ประสิทธิภาพของวัคซีนป้องกันโรคไข้หวัดนกชนิดเชื้อตาย (H5N2) ต่อการป้องกันโรคและการแพร่ของเชื้อไวรัสไข้หวัดนก (H5N1) ในนกกระทาญี่ปุ่น

นายธวัช เล็กดำรงศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2555

EFFICACY OF INACTIVATED AVIAN INFLUENZA (H5N2) VACCINE ON THE PROTECTION AND TRANSMISSION OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS (H5N1) IN JAPANESE QUAIL

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ธวัช เล็กดำรงศักดิ์: ประสิทธิภาพของวัคซีนป้องกันโรคไข้หวัดนกชนิดเชื้อตาย (H5N2) ต่อการป้องกันโรคและการแพร่ของเชื้อไวรัสไข้หวัดนก (H5N1) ในนกกระทาญี่ปุ่น (EFFICACY OF INACTIVATED AVIAN INFLUENZA (H5N2) VACCINE ON THE PROTECTION AND TRANSMISSION OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS (H5N1) IN JAPANESE QUAIL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร.อลงกร อมรศิลป์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร.จิโรจ ศศิปรียจันทร์, 127 หน้า.

วิทยานิพนธ์นี้ได้ศึกษาประสิทธิภาพของวัคซีนป้องกันโรคไข้หวัดนกชนิดเชื้อตาย (H5N2) ต่อการป้องกันโรคและการแพร่ของเชื้อไวรัสไข้หวัดนกชนิดรุนแรง (H5N1) ในนกกระทาญี่ปุ่น โดยแบ่งออกเป็น 3 การทดลอง คือ การทดลองที่ 1 นกกระทาอายุ 9 สัปดาห์ จำนวน 107 ตัว ออกเป็น 4 กลุ่ม กลุ่มที่ 1-3 กลุ่มละ 32 ตัว เป็นกลุ่มทดลอง กลุ่มที่ 4 จำนวน 11 ตัว เป็นกลุ่มควบคุมลบ ให้เชื้อไวรัสไข้หวัดนกชนิดรุนแรง A/chicken/Thailand/CUK2/04 (H5N1) ปริมาณ 10^7, 10^5 และ 10^3 EID_{50} ต่อนก 1 ตัว กับนกกลุ่มที่ 1-3 กลุ่มละ 16 ตัว ตามลำดับ 24 ชั่วโมงต่อมา นำนก 16 ตัวที่เหลือในกลุ่มที่ 1-3 มาเลี้ยงรวมกับนกที่ได้รับเชื้อ บันทึกอาการทางคลินิกและการตาย ทุกวัน เป็นเวลา 21 วัน เก็บ oropharyngeal และ cloacal swabs เพื่อศึกษาการแพร่ของเชื้อไวรัส จากระยะเวลาที่ใช้ในการก่อการตายและเชื้อไวรัสที่แพร่ออกมา พบว่าไวรัสปริมาณ 10^5 EID_{50} ต่อนก 1 ตัวเป็นปริมาณที่เหมาะสมสำหรับการศึกษาประสิทธิภาพของวัคซีน การทดลองที่ 2 นกกระทาอายุ 3 สัปดาห์ จำนวน 60 ตัว แบ่งออกเป็น 3 กลุ่ม กลุ่มละ 20 ตัว กลุ่มที่ 1 และ 2 เป็นกลุ่มที่ได้รับวัคซีน กลุ่มที่ 3 เป็นกลุ่มที่ไม่ได้รับวัคซีน ที่อายุ 3 สัปดาห์ (ครึ่งโดส) และ 7 สัปดาห์ (1 โดส) ให้วัคซีนซึ่งเตรียมจากเชื้อ A/chicken/Mexico/232/94 (H5N2) กับนกกลุ่มที่ได้รับวัคซีน เก็บตัวอย่างเลือดจากนกก่อนและหลังได้รับวัคซีน เพื่อตรวจการตอบสนองทางซีรัมวิทยา พบว่าวัคซีนเชื้อตายสามารถกระตุ้นการตอบสนองทางซีรัมวิทยาได้อย่างมีนัยสำคัญทางสถิติ การทดลองที่ 3 นกกระทาอายุ 3 สัปดาห์ จำนวน 48 ตัว แบ่งออกเป็น 3 กลุ่ม กลุ่มละ 16 ตัว กลุ่มที่ 1 และ 2 เป็นกลุ่มที่ได้รับวัคซีน กลุ่มที่ 3 เป็นกลุ่มที่ไม่ได้รับวัคซีน ที่อายุ 3 สัปดาห์ (ครึ่งโดส) และ 7 สัปดาห์ (1 โดส) ให้วัคซีนซึ่งเตรียมจากเชื้อ H5N2 กับนกกลุ่มที่ได้รับวัคซีน ที่ 10 สัปดาห์ ให้เชื้อไวรัส CUK2 ปริมาณ 10^5 EID_{50}ต่อนก 1 ตัว กับนกทุกกลุ่ม พบว่าวัคซีนสามารถลดอัตราการตายของนกกระทา และลดการแพร่ของเชื้อไวรัส ทั้งในแง่ของจำนวนนกที่แพร่เชื้อไวรัส และปริมาณไวรัสที่นกแพร่ออกมา โดยสรุป วัคซีนสามารถกระตุ้นการตอบสนองทางซีรัมวิทยาและให้การป้องกันโรคบางส่วน แต่อาจไม่สามารถหยุดการแพร่ของโรค เนื่องจากนกกระทาที่ติดเชื้อยังมีการแพร่เชื้อไวรัสออกมา

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: VACCINE, AVIAN INFLUENZA, JAPANESE QUAIL

THAWAT LEKDUMRONGSAK: EFFICACY OF INACTIVATED AVIAN INFLUENZA (H5N2) VACCINE ON THE PROTECTION AND TRANSMISSION OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS (H5N1) IN JAPANESE QUAIL. ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., CO-ADVISOR : PROF. JIROJ SASIPREEYAJAN, D.V.M., Ph.D., 127 pp.

This dissertation is divided into 3 parts. First, the effect of inoculation dose of a Thai HPAI-H5N1 (CUK2) virus was evaluated, 107 quails were divided into 4 groups. At 9-week-old, 16 quails in groups 1-3 (*n*=32 each) were inoculated with 10^7, 10^5 and 10^3 EID_{50} of A/chicken/Thailand/CUK2/04 virus, respectively. One day later 16 quails in each group were added. Group 4 (*n*=11) was negative control group. Quails have been observed for 3 weeks. Oropharyngeal and cloacal swabs were analyzed for viral shedding. The death time and viral shedding suggested that 10^5 EID_{50} of virus per quail was selected for animal challenge in vaccination experiment. Second, serological response to inactivated vaccine was investigated, 60 quails were divided into 3 groups (*n*=20 each), duplicate vaccinated groups and unvaccinated group. The inactivated vaccine, A/chicken/Mexico/232/94 (H5N2) virus, was administered at the age of 3 (half dose) and 7 (full dose) weeks. Blood samples were analyzed for serological response. The result indicated that twice vaccination induced significantly increase HI antibody. Third, efficacy of inactivated vaccine was evaluated, 48 quails were divided into 3 groups (*n*=16 each), duplicate vaccinated groups and unvaccinated group. The inactivated vaccine was administered at the age of 3 (half dose) and 7 (full dose) weeks. At the age of 10 weeks, 8 quails in each group were inoculated with CUK2 virus, One day later 8 quails in each group were added. Quails have been observed for 3 weeks. Oropharyngeal and cloacal swabs were analyzed for viral shedding. The results indicated that twice vaccination could reduce the mortality and the viral shedding. In conclusion, this dissertation demonstrated that given vaccination program induced specific antibody response and partially protected quail from HPAI-H5N1 virus but not effectively prevented transmission of the virus.

Department : Veterinary Public Health Student's Signature .....

Field of Study : Veterinary Public Health Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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AI	Avian influenza
AIVs	Avian influenza viruses
AGID	Agar gel immunodiffusion test
CUK2	A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04
DIVA	Differentiating infected from vaccinated animals
dpc	Day post contact
dpi	Day post inoculation
EID_{50}	50 percent embryo infectious dose
ELD_{50}	50 percent embryo lethal dose
FDA	Food and Drug Aministration
HA test	Hemmaglutination test
HA	Hemagglutinin
HAU	Hemmaglutination units
HI	Hemmaglutination inhibition test
HPAI	High pathogenic avian influenza
hpc	Hour post contact
hpi	Hour post inoculation
H&E	Hematoxylin and eosin
IACUC	Institutional Animal Care and Use Committee
IVPI	Intravenous pathogenicity index
LPAI	Low pathogenic avian influenza
MDT	Mean death time
M	Matrix
NA	Neuraminidase
NP	Nucleoprotein
OIE	The office International des Epizooties
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1

PB2	Polymerase basic protein 2
RBS	receptor binding site
SA α2,3-gal	α2,3-galactose
SA α2,6-gal	α2,6-galactose
SAN	Specific antibody negative
SPF	Specific pathogen free
VTM	Viral transport media

CHAPTER I

Introduction

1.1 Introduction

Avian influenza (AI) is a contagious disease caused by influenza viruses. Influenza viruses are classified into 3 types; type A, B and C. Only type A viruses are known to infect birds. Influenza type A viruses are further divided into hemagglutinin (HA) and neuraminidase (NA) subtypes based on these surface proteins. To date, there are 16 known HA subtypes and 9 known NA subtypes (Fouchier et al., 2005). In addition, a distinct HA subtype from bat has been reported (Tong et al., 2012). On the other hand, avian influenza viruses can also be differentiated to low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) based on their virulence for chicken or HA0 cleavage site amino acid sequences. Currently, HPAI have been restricted to H5 and H7 subtypes. These viruses could infect human and caused fatal disease. In 1997, HPAI-H5N1 caused serious outbreaks in both poultry and human in Hong Kong. Eighteen humans were infected with 6 deaths (Claas et al., 1998). In 2003, the Netherlands reported outbreaks of HPAI-H7N7 in poultry. Infections in human were also reported and one death was recorded (Fouchier et al., 2004).

Highly pathogenic avian influenza (HPAI) subtype H5N1 was first reported in China, in 1996. The first virus (A/Goose/Guangdong/2/96) was isolated from sick geese in southern China (Xu et al., 1999). In 1997, HPAI-H5N1 was diagnosed in Hong Kong (Claas et al., 1998). From 1997 to 2003, sporadic cases of HPAI-H5N1 have been reported in Hong Kong and China. From 2003 to 2004, outbreaks of HPAI-H5N1 have been reported in China, Korea, Japan and Southeast Asia. Since then, the virus has spread to Middle East Asia, Africa and Europe (Alexander, 2007). The first HPAI-H5N1 outbreak in Thailand was reported in early 2004. From 2004 to 2008, at least 7 waves of HPAI-H5N1 outbreaks were recorded in the country (Suwannakarn et al., 2009). The HPAI-H5N1 outbreaks caused serious economic losses not only from high mortality of birds but also from eradication and control programs. More than 62 million birds were killed by disease or culled in Thailand (Tiensin et al., 2005). Moreover, HPAI-H5N1 virus

has also been used as a non tariff trade barrier. European Union and Japan ban poultry products from countries with poultry outbreaks or vaccination (Pongcharoensuk et al., 2011).

Since the disease caused by HPAI-H5N1 reported in several bird species as well as human. Moreover, there were reports of HPAI-H5N1 virus infection in many mammalian species, including tiger and leopard (Keawcharoen et al., 2004), cat (Songserm et al., 2006a), dog (Songserm et al., 2006b), Owston's palm civet (Roberton et al., 2006), Asiatic golden cat and clouded leopard (Desvaux et al., 2009), mink (Zohari et al., 2008), black-lipped pika (Zhou et al., 2009) and donkey (Abdel-Moneim et al., 2010). In Thailand, between January 2004 and December 2005, twenty two human cases have been reported and six of them died (Suwannakarn et al., 2009). Most cases were healthy children and young adults (WHO, 2005). Initially, most cases had influenza like symptoms including high fever. Severe cases had respiratory distress, tachypnea and pneumonia. About 60 % of human cases died (Uyeki, 2008). Infected poultry were the primary sources of infection and probable human-to-human transmission has been reported (Ungchusak et al., 2005).

HPAI-H5N1 outbreaks were mainly found in backyard poultry including Japanese quail (*Coturnix coturnix japonica*). HPAI-H5N1 outbreaks have been reported in Japanese quail in several countries, including Thailand, Vietnam and Indonesia (FAO, 2012). Japanese quail is the top five major poultry species produced in Thailand (Chantong and Kaneene, 2011). In Thailand, poultry production systems are categorized into four sectors based on farm biosecurity and the system used to market product. Sector 1 is industrial integrated system with high biosecurity. Poultry is kept in closed house. Products from this sector are marketed commercially. Sector 2 is commercial poultry production system with moderate to high biosecurity. Poultry is kept in closed house or open house with net. Products from this sector are also marketed commercially. Sector 3 is commercial poultry production system with low to minimal biosecurity. Poultry is kept in open house without net. Products from this sector usually

enter live bird markets or local markets. Japanese quail raising system is categorized in this sector. Sector 4 is village or backyard production with minimal biosecurity. Products from this sector are consumed locally (Chantong and Kaneene, 2011). In Thailand, no HPAI-H5N1 outbreak was reported in sector 1. HPAI-H5N1 infections were found in sector 2, 3 and especially in sector 4 (Tiensin et al., 2007). In addition, industrial large-scale producers opposed poultry vaccination from the impact on international trade. On the other hand, small-scale producers supported poultry vaccination (Pongcharoensuk et al., 2011).

After the first outbreak of HPAI-H5N1 virus in Thailand in 2004, routine surveillance, movement restriction and culling of infected and adjacent flocks have been implemented as control strategies for early detection, control spread and eliminate source of virus. After 2008, there is no evidence of HPAI-H5N1 outbreak in Thailand (Suwannakarn et al., 2009). However, several HPAI-H5N1 outbreaks have been found in domestic poultry and wild birds in neighbouring countries in 2012 (OIE, 2012). Since the viruses can re-emerge in the region by several factors such as animal movement by trading, wild bird migration especially in non-commercial poultry including backyard chicken, duck, and quail (Sims et al., 2005; Keawcharoen et al., 2011). The HPAI-H5N1 outbreak surveillance, monitoring and prevention are still importance and should be continued.

Several disease control and prevention strategies are recommended such as culling, movement control, disease surveillance, strict biosecurity and education, as well as vaccination. Biosecurity is one of the recommendations for disease outbreak prevention and control, however it may not be sufficient because poultry raising systems and biosecurity standards are different among countries (Rushton et al., 2006; Chantong and Kaneene, 2011). Disease control by depopulation of infected flocks and preemptive culling of neighboring farms alone may restrict due to socio-economic limitation. Vaccination may be one of the preferred options in some countries, since vaccine can protect birds against morbidity and mortality and also reduce the spread of virus (Swayne, 2006). Poultry vaccine policy is usually based on the national economy

associated with poultry production and public health. For Thailand, the export market plays an important role for poultry production. Importers may ban poultry products due to the concern of HPAI-H5N1 virus contamination in vaccinated poultry products. Thus, policy makers concern about export industry and vaccination is not permitted (Peyre et al., 2009; Pongcharoensuk et al., 2011).

In previous reports, inactivated avian influenza vaccines could protect several poultry species including chicken, duck and goose from HPAI-H5N1 viruses (Swayne et al., 2006; Rudolf et al., 2009; Pfeiffer et al., 2010). In chicken and goose, vaccines could prevent not only morbidity and mortality, but also reduce viral shedding through respiratory and gastrointestinal tracts. In duck, HPAI-H5N1 typically does not cause disease or death but the vaccines can reduce replication of the virus. However, the information of inactivated avian influenza vaccination in Japanese quail is still limited. Since little is known about transmissibility of HPAI-H5N1 virus in vaccinated Japanese quail. Then the questions arose whether Japanese quail could be protected by vaccination or could transmit the virus into the population.

In this study, Japanese quail were focused due to they are highly susceptible to HPAI-H5N1 viruses and their potential role in virus reassortment. Thus, influenza infection in Japanese quail was investigated to provide information for pathogenicity and transmissibility in Japanese quail. Transmission experiment was performed by mingling inoculated Japanese quail with susceptible contact Japanese quail and investigated the transmission chain. The result obtained in this study provided useful information for improving surveillance and eradication strategies and also for vaccination study.

Serological response of Japanese quail to inactivated avian influenza vaccine was monitored by HI test. An inactivated avian influenza (H5N2) vaccine was used because the H5 hemagglutinin protein in vaccine can induce protective immunity against HPAI-H5N1 virus. HI test has been used to detect H5 specific antibodies against influenza virus. If the vaccine induces high level of neutralizing antibody similar to other poultry, vaccination may be used to protect Japanese quail from HPAI-H5N1.

Vaccine efficacy study was performed by mingling inoculated vaccinated Japanese quail with contact vaccinated Japanese quail and investigating the mortality rate, viral shedding and immune response. If the vaccine protect and reduce viral shedding in Japanese quail similar to other poultry, vaccination can be used as an alternative prevention and control program of HPAI-H5N1 in Japanese quail.

1.2 Objectives

The objectives of this study are:

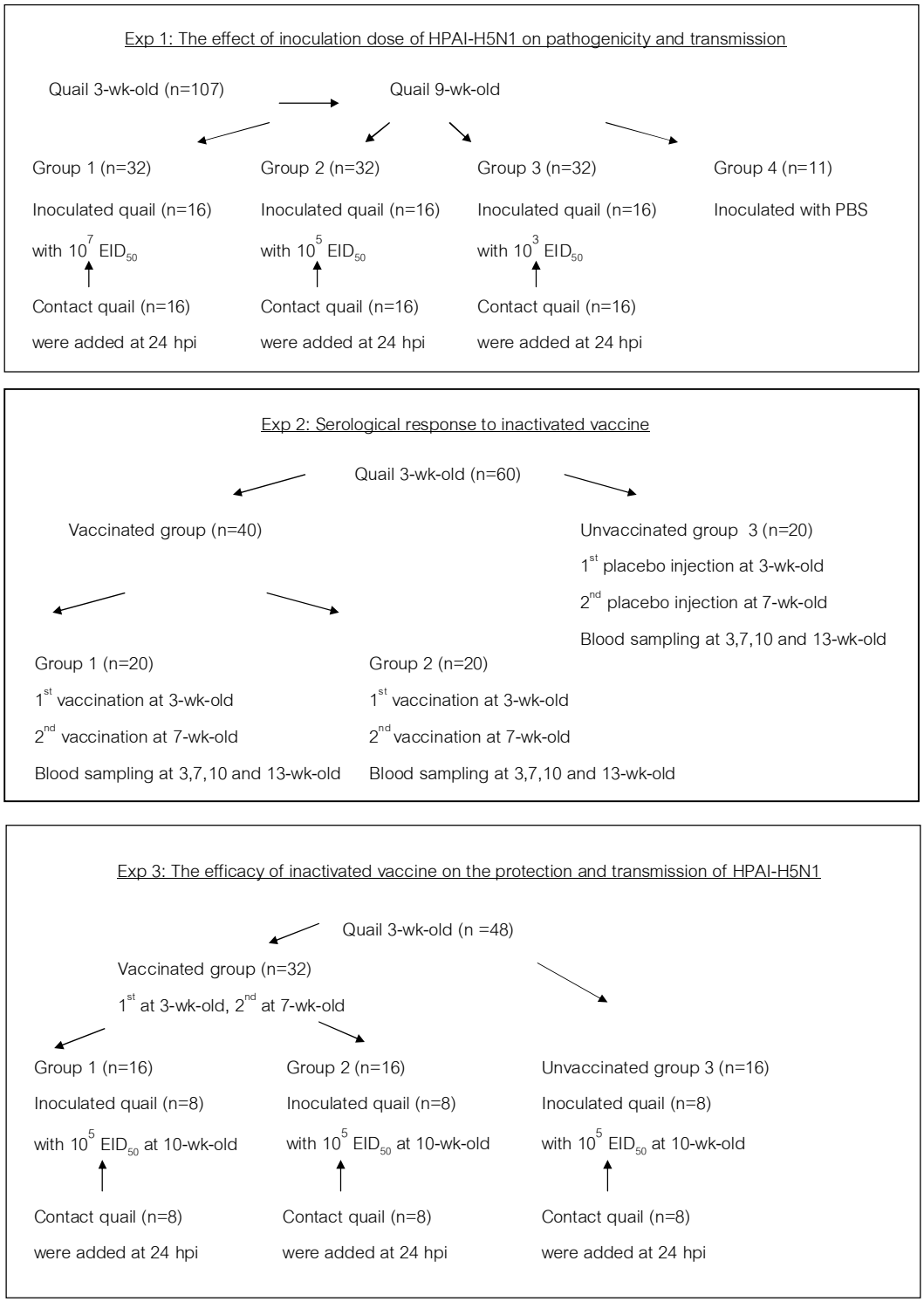
1. To determine the effect of inoculation doses of HPAI-H5N1 virus on the infection, pathogenicity, viral transmission and viral shedding in Japanese quail

2. To determine humeral antibody response to inactivated avian influenza (H5N2) vaccine in Japanese quail

3. To evaluate the efficacy of inactivated avian influenza (H5N2) vaccine on the protection and transmission of HPAI-H5N1 virus in vaccinated Japanese quail

To achieve these objectives, the study was divided into 3 experiments (figure 1.1).

Figure 1.1 Schematic of experimental design



1.3 Literature review

1.3.1 Avian influenza virus

Influenza viruses are the members of the Orthomyxoviridae family. Orthimyxoviridae viruses are divided into 5 genera, including influenza A, B, C, Isavirus and Thogovirus. Classification of influenza viruses into type A, B, and C is measured by the difference of NP and M proteins (Lee and Saif, 2009). Type B and C viruses are human pathogens that can cause infections in some mammals, including pig, dog and seal (Guo et al., 1983; Manuguerra and Hannoun, 1992; Osterhaus et al., 2000). Isavirus is the fish pathogen and Thogovirus is a tick-borne virus. Only type A viruses can infect several species of birds and mammals and can transmit between species.

The influenza A viruses are pleomorphic enveloped viruses with a size ranging from 80-120 nm. Influenza A viruses are negative sense, single strand, segmented RNA viruses. Influenza A viruses have 8 gene segments that encode 10 proteins. Hemagglutinin (HA), neuraminidase (NA) and membrane ion protein (M2) are the surface proteins. Nucleoprotein (NP), matrix protein (M1), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) are the internal proteins. Nonstructural protein 1 (NS1) and nonstructural protein 2 (NS2) are the nonstructural proteins (table 1.1). The structure of virus consists of lipid membrane with 3 viral membrane proteins, the HA, NA and M. HA forms the trimeric spikes and NA forms the tetrameric spikes. HA is responsible for host cell binding and NA functions in released of new viral particles. M2 acts as an ion channel. The inner side of lipid membrane is lined with M1. The genome segments are packed in the viral ribonucleoprotein complex. Negative stranded RNA is covered with NP and attached with 3 polymerase proteins (PB1, PB2 and PA) (figure 1.2). Type A viruses are subdivided into subtypes based on 2 surface proteins that are HA and NA. 16 HA and 9 NA subtypes have been identified. More than 100 combinations have been found (Taubenberger and Kash, 2010). All 16 HA and 9 NA subtypes are found in waterfowl, gulls and shorebirds which are their natural hosts and reservoirs (Webster et al., 1992).

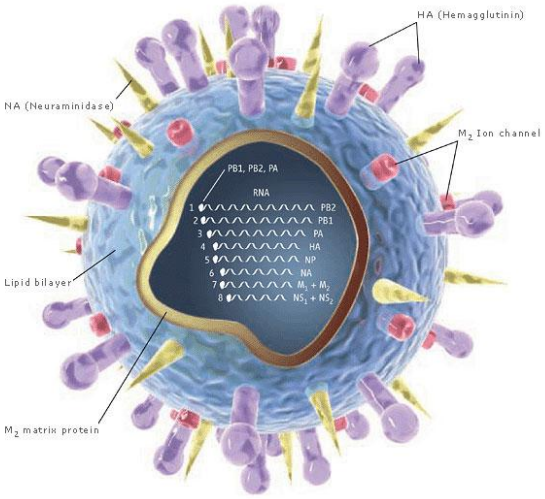


Figure 1.2 Structure of influenza A virus (Kaiser, 2006). HA and NA are found on the surface.

Table 1.1 The function of 10 proteins of influenza A virus encoded by 8 RNA segmented (modified from (Christman et al., 2011))

Segment	Protein	Function
1	PB2	Transcriptase: cap binding
2	PB1	Transcriptase: elongation
3	PA	Transcriptase: protease activity
4	HA	Binding the virus to the host cell receptor, antigenic determinant
5	NP	RNA binding: part of transcriptase complex
6	NA	Release of progeny virions from the host cell, antigenic determinant
7	M1	Component of viral envelope
	M2	Integral membrane protein: ion channel
8	NS1	Interferon antagonist
	NS2	Nuclear export of RNPs

The problem of influenza A virus prevention is high mutability which allowed them to evade immune response. Influenza viruses can evolve by 2 mechanisms, antigenic drift or antigenic shift. Antigenic drift results from the random accumulation of mutation in the HA or NA gene because of the lack of prove reading mechanism in RNA polymerase. Changes in the antigenic site of the HA or NA reduce efficacy of neutralizing antibody. Antigenic drift may allow the virus to cross the species barrier (Lee and Saif, 2009). Although antigenic drift occurs more frequently than antigenic shift, antigenic shift is the greater concern.

