Original Article

Seroprevalence of Influenza A in Domestic Dogs in Thailand, 2013

Sunicha Chanvatik[1,2] Ratanaporn Tangwangvivat[1,2] Supassama Chaiyawong[1,2]

Duangduean Prakairungnamthip[3] Ranida Tuanudom[1] Aunyaratana Thontiravong[1,3]

Alongkorn Amonsin[1,2*]

Abstract

Canine influenza viruses (CIVs) have been reported in dogs worldwide. Due to the relatively high risk of influenza A virus (IAV) infections in dogs and humans through dog-human interface, the seroprevalence of IAVs in dogs in Thailand was surveyed. A serological survey of IAV infections in dogs in Thailand was conducted from December 2012 to November 2013 using ELISA assays for detecting anti-nucleoprotein (NP) antibodies (NP-ELISA) and hemagglutination inhibition (HI) tests for detecting HA-specific antibodies using canine H3N2 (CIV-H3N2), human pandemic H1N1 2009 (Human-pH1N1) and human seasonal H3N2 (Human-H3N2) as viral antigens. A total of 9,891 serum samples were obtained from healthy and sick dogs of any breed, age and gender. The samples were collected weekly for 52 weeks from 467 veterinary clinics and hospitals located in 15 central provinces of Thailand. The survey of IAV infections in dogs showed that 164 (1.66 %) of the 9,891 serum samples had antibodies to IAV by NP-ELISA. Of the 164 positive samples from NP-ELISA, 12.20%, 23.17% and 1.22% had HI titers for CIV-H3N2, Human-pH1N1 virus and Human-H3N2, respectively. Thai dogs showed the highest seropositivity rate for Human-pH1N1. These results suggest that Human-pH1N1 is the dominant subtype circulating in Thai pet dogs. Our study highlights the risk of pH1N1 and CIV infections in dogs. Routine monitoring of IAV infections in dogs should be conducted.

Keywords: dogs, influenza A virus, seroprevalence, Thailand

[1]*Emerging and Re-emerging Infectious Diseases in Animals, Center of Excellence, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand*

[2]*Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand*

[3]*Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand*

***Correspondence**: Alongkorn.A@chula.ac.th*

Introduction

Canine Influenza Virus (CIV) causes influenza outbreaks in dog populations worldwide. CIV subtype H3N8 (canine-H3N8), for example, was first isolated in January 2004 from racing greyhounds in the US (Crawford et al., 2005). Since then, it has been reported in several US states (Crawford et al., 2005; Holt et al., 2010; Payungporn et al., 2008; Serra et al., 2011). In Asia, CIV subtype H3N1 (canine-H3N1) has been reported in Korea, while CIV subtype H3N2 (canine-H3N2) has been reported in both Korea and China (Li et al., 2010; Song et al., 2008; Song et al., 2012). In 2012, canine-H3N2 was first isolated in Thailand from a pet dog (Bunpapong et al., 2014). At present, at least six subtypes of the influenza A virus (IAV) have been reported to infect dogs worldwide; H1N1, H3N8, H3N2, H5N1, H5N2 and H3N1 (Songserm et al., 2006).

In experimental setting, dog-to-dog transmissions of CIV subtypes H5N2, H3N2 and H3N8 have been documented (Jirjis et al., 2010; Song et al., 2013; Songserm et al., 2006). Furthermore, there is evidence of cross-species transmission of canine-H3N2 from dogs to cats (Kim et al., 2013). Since its emergence, pandemic H1N1 2009 (pH1N1) influenza virus has been circulating in human populations and has also been reported in dogs since November 2009 (Lin et al., 2012a). Evidence of the reassortment between CIV H3N2 and pH1N1 was documented (Song et al., 2012). Due to the relatively high risk of infection from dog-human interface, the monitoring of IAV infections in dogs has been routinely conducted. In Thailand, information on the serological status of IAVs in dogs is limited. In this study, a serological survey of IAV infections in dogs was conducted from December 2012 to November 2013 using an ELISA assay for detecting anti-nucleoprotein (NP) antibodies (NP-ELISA) and hemagglutination inhibition (HI) test using canine H3N2 (CIV-H3N2), human pandemic H1N1 2009 (Human-pH1N1) and human seasonal H3N2 (Human-H3N2) as viral antigens.

