การสำรวจทางซีรัมวิทยาของการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ (H3) ในสุนัขในกรุงเทพมหานคร
โดยวิธี NP-ELISA ร่วมกับวิธี HI TEST

นางสาวสุณิชา ชานวาทิก

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

SEROLOGICAL SURVEY OF INFLUENZA A VIRUS (H3) INFECTION IN DOGS IN BANGKOK BY USING NP-ELISA TOGETHER WITH HI TEST

Miss Sunicha Chanvatik

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Public Health

Department of Veterinary Public Health

Faculty of Veterinary Science

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สุณิชา ชานวาทิก : การสำรวจทางซีรัมวิทยาของการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ (H3) ในสุนัขในกรุงเทพมหานครโดยวิธี NP-ELISA ร่วมกับวิธี HI TEST (SEROLOGICAL SURVEY OF INFLUENZA A VIRUS (H3) INFECTION IN DOGS IN BANGKOK BY USING NP-ELISA TOGETHER WITH HI TEST) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. อลงกร อมรศิลป์ , 71 หน้า.

การระบาดของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ H3N2 ในสุนัข มีรายงานในประเทศจีนและเกาหลีตั้งแต่ปี ค.ศ. 2009 ในประเทศไทยมีรายงานการติดเชื้อไวรัสนี้เป็นครั้งแรกในปี ค.ศ. 2012 การศึกษาครั้งนี้ได้ทำการสำรวจทางซีรัมวิทยาของการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในสุนัข ในพื้นที่กรุงเทพฯและปริมณฑล จากตัวอย่างซีรัมสุนัขจำนวน 9,891 ตัวอย่าง ระหว่างเดือนธันวาคม 2012 ถึงพฤศจิกายน 2013 วิธีตรวจการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ ประกอบด้วยวิธี NP-ELISA และวิธี HI test กับไวรัสไข้หวัดใหญ่ชนิด canine-H3N2, human-H3N2 และ human-pH1N1 ผลการศึกษาด้วยวิธี NP-ELISA จาก 9,891 ตัวอย่าง พบว่า 164 ตัวอย่าง คิดเป็น 1.66% มีภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ และเมื่อทดสอบด้วยวิธี HI test พบว่า 36.56% ของตัวอย่างที่บวก และต้องสงสัยจากวิธี NP-ELISA มีภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่ชนิดเอจำเพาะสายพันธุ์คือ 12.20% มีภูมิคุ้มกันจำเพาะต่อเชื้อไวรัส canine-H3N2 และ 1.22% มีภูมิคุ้มกันจำเพาะต่อเชื้อไวรัส human-H3N2 และ 23.17% มีภูมิคุ้มกันจำเพาะต่อเชื้อไวรัส human-pH1N1 การศึกษาครั้งนี้ยังพบการติดเชื้อร่วมระหว่างเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ 2 ชนิด คิดเป็น 2.44% ของตัวอย่างที่ตรวจโดยวิธี HI คือ 1.22% เป็นตัวอย่างที่ติดเชื้อร่วมกันระหว่างเชื้อไวรัสไข้หวัดใหญ่ชนิด canine-H3N2 และ human-pH1N1 และอีก 1.22% เป็นตัวอย่างที่มีการติดเชื้อร่วมกันระหว่างเชื้อไวรัสไข้หวัดใหญ่ชนิด human-H3N2 และ human-pH1N1 โดยสรุปการศึกษาครั้งนี้บ่งชี้ว่า ประชากรสุนัขในพื้นที่กรุงเทพฯและปริมณฑล มีภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ และเคยติดเชื้อไวรัส canine-H3N2, human-H3N2 และ human-pH1N1 มาก่อน ทำให้มีความเสี่ยงของการแลกเปลี่ยนทางพันธุกรรมระหว่างเชื้อไวรัสไข้หวัดใหญ่ชนิดเอที่เกิดขึ้นภายในสัตว์ตัวเดียวกัน ดังนั้นจึงควรจะมีการเฝ้าระวังการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอระหว่างสุนัขและคนอย่างใกล้ชิด เพื่อลดโอกาสการติดเชื้อไข้หวัดใหญ่ชนิดเอ

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ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต ....
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SUNICHA CHANVATIK: SEROLOGICAL SURVEY OF INFLUENZA A VIRUS (H3) INFECTION IN DOGS IN BANGKOK BY USING NP-ELISA TOGETHER WITH HI TEST. ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, 71 pp.

Canine influenza virus subtype H3N2 (canine-H3N2) outbreaks have been reported in dogs in China and Korea since 2009. In Thailand, canine-H3N2 infection in pet dogs was first reported in 2012. In this study, a serological survey of influenza A virus (IAV) infection in dogs in Bangkok and vicinities was conducted during December 2012 to November 2013. A total of 9,891 sera samples was examined for IAV antibodies by using an anti-nucleoprotein antibodies ELISA assay (NP-ELISA) and haemagglutination inhibition (HI) testing with canine-H3N2, human-H3N2 and human-pH1N1 virus antigens. Our result demonstrated that 164 (1.66%) of 9,891 sera samples posed antibody against IAV by NP-ELISA test. For HI test, 36.56% of the positive and suspected NP-ELISA samples posed HI titer to IAV subtypes canine-H3N2 (12.20%), human-H3N2 (1.22%) and human-pH1N1 (23.17%). Interestingly, the evidence of co-infection of IAVs (2.44%) were detected in pet dogs composing co-infections between canine-H3N2 and human-pH1N1 viruses (1.22%) as well as human-H3N2 and human-pH1N1 (1.22%), suggesting the risk for reassortment among IAVs. In summary, IAV infection could be observed in Thai dog populations and evidence of exposures to canine-H3N2, human-H3N2 and human-pH1N1 was demonstrated. Therefore, the transmission of IAVs between dogs and humans should be closely monitored and minimized.

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List of Abbreviations

CIs	Confidence Intervals
CIV	Canine influenza virus
CRBCs	Chicken Red Blood Cells
°C	degree Celsius
EIV	Equine Influenza Virus
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	et alibi, and other
GMTs	Geometric Mean Titers
HA	Haemagglutinin
HAU	Haemagglutination units
HI	Haemagglutination Inhibition Test
H	hour(s)
IAV	Influenza A Virus
MDCK	Madin-Darby canine kidney
MEM	Minimum Essential Medium
NA	Neuraminidase
NP	Nucleoprotein

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NP-ELISA	Nucleoprotein-based Enzyme-Linked Immunosorbent Assay
OIE	World Organisation for Animal Health
PBS	Phosphate Buffered Saline
pH1N1	pandemic H1N1
RBC	Red Blood Cell
RDE	Receptor Destroying Enzyme
TPCK	Tosylsulfonyl Phenylalanyl Chloromethyl Ketone
TRBCs	Turkey Red Blood Cells

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Chapter 1

Introduction

Influenza is an infectious respiratory disease affecting humans and several animal species worldwide. Influenza virus causes mild to severe illness and even death in humans. The clinical symptoms include high fever, headache, coughing, rhinitis and pneumonia. Influenza virus can spread easily among human and animal populations via aerosol and direct contact. In the 20th century, there were three influenza pandemics including the Spanish flu of 1918-1919, the Asian flu of 1957-1958 and the Hong Kong flu of 1968-1969. The influenza virus subtypes H1N1, H2N2 and H3N2 were responsible for these pandemic outbreaks, respectively (Widelock et al., 1958; Cockburn et al., 1969; Taubenberger et al., 1997). To date, H1 and H3 subtypes have become the dominant strains of annual flu and cause seasonal human influenza worldwide (WHO, 2013). The emergence of the outbreak of novel influenza A pandemic H1N1 in 2009 has increasingly recognized as a worldwide public health concern and this outbreak had become the first pandemic of the 21st century in human populations (CDC, 2010). As a result, it can also affect in widespread illness and death, social disruption and economic loss. Because the influenza virus can change themselves easily through the antigenic drift and antigenic

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shift and the outcome are unpredictable, it is a great concern of a new virus emerging that can threat to human life.

Influenza A virus (IAV) is classified into subtypes based on 2 surfaced proteins, Haemagglutinin (HA) and Neuraminidase (NA). Up to date, 18 HA and 11 NA subtypes have been reported. IAV subtype 1-16 have been found in shorebirds and waterfowls that are considered to be a primary reservoir of influenza A virus, while IAV subtypes 17-18 are found in bats (Tong et al., 2013). Several subtypes of IAVs can infect several avian species. However, only selected IAV subtypes can infect both human and animal species (Webby et al., 2004).

Canine IAV caused influenza outbreaks in dog populations in many countries worldwide. Canine influenza virus subtype H3N8 (canine-H3N8) was first isolated from racing greyhound dogs in the United States in January 2004 (Crawford et al., 2005). Since then, canine-H3N8 infection has been reported in several states in the US (Crawford et al., 2005; Dubovi and Njaa, 2008; Payungporn et al., 2008; Beeler, 2009; Barrell et al., 2010; Dubovi, 2010; Hayward et al., 2010; Holt et al., 2010; Rivailler et al., 2010; Seiler et al., 2010; Serra et al., 2011). In Asia, canine-H3N2 has been reported in both Korea and China, while canine influenza virus subtype H3N1 (canine-H3N1) has only been reported in Korea (Song et al., 2008; Li et al., 2010; Song et al., 2012). In 2012, canine-H3N2 was first reported from a dog in Thailand (Bunpapong et al., 2014). At present, at least six IAV subtypes have been reported of infection in dogs worldwide including H1N1 (Lin et al., 2012), H3N8 (Crawford et al.,

2005), H3N2 (Song et al., 2008), H5N1 (Songserm et al., 2006), H5N2 (Zhan et al., 2012) and H3N1 (Song et al., 2012). Intra-species transmission of canine IAV subtype H5N2, H3N2 and H3N8 has also been reported in the experimental dogs (Jirjis et al., 2010; Song et al., 2011; Song et al., 2013). Since IAVs have been proved to cross the species barrier, many scientists have been awakened to study on a cross-species transmission of the viruses. In addition, there is an evidence of experimental study in inter-species transmission of canine-H3N2 from dogs to cats (Kim et al., 2013).

Since canine influenza has been circulating in dog populations and human-pH1N1 and human-H3N2 have been circulating in human populations, the possibility of interspecies transmission among human and canine influenza viruses could not be ignored. The major concern is human and dog interface. In Thailand, the information of serological status of IAV infection in dogs is limited. In this study, a serological survey of IAV infection in pet dogs was conducted to determine the occurrence of IAV infection and to investigate the seroprevalence of canine-H3N2, human-H3N2 and human-pH1N1 influenza in pet dogs in Bangkok and vicinities during December 2012 to November 2013 by using two serological assays (NP-ELISA and HI test).

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Hypothesis

The occurrence of IAV infection in pet dogs in Bangkok detecting by NP-ELISA and HI test can be reported in Thai dog populations.

Objectives of study

1. To monitor IAV infection status in Thai dog populations

2. To determine the occurrence of canine IAV (H3) and human IAV (H1 and H3) infections in pet dogs in Bangkok and vicinities by using NP-ELISA and HI test

Chapter 2

Literature review

The structure, classification and properties of influenza virus

Influenza virus contains 8 segments of negative sense, single-stranded RNA, belonging to the family Orthomyxoviridae (Webster et al., 1992). There are three main types of influenza virus; types A, B and C. The outbreaks of influenza viruses are mostly associated with influenza virus type A and B, while influenza virus type C is rarely reported and causes minor symptoms (Nicholson et al., 2003). IAV can be isolated from several animal species, including pigs, horses, dogs, minks, seals, whales, birds as well as humans (Webster et al., 1992). IAVs have been classified into subtypes based on two surface glycoproteins; the haemagglutinin (HA) and the neuraminidase (NA) proteins. Nowadays, 18 HA (H1-18) and 11 NA (N1-11) subtypes have been identified (Tong et al., 2013). The antigenic variation of IAVs has been occurred from 2 main evolutionary mechanisms; antigenic drift and antigenic shift (Nicholson et al., 2003). Antigenic drift results from accumulative mutations in viral genome that continuously occur in nature, resulting in the emergence of new virus strains (Gerhard and Webster, 1978). Antigenic shift occurs through the process of genetic reassortment in which the gene segments between different subtypes are

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exchanged. This may result in novel subtypes of IAVs with pandemic potential (Webster et al., 1992).

