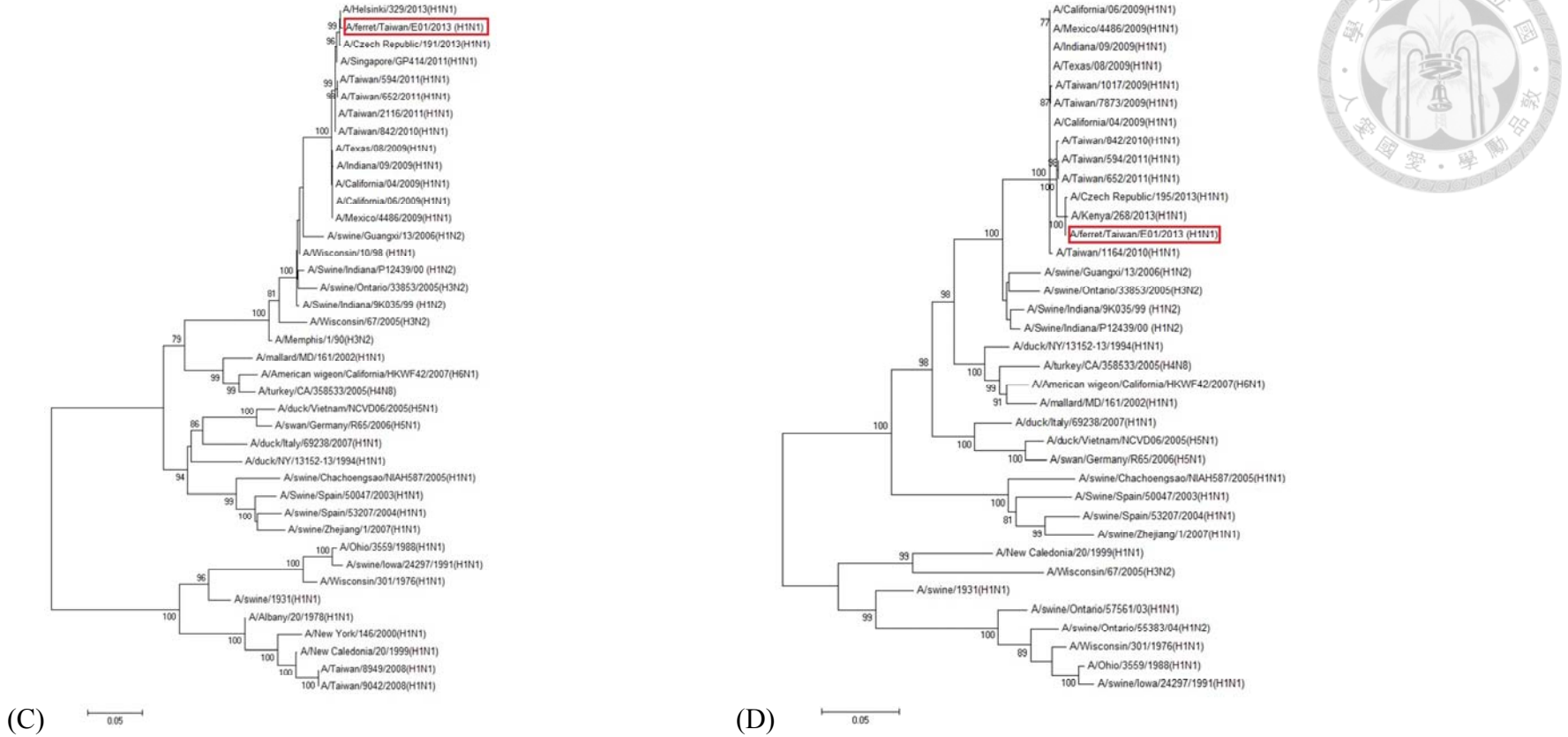
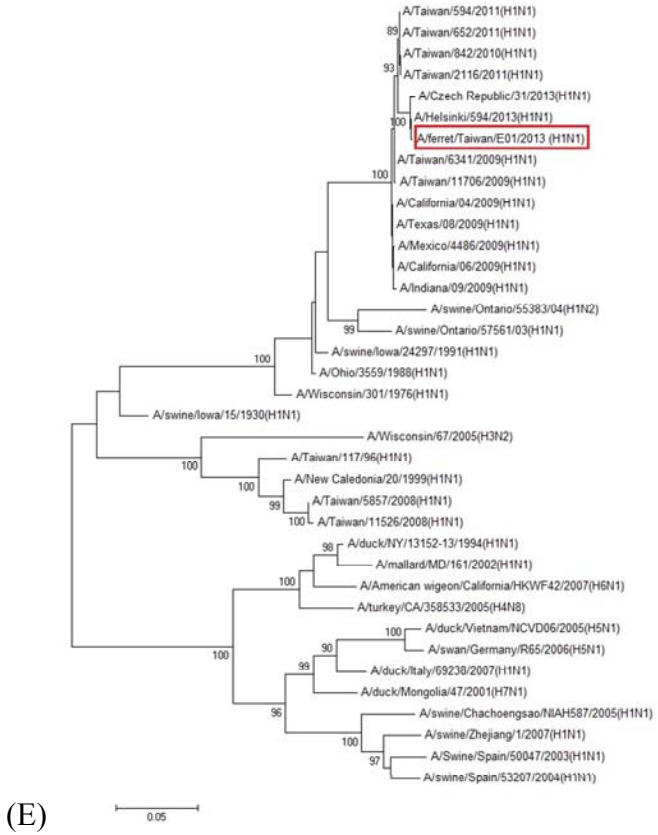
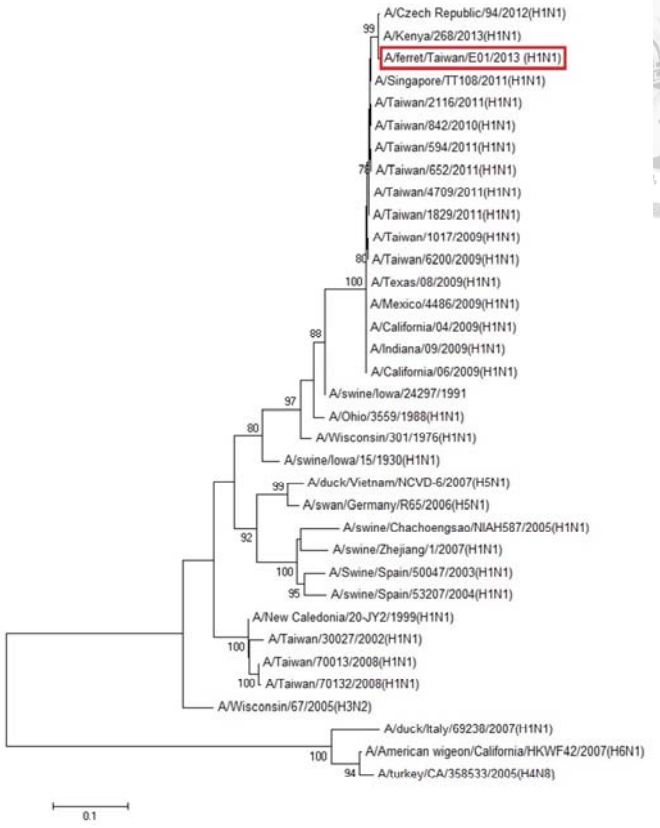