Materials and Methods

Sample Collection: Canine blood samples were obtained from private and university veterinary diagnostic laboratories located in the vicinity and city of Bangkok, Thailand. The samples were collected weekly for 52 weeks from 467 veterinary clinics and hospitals in 15 provinces. Veterinary diagnostic laboratories were selected based on geographic locations, collaboration and number of cases. In total, 9,891 serum samples were obtained from healthy and sick dogs of any breed, age and gender. After collection, all samples were stored and transported at 4^{o}C to the laboratory of the Faculty of Veterinary Science, Chulalongkorn University, where the serum samples were aliquot and kept at -20^{o}C until further examined.

Detection of Antibodies to IAVs : All 9,891 serum samples were screened for the presence of antibodies against the influenza A nucleoprotein (NP) using ID Screen® Influenza A Antibody Competition ELISA kits (ID VET, Montpellier, France) according to the manufacturer's instructions (Dundon et al., 2010). ID Screen® Influenza A Antibody Competition ELISA kits (ID VET) are used as diagnostic tools based on the competitive principle and are designed to detect antibodies directed against the internal nucleoproteins of IAVs. They can be used on multiple species, including dogs (De Benedictis et al., 2010). For serum preparation, the samples were diluted with a dilution buffer. Prepared samples were incubated in 96-well microplates coated with antigen A at 37°C for 1 hr, then each well was washed for 5 times with 300 µl of the wash solution/wash. Then, 50 µl of the recommended dilution of conjugate was added to each well and the plate was incubated for 30 min at 25°C. After washing for three times with the wash solution, 50 µl of the substrate solution was added to each well, followed by incubation in the dark room for 10 min at 25°C. Finally, 50 µl of the stop solution was added before reading and recording the O.D. at 450 nm. ELISA scores were interpreted based on competition percentage. The competition percentage for each sample was calculated using the formula: Competition % = (OD specimen / OD negative control) × 100. Serum samples with a competition percentage less than or equal to 45% were considered positive, while those between 45% and 50% were considered doubtful and those greater than or equal to 50% were considered negative.

Hemagglutination Inhibition Assay: All positive and suspected ELISA samples were further examined by an HI test using IAVs including CIV-H3N2 [A /canine /Thailand/ CU-DC5299/ 2012(H3N2)], Human-pH1N1 [A/Thailand/CUH1N1/2012(p H1N1)] and Human-H3N2 [A/Thailand/ CUH3N2/2012(H3N2)] to determine specific antibodies against influenza subtype infections. The HI tests were performed as previously described (Bunpapong et al., 2014). In brief, the serum samples were pretreated with receptor-destroying enzymes (Denka Seiken Co., Ltd., Tokyo, Japan), and then absorbed with 50% turkey red blood cells (TRBC). Each treated serum sample was serially two-fold diluted with phosphate-buffered saline (PBS) and incubated with 50 µl of 8 hemagglutination units (HAU) of each virus for 45 min at room temperature. Fifty µl of 0.5% suspension of turkey RBCs was added into each well and the plate was incubated at room temperature. The HI titer was determined by the reciprocal of the last dilution that showed no agglutination and was reported as geometric means. Samples with HI titers ≥ 40 were considered positive as evidence of previous exposure (Eichelberger et al., 2008).

Results

In this study, the serum samples were collected from dogs in Bangkok (87.19%, 8,624/9,891), Bangkok's vicinity (9.82%, 971/9,891), and provinces in 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 (0.16%, 16/9,891) Thailand. Results from our 52-week survey showed that 164 (1.66 %) of the 9,891 samples tested positive for antibodies to IAVs by NP-ELISA (Table 1). It is noteworthy that IAV infections in the dogs were found throughout the year. The highest seropositivity rate of IAVs in dogs

occurred in December 2012 (2.9%) and January 2013 (2.73%), while the lowest was found in October 2013 (0.89%) (Fig 1A).