Epidemiology of influenza A virus infection in dogs

Canine influenza A virus (canine IAV), an equine origin influenza A subtype H3N8, was first isolated in the US in January 2004 (Crawford et al., 2005). The first outbreak was reported in racing greyhounds in Florida and was then expanded to both racing greyhounds and pet dogs in 11 states in the US between 2004 and 2005 (Yoon et al., 2005). In addition, the evidences of the equine influenza viruses subtype H3N8 infection in dogs were reported in many countries, including Australia (Kirkland et al., 2010) and the United Kingdom (Daly et al., 2008). During the outbreaks of avian influenza A (H5N1) virus, a fatal case of dog naturally infected with avian influenza H5N1 virus was reported in Thailand (Songserm et al., 2006). Subsequently, canine IAV subtype H3N2 was first isolated from both pet dogs and farmed dogs in South Korea in 2007 (Song et al., 2008). Moreover, canine IAV subtype H3N2 has also been reported in Guangdong province in China (Li et al., 2010). In 2009, either H1N1/2009 influenza virus or canine IAV subtype H5N2 were identified in sick dogs in China (Lin et al., 2012; Zhan et al., 2012). Recently, the novel canine IAV subtype H3N1 was isolated from dogs in South Korea during May 2007 to December 2010. The molecular analysis of this novel canine IAV subtype

H3N1 showed the evidence of genetic reassortment between pandemic H1N1 2009 and canine IAV H3N2 (Song et al., 2012).

Nowadays, canine IAV infection in dogs has been reported in many parts of the world. Most of dogs infected with canine IAV usually show respiratory signs with low mortality rate (Dubovi and Njaa, 2008; Harder and Vahlenkamp, 2010). A previous study demonstrated that canine IAV subtype H3N8 can experimentally transmit among dogs via direct contact (Jirjis et al., 2010). In addition, all dog breeds are prone to be infected with these viruses (Dubovi and Njaa, 2008). Although there is no evidence of canine IAV transmission from dogs to humans, a recent study showed that canine IAV H3N2 can be experimentally transmitted from dogs to cats via airborne (Kim et al., 2013). However, in 2012, Lin et al. suggested the inter-species transmission of IAV from humans to dogs was possible. Two pH1N1/2009 influenza viruses were isolated from domestic dogs in China and the sequence analysis of all eight genes in two viruses characterized as human-like influenza viruses pH1N1/2009, pointing out the possibility of IAV transmission from humans to dogs (Lin et al., 2012).

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Epidemiology of influenza A virus infection in humans

Influenza A viruses are one of the causative agents of human influenza and can be found in human populations worldwide. At present, only 7 subtypes of IAVs

have been reported to infect human including H1N1, H2N2, H3N2, H5N1, H7N7, H7N9 and H9N2 (Payungporn et al., 2010). Influenza viruses are changing all the time by antigenic drift and sometimes antigenic shift occurs. The influenza pandemic emerges when the new virus has occurred and most people have no antibodies against it. The outcome of influenza pandemic affects in human health status and threatens to global public health. Not all subtypes of IAV have turned out to be influenza pandemic. In the past century, there were three influenza pandemics in the 20th century, including the Spanish flu of 1918-1919, the Asian flu of 1957-1958 and the Hong Kong flu of 1968-1969. The H1N1, H2N2 and H3N2 strains of influenza viruses were responsible for these pandemic outbreaks (Stephenson and Zambon, 2002). The first pandemic of the 21st century in human populations was identified in April 2009 and it caused by the novel pandemic H1N1 influenza virus, a triple reassortant among human, avian and swine influenza viruses (Ison and Lee, 2010). At first, this outbreak has emerged in Mexico in April 2009 and was then expanded to over 208 countries, including China, Japan and Thailand (de Silva et al., 2009; Dapat et al., 2012; Fang et al., 2014).

In Thailand, the epidemiological survey between June 2009 and July 2012 was shown that 42% (2697 of the 6417) of patients with influenza-like illnesses in Bangkok were positive for influenza viruses, with identification in pH1N1 (30%), H3N2 (10%) and influenza B (3%) of tested samples. In details, pH1N1 was the

predominant subtypes of human influenza in Bangkok during the 2009-2010. After that, a high prevalence of seasonal influenza H3N2 was detected in 2011. Then, in 2012, human-pH1N1, human-H3N2, and influenza B viruses had been co-circulated in human populations continuously (Prachayangprecha et al., 2013). At present, pH1N1 virus has become the seasonal flu virus that also co-circulates with human-H3N2 in human populations (Poovorawan et al., 2013).

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Serological survey of influenza A virus in dogs

Since IAVs have been proved to cross the species barrier, many countries have been awakened to take action on IAV surveillance and monitoring program especially on the serological profile. Serology tests, including ELISA and haemagglutination inhibition test (HI test), are diagnostic tools used for confirmation of canine IAV infection. Nucleoprotein (NP) based ELISA is used to detect anti-influenza A NP-antibodies in human and animal sera, whereas HI test is able to determine subtype specific antibodies. Nowadays, there are several reports of serological survey of canine IAV infection in dogs in many countries worldwide.

- Influenza A subtype H3N2

In South Korea, the avian origin canine influenza virus (H3N2) infection was identified in farm dogs (19%) and pet dogs (0.5%) by using NP-specific ELISA. This

finding indicated that canine IAV was circulated among dog populations in this country (Lee et al., 2009).

In china, a previous study showed that avian origin canine IAV H3N2 in pet dogs was determined in 5 different animal hospitals in Shenzhen city from May to July 2009. In this study, 6.71% of sera samples of pet dogs were positive for canine influenza virus by using ELISA. Moreover, the results from HI test showed that 6.06% of tested sera contained anti-H3 antibodies. Therefore, it can be concluded that avian origin canine IAV infection could be found in dog populations in China (Zhao et al., 2011). Three years later, another study was aimed to investigate the seroprevalence of avian-origin canine-H3N2 virus in China dogs between January 2012 and June 2013. The results showed that 3.5% (31 out of 882 dogs) of the tested sera had antibodies against H3 antibodies by using HI test (Sun et al., 2014).

- Influenza A subtype H3N8

In Canada, the serological surveys of H3N8 infection in dogs were conducted in 9 veterinary practices in Ontario from January 15 to June 20, 2006. This study showed that anti-canine influenza H3N8 virus antibodies was detected in only one sample of the total 225 dog sera (0.4%) by using haemagglutination inhibition test (Kruth et al., 2008).

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In the US, a previous study showed that 42 % (31 out of 74 dogs) of shelter dogs were seropositive for canine influenza virus (H3N8) (Holt et al., 2010). In addition, a previous serological survey indicated that H3N8 canine IAV infection had been identified in racing greyhounds in the US since 1999 (Anderson et al., 2012a). Subsequently, serologic prevalence of canine IAV subtype H3N8 infection in dogs in the US was noted again between 2005 and 2009. The results showed that 49% of 1,268 dog sera collected from pet and shelter dogs in 42 states was seropositive for canine IAV H3N8 virus by using HI test (Anderson et al., 2013). However, a previous study demonstrated that none of the total 399 sera samples obtained from racing sled dogs were seropositive for canine IAV H3N8 virus (Pecoraro et al., 2012).

- Influenza A subtype H1N1 2009 (pandemic H1N1)

In Italy, serological survey of 2009 pandemic H1N1 (pH1N1) in companion animals was conducted from October to December 2009. The results from this study showed a low prevalence of influenza virus infection (0.7%) in dogs. However, none of the cat sera samples were seropositive for IAV by using a commercial competitive ELISA. In addition, 7 out of 29 canine sera samples showed seropositive against pandemic 2009 H1N1 virus by using HI test (Dundon et al., 2010).

In Germany, 7 out of the 736 dog sera (0.95%) and 10 out of the 414 cat sera (2.42%) were serologically positive for IAVs by using the NP-ELISA. Moreover, this study also revealed that the seroprevalence of pandemic 2009 H1N1-specific

antibodies in dogs and cats was 0.13% and 1.93%, respectively determined by the N1 ELISA and a virus neutralization (VN) test (Damiani et al., 2012).

In China, serological survey of 2009 pandemic H1N1 (pH1N1) in domestic pet dogs was conducted between January 2012 and June 2013. Of the tested sera, 1.5% (13 out of 882 dogs) had pH1N1-specific antibodies by using HI test (Sun et al., 2014).

- Influenza A subtype H9N2

In Iran, 45.05% of the Iranian domestic dogs were seropositive for IAV by using ELISA. Of all seropositive, 81.7 % of these dogs had antibodies against H9N2 influenza viruses by using HI test (Hasiri et al., 2012).

Haemagglutination inhibition (HI) test

Haemagglutination inhibition (HI) test is considered as a gold standard for serological diagnosis of IAV infection. This assay can be used to classify the HA subtypes of the virus by detecting anti-HA specific antibodies. However, nonspecific factors presented in some animal sera, including dog sera might cause inaccurate results of HI test. Therefore, animal sera must be first treated with different methods before performing HI test in order to eliminate the nonspecific factors. The process of sera treatment is composed of 2 steps; the first is the inactivation of non-specific inhibitors of haemagglutination in sera by using receptor-destroying enzyme (RDE),

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trypsin or kaolin. Another step is the elimination of non-specific haemagglutinin in sera by using erythrocytes.

Receptor-destroying enzyme (RDE), an enzyme neuraminidase from Vibrio cholera, has been used in sera treatment for inactivation of non-specific inhibitors of haemagglutination in sera. According to, the OIE recommendations (Edwards, 2006), sera treatment with RDE should be done before starting the process of HI test. In addition, many substances can be used in practical for inactivation of non-specific inhibitors of haemagglutination in sera such as kaolin. Kaolin is a natural clay particle for removing several sera proteins such as lipoproteins and glycoproteins (Haukenes and Hjeltnes, 1991a; Haukenes and Hjeltnes, 1991b). The amount of kaolin particles using in sera treatment is about 5 to about 20%.

To date, there are many recommended protocols for dog sera treatment (Table 1). The variations in each step, including the type of additional sera treatment and the use of different red blood cell (RBC) species in the process of sera treatment, may effect on the validity and reliability of the assay.

Table 1 Inter-laboratory variations of protocols for dog sera treatment and HI test

Canine IAV	Sera treatment		Reference
	Type of additional sera treatment	Type of erythrocyte for sera adsorption	
H3N8	RDE**	-	Pecoraro et al., 2012
pH1N1,H3N8	RDE	-	Dundon et al., 2010
H3N8	RDE, calcium saline, sodium citrate solution	turkey RBCs*	Kruth et al., 2008
H3N8, H3N2	RDE	-	Said et al., 2011
H1N2,H3N2, H5N1, H9N2	RDE	-	Zhao et al., 2011
H3N8	trypsin, potassium metaperiodate	chicken RBCs(Edwards, 2006)	Yamanaka et al., 2012
H9N2	-	chicken RBCs	Hasiri et al., 2012
H3N8	Kaolin	chicken RBCs	Oien et al., 2012

* RBCs: Red Blood Cells, **RDE: Receptor Destroying Enzyme

Up to date, there is no standard protocol recommended for sera treatment in dog sera. Therefore, standardization of sera treatment and HI methods for dog sera in laboratories are necessary before performing HI tests

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Chapter 3

Material and Methods

This study was composed of 3 steps; Step I: The sample collection from pet dogs in Bangkok and vicinities, Step II: The screening of influenza A specific antibody in dog sera by using NP-ELISA and Step III: The evaluation of anti-HA specific antibodies in dog sera by using HI test. The conceptual framework of this study is shown in Figure 1.

