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Monitoring of influenza a viruses in Thailand and pathogenicity in dogs and guinea pigs

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MONITORING OF INFLUENZA A VIRUSES IN THAILAND AND PATHOGENICITY IN DOGS AND GUINEA PIGS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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การเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ ในสุนัขในประเทศไทย และศักยภาพการก่อโรคของเชื้อในสุนัขและหนูตะเภา

น.ส.รัตนพร ตั้งวังวิวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	MONITORING OF INFLUENZA A VIRUSES IN THAILAND AND PATHOGENICITY IN DOGS AND GUINEA PIGS
By	Miss Ratanaporn Tangwangvivat
Field of Study	Veterinary Public Health
Thesis Advisor	Professor ALONGKORN AMONSIN, D.V.M., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

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รัตนพร ตั้งวังวิวัฒน์ : การเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ ในสุนัขในประเทศไทย และศักยภาพการก่อโรคของเชื้อในสุนัขและหนูตะเภา. (MONITORING OF INFLUENZA A VIRUSES IN THAILAND AND PATHOGENICITY IN DOGS AND GUINEA PIGS) อ.ที่ปรึกษาหลัก : ศ. น.สพ.ดร.อลงกร อมรศิลป์

เชื้อไวรัสไข้หวัดใหญ่ (Influenza) เป็นสาเหตุหนึ่งของโรคทางระบบทางเดินหายใจในมนุษย์ และสัตว์หลายชนิด เช่น นก ม้า หมู สุนัข และแมว เนื่องจากสุนัขและแมวเป็นสัตว์ที่ใกล้ชิดกับมนุษย์ ดังนั้นการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ในสุนัขและแมวจึงมีความสำคัญ การศึกษาในวิทยานิพนธ์นี้มี 5 ขั้นตอน ประกอบด้วย ขั้นตอนที่ 1 การเฝ้าระวังโรคไข้หวัดใหญ่ในสุนัขและแมวในประเทศไทย ขั้นตอนที่ 2 การพิสูจน์ความรุนแรงของเชื้อไข้หวัดใหญ่สายพันธุ์ Thai CIV-H3N2 ในสุนัข โดยใช้วิธี intravenous pathogenicity test ขั้นตอนที่ 3 การศึกษาศักยภาพการก่อโรคของเชื้อไวรัสในสุนัข ขั้นตอนที่ 4 ในไก่ และขั้นตอนที่ 5 ในหนูตะเภา ผลการศึกษาในขั้นตอนที่ 1 พบว่า สุนัขร้อยละ 0.97 (9/932) มีภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่โดยวิธี NP-ELISA และ สุนัขร้อยละ 0.64 (6/932) และแมวร้อยละ 1.20 (1/79) มีภูมิคุ้มกันชนิดที่จำเพาะต่อเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ pandemic H1N1-2009 และพบว่าการติดเชื้อไข้หวัดใหญ่ในสุนัข มีลักษณะการติดเชื้อตามฤดูกาล สำหรับขั้นตอนที่ 2 การพิสูจน์ความรุนแรงของเชื้อไวรัส โดยใช้วิธี intravenous pathogenicity test พบว่าเชื้อไวรัสไข้หวัดใหญ่ในสุนัขชนิดนี้ เป็นชนิดที่มีความรุนแรงต่ำ อย่างไรก็ตามผลการศึกษาในขั้นตอนที่ 3 พบว่า เชื้อไวรัสนี้มีศักยภาพในการก่อโรคในสุนัขได้ ในขั้นตอนที่ 4 พบว่า เชื้อไวรัสนี้ไม่มีศักยภาพในการก่อโรคในไก่ ในขั้นตอนที่ 5 พบว่าเชื้อไวรัสนี้มีศักยภาพในการก่อโรคในหนู โดยหนูตะเภาที่ติดเชื้อจะแสดงอาการไม่รุนแรง แต่มีการตอบสนองทางภูมิคุ้มกัน โดยสรุปผลการศึกษา ได้แสดงข้อมูลที่เป็นประโยชน์ของการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ Thai CIV-H3N2 ในสุนัข และแสดงถึงศักยภาพในการก่อโรคของเชื้อไวรัสในสุนัขและหนูตะเภา สามารถนำมาใช้พัฒนาและวางแผนยุทธศาสตร์ในการควบคุมและป้องกันโรค การสำรวจเชื้อไข้หวัดใหญ่ในสุนัขและแมวอย่างต่อเนื่อง และควรนำหลักการสุขภาพหนึ่งเดียวมาใช้

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Ratanaporn Tangwangvivat : MONITORING OF INFLUENZA A VIRUSES IN THAILAND AND PATHOGENICITY IN DOGS AND GUINEA PIGS. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Influenza A virus causes respiratory disease in many species such as birds, horses, pigs, dogs and cats as well as humans. This thesis consists of 5 phases. Phases 1 was monitoring of canine influenza virus infection in Thailand. Phase 2 was determining the intravenous pathogenicity index of Thai canine influenza virus. Phase 3, 4 and 5 were investigating the pathogenicity of canine influenza viruses in dogs, chickens and guinea pigs, respectivly. Our results showed that 0.97% of canine serum samples (9/932) and 1.20% of feline serum sample (1/79) were tested positive for influenza A antibodies by NP-ELISA. Six serum samples (0.64%, 6/932) had HA specific antibodies against pandemic H1N1-2009 by HI assay. Seasonal pattern was also observed. In phase 2, Thai CIV-H3N2 was identified as LPAI based on intravenous pathogenicity test (IVPI), however in phase 3, the intra-species transmission of Thai CIV-H3N2 was confirmed. The CIV-H3N2 infected dogs showed significant clinical signs and H3 specific antibodies. In phase 4, the transmission of Thai CIV-H3N2 in chickens could not be efficiently detected in chickens. In phase 5, the CIV-H3N2 infected guinea pigs and developed mild clinical signs and H3 specific antibodies. In conclusion, our results provided useful information of CIV infection in dogs and cats and the pathogenicity of the Thai CIV-H3N2. These information could be used to develop a strategic plan for influenza prevention and control using One Health approach.

Field of Study: Veterinary Public Health Student's Signature .....
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Ratanaporn Tangwangvivat

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CHAPTER 1

INTRODUCTION

Influenza virus is a single-stranded, negative sense RNA virus of the family *Orthomyxoviridae* consisting of 8 gene segments (Webster et al., 1992). There are 4 types of influenza virus including type A, B, C and D or newly designated as genus alphainfluenza, betainfluenza, deltainfluenza and gammainfluenza viruses (ICTV, 2018). Influenza A virus (IAV) causes respiratory disease in several animal species such as horse, pig, dog, cat and avian as well as human. Influenza type B can infect in human, ferret and seal while influenza type C can infect human, dog and pig. Influenza type A has high genetic diversity and most virulent among three types causing widespread and sometimes severe respiratory diseases in humans and animals.

In 2004, the first canine influenza virus subtype H3N8 (CIV-H3N8) was reported in racing greyhound dogs with respiratory symptoms in Florida and nine other states in the US. The genetic analysis of the CIV-H3N8 revealed that the virus was closely related to influenza viruses subtype H3N8 in horse but not influenza viruses in human or avian species (Crawford et al., 2005; Zhang et al., 2007). Since 2004, there were several reports of CIV-H3N8 outbreaks in many countries such as England (Daly et al., 2008), Canada (Kruth et al., 2008), and Australia (Kirkland et al., 2010).

In Thailand, the first influenza virus subtype H5N1 (IAV-H5N1) infection in dogs was reported in 2004. This IAV-H5N1 infection in dogs was considered as spill over event and there was no intra-species transmission among dogs (Songserm et al., 2006).

Interestingly, in experimentally study of H5N1 infection in dogs, the animals showed subclinical signs and can shed the virus (Giese et al., 2008). Since 2007, the widespread of canine influenza virus subtype H3N2 (CIV-H3N2) outbreaks have been reported in Korea, China (Song et al., 2008; Li et al., 2010) and Thailand (Bunpapong et al., 2014). The experimental study of inter-species transmission of CIV-H3N2 from dogs to cats was also reported (Song et al., 2011a; Kim et al., 2013). As of 2014, at least 6 influenza subtypes have been reported to infect dogs 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).

To date, there are several canine influenza virus infections reported worldwide but there is limited information of canine influenza viruses in Thailand especially routine surveillance data on canine influenza virus infection in dogs in Thailand. The information gained from this study provided useful information on the status of canine influenza virus infection in dogs in Thailand from October 2012 to July 2015. Moreover, the pathogenicity of canine influenza virus in dogs and mammal models was investigated. The results in the thesis have provided supporting data for identifying zoonotic and pandemic potential viruses. The information of intra-species and inter-species transmission will help understanding influenza infection in dogs and other mammals. Overall, the results from this thesis will be useful for strategic planning for canine influenza prevention and control in the future.

Research questions

The research questions of the thesis are as following:

1. What is the occurrence of influenza A virus infection in dogs in Thailand?
2. What is/are the subtypes of canine influenza A viruses circulating in Thailand?
3. What is the pathogenicity of canine influenza A viruses in dogs?
4. What is the pathogenicity of canine influenza A viruses in guinea pigs?

Objectives

The objectives of this thesis are as following

1. To monitor influenza A virus infection in dogs in Thailand from October 2012 to July 2015
2. To determine the intravenous pathogenicity score of Thai canine influenza virus
3. To investigate the pathogenicity of canine influenza in dog
4. To investigate the pathogenicity of canine influenza in chickens

To investigate the pathogenicity of canine influenza in guinea pigs

CHAPTER II

LITERATURE REVIEW

2.1 Virology of influenza virus

Influenza virus is a single-stranded, negative sense RNA virus, belongs to *Orthomyxoviridae* family. The virus particle is 80-120 nm in diameter, spherical and filamentous forms containing 8 gene segments (Webster et al., 1992). Influenza viruses are classified into 4 types (A, B, C and D). Recently, genus of influenza viruses is classified and renamed into Alphainfluenzavirus, Betainfluenzvirus, Deltainfluenzavirus and Gammainfluenzavirus (ICTV, 2018). The most virulent type is influenza type A causing respiratory diseases in humans and many animal species such as horse, pig, dog, cat and avian. The influenza virus composes of 8 single RNA strands encoding for eleven proteins consisting of HA, NA, NP, M1, M2, NS1, NS2, PA, PB1, PB1-F2 and PB2. Influenza A virus can be classified into subtypes based on two surface envelop proteins, HA and NA. To date, the scientist discovered 18 HA (H1-18) and 11 NA (N1-11) subtypes (Tong et al., 2012).

2.2 Epidemiology of influenza A virus infection in humans and dogs

Influenza is one of the common respiratory diseases occurring in many animal species and human worldwide. This virus was discovered more than a century. Some influenza subtypes are species preference, but some subtypes can cross species barrier and infect other animal species. In the last century, there were three virulence

influenza pandemics in humans. First pandemic influenza, Spanish flu (influenza A H1N1) was emerged in 1918. Second pandemic influenza, influenza A H2N2 or Asian flu caused pandemic influenza outbreaks in 1957. Third pandemic influenza, influenza A H3N2 or Hongkong flu in 1968. The recent pandemic influenza outbreaks in this century was emerged in 2009, influenza A H1N1 or pandemicH1N1-2009 was the reassortment of influenza A viruses of human, swine and avian (Garten et al., 2009).

For influenza in dog, the first canine influenza virus emerged in racing greyhounds in Florida, United States, in January 2004. Most of infected dogs showed upper respiratory tract infection such as cough, nasal discharge, fever and subsequently self-recovery. This canine influenza outbreak caused by influenza virus subtype H3N8 which almost homologous to equine influenza subtype H3N8 isolated in 2003 and 2004 (Crawford et al., 2005; Zhang et al., 2007). After the outbreak in 2004, the canine influenza virus H3N8 spread out to other states in various dog breeds not only in the racing greyhound dogs (Payungporn et al., 2008). Moreover, the canine influenza H3N8 in United Kingdom was reported and linked to canine influenza viruses in the US (Newton et al., 2007; Daly et al., 2008).

In 2008, another canine influenza virus subtype H3N2 was emerged in South Korea. The phylogenetic analysis demonstrated that the canine influenza virus subtype H3N2 had 95.5% nucleotide identities or closely related to avian viruses (Song et al., 2008). Avian-origin canine influenza viruses subtype H3N2 were subsequently reported

in dogs in Southern China. The viruses were closely related to Korean canine influenza H3N2 (Li et al., 2010; Lin et al., 2012).

Apart from canine influenza subtype H3N8 and H3N2, dogs can be infected with several influenza subtypes. For example, the evidence of influenza A subtype H5N1 was reported in dogs during H5N1 outbreaks in Thailand in 2004 (Songserm et al., 2006). Although there was no report about influenza A subtype H9N2 infection in dogs, a study revealed that dog is susceptible to influenza A subtype H9N2 virus in the experiment setting (Zhang et al., 2012). In 2009, canine influenza subtype H5N2 was reported in dogs in China. The viruses were closely related with swine influenza H5N1 that originated from Asian avian lineage (Zhan et al., 2012). After 2009, there were many reports of pandemic H1N1/2009 (pH1N1/2009) virus infection in dogs. In China reported indicated that the pH1N1/2009 viruses isolated from dogs were closely related to pH1N1/2009 from the dog's owner (Lin et al., 2012). In South Korea, the reassortment of pH1N1/2009 and canine influenza H3N2 resulted in novel canine influenza H3N1 and caused infection in dogs (Song et al., 2012). Currently, there are at least six influenza subtypes (H1N1, H3N1, H3N2, H3N8, H5N1 and H5N2) ever reported infecting in dogs.

2.3 Transmission of influenza in dogs

2.3.1 Intra-species transmission of canine influenza viruses in dogs

The evidences of intra-species transmission of canine influenza virus were presented in many experimental studies. From the outbreak of canine influenza virus subtype H3N2 in South Korea, the genetic of the virus is closely related to the avian influenza viruses. The experiment of canine influenza virus subtype H3N2 virus infection in dogs demonstrated influenza-liked symptoms and virus shedding in infected dogs (Song et al., 2008). From literature review, it has been documented that dogs to dog transmission for influenza can be observed such as influenza subtype H3N8 (Jirjis et al., 2010), influenza subtype H5N1 (Giese et al., 2008), influenza subtype H5N2 (Song et al., 2013), and pandemic H1N1 2009 (Lin et al., 2012). Dog-to-dog transmission was reported in dog shelters in the US (Pecoraro et al., 2014). Many studies reported of the risk of pet dogs to be infected with pandemic influenza H1N1 2009 (Lin et al., 2012; Su et al., 2014).

2.3.3 Inter-species transmission of canine influenza viruses in other mammal models

The experimental studies of inter-species transmission of canine influenza viruses to other mammal species can be used to predict virulence and pandemic potential of the viruses. Influenza pandemics emerge every 10-40 years that require 3 factors (i) novel to the human immune system (ii) virulent in the human host, and (iii)

transmissible from person to person (Lowen et al., 2006). The first case of inter-species transmission of canine influenza virus was reported in Korea. Cats were infected and died with severe respiratory signs. The genetic characterization of the influenza virus subtype H3N2 revealed the identical genetic composition with canine influenza virus subtype H3N2 in Korea. In experimental challenge study, cats can infect with influenza virus subtype H3N2 and developed the same respiratory signs (Song et al., 2011b).

2.4 Animal model for canine influenza virus infection

Several mammal models have been used for influenza research. For example, ferret is an excellent model of influenza study. Many studies reported that ferret possibly transmit influenza virus from infected to non-infected ferrets by housing together (Herlocher et al., 2001; Belser et al., 2011). However, the ferret model has several disadvantages such as expensive, limited suppliers and difficult to handling, thus the researchers choose other alternative animal models for influenza research (Lowen et al., 2006). Mice model was used as an influenza mammal model for influenza research because mice are inexpensive and easy to handle. However, mice model does not suitable for influenza transmission study (Schulman and Kilbourne, 1963; Bouvier and Lowen, 2010). Another alternative mammal model, guinea pig is a useful mammal model for study of influenza virus (Azoulay-Dupuis et al., 1984). The guinea pig is suitable for both large droplet and air-born viral transmission in mammalian host (Mubareka et al., 2009). Influenza research in guinea pigs could help

understanding influenza infection in mammal and potential epidemic/virulence influenza viruses in the future.

CHAPTER III

MATERIALS AND METHODS

This study was divided into 5 phases; phase I: Monitoring of canine influenza virus infection in Thailand during September 2011 to 2014, phase II: Intravenous pathogenicity index test, phase III: Investigating the pathogenicity of canine influenza viruses in dogs, phase IV: Investigating the pathogenicity of canine influenza viruses in chickens and phase V: investigating the pathogenicity of canine influenza viruses in guinea pigs.

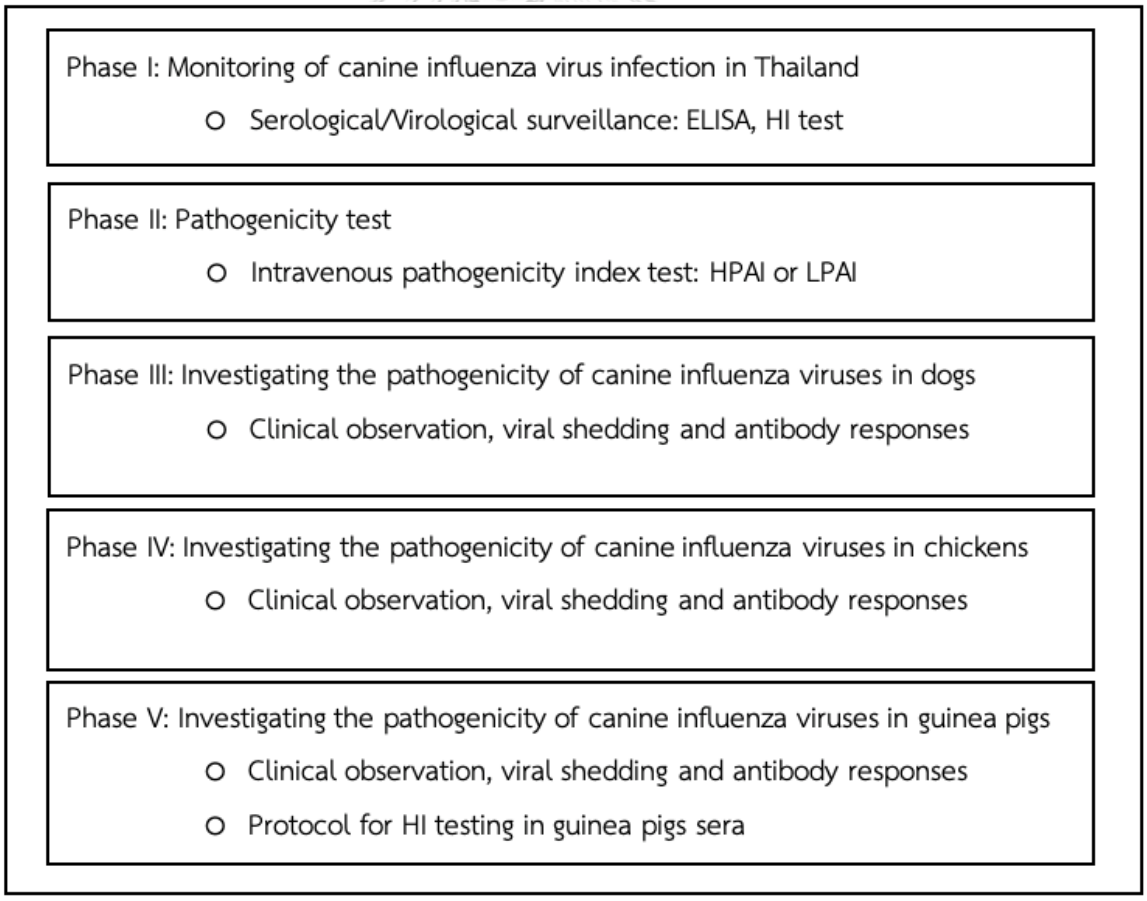


Figure 1 The conceptual framework of this study

3.1 Phase I: Monitoring of canine influenza virus infection in Thailand

3.1.1 Sample collection from dogs and cats

The cross-sectional nasal swabs and serum samples collected from dogs and cats was conducted. The samples were collected from both healthy and sick dogs and cats from September 2011 to September 2014. The sites for sample collection were animal shelters, temples and animal hospitals in 19 provinces of Thailand. Sample collection sites were chosen base on the criteria 1) overcrowded condition (such as shelters, temples), 2) report of sick animals with respiratory signs (animal hospitals) and 3) animal/owner cooperation. In this study, sample collection in dogs was conducted as cross-sectional study, at least 60 locations were visited during the course of study. Places for sample collection were visited only one time and the samples were collected from dogs and cats at the sites.

3.1.2 Detection of canine influenza virus antibodies by serological testing

Dog blood samples were collected in 2 ml tubes. Serum samples were separated from blood by centrifugation at 3,000 rpm for 10 minutes. The serum samples were then be divided into 2 tubes, 150 µl for ELISA and the rest for stock. The serum stocks were kept at -20°C (Figure 3.2). In this study, the Enzyme-Linked Immunosorbent Assay (ELISA), ID VET innovative diagnostics, FRANCE (ID Screen®) was used for the screening of canine influenza A antibody. The serum samples were also tested by Hemagglutination Inhibition testing for specific influenza A antibodies.

3.1.3 Sample preparation for viral detection

Dog nasal swab samples were collected in 2 ml viral transport media (Minimum Essential media, MEM) and were kept at 4°C then transported to the laboratory. The nasal samples were divided into 3 aliquots, 150 µl for RNA extraction, 500 µl for viral isolation and the rest for stock (Figure 3.2). All samples were kept in -80°C.

To extract the canine influenza virus RNA, the RNA extraction kit (QIAamp®, Germany) was used for RNA extraction. Briefly, 150 µl of nasal swab sample was added into 600 µl lysis buffers with RNA carrier, then incubated at 70 °C for 5 minutes to lysis the virus. Ethanol 600 µl was added after inoculation. Mixture was loaded into silica gel membrane column and centrifuged at 8,000 g for 1 minute to bind viral RNA. The column was washed 3 times using washing buffer RAW and centrifuged at 8,000 g for 1 minute then washed once with RAV3 and centrifuged at 8,000 g for 1 minute. Then, column was added with 200 µl RAV3 and centrifuged at 11,000 g for 5 minutes to dry silica membrane. The dry column was placed into 1.5 ml tube and added 30 µl of 70 °C of RNase-free water to elute RNA. The column was incubated at room temperature for a minute and centrifuged at 11,000 g for 1 minute. The elution contained viral RNA was kept in 1.5 ml tube at -20 °C until use.