To determine the anti-HA specific antibodies, the 164 positive and suspected samples from NP-ELISA testing were examined by the HI test to determine anti-canine influenza H3 and anti-human influenza H1 & H3 specific antibodies. The samples with an HI titer ≥ 40 were considered positive, as they indicated previous exposure to IAVs. Results showed that 60 out of the 164 NP-ELISA positive and suspected samples were positive by the HI test. The serum samples were specific to CIV-H3N2 (n=20), Human-H3N2 (n=2) and Human-pH1N1 (n=38). The GMT HI positive titers against CIV-H3N2, Human-H3N2 and Human-pH1N1 were 80.00 (95%CI, 52.42-122.10), 40.00 (95%CI, 40.00-40.00) and 156.71 (95%CI, 118.76-208.21), respectively. Interestingly, evidence from our HI test results showed that serum sample positive for two IAV subtypes occurred, including infections between CIV-H3N2 and Human-pH1N1 (1.22%) and infections between Human-H3N2 and Human-pH1N1 (1.22%).

Table 1 Prevalence of antibodies against influenza A virus in Thai dogs, 2012-2013

Serological assays	Positive/ tested	% positive (95% CI)
ELISA test		
Positive, S/N% ≤45%	129/9,891	1.30%
Suspected,	35/9,891	0.35%
Positive and suspected	164/9,891	1.66%
Negative, S/N%≥50%	9,727/9,891	98.34%
HI test		
CIV H3N2	20/164	12.20% (52.42-122.10)
Human-H3N2	2/164	1.22% (40.0-40.0)
Human-pH1N1	38/164	23.17% (118.76-208.21)

The percentages of HI positive dog serum samples against CIV-H3N2, Human-H3N2 and Human-pH1N1 viruses by month (Fig 1B) were also analyzed. Human-pH1N1 seropositive dogs were observed all year round, except in May 2013. Similarly, CIV-H3N2 seropositive samples were observed throughout the year except in April, July and November 2013. In contrast, Human-H3N2 seropositive dogs were observed only in April and July 2013.

Discussion

CIV-H3N2 and Human-pH1N1 infected dogs have been reported in many countries in Asia, including China and Korea (Li et al., 2010; Lin et al., 2012b; Song et al., 2008; Su et al., 2013; Sun et al., 2013). Recently, CIV-H3N2 has first been isolated in Thailand from a pet dog (Bunpapong et al., 2014). Sporadic transmission and subclinical infections of Human-H1N1 and Human-H3N2 have been reported in dogs, suggesting that they can be infected with seasonal human influenza viruses (Chang et al., 1976; Houser and Heuschele, 1980; Sun et al., 2014). The emergence of avian and human influenza virus infections in dogs raises a public health concern that they may become intermediate hosts for the viruses, which might result in the emergence of a novel influenza virus through genetic reassortment.

In this study, a serological survey of IAV infections in dogs in Thailand was conducted. NP-ELISA results showed that 1.66% of the serum samples had antibodies to IAVs, which is lower than those reported in Italy, Korea and China. In Italy, NP-ELISA results showed that 3% of 964 dog serum samples had antibodies to IAVs (Dundon et al., 2010). In Korea, the serological evidence of IAV infections in dogs was reported in 2009 (19%) and 2010 (34.8%) (Lee et al., 2009; Songserm et al., 2006). In China, the seroprevalence of IAVs in pet dogs was 6.71% in 2011 (Zhao F et al., 2011) and 5.3% in 2013 (Su et al., 2013). Overall, the IAV seropositivity rate among dogs in Thailand is relatively low compared to other countries.

Possible explanations for this observation are the differences in sample collection periods and target animal populations. This study collected samples from both healthy and sick dogs throughout the year from animal hospitals that did not experience influenza outbreaks, while others obtained samples from dogs showing clinical signs of respiratory infection or collected samples after influenza outbreaks in their countries (Dundon et al., 2010; Lee et al., 2009; Zhao F et al., 2011). Several studies have suggested that human influenza occurs most frequently in winter, compared to other seasons (Potter, 2001). The morbidity rates among IAV-infected humans increase in winter due to cold weather and high humidity (Reichert et al., 2004). In this study, the highest IAV seropositive rate among dogs was found during the winter season in Thailand (December, 2.9%; January, 2.73%). These results correspond with those of other studies, suggesting that the prevalence of canine influenza increases in the winter season and correlates with human influenza prevalence in Thailand.