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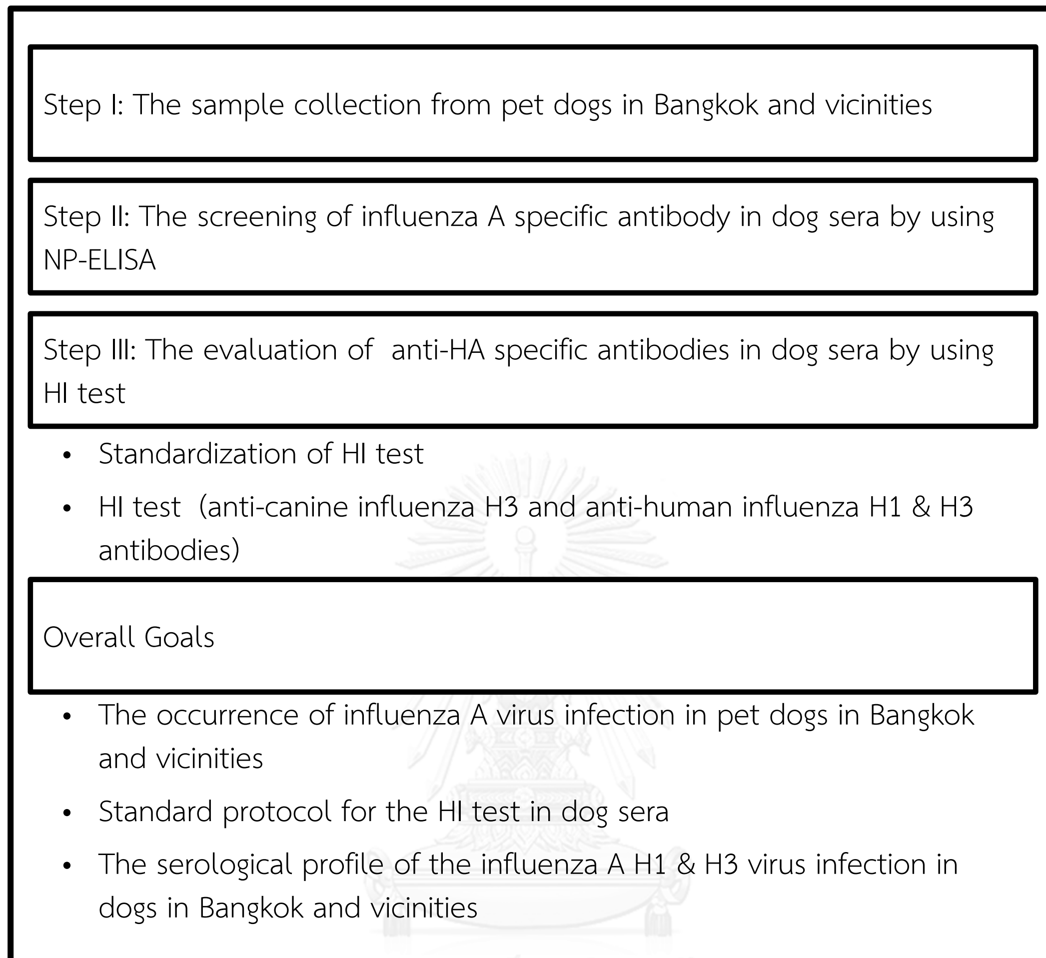

Figure 1 The conceptual framework of this study

Step I: The sample collection from pet dogs in Bangkok and vicinities

Blood samples were collected from December 2012 to November 2013. The samples were obtained from CU-veterinary diagnostic laboratory and private veterinary diagnostic laboratories. The samples were obtained from at least 467 veterinary clinics and hospitals located in 15 main provinces of Thailand, including Bangkok, Chiang Mai, Lamphun, Lop Buri, Mukdahan, Nakhon Nayok, Nakhon Pathom,

Nonthaburi, Pathum Thani, Prachin Buri, Ratchaburi, Samut Prakan, Samut Sakhon, Sing Buri and Udon Thani. The selected animal hospitals and veterinary diagnostic laboratories were chosen based on geographical locations, collaboration and high number of cases in the hospitals or laboratories. In total, 9,891 sera samples were obtained from both healthy and sick dogs of any breed, age and gender. After blood collection, all samples were sent to laboratory of Virology Unit, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University for performing the serological tests.

Step II: The screening of influenza A specific antibody in dog sera by using NP-ELISA

All of the 9,891 sera samples were screened for antibodies against influenza A nucleoprotein (NP) by using ID Screen® Influenza A Antibody Competition ELISA kit (ID VET, Montpellier, France), following the manufacturer's instructions (Bunpapong et al., 2014).

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ID Screen® Influenza A Antibody Competition ELISA kit (ID VET) is used as a diagnostic tool based on competitive principle. It is designed for detection of antibodies directed against the internal nucleocapsid protein of the IAV. It can be used in multi-species including dogs (De Benedictis et al., 2010). For sera preparation, dog sera sample was prepared by diluting each sera sample with

dilution buffer. After the process of sera preparation, each prepared sample was incubated in 96-well microplates coated with Antigen A (Ag A) at 37°C (± 2°C) for 1 hour ± 5 min, followed by washing each well for 5 times with 300 µl of the wash solution/wash. After that, the 50 µl of the conjugate 1X was added to each well and was then incubated for 30 min ± 2 min at 21°C (± 5°C). After an additional 3 times washing with the wash solution, add 50 µl of the substrate solution to each well, followed by incubation for 10 min ± 1 min at 21°C (± 5°C) in the dark room. Then the 50 µl of the stop solution was added before reading and record the O.D. at 450 nm. Both positive and negative controls were included. The ELISA results were interpreted by the competition percentage (competition %). The competition percentage for each sample was calculated from the formula:

$$\text{Competition \%} = (\text{OD specimen} / \text{OD negative control}) \times 100$$

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Sera samples with the competition percentage less than or equal to 45% were considered positive, those of between 45% and 50% were considered doubtful and those of greater than or equal to 50% were considered negative.

Step III: The evaluation of anti-HA specific antibodies in dog sera by using HI test

1. Standardization of HI test

Because HI standard protocol for detecting anti-HA specific antibodies in dog sera was not available, HI test was first standardized by following the OIE standard protocol for avian influenza virus with some modifications (Hirst, 1942; Shortridge and Lansdell, 1972; Rowe et al., 1999; Edwards, 2006). Standardization of HI test was divided into 2 phases; Phase I: Standardization of sera treatment and Phase II: Standardization of HI test. Three known anti-H3 CIV positive control dog sera and three known negative control dog sera were used to standardize HI test in dog sera.

Phase I: Standardization of sera treatment

Normally, there are 2 non-specific factors, non-specific inhibitors of haemagglutination and non-specific haemagglutinin, which need to eliminate by process of sera treatment before performing HI test. In this study, various techniques of sera treatment were compared for determining their efficacy in elimination of non-specific factors in dog sera (Table 1).

- Elimination of non-specific inhibitors of haemagglutination in dog sera

Two techniques were used to eliminate non-specific inhibitors, including 1) receptor destroying enzyme (RDE) treatment and 2) 20% kaolin treatment. The efficacy between these 2 techniques was then compared. For RDE treatment, 100 µl

of sera was treated with 300 µl of RDE working solution (Denka Seiken, Tokyo, Japan) and incubated overnight (20 hours) in a 37°C water bath. Then RDE was inactivated by incubation at 56°C for 30 min. For Kaolin treatment, 100 µl of sera was treated with 400 µl of 20% Kaolin (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 30 min.

- Elimination of non-specific haemagglutinin in dog sera

Two different types (chicken and turkey) and concentrations (50% and 100%) of erythrocytes (RBC) were compared for determining the efficacy in elimination of non-specific haemagglutinin in dog sera. Treated dog sera from the step of elimination of non-specific inhibitors were absorbed with 100 µl of 50% or 100% of chicken or turkey RBCs at room temperature for 1 hour.

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Table 2 The techniques of sera treatment of HI test in dog sera used in this study

Sera treatment		
1. Techniques used for elimination of non-specific inhibitors of haemagglutination	2. Types and percentages of RBC used for non-specific haemagglutinin elimination	
	Type of erythrocyte	% of erythrocyte
RDE**	Chicken RBCs*	50%
		100%
	Turkey RBCs	50%
		100%
20% Kaolin	Chicken RBCs	50%
		100%
	Turkey RBCs	50%
		100%

* RBCs: Red Blood Cells, **RDE: Receptor Destroying Enzyme

According to Table 2, the techniques used to standardize HI test in dog sera can be classified into 8 groups as follows;

Group A: Canine known H3-positive sera and three known negative control dog sera samples were first treated with RDE (1 part sera, 3 parts of RDE) for 20 hour at 37°C, followed by heat inactivation at 56°C for 1 hour. RDE-treated sera were then absorbed with 100 µl of 50% chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour. (RDE + 50% CRBCs)

Group B: Canine known H3-positive sera and three known negative control dog sera samples were first treated with RDE (1 part sera, 3 parts of RDE) for 20 hour at 37°C, followed by heat inactivation at 56°C for 1 hour. RDE-treated sera were then absorbed with 100 µl of 100% chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour. (RDE + 100% CRBCs)

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Group C: Canine known H3-positive sera and three known negative control dog sera samples were first treated with RDE (1 part sera, 3 parts of RDE) for 20 hour at 37°C, followed by heat inactivation at 56°C for 1 hour. RDE-treated sera were then absorbed with 100 µl of 50% Turkey red blood cells (TRBCs) and incubated at room temperature for 1 hour. (RDE + 50% TRBCs)

Group D: Canine known H3-positive sera and three known negative control dog sera samples were first treated with RDE (1 part sera, 3 parts of RDE) for 20 hour at 37°C, followed by heat inactivation at 56°C for 1 hour. RDE-treated sera were then absorbed with 100 µl of 100% Turkey red blood cells (TRBCs) and incubated at room temperature for 1 hour. (RDE + 100% TRBCs)

Group E: Canine known H3-positive sera and three known negative control dog sera samples were first treated with 20% Kaolin (1 part sera, 4 parts of 20% Kaolin) for 30 min at room temperature, followed by centrifuge at 2000 rpm for 10 min. 20% kaolin-treated sera were then absorbed with 100 µl of 50% chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour. (20% Kaolin + 50% CRBCs)

Group F: Canine known H3-positive sera and three known negative control dog sera samples were first treated with 20% Kaolin (1 part sera, 4 parts of 20% Kaolin) for 30 min at room temperature, followed by centrifuge at 2000 rpm for 10 min. 20% kaolin-treated sera were then absorbed with 100 µl of 100% chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour. (20% Kaolin + 100% CRBCs)

Group G: Canine known H3-positive sera and three known negative control dog sera samples were first treated with 20% Kaolin (1 part sera, 4 parts of 20% Kaolin) for 30 min at room temperature, followed by centrifuge at 2000 rpm for 10 min. 20% kaolin-treated sera were then absorbed with 100 µl of 50% Turkey red blood cells (TRBCs) and incubated at room temperature for 1 hour. (20% Kaolin + 50% TRBCs)

Group H: Canine known H3-positive sera and three known negative control dog sera samples were first treated with 20% Kaolin (1 part sera, 4 parts of 20% Kaolin) for 30 min at room temperature, followed by centrifuge at 2000 rpm for 10 min. 20% kaolin-treated sera were then absorbed with 100 µl of 100% Turkey red blood cells (TRBCs) and incubated at room temperature for 1 hour. (20% Kaolin + 100% TRBCs)

Phase II: Standardization of HI test

After sera treatment process, HI tests were performed in V-bottom 96-well microtiter plates (Bunpapong et al., 2014). Each treated sera was serially two-fold diluted with phosphate buffered saline (PBS) and was then incubated with 8 haemagglutination units (HAU) per 50 microliter of an avian origin canine influenza virus antigen (A/canine/Thailand/CUDC5299/2012(H3N2)) for 45 min at room temperature. A 0.5% suspension of chicken RBCs or turkey RBCs were added before reading the titer after 1 hour incubation period. Type of RBCs used in HI test was similar to type of RBCs used in the process of sera treatment. All sera were tested in duplication. Both positive (canine IAV H3-seropositive canine sera) and negative (canine IAV seronegative canine sera) control sera were included. The HI titer was determined by the reciprocal of the last dilution that shows no agglutination and reported as geometric means. Samples with a titer ≥ 40 were considered positive

(Mancini et al., 2012). The flow chart of various techniques used for standardization of HI test in this study is showed in Figure 2.