Antigenic shift can occur through the process called genetic reassortment. This can happen when two different influenza viruses infect the same host cell and exchange genome segments during replication. This new combination is a unique strain of virus then hosts have no immunity. This mechanism is responsible for at least two pandemic influenza viruses in human. For example, H2N2 subtype or Asian flu in 1975 was the result of reassortment between human H1N1 and avian H2N2 viruses. H3N2 subtype or Hong Kong flu in 1968 was the result of reassortment between human H2N2 and avian H3 viruses (Scholtissek et al., 1978; Gething et al., 1980; Kawaoka et al., 1989). Intermediate hosts are believed to play a role in reassortment between human and avian viruses before they could be transmitted swiftly among human (Webster et al., 1997). Suitable intermediate hosts have to be easily infected by influenza viruses from various origins including both mammalian and avian viruses.

Antigenic shift can also occur through the process of adaptive mutation of the virus that can replicate and be transmitted in the new host species. Adaptive mutation may increase the capacity of the virus to bind new host cells then virus can spread easily. Example for this phenomenon include H5N1 (Claas et al., 1998), H7N7(Koopmans et al., 2004) and H9N2 (Peiris et al., 1999) transmissions from animals to humans.

Up until now, only H5, H7 and H9 subtypes have been transmitted from birds to humans and associated with mild to fatal diseases (Koopmans et al., 2004; Butt et al.,

2005). Avian influenza viruses are classified into two pathotypes, low pathogenic avian influenza (LPAI) virus and high pathogenic avian influenza (HPAI) virus, based on the amino acid sequences at the HA cleavage site or severity of the disease in chicken. The virus is classified as HPAI if it has intravenous pathogenicity index (IVPI) in 6-week-old chickens greater than 1.2 or causes more than 75 % mortality of at least 8 susceptible 4 to 8-week-old chickens within 10 days post inoculation (Alexander, 2008) by intravenous route or has multiple basic amino acid sequences at the HA cleavage site, which can be cleaved by furin proteases expressed in various cells, leading to systemic infection. All other viruses are classified as LPAI. LPAI viruses cause localized infections in respiratory and gastrointestinal tracts because the HA cleavage sites are cleaved by trypsin-like proteases restricted in respiratory and gastrointestinal tracts. To date, HPAI have been restricted to the H5 and H7 subtypes. LPAI H5 and H7 subtypes have the potential to mutate to become HPAI. Alteration in pathotype is primarily associated with changes in HA cleavage site. This phenomenal has been documented in poultry outbreaks (Garcia et al., 1996; Suarez et al., 2004).

1.3.2 HA characteristic of HPAI-H5N1 in Thailand

Base on the phylogenetic characterization and sequence homology of the HA gene, the H5N1 viruses were classified into clades. From this nomenclature system, H5N1 viruses which evolved from Gs/GD/1/96 have been classified into 10 distinct clades (WHO/OIE/FAO, 2008). Two clades of influenza A virus (H5N1) have been reported in Thailand. First, clade 1 viruses were detected in lower northern region and central region. Second, clade 2.3.4 viruses were detected in northeast region, which limited found in 2006–2007. The clade 1 viruses in Thailand consist of three sub-lineages, including CUK2-like, PC168-like, and PC170-like lineages. PC168-like and PC170-like virus circulations were also limited between 2005 and 2007. The CUK2-like viruses were the only predominant lineage and had circulated from the first outbreak in 2004 till 2008 (Suwannakarn et al., 2009). In this study, virus used for challenged study is influenza A subtype H5N1 “A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1)

(CUK2)" isolated in 2004 from the infected laying hen in Nakorn Pathom province, central region of Thailand. The virus belongs to clade 1 H5N1. CUK2 virus was classified as HPAI virus because the HA gene contained the polybasic amino acids at the HA cleavage site (SPQRERRRKKR) (Viseshakul et al., 2004).

1.3.3 Japanese quail

The Japanese quail (*Coturnix coturnix japonica*) belong to the order *Galliformes.* The Japanese quail are found in East Asia as migratory birds. Natural habitats of the birds are grasslands and cultivated fields. The average life span of quail is about 3-4 years. The plumage color of Japanese quail is predominantly speckled yellow-brown. Normally, adult male Japanese quail are around 100-130 g. and adult female are around 120-160 g. Japanese quail egg are mottled brown in color. Eggs weight around 9-10 g. Nowadays, Japanese quail are raised commercially for meat and egg productions.

It has been known that Japanese quail pose both sialic acid α2,3-galactose (SA α2,3-gal) and α2,6-galactose (SA α2,6-gal) linked receptors in the trachea and intestine which prefer to bind with both avian and mammalian viruses. Avian influenza viruses generally bind to SA α2,3-gal, whereas mammalian viruses preferentially bind to SA α2,6-gal. Receptor binding specificity is determined by amino acid residues at receptor binding site of viral HA (Baigent and McCauley, 2003). It is noted that the majority of epithelial cells of Japanese quail trachea contain SA α2,6-gal, while the epithelial cells of Japanese quail colon pose more SA α2,3-gal than SA α2,6-gal (Wan and Perez, 2006). From previous reports, swine influenza viruses (H1N1, H3N2 and H1N2) can replicate in Japanese quail (Makarova et al., 2003). Moreover, pandemic H1N1 2009 virus (pH1N1) can also replicate in Japanese quail (Swayne et al., 2009). Japanese quail provide a suitable environment for the reassortment of avian and mammalian influenza viruses (Makarova et al., 2003). Japanese quail may play an important role in influenza infection as an intermediate host for the reassortment of influenza viruses and generating the variant viruses (Perez et al., 2003b).

1.3.4 Japanese quail production in Thailand

In 2011, there are approximately 8,300,000 Japanese quail in 3,200 farms in Thailand (DLD, 2011). The Japanese quail farms are concentrated in the central region of the country. Female Japanese quail are raised for egg production. Male Japanese quail are farmed for meat production. In addition, quail manure is used as fertilizer. Farmers can differentiate female from male at 3-week-old. The female are identified by light tan feathers with black speckling on the throat and upper breast. The male have rusty brown throat and breast feathers. Normally, farmers buy Japanese quail after sexing. Japanese quail can be kept in small cages. Adult Japanese quail need 125 cm^2 of floor space per bird. Laying Japanese quail are raised in battery cages. Female start to lay egg at about 6-week-old and end of egg production cycle at 1-year-old. Normally, Japanese quail are raised in small farms with low biosecurity. They are kept in an open house without vaccination. Local house birds such as sparrow, pigeon can enter the house and can transmit infectious diseases. Moreover, some Japanese quail farms also raised backyard poultry in the premises. Thus, Japanese quail farms pose a high risk for influenza infection.

1.3.5 Avian influenza viruses in Japanese quail

The first reported case of influenza A infection in Japanese quail occurred in Italy during 1966-1968 (Nardelli et al., 1970). Several subtypes of influenza A viruses, such as H3N2, H3N6, H4N6, H5N1, H5N2, H6N1, H7N1, H7N2, H7N3, H9N2 and H10N8, have been isolated from Japanese quail around the world (Saito et al., 1993; Guan et al., 1999; Suarez et al., 1999; Capua and Marangon, 2000; Swayne and Suarez, 2000; Liu et al., 2003; Cheung et al., 2007; Saito et al., 2009; Nfon et al., 2011). Several studies reported that Japanese quail supported the replication of at least 14 subtypes of influenza A viruses and the viruses replicated predominantly in the respiratory tract (Makarova et al., 2003; Saito et al., 2009).

Interestingly, Japanese quail infected with HPAI-H5N9 virus from turkey showed no symptoms but could transmit virus to chicken and cause severe infection and death (Tashiro et al., 1987). Moreover, Japanese quail played a role in the host adaptation of virus from duck to chicken and from duck to mouse (Perez et al., 2003a; Sorrell and Perez, 2007; Hossain et al., 2008). From molecular characterization, a quail H9N2 virus may be the internal gene donor of HPAI-H5N1 virus which caused outbreak in Hong Kong in 1997 (Guan et al., 1999).

1.3.6 HPAI-H5N1 in Japanese quail

Japanese quail are susceptible to common poultry diseases and are highly susceptible to HPAI-H5N1 virus. 100 % mortality has been reported in Japanese quail inoculated with HPAI-H5N1. The experimented quail showed depression before death and displayed neurological signs (L. Perkins and Swayne, 2001; Jeong et al., 2009). Japanese quail were more susceptible than chicken to viruses isolated from China and Thailand (Webster et al., 2002; Saito et al., 2009). Japanese quail shed high amount of virus for longer periods than chicken then Japanese quail may play an important role in HPAI-H5N1 transmission (Jeong et al., 2009). The viruses replicate predominantly in respiratory tract and primary spread by aerosol (Webster et al., 2002; Saito et al., 2009). In 2004, HPAI-H5N1 viruses have been isolated from Japanese quail farms (Tiensin et al., 2005) which viruses can spread via the movement of quail , eggs, manure and via accidental transfer of contaminated equipments and vehicles. In 2006, HPAI-H5N1 viruses have been isolated from Japanese quail from food markets (Amonsin et al., 2008).

At necropsy, the most prominent lesion of Japanese quail infected with HPAI-H5N1 was lung consolidation with edema and congestion. Splenomegaly with parenchymal mottling, renomegaly with parenchymal palor and accentuated lobular surface were also found. Hemorrhage in the gastrointestinal tract mucosa, the epicardium and serosal surface of liver and intestine were also observed (L. Perkins and Swayne, 2001).

1.3.7 Avian influenza vaccine

Avian influenza vaccines have been used in three vaccination strategies (Bruschke et al., 2007; Capua and Alexander, 2008). The first strategy is systematic vaccination or the routine use of vaccine which applied in endemic countries. Systematic vaccination is a long term strategy. However if the routine use of vaccine is applied without other monitoring and control strategies, long term circulation of influenza virus in vaccinated population may be pressured by vaccine and result in antigenic drift away from vaccine strain (Lee et al., 2004). The second strategy is preventive vaccination which employed in disease free country when AI viruses are serious treat and other prevention and control strategies seem not to succeed. All birds at high risk should be vaccinated. The duration of vaccination varies according to the threat. If the preventive vaccination is applied, DIVA (Differentiating Infected from Vaccinated Animals) strategy has been used to detect vaccinated infected birds to eradicate subsequence infection. The last strategy is emergency vaccination which employed when outbreaks have occurred and used with other control and eradication strategies. Emergency vaccination has been introduced as a short term measure to contain an outbreak. All unaffected birds in restriction zone should be vaccinated to reduce spread within the area. Without DIVA strategy, vaccinated birds should be culled. On the other hand, with DIVA strategy, vaccinated birds could be marketed after excluding infected birds.

OIE has proposed that the inactivated vaccine should be used as a part of eradication and prevention program in co-operate with elimination of infected birds, quarantine, and strict biosecurity. It is possible that virus can become endemic if vaccine is used without monitoring systems (OIE, 2009). Infected vaccinated birds still shed virus without showing any clinical signs (Capua and Marangon, 2006), this increases the time taken to detect the viruses and allows them to circulate in population. Long-term circulation of the virus may result in genetic changes (Lee et al., 2004). Vaccine pressure may also increase genetic drift evolution of the field viruses away from the vaccine strain (Abdel-Moneim et al., 2011).

Protection of influenza vaccines is based on neutralizing antibody, which response to hemagglutinin protein. Vaccines provide protection against homologous HA viruses (same HA subtype) but not heterologous HA viruses (different HA subtype). Immune response against neuraminidase protein provides some protections from avian influenza virus. Antibodies to NA reduce the amount of virus released from infected cell but do not prevent infection (Sylte and Suarez, 2009). On the other hand, immune responses to other internal proteins of the influenza virus are insufficient protection. Then, there are no universal vaccines available at this time. Up to date, there are two types of influenza vaccines, which are licensed and used in poultry. The first one is the recombinant fowl pox virus vectored vaccine with HA gene insertion and the other one is an inactivated whole avian influenza virus vaccine. The recombinant fowl pox vaccine can only be used in chicken and chicken must be naive to poxvirus, while the inactivated vaccine can provide protection in multiple poultry species (OIE, 2009).

Recombinant vaccines have been prepared by inserting HA gene of influenza virus into nucleic acid of poxvirus. Recombinant viruses that express HA antigen have been used to produce vaccine. Recombinant fowl pox vaccines have some advantages over inactivated vaccine. They are live vaccines that stimulating both humoral and cellular immunity and can be used in young chick to induce early protection. Distinction between natural infected and vaccinated birds can be easily done by DIVA. Detection of antibodies against vaccine HA and lack of antibodies against NP in natural infected birds by existing serological tests can be used to differentiate between natural infected and vaccinated birds.

About the inactivated vaccine, avian influenza viruses selected for production is based on low pathogenicity avian influenza (LPAI) viruses. In theory, LPAI protects against HPAI viruses of the same HA subtype. The viruses used in inactivated vaccines have broad and long term protection efficacy (Swayne et al., 2000). Reverse genetics have also been used to produce inactivated vaccines. Inactivated vaccines are produced by growing the virus in 9 to 11-day-old embryonated chicken egg. Then,

infected allantoic fluids are harvested and viruses are inactivated by chemical such as beta-propiolactone or formalin and emulsified with oil.

The inactivated vaccines can prevent disease and mortality in chicken, goose and duck. Moreover, vaccines also reduce the ability of the virus to replicate in respiratory and gastrointestinal tracts. Vaccination reduces viral shedding in the case of number of birds shedding virus, duration of viral shedding and the virus titer shed via oropharynx and via cloaca. On the other hand, inactivated avian influenza vaccination increases host resistance to infection with infectious virus. Then infectious cycle may be blocked because not enough virus is shed from infected vaccinated birds to vaccinated birds which are more resistance to infection. However, the inactivated vaccine does not prevent infection and the virus can still replicate and transmit. Extrapolating results from other species should be concerned because effect on the excretion of virus and influence on the transmission may vary from specie to specie even in the same family (Tian et al., 2005). Moreover, the response to the vaccine depends upon many factors, including, health and responsiveness of the hosts, appropriately stored vaccine, administration of the vaccine, vaccine strain, vaccine quality, vaccination program, maternal antibody and also immunosuppression.

For the inactivated vaccines against HPAI-H5N1 viruses, the H5 hemagglutinin protein is used to induce protective immunity against HPAI-H5N1 viruses. The difference neuraminidase such as N2 or N3 is used to allow distinction between natural infected and vaccinated birds by the DIVA strategy. The DIVA strategy is based on the use of an inactivated oil emulsion vaccine containing the same hemagglutinin as the field virus but different neuraminidase (Capua et al., 2003). Antibodies to the other NA except NA of vaccine suggest that natural infections occur.

In 1995, inactivated H5N2 vaccine has been used in HPAI outbreak for the first time during HPAI-H5N2 outbreak in Mexico and the virus was eradicated (Garcia et al., 1998). In Pakistan, inactivated H7N3 vaccine was used in HPAI-H7N3 outbreak in 1995 but had limited success. Since, outbreaks of HPAI-H7N3 in Pakistan in 2004 were

caused by genetically related virus in 1995 (Abbas et al., 2010). Following the widespread outbreaks of HPAI-H5N1, inactivated H5N2 vaccines have been use in Hong Kong in 2002, Indonesia in 2003, China in 2004, Vietnam and Russia in 2005, India, Pakistan and Egypt in 2006 (Capua and Alexander, 2008; Swayne, 2009). Although vaccination has had success in some countries such as Honk Kong (Ellis et al., 2006; Capua and Alexander, 2008), outbreaks still occurred in the other countries. In addition, inactivated H7N7 vaccine has been used in Korea to control HPAI-H7N7 outbreak in 2005 (Swayne et al., 2011).

1.3.8 Influenza vaccine in Japanese quail

In previous reports, inactivated avian influenza (H5N2) vaccine could induce H5 antibody response on the first week post vaccination. The antibody response was gradually increased up to the 4 week post vaccination then declined at 5 week post vaccination (Saad et al., 2010). In one hand, inactivated avian influenza vaccine H9N2 can induce protection against morbidity and clinical signs. Moreover, vaccine can decrease viral titers in the lungs and also lower potential of transmission. On the other hand, vaccine did not prevent the infection (Ebrahimi et al., 2011). The information of inactivated avian influenza vaccination in Japanese quail is still limited. Then, inactivated avian influenza vaccination in Japanese quail needs to be better understood.

1.3.9 Avian influenza diagnosis

Avian influenza infection cannot be diagnosed by clinical signs or lesions alone because no specific symptoms or pathognomonic lesions. Then, laboratory diagnosis is necessary. The conventional laboratory diagnosis consists of isolation and identification of the virus. Generally, virus isolation is done by the inoculating the sample into the embryonated specific pathogen free (SPF) chicken eggs or specific antibody negative (SAN) eggs. Oropharyngeal and cloacal swabs should be collected from live and dead birds. Sample from dead birds should include tissue samples (spleen, lung and any

abnormal tissues) and feces. Samples should be kept in viral transport media (VTM) at -80 °C until use.

Suspensions in VTM are inoculated into allantoic cavity of 9 to 11-day-old embryonated eggs. The eggs are incubated at 35-37 °C for 4-7 days. The allantoic fluids from dead embryos and all eggs remaining at the end of incubation period are tested with hemmaglutination (HA) test. The positive results from bacteria-free allantoic fluids indicate the presence of influenza A virus or avian paramyxo virus. Antibody specific for Newcastle disease virus is used to detect the antigen by hemmaglutination inhibition (HI) test. Allantoic fluids that give negative reaction should be inoculated into at least one further batch of eggs. The presence of influenza A virus can be confirmed by agar gel immunodiffusion test (AGID) for nucleocapsid or matrix antigens. Influenza virus should be subtyped by highly specific antisera directed against H and N. Alternatively, subtyping can be done by using polyclonal antisera raised against a set of influenza viruses or using sequence analysis of HA and NA gene. Even conventional virus isolation and identification is the method of choice for avian influenza diagnosis but this method is time and labor intensive and requires maintenance of large stocks of antisera.

Alternative techniques to evaluate avian influenza virus presence include reverse-transcription polymerase chain reaction (RT-PCR) or real time RT-PCR using nucleocapsid or matrix-specific primers. These methods can save time and labor. RT-PCR technique on clinical specimens can use for rapid detection after virus from primary outbreak was characterized and appropriated primers are used (Tsukamoto et al., 2008; Tsukamoto et al., 2009). This method is also sensitive enough for direct amplification from swab samples. Due to the high sensitivity of RT-PCR technique, contaminations from non-template present in the environment or cross-contaminations between clinical samples are also disadvantage. In case that there are a lot of samples, RT-PCR technique still consumes time and labor.

Real-time RT-PCR technique offers more rapid result. Moreover, this technique minimizes the chance of contamination since both amplification and detection take place in a single close tube. Real-time RT-PCR technique offers an alternative method for both qualitative and quantitative analysis. Real-time RT-PCR technique is based on the hydrolysis probe for generation of the target-specific fluorescence signal. For avian influenza diagnosis, primers have been designed for the detection of matrix (M) gene which is highly conserved for all influenza A viruses (OIE, 2009). The primers specific for the M gene was able to detect all influenza A viruses. This method was shown to have sensitivity and specificity equivalent to virus isolation (Spackman et al., 2002).

While virus isolation in chicken embryo or detection of viral RNA provide current information about active infection, serology which tested for the presence of antibodies can be used to identify post exposure to influenza viruses. AGID test has been used to detect group specific antibodies to influenza A viruses because all of them have common nucleocapsid and matrix antigens. The basis of the AGID test is the diffusion of antigen and antibody through the agar. When the antigen and specific antibody come in contact, they will bind and form immune complex which can be seen as precipitin line. Nevertheless, AGID test are less reliable in antibodies detection in other poultry except chicken and turkey.

HI test has been used to detect subtype specific antibodies against influenza virus. Antibody may be detected as early as 7 days after infection (Swayne and Halvorson, 2003). HI test can also be used to quantitate serum antibody. The basis of the HI test is the agglutination of RBC by influenza virus from the interaction between the receptor binding site (RBS) in HA and sialic acid receptor. Then, agglutination could be block by subtype specific antibody. Although the HI test is the gold standard for demonstration of strain specific antibodies, serum from other poultry except chicken may cause auto-agglutination when chicken RBC was used. Auto-agglutination should be inhibited by adsorption of the serum with chicken RBC. The antibody level measured by HI test correlate with protection against clinical disease and can be used to evaluate vaccine efficacy in chicken (Tian et al., 2005).

CHAPTER II

The effect of inoculation dose of a Thai HPAI-H5N1 (CUK2) virus on the pathogenicity and transmission in Japanese quail

2.1 Introduction

Avian influenza (AI) is an infectious disease of birds caused by avian influenza viruses (AIVs). Most AIVs circulated in avian species and only the H5, H7 and H9 subtypes are known to cause disease in human (Koopmans et al., 2004; Butt et al., 2005). The most well known AIVs is highly pathogenic avian influenza (HPAI) subtype H5N1. Although HPAI-H5N1 infection in human is limited, HPAI-H5N1 viruses pose health risk to human, because these viruses cause severe illness including pneumonia, respiratory failure and death. Up until now, most human cases have occurred due to close contact with infected poultry. Moreover, human-to-human transmission is believed to have occurred (Ungchusak et al., 2005; Wang et al., 2008). Since, influenza A viruses mutate rapidly, thus they have potential to generate new variant viruses which spread easily among human.

The first HPAI-H5N1 outbreak in Thailand was reported in early 2004. From 2004 to 2008, at least 7 waves of HPAI-H5N1 outbreaks were reported in the country (Suwannakarn et al., 2009). Routine surveillance, movement restriction and culling of infected and adjacent flocks have been implemented as control strategies for early detection, control, spread and eliminate sources of the virus. Since then, there is no evidence of HPAI-H5N1 outbreak in Thailand. However, several HPAI-H5N1 outbreaks have been found in domestic poultry and wild birds in neighbouring countries in 2012 (OIE, 2012). Since the viruses can re-emerge in the region by several factors such as animal movement by trading, wild bird migration especially in non-commercial poultry including backyard chicken, duck, and quail (Sims et al., 2005; Keawcharoen et al., 2011). For example, in 2008, HPAI-H5N1 viruses were isolated from quail in live-bird markets (Amonsin et al., 2008). Up to date, there are several reports on HPAI-H5N1

viruses in chicken or even duck and goose. However, the information of HPAI-H5N1 virus in quail is still limited.

It has been known that, the pathotype of HPAI-H5N1 viruses in different bird species are varied (Perkins and Swayne, 2003). The variation of pathobiological features was also found in different breeds of the same species of birds (Saito et al., 2009). In each host, the pathobiological features of HPAI-H5N1 viruses vary among different strains even their genetic compositions are very similar. These differences include lethal dose, mortality rate, symptom, viral shedding (Saito et al., 2009). Thus, the pathotype of each strain of the viruses in each host should be evaluated. From previous study, the inoculation doses are correlated with the infectiousness and mortality. For example, some strains of HPAI-H5N1 viruses, low dose inoculation do not cause 100% infection and mortality (Middleton et al., 2007). In addition, the infection chain may not occur (Spekreijse et al., 2011). Thus, the effect of inoculation dose of virus should be investigated.

In this study, Japanese quail were focused due to they are highly susceptible to HPAI-H5N1 virus. Japanese quail may play an important role in influenza infection as an intermediate host for the reassortment of influenza viruses and generating the variant viruses (Perez et al., 2003b). Several studies reported that Japanese quail supported the replication of at least 14 subtypes of influenza A viruses and the viruses replicated predominantly in the respiratory tract (Makarova et al., 2003; Saito et al., 2009). It has been known that Japanese quails pose sialic acid α2,3-galactose (SA α2,3-gal) and α2,6-galactose (SA α2,6-gal) linked receptors in the trachea and intestine which prefer to bind avian and mammalian viruses, respectively (Wan and Perez, 2006; Guo et al., 2007). Thus, Japanese quail provide a suitable environment for the reassortment of avian and mammalian influenza viruses (Makarova et al., 2003).

Virus used in this study was HPAI subtype H5N1 “A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1) (CUK2)” isolated in 2004 from the infected laying hen in Nakorn Pathom province, central region of Thailand. This virus is commonly used

in our laboratory as Thai HPAI-H5N1 reference strain. In Thailand, two clades of influenza A virus (H5N1) have been reported. The clade 1 viruses are the only predominant clade which consists of three sub-lineages. The CUK2 virus is a member of the predominant lineage (Clade 1 – CUK2-like) (Suwannakarn et al., 2009).

The aim of this study was to determine the effect of inoculation dose of a Thai HPAI-H5N1 (CUK2) virus on the pathogenicity and transmission in Japanese quail. Various inoculation doses were used to determine the susceptibility and relation with transmission. The numbers of successful infections, mortality and viral shedding were investigated. In addition, transmission experiments were performed by mingling inoculated Japanese quail with contact Japanese quail. The result obtained from the study can provide useful information for improving surveillance, eradication and vaccine strategies.

2.2 Materials and methods

2.2.1 Animals

A total of 107 3-week-old Japanese quails (*Coturnix coturnix japonica*) were acquired from a commercial Japanese quail farm with a history of non-vaccinated parent stock. Oropharyngeal and cloacal swabs as well as blood samples were collected and tested to ensure that the quail were naive to influenza virus by egg inoculation and hemagglutination inhibition test (HI). Japanese quail were wing-banded for individual identification and provided feed and water ad libitum. The quail were housed in the biosafety cabinet under biosafety level 3 conditions (figure 2.1). The animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Faculty of Veterinary Science, Mahidol University (Approval number MUVS-2011-35).

Figure 2.1 Animal containment facility in BSL-3 at Faculty of Veterinary Science, Mahidol University

2.2.2 Virus

The HPAI-H5N1 virus, A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1), was used in the study. The virus was isolated from the index chicken case of the 2004 HPAI outbreak in Thailand. Stock virus was propagated in 10-day-old embryonated chicken eggs. The eggs were incubated at 37 $^{\circ}$C for 4 days. Following egg death, the allantoic fluid was harvested. The haemagglutination assay (HA) and real time RT-PCR were performed to confirm influenza virus infection. Virus titer was determined by inoculation of serial 10-fold dilutions of pooled allantoic fluid in embryonated chicken eggs. The mortality of eggs was recorded and 50 percent Embryo Lethal Dose (ELD_{50}) was calculated following Reed and Muench protocol (Reed and Muench, 1938). Allantoic fluid from eggs with dead embryo and all eggs remaining at the end of incubation period were tested with HA and 50 percent Embryo Infectious Dose was calculated (EID_{50}) using the method of Reed and Muench. All work with the HPAI-H5N1 virus was performed in biosafety level 2 containment facilities. Stock virus had a viral titer of $10^{8.5}$ EID_{50}/ml. The virus was kept at -80 $^{\circ}$C until use. Stock viruses were diluted with PBS to $10^{7.0}EID_{50}$ (group 1), $10^{5.0}EID_{50}$ (group 2) and $10^{3.0}$ EID_{50} (group 3)/0.1 ml before inoculation.

