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Original Article

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Abstract

Pathogenicity and transmission of Highly Pathogenic Avian Influenza subtype H5N1 viruses (HPAI-H5N1) depend on the strains and infection doses of the viruses. In this study, experimental infection was performed to determine pathogenicity and transmission of the Thai HPAI-H5N1 in Japanese quails. One hundred and seven quails were divided into 4 groups, experimental groups 1-3 (n = 32 per group) and negative control group (n = 11). At the age of 9 weeks, 16 quails each from groups 1-3 were inoculated with 10^7, 10^5 and 10^3 EID_{50} of Thai HPAI-H5N1, A/chicken/Thailand/CUK2/04(CU-K2) virus. Twenty-four hours later, 16 quails were added as contact quails to each group. Group 4 served as negative control. The quails were observed for clinical presentation, morbidity and mortality. Histopathology and immunohistochemistry were evaluated to confirm viral infection. Oropharyngeal and cloacal swabs were collected and analyzed for virus shedding. Results showed that Thai HPAI-H5N1 (CU-K2) was highly pathogenic for quails similar to other HPAI-H5N1 strains but different in histopathology and tissue tropism. A negative correlation was observed between inoculated doses and mean death time, while a positive correlation was observed between inoculated doses and virus shedding. In addition, the virus can be detected earlier and at a higher titer in oropharyngeal swab than in cloacal swab. This result suggests that oropharyngeal virus excretion exceeds cloacal excretion in quails. Thus, oropharyngeal sampling is superior to cloacal sampling for HPAI-H5N1 surveillance in quails.

Keywords: HPAI-H5N1, pathogenicity, quails, transmission

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บทคัดย่อ

พยาธิกำเนิดและการแพร่ของเชื้อไวรัสไข้หวัดนกชนิดรุนแรงสายพันธุ์ H5N1 ที่แยกได้ในประเทศไทย ในนกกระทาญี่ปุ่น

ธวัช เล็กดำรงศักดิ์[1,4] วิจิตร บรรลุนารา[2] รชฎ ตันติเลิศเจริญ[3,5] ณัฐวัลย์ นนทเบญจวรรณ[4,5] คมเคียว พิณพิมาย[2]
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พยาธิกำเนิดและการแพร่ของเชื้อไวรัสไข้หวัดนกชนิดรุนแรงสายพันธุ์ H5N1(HPAI-H5N1) เกี่ยวข้องกับเสตรนและปริมาณของเชื้อไวรัสที่สัตว์ได้รับ การศึกษาครั้งนี้เป็นการศึกษาพยาธิกำเนิดและการแพร่ของเชื้อ HPAI-H5N1 ที่แยกได้ในประเทศไทยในนกกระทาญี่ปุ่น โดยทดสอบในนกกระทาอายุ 9 สัปดาห์ จำนวน 107 ตัวซึ่งถูกแบ่งออกเป็น 4 กลุ่ม คือ กลุ่มทดลองที่ 1-3 กลุ่มละ 32 ตัวเป็นกลุ่มทดสอบและกลุ่มที่ 4 จำนวน 11 ตัว เป็นกลุ่มควบคุมลบ ให้เชื้อไวรัส A/chicken/Thailand/CUK2/04(CU-K2) กับนกกระทาในกลุ่มที่ 1-3 กลุ่มละ 16 ตัว ในปริมาณ 10^7, 10^5 และ 10^3 EID_{50} ตามลำดับ จากนั้น 24 ชั่วโมงต่อมา นำนกกระทาที่ไม่ได้รับเชื้อกลุ่มละ 16 ตัว มาเลี้ยงรวมกับนกกระทาที่ได้รับเชื้อในกลุ่มที่ 1-3 การศึกษานี้ได้สังเกตอาการทางคลินิกการป่วย การตาย ผลการติดเชื้อไวรัสทางจุลพยาธิวิทยาและอิมมูโนฮิสโตเคมี ผลการแพร่เชื้อไวรัสในตัวอย่างป้ายปากและทวารร่วม ผลการศึกษาแสดงให้เห็นว่าเชื้อ HPAI-H5N1 (CU-K2) สามารถก่อให้เกิดโรครุนแรงในนกกระทาญี่ปุ่นเช่นเดียวกับเชื้อ HPAI-H5N1 สายพันธุ์อื่น แต่มีความแตกต่างทางจุลพยาธิวิทยาและการกระจายของเชื้อไวรัสในเนื้อเยื่อต่างๆ นอกจากนี้ยังพบความสัมพันธ์เชิงลบระหว่างปริมาณของเชื้อไวรัสที่นกกระทาได้รับกับระยะเวลาการตายเฉลี่ยและความสัมพันธ์เชิงบวกระหว่างปริมาณของเชื้อไวรัสที่นกกระทาได้รับกับการแพร่ของเชื้อ โดยพบว่านกกระทาแพร่เชื้อไวรัสออกมาทางปากก่อนและมากกว่าทางทวารร่วม ดังนั้นจึงสรุปได้ว่าในการเฝ้าระวังโรคไข้หวัดนกในนกกระทา ควรเก็บตัวอย่างป้ายปากซึ่งเหมาะสมกว่าตัวอย่างป้ายทวารร่วม