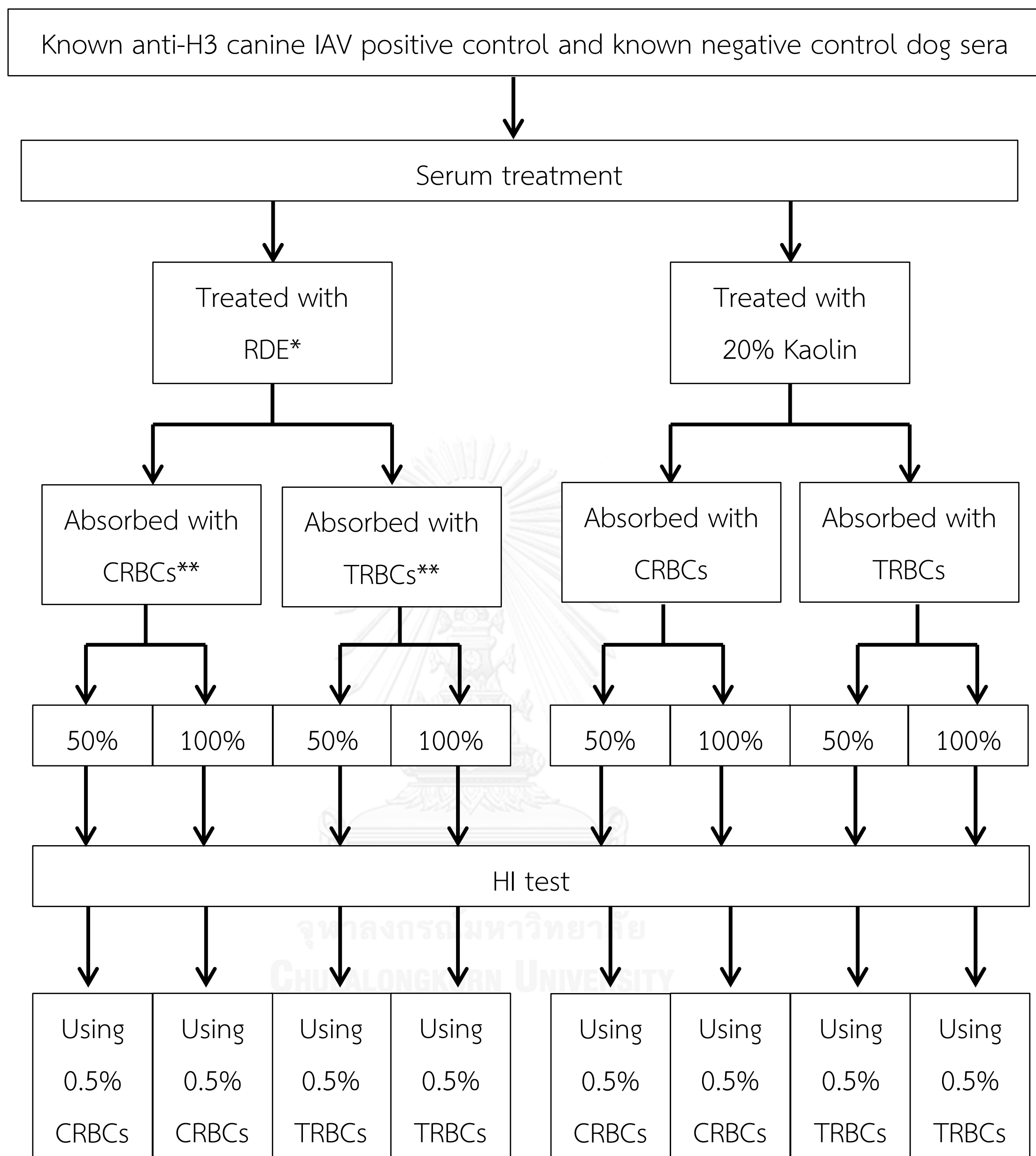

Figure 2 The flow chart of protocols used for standardization of HI test in this study

*RDE: Receptor Destroying Enzyme, **CRBCs: Chicken Red Blood Cells,

***TRBCs: Turkey Red Blood Cells

2. **HI test**

In this study, from HI optimization, the HI protocol of group C was used as standard protocol and applied to evaluate anti-HA specific antibodies in dog sera. Briefly, the NP-ELISA suspect and positive sera samples were first treated with RDE (1 part sera, 3 parts of RDE) for 20 hour at 37°C, followed by heat inactivation at 56°C for 1 hour. RDE-treated sera were then absorbed with 100 μl of 50% Turkey red blood cells (TRBCs) and incubated at room temperature for 1 hour. After the process of sera treatment, each sera sample was serially two-fold diluted with phosphate-buffered saline (PBS) in 96-well micro-titer plates and was incubated with 8 haemagglutination units (HAU) per 50 microliter of each virus for 45 min at room temperature. A 0.5% of turkey RBCs suspension was added before reading the titer after 1 hour of incubation period. All sera were tested in duplicate. Both positive (canine IAV seropositive canine sera) and negative (canine IAV seronegative canine sera) sera controls were included. The HI titer was determined by the reciprocal of the last dilution that shows no agglutination and reported as geometric means. Samples with a titer ≥ 40 were considered positive (Bunpapong et al., 2014).

The virus antigens used in this study included canine H3N2 virus (canine-H3N2) (A/canine/Thailand/CU-DC5299/2012(H3N2)), human pandemic H1N1 2009 (A/Thailand/CUH1N1/2012(pH1N1)) and human H3N2 virus (A/Thailand/CUH3N2/2012(H3N2)). An avian origin canine-H3N2 was isolated from a

pet dog in Thailand and propagated in 10-day-old embryonated chicken eggs (Bunpapong et al., 2014). Human-H3N2 and human-pH1N1 viruses were isolated from patients with an influenza-like illness in February and May 2013, respectively, and propagated in Madin-Darby canine kidney (MDCK) cells maintained in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA) in the presence of 1 μg/mL tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO) as described previously (Thontiravong et al., 2012).

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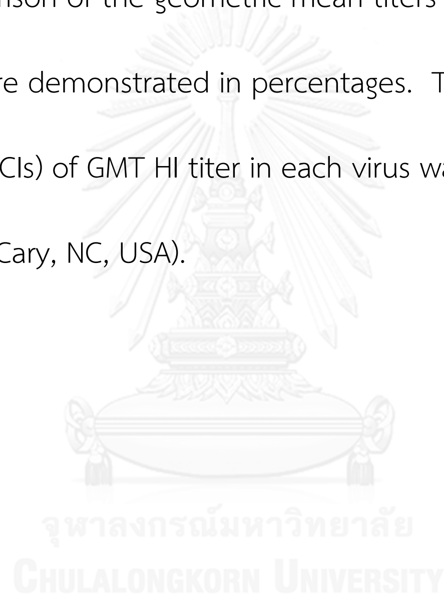
Statistical analysis

Descriptive statistics were used to illustrate the geographic locations and the numbers of IAV infected dogs in Bangkok and vicinities. Data were analyzed with respect to the collection months and geographic locations. To evaluate the standard protocol for sera treatment and HI test in dog sera, the canine sera antibodies against canine-H3N2 in different protocols of sera treatment were assessed by a comparison of the geometric mean titers (GMTs) of HI test. In all tests, seropositive rates were demonstrated in percentages. The estimation of 95% confidence intervals (CIs) of GMT HI titer in each virus was performed by using SAS version 9.3 (SAS Inc., Cary, NC, USA).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Chapter 4

Results

From December 2012 to November 2013, 9,891 sera samples were obtained from CU-veterinary diagnostic laboratory, a private veterinary diagnostic laboratory in Bangkok, and 467 veterinary clinics and hospitals located in 15 main provinces of Thailand, including Bangkok (n=8,624), Chiang Mai (n=15), Lamphun (n=1), Lop Buri (n=19), Mukdahan (n=23), Nakhon Nayok (n=17), Nakhon Pathom (n=160), Nonthaburi (n=566), Pathum Thani (n=159), Prachin Buri (n=150), Ratchaburi (n=22), Samut Prakan (n=6), Samut Sakhon (n=80), Sing Buri (n=16) and Udon Thani (n=33) (Table 3). In total, the highest number of sera samples were collected from dogs in Bangkok (87.19%, 8,624/9,891) followed by vicinities (9.82%, 971/9,891), Eastern (1.52%, 150/9,891), Northeastern (0.57%, 56/9,891), Central (0.53%, 52/9,891), Western (0.22%, 22/9,891) and Northern Thailand (0.16%, 16/9,891), respectively.

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Table 3 Geographic distribution and number of dog sera collected in this study

Provinces/Regions	No. of dogs sera (%)
Bangkok	8,624 (87.19%)
Vicinities	
- Nakhon Pathom	160 (1.62%)
- Nonthaburi	566 (5.72%)
- Pathum Thani	159 (1.61%)
- Samut Prakan	6 (0.06%)
- Samut Sakhon	80 (0.81%)
Northern Thailand	
- Chiang Mai	15 (0.15%)
- Lamphun	1 (0.01%)
Northeastern Thailand	
- Mukdahan	23 (0.23%)
- Udon Thani	33 (0.33%)
Eastern Thailand	
- Prachin Buri	150 (1.52%)
Central Thailand	
- Nakhon Nayok	17 (0.17%)
- Lop Buri	19 (0.19%)
- Sing Buri	16 (0.16%)
Western Thailand	
- Ratchaburi	22 (0.22%)

The number of sera samples collected in each month was shown in Figure 3. The highest number of sera samples was in June 2013 (n=1,150), while the lowest number of sera samples was in August 2013 (n=210). The average number of 600 sera samples was collected per month.

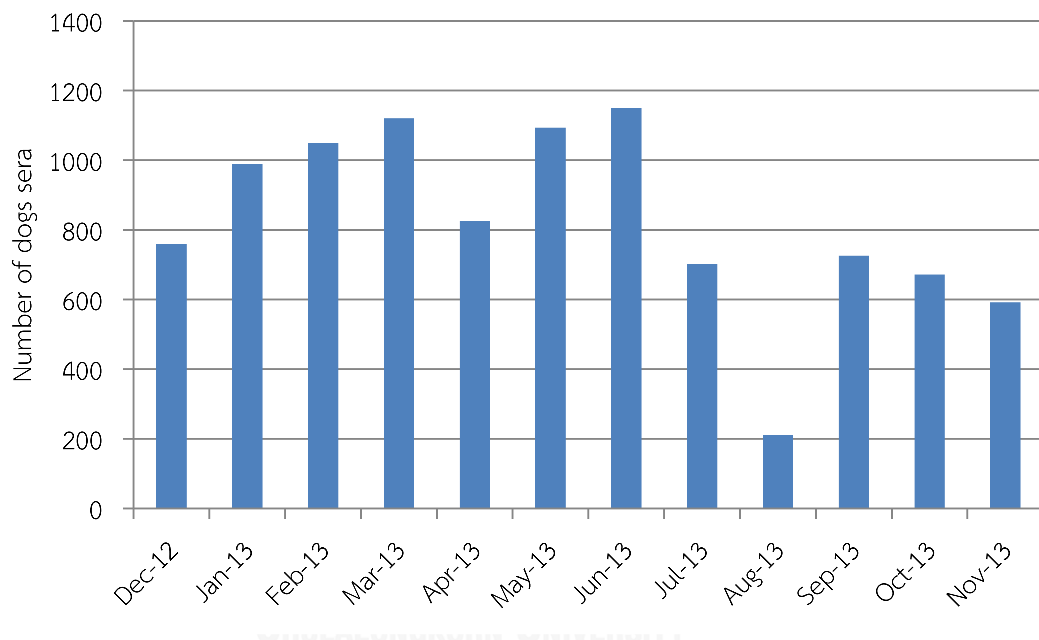

Figure 3 Number of dog serums collected in each month

Screening of influenza A antibodies in dog sera

In this study, 9,891 dog sera samples collected from Bangkok, vicinities and main provinces of Thailand during December 2012 to November 2013 were subjected for IAV antibody detection by using NP-ELISA. The percentages of IAV seropositive dogs detected by NP-ELISA test in each month were shown in Figure 4.

Overall, our results showed that 164 out of the 9,891 sera samples (1.66%) were positive for antibodies against IAV. Our results also showed that seropositive ELISA samples were found in samples collected throughout the year. The highest prevalence of IAV seropositive dogs was found in December 2012 (2.9%) and January 2013 (2.73%), while the lowest prevalence of IAV in dogs was found in October 2013 (0.89%). The number of IAV seropositive samples in each month was shown in Table 4. All of the IAV seropositive samples were collected from dogs located in 11 provinces of Thailand, including Bangkok, Chiang Mai, Lop Buri, Mukdahan, Nakhon Nayok, Nakhon Pathom, Nonthaburi, Pathum Thani, Ratchaburi, Samut Sakhon and Sing Buri. The number of IAV seropositive samples by geographic distribution was demonstrated in Table 5.