To detect canine influenza virus, the extracted RNA samples were examined to detect Matrix (M) gene of influenza virus by Real time RT-PCR (Spackman et al., 2002). Briefly, the extracted RNA sample was used for testing by Real time RT-PCR with forward primers, reverse primers, M64 probe, 2x Master mix, Superscript III and MgSO4.

After mixing all reagents then place the tube in the machine with the condition 50ºC for 30 minutes for 15 minutes followed by 50 cycles of amplification then 95ºC for 15 seconds and 60ºC for 30 seconds. The detail is in the appendix E. The positive real time RT-PCR samples (Ct< 36) were subjected to viral isolation. The positive nasal swab samples were then inoculated into embryonated chicken eggs (9 to 11 day-old) and incubated at 37ºC for 48 hours. The suspected allantoic fluid were tested by Hemagglutination (HA) test using 1% of chicken red blood cell and serial two-fold dilutions of the allantoic fluid. After an hour of incubation period, hemagglutination of RBCs was observed. Positive HA test samples which presented titer $\geq 2^2$ HA unit recommended by WHO, 2002 were reconfirmed for influenza A virus by real time RT-PCR.

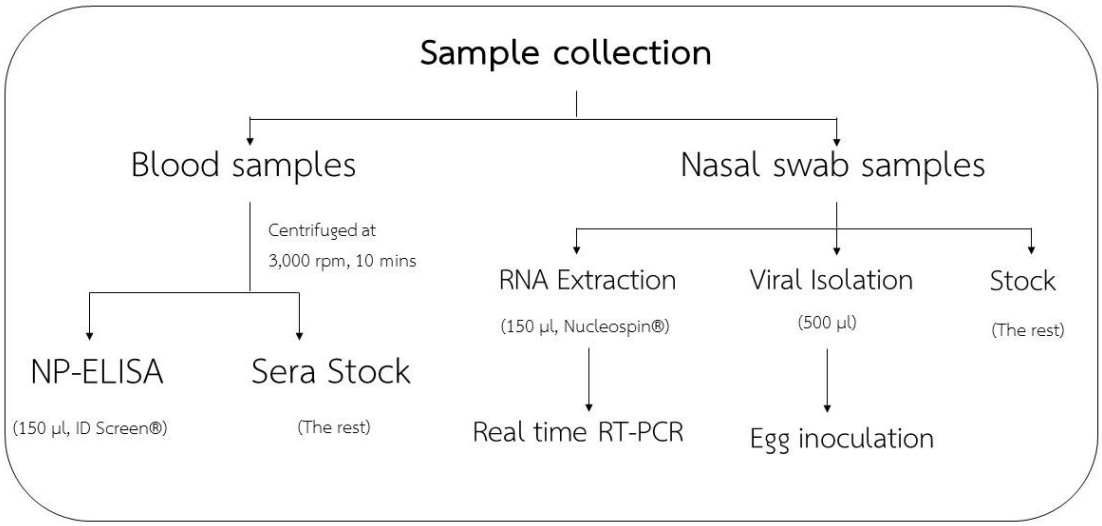


Figure 2 Sample collection and preparation protocol

3.2 Phase II: Intravenous Pathogenicity index test (IVPI) for canine influenza virus

According the OIE protocol for avian influenza virus, the intravenous pathogenicity index has been using for classification of highly pathogenic influenza virus (HPAI) and low pathogenic influenza virus (LPAI). In this thesis, a Thai CIV-H3N2 virus designated as "A/canine/Thailand/CU-DC5299/2012/H3N2" was subjected to intravenous pathogenicity index test (IVPI) following by the Office International Des Epizooties recommendation (OIE, 2014). The CIV-H3N2 (CU-DC5299) was selected based on the following criteria; 1) the virus was isolated from dog in Thailand 2) the virus was previous genetic characterized and the nucleotide sequences were available in the GenBank database. In detail, 12 six-week-old chickens were used in the experiment. The chickens were separated into 2 groups, inoculated and control groups. In group 1 (inoculated group), 10 chickens were intravenously injected through jugular vein with 0.1 ml of 1:10 dilution of the virus in sterile isotonic saline. In group 2 (control group), 2 chickens were injected with 0.1 ml of 1X phosphate buffer solution (PBS) intravenously (Figure 3.3). All chickens were examined daily for 10 days and scored, 0 (normal), 1 (sick), 2 (very sick), and 3 (dead). Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and head, nervous signs. Dead individual birds must be scored as 3 at each of the remaining daily observations after death. After the experiment IVPI score was calculated following OIE/WHO guideline.

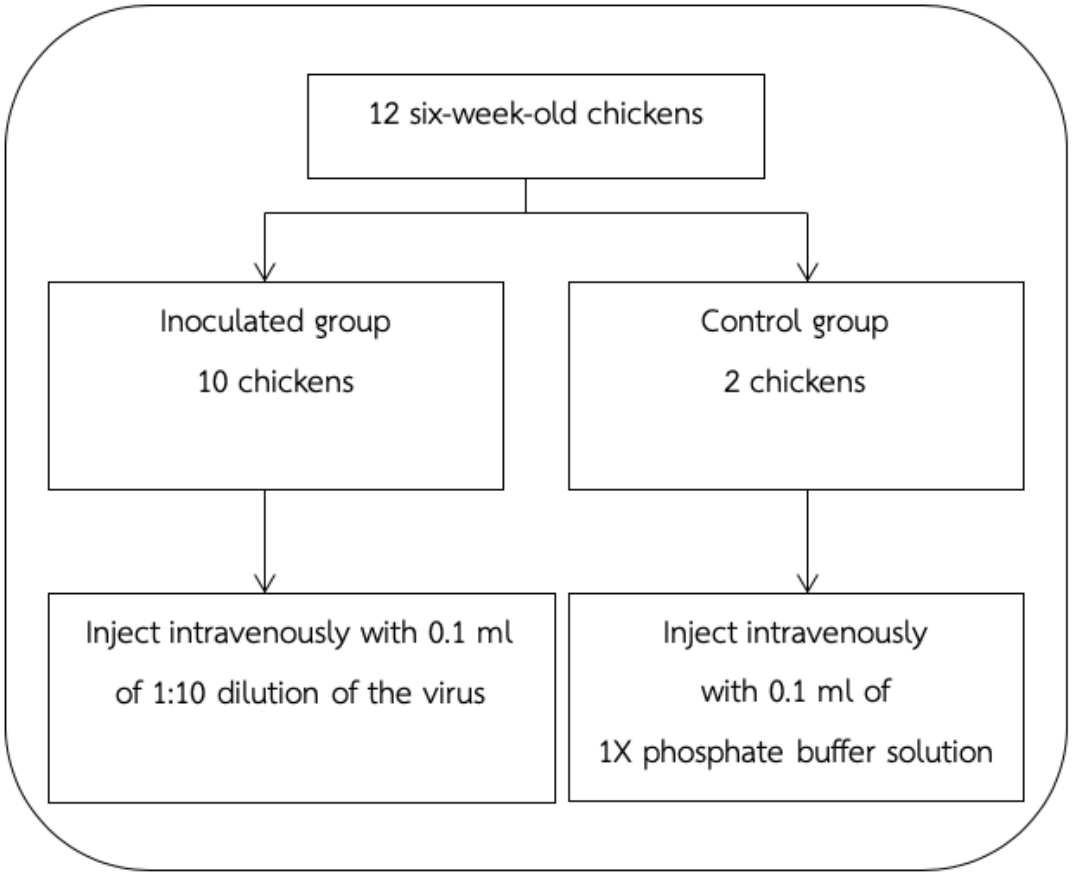


Figure 3 Outline of Intravenous pathogenicity index test protocol

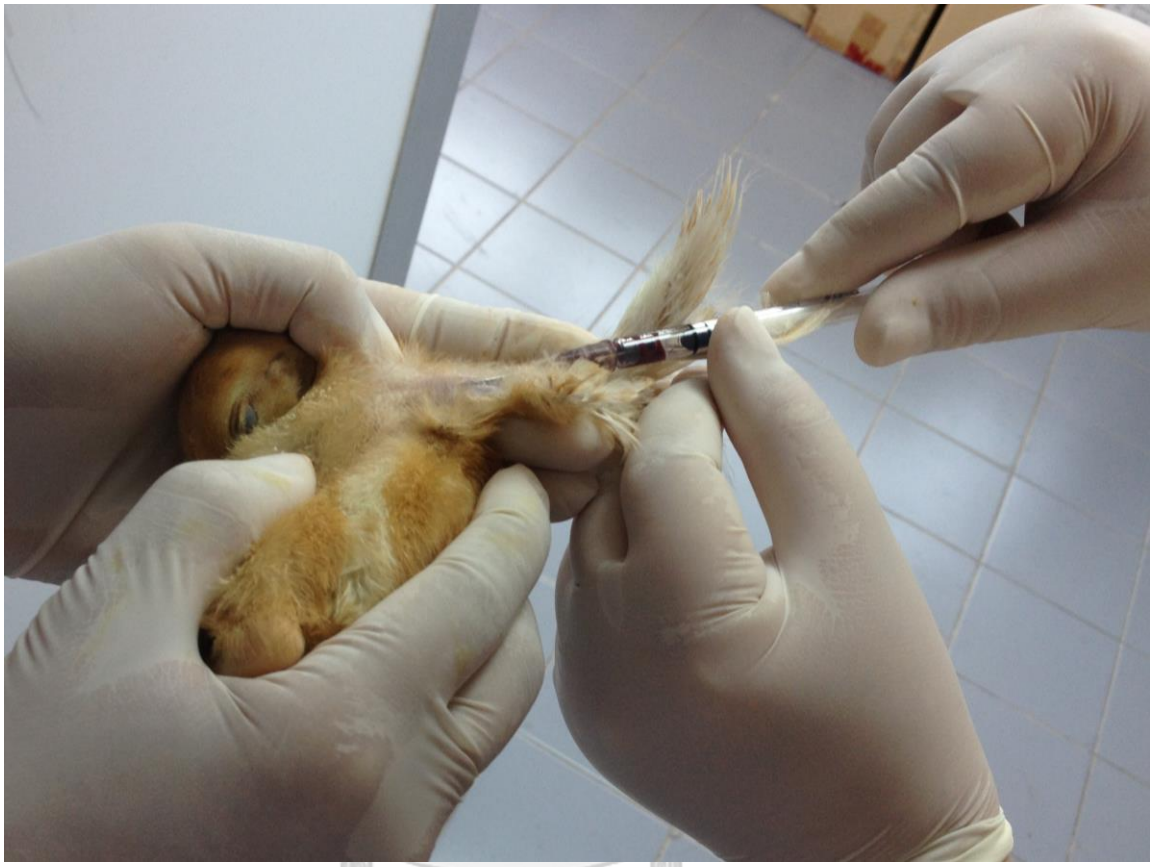


Figure 4 Blood collection from experimental chickens for intravenous pathogenicity index test

3.3 Phase III: Investigating the pathogenicity of canine influenza viruses in dogs

In the thesis, dog model was used for studying infection and pathogenicity of canine influenza viruses in dogs. In this study, a Thai CIV-H3N2 virus (A/canine/Thailand/CU-DC5299/2012/H3N2) was selected for the animal model experiment. In this experimental, 13 8-week-old, influenza-free beagle dogs were used in the experimental dog model. The dogs (n=13) were tested and free of influenza virus from blood and nasal swab samples before the experiment. Dogs were randomly divided into 3 groups including inoculated, contact and control groups. The inoculated group (n=5) was challenged intranasally with 1 ml (500 µl per nare) of filtrated canine influenza A virus (CU-DC5299) with a 10^6 EID_{50}. Before virus inoculation, dogs were sedated with a mixture of xylazine and atropine administrated intramuscularly. The control group dogs (n=3) was challenged with 1 ml (500 µl per nare) of Phosphate Buffer Solution (PBS). The contact group (n=5), dog was placed one by one in the same cage as inoculated dogs since day 1 after inoculation (Figure 3.5, 3.6 and 3.7). Parameters for canine influenza infection and pathogenicity in dogs include clinical signs observation, pathological changes, viral shedding, and antibody response, were recorded and analyzed.

3.3.1 Clinical sign observation

All dogs were observed for clinical signs including rectal temperature, ocular discharge, nasal discharge, coughing, sneezing, panting, and abdominal breathing. The apparent clinical signs were graded as mild, moderate, and severe and were recorded in daily examination chart. The clinical signs were analyzed by comparing the clinical score within groups (Day post inoculation; DPI) and among groups (inoculated, contact and control groups.

3.3.2 Pathological changes

One dog from each group was euthanized randomly at 7 and 14 dpi to observe pathological lesions (gross and histopathological lesions) by using hematoxylin and eosin (H&E) and immunohistochemistry staining. The organs including trachea, lungs, heart, liver, spleen, and kidneys were collected to examine the histopathological lesions. Euthanasia was performed on the rest of dogs at the end of experiment. The pathological changes were analyzed by description of gross and histopathological changes of each tissues/organs within groups (Day post inoculation; DPI) and among groups (inoculated, contact and control groups.

3.3.3 Viral shedding and antibody response

The nasal samples of dogs in each group were collected daily at 1 – 10, 14 and 21 dpi, respectively. The nasal and rectal swab samples were tested for influenza A viruses by Real time RT-PCR to analyze viral shedding. The blood samples were collected at 7, 10, 14 and 21 dpi. Serum samples were tested for influenza antibodies by the Enzyme-Linked Immunosorbent Assay (ELISA) and Haemagglutinin inhibition (HI) assay.

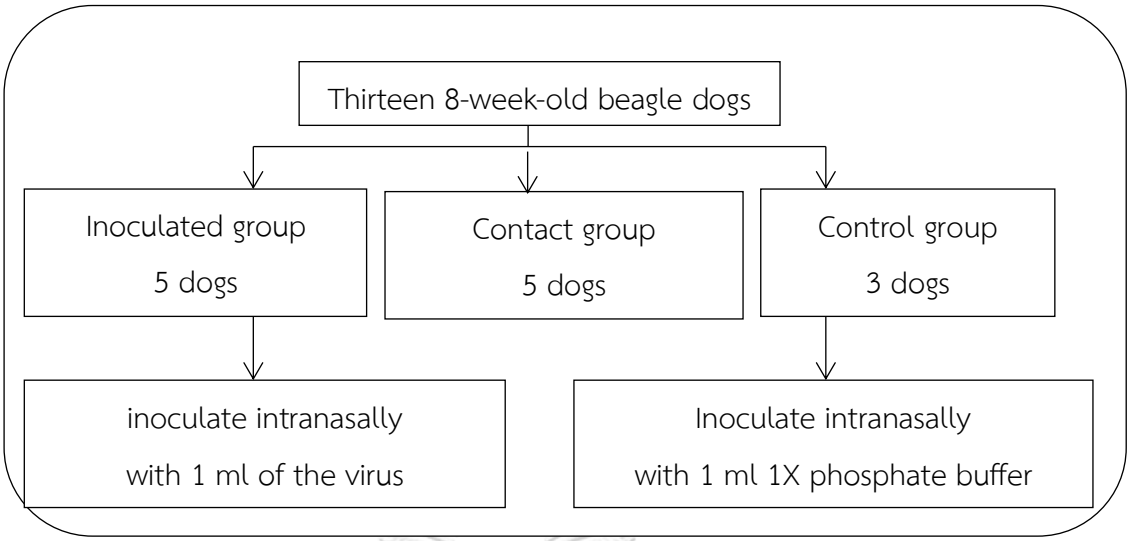


Figure 5 Outline of the study designs for infection and pathogenicity of canine influenza viruses in dogs

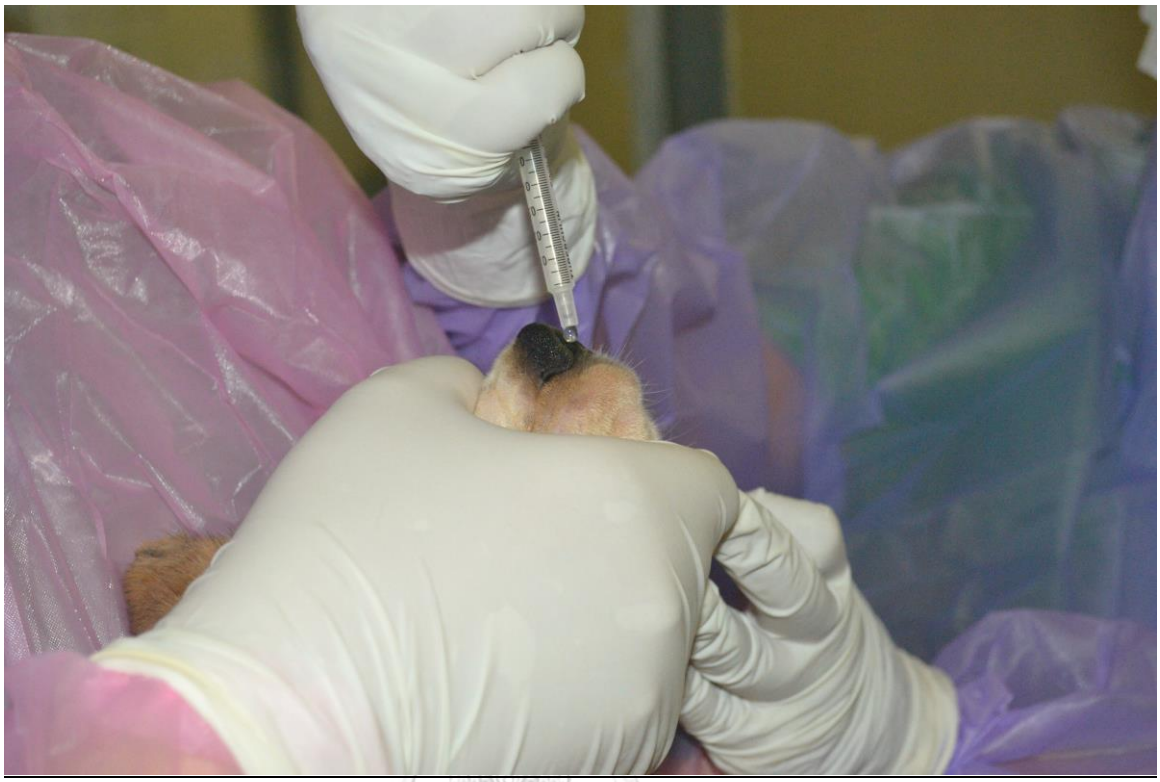


Figure 6 Intranasal injection of CIV-H3N2 (CU-DC5299) challenged dogs.

Figure 7 Adaptable personal protective equipment and sample collection in experimental room

3.4 Phase IV: Investigating the pathogenicity of canine influenza viruses in chickens

In this thesis, chicken model was used for studying infection and pathogenicity of canine influenza viruses in avian species. The SPF female chickens (n=9) were tested and free of influenza virus from blood and nasal swabs samples before experiment. The experiments were performed in the animal isolators in Biosafety level 2+ at the Faculty of veterinary sciences, Chulalongkorn University. Chickens were divided randomly into 3 experimental groups (inoculated, contacted and control groups). For inoculated group, the chicken (n=3) were inoculated with 200 μl of CIV-H3N2 with a 10^6 EID_{50}. For contact group, the chickens (n=3) were placed at 1 dpi. For control group, the chickens (n=3) were inoculated with 200 μl of phosphate buffer solution (PBS) (Figure 3.8 and 3.9). All chickens were euthanized at the end of the experiment. Parameters for canine influenza infection and pathogenicity in chickens including clinical sign observation, pathological changes, viral shedding, and antibody response were recorded and analyzed. The material and methods for clinical sign observation, pathological changes, and viral shedding were performed as previous sections 3.3.1-3.3.3. The blood samples were collected at 7 and 12 dpi. Serum samples were tested for influenza antibodies by the Enzyme-Linked Immunosorbent Assay (ELISA).

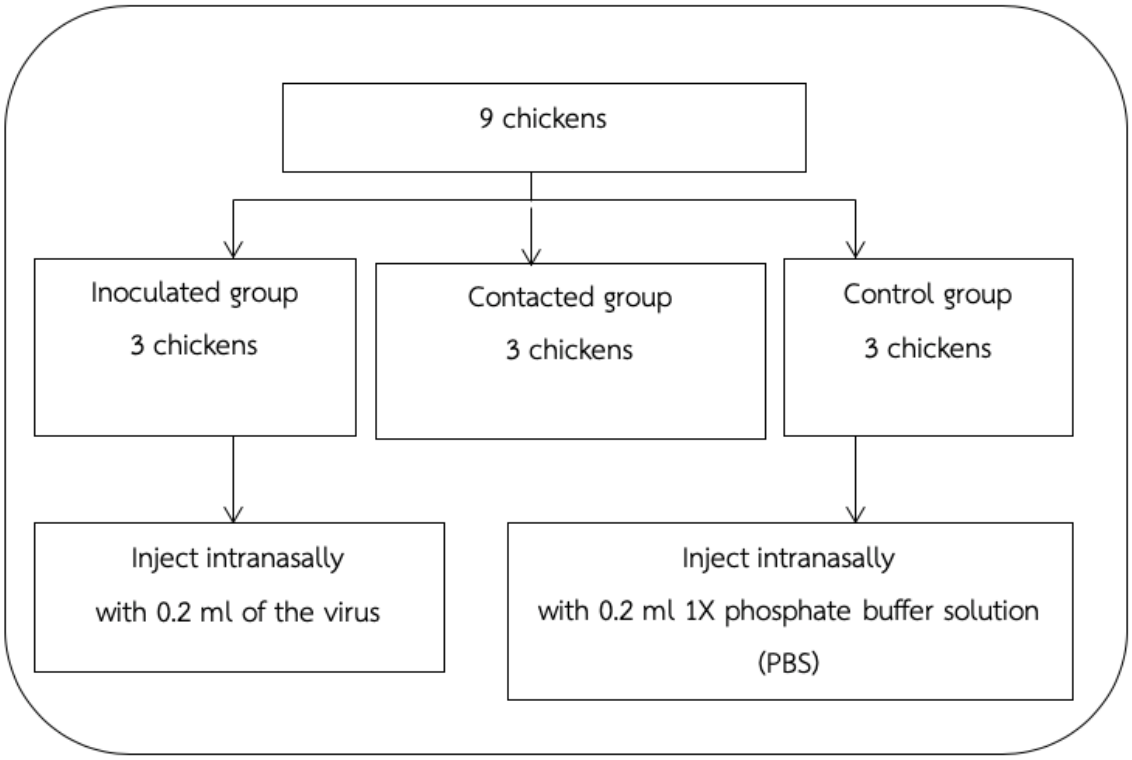


Figure 8 Outline of the study design for infection and pathogenicity of canine influenza viruses in chickens.