คำสำคัญ: การแพร่เชื้อ พยาธิกำเนิด นกกระทา HPAI-H5N1

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Introduction

In Thailand, the first Highly Pathogenic Avian Influenza (HPAI-H5N1) outbreak was reported in early 2004. Since then, at least 7 waves of HPAI-H5N1 outbreaks were reported in the country during 2004-2008. The HPAI-H5N1 outbreaks caused serious economic losses not only from high mortality of birds, but also from eradication and control program. In addition, 22 confirmed human cases with 6 deaths have been reported. At least two clades of HPAI-H5N1 viruses, clade 1 and 2.3.4, were reported in Thailand. The predominant clade was clade 1 (CU-K2-like viruses) found in central Thailand, while clade 2.3.4 was only found in north-eastern Thailand (Suwannakarn et al., 2009). The outbreaks were mainly reported to affect non-commercial poultry including backyard chickens, ducks, and quails (Tiensin et al., 2005; Amonsin et al., 2008). After 2008, there was no evidence of HPAI-H5N1 outbreak in Thailand. However, clade 1 HPAI-H5N1 viruses have been reported in neighboring countries in 2012 (Horm et al., 2013). To date, there have been several reports on experimental infection of Thai HPAI-H5N1 viruses in chickens and ducks (Bublot et al., 2007; Saito et al., 2009). However, information on experimental infection of Thai HPAI-H5N1 viruses in quails is still limited.

It has been reported that the pathogenicity of HPAI-H5N1 viruses varies among different breeds and species of birds (Perkins and Swayne, 2001; Perkins and Swayne, 2003; Brown et al., 2009; Saito et al., 2009). Differences in breeds and species of birds as well as strains of HPAI-H5N1 viruses result in different infectious doses, lethal doses, morbidity rates, mortality rates, clinical symptoms, pathobiological features and virus shedding. Thus, the pathogenicity and transmission of individual

HPAI-H5N1 strain in specific hosts should be investigated. In addition, inoculation doses are correlated with infectiousness, mortality and virus shedding in chickens and ducks (Middleton et al., 2007; Saito et al., 2009; Spekreijse et al., 2011). Based on previous studies in chickens and ducks, a low inoculation dose of HPAI-H5N1 virus did not cause 100% infection and mortality (Middleton et al., 2007; Saito et al., 2009; Spekreijse et al., 2011). Thus, the effect of inoculation dose of virus in quails should also be investigated.

The aim of this study was to determine the pathogenicity and transmission of the Thai HPAI-H5N1 strain CU-K2 in Japanese quails by using experimental infection with three different infectious doses (10^7, 10^5 and 10^3 EID_{50}). Clinical presentation, morbidity, mortality, gross pathology, histopathology, immunohistochemistry and virus shedding in quails were evaluated.

Materials and Methods

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

Virus: HPAI-H5N1 virus, A/chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1)(CU-K2), was used in the study. The virus was isolated from the index chicken case of the 2004 HPAI-H5N1 outbreak in Thailand (Viseshakul et al., 2004). Stock virus was propagated in embryonated chicken eggs and median embryo infective dose (EID_{50}) was determined following Reed and Muench protocol (Reed and Muench, 1938).

Experimental infection of quails with Thai HPAI-H5N1: At 9 weeks old, one hundred and seven quails were allocated into 4 groups. Groups 1-3 (n = 32 per group) were experimental groups. Group 4 (n = 11) was the negative control group. Sixteen quails each from the experimental groups 1-3 (inoculated quails) were inoculated intranasally and intraorally 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 CU-K2 virus, respectively. Twenty four hours later, 16 quails (contact quails) were added to each group. Quails in group 4 were inoculated with phosphate buffer saline (PBS) as placebo.