In addition to the ELISA results, our HI results indicated that dogs in Thailand showed antibodies against CIV-H3N2, Human-pH1N1 and Human-H3N2, suggesting previous viral exposure. It is noted that Thai dogs showed the highest seropositivity rate for Human-pH1N1 (23.17%), followed by CIV-H3N2 (12.20%) and Human-H3N2 (1.22%). Compared to the study in Korea, Thai dogs had a higher seropositivity rate for Human-pH1N1 and CIV-H3N2 (1.5% and 3.5%, respectively). The seropositivity rate for Human-H3N2 was comparable (1.2%) between Thai and Korean pet dogs (Sun et al., 2014). From our results, Human-pH1N1 was the dominant subtype found in Thai pet dogs, while CIV-H3N2 was dominant in Chinese pet dogs (Sun et al., 2014). Similar to China, CIV-H3N2 was dominant in Korean pet dogs (Sun et al., 2014). It is noteworthy that

CIV-H3N2 was first introduced to dogs in Thailand in 2012 and only 1 confirmed canine-H3N2 case was reported (Bunpapong et al., 2014). As CIV-H3N2 is less common, information related to immunity against CIV-H3N2 in Thai dog populations is very limited.

Previous studies of humans from June 2009 to July 2012 demonstrated that the predominant IAV subtype in humans in Thailand was Human-pH1N1, followed by Human-H3N2 and influenza B viruses (Prachayangprecha et al., 2013). Human-pH1N1 virus has been circulating in human populations and now it has become an endemic seasonal human influenza virus that is usually found to co-infect with Human-H3N2 virus (Poovorawan et al., 2013). Close contact between human and dog increases the risk of cross-species transmission. Consequently, it may be possible that humans can transmit pH1N1 to dogs, which would explain the highest seropositivity rate of pH1N1 among Thai dogs. However, to date, no Human-pH1N1 virus has been isolated from dogs in Thailand. On the other hand, only 1.2% of dogs were positive for Human-H3N2 virus by HI testing. Similarly, the results from serological surveys on dogs in China showed the lowest seropositivity for Human-H3N2 among Chinese dog populations, compared to other subtypes of IAVs (Sun et al., 2014). However, the percentage of seropositive dogs to Human-H3N2 virus was relatively low.