Figure 9 Investigating the pathogenicity of canine influenza viruses in chickens at the animal isolators in Biosafety level 2+ at the Faculty of veterinary sciences, Chulalongkorn University

3.5 Phases V: Investigating the pathogenicity of canine influenza viruses in guinea pigs

In this thesis, the guinea pig (*Cavia porcellus)* was used as a mammal model for infection and pathogenicity of canine influenza virus. Since influenza antibody detection for guinea pig serum by HI test do not available. In this thesis, HI test protocol for guinea pig serum was developed and standardized.

3.5.1 HI protocol standardization for guinea pig sera

To acquired known positive influenza A serum of guinea pig, blood samples were collected from CIV-H3N2 infected SPF guinea pigs at 14 day-post-inoculation. The guinea pigs were inoculated with 300 μl of canine influenza virus subtype H3N2 (A/canine/Thailand/CU-DC5299/12). Blood samples were collected at 14 dpi and centrifuged at 3,000 rpm for 10 minutes to separate serum. The serum samples were then tested for antibodies against influenza A virus using ELISA, ID VET innovative diagnostics, FRANCE (ID Screen®).

For HI protocol standardization, the serum samples were processed for HI assay against specific influenza H3 antibodies. Four different factors were evaluated including a) elimination of non-specific inhibitors (20% kaolin or receptor destroying enzyme (RDE)), b) type of red blood cells (RBCs), and c) percentage of RBCs, and d) hemagglutination unit (HAU) of virus totally 16 experimental groups (A-P).

For sera treatment, the positive serum samples of guinea pigs from the experiment were divided into 16 groups including group A – P (Table 3.1). The serum samples were treated by either receptor destroying enzyme (RDE) or 20% Kaolin with different types (chicken or turkey RBCs) and different concentration (50% or 100% RBCs). In detail, for the first eight groups (A-H), with 20% Kaolin at room temperature for 30 minutes and centrifuges at 2,000 rpm for 10 minutes. Treated samples were absorbed with 100 microliters of 50% turkey red blood cells or 50% chicken red blood cells. All samples were incubated at room temperature for an hour. For the other eight groups of positive sera samples were treated the sera were treated with receptor destroying enzyme (RDE) at 37°C for 20 hours then inactivation by heat at 56 °C for an hour and used 100 microliters of 50% chicken red blood cells or 50% turkey red blood cells. All samples were incubated at room temperature for an hour. Phosphate-buffered saline (PBS) was used for two-fold dilution in each sample in 96-well micro-titer plates. Samples were incubated with 4 or 8 haemagglutination unit (HAU) per 50 μl of each virus for 45 min at room temperature. RBCs with 0.5% or 1% of Chicken RBCs or Turkey RBCs were added and incubation for 1 hour. Then, samples were read the titer. The HI titer was determined by the reciprocal of the last dilution which presented non-agglutination. Positive samples were identified with samples showing a titer $\geq$ 40 (Bunpapong et al., 2014).

Table 1 Experimental groups of sera treatment step and HI protocol (types and concentration of RBCs) for HI protocol standardization.

Sample	Eliminated technique used	Types of RBCs	Hemagglutination unit (HAU) of virus	Percentage of RBCS	Experimental group
Known positive guinea pig sera	20% Kaolin	Turkey RBCs (50%)	4HAU/25μl	1% RBC	A
				0.5% RBC	B
			8HAU/50μl	1% RBC	C
				0.5% RBC	D
		Chicken RBCs (50%)	4HAU/25μl	1% RBC	E
				0.5% RBC	F
			8HAU/50μl	1% RBC	G
				0.5% RBC	H
	RDE	Turkey RBCs (50%)	4HAU/25μl	1% RBC	I
				0.5% RBC	J
			8HAU/50μl	1% RBC	K
				0.5% RBC	L
		Chicken RBCs (50%)	4HAU/25μl	1% RBC	M
				0.5% RBC	N
			8HAU/50μl	1% RBC	O
				0.5% RBC	P

3.5.2 Investigating the pathogenicity of canine influenza virus in Guinea pigs

In this thesis, the 4-week-old guinea pigs *(Cavia porcellus)* were used as mammal model for influenza infection and pathogenicity study. Twenty 8-week-old, influenza-free guinea pigs (Hartley strain), were used and randomly divided into 4 groups (inoculated, direct contacted, aerosol contacted and control groups). In inoculated group, the guinea pigs (n=5) were intranasally inoculated with 300 µl (150 µl per nare) of canine influenza virus (CIV-H3N2) with a 10^6 EID_{50}. In control group, the guinea pigs (n=5) were inoculated with 300 µl (150 µl per nare) of PBS. In direct contact group, at day 1 after challenge the virus (1 dpi), the guinea pigs (n=5) were placed in caged together with inoculated group as the contact group. In aerosol contact group, the influenza-free guinea pigs (n=5) were placed in adjacent cage 20 cms apart from inoculated and contacted guinea pigs (Figure 3.10 – 3.16). All guinea pigs were sedated with xylazine and ketamine administrated (IM) before experiment challenge. Parameters for canine influenza infection and pathogenicity in guinea pigs including clinical sign observation, pathological changes, viral shedding, and antibody response were recorded and analyzed. The material and methods for clinical sign observation, pathological changes, and viral shedding and antibody response were performed as previous sections 3.3.1-3.3.3.

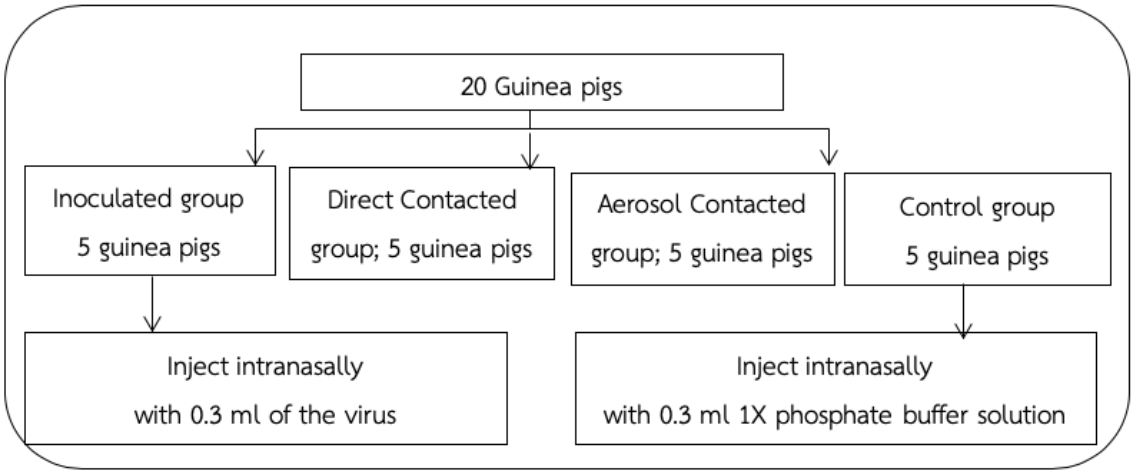


Figure 10 Outline of the study design for infection and pathogenicity of canine influenza viruses in guinea pigs.

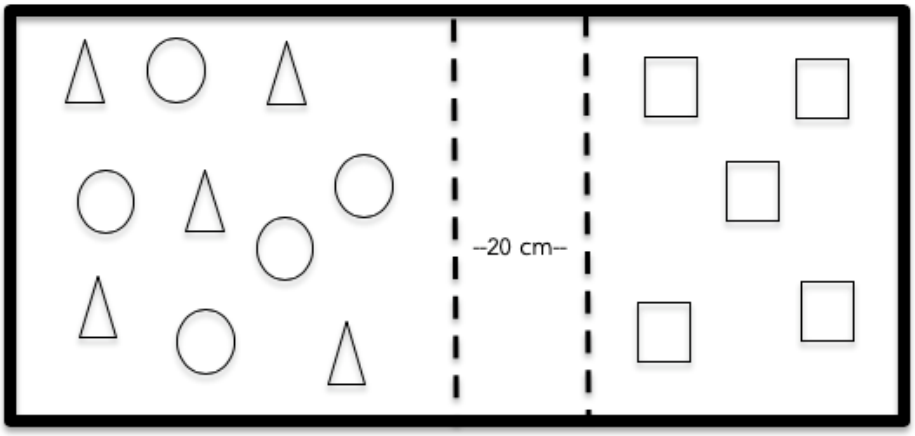


Figure 11 Cage layout of guinea pigs in the experiment (△ = Inoculated group, ○ = Direct contact group, □ = Aerosol contact group).

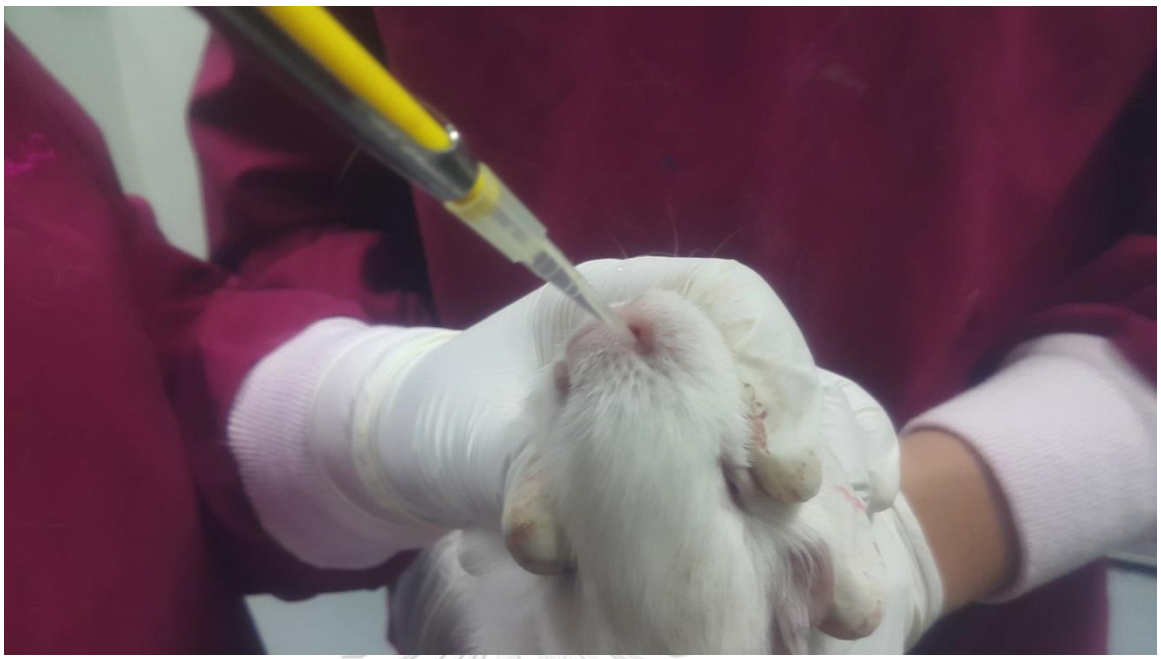


Figure 12 Intranasal inoculation in CIV-H3N2 challenged guinea pigs.

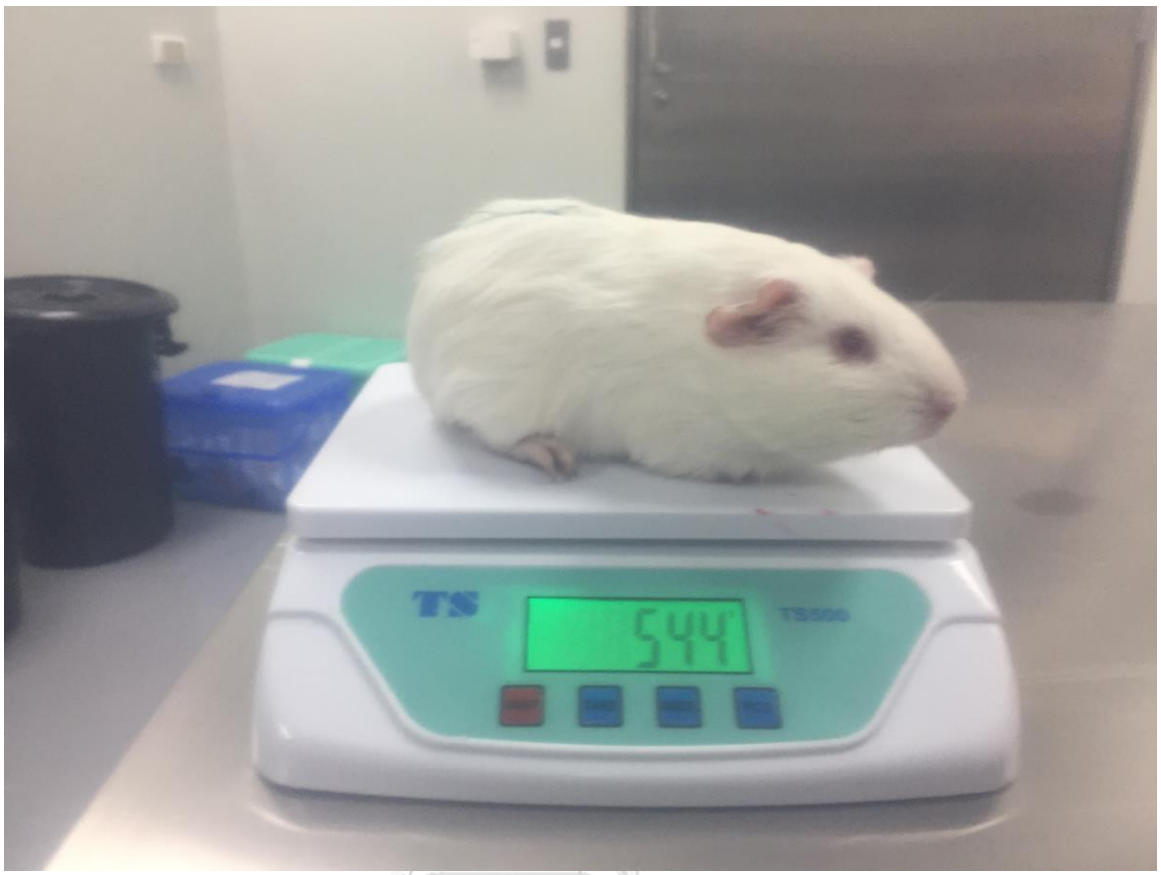


Figure 13 Guinea pig was weighted in the experiment room.

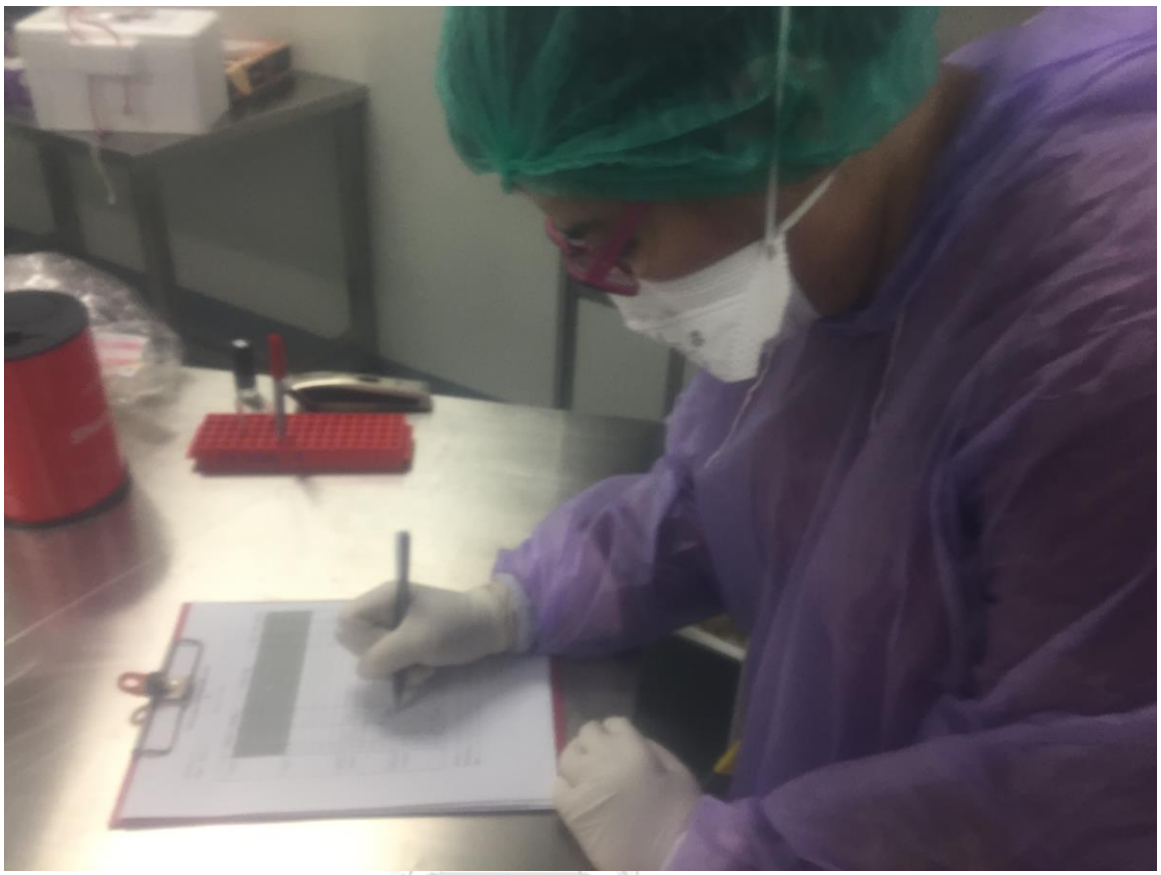


Figure 14 Recording clinical sign, rectal temperature and weight measurement daily in the experiment room.

Figure 15 Collecting blood sample in the experimental room

Figure 16 Experimental cage

CHAPTER 4

RESULTS

4.1 Phase I: Monitoring of canine influenza virus infection in Thailand

In this study, dogs from 47 animal shelters from 19 provinces in five regions of Thailand were surveyed for influenza infection. The 19 provinces include provinces from northern part (4 provinces), northeastern region (2 provinces), western region (3 provinces), eastern region (1 province) and central region (9 provinces) (Figure 4.1). During September 2011 to September 2014, 932 canine serum samples were collected from male dogs (n = 418, 44.85%) and female dogs (n = 514, 55.15%) from 45 shelters in 18 provinces. For cats, 79 feline serum samples were collected from male cats (n = 34, 43.0%) and female cats (n = 45, 57.0%) from 16 shelters in 11 provinces. The serum samples were tested for antibodies against influenza A virus by NP-ELISA assay. Our results showed that 0.97% (9/932) of canine serum samples were positive for influenza A antibodies. In detail, the positive samples were collected from central provinces (Bangkok [n = 2], Nakhon Nayok [n = 2], Nakhon Pathom [n = 2], Nonthaburi [n = 1], Ayutthaya [n = 1]) and eastern province (Chonburi [n = 1]). On the other hand, all 79 feline serum samples were negative for influenza A antibodies by NP-ELISA test.

The serum samples were also tested for specific antibodies against each influenza subtype (pdmH1N1, human H3N2 and canine H3N2) by HI test. The result of HI test showed that 0.64% of dog serum samples (n=6/932) and 1.20% (1/79) of cat serum samples posed antibodies against influenza virus (pdmH1N1-2009) with HI titers

ranging from 40 to 160 (Table 4.1). However, from our record during sample collection, seropositive animals did not show any respiratory signs at the sampling time. Moreover, some animals with respiratory signs might not show sero-positive. Three positive dog samples and one positive cat samples were collected from Bangkok and Nakorn Pathom provinces and 2 positive dog samples from Chonburi province. It should be noted that from 3-year survey, all positive samples were from dogs and cats sampled during the winter season in Thailand (September to January; Figure 4.2)

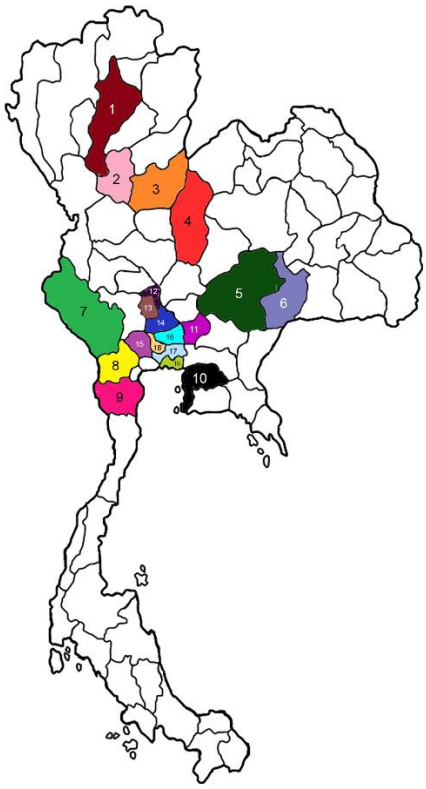


Figure 17 Sample collection site and number of collected samples from dogs and cats in Thailand.