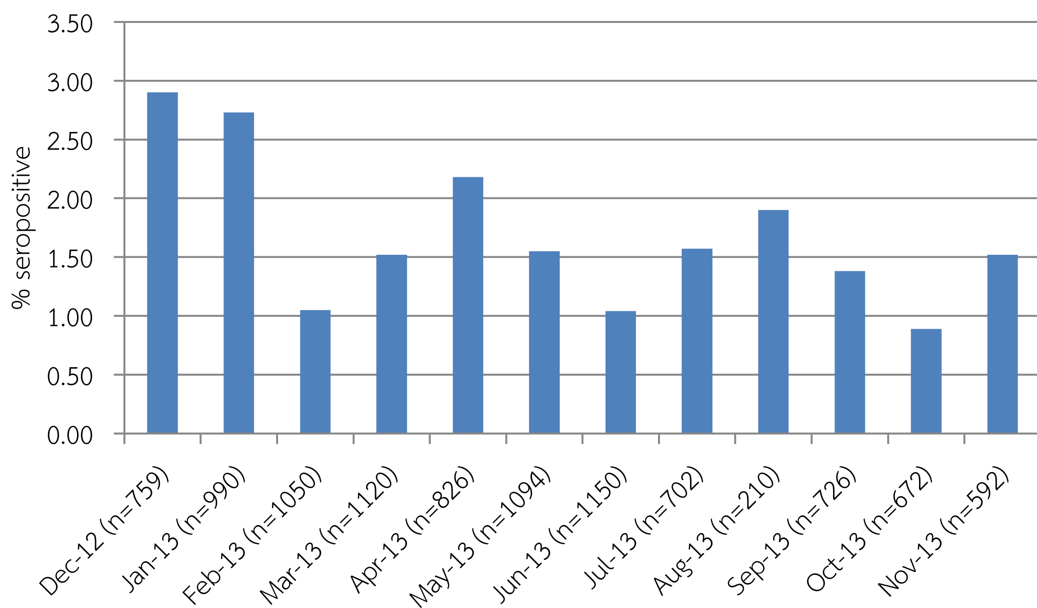

Figure 4 Monthly percentage of IAV seropositive dogs detected by NP-ELISA test

Table 4 Number and percentage of IAV seropositive dog samples detected by NP-ELISA

Collection month/year	No. of sera samples	No. of positive ELISA (%)
Dec-12	759	22 (2.90)
Jan-13	990	27 (2.73)
Feb-13	1,050	11 (1.05)
Mar-13	1,120	17 (1.52)
Apr-13	826	18 (2.18)
May-13	1,094	17 (1.55)
Jun-13	1,150	12 (1.04)
Jul-13	702	11 (1.57)
Aug-13	210	4 (1.90)
Sep-13	726	10 (1.38)
Oct-13	672	6 (0.89)
Nov-13	592	9 (1.52)

Table 5 Geographic distribution of IAV seropositive dog samples

Provinces	No. of IAV seropositive dogs (%)
Bangkok	141 (85.98%)
Chiang Mai	1 (0.61%)
Nakhon Nayok	1 (0.61%)
Nakhon Pathom	1 (0.61%)
Nonthaburi	12 (7.32%)
Pathum Thani	1 (0.61%)
Mukdahan	1 (0.61%)
Ratchaburi	2 (1.22%)
Lop Buri	2 (1.22%)
Samut Sakhon	1 (0.61%)
Sing Buri	1 (0.61%)

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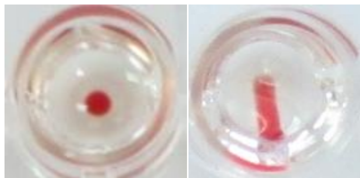
Determination of anti-HA specific antibodies in dog sera detected by using HI test

In this study, we standardized HI test in dog sera since there was no HI standard protocol for dog sera. Three known anti-H3 canine IAV positive and canine IAV negative control dog sera were used to standardize the HI test by using canine-H3N2 antigen. Eight protocols of sera treatment used for HI test standardization were examined (Group A-H). The patterns of agglutination and the GMT HI positive titer were compared to determine the suitable HI protocol for dog sera.

Our results showed that the canine IAV negative control dog sera presented HI negative result and there was no evidence of partial haemagglutination in sera control of all groups. This result indicated that non-specific factors in dog sera were completely eliminated (Figure 5). It should be noted that 50% and 100% of TRBCs showed unambiguous inhibition pattern and more easily to interpret the result than those from CRBCs (Figure 6). Moreover, dog sera treated with RDE (group A, B, C, D) showed more consistent GMT HI positive titer (254.57) than those treated with 20% kaolin treatment (group E, F, G, H) (Table 6). It is important to note that RDE treated dog sera absorbed with TRBCs yielded higher percentage of positive samples and GMT HI positive titer than those absorbed with CRBCs when high number of dog sera samples (n=112) were used to compare between these 2 techniques (data not shown). Our result demonstrated that RDE treatment, followed by TRBCs absorption is the most appropriate technique for elimination of non-specific factors in dog sera.

It is noted that 50% and 100% of TRBCs showed similar GMT HI positive titer. Overall, it can be concluded that RDE and 50% Turkey RBCs were chosen to be a standard protocol for sera treatment of HI test in dog sera (Table 7).

A) Complete treatment



B) Partial agglutination

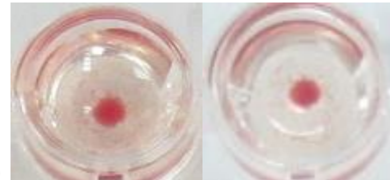


Figure 5 The patterns of haemagglutination in treated sera

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Table 6 The HI results of known anti- H3 CIV positive sera samples treated with different techniques of sera treatment of HI test

Group	HI Protocol	GMT HI positive titer*	Agglutination (partial/complete)
A	RDE** + 50% CRBCs***	254.57	Complete
B	RDE + 100% CRBCs	254.57	Complete
C	RDE + 50% TRBCs****	254.57	Complete
D	RDE + 100% TRBCs	254.57	Complete
E	20% Kaolin + 50% CRBCs	254.57	Complete
F	20% Kaolin + 100% CRBCs	320.00	Complete
G	20% Kaolin + 50% TRBCs	100.56	Complete
H	20% Kaolin + 100% TRBCs	160.00	Complete

*GMT HI positive titer = 2^n x 5; n = mean HI positive score,

**RDE: Receptor Destroying Enzyme, ***CRBCs: Chicken Red Blood Cells,

****TRBCs: Turkey Red Blood Cells

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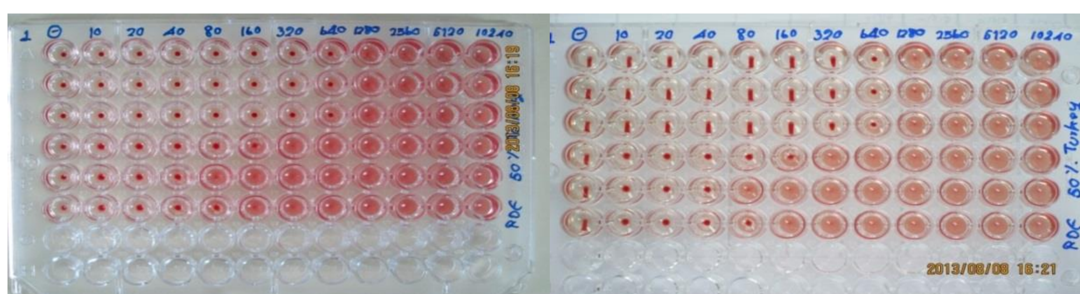

Figure 6 The HI tested plate from sera samples treated with RDE and 50% Turkey RBCs

Table 7 The standard protocol for sera treatment of HI test in dog sera

HI protocol	Canine sera
Sera treatment	
- Elimination of non-specific inhibitors of haemagglutination	RDE
- Elimination of non-specific haemagglutinin	50% Turkey RBCs
HI test	
- RBC source & concentration	0.5% Turkey RBCs

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To determine specific HA antibodies, 164 NP-ELISA suspect and positive sera samples were subjected to determine anti-canine influenza H3 and anti-human influenza H1 & H3 specific antibodies by using HI test. The samples with a HI titer ≥ 40 were considered positive, indicating the evidence of previous exposure to IAVs. The HI test results showed that approximately 37% of all positive and suspected sera samples from NP-ELISA test were positive HI titer to IAV subtypes canine-H3N2 (20, 12.20%), human-H3N2 (2, 1.22%) and human-pH1N1 (38, 23.17%). The GMT HI positive titer against canine-H3N2, human-H3N2 and human-pH1N1 were 80.00, 40.00 and 156.71, respectively (Table 8) (Figure 7). Interestingly, from HI test results, the evidence of co-infection of IAVs including co-infections between canine-H3N2 and

human-pH1N1 viruses (1.22%) and co-infections between human-H3N2 and human-pH1N1 (1.22%) have been observed.

Table 8 Percentage of seropositive sera samples and GMT HI positive titer detected by HI tests

Virus	Total No. of tested sera samples	No. & (%) of HI positive samples	GMT HI positive titer*
Canine-H3N2	164	20 (12.20)	80.0
Human-H3N2	164	2 (1.22)	40.0
Human-pH1N1	164	38 (23.17)	156.71

*GMT HI positive titer = 2^n x 5; n = mean HI positive score

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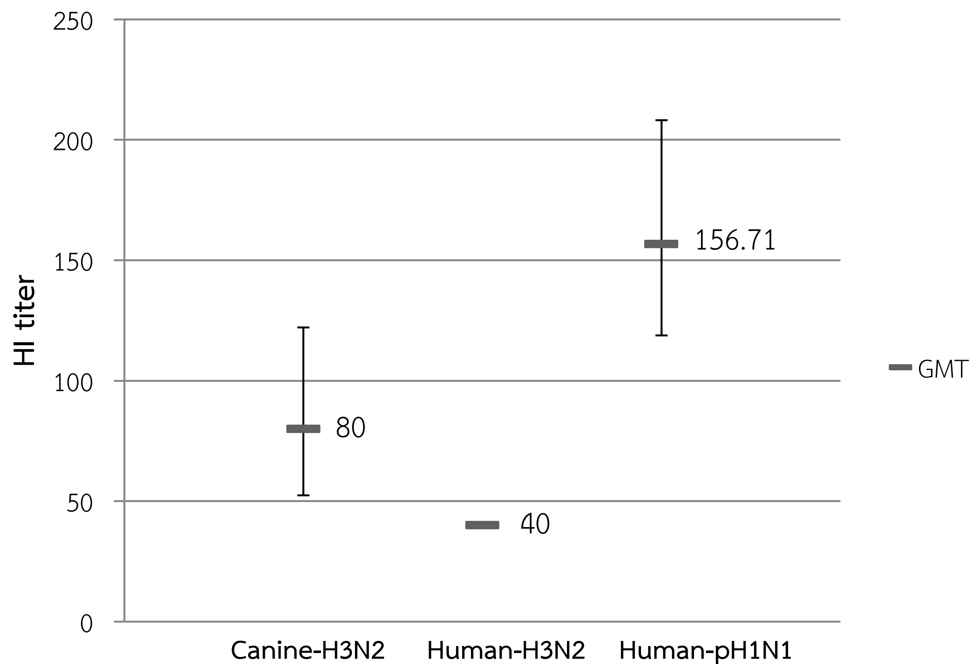

Figure 7 HI titers of seropositive sera samples from dogs against canine-H3N2, human-H3N2 and human-pH1N1 viruses. GMT and 95% confidence intervals are indicated by long and short horizontal lines, respectively

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The percentages of HI positive dog sera samples against canine-H3N2, human-H3N2 and human-pH1N1 viruses were analyzed by month (Figure 8). The positive sera samples for human-pH1N1 virus were observed all year round (except in May 2013). Moreover, the canine-H3N2 seropositive samples were observed throughout the year except in April, July and November 2013. In contrast, the positive sera samples for human-H3N2 virus were observed only in April and July 2013.

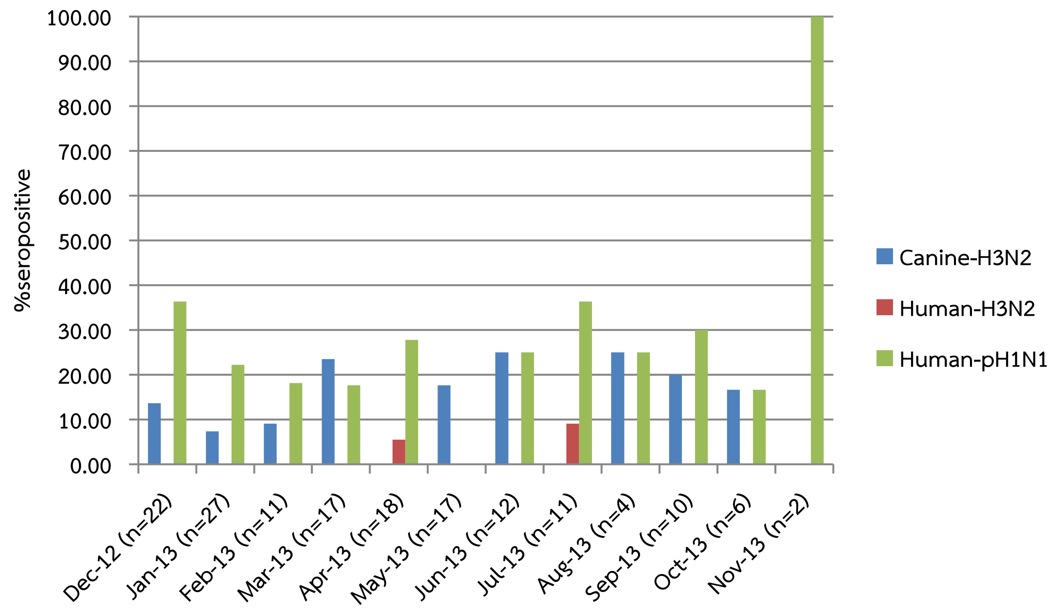

Figure 8 Percentages of HI seropositive sera samples from dogs against canine-H3N2, human-H3N2 and human-pH1N1 viruses, by month. Samples were positive when titer was ≥ 40.