2.2.3 Serological test

Hemagglutination inhibition assay (HI) was performed following the OIE standard (OIE, 2009). Briefly, all sera were pretreated with chicken red blood cells. 10 µl of packed chicken RBCs was added in 200 µl of serum then pelleted and treated serum was used. Chicken RBCs were washed and resuspended to the final concentration of 1 % (v/v) in PBS. H5 virus/antigen was adjusted to 4 hemmaglutination units (HAU) per 25 µl in PBS. 25 µl of treated sera were serially diluted two-fold with PBS in plastic V-bottom micro-titer plates. Serially diluted sera were incubated with 4 HAU of viral antigen for 30 minutes at room temperature. After incubation, 25 µl of 1% chicken RBCs were added. Plates were incubated at room temperature for 40 minutes. HI positive wells were defined by the button of unagglutinated chicken RBCs at the bottom of the

wells. HI negative wells had diffuse sheet of agglutinated chicken RBCs covering the bottom. The HI titer was the highest dilution that agglutination was not observed.

2.2.4 Real-time RT-PCR specific for the influenza A virus matrix (M) gene

RNA was extracted with Viral NA Extraction Kit (Beckman Coulter®, California, USA). To identify and titrate influenza A virus, real-time RT-PCR specific for the influenza A virus matrix (M) gene was conducted (Spackman et al., 2002). Briefly, the cocktail, composed of forward/reverse primers 0.4 µM per reaction, probe 0.1 µM per reaction, tag 0.3 µl per reaction, master mix 7.5 µl ($MgSo_4$ 4 mM) per reaction (SuperScript™ III Platinum® One-Step Quantitative RT-PCR System, Invitrogen™, California, USA) and distilled water, was made for all the reactions (table 2.1). 11 µl of the cocktail and 4 µl of RNA were added to 0.2 ml. tube. One step real-time RT-PCR was performed on Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Cycling conditions included a reverse transcription step at 50°C for 30 minutes. After an initial denaturation step at 95°C for 15 minutes, amplification was performed for 50 cycles including denaturation (95°C for 15 seconds), annealing (60°C for 30 seconds). Multiple fluorescent signals were obtained once per cycle at the end of the annealing step with detectors to FAM channel. Data acquisition and analysis of the real-time RT-PCR assay were performed using the Rotor-Gene Version 6.0.19 software (Corbett Research, Sydney, Australia). The standard curve and threshold were computed from three different concentration standard reactions and negative template control (NTC). The viral titers were calculated from a standard curve.

Table 2.1 Primer and probe used in this study (Spackman et al., 2002)

Primer and probe	Sequence (5' to 3')	Working concentration
MF25	AGATGAGTCTTCTAACCGAGGTCG	10 µM
MR124	TGCAAAGACATCTTCAAGTCTCTG	10 µM
M64 Probe	[6FAM] TCAGGCCCCCTCAAAGCCGA[TAM]	2.5 µM

2.2.5 Histopathology and immunohistochemical staining

For histopathology and immunohistochemical staining, tissues were fixed by 10 % buffered formalin and embedded in paraffin. Duplicated sections were cut at 5 µm. The first section was stained with hematoxylin and eosin (H&E). The histologic lesions were scored according to the distribution of lesions and severity of pathologic lesions, for example, congestion, hemorrhage and necrosis : - = no lesion, $\pm$ = minimal; few of focal lesions (<10%), + = mild; multifocal distribution (10-30%) and low pathologic lesions, ++ = moderate; diffuse distribution (30-60%) and moderate pathologic lesions , +++ = severe; diffuse distribution (>60%) and widespread pathologic lesions (Perkins and Swayne, 2003).

The second section was immunohistochemical stained for the detection of influenza virus antigen in the tissues. Immunohistochemical staining was performed as previously reported (Thontiravong et al., 2012). Briefly, section was deparaffinized 3 times with xylene, 5 minutes each and rehydrated by 50-50 mix of xylene and alcohol, absolute alcohol, 95 % alcohol, 80% alcohol, 75 % alcohol, 2 minutes each, distilled water and phosphate buffered saline (PBS), 5 minutes each. Endogenous peroxidase was blocked with 0.3 % H_2O_2 for 30 minutes at room temperature. 0.05 % Proteinase K was used to retrieve the epitope for 10 minutes at 37 ^{o}C then wash 3 times with PBS, 5 minutes each. Unspecific binding of antibody was blocked with 1 % bovine serum albumin for 45 minutes at 37 ^{o}C then wash 3 times with PBS. The primary antibody (mouse anti-Influenza A virus (NP) monoclonal antibody clone EVS 238, 1:300 dilution,

BV European Veterinary Laboratory, The Netherlands) was applied for 12 hours at 4 ^{o}C then wash 3 times with PBS. The primary antibody was detected by chain polymer kit (Dako REAL^{TM} envision–HRP system, anti-rabbit/mouse, Glostrup, Denmark) for 45 minutes at 37 ^{o}C then wash 3 times with PBS and followed by 3,3' diaminobenzidine tetrahydrochlorid (DAB) substrate (Sigma-Aldrich, USA) (DAB 0.075 g + Tris buffer 150 ml + 30% H_2O_2 50 µl). Section was counterstained with H&E and dehydrated with 75 % alcohol, 80% alcohol, 95 % alcohol, absolute alcohol, 50-50 mix of xylene and and xylene.

The distribution of viral antigen in tissue samples was scored according to the number of positive immunostaining cells (brown color) : - = no positive cells, $\pm$ = rare; a few number of positive cells (<2 cells/HPF(high power field)), + = infrequence; low number of the positive cells (2-5 cells/HPF), ++ = common; moderate number of positive cells (5-10 cells/HPF), +++ = widespread; diffuse positive cells (>10 cells/HPF) (Perkins and Swayne, 2003).

2.2.6 Experimental infection and transmission experiment

One hundred and seven 3-week-old Japanese quails were used for the analysis of the effect of inoculation dose of CUK2 virus on the pathogenicity and transmission. Japanese quail were raised until 9-week-old and then allocated into 4 groups. Group 1-3 (n=32) were experimental groups. Group 4 (n=11) was negative control group. At 9-week old, 16 Japanese quails in group 1-3 (inoculated quail) were inoculated intranasal and intraoral with 0.1 ml (0.05 ml for each route) of diluted allantoic fluid containing $10^{7.0}$ (group 1), $10^{5.0}$ (group 2) and $10^{3.0}$ (group 3) EID_{50} of HPAI-H5N1 virus, respectively. Japanese quail in group 4 were inoculated with PBS as placebo. Twenty four hours later, 16 Japanese quails (contact quail) were added into group 1-3 (van der Goot et al., 2005)(figure 2.2).

After inoculation, quail were kept and observed for 3 weeks. In group 1, 2 and 3, inoculated quail were euthanized at 12 (n=3), 24 (n=3) and 36 (n=3) hour post

inoculation (hpi) and contact quail were euthanized at 12 (n=3), 24 (n=3) and 36 (n=3) hour post contact (hpc). In group 4, inoculated quail were euthanized at 12 (n=2), 24 (n=2) and 36 (n=2) hpi. Tissue samples were collected in 10% buffered formalin for histopathologic evaluation and immunohistochemical staining to confirm viral infection. Oropharyngeal and cloacal swabs were also collected to confirm viral shedding. All quail were monitored on a daily basis for clinical signs and mortality. Dead quail were necropsied and tissue samples were collected. Oropharyngeal and cloacal swabs were also collected.

The Japanese quail were monitored for viral shedding by sampling oropharyngeal and cloacal swabs daily for the first 10 days and 14, 16 and 21 day post inoculation. Swab was placed into tube containing 2 ml of viral transport media (VTM). Determination of virus titers was performed by real-time RT-PCR. The experiment was terminated 3 weeks after the challenge. Blood samples were collected from all remaining quail for serological examination. The serological response was studied by the HI test. Quail were euthanized by intramuscular administration of Zolazepam/Tiletamine. Euthanized quail were necropsied. Tissue samples, oropharyngeal and cloacal swabs were collected.

2.2.7 Statistical analysis

The statistical significant differences ($p<0.05$) in death time were compared by analysis of variance (ANOVA) with LSD. Viral titers and HI titers were analyzed for statistical significant differences ($p<0.05$). Viral titers and HI titers were compared between groups by ANOVA with LSD and between days by paired t-test. Viral titer from oropharyngeal and cloacal swab were compare by paired-t test. The statistical significant differences ($p<0.05$) in numbers of quail shedding virus between groups and days were evaluated by Fisher's exact test. The data was analyzed by SAS 9.2 software package (SAS Institute Inc., North Carolina, USA).

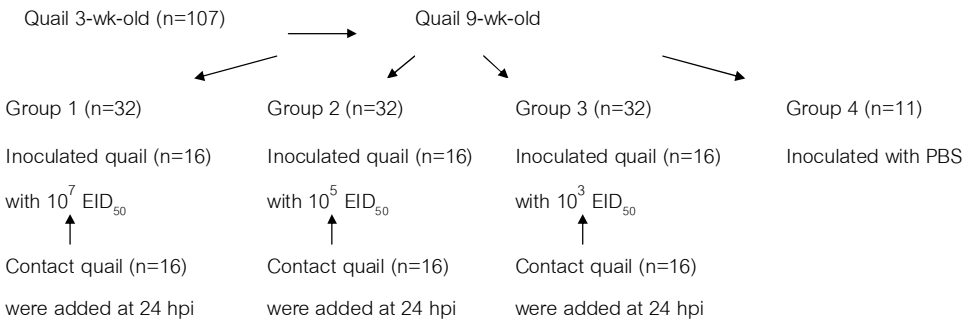


Figure 2.2 Schematic of experimental design

2.3 Results

2.3.1 Effect of inoculation dose of HPAI-H5N1 (CUK2) on mortality of Japanese quail

In negative control group (group 4), there was no mortality of quail. The quail did not show any clinical sign until the end of experiment. In group 1 (inoculated with $10^{7.0}$ EID_{50}) and group 2 (inoculated with $10^{5.0}$ EID_{50}), 6 % (1/16) of the inoculated quail show depression and ruffed feather at 1 dpi. At 2 dpi, 30 % (3/10) of inoculated quail in group 1 and 20 % (2/10) of inoculated quail in group 2 showed depression and ruffed feather. Diarrhea was observed in 20 % (2/10) of inoculated quail in group 1 and 10 % (1/10) of inoculated quail in group 2 at 2 dpi. 10 % (1/10) of inoculated quail in group 1 and 75 % (3/4) of inoculated quail in group 3 which inoculated with $10^{3.0}$ EID_{50} display neurologic sign such as tremors and paralysis at 2 dpi and 3 dpi, respectively. All inoculated quail died within 3.5 dpi. The quail in group 1 ($10^{7.0}$ EID_{50}) died at 1.5-2.5 dpi. The quail in group 2 ($10^{5.0}$ EID_{50}) died at 2-3.5 dpi. The quail in group 3 ($10^{3.0}$ EID_{50}) died at 1.5-3 dpi (figure 2.3). There was no statistical difference in death time between inoculated groups. The mean death time (MDT) was displayed in table 2.2.

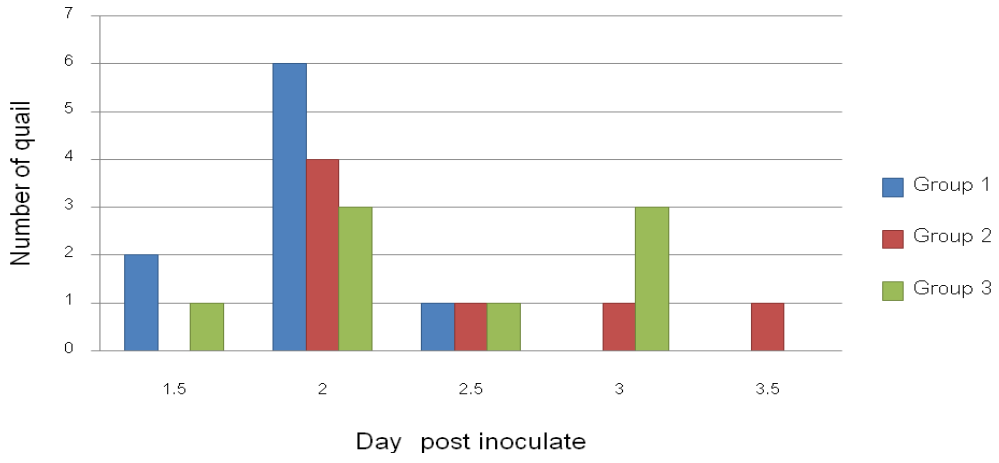


Figure 2.3 Number of inoculated quail that died after inoculation

For contact groups, all contact quail died within 6.5 dpc. The contact quail in group 1 died at 1.5-2.5 dpc. The contact quail in group 2 died at 2.5-3 dpc. The contact quail in group 3 died at 2.5-6.5 dpc (figure 2.4). MDT of contact quail in group 3 was statistically significant longer than group 1. The mean death time (MDT) was displayed in table 2.2.

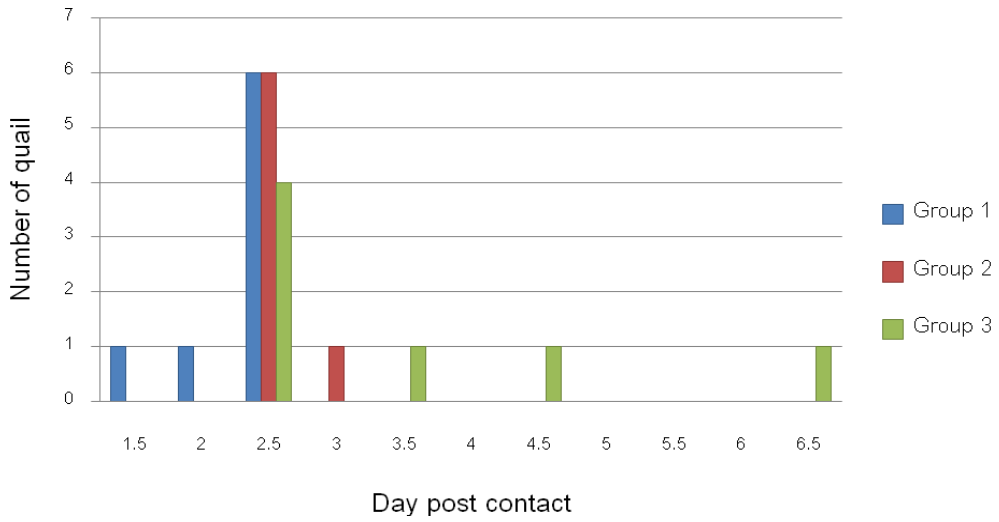


Figure 2.4 Number of contact quail that died after contact

Table 2.2 Mean death time of inoculated* and contact quail

MDT	Group 1 inoculated (10^7 EID_{50})	Group 2 inoculated (10^5 EID_{50})	Group 3 inoculated (10^3 EID_{50})	Group 1 contact	Group 2 contact	Group 3 contact
dpi or dpc	1.94	2.43	2.38	2.31 ^{A}	2.57	3.50 ^{B}

* Quail in group 4 (negative control) survived until the end of experiment

Different alphabet in the same row means significant at 95 % confidence interval

2.3.2 Gross lesions

At necropsy, quail in negative control group (group 4) showed no gross lesions. Gross lesions of inoculated quail in group 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50}, respectively) and contact quail in each group were similar. The primary gross lesions in all quail were lung edema, congestion, hemorrhage, focal pneumonia and severe acute diffuse pneumonia. The second most prominent lesion was found in pancreas, including edema, congestion, hemorrhage and multifocal necrosis (figure 2.5). The third most common lesion was kidney congestion (table 2.3). Splenomegaly with congestion (5/32) and hemorrhage, hepatomegaly with congestion and necrosis (4/32), hemorrhage in the epicardium (2/32) were observed. Congestion and hemorrhage were also found in the bursa of fabricious (2/32), intestine (1/32) and ovary (1/32).

Table 2.3 Percentage of gross lesion positive in visceral organ samples

Organ	Gross lesion positive samples (%)					
	Group 1 inoculated (10^7 EID_{50})	Group 2 inoculated (10^5 EID_{50})	Group 3 inoculated (10^3 EID_{50})	Group 1 contact	Group 2 contact	Group 3 contact
Lung	100 (16/16)*	100 (16/16)	100 (16/16)	100 (16/16)	100 (16/16)	100 (16/16)
Pancreas	12.5 (2/16)	25 (4/16)	12.5 (2/16)	25 (4/16)	12.5 (2/16)	0 (0/16)
Kidney	18.75 (3/16)	0 (0/16)	0 (0/16)	18.75 (3/16)	0 (0/16)	6.25 (1/16)

* Number of positive quail / total quail

(A) (B)

(C) (D)

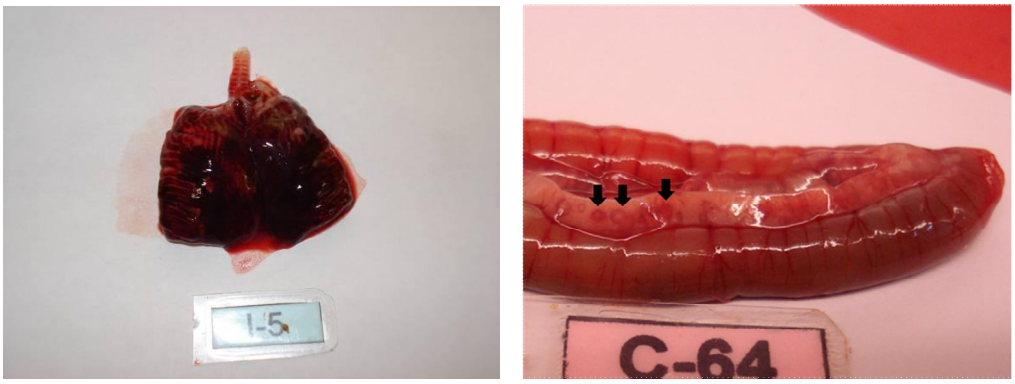


Figure 2.5 Japanese quail after inoculation (A) Japanese quail showed depression and ruffed feather at 1 dpi (B) All Japanese quail died within 6.5 dpc (C) Lung, severe congestion, pulmonary edema and pneumonia (D) Pancreas, multifocal necrosis (arrow)

2.3.3 Histopathology

Histopathological examination by H&E staining of tissue sections revealed that lesions were found in tissues of both inoculated and contact quail. Lesions from inoculated quail in group 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50}, respectively) and contact quail were similar except for lung. Histologic lesions of lung of inoculated quail in group 2 and 3 were more severe than contact quail (table 2.4). The quail in negative control group (group 4) showed no histopathological lesions.

Table 2.4 Average severity of histologic lesion in visceral organ samples

Organ	Group 1* inoculated (10^7 EID_{50})	Group 2 inoculated (10^5 EID_{50})	Group 3 inoculated (10^3 EID_{50})	Group 1 contact	Group 2 contact	Group 3 contact
Trachea	+**	++	+	+	++	++
Lung	+++	+++	+++	+++	++	++
Heart	±	±	+	+	±	±
Brain	+	±	+	±	+	±
Intestine	+	+	++	++	++	++
Pancreas	++	++	++	++	++	++
Liver	++	+	++	++	++	+
Kidney	+	+	++	++	+	+
Spleen	+	+	++	+	+	+
Oviduct	-	±	+	+	-	-
Ovary	-	±	+	+	-	-
Bursa	-	+	±	+	+	+

* n = 5 in group 1 inoculated, n = 7 in other groups

**- = no lesion; ± = minimal; + = mild; ++ = moderate; +++ = severe

In summary, lesions were more prominent in inoculated and contact quail found dead than euthanized quail. The most important lesion was found in the lung. In the lungs, severe congestion, focal hemorrhage and pulmonary edema were noted especially in alveoli and around the vessel. These lesions were observed in 100% of lung from inoculated and contact quail in group 1, 2 and 3 euthanatized from 12 to 36 hpi and hpc and from quail found dead. Acute inflammation with focal infiltration of heterophils and lymphocytes of pneumonia was also found. Pneumonia was found in contact quail in group 3 more often than group 1 and 2 (table 2.5).

Table 2.5 Percentages of pneumonia positive sample

Time after inoculated or contact	Pneumonia positive sample (%)					
	Group 1 inoculated (10^7 EID_{50})	Group 2 inoculated (10^5 EID_{50})	Group 3 inoculated (10^3 EID_{50})	Group 1 contact	Group 2 contact	Group 3 contact
36 hpi or hpc	0 (0/3)*	0 (0/3)	0 (0/2)	0 (0/3)	33 (1/3)	0 (0/3)
Death	0 (0/5)	14 (1/7)	14 (1/7)	0 (0/7)	14 (1/7)	86 (6/7)

* number of positive quail / total quail

In respiratory tract and cardiovascular system, lesions that were found in trachea include detachment of the epithelium and subepithelial edema, infiltration of heterophils and lymphocytes in subepithelial layer, focal death of epithelial cells. Tracheitis and epithelial detachment were first observed in inoculated quail (3/9) at 12 hpi and first observed in contact quail (2/9) at 12 hpc. Lesions in the hearts were mild to moderate and sporadic including congestion, haemorrhage and lymphocyte infiltration.

Focal necrosis of pancreatic acinar cells without an inflammatory response was observed. Pancreatic necrosis was first observed in inoculated quail (2/9) at 12 hpi but

found more frequently (6/8) at 36 hpi and first observed in contact quail (2/9) at 36 hpc. 89.5 % (16/19) of inoculate quail found dead and 67 % (14/21) of contact quail found dead had pancreatic necrosis (figure 2.6).

Renal congestion was first observed at 12 hpi (4/9) and 12 hpc (4/9) but found more frequently at 36 hpi (7/8) and 36 (6/9) hpc. Lymphoid depletion and necrosis in spleens start at 24 hpi in inoculated quail (1/9) and 36 hpc in contact quail (3/9) but found more frequently in inoculated (15/19) and contact quail (16/21) found dead. Fatty infiltration and liver congestion occurred in 77 % (7/9) of inoculate quail at 12 hpi and 88 % (8/9) of contact quail at 12 hpc.

Lymphoid depletion and necrosis was first observed in bursa of inoculated (1/8) and contact quail (1/9) at 36 hpi and 36 hpc but found more frequently in inoculated (6/19) and contact (11/21) quail found dead. Desquamation of mucosal epithelium and enteritis were first observed in of inoculated (3/9) and contact quail (1/9) duodenums at 12 hpi and 12 hpc but found more frequently in inoculated quail (6/8) at 36 hpi and contact (18/21) quail found dead. Desquamation of mucosal epithelium was also observed in cecum. Congestion, heterophil and lymphocyte infiltration and necrosis of oviducts were mild to moderate and sporadic. Hemorrhage, heterophil and lymphocyte infiltration and necrosis of ovary were mild to moderate and sporadic. Lesions in the brains were mild and sporadic including non-suppurative encephalitis, gliosis in cerebrum, cerebellum liquefaction, vacuolation of brain stem and congestion.