After inoculation, the quails were observed for 3 weeks. All quails were monitored on a daily basis for clinical signs, morbidity and mortality. For pathogenicity study, the inoculated quails (groups 1-3) were euthanized by cervical dislocation (sedated with tiletamine-zolazepam (Zoletil, Virbac, Carros, France) at 0.5, 1.0 and 1.5 days post inoculation (dpi) (n = 3 each) and the contact quails (groups 1-3) were euthanized at 0.5, 1.0 and 1.5 days post contact (dpc) (n = 3 each). In group 4, the inoculated quails were euthanized at 0.5, 1.0 and 1.5 dpi (n = 2 each). Dead and euthanized quails were necropsied to collect tissue samples (trachea, lung, heart, brain, intestine, pancreas, liver, kidney, spleen, oviduct, ovary and bursa of Fabricius). Gross lesions were observed and tissue samples were collected in 10% buffered formalin for hematoxylin & eosin (H&E) and immunohistochemical (IHC) staining. To determine virus shedding, oropharyngeal and cloacal swabs were collected daily for the first 10 days and 14, 16 and 21 dpi. Detection and quantitation of CU-K2 virus titers were performed by real-time RT-PCR assay. To determine antibody response, blood samples were collected from all quails until day 21 for serological analysis by HI test.

Histopathology* and *Immunohistochemistry : Tissue samples were fixed by 10% buffered formalin, processed and embedded in paraffin blocks. The tissue sections were cut at 5 µm and stained with H&E. For detection of influenza viral antigen, IHC staining was performed as previously described (Thontiravong et al., 2012). Briefly, epitopes were retrieved from tissue sections by proteinase K (Invitrogen™, Germany). Primary antibody (mouse anti-Influenza A virus monoclonal antibody clone EVS 238, 1:300 dilution, BV European Veterinary Laboratory, The Netherlands) was added, followed by incubation with a chain polymer kit (Dako REAL™ envision–HRP system, anti-rabbit/mouse antibody, Glostrup, Denmark) and visualized by adding 3,3′ diamino benzidine tetrahydrochlorid (DAB) substrate (Sigma-Aldrich, USA). The sections were counterstained with hematoxylin. Lesion scoring and viral antigen distribution by IHC were based on previous report (Perkins and Swayne, 2003).

Virus detection and quantitation: RNA of HPAI-H5N1 virus was extracted using Viral NA Extraction Kit (Beckman Coulter®, California, USA). To identify and quantify influenza A virus, real-time RT-PCR specific for the matrix (M) gene was conducted. Briefly, reaction mixture comprised forward/reverse primers 0.4 µM per reaction, probe 0.1 µM per reaction (Spackman et al., 2002), 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, to a final volume of 11 µl. To 11 µl of this reaction mixture, 4 µl of RNA were added. One step real-time RT-PCR was performed in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Cycling conditions included a reverse transcription step at 50^0C for 30 min. After an initial denaturation step at 95^0C for 15 min, amplification was performed for 50 cycles including denaturation (95^0C for 15 sec) and annealing (60^0C for 30 sec). Multiple fluorescent signals were obtained once per cycle at the end of the annealing step with FAM channel detectors. 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). Standard curve was constructed from three standard reactions (serial dilution) and negative template control (NTC) by plotting threshold cycle value against viral concentration. The viral concentration in swab samples was calculated with reference to the standard curve.

Serological test: HI assay to detect the presence of specific antibody against the H5 virus was performed following the OIE standard (OIE, 2012) using chicken red blood cells and H5 antigen (CU-K2 virus). The HI titer was the highest dilution of the antiserum that caused complete inhibition of agglutination by 4 HAU of the H5 antigen.

Statistical analyses: Mean death time (MDT), virus titers and HI titers were analyzed for statistically significant differences between groups by analysis of variance (ANOVA) with LSD. The statistically significant differences in numbers of quails shedding virus among groups were evaluated by Fisher's exact test and pair *t*-test. Virus titers and numbers of quail shedding virus between oropharyngeal and cloacal swabs in each group were analyzed for statistically significant differences by pair *t*-tests and Fisher's exact test, respectively. The data was analyzed by SAS 9.2 software package (SAS Institute Inc, North Carolina, USA). *P*-values < 0.05 were considered to be statistically significant.