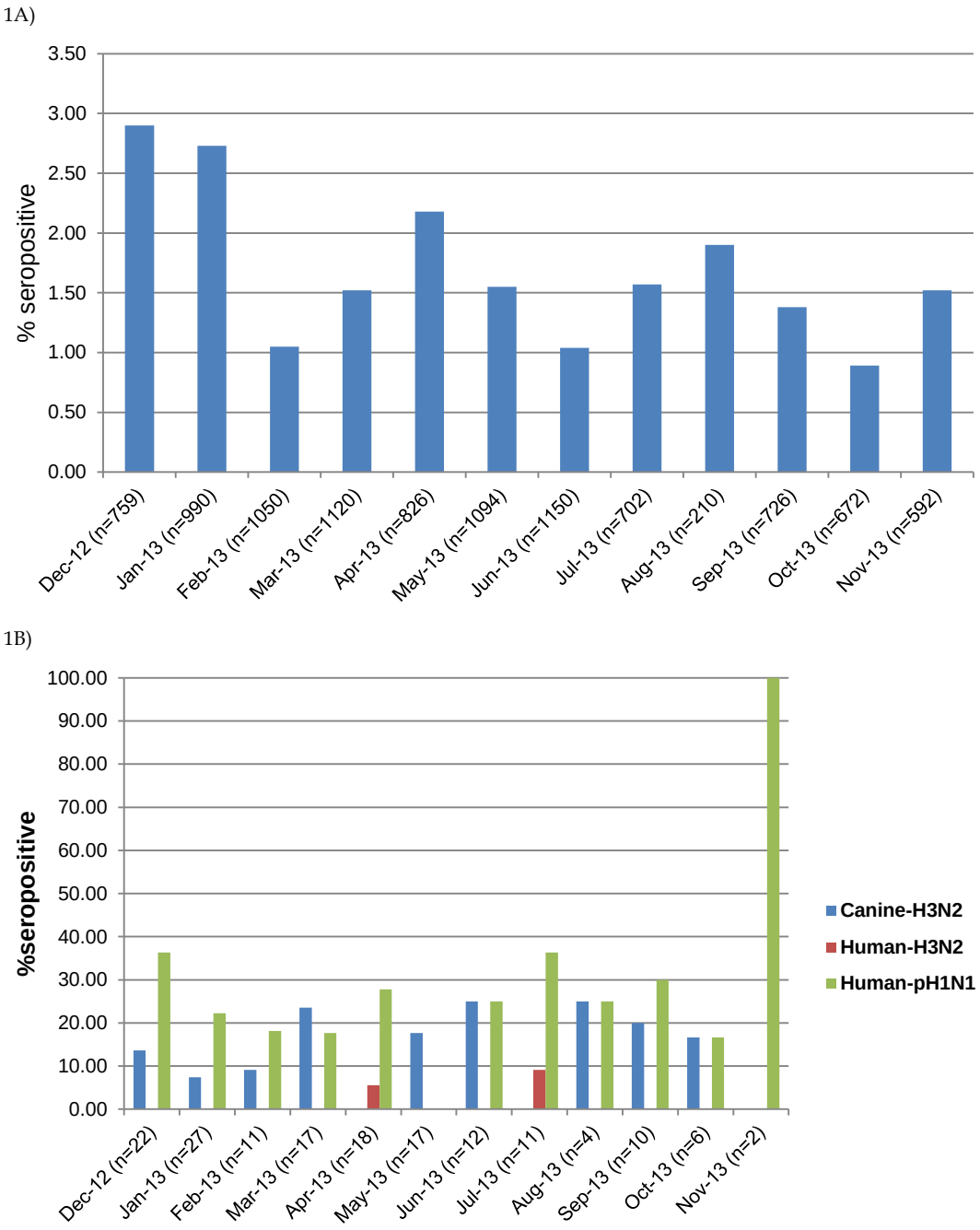

Figure 1 A). Percentage of NP-ELISA seropositive serum, by month. Samples were positive when ELISA competition % was less than 45%, and B) Percentage of HI seropositive serum samples from dogs against CIV-H3N2, Human-H3N2 and Human-pH1N1 viruses, by month. Samples were positive when titer was ≥ 40.

Co-infection with different IAVs in the same host may increase the risk for genetic reassortment, resulting in the exchange of gene segments to create novel reassortant IAVs (Peacey et al., 2010). Recently, H3N1 influenza virus, a reassortant between an avian-origin CIV-H3N2 and the pH1N1 viruses, was isolated from dogs in Korea (Song et al., 2012). In this study, based on the HI test results, 2 dogs showed antibodies against both CIV-H3N2 and Human-pH1N1 and 2 dogs had antibodies against both Human-H3N2 and Human-pH1N1. It is interesting to note that previous infection with two subtypes of IAVs was observed in Thai dogs. However, cross-reaction between both IAV subtypes should not be ruled out, even if it rarely occurs. Therefore, the risk 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 is the first report of the seroprevalence of CIV-H3N2, Human-pH1N1 and Human-H3N2 in dogs in Thailand. Our results showed that 1.66% of dogs in Thailand had IAV infections. Further investigation into anti-HA specific antibodies revealed that the dogs showed the highest seropositivity to Human-pH1N1. In fact seropositives to Human-pH1N1 could be detected all year round, but evidence of a seasonal pattern could not be concluded in the study. Therefore, the results from our study suggest that the risk of pH1N1 and CIV infections in dogs should not be ignored. Monitoring of IAV infection in dogs should be routinely conducted. Influenza prevention and control between dogs and humans should also be emphasized.