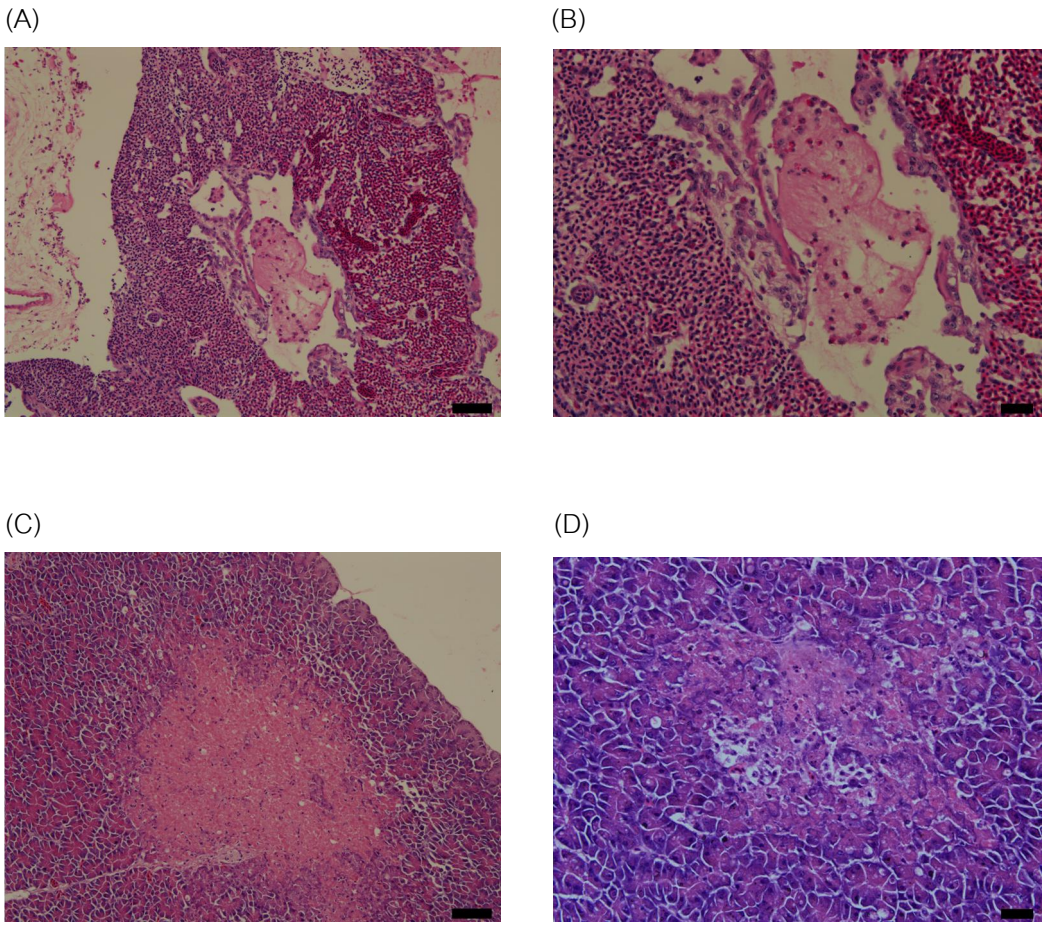


Figure 2.6 Hematoxylin and eosin stained tissue sections from HPAI-H5N1 inoculated Japanese quail (A) Lung, pneumonia with infiltration of inflammatory cells, severe congestion and pulmonary edema (scale bar = 50 µm) (B) Lung, acute inflammation with focal infiltration of heterophils and lymphocytes (scale bar = 20 µm) (C) Pancreas, large focal necrosis (scale bar = 50 µm) (D) Pancreas, focal necrosis of pancreatic acinar cells (scale bar = 20 µm)

2.3.4 Immunohistochemistry

Viral antigen was found in all tissues which had histologic lesions (figure 2.7 and 2.8). Viral antigen was found in the nucleus of infected cells. Viral particles were most commonly observed in the brain (14/19 of inoculated quail and 15/21 of contact quail). Many infected cells were seen in all parts of the brain, including cerebrum, brain stem and cerebellum. Neurons, Purkinje cells and glia cells were infected. Viral particles were commonly found in pancreas, heart and liver (table 2.6). Viral particles were found in the pancreatic acinar cells around necrotic foci, cardiac mycocytes and hepatocytes. Viral antigen was found in tissues from all inoculated quail (100%) died after 36 hpi and contact quail died after 36 hpc. Viral antigen was first observed in inoculated quail in group 1 ($10^{7.0}$ EID_{50}) (1/3) at 24 hpi and inoculated quail in group 2 ($10^{5.0}$ EID_{50}) (1/3) at 36 hpi. Viral antigen was first observed in contact quail in group 1 (2/3) at 36 hpc. No viral antigen was detected in tissues from quail in negative control group (group 4).

Table 2.6 Average distribution of viral antigen in tissue samples

Organ	Group 1* inoculated (10^7 EID_{50})	Group 2 inoculated (10^5 EID_{50})	Group 3 inoculated (10^3 EID_{50})	Group 1 contact	Group 2 contact	Group 3 contact
Trachea	+**	±	±	±	+	±
Lung	++	++	++	++	++	++
Heart	++	++	++	+++	+++	++
Brain	+++	+++	+++	+++	+++	+++
Intestine	+	+	+	+	+	+
Pancreas	+	++	++	++	+++	+++
Liver	+++	+++	++	++	+++	++
Kidney	+	++	+	+	++	+
Spleen	++	++	+	+	++	+
Oviduct	++	++	+	++	+++	++
Ovary	+	±	±	±	±	±
Bursa	±	±	±	±	±	±

* n = 5 in group 1 inoculated, n = 7 in other groups

** - = none; ± = rare; + = infrequence; ++ = common; +++ = widespread

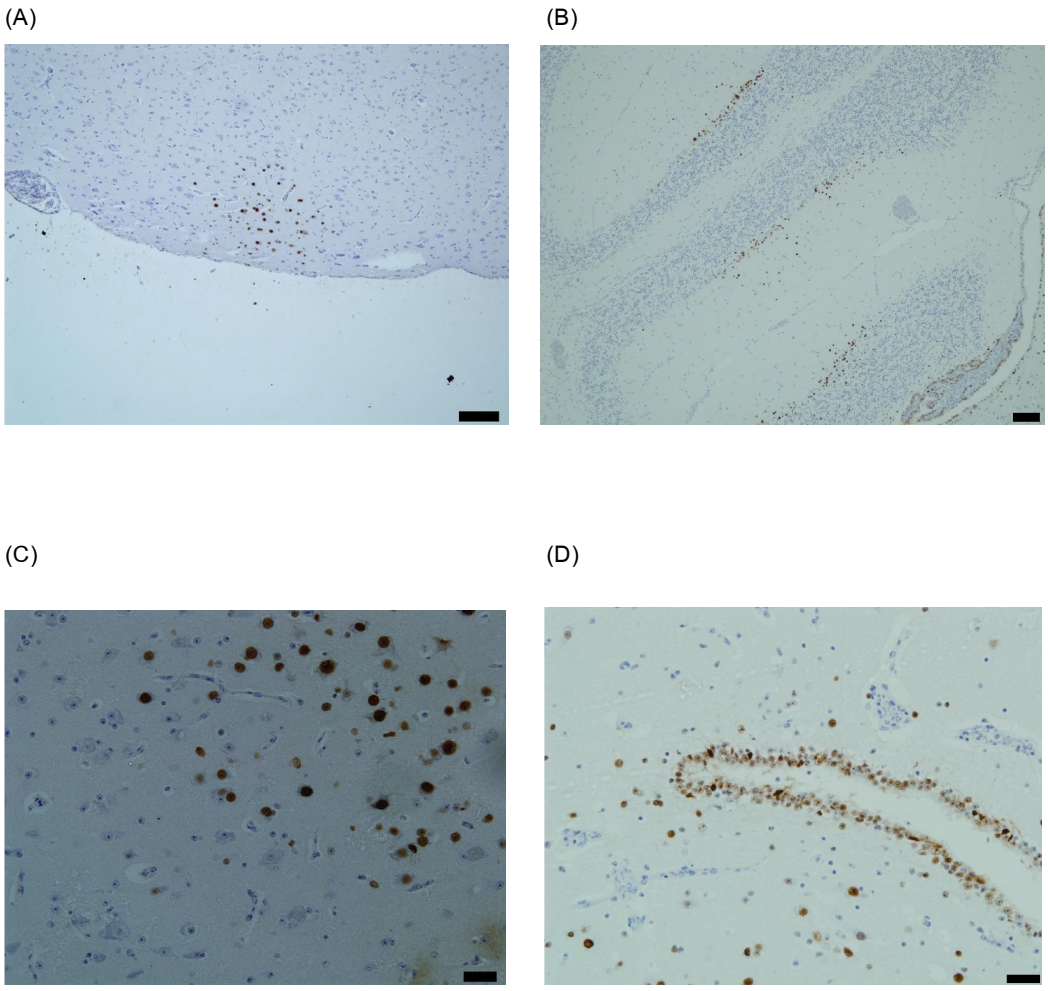
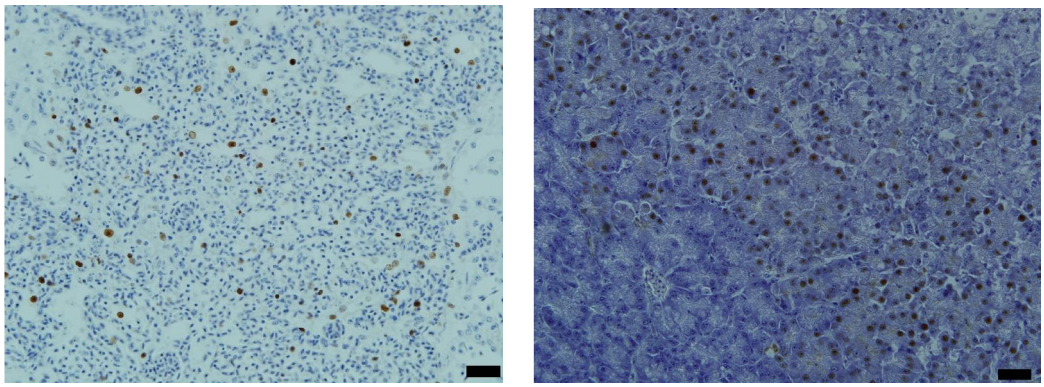


Figure 2.7 Immunohistochemical stained sections from HPAI-H5N1 inoculated Japanese quail, viral antigen was stained brown in nucleus. (A) Brain, cerebrum, wide spread of virus-infected cells (scale bar = 100 µm) (B) Brain, cerebellum, wide spread of virus-infected cells (scale bar = 100 µm) (C) Brain, cerebrum, viral antigen in nucleus of neurons and glia cells (scale bar = 20 µm) (D) Brain, cerebellum, viral antigen in nucleus of Purkinje cells (scale bar = 20 µm)

(A) (B)



(C) (D)

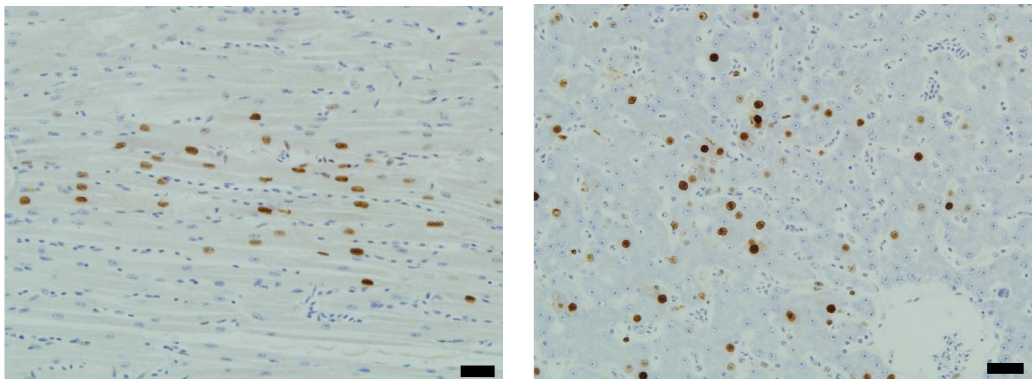


Figure 2.8 Immunohistochemical stained sections from HPAI-H5N1 inoculated Japanese quail, viral antigen was stained brown (scale bars = 20 µm) (A) Lung, viral antigen in nucleus of infected cells (B) Pancreas, viral antigen in the pancreatic acinar cells around necrotic foci (C) Heart, viral antigen in cardiomycocytes (D) Liver, viral antigen in hepatocytes

2.3.5 Viral shedding

In inoculated quail, virus was not detected in oropharyngeal swab and cloacal swab from quail in negative control group (group 4) from 1 dpi until the end of experiment by real-time RT-PCR. Inoculated quail in group 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50} , respectively) shed virus in oropharynx since 1 dpi until death (table 2.7). Virus titer in oropharyngeal swabs obtained from inoculated quail in group 2 was statistically significant increased at 2 dpi and tended to increase until death. Virus titers in oropharyngeal swabs obtained from inoculated quail in group 1 and 2 were statistically significant higher titer than group 3 at 1 and 2 dpi.

Table 2.7 Mean viral titer (log_{10} EID_{50}) and standard error mean of oropharyngeal swabs from inoculated quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Group‡	Virus titer (log_{10} EID_{50}/ml)†			
	1 dpi	2 dpi	3 dpi	4 dpi
1	5.0±0.1 ^{A}	5.9±0.2 ^{A}	5.4	
(10^{7} EID_{50})	(16/16) ††	(7/7)	(1/1)	
2	4.3±0.3 ^{A*}	5.9±0.2 ^{A**}	6.2±0.1	6.6
(10^{5} EID_{50})	(16/16)	(7/7)	(2/2)	(1/1)
3	3.6±0.3 ^{B}	4.6±0.2 ^{B}	5.5±0.4	
(10^{3} EID_{50})	(15/16)	(7/7)	(5/5)	

‡ virus cannot be isolated from quail in group 4

†Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

††Number of positive quail /total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

Inoculated quail in group 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50} , respectively) shed virus in cloaca since 1 dpi until death (table 2.8). Virus titers in cloacal swabs obtained from inoculated quail in group 1, 2 and 3 were statistically significant increased at 2 dpi and virus titer in cloacal swabs obtained from inoculated quail in group 3 was statistically significant increased at 3 dpi. The number of inoculated quail in group 1, 2 and 3 that shed virus from cloaca was statistically significant increased at 2 dpi. The number of inoculated quail in group 1, 2 and 3 that shed virus from cloaca at 1 dpi decreased respectively according to the amount of inoculated virus. Virus titers in cloacal swabs obtained from inoculated quail in group 1 and 2 were statistically significant higher than group 3 at 2 dpi.

Virus titers in oropharyngeal swabs from inoculated quail in group 1, 2 and 3 at 1 dpi were statistically significant higher than those in cloacal swabs. The number of inoculated quail that shed virus from oropharynx at 1 dpi in group 1, 2 and 3 was statistically significant higher than those from cloaca (table 2.7 and 2.8).

Table 2.8 Mean viral titer (log_{10} EID_{50}) and standard error mean of cloacal swabs from inoculated quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Group‡	Virus titer (log_{10} EID_{50}/ml)†			
	1 dpi	2 dpi	3 dpi	4 dpi
1	2.3±0.3 *	6.1±0.2 ^{A}**	6.5	
(10^7 EID_{50})	(5/16) *	(7/7) **	(1/1)	
2	2.2±1.1 *	5.3±0.6 ^{A}**	4.7±0.7	6.5
(10^5 EID_{50})	(3/16) *	(7/7) **	(2/2)	(1/1)
3	3.2 *	3.7±0.6 ^{B}**	5.2±0.4 ***	
(10^3 EID_{50})	(1/16) *	(7/7) **	(5/5)	

‡ virus cannot be isolated from quail in group 4

†Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

†Number of positive quail /total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

In contact quail, contact quail in group 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50}, respectively) shed virus in oropharynx since 1 dpc until death. Virus titers in oropharyngeal swabs obtained from contact quail in group 1, 2 and 3 were statistically significant increased at 2 dpc and at 3 dpc in group 1 and 2 (table 2.9). Virus titer in oropharyngeal swabs obtained from contact quail in group 1 and 2 were statistically significant higher than group 3 at 1 and 2 dpc. The number of contact quail in group 1 and 2 that shed virus from oropharynx were statistically significant higher than group 3 at 1 dpc.

Table 2.9 Mean viral titer (log_{10} EID_{50}) and standard error mean of oropharyngeal swabs from contact quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Group	Virus titer (log_{10} EID_{50}/ml)[†]						
	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc
1	3.8±0.4 [A]*	5.1±0.2 [A]**	6.1±0.2 ***				
	(13/13) [a]	(10/10)	(6/6)				
2	4.4±0.1 [A]*	5.1±0.2 [A]**	6.0+0.2 ***				
	(13/13) [a]	(10/10)	(7/7)				
3	2.4±0.5 [B]*	4.7±0.5 [B]**	5.0±0.6	5.7±1.0	4.7±1.4	5.0	5.7
	(8/13) [b]	(7/10)	(7/7)	(2/3)	(2/2)	(1/1)	(1/1)

[†]Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

[†]Number of positive quail /total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

Contact quail in group 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50} , respectively) shed virus in cloaca since 1 dpc until death (table 2.10). Virus titers in cloacal swabs obtained from contact quail in group 1 and 2 were statistically significant increased at 2 dpc. Virus titers in cloacal swabs obtained from contact quail in group 1, 2 and 3 were statistically significant increased at 3 dpc. Virus titer in cloacal swabs obtained from contact quail in group 1 was statistically significant higher than group 3 at 1 and 2 dpc. The number of contact quail in group 2 and 3 that shed virus from cloaca was statistically significant increased at 2 dpc. The number of contact quail in group 1, 2 and 3 that shed virus from cloaca at 1 dpc decreased respectively corresponding to the amount of inoculated virus.

Virus titers in oropharyngeal swabs from contact quail in group 1, 2 and 3 at 1 dpc were statistically significant higher than those in cloacal swabs. Virus titer in oropharyngeal swabs from contact quail in group 2 was statistically significant higher than virus titers in cloacal swabs at 2 dpc. The number of contact birds in group 1, 2 and 3 that shed virus from oropharynx at 1 dpc was statistically significant higher than the number of quail that shed virus from cloaca (table 2.9 and 2.10).

Table 2.10 Mean viral titer (log_{10} EID_{50}) and standard error mean of cloacal swabs from contact quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Group	Virus titer (log_{10} EID_{50}/ml)†						
	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc
1	1.9±0.3 A*	4.5±0.6 A**	7.2±0.2 ***				
	(8/13)	(9/10)	(6/6)				
2	1.8±0.4 *	3.2 +0.4 **	6.2±0.2 ***				
	(4/13) *	(9/10) **	(7/7)				
3	1.0±0.7 B	2.8±0.9 B*	6.1±0.9 **	4.4±1.8	4.4±0.7	4.7	5.8
	(2/13) *	(6/10) **	(6/7)	(3/3)	(2/2)	(1/1)	(1/1)

†Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

†Number of positive quail /total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

2.4 Discussion

Pathologenetic studies of HPAI-H5N1 strains on several animal hosts are important because pathobiological features (pathotypes) of each HPAI-H5N1 virus vary among strains. For example, some studies reported significantly difference of HPAI-H5N1 virulence in different bird species and different breeds (Perkins and Swayne, 2001; Saito et al., 2009). Thus, study of the effects of inoculation dose of HPAI-H5N1 virus in each host is important because different inoculation dosages may affect pathobiological features (Middleton et al., 2007; Spekreijse et al., 2011).

In this study, virus was not detected in oropharyngeal swabs and cloacal swabs from quail in negative control group (group 4) from 1 dpi until the end of experiment. In addition, no viral antigen and antibody titer was detected in euthanized quail at 12, 24, and 36 hpi and at the end of experiment. This result confirmed that experimental quail were naive to influenza virus.

Our experiments demonstrated that A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 was highly pathogenic to Japanese quail similar to other viruses isolated from Thailand, e.g. A/Chicken/Suphanburi/1/2004, A/Duck/Angthong/72/2004, and A/Quail/Angthong/71/2004 (Saito et al., 2009) and also A/Chicken/Korea/IS/06 (Jeong et al., 2009) and A/Chicken/Hong Kong/220/97 (Perkins and Swayne, 2001). CUK2 virus caused 100 % mortality with $10^{3.0}$, $10^{5.0}$ and $10^{7.0}$ EID_{50} inoculants of the virus within 3.5 days post inoculation. The most severe and consistent lesions were lung congestion and pancreatic necrosis. Histologic lesions were prominent in lung, intestine and pancreas. Viral infection was confirmed by immunohistochemical staining. Immunohistochemical staining is commonly used to study viral pathogenesis. Immunohistochemical staining allowed identification of viral replication site in tissue. In this study, viral antigen was found in all tissues with histologic lesion.

Our result demonstrated that different strains of HPAI-H5N1 virus and age of quail may correlate with pathological features. Histologic lesions in pancreas from our

experiment (75%) were found more often than quail inoculated with A/Chicken/Hong Kong/220/97 (55%) (Perkins and Swayne, 2001) even the mortality were compatible. In addition, distribution of viral antigen was in contrast to previous reports (Perkins and Swayne, 2001; Antarasena et al., 2006). For example, viral antigen in brain from our experiment (73%) was detected more often than natural infected Japanese quail (46%) (Antarasena et al., 2006).

It has previously been shown that young duck were more susceptible to HPAI-H5N1 virus than older duck (Tian et al., 2005). After combining the result of previous and this study, age of quail also correlated with incubation period and MDT. The incubation period and MDT is shorter in the younger quail than in the older quail when receiving the similar virus strain. MDT in 4-week-old quail which get $10^{6.0}$ EID_{50} were between 1.0-1.4 day (Saito et al, 2009) while MDT in our study that 6-week-old quail which get $10^{7.0}$ EID_{50} (group 1) is 1.94 day.

In addition, Inoculated quail and contact quail started to shed virus via oropharynx and cloaca at 1 dpi and 1 dpc which earlier than previous report (Jeong et al., 2009). All inoculated quail died within 3.5 dpi and all contact quail died within 6.5 dpc. These results indicated that the incubation period of CUK2 virus in quail was short and the virus was able to spread and transmit rapidly. Previous reports (Makarova et al., 2003; Jeong et al., 2009) and our report revealed that virus titers in oropharyngeal swabs were statistically significant higher than those in cloacal swabs. Interestingly, the virus was isolated from cloacal swabs less frequently than oropharyngeal swabs at 1 dpi and 1 dpc especially in quail of $10^{3.0}$ EID_{50} inoculation group (group 3). The transmission of H5N1 virus in quail via oral-oral route is more important than fecal-oral route. The results support the speculation that oropharyngeal swabs are better as sampling route for HPAI-H5N1 surveillance in Japanese quail than cloacal swabs.

Our experiments demonstrated that difference inoculation dosages correlated with pathobiological features including incubation period, MDT and viral shedding. In previous reports, HPAI-H5N1 viruses with similar genetic composition have varied

incubation period and MDT (Saito et al., 2009). However, our finding indicated that incubation period and MDT correlate with not only the virus strain, but also the viral load. In this study, all inoculated quail inoculated with 10^7 (group 1), 10^5 (group 2) and 10^3 (group 3) EID_{50} of HPAI-H5N1 virus per bird became ill and died. This means the viral titers were high enough to infect and cause death. All contact quail in all groups became ill and died after contact with inoculated quail as well. This mean the amount of virus shed by inoculated quail was sufficient to induce infection to contact quail in all groups (Ebrahimi et al., 2011) but the contact quail in group 3 died significantly later than contact quail in group 1. Our result showed that increasing the dose decreased the MDT. This result correlated well with previous finding in chicken and duck that mean latent period decreased significantly with increasing dose (Middleton et al., 2007; Spekreijse et al., 2011).

In addition, increasing dose resulted in increasing amount of viral shedding. Our result was consistent with previous report that the amount of virus shed from inoculated birds until successful contact infection also increased with dose (Spekreijse et al., 2011). Additionally, the number of inoculated bird shed virus from cloaca increased with dose (Middleton et al., 2007; Zarkov, 2012). In this study, virus titers in oropharyngeal and cloacal swabs obtained from inoculated and contact quail in group 3 ($10^{3.0}$ EID_{50}) were lower than group 1 ($10^{7.0}$ EID_{50}) and 2 ($10^{5.0}$ EID_{50}). Virus titers in oropharyngeal swabs obtained from inoculated quail in group 3 were statistically significant lower than group 1 and 2 at 1 and 2 dpi. Virus titers in cloacal swabs obtained from inoculated quail in group 3 were statistically significant lower than group 1 and 2 at 2 dpi. Virus titers in oropharyngeal swabs obtained from contact quail in group 3 were statistically significant lower than group 1 and 2 at 1 and 2 dpc. Virus titers in cloacal swabs obtained from contact quail in group 3 were statistically significant lower than group 1 at 1 and 2 dpc.

Moreover, virus titers in oropharyngeal and cloacal swabs obtained from inoculated and contact quail in group 1 ($10^{7.0}$ EID_{50}) were higher than group 2 ($10^{5.0}$

EID_{50}) and 3 ($10^{3.0}$ EID_{50}). As the results, $10^{3.0}$ EID_{50} of CUK2 virus per quail may be too low and $10^{7.0}$ EID_{50} of CUK2 virus per quail may be too high. Moreover,$10^{3.0}$ and $10^{7.0}$ EID_{50} of HPAI-H5N1 virus per quail may be too low and too high compared with inoculation dose used in other studies (Perkins and Swayne, 2001; Jeong et al., 2009; Saito et al., 2009) then $10^{5.0}$ EID_{50} of HPAI-H5N1 virus per quail was selected for the study of the efficacy of inactivated avian influenza vaccine in Japanese quail.

In conclusion, even quail which inoculated with $10^{3.0}$, $10^{5.0}$ and $10^{7.0}$ EID_{50} had the same mortality, similar histological lesions and virus distribution in tissues, but contact quail in group 3 which inoculated with $10^{3.0}$ EID_{50} had longer MDT compared with other groups. Moreover, viral shedding from quail in group 3 was lower than other groups. On the other hand, viral shedding from quail in group 1 which inoculated with $10^{7.0}$ EID_{50} was higher than other groups. Based on the results from this study, death time after contact and virus titer in oropharyngeal and cloacal swabs obtained from inoculated and contact quail suggested that 10^{5} EID_{50} of HPAI-H5N1 virus per quail should be selected and used for animal challenge in vaccination experiment.

CHAPTER III

Serological response of Japanese quail to inactivated avian influenza (H5N2) vaccine

3.1 Introduction

Highly pathogenic avian influenza (HPAI) subtype H5N1 (HPAI-H5N1) was first reported in China, in 1996 (Xu et al., 1999). Since then, HPAI-H5N1 is an important emerging disease of animals and humans. To decrease the risk in human, controlling HPAI-H5N1 in poultry is necessary to prevent opportunity for virus to infect human. Moreover, maintaining poultry free from HPAI is essential for international poultry trading. The goals of dealing with HPAI are prevention, control and eradication viral infection in poultry. To achieve these goals, the control strategies including biosecurity, education, surveillance and diagnosis and elimination of infected and adjacent flocks should be implemented. However, stamping-out program may not be effective especially in developing countries due to socioeconomic problems.

Thus, vaccination may be an alternate program for controlling or even eradicating HPAI. Vaccination can reduce viral shedding both duration of viral shedding and the amount of virus. In addition, inactivated avian influenza vaccination may increase host resistance to infection with infectious HPAI. Thus, infectious cycle may be intervened since less viruses are shed from vaccinated birds and at the same time vaccinated birds are more resistance to infection. However, one limitation of inactivated vaccine is that the vaccine does not prevent infection and the virus can still replicate and transmit at low level (Ebrahimi et al., 2011). Moreover, infected vaccinated birds can shed virus without showing any clinical signs (Capua and Marangon, 2007). Based on OIE's recommendation, the avian influenza vaccine should be used as a part of eradication and prevention program in co-operate with elimination of infected birds, quarantine, and strict biosecurity (OIE, 2009).