Results

Clinical presentation, morbidity and mortality: At 1 dpi of the quails inoculated with $10^{7.0}$ EID_{50} (group 1) and $10^{5.0}$ EID_{50} (group 2), 6.3% showed depression and ruffled feathers. At 2 dpi, 30.0% and 20.0% of the inoculated quails in groups 1 and 2 showed depression and ruffled feathers. At 2 dpi, diarrhea was observed in 20.0% and 10.0% of the inoculated quails in groups 1 and 2. At 2 dpi, neurological signs such as tremors and paralysis were also observed in 10.0% of the inoculated quails in group 1. At 3 dpi, neurological signs were observed in 75.0% of the inoculated quails in group 3 ($10^{3.0}$ EID_{50}). It was noted that all inoculated quails died within 3.5 dpi (Fig 1). On the other hand, in the negative control group, there was no morbidity and mortality of quails. The MDT of inoculated quails in groups 1, 2 and 3 was 1.94, 2.43 and 2.38 days, respectively. As for the contact quails, at 1 dpc, 6.3% of quails in group 1 showed depression and ruffled feathers. At 1 dpc, diarrhea was observed in 6.3% of the contact quails in group 3. At 2 dpc, 20.0 % and 10.0% of the contact quails in groups 1 and 2 showed depression and ruffled feathers. At 3 dpc, 66.7% of the contact quails in group 3 showed depression and ruffled feathers. At

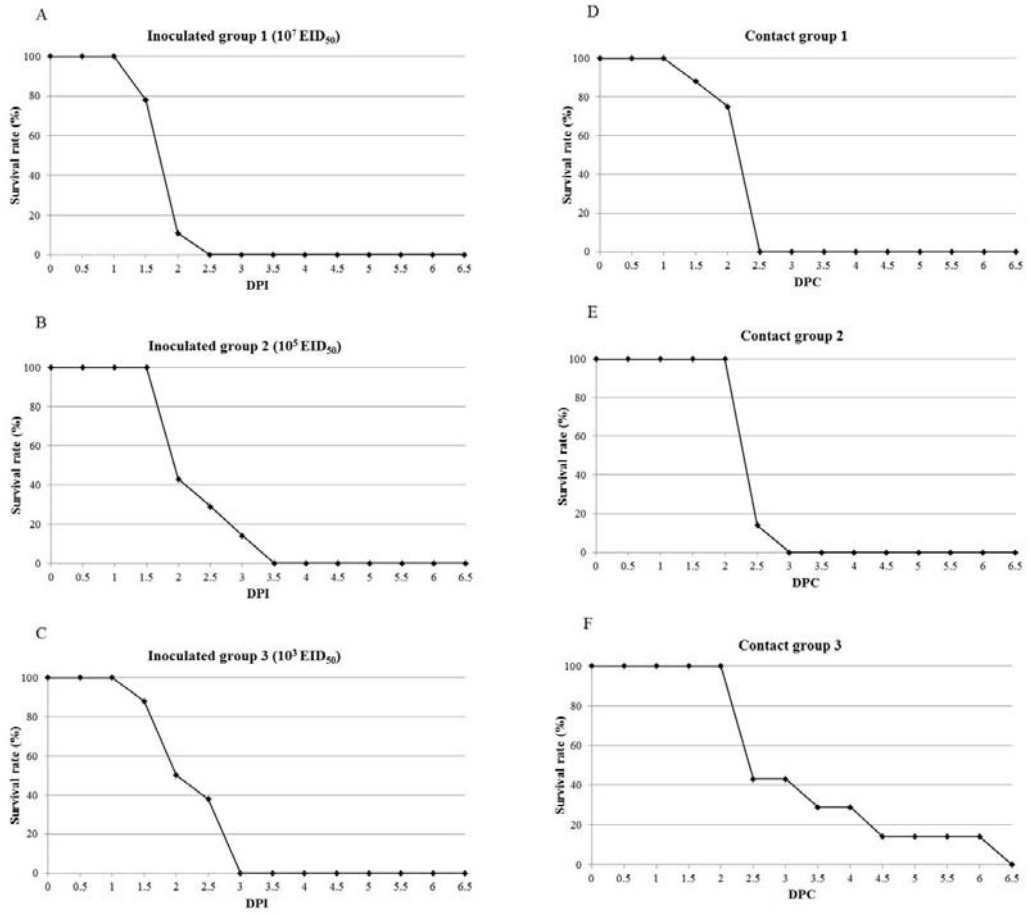

Figure 1 Survival rates of HPAI-H5N1 (CU-K2) infected quails in inoculated and contact groups. (A) Inoculated group 1 with 10^7 EID_{50}, (B) Inoculated group 2 with 10^5 EID_{50}, (C) Inoculated group 3 with 10^3 EID_{50}, (D) Contact group 1, (E) Contact group 2, and (F) Contact group 3.