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Chapter 5

Discussion

Interspecies transmission of IAVs poses a potential threat to global human health since the outbreak of swine-origin pH1N1 2009 virus emerged in human (Garten et al., 2009). At present, seasonal human-H3N2 and human-pH1N1 viruses continue to circulate in human population worldwide (Schrauwen et al., 2011; Komadina et al., 2014). The close contact between humans and dogs increased the possibility of IAVs interspecies transmission. In addition, many subtypes of IAVs, including avian-origin canine-H3N2, have already been documented to cross the species barrier from avian to dogs (Song et al., 2008). However, serological status of IAV infection in dogs especially in Thailand is limited.

Canine-H3N2 and human-pH1N1 infected dogs were reported in many countries in Asia, including China and Korea (Song et al., 2008; Li et al., 2010; Lin et al., 2012; Su et al., 2013; Sun et al., 2013). Recently, canine-H3N2 was first isolated in a dog in Thailand (Bunpapong et al., 2014). The phylogenetic analysis showed that Thai canine-H3N2 has high genetic similarity with those of avian influenza virus (H3N2) from Eastern China (Bunpapong et al., 2014). In addition, the sporadic transmission and subclinical infection of human-H3N2 has been reported in dogs, suggesting that

dogs can infect with human influenza virus (Nikitin et al., 1972; Kilbourne and Kehoe, 1975; Chang et al., 1976; Houser and Heuschele, 1980). The emergence of avian and human influenza virus infection in dogs has raised a public health concern that dogs can become intermediate host between avian and human viruses, resulting in the emerging of a novel influenza virus through reassortment.

Here, our study reported the serological survey of IAV infection in pet dogs in Thailand. NP-ELISA result showed that 164 (1.66 %) out of 9,891 sera samples had antibodies to IAVs, which was lower than those reported in Italy, Korea and China. In Italy, the NP-ELISA result showed that 29 (3%) of the 964 dog sera samples collected in 2009 posed antibodies to IAVs (Dundon et al., 2010). In Korea, the serological evidence of IAV infection in farmed dogs (19%) had been reported in 2009 by NP-ELISA (Lee et al., 2009). Similarly, the percentage of seropositive sera samples to IAVs detected by NP-ELISA test in Korean dogs in 2010 was 34.8% (24/69) (Song et al., 2011). In china, the seroprevalence of IAVs infection in pet dogs in 2011 was 6.71% (31/462) determined by NP-ELISA (Zhao et al., 2011). In 2013, 48 (5.3%) of the 900 sera samples and 66 (12.2%) of the 540 sera sample from pet dogs and farmed dogs in China, respectively, had IAV antibodies by NP-ELISA (Su et al., 2013). Overall, our results indicated that IAV infection was found in dogs in Bangkok and vicinities. However, the rate of IAV exposure in dogs was relatively low (1.66%) comparing to other countries. It seems possible that these results are due to the sample

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collection period and the target population. In this study, we collected the sample from both healthy and sick dogs throughout the year from animal hospitals where did not experience in influenza outbreak, while other studies obtained the sample from dogs showing clinical signs of respiratory infection or collecting after laboratory-confirmed cases of influenza outbreaks in those countries (Lee et al., 2009; Dundon et al., 2010; Zhao et al., 2011).

Several studies have suggested that human influenza frequently occurs in winter, comparing with other seasons (Potter, 2001). The morbidity and mortality rates of IAV infected patients have increased in winter due to the cold weather and high humidity (Crombie et al., 1995; Donaldson and Keatinge, 2002; Reichert et al., 2004). In this study, we found the highest IAV seropositive samples in December (2.9%), followed by January (2.73%). These results corresponded with of the results from other studies (Shih et al., 2005), suggesting that the number of canine influenza has risen in the winter season and corresponded with human influenza.

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As mentioned in the literature review, there is no standard protocol for detecting anti-HA specific antibodies in dog sera. Therefore, standardization of HI test was performed before starting HI test with the field samples. The current study found that the standard protocol for sera treatment of HI test in dog sera was RDE and 50% Turkey RBCs. Comparing RDE with kaolin showed that RDE treatment yielded the most consistent GMT HI positive titer (254.57), while those using kaolin

treatment produced the variation in GMT HI positive titer (100.56-320.00). In accordance with the present results, previous studies have demonstrated that kaolin treatment can cause false positive results when tested for antibodies against Equine influenza virus (EIV) H7N7, comparing with RDE. However, false positive results were not found when tested against EIV-H3N8. These findings suggested that RDE treatment showed the high efficiency in removal of nonspecific inhibitors from equine sera, comparing with kaolin (Boliar et al., 2006). In addition, another study in canine sera suggested that RDE treatment could be helpful to maintain high specificity in HI test against Canine influenza virus (CIV) H3N8 (Anderson et al., 2012b). Moreover, the present study determined the efficacy in elimination of non-specific haemagglutinin in dog sera, by comparing 2 different types and concentrations of RBCs. The results of this study indicated that using 50% and 100% concentrations in both chicken and turkey RBCs were able to eliminate non-specific haemagglutinin in dog sera completely. However, 50% and 100% of turkey RBCs showed unambiguous inhibition pattern and easily to interpret result. This finding is in agreement with Anderson's (2012) findings which indicated using turkey and chicken RBCs provided a highly sensitive and specific assay for serological diagnosis of canine-H3N8 infection in dogs. However, turkey RBCs has also shown the highest GMT HI titer against canine-H3N8, comparing with chicken and canine RBCs (Anderson et al., 2012b). Because of the clear inhibition pattern, the short settling time and the high HI titer, turkey RBCs are frequently chosen for elimination of non-specific haemagglutinin and HI test in

dog sera (Kruth et al., 2008). Therefore, RDE and 50% Turkey RBCs were chosen to be a standard protocol for sera treatment and HI test in dog sera.

The result from HI test indicated that dogs in Bangkok and vicinities showed antibodies against canine-H3N2, human-pH1N1 and human-H3N2 viruses suggesting previous exposure to these viruses. The seropositivity rate for human-pH1N1 in Thai dogs (23.17%) was greater than those Italian dogs in 2009 (0.7%) (Dundon et al., 2010). In addition, this study showed that the seropositivity rate for canine-H3N2 (12.20%) and human-pH1N1 (23.17%) in Thai dogs was higher than those for canine-H3N2 (3.5%) and human-pH1N1 (1.5%) in Chinese pet dogs in 2012, respectively. Although, the seropositivity rate for human-H3N2 in Thai dogs (1.2%) was similar to those of Chinese pet dogs in 2012 (1.2%) (Sun et al., 2014). However, these data must be interpreted with caution because the results from current study are based on NP-ELISA and HI test, while those from the previous studies were tested based on HI test and MN test (Sun et al., 2014).

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From our results, we observed that Thai dogs posed the highest seropositivity rate for human-pH1N1, comparing with canine-H3N2 and human-H3N2, whereas the previous study in 2012 showed the canine-H3N2 positive sera samples was the greatest proportion among Chinese pet dogs, comparing with human-pH1N1 and human-H3N2 (Sun et al., 2014). Since 2006, canine-H3N2 virus was frequently isolated from dogs in China and the serological survey of IAV infection in China

indicated this canine-H3N2 was the predominant subtype of IAVs circulating in Chinese dog populations (Li et al., 2010; Su et al., 2013; Sun et al., 2013; Sun et al., 2014). In contrast, canine-H3N2 was first introduced to a dog in Thailand in 2012 and now there is just only 1 confirmed canine-H3N2 infected dog in Thailand (Bunpapong et al., 2014). Due to canine-H3N2 case is less common, the information related to immunity against canine-H3N2 in Thai dog populations is very limited. In accordance with the present results, previous studies in human during June 2009 and July 2012 have demonstrated that the predominant IAV subtype in human in Bangkok, Thailand was influenza A (pH1N1) virus, followed by influenza A (H3N2) and influenza B viruses (Prachayangprecha et al., 2013). Human-pH1N1 virus has been circulating in human populations and now this virus is becoming an endemic seasonal human influenza virus, which usually found to co-infect with human-H3N2 virus (Poovorawan et al., 2013). The close contact between human and dog has increasing the risk of specie-to-specie transmission. Consequently, it can be possible that human can transmit pH1N1 to dogs, as indicated by the highest seropositivity rate to pH1N1 among Thai dog populations. However, to date, no human-pH1N1 virus has been isolated from dogs in Thailand. In this study, we provided the serological evidence of human-H3N2 infection in dogs. Only 1.2% was positive to human-H3N2 virus by HI test, indicating in limited evidence of prior exposure to human-H3N2 in Thai dog populations. Similarly, the result from serological survey in dogs in China showed the lowest percentage of human-H3N2 seropositive dogs among Chinese dog populations,

comparing with other subtypes of IAVs (Sun et al., 2014). Our results indicated that human-H3N2 virus circulated among dog populations. However, the percentage of seropositive dogs to human-H3N2 virus was relatively low.

Co-infection with different IAVs in the same host may increase the risk for IAVs reassortment, resulting in exchange of gene segments and becoming a novel reassortant influenza virus (Peacey et al., 2010). Recently, H3N1 influenza virus, a reassortant between an avian-origin canine-H3N2 and the pH1N1 viruses, was isolated from dogs in Korea (Song et al., 2012). Based on the HI results, we found that 2 dogs posed antibodies against canine-H3N2 and human-pH1N1 and 2 dogs posed antibodies against human-H3N2 and human-pH1N1. It is interesting to note that co-infection of IAVs could have been observed in Thai dogs. However, cross-reaction between 2 IAV subtypes could not be rule out even it rarely happened. Therefore, the event of genetic reassortment between different IAV subtypes occurring in dogs should not be ignored. The transmission of IAVs between dogs and humans should be closely monitored and minimized.

In summary, this study provided the occurrence of IAV infection and the serological profile of influenza A H1 and H3 virus infection in pet dogs in Bangkok and vicinities. These findings provided significant information for public health awareness and raised the need for continuous monitoring for IAV infection in domestic animals.

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In addition, these findings have important implications for developing standard protocol for HI test in dog sera.

Chapter 6

Conclusions

In this study, the occurrence of IAV infection and the serological profile of the influenza A H1 & H3 virus infections in pet dogs in Bangkok and vicinities between December 2012 and November 2013 were elucidated.

- Serological survey confirmed IAV exposure in dogs in Bangkok and vicinities. The rate of IAV exposure in dogs was relatively low (1.66%).
- HI antibodies against canine-H3N2, human-pH1N1 and human-H3N2 viruses from dogs demonstrated previous exposure of canine-H3N2, human-pH1N1 and human-H3N2 viruses in dog population in Bangkok and vicinities. A high percentage of dogs exposed with human-pH1N1 virus, suggesting that dogs can act as a potential host for the transmission of human-pH1N1 among human and canine species.
- This study showed noteworthy that dogs exposed with different subtypes of IAVs, pointing out the potential emergence of new IAV viruses in Thai dogs. These raise a public health concern due to human and dog interface.

Therefore, it is important to keep in place the routine surveillance and monitoring program on IAV infection in pet dogs and domestic mammals. Further

studies should focus on serological and virological surveys on a larger scale in companion animals, including in dogs and cats, as well as in human. The information will provide better understanding of dynamic of IAV infection in animals and human in Thailand.