Up to date, avian influenza vaccines have been used for three vaccination strategies (Bruschke et al., 2007; Capua and Alexander, 2008). The first strategy is

systematic vaccination or the routine use of vaccine. This strategy is recommended to apply in HPAI-H5N1 endemic country. The second strategy is preventive vaccination which should be employed in HPAI-H5N1 free country when AI viruses are serious treat and other prevention and control strategies may not succeed. The third strategy is emergency vaccination which should be employed when outbreaks have occurred and used with other control and eradication strategies.

In 1995, inactivated H5N2 vaccine has been used in HPAI outbreak for the first time during HPAI-H5N2 outbreak in Mexico and the virus had been eradicated (Garcia et al., 1998). Following the widespread outbreaks of HPAI-H5N1, inactivated H5N2 vaccines have been used in Hong Kong in 2002, Indonesia in 2003, China in 2004, Vietnam and Russia in 2005, India, Pakistan and Egypt in 2006 (Capua and Alexander, 2008; Swayne, 2009). Although vaccination has had success in some countries such as Hong Kong (Ellis et al., 2006; Capua and Alexander, 2008), outbreaks still occurred in some countries.

Protection of avian influenza vaccines is based on neutralizing antibody, which response to hemagglutinin protein (HA). Vaccines provide protection against homologous HA viruses but not heterologous HA viruses. While, antibodies to NA reduce the amount of virus released from infected cell but do not prevent infection (Sylte and Suarez, 2009). Up to date, there are two types of avian influenza vaccines which are licensed and used in poultry. First is the inactivated whole avian influenza virus vaccine and second is recombinant fowl pox virus vector vaccine with HA gene insertion. The inactivated vaccine can provide protection in multiple poultry species, while the recombinant fowl pox vaccine can only be used in chicken (OIE, 2009).

In previous reports, inactivated avian influenza vaccines could protect several poultry species including chicken, duck and goose from HPAI-H5N1 viruses (Swayne et al., 2006; Rudolf et al., 2009; Pfeiffer et al., 2010). In chicken and goose, vaccines could prevent not only morbidity and mortality, but also reduce viral shedding through respiratory and gastrointestinal tracts. Moreover vaccines also reduce the duration of

viral shedding. In duck, HPAI-H5N1 typically does not cause disease or death but the vaccine can reduce replication of the virus. In addition, vaccine increases the infectious dose needed to infect vaccinated turkey (Capua et al., 2004). However, the information of inactivated avian influenza vaccination in Japanese quail (*Coturnix coturnix japonica*) which is highly susceptible to HPAI-H5N1 viruses is still limited. Extrapolating results from other species should be concerned because effect on the excretion of virus and influence on the transmission may vary from specie to specie even in the same family (Tian et al., 2005).

The vaccine used in this study is Nobilis® Influenza H5, an inactivated avian influenza Type A H5N2 virus (A/Chicken/Mexico/232-CPA/94) water-in-oil emulsion vaccine. The H5 hemagglutinin protein is used to induce protective immunity against HPAI-H5N1 viruses. The difference neuraminidase, N2 is used to allow distinction between natural infected and vaccinated birds by the DIVA (Differentiating Infected from Vaccinated Animals) strategy. The DIVA strategy is based on the use of an inactivated oil emulsion vaccine containing the same hemagglutinin (HA) as the field virus but different neuraminidase (NA) (Capua et al., 2003). Antibodies to the other NA except NA of vaccine suggest that natural infections occur.

In this study, serological response of Japanese quail to inactivated avian influenza vaccine was monitored by HI test before and after vaccination. If the vaccine induces high level of neutralizing antibody similar to other poultry species, vaccination could be used as an alternative prevention and control program of HPAI-H5N1 in Japanese quail.

3.2 Materials and methods

3.2.1 Animals

A total of 60 3-week-old Japanese quails (*Coturnix coturnix japonica*) were acquired from a commercial Japanese quail farm with a history of non-vaccinated parent stock. Oropharyngeal and cloacal swabs as well as blood samples were collected and tested to ensure that the quail were naive to influenza virus by egg inoculation and hemagglutination inhibition test (HI). Japanese quail were wing-banded for individual identification and provided feed and water ad libitum. The quail were housed in the biosafety cabinet under biosafety level 3 conditions. In this study, animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Veterinary Science, Mahidol University (Approval number MUVS-2011-35).

3.2.2 Vaccine

The inactivated avian influenza vaccine in the study was a commercial vaccine (Nobilis® Influenza H5N2, Intervet International, Boxmeer, the Netherlands) which prepared from influenza A virus (A/Chicken/Mexico/232/94 (H5N2)). Vaccination was permitted for experimental purpose by FDA Thailand. The vaccine titer was 1:40 HA unit /0.5 ml. The lot number of vaccine was B382A01 and the expiration date was 05/2011 (figure 3.1). The vaccine was kindly provided by Dr. Thaweesak Songserm (Faculty of Veterinary Medicine, Kasetsart University, Thailand). The administration route of vaccine was subcutaneous injection for an individual quail.

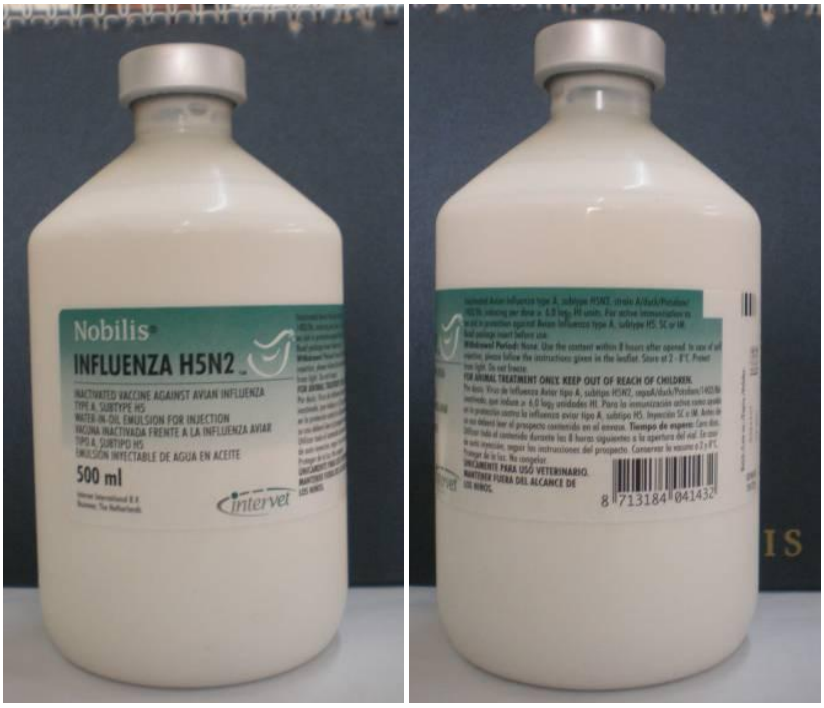


Figure 3.1 The inactivated avian influenza vaccine, Nobilis® Influenza H5N2

3.2.3 Serological test

Hemagglutination inhibition assay (HI) was performed following the OIE standard (OIE, 2009). Briefly, all sera were pretreated with chicken red blood cells. 10 µl of packed chicken RBCs was added in 200 µl of serum then pelleted and treated serum was used. Chicken RBCs were washed and resuspended to the final concentration of 1 % (v/v) in PBS. H5 virus/antigen was adjusted to 4 hemmaglutination units (HAU) per 25 µl in PBS. 25 µl of treated sera were serially diluted two-fold with PBS in plastic V-bottom microtitre plates. Serially diluted sera were incubated with 4 HAU of viral antigen for 30 minutes at room temperature. After incubation, 25 µl of 1% chicken RBCs were added. Plates were incubated at room temperature for 40 minutes. HI positive wells were defined by the button of unagglutinated chicken RBCs at the bottom of the wells. HI negative wells had diffuse sheet of agglutinated chicken RBCs covering the bottom. The HI titer was the highest dilution that agglutination was not observed.

3.2.4 Serological response to vaccination

Sixty quails were used for the analysis of serological response to inactivated avian influenza (H5N2) vaccine. Japanese quail were divided into 3 groups (figure 3.2). Vaccination groups were done in duplicate (vaccinated group 1 and 2). Japanese quail in vaccinated group (n=20 each) were vaccinated subcutaneous with commercial vaccine (H5N2) which prepared at 1:40 HA unit per 0.5 ml. The dosage was 0.25 ml at 3-week-old and 0.5 ml at 7-week-old. Japanese quail in unvaccinated group, group 3 (n=20), were vaccinated with a placebo injection of normal saline. Blood samples were collected at 3, 7, 10 and 13-week-old from each quail. Hemagglutination-inhibition (HI) test was performed to determine the serum-antibody titers. The experiment was terminated 6 weeks after second vaccination. Quail were euthanized by intramuscular administration of Zolazepam/Tiletamine. Euthanized quail were necropsied. Oropharyngeal and cloacal swabs were collected.

3.2.5 Statistical analysis

HI titers were analyzed for statistical significant differences ($p<0.05$). HI titers were compared between groups by analysis of variance (ANOVA) with LSD and between days by paired t-test. The statistical significant differences ($p<0.05$) in the number of positive samples were evaluated by Fisher's exact test. The data was analyzed by SAS 9.2 software package (SAS Institute Inc., North Carolina, USA).

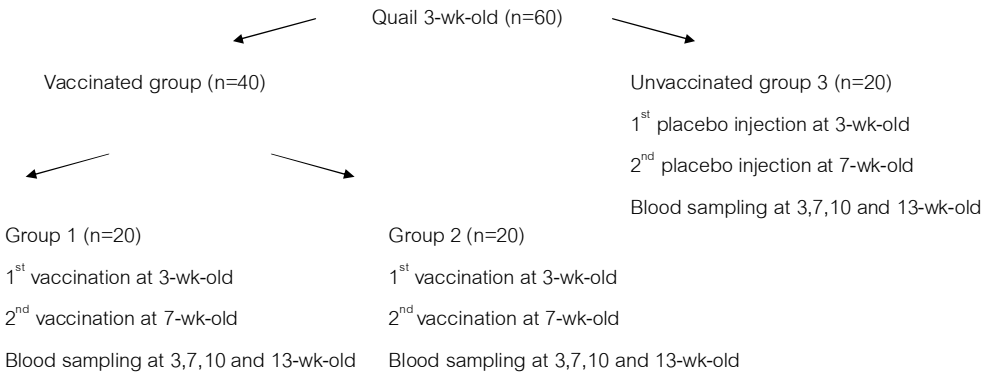


Figure 3.2 Schematic of experimental design

3.3 Results

Serological response of Japanese quail to inactivated avian influenza (H5N2) vaccine

In this study, virus was not detected in oropharyngeal swabs and cloacal swabs from quail at 3-week-old. In addition, no antibody titer was detected in blood samples of 3-week-old quail. In unvaccinated group (group 3), swabs and blood samples of quail at 7, 10 and 13 weeks showed no viral antigen and antibody titer. This result confirmed that experimental quail were naive to influenza virus. In vaccinated group 1 and 2, Japanese quail, showed normal appetite and appeared active after both vaccinations. In unvaccinated group, Japanese quail were also normal after placebo injection of normal saline. No clinical signs or mortality were observed in quail in vaccinated group 1 and 2 after vaccination and quail in unvaccinated group after placebo injection until the end of experiment. No remarkable lesion was found in all quail at the end of experiment.

To evaluate serological response to inactivated avian influenza (H5N2) vaccine, serum samples were evaluated for antibody against H5 avian influenza virus with HI test. In this study, no antibody titer was detected in vaccinated group 1, 2 and unvaccinated group (group 3) at 3-week-old. HI antibody was detected at 4 weeks post first vaccination (7-week-old). HI titers were 0.75 ± 0.34 (5/20) and 0.15 ± 0.11 (2/20) in vaccinated group 1 and 2, respectively. HI titer increased to 4.7 ± 0.49 (18/20) and 3.95 ± 0.53 (18/20) at 3 week post second vaccination (10-week-old) and declined to 4.3 ± 0.53 (18/20) and 3.1+0.57 (16/20) at 6 week post second vaccination (13-week-old) (table 3.1 and figure 3.3). These results showed scatter serological response to inactivated avian influenza (H5N2) vaccine. No antibody titer was detected in unvaccinated group until the end of experiment.

At 4 week post first vaccination (7-week-old), the HI titer of the vaccinated group 1 was statistically significant higher than vaccinated group 2 and unvaccinated group (group 3). At 3 and 6 week post second vaccination (10 and 13-week-old), the HI titer

and the number of HI positive samples of the vaccinated group 1 and 2 were statistically significant higher than unvaccinated group. HI titers and the number of HI positive samples were statistically significant increased after first and second vaccination in vaccinated group 1. HI titer and the number of HI positive samples increased after first vaccination and statistically significant increased after second vaccination in vaccinated group 2 (table 3.1).

Table 3.1 Mean HI titer (log_2) and standard error mean and number of HI positive samples

Group	HI titer (log_2)†			
	3 wk	7 wk	10 wk	13 wk
Vaccinated	0 *	0.75±0.34 ^{A} **	4.7±0.49 ^{A}***	4.3±0.53 ^{A}
group 1	(0/20††) *	(5/20) **	(18/20) ^{a}***	(18/20) ^{a}
Vaccinated	0	0.15±0.11 ^{B} *	3.95±0.53 ^{A}**	3.1±0.57 ^{A}
group 2	(0/20)	(2/20) *	(18/20) ^{a}**	(16/20) ^{a}
Unvaccinated	0	0 ^{B}	0 ^{B}	0 ^{B}
group 3	(0/20)	(0/20)	(0/20) ^{b}	(0/20) ^{b}

†Mean HI titer ± S.E.M expressed as log_2

††Number of HI positive samples

Different alphabet in the same column and different number of star in the same row mean significant at 95 % confidence interval

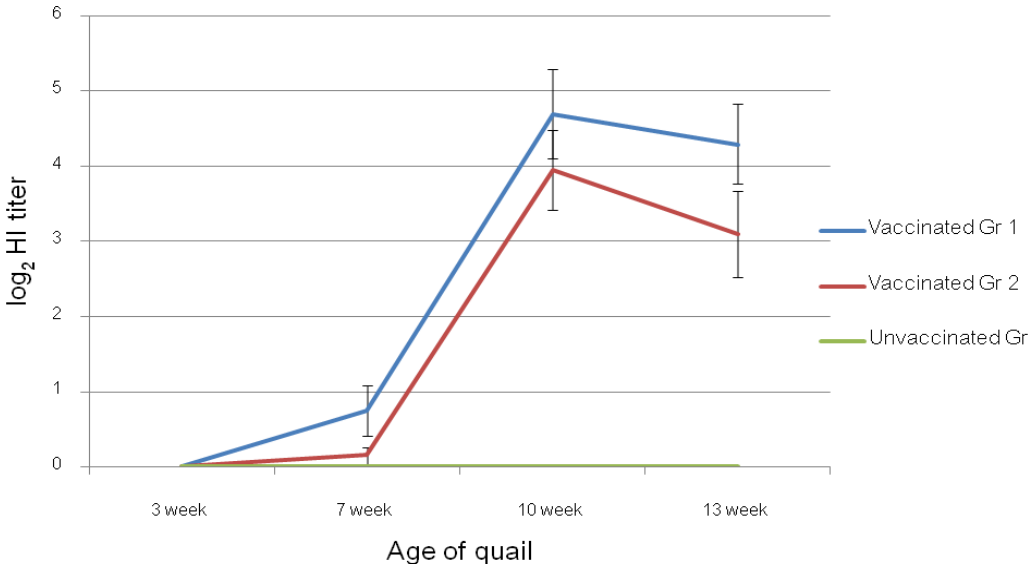


Figure 3.3 Mean HI titer (log_2) in vaccinated and unvaccinated quail

3.4 Discussion

The result from this study indicated that two administrations of a half and a full dose of inactivated H5N2 virus (A/Chicken/Mexico/232-CPA/94) water-in-oil emulsion vaccine induced serological response but may not resulted in protective immunity. No adverse reactions related to vaccine were found. This result indicated that the inactivated avian influenza vaccine was safe to administer to Japanese quail.

In this study, HI tests have been used to detect subtype specific antibodies against influenza virus. The HI test can also be used to quantitate serum antibody. The antibody level measured by HI test correlate with protection against clinical disease and can be used to evaluate vaccine efficacy (Tian et al., 2005). At 4 week post first vaccination (7-week-old), H5 specific antibody could be detected at low level in only 25 % and 10 % of Japanese quail in vaccinated group 1 and 2, respectively. This finding indicated that a half dose of inactivated avian influenza (H5N2) vaccine could not provide adequate protection for Japanese quail against HPAI-H5N1 virus, if 4 log 2 is considered protective titer of Japanese quail the same as chicken (Tian et al., 2005).

In vaccinated group 1, a half dose of inactivated avian influenza (H5N2) vaccine induced significantly increase HI antibody against H5 influenza virus and the number of HI positive samples at 4 week post vaccination. On the other hand, in vaccinated group 2 inactivated avian influenza vaccine induced non-significantly increase HI titer and the number of HI positive samples. The difference between vaccinated groups can be explained by natural variations among quail. Natural variation in immune response can be found even in SPF birds (Kumar et al., 2007). Moreover, poor serological responses were also found in Japanese quail infected with influenza virus (Lavoie et al., 2007).

At 3 week post second vaccination (10-week-old), the HI titers of the vaccinated group 1 and 2 were statistically significant higher than unvaccinated group (group 3). In addition, the HI titers were higher than 4 log 2 or equal which may protect Japanese quail against morbidity and mortality. This result indicated that booster vaccination with

a full dose of inactivated avian influenza vaccine induced significantly increase HI antibody.

At 6 week post second vaccination (13-week-old), the HI titers of the vaccinated group 1 and 2 were still statistically significant higher than unvaccinated group. The HI titers of both vaccinated groups decreased which HI titer of vaccinated group 2 was lower than 4 log 2 which may not protect Japanese quail from the disease. Our result was similar to previous report that HI titers decreased at 5 weeks after vaccination (Saad et al., 2010). This observation indicated that inactivated avian influenza vaccine did not provide long term immunity in Japanese quail.

Japanese quail in other studies showed higher HI antibody response to avian influenza vaccination than our results. These may be accounted by different age, type and dose of vaccine (Saad et al., 2010). There were age related differences in immune responses in Japanese quail. The HI antibody titers after secondary infection with influenza virus in pubescent quail were lower than juvenile quail (Lavoie et al., 2007). Different antigen and adjuvant in vaccine can also affect serological response to influenza vaccine (Swayne et al., 2006; Webster et al., 2006). Serological response to inactivated avian influenza vaccine also correlated with amount of antigen in vaccine (Maas et al., 2009; Sasaki et al., 2009). Reduced vaccine dose decreased HI antibody response and increased viral shedding in chicken (Goetz et al., 2008).

Comparing with serological response of chicken to inactivated avian influenza vaccines, Japanese quail developed HI antibody lower than chicken and also declined faster. According to previous report, significant differences in serological response to inactivated avian influenza vaccine have been found among species of birds even in the same order (Tian et al., 2005). In addition, serological response of Japanese quail to inactivated Newcastle disease vaccines was lower and declined faster than chicken (Stone et al., 1981; Paullio et al., 2009).

Upon booster vaccination with a full dose of inactivated avian influenza vaccine, HI antibody titers in both vaccinated groups significantly increased and were higher than unvaccinated group at 3 week post second vaccination (10-week-old). However, two administration of a half and a full dose of inactivated avian influenza vaccine could induce protective HI antibody titer in only 70 % and 55 % of Japanese quail in vaccinated group 1 and 2, respectively. Moreover, at 6 week post second vaccination (13-week-old), the HI antibody titer declined. This finding implied that Japanese quail vaccinated twice with a half and a full dose of inactivated avian influenza (H5N2) vaccine developed unsatisfactorily high HI antibodies with individual variability. According to previous report, the serological response depended on the antigen content in the vaccine. Antibody titer increased when antigen content in the vaccine increased (Maas et al., 2009). The administration of a full dose vaccine at first vaccination may give a better sero-conversion and higher antibody response.

Our study revealed that few of vaccinated Japanese quail sero-converted after the first vaccination with a half dose of inactivated avian influenza vaccine. Moreover, sero-converted quail had low antibody response. The result suggested that a half dose of inactivated avian influenza vaccine might not protect Japanese quail from HPAI-H5N1 virus and not recommend.

On the other hand, most of vaccinated Japanese quail sero-converted at 4 weeks after the second vaccination with a full dose of vaccine. In addition, antibody titers in vaccinated quail were statistically significant higher than those in unvaccinated quail and may reach protective level. Thus, twice vaccination with a half and a full dose of vaccine may protect Japanese quail from HPAI-H5N1 virus. Interestingly, antibody titers declined at 6 weeks after the second vaccination. Our observation suggested that inactivated avian influenza vaccine may not provide long term protection in Japanese quail.

As the result of this study, inactivated avian influenza vaccine should not be used for systemic or preventive vaccination but may be used for emergency vaccination

in Japanese quail especially for Thailand which no evidence of HPAI-H5N1 outbreak since 2008. Emergency vaccination may be used to assist in the control of HPAI-H5N1 virus before eradication by stamping out of animals in the infected and neighboring farms. Our results also provided useful information of vaccination in Japanese quail for the country used prophylactic vaccination in evaluation the use of vaccines.

CHAPTER IV

The efficacy of inactivated avian influenza (H5N2) vaccine on the protection and transmission of HPAI-H5N1 virus in vaccinated Japanese quail

4.1 Introduction

HPAI-H5N1 subtype causes severe diseases in several bird species as well as human. Infected poultry has been reported of primary source of infection. Controlling avian influenza at the source should be concerned. From the first outbreak of HPAI-H5N1 virus in Thailand in 2004, routine surveillance, movement restriction and culling of infected and adjacent flocks have been implemented as control strategies. After 2008, there is no evidence of HPAI-H5N1 outbreak in Thailand (Suwannakarn et al., 2009).

Since the viruses can re-emerge in the country by several factors such as animal movement by trading, wild bird migration (Sims et al., 2005; Keawcharoen et al., 2011). For example, in 2008, HPAI-H5N1 viruses were isolated from Japanese quail in live-bird markets in Thailand (Amonsin et al., 2008). HPAI-H5N1 outbreaks have been reported in quail in several countries, including Vietnam and Indonesia (FAO, 2012).

Japanese quail are included in five major species of poultry produced in Thailand (Chantong and Kaneene, 2011). Normally, they are raised in small farms with low biosecurity. Thus, Japanese quail farms pose a high risk for influenza infection. Japanese quail are highly susceptible to HPAI-H5N1 virus. 100 % mortality has been reported in Japanese quail inoculated with HPAI-H5N1 (Perkins and Swayne, 2001). Moreover, Japanese quail shed high amount of virus for longer periods than chicken, then Japanese quail may play an important role in HPAI-H5N1 transmission (Jeong et al., 2009).

From previous reports, Intermediate hosts are believed to play a role in reassortment between human and avian viruses before they could be transmitted among human (Webster et al., 1997). Suitable intermediate hosts have to be easily infected by influenza viruses from various origins including both mammalian and avian viruses. It has been known that Japanese quail pose sialic acid α2,3-galactose (SA

α2,3-gal) and α2,6-galactose (SA α2,6-gal) linked receptors in the trachea and intestine which prefer to bind avian and mammalian viruses, respectively. Thus, Japanese quail provide a suitable environment for the reassortment of avian and mammalian influenza viruses (Makarova et al., 2003). Japanese quail may play an important role in influenza infection as an intermediate host for the reassortment of influenza viruses and generating the variant viruses (Perez et al., 2003b). Thus, protection of Japanese quail from influenza virus infection is important.

Disease control by depopulation of infected flocks and preemptive culling of neighboring farms alone may restrict due to socio-economic limitation. Vaccination may be one of the preferred options in some countries (Marangon et al., 2008). In previous reports, inactivated avian influenza vaccine can protect chicken, duck and goose against morbidity and mortality and also reduce the spread of virus (Swayne, 2006). However, the information of inactivated avian influenza vaccination in Japanese quail is still limited. Then the questions arose whether Japanese quail could be protected by vaccination or could transmit the virus into the population.

In this study, virus used for animal challenge was HPAI subtype H5N1 "A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1) (CUK2)". From our result in chapter 2, CUK2 virus caused 100 % mortality in inoculated Japanese quail. All contact Japanese quail became ill and died after contacting with inoculated Japanese quail. An inactivated avian influenza (H5N2) vaccine was used because the H5 hemagglutinin protein in vaccine can induce protective immunity against HPAI-H5N1 virus. From our result in chapter 3, two administration of a half and a full dose of inactivated avian influenza vaccine could induce significantly increased HI antibody titer. It is noted that 4 log 2 is considered protective titer of chicken (Tian et al., 2005). From the result, we could not conclude the protective titer of the vaccine in Japanese quail. Extrapolating results from other species should be concerned.

In this study, vaccine efficacy study was performed by mingling inoculated vaccinated Japanese quail with contact vaccinated Japanese quail and investigating the mortality rate, viral shedding and immune response. Viral shedding was

investigated by real time RT-PCR. Real-time RT-PCR technique offers an alternative method for both qualitative and quantitative analysis. The method was shown to have sensitivity and specificity equivalent to virus isolation (Spackman et al., 2002). Viral infection was confirmed by immunohistochemical staining. HI tests have been used to detect subtype specific antibodies against influenza virus. From previous report, antibody may be detected as early as 7 days after infection (Swayne and Halvorson, 2003).

The aims of this study were to determine the efficacy of inactivated avian influenza (H5N2) vaccine on protection and transmission of HPAI-H5N1 virus in Japanese quail. If the vaccine protect and reduce viral shedding in Japanese quail similar to other poultry species, vaccination could be used as an alternative prevention and control program of HPAI-H5N1 in Japanese quail. Our results provide useful information about protection by vaccination in Japanese quail for country where the use of prophylactic vaccination in evaluation of vaccine uses because there are limited studies in Japanese quail. Even country which poultry vaccination against HPAI is prohibited but virus still re-emerge, the use of vaccine may be re-evaluated then emergency vaccination may be used to assist in the control of HPAI-H5N1 virus before eradication by stamping out of animals in the infected and neighboring farms.