Figure 3-5. (C) Phylogenetic tree of complete PB1 gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. (D) Phylogenetic tree of complete PA gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. A/ferret/Taiwan/E01/2013 is grouped with A(H1N1)pdm09 viruses. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree.



(E)



(F)

Figure 3-6. (E) Phylogenetic tree of complete NP gene of A/ferret/Taiwan/E01/2013 with other influenza A virus lineages. (F) Phylogenetic tree of complete NS gene of A/ferret/Taiwan/E01/2013 (H1N1) with other influenza A virus lineages. A/ferret/Taiwan/E01/2013 is grouped with A(H1N1)pdm09 viruses. It was conducted using maximum likelihood with bootstrap analysis with 1,000 replications. Only branches with bootstrap values of more than 75% are denoted on the phylogenetic tree.

4. Conclusion

In this study, the positive rate of serum influenza A antibody was 1.1% (3/281) in household dogs and 3.1 % (6/193) in rural free-ranging dogs using blocking ELISA assay. HI assay against avian H6 subtype also showed H6 antibody presence in three of nine bELISA positive serum specimens. The serological survey indicated that influenza A virus infection indeed existed in dogs in Taiwan, especially the H6 subtype . Phylogenetic analysis for the canine isolate demonstrated that the pathogen was avian influenza H6N1 virus. Besides, a unique E627K substitution of PB2 protein was found in A/canine/Taiwan/E01/2014, indicating an increase in potential risk of dog to dog transmission . This is the first report on dog natural infection with avian influenza H6N1 virus in the world. Additionally, a natural infection case of influenza A(H1N1)pdm09 with the unique features of localized influenza virus in Taiwan was also demonstrated in a pet ferret. The study results could provide the preliminary information on influenza prevalence in companion animals in Taiwan.

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