Suggestion for CIV prevention and control

Because the influenza is a contagious disease and commonly affects population widespread, prevention and control has become an important ways to reduce the transmission of influenza. The principles of prevention and control in canine influenza are the same as those in other respiratory diseases, including the following precautions:

- Keep pet dog away from overcrowded condition
- Avoid pet dog to contact with infected dog or any items that may have been exposed to an infected dog
- Wash hands before and after handling dogs
- Clean and sanitize contaminated surfaces with disinfectants such as alcohol, quaternary ammonium compounds or bleach
- Monitor your dog’s health for respiratory signs and check-up health status annually

Moreover, the most effective way to prevent and control influenza is vaccination. The first canine influenza H3N8 vaccine was licensed by the United States Department of Agriculture (USDA) in June 2009. The aim of vaccination is to control canine influenza infection with significantly reduces the severity and duration of clinical signs. It results in reduce clinical signs in infected dog. However, the vaccine may not prevent the infection. The canine influenza vaccine is recommended for dogs living in areas with high canine influenza incidences or high risk areas to infect this virus.

To date, there is no evidence of human infected with canine influenza virus. However, the occurrence of human infection with the new strain of influenza viruses should be concerned. Avoiding the close contact with infected dogs with respiratory signs may reduce a chance in interspecies transmission of influenza viruses and also prevent the occurrence of genetic reassortment between different IAV subtypes. Consequently, human and other animal influenza viruses should be closely monitored and minimized.

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REFERENCES

Anderson TC, Bromfield CR, Crawford PC, Dodds WJ, Gibbs EP and Hernandez JA 2012a. Serological evidence of H3N8 canine influenza-like virus circulation in USA dogs prior to 2004. Vet J. 191(3): 312-316.

Anderson TC, Crawford PC, Dubovi EJ, Gibbs EP and Hernandez JA 2013. Prevalence of and exposure factors for seropositivity to H3N8 canine influenza virus in dogs with influenza-like illness in the United States. J Am Vet Med Assoc. 242(2): 209-216.

Anderson TC, Crawford PC, Katz JM, Dubovi EJ, Landolt G and Gibbs EP 2012b. Diagnostic performance of the canine influenza A virus subtype H3N8 hemagglutination inhibition assay. J Vet Diagn Invest. 24(3): 499-508.

Barrell EA, Pecoraro HL, Torres-Henderson C, Morley PS, Lunn KF and Landolt GA 2010. Seroprevalence and risk factors for canine H3N8 influenza virus exposure in household dogs in Colorado. J Vet Intern Med. 24(6): 1524-1527.

Beeler E 2009. Influenza in dogs and cats. Vet Clin North Am Small Anim Pract. 39(2): 251-264.

Boliar S, Stanislawek W and Chambers TM 2006. Inability of kaolin treatment to remove nonspecific inhibitors from equine serum for the hemagglutination inhibition test against equine H7N7 influenza virus. J Vet Diagn Invest. 18(3): 264-267.

Bunpapong N, Nonthabenjawan N, Chaiwong S, Tangwangvivat R, Boonyapisitsopa S, Jairak W, Tuanudom R, Prakairungnamthip D, Suradhat S, Thanawongnuwech R and Amonsin A 2014. Genetic characterization of canine influenza A virus (H3N2) in Thailand. Virus Genes. 48(1): 56-63.

CDC 2010. "Subject: 2009 H1N1 Flu" (online). Available: http://www.cdc.gov/h1n1flu/.

Chang CP, New AE, Taylor JF and Chiang HS 1976. Influenza virus isolations from dogs during a human epidemic in Taiwan. Int J Zoonoses. 3(1): 61-64.

Cockburn WC, Delon PJ and Ferreira W 1969. Origin and progress of the 1968-69 Hong Kong influenza epidemic. Bull World Health Organ. 41(3): 345-348.

Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM and Donis RO 2005. Transmission of equine influenza virus to dogs. Science. 310(5747): 482-485.

Crombie DL, Fleming DM, Cross KW and Lancashire RJ 1995. Concurrence of monthly variations of mortality related to underlying cause in Europe. J Epidemiol Community Health. 49(4): 373-378.

Daly JM, Blunden AS, Macrae S, Miller J, Bowman SJ, Kolodziejek J, Nowotny N and Smith KC 2008. Transmission of equine influenza virus to English foxhounds. Emerg Infect Dis. 14(3): 461-464.

Damiani AM, Kalthoff D, Beer M, Muller E and Osterrieder N 2012. Serological survey in dogs and cats for influenza A(H1N1)pdm09 in Germany. Zoonoses Public Health. 59(8): 549-552.

Dapat IC, Dapat C, Baranovich T, Suzuki Y, Kondo H, Shobugawa Y, Saito R, Suzuki H and Japanese Influenza Collaborative Study G 2012. Genetic characterization of human influenza viruses in the pandemic (2009-2010) and post-pandemic (2010-2011) periods in Japan. PLoS One. 7(6): e36455.

De Benedictis P, Anderson TC, Perez A, Viale E, Veggiato C, Tiozzo Caenazzo S, Crawford PC and Capua I 2010. A diagnostic algorithm for detection of antibodies to influenza A viruses in dogs in Italy (2006-2008). J Vet Diagn Invest. 22(6): 914-920.

de Silva UC, Warachit J, Waicharoen S and Chittaganpitch M 2009. A preliminary analysis of the epidemiology of influenza A(H1N1)v virus infection in Thailand from early outbreak data, June-July 2009. Euro Surveill. 14(31).

Donaldson GC and Keatinge WR 2002. Excess winter mortality: influenza or cold stress? Observational study. BMJ. 324(7329): 89-90.

Dubovi EJ 2010. Canine influenza. Vet Clin North Am Small Anim Pract. 40(6): 1063-1071.

Dubovi EJ and Njaa BL 2008. Canine influenza. Vet Clin North Am Small Anim Pract. 38(4): 827-835, viii.

Dundon WG, De Benedictis P, Viale E and Capua I 2010. Serologic evidence of pandemic (H1N1) 2009 infection in dogs, Italy. Emerg Infect Dis. 16(12): 2019-2021.

Edwards S 2006. OIE laboratory standards for avian influenza. Dev Biol (Basel). 124: 159-162.

Fang Q, Gao Y, Chen M, Guo X, Yang X, Yang X and Wei L 2014. Molecular epidemiology and evolution of A(H1N1)pdm09 and H3N2 virus during winter 2012-2013 in Beijing, China. Infect Genet Evol. 26: 228-240.

Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr., Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI and Cox NJ 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 325(5937): 197-201.

Gerhard W and Webster RG 1978. Antigenic drift in influenza A viruses. I. Selection and characterization of antigenic variants of A/PR/8/34 (HON1) influenza virus with monoclonal antibodies. J Exp Med. 148(2): 383-392.

Harder TC and Vahlenkamp TW 2010. Influenza virus infections in dogs and cats. Vet Immunol Immunopathol. 134(1-2): 54-60.

Hasiri MA, Nazifi S, Mohsenifard E and Ansari-Lari M 2012. Seroprevalence of avian H9N2 influenza virus in a population of Iranian domestic dogs. J Bacsic Appl Sci. 8: 339-343.

Haukenes G and Hjeltnes BK 1991a. Heterogeneity in the reactivity of various groups of IgM antibodies with kaolin. Biologicals. 19(1): 49-52.

Haukenes G and Hjeltnes BK 1991b. Kinetics of the binding of immunoglobulins, antibodies and virus haemagglutination inhibitors to kaolin. Biologicals. 19(1): 31-35.

Hayward JJ, Dubovi EJ, Scarlett JM, Janeczko S, Holmes EC and Parrish CR 2010. Microevolution of canine influenza virus in shelters and its molecular epidemiology in the United States. J Virol. 84(24): 12636-12645.

Hirst GK 1942. The Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination. J Exp Med. 75(1): 49-64.

Holt DE, Mover MR and Brown DC 2010. Serologic prevalence of antibodies against canine influenza virus (H3N8) in dogs in a metropolitan animal shelter. J Am Vet Med Assoc. 237(1): 71-73.

Houser RE and Heuschele WP 1980. Evidence of prior infection with influenza A/Texas/77 (H3N2(virus in dogs with clinical parainfluenza. Can J Comp Med. 44(4): 396-402.

Ison MG and Lee N 2010. Influenza 2010-2011: lessons from the 2009 pandemic. Cleve Clin J Med. 77(11): 812-820.

Jirjis FF, Deshpande MS, Tubbs AL, Jayappa H, Lakshmanan N and Wasmoen TL 2010. Transmission of canine influenza virus (H3N8) among susceptible dogs. Vet Microbiol. 144(3-4): 303-309.

Kilbourne ED and Kehoe JM 1975. Demonstration of antibodies to both hemagglutinin and neuraminidase antigens of H3N2 influenza A virus in domestic dogs. Intervirology. 6(4-5): 315-318.

Kim H, Song D, Moon H, Yeom M, Park S, Hong M, Na W, Webby RJ, Webster RG, Park B, Kim JK and Kang B 2013. Inter- and intraspecies transmission of canine influenza virus (H3N2) in dogs, cats, and ferrets. Influenza Other Respir Viruses. 7(3): 265-270.

Kirkland PD, Finlaison DS, Crispe E and Hurt AC 2010. Influenza virus transmission from horses to dogs, Australia. Emerg Infect Dis. 16(4): 699-702.

Komadina N, McVernon J, Hall R and Leder K 2014. A historical perspective of influenza A(H1N2) virus. Emerg Infect Dis. 20(1): 6-12.

Kruth SA, Carman S and Weese JS 2008. Seroprevalence of antibodies to canine influenza virus in dogs in Ontario. Can Vet J. 49(8): 800-802.

Lee C, Song D, Kang B, Kang D, Yoo J, Jung K, Na G, Lee K, Park B and Oh J 2009. A serological survey of avian origin canine H3N2 influenza virus in dogs in Korea. Vet Microbiol. 137(3-4): 359-362.

Li S, Shi Z, Jiao P, Zhang G, Zhong Z, Tian W, Long LP, Cai Z, Zhu X, Liao M and Wan XF 2010. Avian-origin H3N2 canine influenza A viruses in Southern China. Infect Genet Evol. 10(8): 1286-1288.

Lin D, Sun S, Du L, Ma J, Fan L, Pu J, Sun Y, Zhao J, Sun H and Liu J 2012. Natural and experimental infection of dogs with pandemic H1N1/2009 influenza virus. J Gen Virol. 93(Pt 1): 119-123.

Mancini DA, Mendonca RM, Pereira AS, Kawamoto AH, Vannucchi CI, Pinto JR, Mori E and Mancini Filho J 2012. Influenza viruses in adult dogs raised in rural and urban areas in the state of Sao Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 54(6): 311-314.

Nicholson KG, Wood JM and Zambon M 2003. Influenza. Lancet. 362(9397): 1733-1745.

Nikitin A, Cohen D, Todd JD and Lief FS 1972. Epidemiological studies of A-Hong Kong-68 virus infection in dogs. Bull World Health Organ. 47(4): 471-479.

Payungporn S, Crawford PC, Kouo TS, Chen LM, Pompey J, Castleman WL, Dubovi EJ, Katz JM and Donis RO 2008. Influenza A virus (H3N8) in dogs with respiratory disease, Florida. Emerg Infect Dis. 14(6): 902-908.

Payungporn S, Panjaworayan N, Makkoch J and Poovorawan Y 2010. Molecular characteristics of the human pandemic influenza A virus (H1N1). Acta Virol. 54(3): 155-163.

Peacey M, Hall RJ, Sonnberg S, Ducatez M, Paine S, Nicol M, Ralston JC, Bandaranayake D, Hope V, Webby RJ and Huang S 2010. Pandemic (H1N1) 2009 and seasonal influenza A (H1N1) co-infection, New Zealand, 2009. Emerg Infect Dis. 16(10): 1618-1620.

Pecoraro HL, Lee JS, Achenbach J, Nelson S, Jr. and Landolt GA 2012. Seroprevalence of canine influenza virus (H3N8) in Iditarod racing sled dogs. Can Vet J. 53(10): 1091-1094.

Poovorawan Y, Pyungporn S, Prachayangprecha S and Makkoch J 2013. Global alert to avian influenza virus infection: from H5N1 to H7N9. Pathog Glob Health. 107(5): 217-223.

Potter CW 2001. A history of influenza. J Appl Microbiol. 91(4): 572-579.

Prachayangprecha S, Makkoch J, Suwannakarn K, Vichaiwattana P, Korkong S, Theamboonlers A and Poovorawan Y 2013. Epidemiology of seasonal influenza in Bangkok between 2009 and 2012. J Infect Dev Ctries. 7(10): 734-740.

Reichert TA, Simonsen L, Sharma A, Pardo SA, Fedson DS and Miller MA 2004. Influenza and the winter increase in mortality in the United States, 1959-1999. Am J Epidemiol. 160(5): 492-502.