4.2 Materials and methods

4.2.1 Animals

A total of 48 3-week-old Japanese quails (*Coturnix coturnix japonica*) were acquired from a commercial Japanese quail farm with a history of non-vaccinated parent stock. Oropharyngeal and cloacal swabs as well as blood samples were collected and tested to ensure that the quail were naive to influenza virus by egg inoculation and hemagglutination inhibition test (HI). Japanese quail were wing-banded for individual identification and provided feed and water ad libitum. The quail were housed in the biosafety cabinet under biosafety level 3 conditions. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Faculty of Veterinary Science, Mahidol University (Approval number MUVS-2011-35).

4.2.2 Virus

The HPAI-H5N1 virus, A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1), was used in the study. The virus was isolated from the index chicken case of the 2004 HPAI outbreak in Thailand. Stock virus was propagated in 10-day-old embryonated chicken eggs. The eggs were incubated at 37 $^{\circ}$C for 4 days. Following egg death, the allantoic fluid was harvested. The haemagglutination assay (HA) and real time RT-PCR were performed to confirm influenza virus infection. Virus titer was determined by inoculation of serial 10-fold dilutions of pooled allantoic fluid in embryonated chicken eggs. The mortality of eggs was recorded and 50 percent Embryo Lethal Dose (ELD_{50}) was calculated following Reed and Muench protocol (Reed and Muench, 1938). Allantoic fluid from eggs with dead embryo and all eggs remaining at the end of incubation period were tested with HA and 50 percent Embryo Infectious Dose was calculated (EID_{50}) using the method of Reed and Muench. All work with the HPAI-H5N1 virus was performed in biosafety level 2 containment facilities. Stock virus had a viral

titer of $10^{8.5}$ EID_{50}/ml. The virus was kept at -80 ^{o}C until use. Stock viruses were diluted with PBS to $10^{5}EID_{50}$ /0.1 ml before inoculation.

4.2.3 Vaccine

The inactivated avian influenza vaccine in the study was a commercial vaccine (Nobilis® Influenza H5N2, Intervet International, Boxmeer, the Netherlands) which prepared from influenza A virus (A/Chicken/Mexico/232/94 (H5N2)). Vaccination was permitted for experimental purpose by FDA Thailand. The vaccine titer was 1:40 HA unit /0.5 ml. The lot number of vaccine was B382A01 and the expiration date was 05/2011. The vaccine was kindly provided by Dr. Thaweesak Songserm (Faculty of Veterinary Medicine, Kasetsart University, Thailand). The administration route of vaccine was subcutaneous injection for an individual quail.

4.2.4 Serological test

Hemagglutination inhibition assay (HI) was performed following the OIE standard (OIE, 2009). Briefly, all sera were pretreated with chicken red blood cells. 10 µl of packed chicken RBCs was added in 200 µl of serum then pelleted and treated serum was used. Chicken RBCs were washed and resuspended to the final concentration of 1 % (v/v) in PBS. H5 virus/antigen was adjusted to 4 hemmaglutination units (HAU) per 25 µl in PBS. 25 µl of treated sera were serially diluted two-fold with PBS in plastic V-bottom micro-titer plates. Serially diluted sera were incubated with 4 HAU of viral antigen for 30 minutes at room temperature. After incubation, 25 µl of 1% chicken RBCs were added. Plates were incubated at room temperature for 40 minutes. HI positive wells were defined by the button of unagglutinated chicken RBCs at the bottom of the wells. HI negative wells had diffuse sheet of agglutinated chicken RBCs covering the bottom. The HI titer was the highest dilution that agglutination was not observed.

4.2.5 Real-time RT-PCR specific for the influenza A virus matrix (M) gene

RNA was extracted with Viral NA Extraction Kit (Beckman Coulter®, California, USA). To identify and titrate influenza A virus, real-time RT-PCR specific for the influenza A virus matrix (M) gene was conducted (Spackman et al., 2002). Briefly, the cocktail, composed of forward/reverse primers 0.4 µM per reaction, probe 0.1 µM per reaction, tag 0.3 µl per reaction, master mix 7.5 µl ($MgSo_4$ 4 mM) per reaction (SuperScript™ III Platinum® One-Step Quantitative RT-PCR System, Invitrogen™, California, USA) and distilled water, was made for all the reactions (table 2.1). 11 µl of the cocktail and 4 µl of RNA were added to 0.2 ml. tube. One step real-time RT-PCR was performed on Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Cycling conditions included a reverse transcription step at 50°C for 30 minutes. After an initial denaturation step at 95°C for 15 minutes, amplification was performed for 50 cycles including denaturation (95°C for 15 seconds), annealing (60°C for 30 seconds). Multiple fluorescent signals were obtained once per cycle at the end of the annealing step with detectors to FAM channel. Data acquisition and analysis of the real-time RT-PCR assay were performed using the Rotor-Gene Version 6.0.19 software (Corbett Research, Sydney, Australia). The standard curve and threshold were computed from three different concentration standard reactions and negative template control (NTC). The viral titers were calculated from a standard curve.

4.2.6 Histopathology and immunohistochemical staining

For histopathology and immunohistochemical staining, tissues were fixed by 10 % buffered formalin and embedded in paraffin. Duplicated sections were cut at 5 µm. The first section was stained with hematoxylin and eosin (H&E). The second section was immunohistochemical stained for the detection of influenza virus antigen in the tissues. Immunohistochemical staining was performed as previously reported (Thontiravong et al., 2012). Briefly, section was deparaffinized 3 times with xylene, 5 minutes each and rehydrated by 50-50 mix of xylene and alcohol, absolute alcohol, 95 % alcohol, 80% alcohol, 75 % alcohol, 2 minutes each, distilled water and phosphate buffered saline

(PBS), 5 minutes each. Endogenous peroxidase was blocked with 0.3 % H_2O_2 for 30 minutes at room temperature. 0.05 % Proteinase K was used to retrieve the epitope for 10 minutes at 37 ^{o}C then wash 3 times with PBS, 5 minutes each. Unspecific binding of antibody was blocked with 1 % bovine serum albumin for 45 minutes at 37 ^{o}C then wash 3 times with PBS. The primary antibody (mouse anti-Influenza A virus (NP) monoclonal antibody clone EVS 238, 1:300 dilution, BV European Veterinary Laboratory, The Netherlands) was applied for 12 hours at 4 ^{o}C then wash 3 times with PBS. The primary antibody was detected by chain polymer kit (Dako REAL^{TM} envision–HRP system, anti-rabbit/mouse, Glostrup, Denmark) for 45 minutes at 37 ^{o}C then wash 3 times with PBS and followed by 3,3' diaminobenzidine tetrahydrochlorid (DAB) substrate (Sigma-Aldrich, USA) (DAB 0.075 g + Tris buffer 150 ml + 30% H_2O_2 50 µl). Section was counterstained with H&E and dehydrated with 75 % alcohol, 80% alcohol, 95 % alcohol, absolute alcohol, 50-50 mix of xylene and and xylene.

4.2.7 The efficacy of inactivated avian influenza (H5N2) vaccine on the protection and transmission of HPAI-H5N1 virus in vaccinated Japanese quail

Forty-eight 3-week-old Japanese quails were used for the analysis of efficacy of vaccine on protective efficacy and transmission of avian influenza virus. Japanese quail were divided into 3 groups (figure 4.1). Vaccinated groups were done in duplicate (vaccinated group 1 and 2). Japanese quail in vaccinated group 1 and 2 (n=16 each) were vaccinated subcutaneous. The dosage was 0.25 ml at 3-week-old and 0.5 ml at 7-week-old. Japanese quail in unvaccinated group, group 3 (n=16) were vaccinated with a placebo injection of normal saline.

At 10-week-old, 8 Japanese quails in each group (inoculated quail) were inoculated both intranasal and intraoral with 0.1 ml (0.05 ml for each route) of diluted allantoic fluid containing of $10^{5}EID_{50}$ of HPAI-H5N1 virus. Twenty four hours later, 8 Japanese quails in each group (contact quail) were added (van der Goot et al., 2005). After inoculation, quail were kept and observed for 3 weeks. All quail were monitored on

a daily basis for clinical sign and mortality. Dead quail were necropsied and tissues were collected in 10% buffered formalin for histopathologic evaluation and immunohistochemical staining. Immunohistochemical staining was used to confirm viral infection. Oropharyngeal and cloacal swabs were collected to confirm viral shedding.

The Japanese quail were monitored for virus shedding by sampling oropharyngeal and cloacal swabs daily for the first 10 days and 14, 16 and 21 day post inoculation. Swab was placed into tube containing 2 ml of viral transport media (VTM). Determination of virus titers was performed by real-time RT-PCR. The experiment was terminated at 3 weeks after the challenge. Blood samples were collected from all remaining quail for serological examination. The serological response was studied by HI test. Quail were euthanized by intramuscular administration of Zolazepam/Tiletamine. Euthanized quail were necropsied. Tissue samples, oropharyngeal and cloacal swabs were collected.

4.2.8 Statistical analysis

Viral titers and HI titers were analyzed for statistical significant differences ($p<0.05$). Viral titers and HI titers were compared between groups by ANOVA with LSD and between days by paired t-test. Viral titer from oropharyngeal and cloacal swab were compare by paired t-test. The statistical significant differences ($p<0.05$) in survival rate, numbers of quail shedding virus between groups and days were evaluated by Fisher's exact test. The data was analyzed by SAS 9.2 software package (SAS Institute Inc., North Carolina, USA).

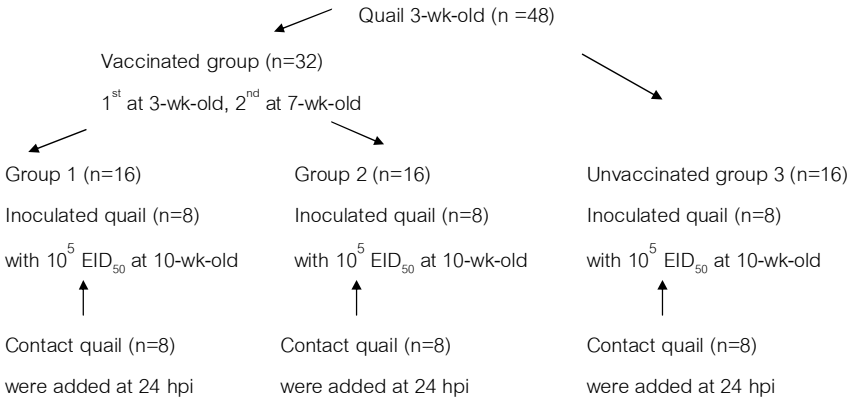


Figure 4.1 Schematic of experimental design

4.3 Results

4.3.1 The efficacy of inactivated avian influenza (H5N2) vaccine on the protection from mortality

For clinical observation, diarrhea was observed in 12 % (2/16) of quail in vaccinated group 1 and 6 % (1/16) of quail in vaccinated group 2 and unvaccinated group (group 3) between 2-6 dpi. Twenty five percent (25 %) of inoculated quail in vaccinated group 1 and 2, 38 % (3/8) of contact quail in vaccinated group 1 displayed neurologic sign such as tremors and paralysis between 4-12 dpi. All inoculated quail in unvaccinated group died between 2-3 dpi. One half of the inoculated quail in vaccinated group 1 died between 3-7 dpi. One half of the inoculated quail in vaccinated group 2 died between 5-10 dpi (figure 4.2). The mean death time (MDT) was displayed in table 4.1.

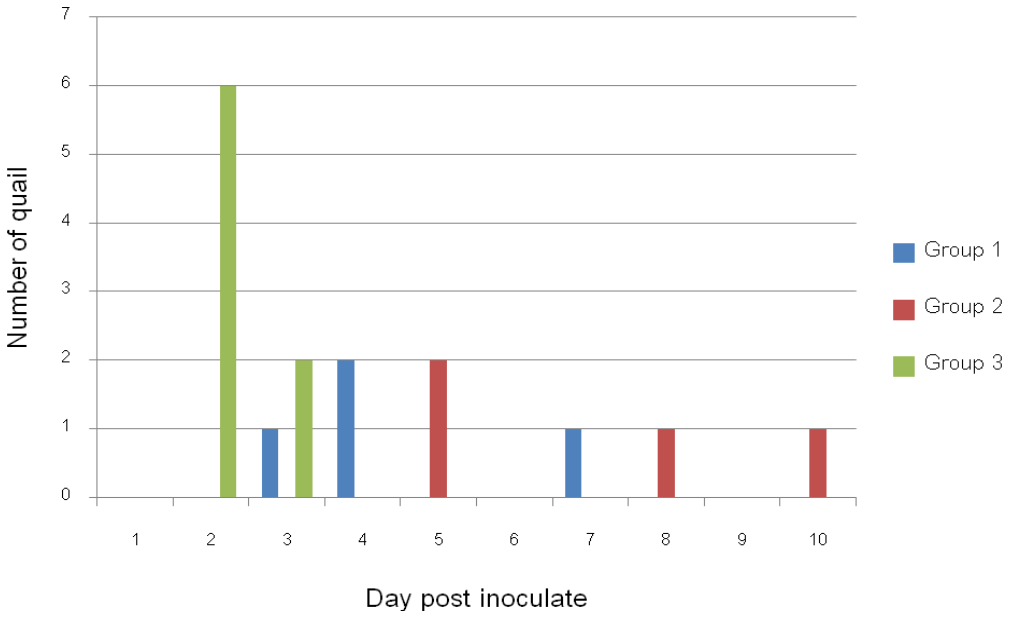


Figure 4.2 Number of inoculate quail that died after inoculation

In contact groups, all contact quail in unvaccinated group died between 2-4 dpc. Seven of 8 contact quail in vaccinated group 1 died between 5-11 dpc. Only 1 quail in vaccinated group 2 died at 18 dpc from other causes (figure 4.3). The mean death time (MDT) was displayed in table 4.1.

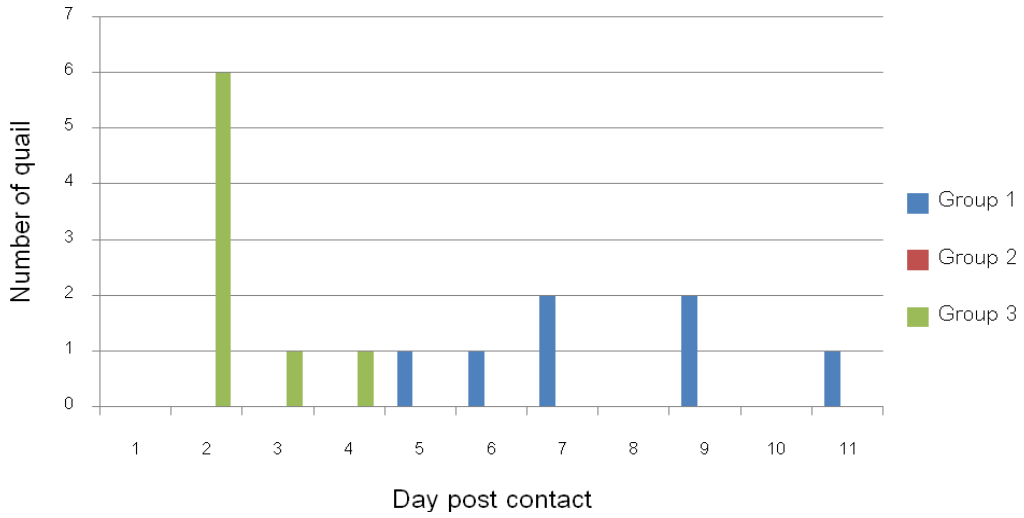


Figure 4.3 Number of contact quail that died after contact

Table 4.1 Mortality and mean death time of inoculated and contact quail

Group	Inoculated quail		Contact quail	
	Mortality (%)	MDT (dpi)	Mortality (%)	MDT (dpc)
1	50 (4/8)	4.5	87.5 (7/8)	7.71
2	50 (4/8)	7	0*	Alive*
3	100 (8/8)	2.25	100 (8/8)	2.38

*1 quail died at 18 dpc from other causes

4.3.2 The efficacy of inactivated avian influenza (H5N2) vaccine on the viral shedding

The viral titers in the oropharyngeal and cloacal swabs were determined by real-time RT-PCR to study transmission of viruses. In unvaccinated group (group3), inoculated quail shed virus via oropharynx from 1 dpi until death and via cloaca from 2 dpi until death. The number of quail that shed virus in oropharyngeal and cloacal swabs were presented in table 4.2 and 4.3, respectively. The result showed that the number of inoculated quail that shed virus from oropharynx was statistically significant higher than the number of quail that shed virus from cloaca at 1 dpi. The number of inoculated quail that shed virus from cloaca significantly increased at 2 dpi. Mean viral titers in oropharyngeal swabs were presented in table 4.2 and figure 4.4. Mean viral titers in cloacal swabs were presented in table 4.3 and figure 4.5. The result showed that virus titers in oropharyngeal swabs were statistically significant higher than virus titers in cloacal swabs at 1 and 2 dpi. Virus titers in oropharyngeal and cloacal swabs significantly increased at 2 dpi and tended to increase until death.

Contact quail shed virus via oropharynx from 1 dpc until death and via cloaca from 2 dpc until death. The number of quail that shed virus in oropharyngeal and cloacal swabs were presented in table 4.4 and 4.5, respectively. The result showed that the number of contact quail that shed virus from oropharynx was statistically significant higher than the number of quail that shed virus from cloaca at 1 dpc. The number of contact birds that shed virus from cloaca significantly increase at 2 dpc. Mean viral titers in oropharyngeal swabs were presented in table 4.4 and figure 4.6. Mean viral titers in cloacal swabs were presented in table 4.5 and figure 4.7. The result showed that the virus titers in oropharyngeal swabs were statistically significant higher than virus titers in cloacal swabs at 1 dpc. Virus titers in oropharyngeal and cloacal swabs significantly increased at 2 dpc and tended to increase until death.

In vaccinated group 1, inoculated quail shed virus via oropharynx from 1 dpi until death and quail that survived shed virus until 9 dpi and shed virus via cloaca from 2 dpi until death and quail that survived shed virus only at 2 dpi. The number of quail that shed virus in oropharyngeal and cloacal swabs were presented in table 4.2 and 4.3,

respectively. The result showed that the number of inoculated quail that shed virus from oropharynx was statistically significant higher than the number of quail that shed virus from cloaca at 1, 4 and 5 dpi. Mean viral titers in oropharyngeal swabs were presented in table 4.2 and figure 4.4. Mean viral titers in cloacal swabs were presented in table 4.3 and figure 4.5. The result showed that virus titers in oropharyngeal swabs were statistically significant higher than virus titers in cloacal swabs at 1-5 dpi.

Contact quail shed virus via oropharynx from 3 dpc until death and quail that survived may shed virus until 8 dpc and shed virus via cloaca from 4 dpc until death and quail that survived shed virus until 8 dpc. The number of quail that shed virus in oropharyngeal and cloacal swabs were presented in table 4.4 and 4.5, respectively. The result showed that the number of contact quail that shed virus from oropharynx significantly increased at 3 dpc. The number of contact quail that shed virus from cloaca significantly increased at 4 dpc. The number of contact quail that shed virus from oropharynx was statistically significant higher than the number of quail that shed virus from cloaca at 3 and 7 dpc. Mean viral titers in oropharyngeal swabs were presented in table 4.4 and figure 4.6. Mean viral titers in cloacal swabs were presented in table 4.5 and figure 4.7. The result showed that virus titers in oropharyngeal swabs significantly increased at 3 and 4 dpc and tended to increase until death. Virus titers in cloacal swabs significantly increased at 4 dpc. Virus titers in oropharyngeal swabs were statistically significant higher than virus titers in cloacal swabs at 3-7 dpc.

In vaccinated group 2, inoculated quail shed virus via oropharynx from 1 dpi until death and quail that survived shed virus until 9 dpi and shed virus via cloaca only at day 9 post inoculation. The number of quail that shed virus in oropharyngeal and cloacal swabs were presented in table 4.2 and 4.3, respectively. The result showed that the number of inoculated quail that shed virus from oropharynx was statistically significant higher than the number of quail that shed virus from cloaca at 2-5 dpi. Mean viral titers in oropharyngeal swabs were presented in table 4.2 and figure 4.4. Mean viral titers in cloacal swabs were presented in table 4.3 and figure 4.5. The result showed that

virus titers in oropharyngeal swabs from inoculated birds were also statistically significant higher than virus titers in cloacal swabs at 2-5 dpi.

Contact quail shed virus via oropharynx from 4 dpc and may shed virus until 13 dpc and shed virus via cloaca only at day 8 post contact. The number of quail that shed virus in oropharyngeal and cloacal swabs were presented in table 4.4 and 4.5, respectively. Mean viral titers in oropharyngeal swabs were presented in table 4.4 and figure 4.6. Mean viral titers in cloacal swabs were presented in table 4.5 and figure 4.7.

Table 4.2 Mean viral titer (log_{10} EID_{50}) and standard error mean of oropharyngeal swabs from inoculated quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Group	Virus titer (log_{10} EID_{50}/ml)†								
	dpi								
	1	2	3	4	5	6	7	8	9
1	5.3 ±0.4 (5/8)††	5.6 ±0.4 (6/8)	5.1 ±0.5 (7/8)	5.2 ±0.6 (6/7)	3.1 ±1.0 (5/5)	4.3 ±0.8 (2/5)	0 (0/5)	0 (0/4)	3.6 (1/4)
2	2.8 ±0.8 (3/8)	4.1 A ±0.8 (5/8)	3.0 ±0.6 (7/8)	4.3 ±0.5 (5/8)	5.2 ±0.2 (5/8)	4.7 ±0.4 (3/6)	4.0 ±0.8 (3/6)	2.4 ±1.8 (2/6)	3.3 ±1.2 (3/5)
3	4.1 * ±0.6 (7/8)	5.8 B** ±0.5 (8/8)	6.4 ±0.8 (2/2)						

†Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

††Number of positive quail/total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

Table 4.3 Mean viral titer (log_{10} EID_{50}) and standard error mean of cloacal swabs from inoculated quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Group	Virus titer (log_{10} EID_{50}/ml)[†]								
	dpi								
	1	2	3	4	5	6	7	8	9
1	0	2.7 [A] ±0.9	5.6 ±0.1	7.0	0	0	0	0	0
	(0/8)[††]	(2/8) [a]	(3/8)	(1/7)	(0/5)	(0/5)	(0/5)	(0/4)	(0/4)
2	0	0 [A]	0	0	0	0	0	0	3.0 ±0.9
	(0/8)	(0/8)[a]	(0/8) [a]	(0/8)	(0/8)	(0/6)	(0/6)	(0/6)	(2/5)
3	0 *	5.3 [B]** ±0.3	6.0 ±0.2						
	(0/8) *	(7/8) [b]**	(2/2) [b]						

[†]Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

[††]Number of positive quail/total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

Figure 4.4 Mean viral titer (log_{10} EID_{50}) and standard error mean of oropharyngeal swabs from inoculated quail by real time RT-PCR

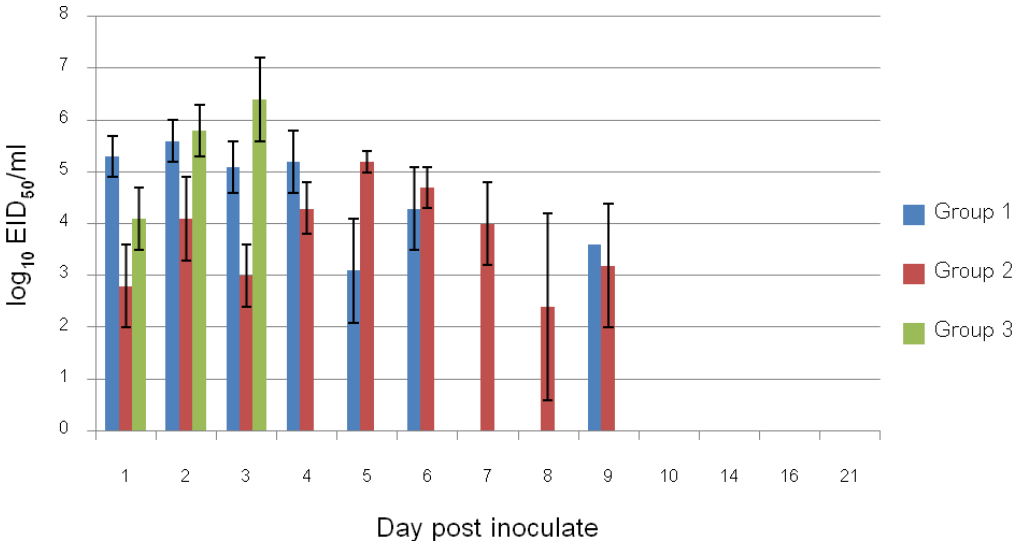


Figure 4.5 Mean viral titer (log_{10} EID_{50}) and standard error mean of cloacal swabs from inoculated quail by real time RT-PCR