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บทคัดย่อ

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สุณิชา ชานวาทิก[1,2] รัตนพร ตั้งวังวิวัฒน์[1,2] สุพัศมา ไชยวงษ์[1,2]
ดวงเดือน ประกายรุ้งน้ำทิพย์[3] รนิดา ต่วนอุดม[1] อัญญรัตน์ ต้นธีรวงศ์[1,3] อลงกร อมรศิลป์[1,2]*

เชื้อไวรัสไข้หวัดใหญ่สุนัขทำให้เกิดโรคไข้หวัดใหญ่ในสุนัขทั่วโลก เนื่องจากสุนัขและคนมีความใกล้ชิดกัน จึงมีความเสี่ยงในการติดเชื้อระหว่างคนและสุนัข การศึกษาครั้งนี้เป็นการสำรวจทางซีรัมวิทยาของการติดเชื้อไวรัสไข้หวัดใหญ่ในสุนัขระหว่างเดือนธันวาคม พ.ศ. 2555 ถึงเดือนพฤศจิกายน พ.ศ. 2556 โดยใช้วิธี ELISA เพื่อตรวจภูมิคุ้มต่อโปรตีน NP (NP-ELISA) และวิธี hemagglutination inhibition test (HI test) เพื่อตรวจภูมิคุ้มที่จำเพาะต่อเชื้อ canine H3N2 (CIV-H3N2), human pandemic H1N1 2009 (Human-pH1N1) และ human seasonal H3N2 (Human-H3N2) การศึกษาครั้งนี้ได้เก็บตัวอย่างซีรัมจำนวน 9,891 ตัวอย่างจากสุนัขป่วยและสุนัขปกติ ทุกช่วงอายุ เพศ และพันธุ์ โดยเก็บตัวอย่างเป็นเวลา 52 สัปดาห์จากโรงพยาบาลสัตว์และคลินิกรักษาสัตว์จำนวน 467 แห่งใน 15 จังหวัด จากการตรวจตัวอย่างซีรัมเพื่อหาภูมิคุ้มต่อเชื้อไข้หวัดใหญ่โดย ID Screen® Influenza A Antibody Competition ELISA kits พบว่ามีสุนัขร้อยละ 1.66 (164/9,891) ที่มีภูมิคุ้มต่อเชื้อไวรัสไข้หวัดใหญ่ และเมื่อตรวจตัวอย่างซีรัมจำนวน 164 ตัวอย่างนี้ด้วยวิธี HI Test พบว่าสุนัขมีภูมิคุ้มต่อเชื้อไวรัสไข้หวัดใหญ่ที่จำเพาะต่อเชื้อ CIV-H3N2, Human-pH1N1 virus and Human-H3N2 คิดเป็นร้อยละ 12.20, 23.17 และ 1.22 ตามลำดับ โดยพบว่าสุนัขในประเทศไทยมีภูมิคุ้มที่จำเพาะต่อเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ (Human-pH1N1) มากที่สุด ผลการศึกษาครั้งนี้แสดงให้เห็นว่าเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ (Human-pH1N1) เป็นสายพันธุ์หลักที่ติดเชื้อวนเวียนอยู่ในประชากรสุนัขในประเทศไทย ซึ่งการศึกษาครั้งนี้แสดงให้เห็นความเสี่ยงในการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่และไข้หวัดใหญ่สุนัขในสุนัขในประเทศไทย ดังนั้นการตรวจติดตามการติดเชื้อไวรัสไข้หวัดใหญ่ในสุนัขจึงควรดำเนินการอย่างต่อเนื่อง

คำสำคัญ: สุนัข เชื้อไวรัสไข้หวัดใหญ่ อุบัติการณ์ทางซีรัมวิทยา ประเทศไทย

[1]ศูนย์เชี่ยวชาญเฉพาะทางโรคอุบัติใหม่และอุบัติซ้ำในสัตว์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนอังรีดูนังต์ เขตปทุมวัน กรุงเทพฯ ประเทศไทย 10330
[2]ภาควิชาสัตวแพทยสาธารณสุข คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนอังรีดูนังต์ เขตปทุมวัน กรุงเทพฯ ประเทศไทย 10330
[3]ภาควิชาจุลชีววิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนอังรีดูนังต์ เขตปทุมวัน กรุงเทพฯ ประเทศไทย 10330
***ผู้รับผิดชอบบทความ** E-mail: Alongkorn.A@chula.ac.th*