The sample collection sites include the northern provinces; 1: Lampang (n = 4), 2: Sukhothai, 3: Phitsanulok (n = 15), 4: Phetchabun (n = 60), the north-eastern provinces; 5: Nakhon Ratchasima (n = 43), 6: Buri Ram (n = 49), the western provinces; 7: Kanchanaburi (n = 20), 8: Ratchaburi (n = 8), 9: Phetchaburi (n = 13), the Eastern province; 10: Chon Buri (n = 104) and the central provinces; 11: Nakhon Nayok (n = 58), 12: Sing Buri (n = 8), 13: Ang Thong (n = 4), 14: Ayutthaya (n = 46), 15: Nakhon Pathom (n = 190), 16: Pathum Thani (n = 119), 17: Bangkok (n = 85), 18: Nonthaburi (n = 140), 19: Samut Prakan (n = 21)

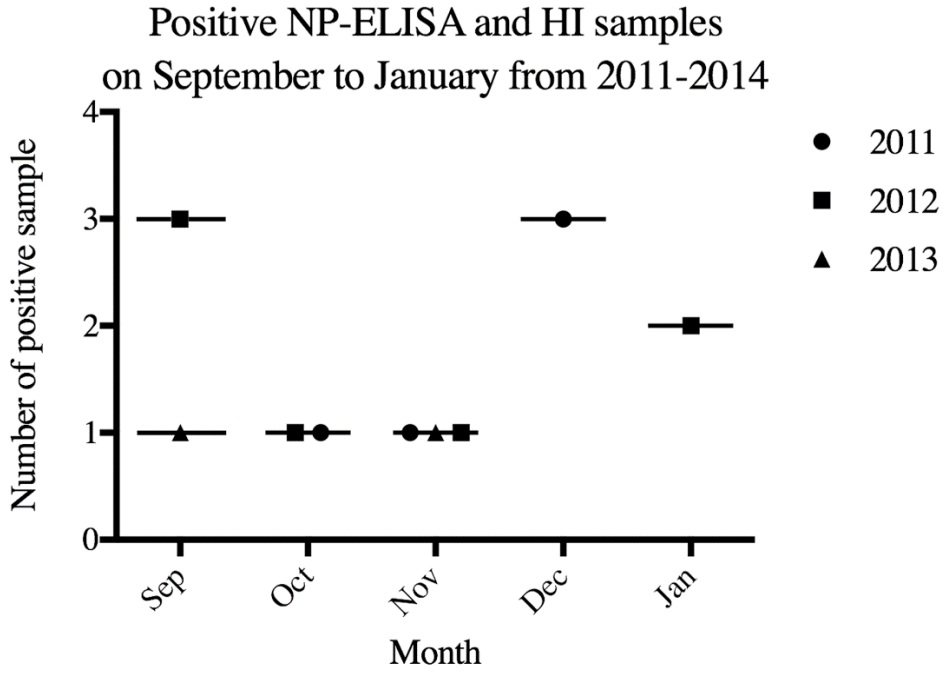


Figure 18 NP-ELISA and HI results. NP-ELISA and HI results from September to January from 2011 to 2014.

Table 2 Detail descriptions of NP-ELISA and HI positive samples in dogs and cats in Thailand.

Province	Region	Species	NP-ELISA test	
			S/N value	Result
ELISA test				
Ayutthaya	Central	Canine	44.58	Positive
Bangkok	Central	Canine	38.82	Positive
Bangkok	Central	Canine	30.48	Positive
Nakhon Pathom	Central	Canine	42.81	Positive
Nakhon Pathom	Central	Canine	38.92	Positive
Nakhon Nayok	Central	Canine	41.84	Positive
Nakhon Nayok	Central	Canine	30.15	Positive
Nonthaburi	Central	Canine	44.69	Positive
Chonburi	Eastern	Canine	38.46	Positive

Province	Region	Species	HI test		
			pH1N1	hH3N2	cH3N2
HI test					
Bangkok	Central	Canine	40	(–)	(–)
Bangkok	Central	Canine	80	(–)	(–)
Nakhon Pathom	Central	Feline	40	(–)	(–)
Nakhon Pathom	Central	Canine	160	(–)	(–)
Chonburi	Eastern	Canine	160	(–)	(–)
Chonburi	Eastern	Canine	40	(–)	(–)

4.2 Phase II: Intravenous pathogenicity index test (IVPI) for canine influenza virus

The IVPI test was performed following the OIE recommendation. In this study, the IVPI test was performed to determine the pathogenicity of the Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2). The 12 six-week-old chickens were divided into 2 groups, inoculated group (n=10) and control groups (n=2). All chickens were intravenously challenged with Thai CIV-H3N2 and examined daily for 10 days and scored, 0 (normal), 1 (sick), 2 (very sick), and 3 (dead). In this experiment, all chickens did not develop any clinical signs, thus all clinical score were 0. The IVPI scores were calculated based on OIE/WHO guideline. The IVPI is the mean score per bird per observation over the 10-day period. The IVPI index of Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) was 0.00 and classified as Low Pathogenic Influenza Viruses (Table 4.2).

Table 3 The IVPI scores, which calculated from the mean score per bird per observation.

Clinical signs	D1	D2	D3	D3	D4	D5	D6	D7	D8	D9	D10	Total	Score
Normal	10	10	10	10	10	10	10	10	10	10	10	100x0	=0
Sick	0	0	0	0	0	0	0	0	0	0	0	0x1	=0
Paralyze	0	0	0	0	0	0	0	0	0	0	0	0x2	=0
Died	0	0	0	0	0	0	0	0	0	0	0	0x3	=0
												Total	=0

4.3 Phase III: Investigating the pathogenicity of canine influenza viruses in dogs

To examine the pathogenicity of canine influenza viruses in dogs, the Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) was challenged in experimental dogs. All dogs in inoculated group (n=5), contact group (n=5) and control groups (n=3) showed clinical signs including fever, depression, nasal discharge, ocular discharge and coughing (Table 4.3).

In detail, dogs in inoculated group showed clinical signs since 2 dpi with mild depression (loss of appetite and less activity) (2 dpi – 6 dpi) and serous nasal discharge (2 dpi - 10 dpi). One dog developed coughing at 3 dpi and the others presented coughing at 4 dpi - 10 dpi. Ocular discharge was observed at 4 dpi - 7 dpi. Clinical presentations (depression, nasal discharge, coughing, and ocular discharge) of CIV-H3N2 challenged dogs in inoculated group are shown in Figure 4.3.

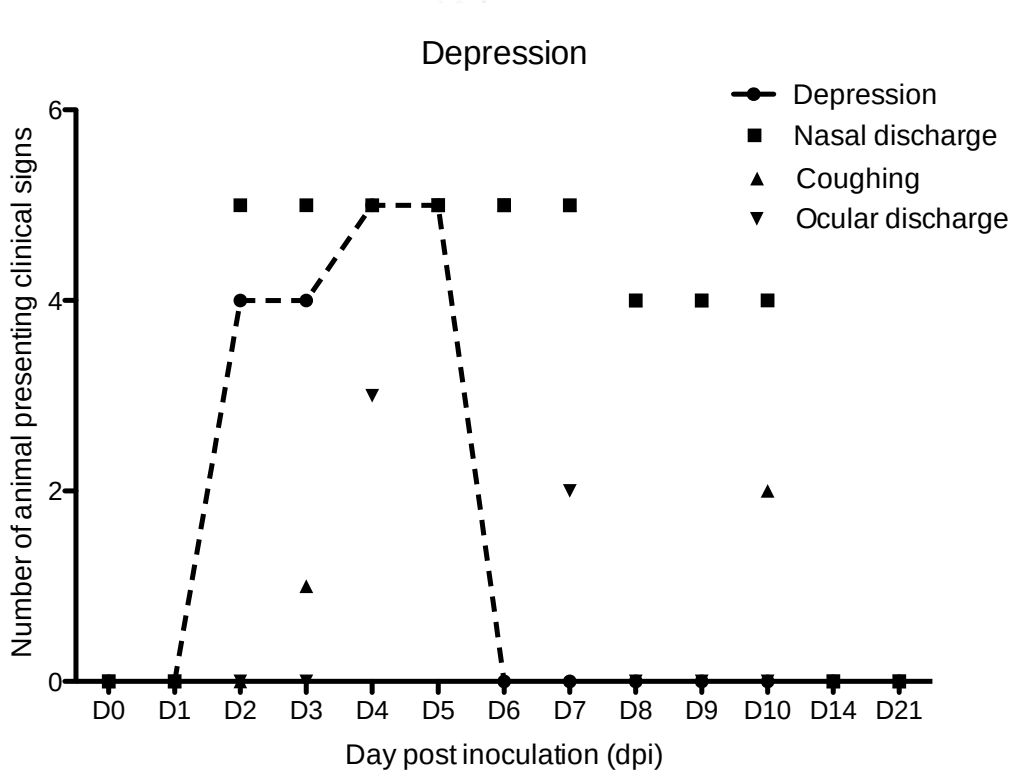
Table 4 Number and percentage of dogs showed clinical signs in CIV-H3N2 challenged experiment.

Number of dogs showed clinical signs (%)													
	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D14	D21
Inoculated group	0/5 (0%)	0/5 (0%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	4/4 (100%)	0/4 (0%)	0/3 (0%)
Contact group	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	4/4 (100%)	2/4 (50%)	0/3 (0%)
Control group	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/1 (0%)

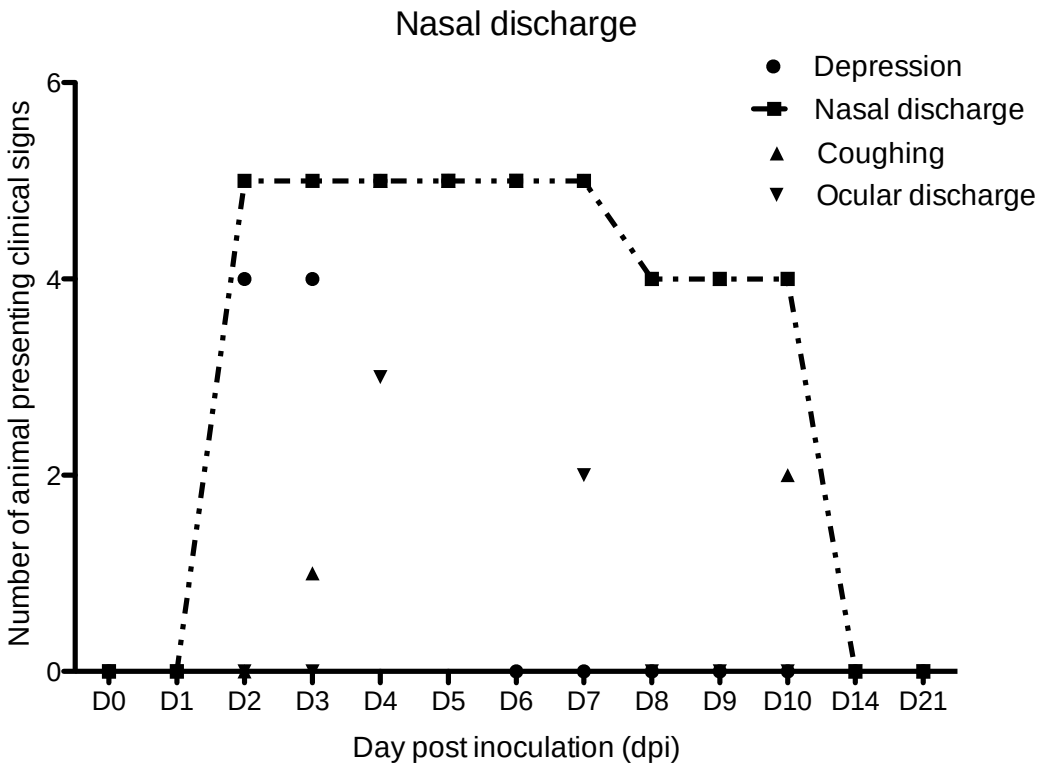
Figure 19 Clinical presentations of CIV-H3N2 challenged dogs in inoculated group,

a) Depression (at 2 dpi – 5 dpi) b) Nasal discharge (at 2 dpi – 10 dpi) c) Coughing (at 3 dpi – 10 dpi) and d) Ocular discharge (at 4 dpi – 7 dpi).

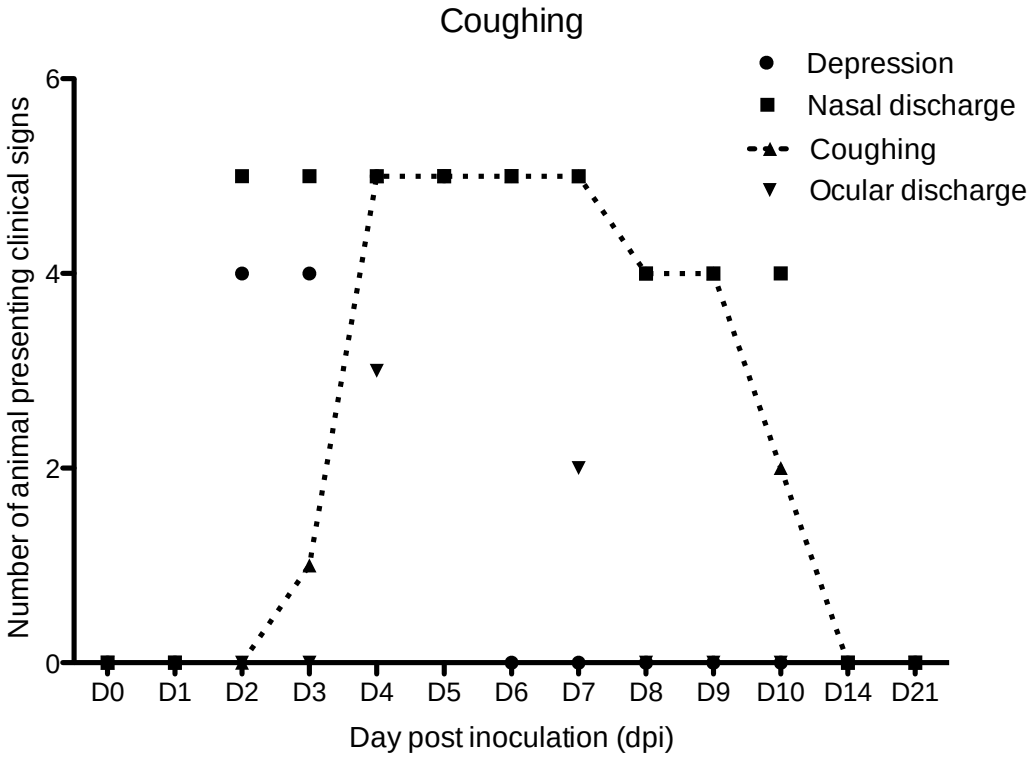
a) Depression;



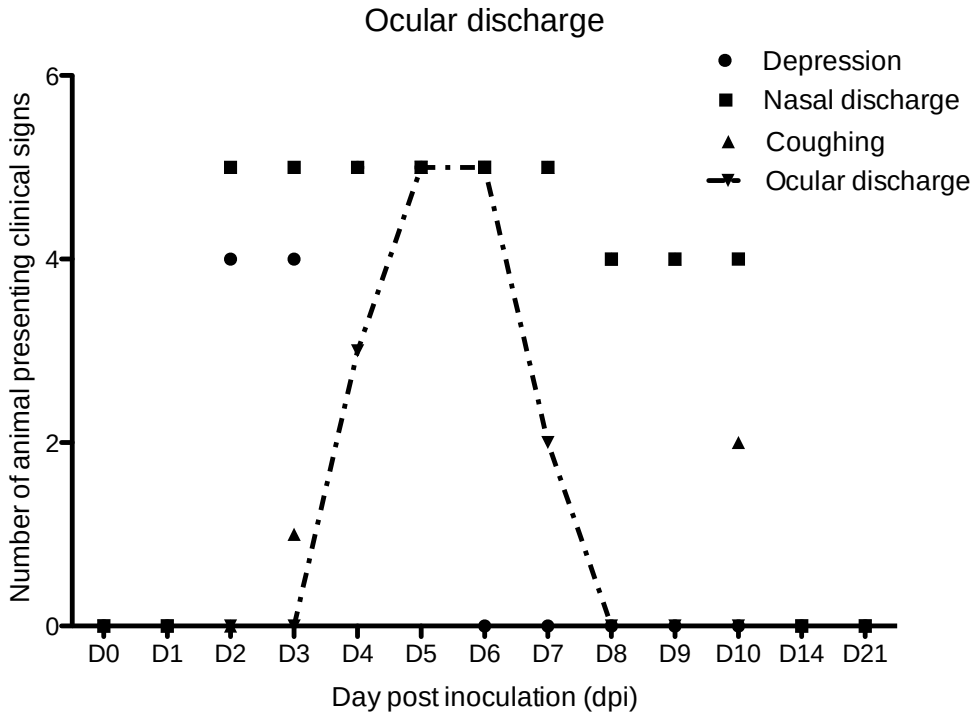
b) Nasal discharge;



c) Coughing;



d) Ocular discharge;



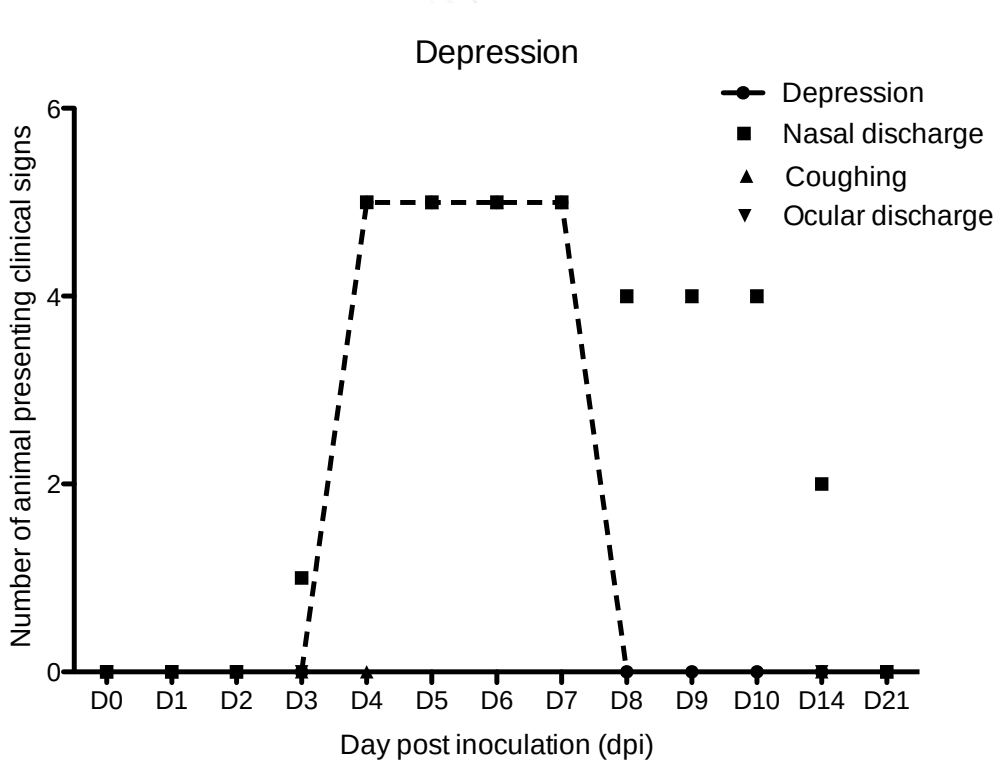
In contact group, one dog (n=1) showed serous nasal discharge start at 2 dpc (days post contact), but the other dogs (n=4) demonstrated clinical signs at 3 dpc to 13 dpc. Depression also observed at 2 dpc to 6 dpc. All dogs in contact group showed coughing at 4 dpc – 9 dpc. Moreover, ocular discharge was observed at 3 dpc to 9 dpc (Figure 4.4). In control group, all dogs (n=3) did not show any clinical signs throughout the experiment.

All dogs in inoculated and contact groups developed fever since 3 dpi and 3 dpc, respectively. Dogs developed fever approximately 3 - 4 days and then return to normal (Figure4.5). The highest rectal temperature was 103.8 F (at 3 dpi) in inoculated group and 103.6 F (at 3 dpc) in contact group.

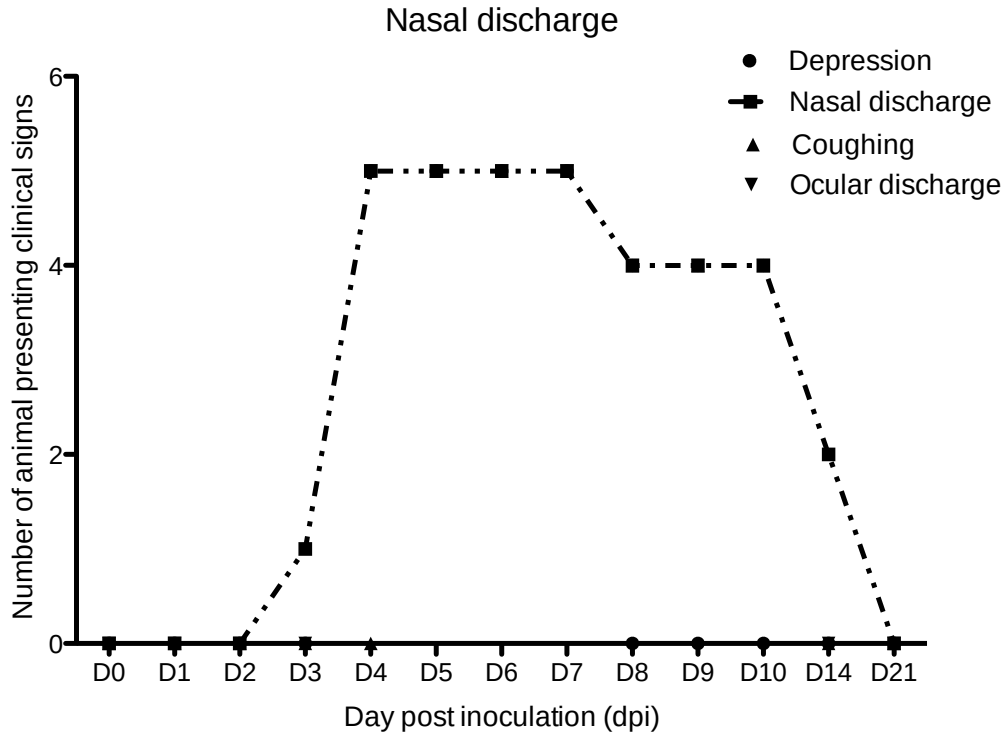
Figure 20 Clinical presentations of CIV-H3N2 challenged dogs in contact group

a) Depression (at 2 dpc - 6 dpc) b) Nasal discharge (at 2 dpc - 13 dpc) c) Coughing (at 4 dpc – 9 dpc) and d) Ocular discharge (at 3 dpc - 9 dpc).