Rivailler P, Perry IA, Jang Y, Davis CT, Chen LM, Dubovi EJ and Donis RO 2010. Evolution of canine and equine influenza (H3N8) viruses co-circulating between 2005 and 2008. Virology. 408(1): 71-79.

Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, Fukuda K, Cox NJ and Katz JM 1999. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol. 37(4): 937-943.

Schrauwen EJ, Herfst S, Chutinimitkul S, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Kuiken T and Fouchier RA 2011. Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment. Emerg Infect Dis. 17(2): 200-208.

Seiler BM, Yoon KJ, Andreasen CB, Block SM, Marsden S and Blitvich BJ 2010. Antibodies to influenza A virus (H1 and H3) in companion animals in Iowa, USA. Vet Rec. 167(18): 705-707.

Serra VF, Stanzani G, Smith G and Otto CM 2011. Point seroprevalence of canine influenza virus H3N8 in dogs participating in a flyball tournament in Pennsylvania. J Am Vet Med Assoc. 238(6): 726-730.

Shih SR, Chen GW, Yang CC, Yang WZ, Liu DP, Lin JH, Chiu SC, Chen HY, Tsao KC, Huang CG, Huang YL, Mok CK, Chen CJ, Lin TY, Wang JR, Kao CL, Lin KH, Chen LK, Eng HL, Liu YC, Chen PY, Lin JS, Wang JH, Lin CW, Chan YJ, Lu JJ, Hsiung CA, Chen PJ and Su IJ 2005. Laboratory-based surveillance and molecular epidemiology of influenza virus in Taiwan. J Clin Microbiol. 43(4): 1651-1661.

Shortridge KF and Lansdell A 1972. Serum inhibitors of A 2 -Hong Kong influenza virus haemagglutination. Microbios. 6(24): 213-219.

Song D, Kang B, Lee C, Jung K, Ha G, Kang D, Park S, Park B and Oh J 2008. Transmission of avian influenza virus (H3N2) to dogs. Emerg Infect Dis. 14(5): 741-746.

Song D, Moon HJ, An DJ, Jeoung HY, Kim H, Yeom MJ, Hong M, Nam JH, Park SJ, Park BK, Oh JS, Song M, Webster RG, Kim JK and Kang BK 2012. A novel reassortant canine H3N1 influenza virus between pandemic H1N1 and canine H3N2 influenza viruses in Korea. J Gen Virol. 93(Pt 3): 551-554.

Song DS, An DJ, Moon HJ, Yeom MJ, Jeong HY, Jeong WS, Park SJ, Kim HK, Han SY, Oh JS, Park BK, Kim JK, Poo H, Webster RG, Jung K and Kang BK 2011. Interspecies transmission of the canine influenza H3N2 virus to domestic cats in South Korea, 2010. J Gen Virol. 92(Pt 10): 2350-2355.

Song QQ, Zhang FX, Liu JJ, Ling ZS, Zhu YL, Jiang SJ and Xie ZJ 2013. Dog to dog transmission of a novel influenza virus (H5N2) isolated from a canine. Vet Microbiol. 161(3-4): 331-333.

Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Pariyothorn N, Payungporn S, Theamboonlers A, Chutinimitkul S, Thanawongnuwech R and Poovorawan Y 2006. Fatal avian influenza A H5N1 in a dog. Emerg Infect Dis. 12(11): 1744-1747.

Stephenson I and Zambon M 2002. The epidemiology of influenza. Occup Med (Lond). 52(5): 241-247.

Su S, Li HT, Zhao FR, Chen JD, Xie JX, Chen ZM, Huang Z, Hu YM, Zhang MZ, Tan LK, Zhang GH and Li SJ 2013. Avian-origin H3N2 canine influenza virus circulating in farmed dogs in Guangdong, China. Infect Genet Evol. 14: 444-449.

Sun Y, Shen Y, Zhang X, Wang Q, Liu L, Han X, Jiang B, Wang R, Sun H, Pu J, Lin D, Xia Z and Liu J 2014. A serological survey of canine H3N2, pandemic H1N1/09 and human seasonal H3N2 influenza viruses in dogs in China. Vet Microbiol. 168(1): 193-196.

Sun Y, Sun S, Ma J, Tan Y, Du L, Shen Y, Mu Q, Pu J, Lin D and Liu J 2013. Identification and characterization of avian-origin H3N2 canine influenza viruses in northern China during 2009-2010. Virology. 435(2): 301-307.

Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE and Fanning TG 1997. Initial genetic characterization of the 1918 "Spanish" influenza virus. Science. 275(5307): 1793-1796.

Thontiravong A, Kitikoon P, Wannaratana S, Tantilertcharoen R, Tuanudom R, Pakpinyo S, Sasipreeyajan J, Oraveerakul K and Amonsin A 2012. Quail as a potential mixing vessel for the generation of new reassortant influenza A viruses. Vet Microbiol. 160(3-4): 305-313.

Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA and Donis RO 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog. 9(10): e1003657.

Webby R, Hoffmann E and Webster R 2004. Molecular constraints to interspecies transmission of viral pathogens. Nat Med. 10(12 Suppl): S77-81.

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

WHO 2013. "Subject: Influenza virus infections in humans" (online). Available: http://www.who.int/influenza/GIP_InfluenzaVirusInfectionsHumans_Jul13.pdf.

Widelock D, Klein S, Peizer LR and Simonovic O 1958. Laboratory analysis of 1957-1958 influenza outbreak (A/Japan) in New York City. I. Preliminary report on seroepidemiologic investigation and variant A/Japan isolate. J Am Med Assoc. 167(5): 541-543.

Yoon KJ, Cooper VL, Schwartz KJ, Harmon KM, Kim WI, Janke BH, Strohbehn J, Butts D and Troutman J 2005. Influenza virus infection in racing greyhounds. Emerg Infect Dis. 11(12): 1974-1976.

Zhan GJ, Ling ZS, Zhu YL, Jiang SJ and Xie ZJ 2012. Genetic characterization of a novel influenza A virus H5N2 isolated from a dog in China. Vet Microbiol. 155(2-4): 409-416.

Zhao F, Li S, Zhou D, Chen N, Zhang Y, Qi W, Jiao P, Liao M, Tong G and Zhang G 2011. Seroprevalence of avian origin H3N2 canine influenza virus infection in pet dogs in Shenzhen, China. Afr J Microbiol Res 5(32): 5960-5963.

APPENDIX

Reagents and preparations

1. Phosphate Buffer Saline (PBS)

Sodium chloride (NaCl)	8.5	g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15	g
Monosodium dihydrogen phosphate (NaH_2PO_4)	0.2	g
Distilled water	1000	ml

2. Alsever’s solution

Dextrose	10.25	g
Sodium citrate	4	g
Sodium chloride	2.1	g
Citric acid	0.275	g
Distilled water	500	ml

Sterilize immediately by autoclave

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Reagents and preparations for NP-ELISA test

- Kit Components for NP-ELISA test
    1. Microplates coated with Ag A
    2. Concentrated Conjugate (10X)
    3. Positive control
    4. Negative control
    5. Dilution Buffer 3
    6. Dilution Buffer 2
    7. Wash Concentrate (20X)
    8. Substrate solution
    9. Stop Solution (H_2SO_4 0.5 M)

- Wash solution preparations

Prepare the wash solution (1X) by diluting the wash concentrate (20X) in distilled water.

- Conjugate 1X preparations

Prepare the conjugate 1X by diluting the concentrated conjugate (10X) to 1/10 in Dilution Buffer 3

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NP-ELISA procedure

Using ID Screen® Influenza A Antibody Competition ELISA kit (ID VET, Montpellier, France), following the manufacturer's instructions

1. For sera preparation, dog sera sample was prepared by diluting 10 µl of each sera sample with 90 µl of Dilution buffer 2 before beginning the assay.
2. After the process of sera preparation, each prepared sample was incubated in 96-well microplates coated with Antigen A (Ag A) at 37°C (± 2°C) for 1 hour ± 5 min.
3. Then empty the wells, wash each well for 5 times with 300 µl of the wash solution/wash.
4. Add 50 µl of the conjugate 1X to each well and incubate at 21°C (± 5°C) for 30 min ± 2 min.
5. Then empty the wells, wash each well for 3 times with 300 µl of the wash solution/wash.
6. After that, add 50 µl of the substrate solution to each well, followed by incubation for 10 min ± 1 min at 21°C (± 5°C) in the dark room.
7. Add 50 µl of the stop solution to each well and before
8. Read and record the O.D. at 450 nm.
9. Both positive and negative controls were included.

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Interpretation of the NP-ELISA results

The ELISA results were interpreted by the competition percentage (competition %). The competition percentage for each sample was calculated from the formula:

Competition % = (OD specimen / OD negative control) × 100

Sera samples with the competition percentage less than or equal to 45% were considered positive, those of between 45% and 50% were considered doubtful and those of greater than or equal to 50% were considered negative.

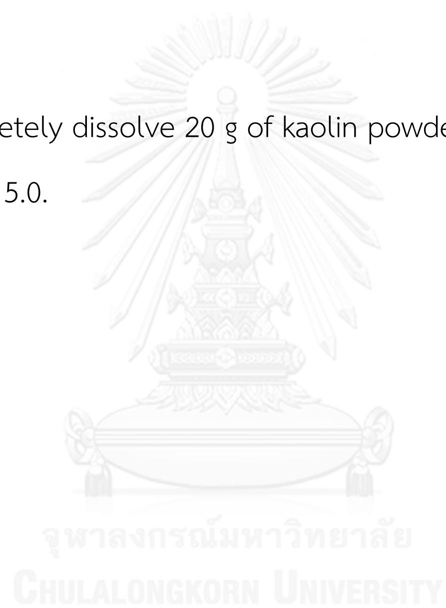
Reagents for standardization of sera treatment and HI test

1. RDE

Completely dissolve the product in 20 ml of sterile physiological saline. This solution should be used immediately.

2. 20% Kaolin

Completely dissolve 20 g of kaolin powder in 80 ml of water and adjust pH to 3.5 - 5.0.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Preparations for standardization of sera treatment and HI test

- Preparation of standardized control antigens for the HI test and back titration. Each control antigen must be standardized to contain 8 HAU per 50 ml.
- Preparation of packed turkey RBCs for the sera treatment and HI test.
  1. Collect 5 ml of turkey blood in 5 ml of Alsever's solution (a ratio of 1 part blood to 1 part Alsever's solution) and mix gently.
  2. Centrifuge at 1500 rpm for 10 minutes at 25°C.
  3. Discard the supernatant using a 1,000 microliter pipette. Be careful to not disturb the pellet of RBCs. After that, add 10 ml of 1X PBS and mix gently by inversion. Then Centrifuge at 1500 rpm for 10 minutes at 25°C.
  4. Repeat two times as in step 3.
  5. Discard the remained supernatant using a 1,000 microliter pipette. Aspirate the remaining supernatant with a 1,000 microliter pipette for final packed turkey RBCs. Keep packed turkey RBCs in 4°C.

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Sera treatment procedure

1. Under sterile conditions, add specimen sera to the RDE solution in the ratio of 1:3, and mix thoroughly.
2. Incubate the mixture at 37°C for 20 hours for the reaction to occur.
3. Then heat at 56°C for 1 hour to inactivate the RDE.
4. After that, absorbed RDE-treated sera with 100 μl of 50% Turkey red blood cells (TRBCs) and incubated at room temperature for 1 hour.
5. Use the treated sera in the HI test for influenza virus.

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HI test procedure

1. After the process of sera treatment, each sera sample was serially two-fold diluted with phosphate-buffered saline (PBS) in 96-well micro-titer plates
2. Add 50 microliter of each virus (8 HAU per 50 microliter) to all wells of plates containing the sets of treated sera.
3. Both positive (CIV seropositive canine sera) and negative (CIV seronegative canine sera) sera controls were included.
4. Mix the contents of the plates and incubate at room temperature for 45 minutes.
5. Then add 0.5% of turkey RBCs suspension to all wells of plates and mix the contents of the plates.
6. Cover the plates and allow the RBCs to settle at room temperature for 1 hour.
7. Record the HI titers and interpret the results.
8. All sera were tested in duplicate.

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Interpretation of the HI results

The HI titer was determined by the reciprocal of the last dilution that shows no agglutination and reported as geometric means. Samples with a titer ≥ 40 were considered positive.

VITA

Miss Sunicha Chanvatik was born on September 26, 1985 in Bangkok, Thailand. She got the Degree of Veterinary Sciences (D.V.M.) from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2009. After that, she enrolled the Master degree of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2011.