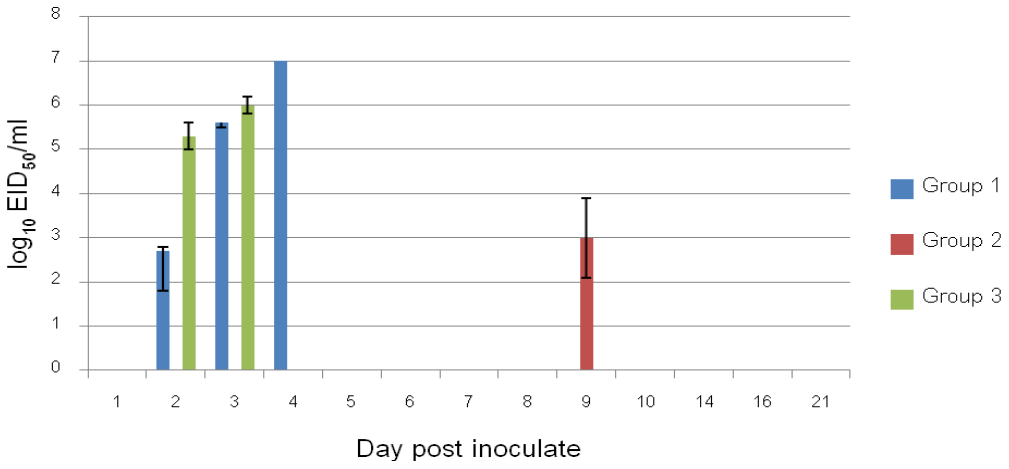


Table 4.4 Mean viral titer (log_{10} EID_{50}) and standard error mean of oropharyngeal swabs from contact quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Gr	Virus titer (log_{10} EID_{50}/ml)†									
	dpc									
	1	2	3	4	5	6	7	8	9	13
1	0 ^{A}	0 ^{A}*	4.0 ^{A}**	4.5 ^{A}***	4.9 ^{A}	5.2 ^{A}	5.2 ^{A}	4.3 ^{A}	5.3	0
			±0.9	±0.6	±0.3	±0.4	±0.5	±1.5	±0.5	
	(0/8)††^{a}	(0/8) ^{a}*	(5/8) ^{a}**	(8/8) ^{a}	(8/8) ^{a}	(7/7) ^{a}	(6/6) ^{a}	(4/4)	(3/4)	(0/1)
2	0 ^{A}	0 ^{A}	0 ^{B}	3.7 ^{B}	1.9 ^{B}	5.4 ^{B}	5.3 ^{B}	2.7 ^{B}	3.8	3.5
					±1.2			±1.3	±1.0	±0.5
	(0/8) ^{a}	(0/8) ^{a}	(0/8) ^{b}	(1/8) ^{b}	(3/8) ^{b}	(1/8) ^{b}	(1/8) ^{b}	(3/8)	(3/8)	(2/8)
3	3.8 ^{B}*	5.5 ^{B}**	6.4	5.6						
	±0.5	±0.1	±0.8							
	(6/8) ^{b}	(8/8) ^{b}	(2/2) ^{a}	(1/1)						

†Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

††Number of positive quail/total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

Table 4.5 Mean viral titer (log_{10} EID_{50}) and standard error mean of cloacal swabs from contact quail by real time RT-PCR and number of quail that shed virus per total quail in each group

Gr	Virus titer (log_{10} EID_{50}/ml)[†]									
	dpc									
	1	2	3	4	5	6	7	8	9	13
1	0	0 [A]	0 *	5.0 [A]** ±0.3	2.2 [A] ±0.5	2.8 ±1.1	6.8	1.8 [A] ±0.1	2.7	0
	(0/8) [††]	(0/8) [a]	(0/8) [a]*	(5/8) [a]**	(7/8) [a]	(3/7)	(1/6)	(4/4) [a]	(1/4)	(0/1)
2	0	0 [A]	0	0 [B]	0 [B]	0	0	0.3 [B]	0	0
	(0/8)	(0/8) [a]	(0/8) [a]	(0/8) [b]	(0/8) [b]	(0/8)	(0/8)	(1/8) [b]	(0/8)	(0/8)
3	0 *	6.0 [B]** ±0.5	6.4 ±0.5	6.8						
	(0/8) *	(7/8) [b]**	(2/2) [b]	(1/1)						

[†]Mean virus titer ± S.E.M expressed as log_{10} EID_{50}/ml calculated only from quail that shed virus

[††]Number of positive quail/total quail

Different alphabet in the same column and different number of star in the same row means significant at 95 % confidence interval

Figure 4.6 Mean viral titer (log_{10} EID_{50}) and standard error mean of oropharyngeal swabs from contact quail by real time RT-PCR

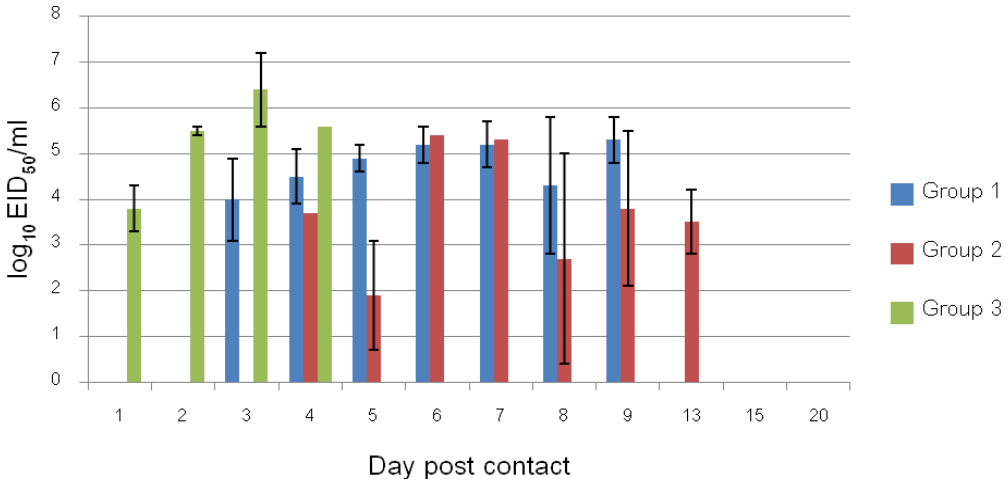
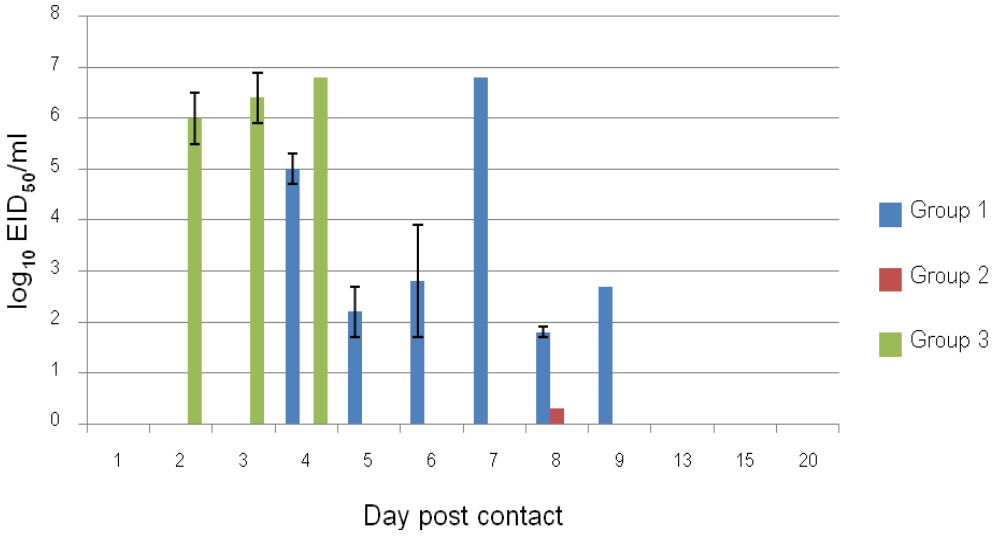


Figure 4.7 Mean viral titer (log_{10} EID_{50}) and standard error mean of cloacal swabs from contact quail by real time RT-PCR



In inoculated quail, table 4.2 and 4.3 showed the number of quail that shed virus and mean viral titers in oropharyngeal and cloacal swabs, respectively. The result showed that the number of inoculated quail in vaccinated group 1 and 2 that shed virus from cloaca was statistically significant lower than unvaccinated group (group 3) at 2 dpi. On the third day post inoculation, the number of inoculated quail in vaccinated group 2 that shed virus from cloaca was statistically significant lower than unvaccinated group. For viral titers, inoculated quail in vaccinated group 2 shed viruses via oropharynx statistically significant lower than unvaccinated group at 2 dpi. Both vaccinated groups shed statistically significant lower viruses via cloaca than unvaccinated group at 2 dpi.

In contact quail, table 4.4 and 4.5 showed the number of quail that shed virus and mean viral titers in oropharyngeal and cloacal swabs, respectively. The result showed that the number of contact quail in vaccinated group 1 and 2 that shed virus from oropharyx was statistically significant lower than unvaccinated group (group 3) at 1 and 2 dpc. On the third day post contact, the number of contact quail in vaccinated group 2 that shed virus from oropharynx was statistically significant lower than unvaccinated group. Between day 3 and day 7 post contact, the number of contact quail in vaccinated group 2 that shed virus from oropharynx was statistically significant lower than vaccinated group 1. In addition, the number of contact quail in vaccinated group 1 and 2 that shed virus from cloaca was statistically significant lower than unvaccinated group at 2 and 3 dpc. On day 4, 5 and 8 post contact, the number of contact quail in vaccinated group 2 that shed virus from cloaca was statistically significant lower than vaccinated group 1.

For viral titer, contact quail in vaccinated group 1 and group 2 shed statistically significant lower viruses via oropharynx than unvaccinated group (group 3) at 1 and 2 dpc. Between day 3 and day 8 post contact, contact quail in vaccinated group 2 shed statistically significant lower viruses via oropharynx than vaccinated group 1. In addition, contact quail in vaccinated group 1 and group 2 shed statistically significant

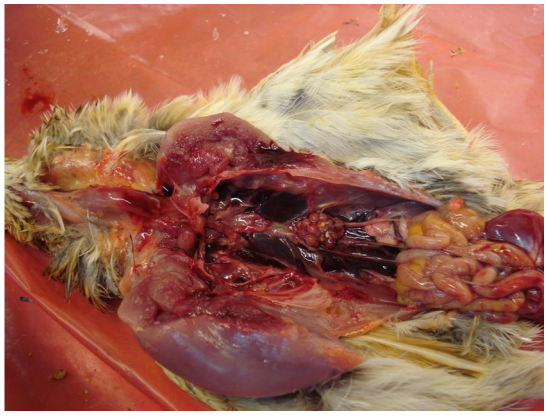
lower viruses via cloaca than unvaccinated group at 2 dpc. On day 4, 5 and 8 post contact, contact quail in vaccinated group 2 shed statistically significant lower viruses via cloaca than vaccinated group 1.

4.3.3 Gross lesions

At necropsy, in the unvaccinated group (group 3), the primary gross lesions in all inoculated (8/8) and contact quail (8/8) were lung edema, congestion, hemorrhage, focal pneumonia and severe acute diffuse pneumonia. The second most prominent lesions were found in pancreas (3/16), including edema, congestion, hemorrhage and multifocal necrosis (figure 4.8). In vaccinated group 1, the same gross lesions were found in inoculated quail that died and contact quail that died before 9 pdc. None of these remarkable lesions were found in inoculated quail that survived and contact quail that survived and died after 9 dpc. In vaccinated group 2, the same gross lesions were found in inoculated quail that died before 10 dpi. None of these remarkable lesions were found in inoculated quail that survived and died after 10 dpi and all contact quail.

(A)

(B)



(C)

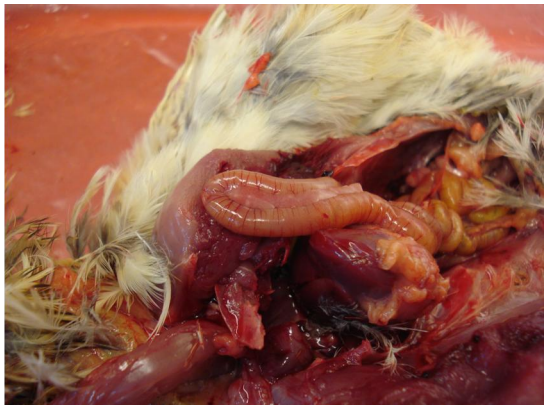


Figure 4.8 Japanese quail after inoculation (A) Japanese quail showed depression and ruffed feather at 1 dpi (B) Lung, severe congestion, pulmonary edema and pneumonia (C) Pancreas, multifocal necrosis

4.3.4 Histopathology

Histophathological examination by H&E staining of tissue sections reveals that lesions were found in tissues of both inoculated and contact quail in unvaccinated group (group 3). In respiratory tract and cardiovascular system, lesions that were found in trachea include detachment of the epithelium and subepithelial edema, infiltration of heterophils and lymphocytes in subepithelial layer, focal death of epithelial cells. The most important lesion was found in the lung. In the lungs, severe congestion, focal hemorrhage and pulmonary edema were noted especially in alveoli and around the vessel. 100% of lung from inoculated and contact quail in unvaccinated group showed these lesions. Acute inflammation with focal infiltration of heterophils and lymphocytes were also found. Lesions in the hearts were mild to moderate and sporadic including congestion, haemorrhage and lymphocyte infiltration.

Focal necrosis of pancreatic acinar cells without an inflammatory response was observed. Renal congestion was observed. Lymphoid depletion and necrosis in spleens were found. Focal necrosis, fatty infiltration and liver congestion occurred (figure 4.9). Lymphoid depletion and necrosis were observed in bursa. Desquamation of mucosal epithelium and enteritis were observed in duodenums. Desquamation of mucosal epithelium was also observed in cecum. Congestion, heterophil and lymphocyte infiltration and necrosis of oviducts were mild to moderate and sporadic. Haemorrhage, heterophil and lymphocyte infiltration and necrosis of ovary were mild to moderate and sporadic. Lesions in the brains were mild and sporadic including, non-suppurative encephalitis, gliosis in cerebrum, cerebellum liquefaction, vacuolation of brain stem and congestion.

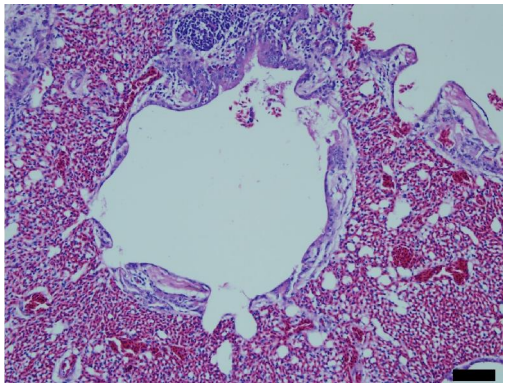
These lesions were observed more often in tissues from inoculated and contact quail in unvaccinated group (group 3) than vaccinated group 1 and 2 (table 4.6). Moreover, lesions were found in both survived and dead quail.

Table 4.6 Percentages of histologic lesion positive samples

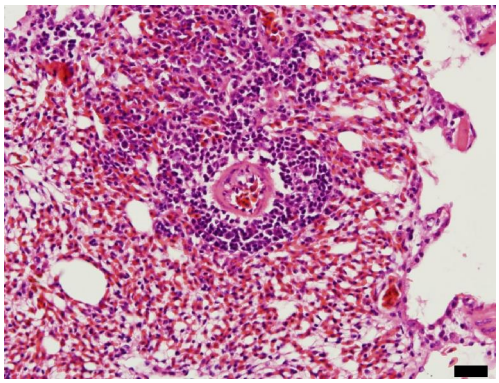
Organ	Histologic lesion positive samples (%)					
	Group 1 inoculated	Group 2 inoculated	Group 3 inoculated	Group 1 contact	Group 2 contact	Group 3 contact
Lung	87.5 (7/8)*	87.5 (7/8)	100 (8/8)	75 (6/8)	87.5 (7/8)	100 (8/8)
Intestine	25 (2/8)	0 (0/8)	37.5 (3/8)	12.5 (1/8)	50 (4/8)	62.5 (5/8)
Pancreas	25 (2/8)	0 (0/8)	75 (6/8)	0 (0/8)	0 (0/8)	25 (2/8)
Liver	0 (0/8)	12.5 (1/8)	62.5 (5/8)	0 (0/8)	0 (0/8)	37.5 (3/8)

* Number of positive quail / total quail

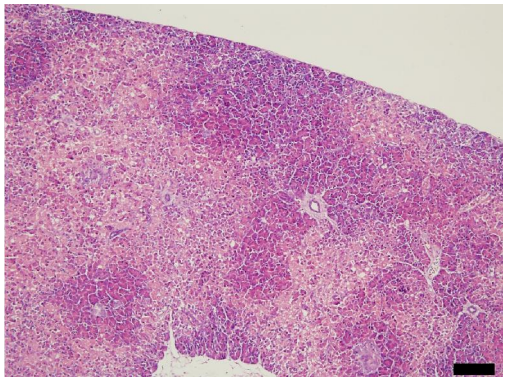
(A)



(B)



(C)



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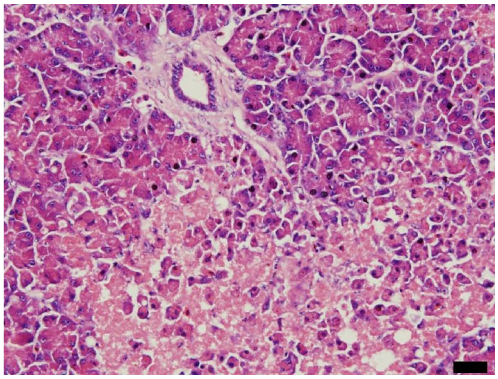

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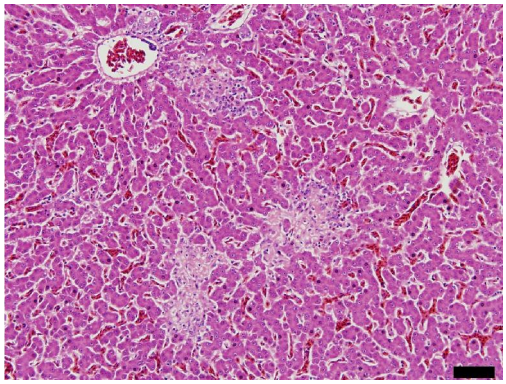


(F)

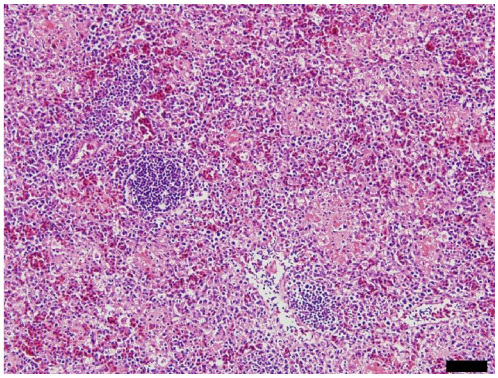


Figure 4.9 Hematoxylin and eosin stained tissue sections (A) Lung, severe congestion, pneumonia with infiltration of inflammatory cells (scale bar = 50 μm) (B) Lung, severe congestion with focal infiltration of lymphocytes around vessel (scale bar = 20 μm) (C) Pancreas, Large focal necrosis (scale bar = 100 μm) (D) Pancreas, focal necrosis of pancreatic acinar cells (scale bar = 20 μm) (E) Liver, focal necrosis of liver cells (scale bar = 50 μm) (F) Spleen, lymphoid depletion (scale bar = 50 μm)

4.3.5 Immunohistochemistry

Viral antigens could be found in tissues from all organ systems which were collected, including brain, heart, lung, liver, pancreas, kidney, proventriculus, gizzard, ovary, oviduct, adrenal gland, spleen. Viral particles were commonly observed in brain and pancreas. Viral antigens were observed in nucleus of neurons and Purkinje cells. No inflammatory response was observed in both cerebrum and cerebellum. In pancreas, viral particle was found in the acinar cells around necrotic foci. In the heart, viral particles were found in nucleus of muscle cells. In gizzard, viral particle was found in nucleus of muscle cells and also mucosal epithelial cells. In kidney, viral antigens were observed in few tubular cells and vascular endothelium in glomeruli. Moreover, viral antigens were also found in liver cells, ovary, spleen and lung (figure 4.10-4.11). It has been noted that only few tissues that positive for influenza viral staining have inflammatory response.

In inoculated quail, viral antigens were found in all quail in unvaccinated group (group 3). Viral antigens could be found in tissue from all organ systems. In vaccinated group 1, viral antigens were found in all dead inoculated quail. Viral antigens were not found in survived inoculated quail. Viral antigens were most prominent in the brain and found more often (3/8) than other tissues. In vaccinated group 2, viral antigens were only found in the brain of dead inoculated quail (3/8). Viral antigens were not found in inoculated quail that survived and died at 10 dpi.

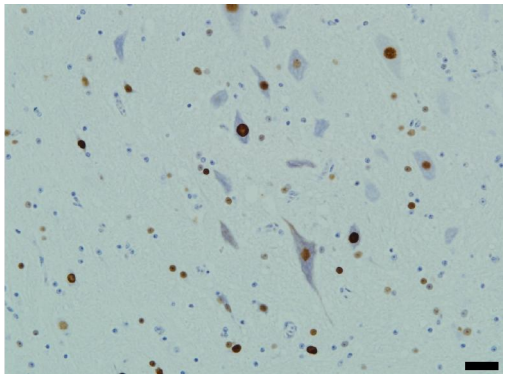
In contact quail, viral antigens were found in all quail in unvaccinated group (group 3). Viral antigens could be found in tissue from all organ systems. In vaccinated group 1, viral antigens were found in all contact quail which dead before 9 dpc. Viral antigens were not found in contact quail that survived and died at 9 and 11 dpc. Viral antigens were most prominent in the brain and found more often (3/8) than other tissues. In vaccinated group 2, there was no viral antigen staining in any tissues from contact quail (table 4.7).

Table 4.7 Mortality and number of immunohistochemical positive sample

Group	Mortality	Immunohistochemical positive sample (%)
1 inoculated	4/8	4/8 (50)
1 contact	7/8	5/8 (62.5)
2 inoculated	4/8	3/8 (37.5)
2 contact	0/8	0/8 (0)
3 inoculated	8/8	8/8 (100)
3 contact	8/8	6/6* (100)

* only 6 contact quail in unvaccinated group (group 3) were immunohistochemical staining

(A)



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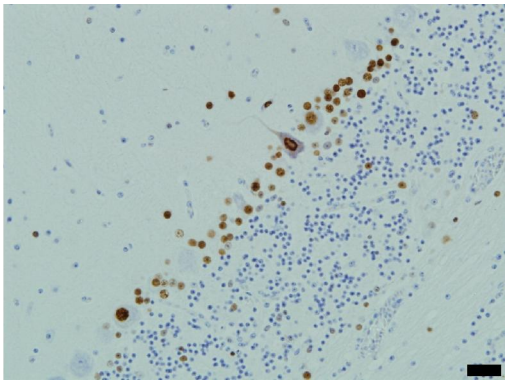

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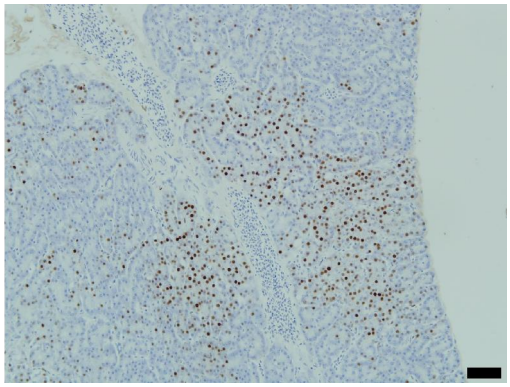

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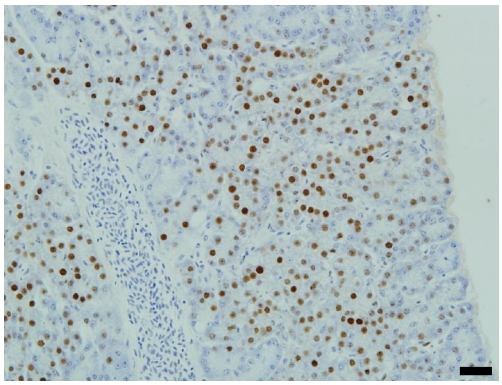

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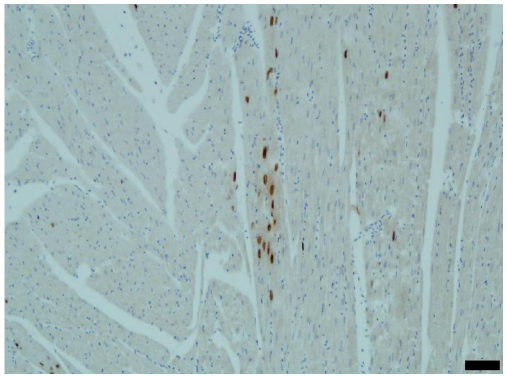

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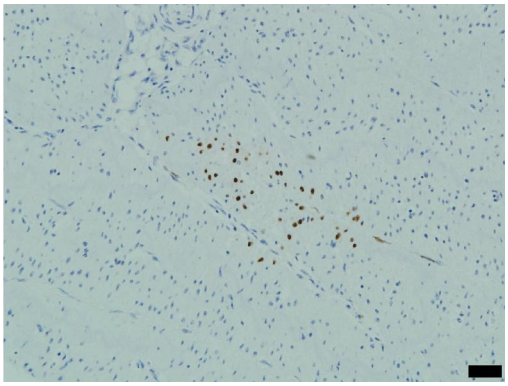
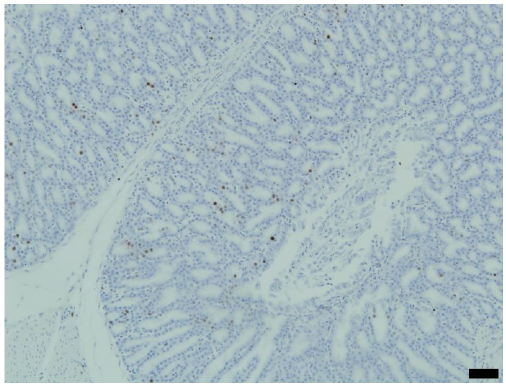


Figure 4.10 Immunohistochemical stained sections (A) Brain, brain stem, viral antigens in nucleus of neurons and glia cells (scale bar = 20 μm) (B) Brain, cerebellum, viral antigens in nucleus of Purkinje cells and glia cells (scale bar = 20 μm) (C) Pancreas, virus-infected cells around necrotic foci (scale bar = 50 μm) (D) Pancreas, viral particles in the acinar cells (scale bar = 20 μm) (E) Heart, viral particles in nucleus of muscle cells (scale bar = 20 μm) (F) Gizzard, viral particles in nucleus of muscle cells and also mucosal epithelial cells (scale bar = 20 μm)