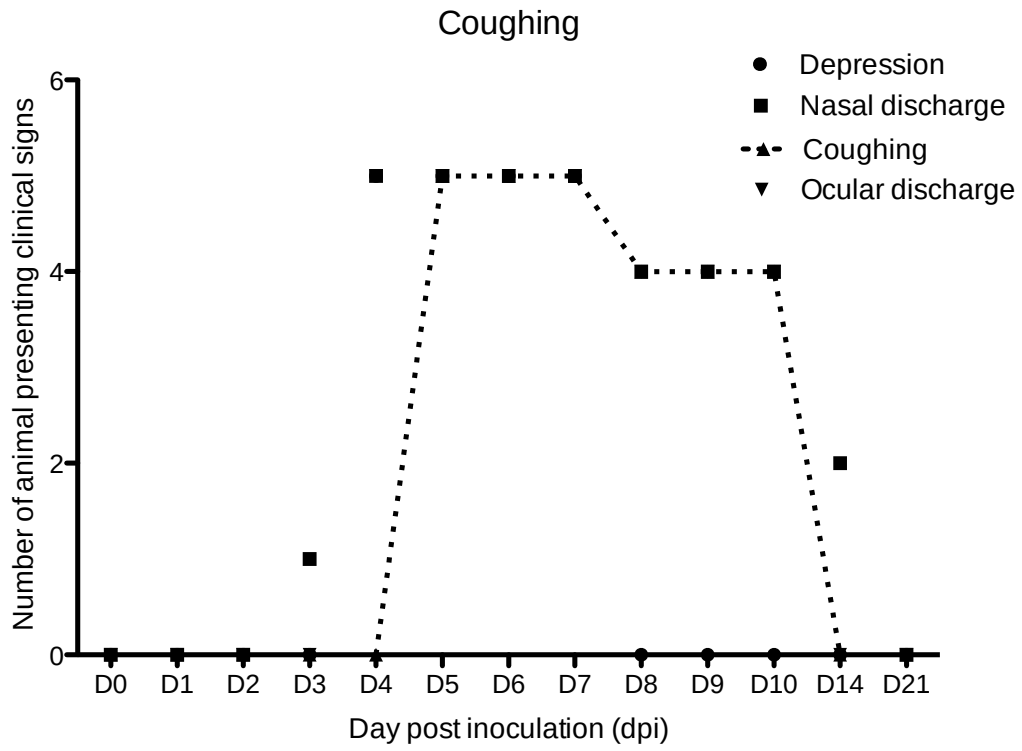
a) Depression; contacted dogs presented depression since at 2 dpc until 6 dpc.



b) Nasal discharge; one dog showed 2 dpc, four dogs at 3 dpc until 13 dpc.



c) Coughing; at 4 dpc – 9 dpc.



d) Ocular discharge; dogs showed ocular discharge at 3 dpc until 9 dpc.

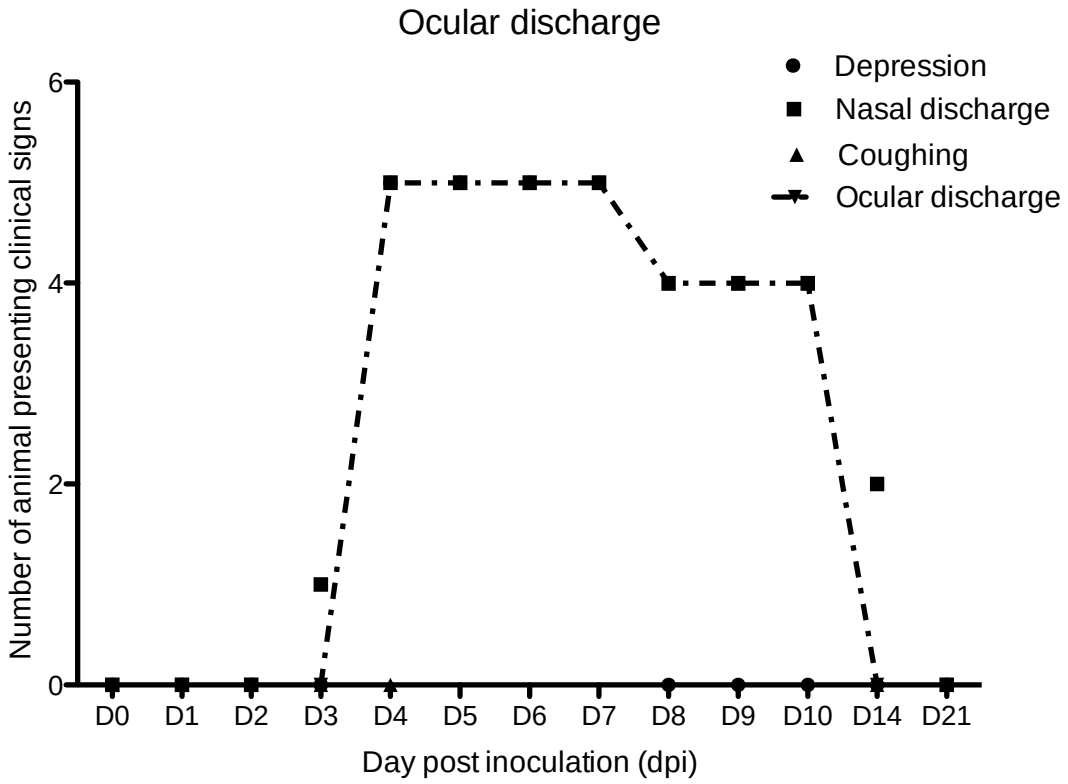
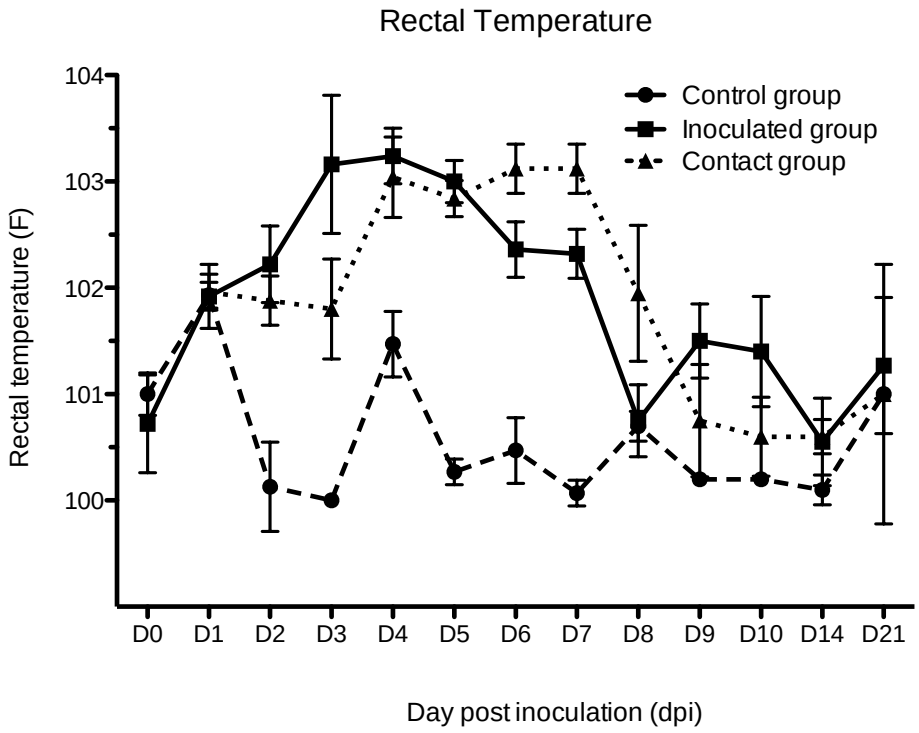


Figure 4.5 Rectal temperatures of CIV-H3N2 challenged dogs in inoculated, contacted and control groups.



Antibody response of CIV-H3N2 challenged dogs

Blood samples were collected from all dogs at day -7, 0, 7, 10, 14 and 21 post inoculation. Serum samples were tested for antibodies against influenza A virus by NP-ELISA and specific antibodies against CIV-H3N2 by Haemagglutinin inhibition (HI) assay. In this experiment, all dogs did not have antibodies against CIV-H3N2 at day -7 dpi and 0 dpi. The dogs in inoculated and contact groups showed seropositive at 10 dpi and 14 dpi, respectively until the end of experiment (Table 4.4). All dogs in inoculated and contact groups presented positive HI titer since day 14 dpi. These results suggested that HI antibody titers against Thai CIV-H3N2 were completely developed at 10 dpi in inoculated group and 14 dpi in contact group. As expected, the dogs in control group did not have HI antibody throughout the experiment (Figure 4.6, Table 4.4 and 4.5).

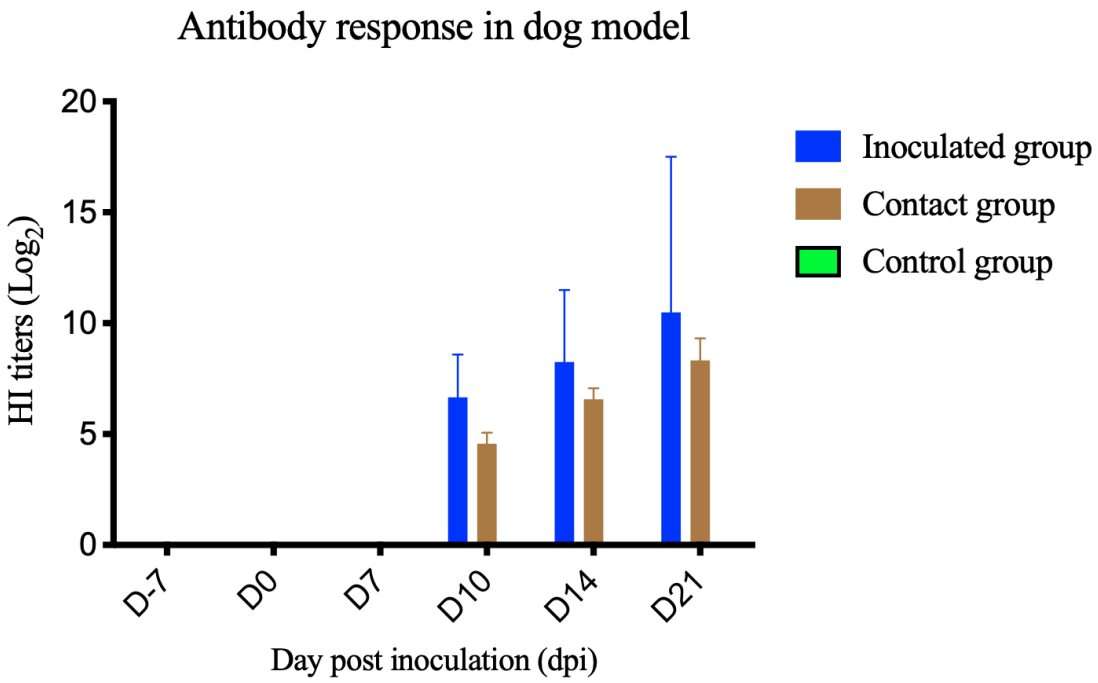
Table 5 Number and percentage of dogs showed antibody response to influenza A virus by NP-ELISA assay.

Number of dogs showed antibody response to influenza A virus (%)						
	D-7	**D0**	**D7**	**D10**	**D14**	**D21**
Inoculated group (n=5)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/4 (100%)	4/4 (100%)	3/3 (100%)
Contact group (n=5)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/4 (25%)	4/4 (100%)	3/3 (100%)
Control group (n=3)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/2 (0%)	0/1 (0%)

Table 6 Antibody response to CIV-H3N2 in challenged dogs by HI assay.

Inoculated group						Mean	SD
D7	0	0	0	10	10	4.000	5.477
D10		80	40	40	80	56.569	23.094
D14		160	80	160	80	113.137	46.188
D21			160	160	80	126.992	46.188
Contacted group						**Mean**	**SD**
D7	0	0	0	0	0	0.000	0.000
D10		20	20	20	40	23.784	10.000
D14		80	160	80	80	95.137	40.000
D21			640	320	160	320.000	244.404
Control group						**Mean**	**SD**
D7			0	0	0	0	0
D10				0	0	0	0
D14				0	0	0	0
D21					0	0	0

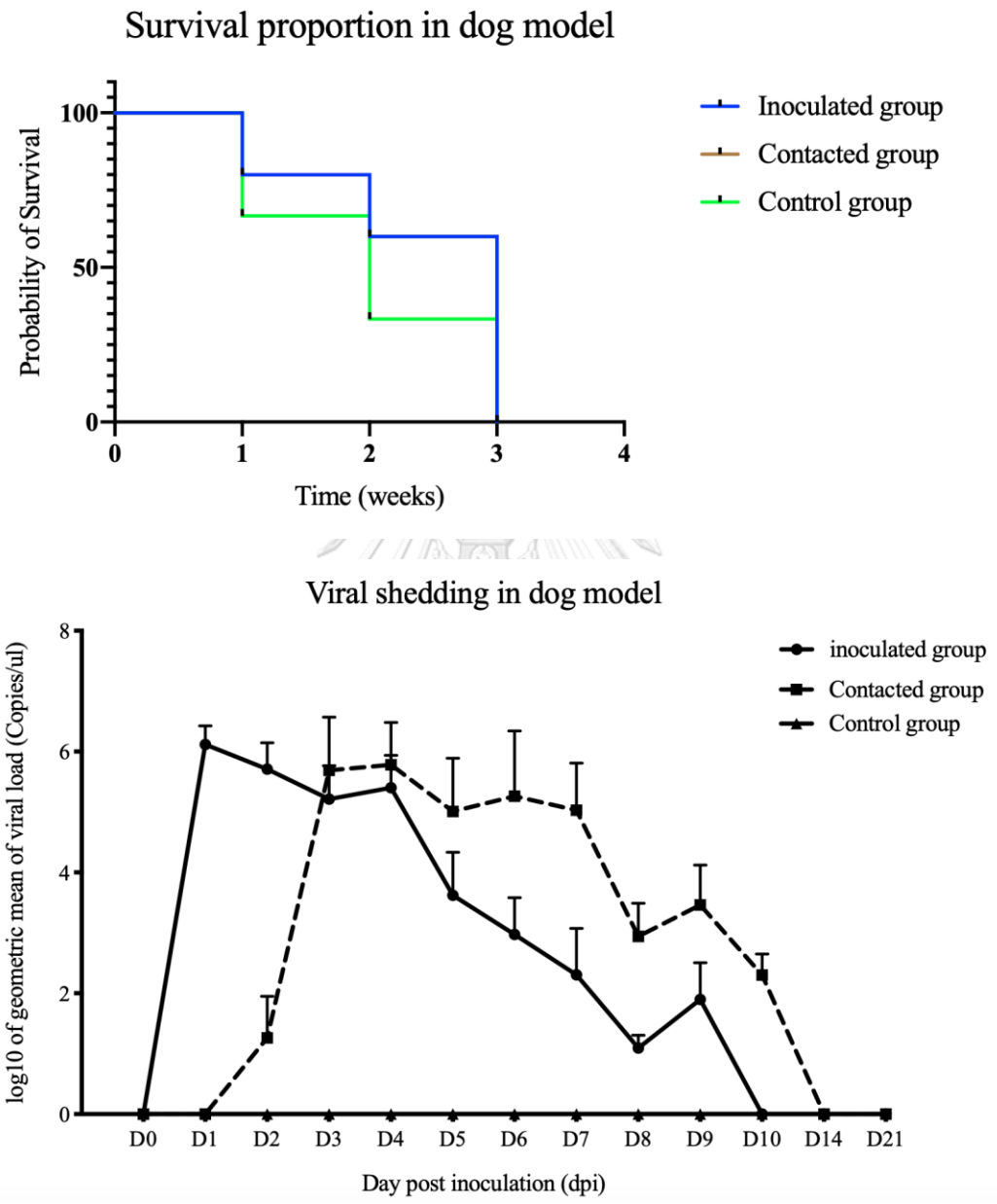
Figure 21 Antibody response to CIV-H3N2 in challenged dogs by HI assay.



Virus shedding of CIV-H3N2 challenged dogs

To confirm viral shedding in dogs, nasal swab samples were collected from day -1, 0, 10, 14 and 21 dpi. The viral titer was detected by using real time RT-PCR. The real time RT-PCR result in Ct value was conversed to viral copies per microliter. Our results showed that all dogs are negative for CIV-H3N2 before inoculation (-1 and 0 dpi). In inoculated group and contact group, CIV-H3N2 could be detected in the respiratory tract (Figure 4.7). In detail, dogs in inoculated group shed CIV-H3N2 in respiratory tract at 1 dpi (highest) and decreased gradually until 9 dpi. Dogs in contact group, some dogs shed the virus since 1 dpc (1 day post contact) and highest at 2 dpc and decreased gradually until 9 dpc. It is noted that the CIV-H3N2 could not be detected in all rectal swab samples of dogs throughout the experiment. As expected, in control group, no viral shedding in all dogs throughout the experiment.

Figure 22 Survival proportion and viral shedding from CIV-H3N2 challenged dogs in inoculated and contact group. Viral shedding was present as log10 of geometric mean (copies per microliter).



Gross and histopathological changes of CIV-H3N2 challenged dogs

For pathological changes of CIV-H3N2 challenged experiment in dogs, a dog from each group was euthanized at 7 dpi and 14 dpi. The organ tissues including lung, heart, liver, spleen and kidney were collected for gross and histopathological examination.

In inoculated group, gross diagnosis was moderate acute diffuse, red hepatization, pneumonia with multifocal petechial hemorrhage, mild splenomegaly and

mild hepatic congestion (Figure 4.8). In detail, at 7 dpi, gross lesion of lungs showed that lung lobes were collapse with moderate red hepatization. Spleen was round edge with mild splenomegaly. Liver was mild hepatic congestion. Kidney did not show any remarkable lesion. In contact group at 7dpi, all lung lobes were collapse moderate red hepatization and multifocal petechial hemorrhage. At 14 dpi, lung lobes showed moderate congestions but no frothy exudate in tracheal lumen in both inoculated and contact groups (Figure 4.9).

The histological examination of inoculated dogs and contacted dogs at 7 dpi (Figure 4.10) showed diffusely interstitial pneumonia. Pneumocyte type II hyperplasia and inflammatory cells were found. The shortening of tracheal epithelial cells was identified as tracheitis in both groups. At 14 dpi, the inoculated dogs showed moderate diffuse pulmonary edema with focally extensive hemorrhage, mild tracheitis and the

contacted dog showed severe diffuse interstitial bronchopneumonia with edema, moderate tracheitis.

Figure 23 Gross examination in CIV-H3N2 challenged dog at 7 dpi.
All lung lobes were collapse with moderate red hepatization. There was multifocal petechial hemorrhage in middle, left cranial and caudal lung lobe. Spleen was round edge with mild splenomegaly. Liver was mild hepatic congestion. Kidney did not show any remarkable lesion.

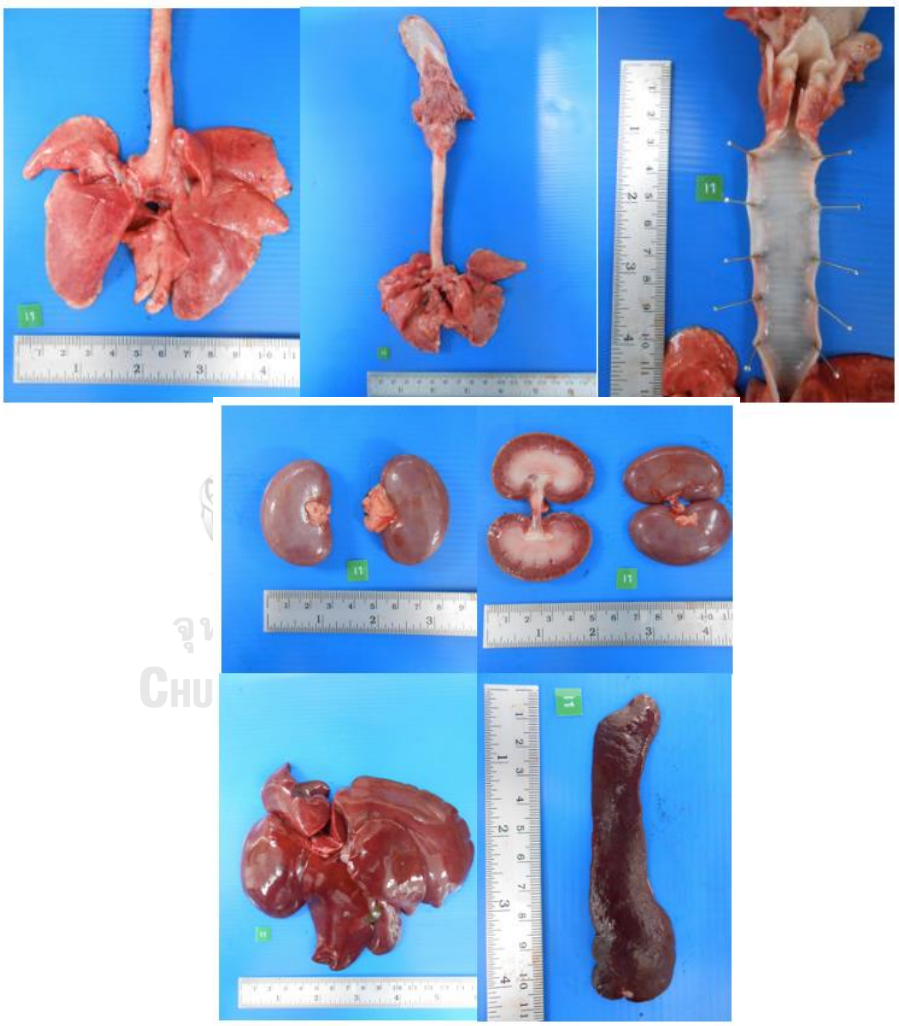


Figure 24 Gross examination in CIV-H3N2 challenged dog at 14 dpi.
There was moderate congestion in caudal lobe of lung. There was no frothy exudate in tracheal lumen. Spleen had round edge spleen with mild splenomegaly. Liver had moderate hepatic congestion with glassy surface. Heart and kidney did not show any remarkable lesion.

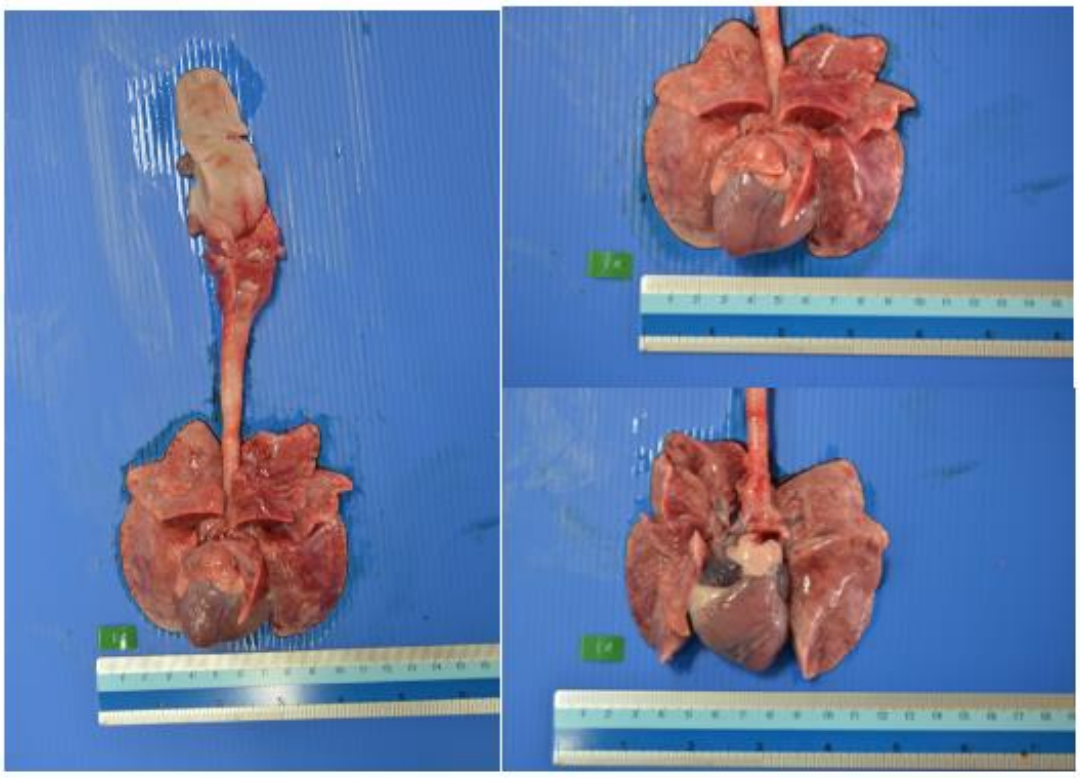
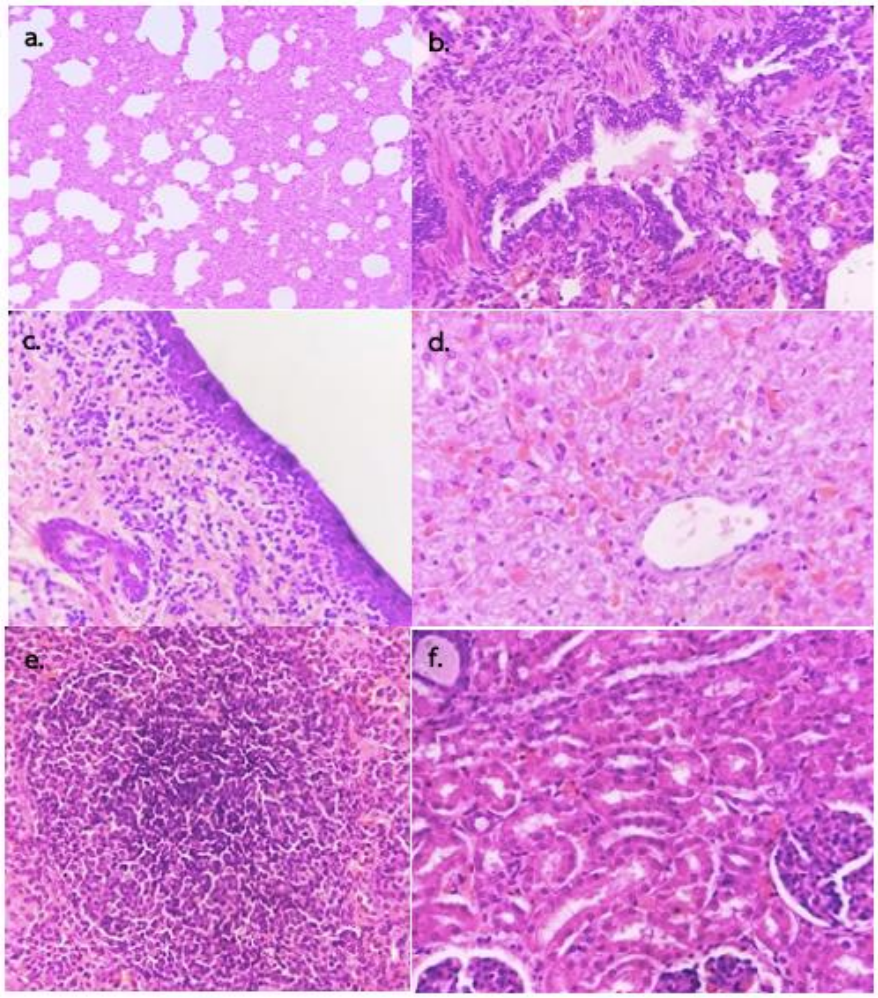


Figure 25 Histological examination in CIV-H3N2 challenged dog.
The findings are a) interstitial pneumonia (4x), b) bronchiolitis obliterans like lesion (40x), c) Inflammatory cell infiltration with shorten tracheal epithelial (10x), d) Centrilobular fatty change degeneration (40x), e) White pulp contained moderate proliferative lymphoid cells and f) mild congestion and tubular degeneration.



4.4 Phase IV: Investigating the pathogenicity of canine influenza viruses in chickens

To examine the pathogenicity of canine influenza virus in chickens, the Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) was challenged in experimental chickens. All chickens in inoculated group (n=3), contact group (n=3) and control groups (n=3) did not show any clinical signs relating to respiratory disease throughout the experiment.

Antibody response of CIV-H3N2 challenged chickens

Blood samples were collected from all chickens at -7, -1, 7 and 12 dpi. Serum samples were tested for antibodies against influenza A virus by NP-ELISA. In this experiment, all chickens did not have antibodies against CIV-H3N2 at day -7 dpi and -1 dpi. The chickens in inoculated and contact groups did not antibody against influenza A virus at 7 and 12 dpi (Table 4.6).

Table 7 Antibodies response to CIV-H3N2 in challenged chickens by NP-ELISA.

Antibodies response to CIV-H3N2 in challenged chicken by NP-ELISA									
Day	Inoculated group			Contact group			Control group		
-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
7 dpi	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
12 dpi	Neg	Neg	39.73	Neg	Neg	Neg	Neg	Neg	Neg

Viral shedding of CIV-H3N2 challenge chickens

To confirm viral shedding in chickens, nasal swab and rectal samples were collected from day -1, 1, 2, 3, 5, 7 and 10 dpi using real time RT-PCR method. The real time RT-PCR results (Ct value) were conversed to viral copies per microliter. Our results showed that all chickens were negative for CIV-H3N2 before inoculation (-1 dpi). In inoculated group, CIV-H3N2 could not be detected in nasal swab and rectal swab samples (Table 4.7). In contact group, the CIV-H3N2 was detected as weak positive at 7 dpi from both oropharyngeal swab and cloacal swab. The rest of swab samples were negative throughout the experiment. As expected, in control group, no viral shedding in all chickens throughout the experiment.

Gross and histopathological changes of CIV-H3N2 challenged chickens

For gross and histopathological lesions, chicken did not show any specific lesion.

Table 8 Real time RT-PCR results (Ct value) of oropharyngeal samples (T) and cloacal samples (C) from CIV-H3N2 challenged chickens in inoculated, contacted and control groups.

	Inoculated group						Contact group						Control group					
No.	2		3		5		4		6		7		1		8		9	
	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C
Day -1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Day 1	(-)	(-)	(-)	(-)	(-)	(-)							(-)	(-)	(-)	(-)	(-)	(-)
Day 2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Day 3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Day 5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Day 7	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	**33.02**	**32.96**	(-)	(-)	(-)	(-)	(-)	(-)
Day 10	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

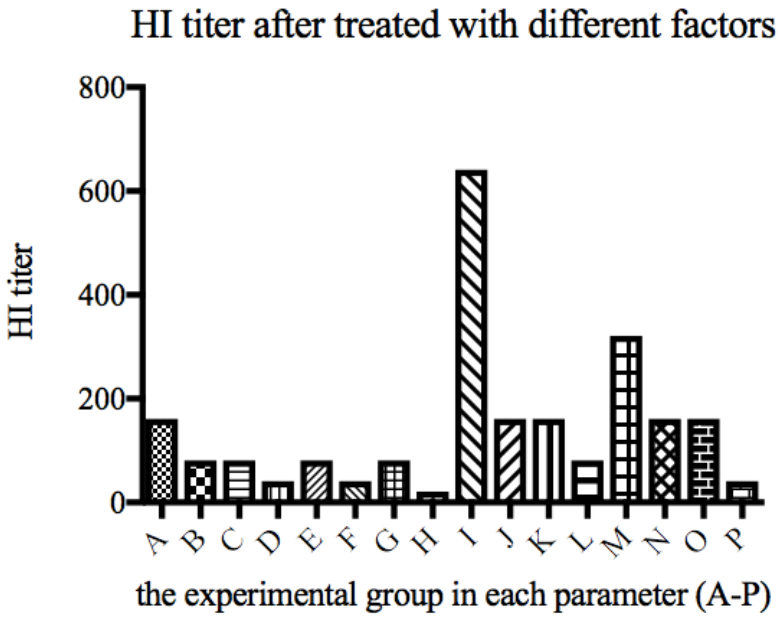
4.5 Phases V: Investigating the pathogenicity of canine influenza viruses in guinea pigs

In this experiment, there were 2 phases including a) HI protocol standardization for guinea pig sera and b) Investigating the pathogenicity of canine influenza virus in Guinea pigs.

4.5.1 HI protocol standardization for guinea pig sera

Known positive guinea pigs sera sample was treated with four different factors including a) elimination of non-specific inhibitors (20% kaolin or receptor destroying enzyme (RDE)), b) type of red blood cells (RBCs), and c) percentage of RBCs, and d) hemagglutination unit (HAU) of virus and classified as 16 experimental groups. The results showed that the serum which was treated with receptor destroying enzyme (RDE), 1% of turkey RBCs and 4HAU/25μl (group I) showed the highest HI titer compared with other 15 groups. The serum which was treated with receptor destroying enzyme (RDE), 1% of chicken RBCs and 4HAU/25μl (group M) showed the second highest HI titer. The detail is shown in Figure 4.11.

Figure 26 HI titers after evaluation by 4 different factors (16 experimental groups; A-P)



4.5.2 Investigating the pathogenicity of canine influenza viruses in guinea pigs

To examine the pathogenicity of canine influenza viruses in guinea pigs, the Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) was challenged in experimental guinea pigs. All guinea pigs in inoculated group (n=5), direct contact group (n=5), aerosol contact group (n=5) show mild clinical signs relating to respiratory disease and significant high rectal temperature compared with control groups (n=5).

In detail, weight measurements, all guinea pigs were weighted at -7, 0-7, 10 and 14 dpi. The mean weight of guinea pigs presented in inoculated group (497.20 – 593.67 g), direct contact group (468.20 – 575.33 g), aerosol contact group (490.40 – 614.67 g) and control group (488.00 – 612.00 g) and no significantly different among groups (Figure 4.12 and Table 4.8).

For rectal temperature, the mean in inoculated group was in the range of 100.44 – 102.12 °F whereas in control group was in the range of 100.07 – 101.00 °F. The direct contacted and aerosol contact group showed in the range of 99.92 – 102.04 °F and 100.23 – 101.47 °F, respectively (Figure 4.13 and Table 4.9). The rectal temperature of guinea pigs in inoculated group was statistical significantly higher than that of the control group at 1 dpi – 3 dpi (Figure 4.13 and Table 4.9). Interestingly, the rectal temperature of direct contacted and aerosol contacted showed statistical significantly higher than the control group only at 1 dpc.

Figure 27 Mean weight (gram) of CIV-H3N2 challenged guinea pigs (inoculated, direct contact, aerosol contact and control groups)

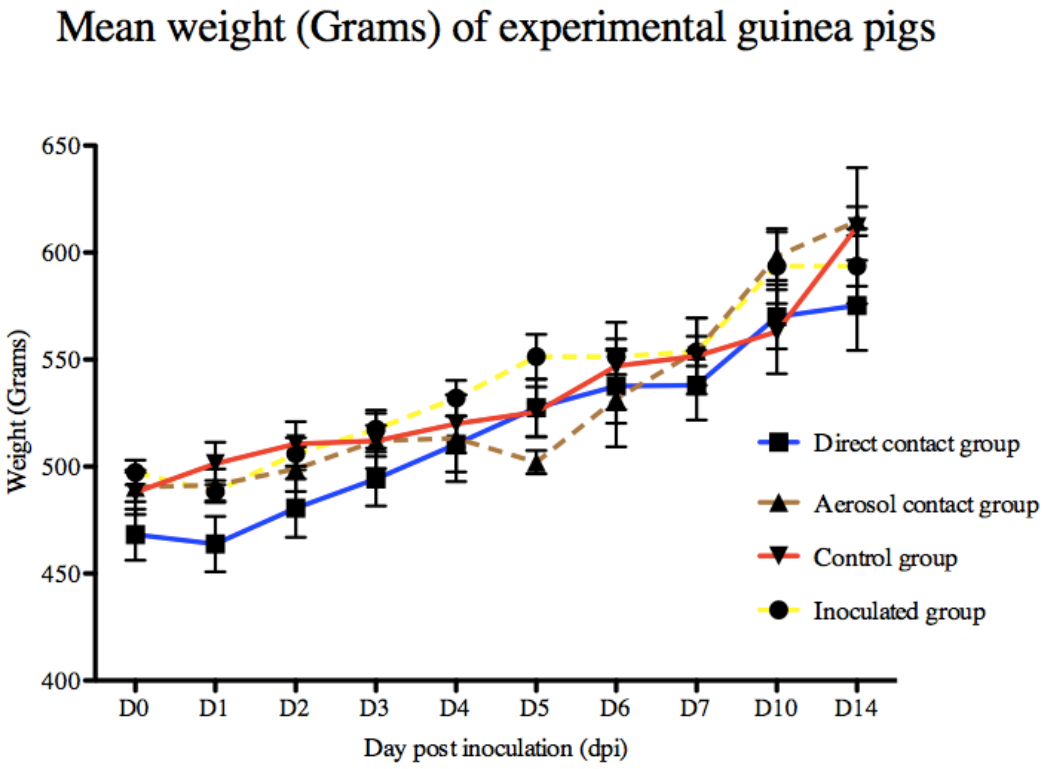


Table 9 Mean weight, standard deviation and number of guinea pig in each experimental group.

	Control			Inoculated gr			Direct contacted gr			Aerosol contacted gr		
Day	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	488.00	23.28	5.00	497.20	12.81	5.00	468.20	26.72	5.00	490.40	15.27	5.00
1	501.40	22.28	5.00	488.40	11.44	5.00	463.80	28.94	5.00	491.40	16.65	5.00
2	510.60	23.16	5.00	506.00	16.78	5.00	480.60	30.61	5.00	498.80	23.41	5.00
3	512.00	28.87	5.00	517.50	19.94	5.00	494.20	28.24	5.00	512.00	16.18	5.00
4	520.00	27.36	4.00	532.00	16.55	4.00	510.50	26.03	4.00	513.25	40.62	4.00
5	525.50	23.59	4.00	551.33	20.84	4.00	527.50	26.81	4.00	502.00	10.86	4.00
6	547.00	35.38	3.00	551.33	14.50	3.00	537.67	30.14	3.00	531.67	38.85	3.00
7	551.67	30.92	3.00	553.67	27.21	3.00	538.00	28.05	3.00	554.00	12.00	3.00
10	563.00	34.07	3.00	593.67	30.37	3.00	570.00	26.06	3.00	598.33	19.66	3.00
14	612.00	48.04	3.00	593.67	30.37	3.00	575.33	36.50	3.00	614.67	11.68	3.00

Figure 28 Mean rectal temperature (F) of CIV-H3N2 challenged guinea pigs (inoculated, direct contacted, aerosol contacted and control groups)

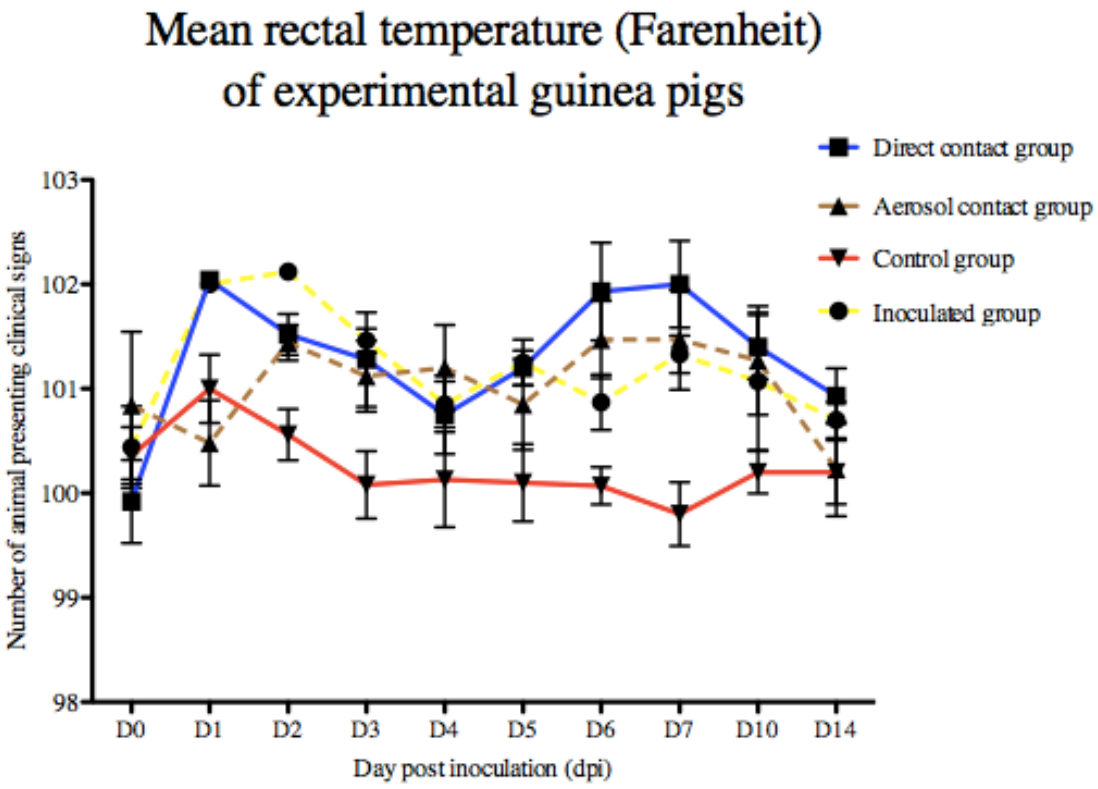


Table 10 Mean rectal temperature, standard deviation and number of guinea pig in each experimental group.

	Control			Inoculated gr			Direct contacted gr			Aerosol contacted gr		
Day	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	100.36	0.61	5.00	100.44	0.88	5.00	99.92	0.89	5.00	100.84	1.58	5.00
1	101.00	0.73	5.00	102.00	0.14	5.00	102.04	0.09	5.00	100.48	0.91	5.00
2	100.56	0.55	5.00	102.12	0.11	5.00	101.52	0.44	5.00	101.44	0.38	5.00
3	100.08	0.72	5.00	101.46	0.26	5.00	101.28	1.01	5.00	101.12	0.76	5.00
4	100.13	0.91	4.00	100.85	0.44	4.00	100.75	0.75	4.00	101.20	0.82	4.00
5	100.10	0.74	4.00	101.25	0.44	4.00	101.20	0.33	4.00	100.85	0.87	4.00
6	100.07	0.31	3.00	100.87	0.46	3.00	101.93	0.81	3.00	101.47	0.64	3.00
7	99.80	0.53	3.00	101.33	0.31	3.00	102.00	0.72	3.00	101.47	0.83	3.00
10	100.20	0.35	3.00	101.07	1.14	3.00	101.40	0.53	3.00	101.27	0.90	3.00
14	100.20	0.53	3.00	100.70	0.30	3.00	100.93	0.46	3.00	100.23	0.78	3.00

Antibody response of CIV-H3N2 challenged guinea pigs

Blood samples were collected from all guinea pigs at -7, 0, 7, 10 and 14 day post inoculation. Serum samples were tested for specific antibodies against CIV-H3N2 by Haemagglutinin inhibition (HI) assay. In this experiment, all guinea pigs did not have antibodies against CIV-H3N2 at day -7 dpi and 0 dpi.

The guinea pigs in inoculated group (2/3; 66.67%) showed seropositive since at 7 dpi but guinea pigs in direct contacted (2/3; 66.67%) and aerosol contacted (3/3; 100%) showed seropositive since 10 dpi. These results suggested that HI antibody titers against Thai CIV-H3N2 were completely developed at 10 dpi in inoculated group. As expected, the guinea pigs in control did not have HI antibody throughout the experiment (Figure 4.14 and Table 4.11). Positive samples were identified with samples showing a titer $\geq$ 40 (Bunpapong et al., 2014).

Figure 29 HI titers from CIV-H3N2 challenged guinea pigs (inoculated, direct contact, aerosol contact and control groups).