(A)



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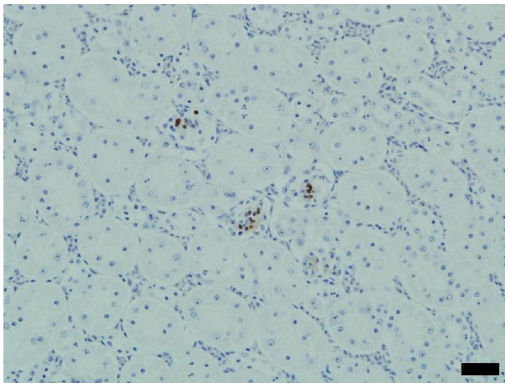

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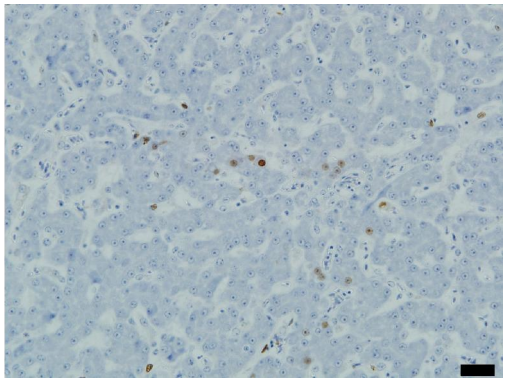

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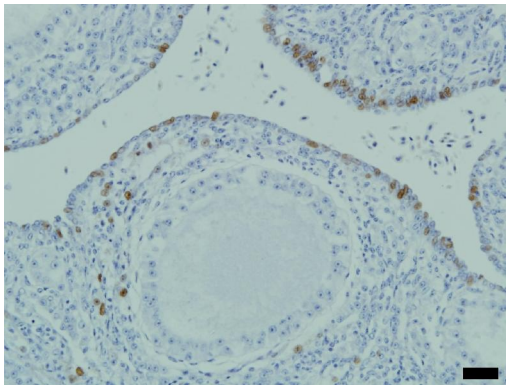

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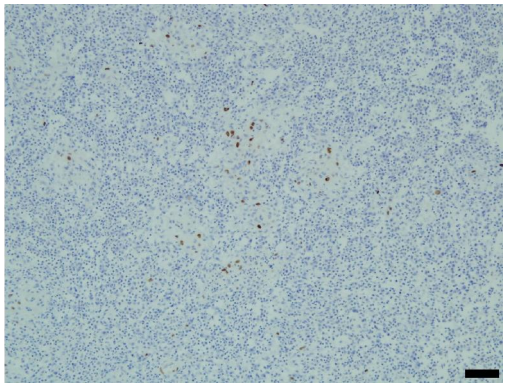

(F)

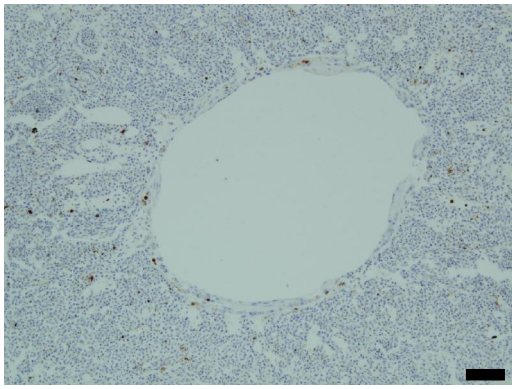

Figure 4.11 Immunohistochemical stained sections (A) Proventriculus, viral particles in the secretory cells (scale bar = 50 µm) (B) Kidney, viral particles in glomerular tuff (scale bar = 20 µm) (C) Liver, viral particles in liver cells (scale bar = 20 µm) (D) Ovary, viral particles in nucleus of infected cells (scale bar = 20 µm) (E) Spleen, viral particles in nucleus of infected cells (scale bar = 50 µm) (F) Lung, viral particles in nucleus of infected cells (scale bar = 50 µm)

4.3.6 Serology

Sero-conversion after inoculation was demonstrated by comparing antibody after inoculation with antibody after vaccination in chapter 3. In this study, all of the surviving quail in vaccinated group 1 and 2 showed sero-conversion after inoculation. HI titers were 6.5±0.3 (4/4) and 4.75±0.9 (4/4) at 3 week post inoculation in inoculated quail in vaccinated group 1 and 2, respectively. HI titer were 7 (1/1) and 4.7±1.1 (6/6) at 3 week post contact in contact quail in vaccinated group 1 and 2, respectively.

4.4 Discussion

In this study, Japanese quail were acquired from a parent flock farm with no vaccination practice. Japanese quail at 3-week-old were confirmed to be free of any H5 specific antibody. None of the oropharyngeal and cloacal swabs from the Japanese quail posed any influenza virus. This result demonstrated that these birds were negative from avian influenza virus.

Efficacy of inactivated H5N2 vaccine on protection of H5N1 virus was demonstrated by mortality rate. 10^5 EID_{50} of HPAI-H5N1 virus per quail caused 100% mortality in unvaccinated group (group 3) demonstrated that the virus were virulence and high enough to infect, cause death and transmit to other birds. Inoculated and contact quail in vaccinated group 1 and 2 survived at higher rate than those of unvaccinated group by which contact quail in vaccinated group 2 survived at statistically significant higher rate than those in unvaccinated group. MDT of inoculated and contact quail in vaccinated group were longer than unvaccinated group the same as chicken (Pfeiffer et al., 2010). MDT of inoculated quail in vaccinated group 2 was longer than vaccinated group 1. The different result between vaccinated group may due to variable serological response to vaccination (data from chapter 3). Variable response to vaccination may due to individual variability. In addition, histopathological lesions were seen more often in tissues from inoculated and contact quail in unvaccinated group than vaccinated group 1 and group 2. This result shows that vaccine can reduce viral severity in vaccinated Japanese quail.

Viral antigens were found in all inoculated and contact quail in unvaccinated group. In inoculated quail, in vaccinated group 1, viral antigens were not found in inoculated quail that survived. In vaccinated group 2, viral antigen was not found in inoculated quail that survived and died at 10 dpi. In contact quail, in vaccinated group 1, viral antigen was not found in contact quail that survived and died at 9 and 11 dpc. In vaccinated group 2, there was no viral antigen staining in any tissues from contact quail. These results demonstrate that immonohistochemistry can use for HPAI diagnosis in

unvaccinated Japanese quail but cannot be use in vaccinated Japanese quail that died late after infection or survived because these quail may infect and shed virus.

Efficacy of inactivated H5N2 vaccine on transmission of H5N1 virus was demonstrated by viral shedding. The inoculated quail in vaccinated groups were infected and shed virus later than unvaccinated group. The inoculated quail in vaccinated groups shed virus via oropharynx and cloaca less than unvaccinated group both for number of quail that shed virus and viral titers in the same period. The results were significantly lower in some days and the difference was more prominent in cloacal swabs for example in cloacal swabs at 2 dpi. Similarly, the contact quail in vaccinated groups were infected and shed virus later than unvaccinated group. The contact quail in vaccinated groups significantly shed virus via oropharynx and cloaca less than contact quail in unvaccinated group in some days, for example in oropharyngeal swabs at 1-2 dpc and in cloacal swabs at 2 dpc. In this study, no viral shedding and no antibody response were observed in some contacted quail in vaccinated group 2 similar to previous report in chicken and duck (Webster et al., 2006; Middleton et al., 2007). This result demonstrated that vaccination not only partial protected Japanese quail against morbidity and mortality but also reduced viral replication in respiratory and gastrointestinal tract and viral shedding (Ebrahimi et al., 2011).

In vaccinated group 1, the number of inoculated quail that shed virus from oropharynx and virus titers in oropharyngeal swabs was statistically significant higher than those from cloaca at 1 and 4 dpi. Similarly, the number of contact quail that shed virus from oropharynx and virus titers in oropharyngeal swabs was statistically significant higher than those from cloaca at 3 and 7 dpc. In vaccinated group 2, inoculated quail shed virus via cloaca only at day 9 post inoculation. Contact quail shed virus via cloaca only at day 8 post contact. The number of inoculated quail that shed virus from oropharynx and virus titers in oropharyngeal swabs was statistically significant higher than cloaca at 2-5 dpi.

Previous reports (Makarova et al., 2003; Jeong et al., 2009) and our report reveal that virus titers in oropharyngeal swabs were statistically significant higher than those in

cloacal swabs. Moreover, virus was isolated from cloacal swabs less frequently than oropharyngeal swabs. Thus transmission of H5N1 virus in Japanese quail via oral-oral route is more important than fecal-oral route especially in vaccinated quail. In addition, oropharyngeal swabs are better samples for HPAI-H5N1 surveillance in Japanese quail than cloacal swabs since the virus tend to shed earlier, more often, longer and higher in oropharynx than in cloaca (Makarova et al., 2003).

Interestingly, variable response to vaccination has been noted between vaccinated groups. Vaccinated group 2, the inoculated quail tend to infect and shed virus later than vaccinated group 1. In correlation, the contact quail in vaccinated group 2 tend to infect and shed virus later than vaccinated group 1. Moreover, the number of contact quail in vaccinated group 2 that shed virus from oropharynx and cloaca and viral titers in oropharyngeal and cloacal swabs were lower than vaccinated group 1. The results showed significantly lower in oropharyngeal swabs at 3-7 dpc and in cloacal swabs 4-5 dpc. The different results between vaccinated groups may due to variable response to vaccination (data from chapter 3). Variable response to vaccination may due to individual variability. Japanese quail used in this study were outbred animals and non SPF which had individual variation (Kumar et al., 2007; Middleton et al., 2007).

Contact quail in both vaccinated groups were infected and shed virus. This result suggested that vaccine was able to reduce mortality and viral shedding in Japanese quail but may not be able to stop the transmission of the virus and hence may not prevent avian influenza infection in quail, This result supported the similar observation in previous study (Ebrahimi et al., 2011). In this study, the amino acid sequence identity of HA protein between vaccine H5 virus (Hidalgo/232/94) and challenged H5 virus (Thailand/CUK2/04) was 84.4%. Some study reported that vaccine with <90% HA protein similarity may provide protection but not consistently reduce virus shedding from respiratory tract (Swayne et al., 2000). In addition, reduced vaccine dose could increase viral shedding in chicken (Goetz et al., 2008).

Sero-conversion after inoculation was confirmed in all of the surviving quail in vaccinated groups. However, this cannot be use as a marker of infection if pair serum

and virus isolation were not performed. The result showed that, some contact quail in vaccinated group 2 posed no virus titer and fail to virus isolation. These results suggested that there was no or low replication of HPAI-H5N1 virus in these quail which similar to previous reports in chicken and duck (Webster et al., 2006; Middleton et al., 2007). This result may be due to contact quail in vaccinated group 2 received lower number of virus than vaccinated group 1 from lower viral shedding from inoculated quail (Swayne et al., 2006). From previous report, inactivated avian influenza vaccination increases host resistance to infection with infectious virus (Capua, 2007). Then infectious cycle may be blocked because low amount of viral shed from vaccinated birds may not enough to infected vaccinated birds which are more resistance to infection. Based on this information, it is possible to formulate the ideal vaccine and vaccination program, which prevents disease, infection and transmission in Japanese quail. This can be done by optimized doses and vaccination time of commercial poultry vaccine in Japanese quail and select suitable types of antigen in vaccine, which will influent the vaccine effectiveness (Swayne, 2006; Webster et al., 2006).

In conclusion, inactivated H5N2 vaccine can not only protect Japanese quail from disease and mortality but also reduce viral shedding in the case of number of quail shedding virus and the virus titer shed via oropharynx and via cloaca.

CHAPTER V

Discussion and conclusion

In 1997, HPAI-H5N1 caused serious outbreaks in both poultry and human in Hong Kong (Claas et al., 1998). Since then, HPAI-H5N1 is an important emerging disease. Because HPAI-H5N1 virus causes serious public health problem, the goals of dealing with HPAI-H5N1 are prevention, control, eradication and monitoring. Several disease control and prevention strategies such as culling, movement control, disease surveillance, strict biosecurity and education are recommended. However, it may not be sufficient because poultry raising systems and biosecurity standards are different among countries (Rushton et al., 2006; Chantong and Kaneene, 2011). HPAI-H5N1 outbreaks are mainly found in backyard poultry and small farms with low biosecurity. Disease control by depopulation of infected flocks and preemptive culling of neighboring farms alone may restrict due to socio-economic limitation. Vaccination could be one of the preferred options.

In Thailand, there are 5 major species of poultry including chicken, duck, goose, quail and ostrich (Chantong and Kaneene, 2011). Japanese quail is focused in this study due to it is highly susceptible to HPAI-H5N1 viruses and shed high amount of virus for longer periods than chicken (Jeong et al., 2009). Moreover, Japanese quail may play a role in the host adaptation of virus (Perez et al., 2003a; Sorrell and Perez, 2007). In addition, Japanese quail provide a suitable environment for the reassortment of avian and mammalian influenza viruses (Makarova et al., 2003), then Japanese quail is important in influenza infection since it can be an intermediate host for the reassortment of influenza viruses and generating the variant viruses (Perez et al., 2003b).

Previous studies demonstrated that inactivated avian influenza vaccines can protect chicken, goose, and duck from HPAI-H5N1 viruses (Swayne et al., 2006; Rudolf et al., 2009; Pfeiffer et al., 2010). The inactivated vaccines prevented disease and mortality in chicken and goose and reduced the ability of the viruses to replicate in respiratory and gastrointestinal tracts. Vaccines can also reduced the ability of the

viruses to replicate in duck, even viruses may not cause disease and mortality (Swayne et al., 2006; Rudolf et al., 2009; Pfeiffer et al., 2010). Up to date, only few reports on the efficacy of inactivated avian influenza vaccines in Japanese quail are available in the literature database.

This dissertation contains three objectives 1) To determine the effect of inoculation doses of HPAI-H5N1 virus on the infection, pathogenicity, viral transmission and viral shedding in Japanese quail 2) To determine humeral antibody response to inactivated avian influenza (H5N2) vaccine in Japanese quail 3) To evaluate the efficacy of inactivated avian influenza (H5N2) vaccine on the protection and transmission of HPAI-H5N1 virus in vaccinated Japanese quail.

For the first objective, the effect of inoculation doses of HPAI-H5N1 virus isolated in Thailand on the susceptibility, pathogenicity and transmissibility in Japanese quail were evaluated. The result showed that, A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 was highly pathogenic to Japanese quail similar to other HPAI viruses isolated from Thailand (Saito et al., 2009), A/Chicken/Korea/IS/06 (Jeong et al., 2009) and A/Chicken/Hong Kong/220/97 (Perkins and Swayne, 2001). This recent study revealed that low dose of CUK2 virus ($10^{3.0}$ EID_{50} per quail) caused 100% mortality in inoculated Japanese quail. At the same dose, CUK2 virus also caused 100% mortality in contact Japanese quail. Histopathological lesions of infected Japanese quail were similar with those of other HPAI-H5N1 strains but lesions in pancreas were found more often (Perkins and Swayne, 2001). It is noted that viral antigen could not be found in tissues in all inoculated quail and contact quail within 36 hpi and hpc. Thus, immunohistochemical test may not a suitable test for HPAI-H5N1 diagnosis in quail in the earlier stage.

In this study, inoculated quail and contact quail started to shed virus via oropharynx and cloaca at 1 dpi and 1 dpc which earlier than previous report (Jeong et al., 2009). This result indicated that the incubation period of CUK2 virus in Japanese quail was short and virus able to spread and transmit rapidly, then CUK2 virus spread

rapidly in Japanese quail under experimental condition. Both inoculated quail and contact quail shed virus until death. In this study, the viral shedding via oropharynx was greater than via clocal, as demonstrated by higher number of isolation and also higher viral titers in oropharyngeal swabs than cloacal swabs. This result was in line with previous study (Makarova et al., 2003). Thus, transmission of HPAI-H5N1 virus in Japanese quail via oral-oral route is more important than fecal-oral route. From this result, it is suggested that the oropharyngeal swab sampling is more suitable for HPAI-H5N1 surveillance in Japanese quail than cloacal swab sampling.

This study showed that virus titers in oropharyngeal and cloacal swabs obtained from inoculated and contact quail inoculated with $10^{3.0}$ EID_{50} of CUK2 virus were lower than those of quail which inoculated with $10^{5.0}$ EID_{50} of virus (statistically significant in some days). Thus, $10^{3.0}$ EID_{50} of virus per quail may be too low for vaccine efficacy study. On the other hand, virus titers in oropharyngeal and cloacal swabs obtained from inoculated and contact quail which inoculated with $10^{7.0}$ EID_{50} of virus were higher than those of quail inoculated with $10^{5.0}$ EID_{50} of virus. Moreover,$10^{7.0}$ EID_{50} of virus per quail may be too high compared with inoculation dose used in other studies (Perkins and Swayne, 2001; Jeong et al., 2009; Saito et al., 2009). Thus, $10^{5.0}$ EID_{50} of HPAI-H5N1 virus per quail was selected for the study of the efficacy of inactivated avian influenza vaccine in Japanese quail.

Overall, the result from the study objective 1 demonstrated that the pathobiological features including incubation period, MDT and viral shedding corresponding to the differences of inoculation dosage. Increasing the dose resulted in decreasing the MDT. The negative correlation was observed between inoculate dose and MDT while the positive correlation was present between inoculation dose and viral shedding in Japanese quail. This finding was similar to previous finding in chicken and duck that mean latent period decreased significantly with increasing dose. In addition, increasing the dose increased amount of viral shedding. The result was consistent with previous report in chicken that the amount of virus shed from inoculated birds until

successful contact infection also increased with dose (Middleton et al., 2007; Spekreijse et al., 2011).

For the second objective, serological responses of Japanese quail to inactivated avian influenza (H5N2) vaccines were evaluated. Our study revealed that no adverse reactions have been observed. This result indicated that inactivated avian influenza (H5N2) vaccine was safe to administer in Japanese quail. After the first vaccination with half-dose vaccine (at 3-week-old), only few of vaccinated Japanese quail sero-converted. This result suggested that a half-dose of inactivated avian influenza vaccine could not provide adequate protection for Japanese quail against HPAI-H5N1 virus (if 4 log 2 is considered protective titer of Japanese quail the same as chicken). Our result was different from inactivated avian influenza vaccination in chicken, duck and goose (Tian et al., 2005). Thus, extrapolating protective titer from other avian species should be concerned. After the second vaccination with full-dose vaccine (at 7-week-old), most of vaccinated Japanese quail were sero-converted. This result suggested that booster vaccination with a full dose of inactivated avian influenza vaccine induced statistically significant increase HI antibody (4.7 and 3.95 $\log_2$ HI titer in vaccinated group 1 and 2, respectively) which may protect quail from HPAI-H5N1 virus (if the HI titers were higher than 4 log 2 or equal). In contrast to other poultry, HI titer in Japanese quail declined faster (after 6 week) (Tian et al., 2005). This observation suggested that inactivated avian influenza vaccine did not provide long term immunity in Japanese quail when comparing with other poultry. Thus, inactivated avian influenza vaccine may not be the good option for systemic or preventive vaccination but may be useful for emergency vaccination because vaccine induced significantly increase HI antibody but not long term immunity.

Japanese quail in other studies showed higher HI antibody response to avian influenza vaccination than our results. These may be account by different age, type and dose of vaccine (Saad et al., 2010). In a previous report, there were age related differences in immune responses in Japanese quail (Lavoie et al., 2007). Different

antigen and adjuvant in vaccine can affect serological response to influenza vaccine (Swayne et al., 2006; Webster et al., 2006). Serological response to inactivated avian influenza vaccine also correlated with amount of antigen in vaccine (Maas et al., 2009; Sasaki et al., 2009). According to previous report, the serological response depended on the antigen content in the vaccine. Antibody titer increased when antigen content in the vaccine increased (Maas et al., 2009). The administration of a full dose of vaccine at first vaccination may give a better sero-conversion and higher antibody response. Further studies should be done.

Overall, the result from the study objective 2 demonstrated that twice vaccination with a half dose at 3-week-old and a full dose at 7-week-old induced statistically significant increased HI antibody at 3 week after second vaccination which may protect quail from HPAI-H5N1 virus.

For the third objective, the efficacy of inactivated avian influenza vaccine on the protection and transmission of HPAI-H5N1 virus in Japanese quail were evaluated. The result showed that the inactivated avian influenza vaccine reduced disease and mortality in Japanese quail. According to previous report, inactivated avian influenza vaccination increased host resistance to infection with infectious virus (Capua et al., 2004). MDT of inoculated and contact quail in vaccinated group were longer than unvaccinated group. Histopathological lesions were observed more often in tissues from inoculated quail in unvaccinated group than vaccinated groups. However, the inactivated avian influenza vaccine did not prevent infection and the virus still replicated and transmitted (Ebrahimi et al., 2011). Moreover, the amount of virus shed from vaccinated Japanese quail was high enough to cause infection in vaccinated Japanese quail and also caused disease and mortality.

Viral antigens were found in all quail in unvaccinated group. In vaccinated group 1, viral antigen was not found in quail that survived and died late after infection. In vaccinated group 2, viral antigen was not found in inoculated quail of that survived and died late after infection. In addition, there was no viral antigen staining in any

tissues from contact quail. These results demonstrated that immonohistochemistry can use for HPAI-H5N1 diagnosis in unvaccinated Japanese quail but cannot be use in vaccinated Japanese quail that died late after infection or survived because these quail may infect and shed virus before test.

For the effect of inactivated avian influenza vaccine on the transmission of virus, the quail in vaccinated groups were infected and shed virus later than unvaccinated group. The quail in vaccinated groups shed virus via oropharynx and cloaca less than unvaccinated group both for number of quail that shed virus and viral titers in the same period. The result was more prominent in contact quail. This result demonstrated that vaccination not only protected Japanese quail against morbidity and mortality but also reduced viral replication in respiratory and gastrointestinal tracts and viral shedding the same as other poultry (Tian et al., 2005; Ebrahimi et al., 2011).

Viral shed from vaccinated quail via oropharynx was greater than via clocal, as demonstrated by higher number of isolation and also higher viral titers in oropharyngeal swabs than cloacal swabs. This result was in line with viral shedding from unvaccinated quail. Furthermore, some vaccinated quail shed virus only in oropharyngeal swabs. Thus, transmission of HPAI-H5N1 virus in vaccinated Japanese quail via oral-oral route is more important than fecal-oral route. Oropharyngeal swabs are better samples for HPAI-H5N1 surveillance in vaccinated Japanese quail than cloacal swabs.

The difference between vaccinated groups in serological response and protective efficacy can be cause by type of birds which were commercial not SPF. From previous reports, serological responses varied even in SPF birds (Kumar et al., 2007). Moreover, poor serological responses were also found in Japanese quail infected with influenza virus (Lavoie et al., 2007). These findings implied that Japanese quail vaccinated twice with a half and a full dose of inactivated avian influenza (H5N2) vaccine developed unsatisfactorily high HI antibodies with individual variability. There were correlation between serological response and protective efficacy from serological experiment and vaccination experiment. Then 4 log 2 may be the protective titer of

Japanese quail the same as chicken. Further study should be performed to confirm the level of protective immunity in Japanese quail.

From viral shedding result, some vaccinated quail shed virus without clinical sign. The vaccines should be used with caution. If vaccination was used as preventive strategies in quail, unvaccinated sero-negative quail should be used as sentinel birds to monitor the infection in vaccinated flock. Clinical disease or mortality of sentinel birds should be investigated daily and serological response should be investigated periodically to ensure that there was no viral circulation in the vaccinated flock (Marangon et al., 2008). On the other hand, no viral shedding was observed in some contacted quail in vaccinated group 2 similar to previous reports in chicken and duck (Webster et al., 2006; Middleton et al., 2007). This result demonstrated that vaccine prevents viral infection and shedding completely in some Japanese quail, then proper vaccination may stop viral replication and outbreak. The administration of a full dose of vaccine at first vaccination may give a better protection. Further studies should be done.

In summary, this study provides information about pathogenicity of Thai HPAI-H5N1 (CUK2) virus in Japanese quail. The viral transmission result will be useful in surveillance program in Japanese quail. This study also provides information about efficacy of inactivated avian influenza (H5N2) vaccine on humeral antibody response, the protection and transmission of HPAI-H5N1 virus in vaccinated Japanese quail. The result can be useful in evaluation the vaccination policy in Japanese quail.

In conclusion, our study demonstrated similar high pathogenic of CUK2 virus the same as other HPAI-H5N1 but difference in some pathobiological features such as histopathology and viral distribution in tissues. Thus, the pathogenicity of each strain of the viruses should be evaluated. From viral shedding result both in unvaccinated and vaccinated quail, we recommend oropharyngeal swab for surveillance program because virus can be detected in oropharyngeal swab more often and earlier than cloacal swab. Moreover, our study demonstrated that, the inoculation doses are correlated with MDT and viral shedding. After evaluation the effect of inoculation dose

and compare with other reports, we suggest $10^{5.0}$ EID_{50} of HPAI-H5N1 virus per quail which virulence to all inoculated and contact quail for vaccination study.

The serological result demonstrated that booster vaccination is necessary but not provide long term immunity in Japanese quail and HI titer can be use to predict protection from vaccine but the protective titer in Japanese quail should be evaluation. From the vaccine efficacy result, inactivated avian influenza (H5N2) vaccine in our vaccination program can partially protect and reduce viral shedding in Japanese quail. Thus, inactivated vaccine should not be used for systemic or preventive vaccination but may be used for emergency vaccination in Japanese quail especially for Thailand which no evidence of HPAI-H5N1 outbreak since 2008. Emergency vaccination may be used to assist in the control of HPAI-H5N1 virus before eradication by stamping out.

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