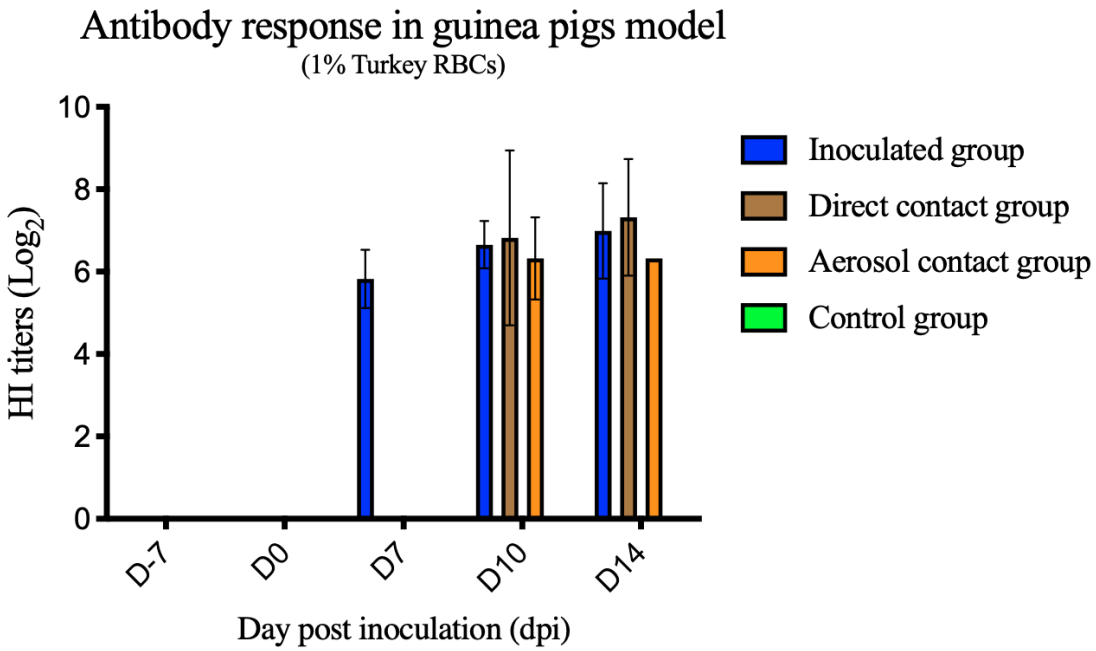


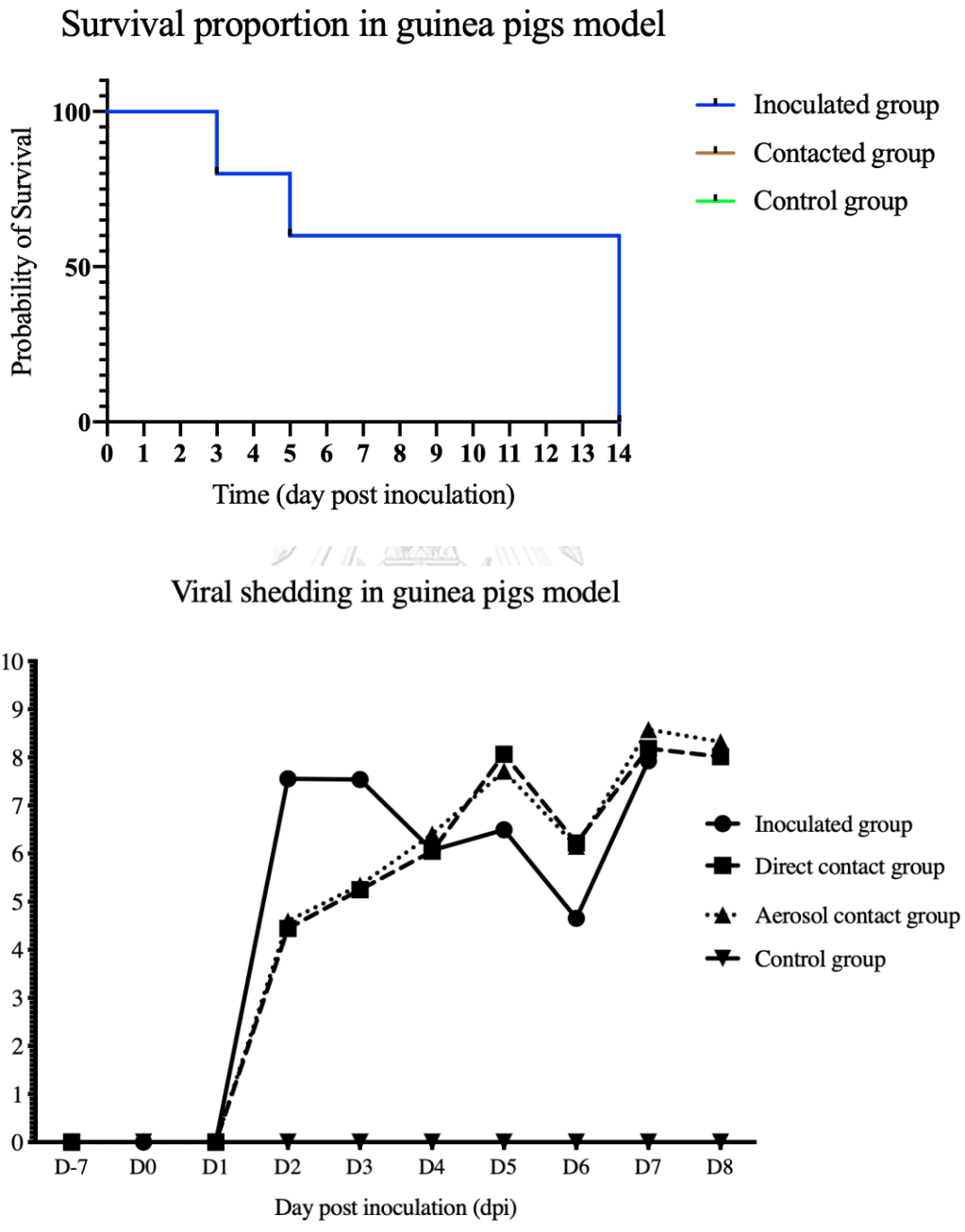
Table 11 HI titers of CIV-H3N2 challenged guinea pigs (inoculated, direct contacted, aerosol contacted and control groups) at 0, 7, 10 and 14 dpi

	Control			Inoculated gr			Direct contacted gr			Aerosol contacted gr		
Day pi	D1	D4	D5	A3	A4	A5	B1	B2	B5	C1	C3	C5
0	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
7	(-)	(-)	(-)	40	(-)	80	(-)	(-)	(-)	(-)	(-)	(-)
10	(-)	(-)	(-)	80	80	160	320	40	(-)	160	80	40
14	(-)	(-)	(-)	80	80	320	320	80	(-)	80	80	80

Virus shedding of CIV-H3N2 challenged guinea pigs

To confirm viral shedding in guinea pigs, nasal wash samples were collected from day -7, 0, 2-7 dpi. The viral titer was detected by using real time RT-PCR. The real time RT-PCR result in Ct value was conversed to viral copies per microliter. Our results showed that all guinea pigs are negative for CIV-H3N2 before inoculation (-7 and 0 dpi). In inoculated group, direct contact group and aerosol contact group, the CIV-H3N2 could be detected in the respiratory tract (Figure 4.15). In detail, guinea pigs in inoculated group shed CIV-H3N2 in respiratory tract at 2 dpi – 3 dpi. Guinea pigs in direct contact group and aerosol contact group shed the virus since 4 dpc - 7 dpc. Interestingly, the viral titers showed the highest titer in the aerosol contact group at 7 dpi. Moreover, the viral titers in directed contact group and aerosol contact group showed higher titers than the inoculated group. As expected, in control group, no viral shedding in all guinea pigs throughout the experiment.

Figure 30 Survival proportion and viral shedding from CIV-H3N2 challenged guinea pigs in inoculated group, direct contact group and aerosol contact group. Viral shedding was present as $\log_{10}$ of geometric mean (copies per microliter).



Gross and histopathological changes of CIV-H3N2 challenged guinea pigs

For pathological changes of CIV-H3N2 challenged experiment in guinea pigs. A guinea pigs from each group was euthanized at 3 dpi and 5 dpi. The organ tissues including lung, heart, liver, spleen and kidney were collected for gross and histopathological examination.

In inoculated group, gross diagnosis was mild lung congestion, moderate hepatic congestion and mild splenomegaly (Figure 4.16). In detail, at 3 dpi, gross lesion of lungs showed that mild congestion in left caudal lobe of lung. Moderate emphysema at periphery was also seen. Spleen was round with mild splenomegaly. Liver had moderate hepatic congestion and reddish kidney. The mild lung congestion was also found in direct contact group and aerosol contact group

Histopathological examination in the inoculation group showed necrotizing and lymphocytic bronchointerstitial pneumonia, mild multifocal BALTs hyperplasia with type II pneumocyte and PAMs hyperplasia with mild tracheitis (Figure 4.21). In direct contacted and aerosol contact groups, the lesions showed bronchointerstitial pneumonia with tracheitis (Figure 4.17 – 4.19).

Figure 31 Gross examination of CIV-H3N2 challenged guinea pig in inoculated group. There was mild congestion in lung. Moderate emphysema at periphery was also seen.

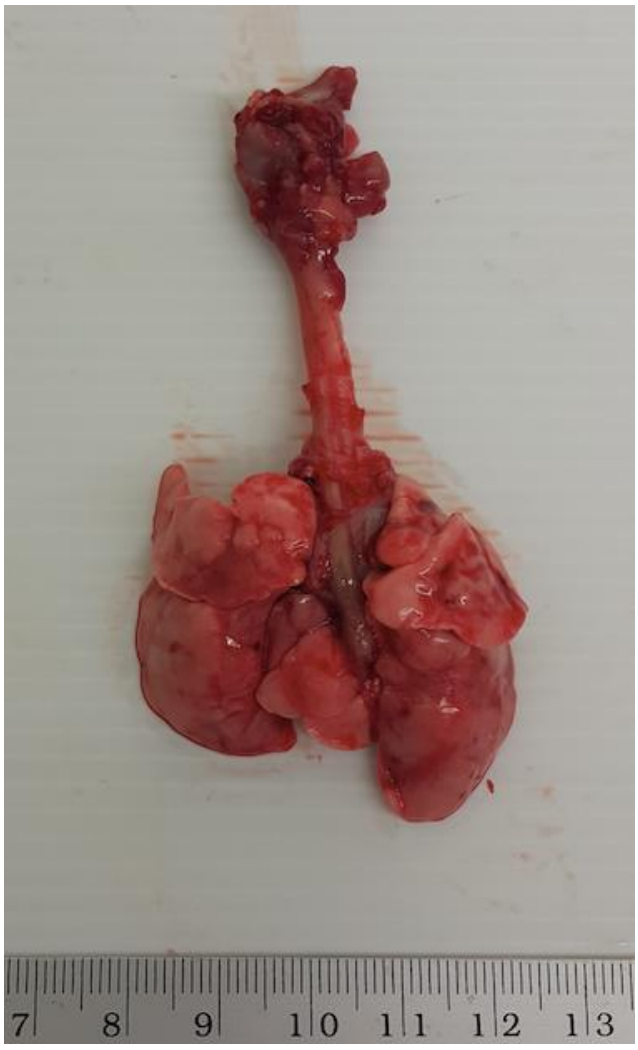
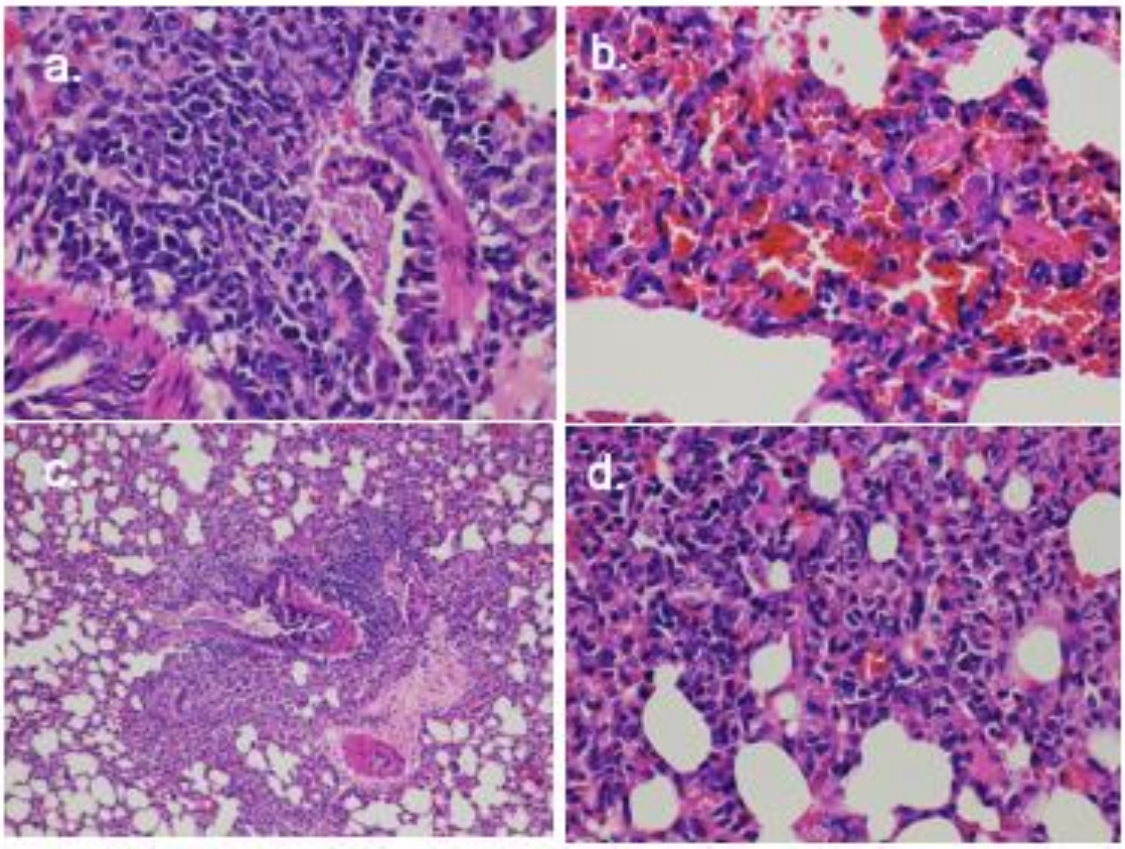


Figure 32 Histological examination of CIV-H3N2 challenged guinea pig in inoculated group.

The findings are a) bronchointerstital pneumonia, BALTs hyperplasia and hemorrhage 40x, b) bronchointerstital pneumonia, BALTs hyperplasia and hemorrhage 40x, c) bronchointerstitial pneumonia and BALTs hyperplasia 10x, d) interstitial pneumonia and type II pneumocyte hyperplasia 40x, e) myocardial degeneration and fibrosis 4x. f) myocardial degeneration and fibrosis 40x, g) myocardial degeneration and fibrosis 10x, h) pulmonary hemorrhage 10x and i) pulmonary hemorrhage 40x.



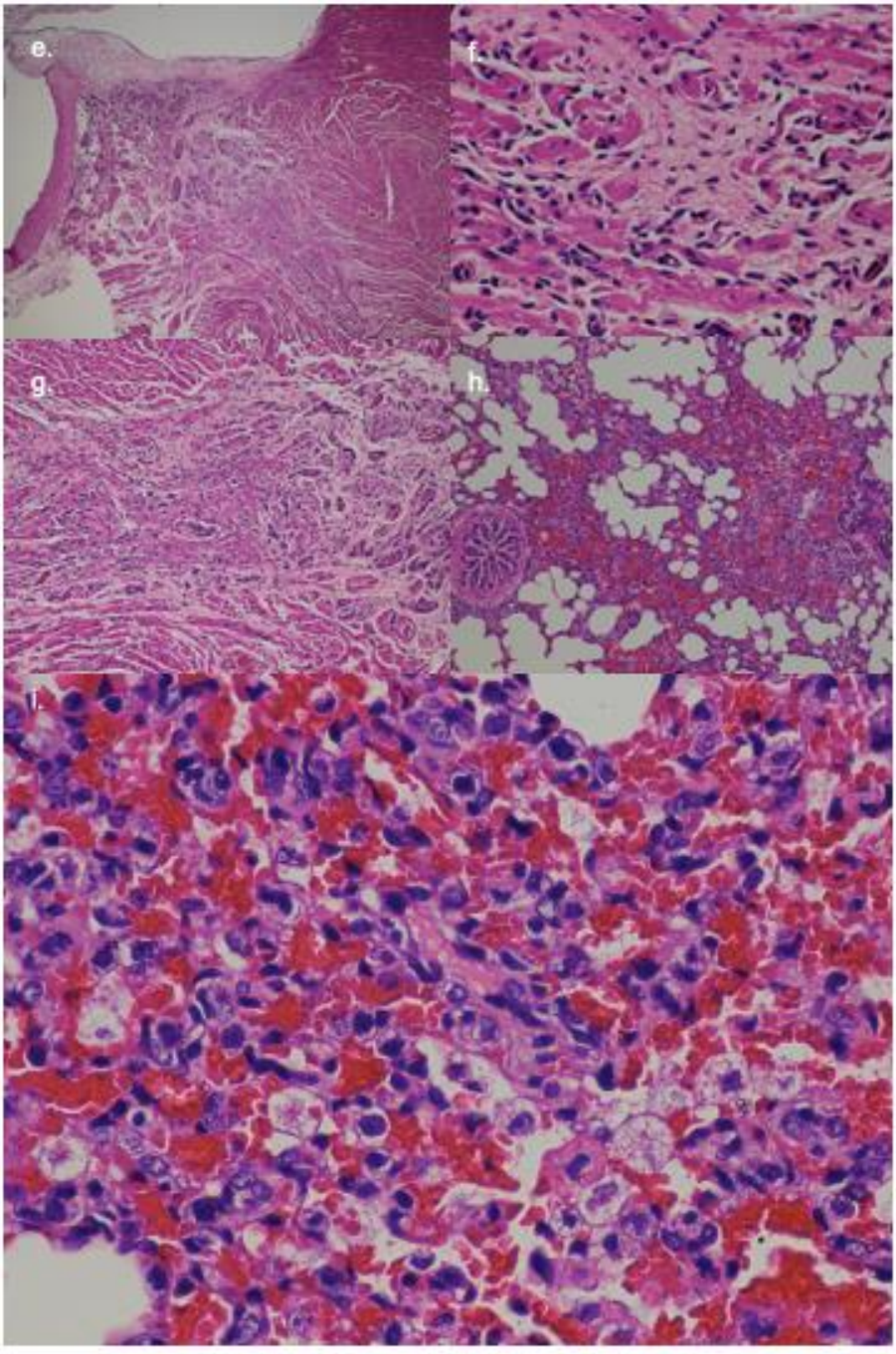

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Figure 33 Histological examination of CIV-H3N2 challenged guinea pig in direct contact group.

The findings are a) interstitial pneumonia 4x b) interstitial pneumonia 10x, c) interstitial pneumonia 40x, d) lung Catarrhal bronchiolitis 40x, e) lung Pulmonary hemorrhage 10x

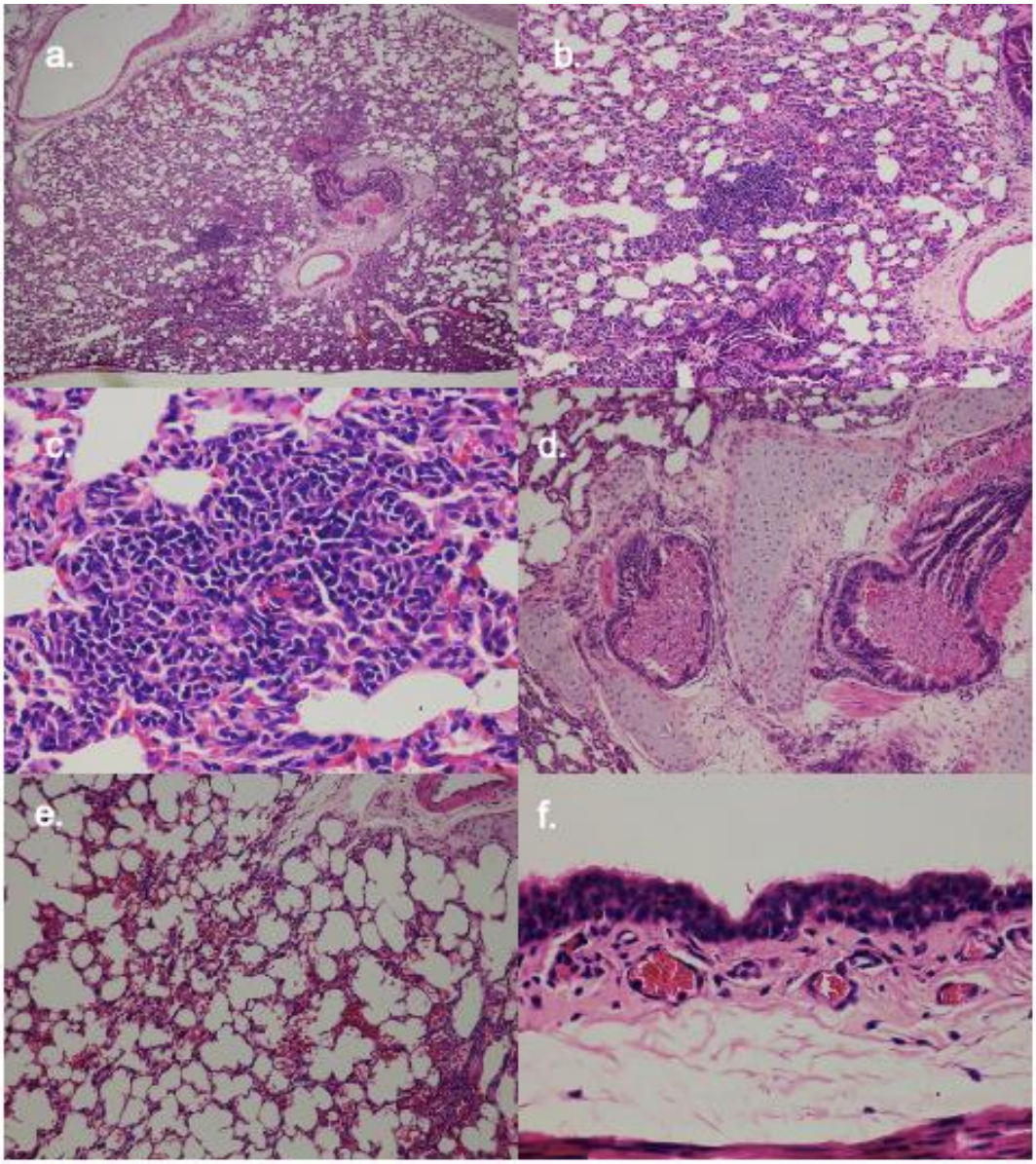
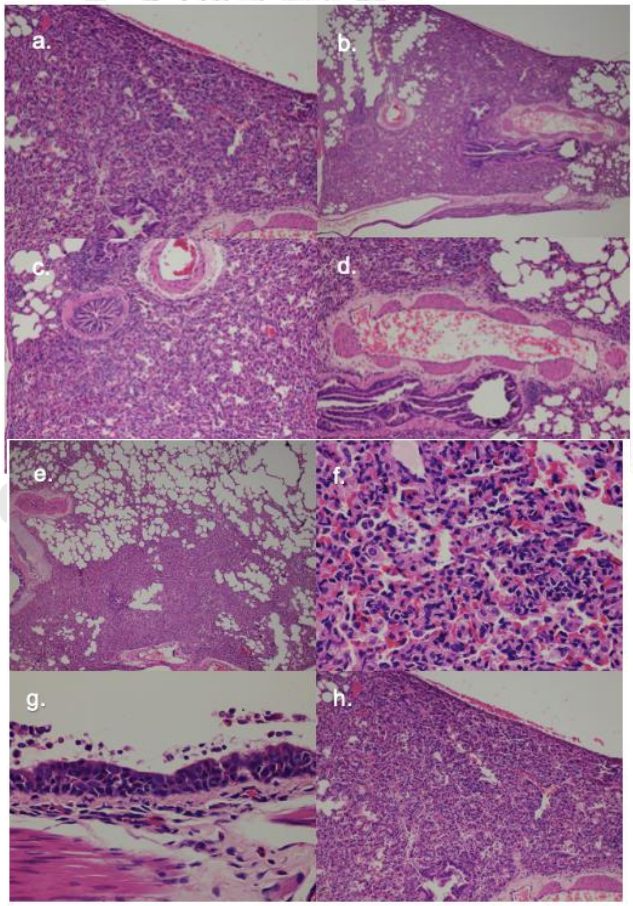


Figure 34 Histological examination of CIV-H3N2 challenged guinea pig in aerosol contact group.

The findings are a) interstitial pneumonia 10x, b) interstitial pneumonia and BALTs hyperplasia 4x, c) interstitial pneumonia and BALTs hyperplasia 10x d) interstitial pneumonia and BALTs hyperplasia 10x e) interstitial pneumonia with type II pneumocyte and PAMs hyperplasia 4x, f) interstitial pneumonia with type II pneumocyte and PAMs hyperplasia 40x, g) tracheitis and mucosal attenuation 40x and h) interstitial pneumonia 10x.



CHAPTER 5

DISCUSSION

5.1 Canine influenza virus infection in dogs and in cats in Thailand

Canine influenza viruses have been reported to infect dogs in many countries. The first report of the outbreak of equine origin CIV-H3N8 was documented in the US, 2004 (Crawford et al., 2005). In Asia, the CIV-H3N2 has been reported in South Korea, China and Thailand in the 2010s (Song et al., 2008; Li et al., 2010; Lin et al., 2012; Bunpapong et al., 2014). The inter-species transmission of CIV-H3N2 between dogs and cats has also been reported (Kim et al., 2013). Thus, zoonotic potential of CIV-H3N2 between humans and pets has been a concern. Many studies have been documented the possibility that dogs can be infected with seasonal human influenza viruses especially pdmH1N1-2009 (Chang et al., 1976; Su et al., 2014; Chanvatik et al., 2016). Thus, zoonotic potential of CIV-H3N2 between humans and pets has been a concern.

In this study, the seroprevalence of influenza A virus infection in dogs in Thailand was 0.97%. And the seroprevalences of specific IAV subtype (pdmH1N1-2009) in dogs and cats were 0.64% and 1.20%, respectively. It should be noted that this study is the first to report of pdmH1N1-2009 infection in sheltered dogs in Thailand. Compared with previous studies, the seroprevalence (0.64%) for pdmH1N1-2009 in dogs in Thailand was comparable to the study conducted in Italy in 2009 (0.7%) (Dundon et al., 2010) but slightly lower than the report from China (1.5%) (Sun et al., 2014). Experimental data have revealed efficient CIV-H3N2 transmission among dogs

whereas pdmH1N1-2009 has shown limited transmission (Song et al., 2009; Lin et al., 2012). In this study, antibodies against canine H3N2 and human H3N2 could not be found. On the other hand, the seropositive of canine H3N2 that have been reported in China (Zhao et al., 2011; Sun et al., 2014) similar to the report in South Korea (Lee et al., 2009). Seropositivity to Human H3N2 in cat has also been reported in China and Japan (Song et al., 2009; Ali et al., 2011; Sun et al., 2014). In this study, all positive samples were collected from September to January (winter season) showed an indication of a possible association with weather and temperature. All samples were collected from sheltered animals, that stay in the free-range area and have unrestricted contact with the environment. Human influenza has been reported in significantly high frequency in winter season (Potter and Jennings, 2011). It is possible that there is a degree of correlation between the seropositive samples from sheltered dogs and cats in this study and the high prevalence of human influenza, but this suggestion will need an independent validation. In this study, there were inconsistencies between NP-ELISA and HI test result. The seropositivity presented by NP-ELISA and HI showed nine positive samples (0.97%) and five positive samples (0.53%) from dog sera samples, respectively. Of these, 4 of the 9 positives by NP-ELISA were positive by HI test. Similar to the previous study, the seropositive prevalence of CIV in dogs by HI assay was lower than NP-ELISA assay. It has been known that NP-ELISA is suitable for rapid and large-scale serological screening of IAV, while HI assay provides information on specific antibodies against influenza subtypes. Some studies

documented that the antibodies with the HI test appeared 2 days later than the NP-ELISA test (Lee et al., 2009; Zhao et al., 2011). It should be noted that cross-reactivity between virus antigens or closely related antigenic viruses could not be ignored; hence, these findings should be taken with some cautions (Tse et al., 2012).

In conclusion, our results revealed antibodies against pdmH1N1-2009 in sheltered dogs and cats in the central and the eastern provinces of Thailand. Potential zoonosis and reverse zoonosis of influenza viruses between domestic animals and human should be a concern particularly to the public and animal health authorities. Thus, surveys of influenza virus infection in domestic animals as well as animal caretakers in the shelters and those living in close proximity should be routinely conducted. Discussion content is part of the publication by Tangwangvivat et al., 2019 (Tangwangvivat et al., 2019).

5.2 Low pathogenic avian influenza (LPAI) of Thai CIV-H3N2

For IVPI test, the chickens were inoculated intravenously with Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) and were monitored for 12 days. The chickens did not present any respiratory symptoms that are related to influenza infection. The IVPI index of Thai CIV-H3N2 in this experiment was 0.00 indicating low pathogenicity of the virus. In general, chickens when inoculated with some LPAI or HPAI will show clinical signs such as depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and head, nervous signs and death in severe cases. The

transmissions of LPAI viruses to domestic animals indicating cross species barriers were found in many virus subtypes and many animal species such as marine mammals and birds (Short et al., 2015). In the previous study revealed that dogs have the receptors for avian influenza virus in the lower part of the respiratory tract, trachea and nose (Maas et al., 2007). Thus, dogs are susceptible to avian influenza virus especially HPAI-H5N1 infection (Giese et al., 2008) but there is still no evidence of the transmission of canine influenza from dog to avian species.

The explanations for LPAI characteristics of Thai CIV-H3N2 in chickens are i) species preference of the CIV and ii) administration route. The specificity of CIV binding to HA receptor of host should be efficient for species preference (Short et al., 2015). Receptors for influenza virus are in respiratory tract of host, thus administration route by intravenous inoculation may affect the outcome of the IVPI test (Ramos et al., 2011).

5.3 Pathogenicity of Thai CIV-H3N2 in dogs

For pathogenicity test of Thai CIV-H3N2 in dogs, the A/canine/Thailand/CU-DC5299/2012/H3N2 was used for the challenge study. This virus was previously characterized virus causing CIV outbreak in dogs in Thailand (Bunpapong et al., 2014). In this challenge experiment, CIV-H3N2 can infect dogs both in the inoculated group and the direct contact group. The results showed evidences that dogs were able to shed the virus from respiratory tract and develop antibody response. CIV-H3N2 can infect and replicate in respiratory tract of dogs in inoculated group (100%) since 2 dpi

and in direct contact group since 2 dpc. The CIV-H3N2 infected dogs showed clinical signs including fever, depression, nasal discharge, ocular discharge and coughing. The previous study supported our result that tracheal, bronchial, and bronchiolar epithelial cells of dogs have receptor (SAα 2,3-gal) for avian influenza viruses suggesting potential transmission of avian influenza virus (H3N2) from poultry to dogs (Song et al., 2008). Interestingly, the viral shedding in this study was prolonged until 9 dpi in inoculated group and 14 dpi in inoculated group. In a previous study the CIV-H3N2 (A/canine/Korea/01/2007) infected dogs began to shed the virus at 1 dpi and continue to 6 dpi (Song et al., 2008). The inconsistency of the viral shedding period might be due to i) the virulence of the virus (Thai CIV-H3N2 and Korea CIV-H3N2). The CIV-H3N2 used in this experiment was isolated from the infected dog in Thailand (Bunpapong et al., 2014), while the study by Song and team in 2008 used CIV-H3N2 from canine influenza outbreak in Korea (Song et al., 2008) and ii) the reinfection among dogs by direct contact in this experiment. In this experiment, there were inoculated, contacted and control groups which inoculated and contacted were placed together, so there is a possibility of the transmission back among dogs. For the serological results, the infected dogs showed antibody response against CIV-H3N2 significantly at 10 dpi compared to those in the previous study at 6 and 8 dpi (Song et al., 2008).

There is no report of CIV-H3N2 identified in humans, however the future reassortment should be considered. Some scientific data showed that influenza viruses

can evolved when they have reassortment with contemporary influenza viruses resulted in higher viral replication, transmissibility and virulence (Schrauwen et al., 2011; Short et al., 2015). Breed of dogs is another factor that contribute to more human-animal interface and lead to viral spillover or transmission (Lit et al., 2010).

Influenza vaccine in human has been used for more than 50 years. They are safe and effective to prevent mild to severe outcomes (WHO, 2002). While, the canine influenza vaccine for dogs has been developed in Korea. The inactivated A/canine/Korea/01/07 (H3N2) was reported by Lee and team in 2010. This vaccine showed to be highly efficient to reduce fever and lung lesions and decrease viral shedding in dogs (Lee et al., 2010). Canine influenza vaccination in dogs will be another option for prevention and control of canine influenza virus among dogs and minimize the intra-species transmission by reducing viral shedding.

5.4 Pathogenicity of Thai CIV-H3N2 in chickens

For pathogenicity test of Thai CIV-H3N2 in chickens, the A/canine/Thailand/CU-DC5299/2012/H3N2 was used for the challenge study. In this experiment, the Thai CIV-H3N2 did not replicate and shed in chickens in both of inoculated and contact groups. The antibody response against CIV-H3N2 was not detected in all chickens. Even though the canine CIV-H3N2 is closely related to avian influenza viruses of the Eurasian lineage but the virus was not efficiently infect and cause diseases in challenged chickens. Thus, the challenged chickens did not show any clinical sign and seroconversion against CIV-H3N2.

It has been known that receptor for influenza virus in mammals is SAα 2,6-gal and avian is SAα 2,3-gal (Rogers et al., 1983). Dogs poses mainly SAα 2,3-gal in trachea, bronchus and bronchioles (Song et al., 2008) which CIV-H3N2 can bind to these receptors and cause respiratory disease in dog. On the other hand, the CIV-H3N2 could not efficiently replicate in avian host (chickens). This result suggested that other factors (not only receptor binding between virus and host cell could contribute to the virulence of the virus.

5.5 Pathogenicity of Thai CIV-H3N2 in guinea pigs

Although there are HI standard protocols for detecting anti haemagglutinin specific antibodies for multi-species, there is no HI standard protocol in guinea pig sera. In this experiment, we have developed the standard protocol for HI test in guinea pig before the pathogenicity challenge study. The appropriate standard protocol for HI test in guinea pig is RDE and 1% of turkey RBCs. In detail, for the non-specific inhibitors (20% kaolin or receptor destroying enzyme (RDE), RDE yielded higher HI titer than those treated by 20% Kaolin. Previous report indicated that using turkey RBCs and chicken RBCs provide a highly sensitive and specific assay in canine H3N8 challenged study (Anderson et al., 2012). In our experiment, the turkey RBCs yielded higher than those used chicken RBCs. Therefore, RDE and 1% of turkey RBCs with 4HAU/25μl were chosen for HI standard protocol for detecting anti haemagglutinin specific antibodies in guinea pig.

For pathogenicity test of Thai CIV-H3N2 in guinea pigs, A/canine/Thailand/CU-DC5299/2012/H3N2 was used for the challenge study. The guinea pig used in the animal challenge model represent the mammal model (Azoulay-Dupuis et al., 1984). In general, ferrets were used as mammal models for influenza infection experiment. Many studies reported that ferret possibly transmit influenza virus from infected to non-infected ferrets by housing together (Herlocher et al., 2001; Belser et al., 2011). However, the ferret model has several disadvantages such as expensive, limited suppliers and handling difficulties (Lowen et al., 2006). In addition, there were many

reports of using guinea pigs as mammal model for influenza infection (Lowen et al., 2006). Guinea pig is suitable for both large droplet and air-born viral transmission in mammalian host (Mubareka et al., 2009). Other advantages for using guinea pigs are susceptibility for both avian and human influenza viruses and transmissibility of influenza viruses. In contrast, the immunology of influenza in guinea pig is still unclear because of the paucity of species-specific reagents (Thangavel and Bouvier, 2014).

The results from this study showed evidences that the CIV-H3N2 can infect guinea pigs and the virus can transmit to other guinea pigs to both direct contact group and aerosol contact group. The guinea pigs in inoculated group can shed the virus since 2 dpi to 3 dpi. The previous study showed the virus titers could be detected in guinea pig challenged with pandemic H1N1-2009 since 2 dpi (Wiersma et al., 2015). At 7 dpi, the virus that had been detected again that might have been from reinfection between inoculated group and direct contact group. It is noted that, intermittent viral shedding has been observed in guinea pigs in all groups.

For the serological results, the guinea pig presented the antibody response against CIV-H3N2 partially at 7 dpi and completely at 10 dpi in inoculated group. In contact group, the guinea pig presented the antibody response since 10 dpi in direct contacted and aerosol contact groups. Our results confirmed and supported that CIV-H3N2 can induce antibody response in guinea pig. Thus, guinea pigs are suitable for mammal model for influenza infection.

Guinea pigs are used as animal model for influenza infection in mammals. It has been reported that both SAα 2,3-gal and SAα 2,6-gal receptors are widely presented in nasal and trachea of guinea pig. And, SAα 2,3-gal receptor is the dominantly presented in the lung (Sun et al., 2010). The results from this study confirmed the guinea pig-to-guinea pig transmission. Thus, the transmission of guinea pigs to human should be concerned in term of the presence of SAα 2,6-gal receptor in both human and guinea pig respiratory tract.

CHAPTER 6

CONCLUSIONS and RECOMMENDATIONS

Canine Influenza virus causes respiratory disease in dogs. In this thesis, we monitored canine influenza virus infection in dogs and cats and investigated the pathogenicity of canine influenza virus subtype H3N2 among chicken, dog, and guinea pig models.

In phase I, we surveyed canine influenza virus infection in 47 shelters of 19 provinces from September 2011 to September 2014. The findings are as following:

1. The 932 serum samples from dogs (n=932) and cats (n=79) were tested for antibodies against Influenza A virus.
2. NP-ELISA results showed that 0.97% of canine serum samples (9/932) were positive for influenza A antibodies.
3. HI results showed evidence of HA-specific antibodies against pandemic H1N1-2009 in dogs (5/932; 0.64%) and cats (1/79; 1.20%).
4. Seasonal pattern (September to January) of influenza A infection in dogs was observed.
5. The result from this study phases is published in “Evidence of pandemic H1N1 influenza exposure in dogs and cats, Thailand: A serological survey”, Zoonoses Public Health, 2019; Volume 66, Issue 3, Page 1-5.

In phase 2, we investigated pathogenicity of the canine influenza virus subtype H3N2 (CU-DC5299). The intravenous pathogenicity index test (IVPI) of the virus was evaluated. The findings are as following:

1. For IVPI test, all experimental chickens inoculated with Thai CIV-H3N2 did not show any specific clinical signs relating to respiratory disease.
2. The IVPI index of Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) in this experiment was 0.00 indicating low pathogenicity of the virus.

In phase 3, we investigated the pathogenicity of the canine influenza virus subtype H3N2 (CU-DC5299) in animal model (dogs). This experiment was to proof pathogenicity and potential intra-species transmission of the virus. The findings are as following:

1. The CIV-H3N2 challenged experiment was conducted in 13 dogs (n=13), including inoculated group (n=5), direct contact group (n=5) and control group (n=3).
2. The CIV-H3N2 infected dogs (both in inoculated group and direct contact group) showed statistically significant clinical signs including fever, serous nasal discharge, ocular discharge, coughing, depression and loss appetite.
3. The CIV-H3N2 infected dogs (both in inoculated group and direct contact group) showed statistically significant H3 specific antibodies since day 10

post inoculation (inoculated group) and day 14 post inoculation (contact group).

4. Our result suggested that CIV-H3N2 could efficiently infect dogs. The CIV-H3N2 infected dogs showed clinical signs and developed antibodies against the virus.

In phase 4, we investigated the pathogenicity of canine influenza virus subtypes H3N2 (CU-DC5299) in animal model (chickens). This experiment was to proof pathogenicity and potential reverse transmission of ancient lineage of the virus to avian species. The findings are as following:

1. The CIV-H3N2 challenged experiment was conducted in 9 chickens (n=9), including inoculated group (n=3), direct contact group (n=3) and control group (n=3).
2. The CIV-H3N2 challenged chickens did not present any specific clinical signs relating to respiratory disease throughout the experiment.
3. There was no viral shedding in the CIV-H3N2 challenged chickens throughout the experiment.
4. Our result suggested that CIV-H3N2 could not efficiently infect chickens. The CIV-H3N2 challenged chickens did not develop any clinical signs or antibodies against the virus.

In phase 5, we investigated the pathogenicity of the canine influenza virus subtype H3N2 (CU-DC5299) in animal model (guinea pigs). This experiment was to proof pathogenicity and potential transmission of the virus in mammal model. The findings are as following:

1. The CIV-H3N2 challenged experiment was conducted in 20 guinea pigs (n=20), including inoculated group (n=5), direct contact group (n=5), aerosol contact group (n=5) and control group (n=5).
2. The CIV-H3N2 challenged guinea pigs showed high fever but no any specific clinical signs relating to respiratory disease throughout the experiment.
3. The CIV-H3N2 infected guinea pigs (inoculated, direct contacted, and aerosol contact groups) developed H3 specific antibodies since day 7 post inoculation (inoculated group) and day 10 post inoculation (direct contacted and aerosol contact group).
4. Our results suggested that CIV-H3N2 could moderately infect guinea pigs. The CIV-H3N2 challenged animals developed fever and the animals also developed antibodies against the virus.

In conclusion our results provided useful information of canine influenza infections in dogs and cats and the pathogenicity of the Thai CIV-H3N2. These information will help develop a strategic planning for influenza prevention and control in companion animals and humans. The significant findings are

1. From September 2011 to September 2014, a serological survey of canine influenza in dogs in Thailand demonstrated the evidence of antibodies against pandemic H1N1-2009.
2. Thai CIV-H3N2 (A/canine/Thailand/CU-DC5299/2012/H3N2) is classified as low pathogenic influenza (LPAI) virus.
3. Thai CIV-H3N2 can infect and transmit from dogs to dogs (intra-species) via direct contact based on dog challenged model.
4. Thai CIV-H3N2 is unable to infect and transmit in avian species based on chicken challenged model.
5. Thai CIV-H3N2 potentially infect and transmit in mammal species based on guinea pig challenged model.

Our findings confirmed that canine influenza virus is a very important respiratory pathogen and has potentially infected to other mammal species. According to the results of this study, the recommendations for canine influenza prevention and control including

1. Surveillance of influenza virus in dogs and cats should be routinely conducted to determine the status of influenza infection in dogs and cats.
2. Human and companion animal influenza vaccines should be considered and routinely practiced.

3. Human-animal interaction is constant, but the personal hygiene should be a practical requirement.

One Health approach should be used for raising awareness of human-domestic animal interface contributing to potential zoonotic transmission of influenza.

APPENDICES

APPENDIX A

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

Sterile immediately by autoclave

APPENDIX B

NP-ELISA Protocol

Step 1: 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

Step 2: NP-ELISA procedure

Using ID Screen Influenza A Antibody competition ELISA kit (ID VET, Montpelier, France), following the manufacturer's instructions

1. For sera preparation, dog sera sample was prepared by diluting 10 ul of each sera sample with 90 ul 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 ℃ (± 2℃) for 1 hour ± 5 min.
3. Then empty the wells, wash each well for 5 times with 300 ul of the wash solution/wash.
4. Add 50 ul of the conjugate 1X to each well and incubate at 21℃ (± 5℃) for 30 min ± 2 min.
5. Then empty the wells, wash each well for 3 times with 300 ul of the wash solution/wash
6. After that, add 50 ul of the substrate solution to each well, followed by incubation for 10 min ± 1 min at 21℃ (± 5℃) in the dark room
7. Add 50 ul of the stop solution to each and before
8. Read and record the O.D at 450 nm.
9. Both positive and negative controls were included.

Step 3: 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) x 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.

APPENDIX C

Haemagglutination Inhibition Test

Step 1: Reagents for standardization of sera treatment and HI test preparation

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 – 5.5

Step 2: Preparations for standardization of sera treatment and HI test

- Preparation of standardized control antigens for the HI test back titration.

  Each control antigen must be standardized to contain 4 HAU per 25 ml or 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 μl pipette. Be careful to not disturb the pellet of RBCs. 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 μl pipette.

   Aspirate the remaining supernatant with a 1,000 μl pipette for final packed turkey RBCs. Keep packed RBCs in 4°C

Step 3: 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 56c for 1 hour to inactivate the RDE.
4. After that, absorbed RDE-treated sera with 100 ul of 50% Turkey red blood cells (TRBCs) or 50% Chicken red blood cells (CRBCs) and incubated at room temperature for 1 hour.
5. Use the treated sera in the HI test influenza virus.

Step 4: 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) or 25 microliter of each virus (4 HAU per 25 microliter) to all wells of pates 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%, 1% of turkey RBCs or 0.5%, 1% of chicken 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.

Step 5: Interpretation of the HI results

The HI titer was determined by the reciprocal of the last dilution that shows on agglutination and reported as geometric means. Samples with a titer 40 were considered positive.

APPENDIX D

Protocol for RNA extraction

Step 1: 600 µl of RAV1 buffer was mixed with 150 µl of allantoic fluid and incubated at 70°C for 5 minutes.

Step 2: 600 µl of ethanol was added to the tube and mixed by vortexing for 15 seconds.

Step 3: 700 µl of lysed samples was added to Nucleospin® RNA virus columns in collection tubes (2ml) and centrifuged for 1 minute at 8,000 x g.

Step 4: 500 µl of RAW buffer was added to the Nucleospin® RNA virus columns in collection tubes (2ml), centrifuged for 1 minute at 8,000 x g, and flow-through was discarded.

Step 5: 600 µl of RAV3 buffer was added to the Nucleospin® RNA virus columns, centrifuged for 1 minute at 8,000 x g, and flow-through was discarded with collection tubes.

Step 6: 200 µl of RAV3 buffer was added to the Nucleospin® RNA virus columns in new collection tubes (2ml) and centrifuged for 3 minutes at 11,000 x g.

Step 7: 50 µl of RNase-free water (preheated to 70°C) was added to the Nucleospin® RNA virus columns in sterile microcentrifuge tube (1.5 ml), incubated for 2 minutes at room temperature, and centrifuged for 1 minute at 11,000 x g.

APPENDIX E

Protocol for real time RT-PCR (rRT-PCR)

Step 1: Reagents preparation

RNA	4	µl
10 µM forward primers	0.5	µl
10 µM reverse primers	0.5	µl
2.5 µM M64 probe	0.5	µl
2x Master mix	6.25	µl
Superscript III	0.25	µl
Distilled water	0.42	µl
50 µM MgSO4	0.08	µl
Final volume	12.5	µl

Step 2: PCR condition for real-time RT-PCR

50ºC for 30 minutes for 15 minutes

followed by 50 cycles of amplification

95ºC for 15 seconds

60ºC for 30 seconds

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