Table 1 Histopathological lesion of visceral organs of HPAI-H5N1 (CU-K2) infected quails

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	Lesion
Trachea	+**	++	+	+	++	++	Severe necrotic tracheitis and subepithelial edema
Lung	+++	+++	+++	+++	++	++	Severe acute diffuse pneumonia and focal hemorrhage
Heart	±	±	+	+	±	±	Congestion, hemorrhage and lymphocyte infiltration
Brain	+	±	+	±	+	±	Non-suppurative encephalitis, gliosis, and encephalomalacia
Intestine	+	+	++	++	++	++	Catarrhal enteritis
Pancreas	++	++	++	++	++	++	Focal necrotic pancreatitis
Liver	++	+	++	++	++	+	Fatty degeneration and congestion
Kidney	+	+	++	++	+	+	Congestion
Spleen	+	+	++	+	+	+	Necrotic splenitis
Oviduct	-	±	+	+	-	-	Congestion, heterophil and lymphocyte infiltration
Ovary	-	±	+	+	-	-	Hemorrhage, heterophil and lymphocyte infiltration and necrosis
Bursa	-	+	±	+	+	+	Lymphoid depletion and necrosis

* n : 5 in group 1 inoculated, n = 7 in other groups
**- : no lesion; ± : minimal; + : mild; ++ : moderate; +++ : severe

3 dpc, dyspnea was observed in 16.7% and 14.3% of the contact quails in groups 1 and 2. At 4 dpc, 100% of contact quails in group 3 showed depression and ruffled feathers. All contact quails died within 6.5 dpc (Fig 1). The MDT of contact quails in groups 1, 2 and 3 was 2.31, 2.57 and 3.50 days, respectively. It was noted that there was no statistically significant difference in the MDT between the groups of inoculated quails, while the MDT of the contact quails in group 3 was statistically significant longer than in group 1.

Gross pathology, histopathology, virus antigen distribution: Gross lesions of the HPAI-H5N1 inoculated and contact quails in groups 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50}) were similar. The prominent gross lesions in all quails were lung edema, congestion, hemorrhage, focal acute pneumonia and severe acute diffuse pneumonia (100%) (Fig 2). The second most prominent lesions were found in the pancreas (14.6%), including edema, congestion, hemorrhage and multifocal necrosis. The third most common lesion was renal congestion (7.3%). Splenomegaly with congestion and hemorrhage (5.2%), hepatomegaly with congestion and focal necrosis (4.2%), and hemorrhage in the epicardium (2.1%) were also observed. Congestion and hemorrhage were found in the bursa of Fabricius (2.1%), intestine (1.0%) and ovary (1.0%). On the other hand, the quails in the negative control group showed no gross lesions.

Histopathological analysis revealed that the lesions of inoculated and contact quails in groups 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50}) were similar (Table 1), while the negative control group showed no histopathological lesions. In this study, histopathological lesions were more prominent in the infected dead quails than the euthanized quails. The most prominent lesions were observed in lung including severe congestion, focal hemorrhage and pulmonary edema, especially in alveoli and aroundthe vessels. Acute lung inflammation with focal infiltration of heterophils and lymphocytes was found (Fig 2). These lung lesions were observed in both euthanatized and dead quails. It was noted that thelung lesions of inoculated quails in groups 2 and 3 were more severe than in the contact quails. Multifocal necrosis of pancreatic acinar cells without an inflammatory response was commonly observed. The liver was commonly affected with fatty degeneration and congestion. Histopathological lesions were also found in trachea, heart, brain, intestine, kidney, spleen, oviduct, ovary and bursa of Fabricius.

As for the IHC assay, viral antigen was first observed in the euthanatized inoculated quails in group 1 at 1.0 dpi, while viral antigen was first observed in the euthanatized contact quails in group 1 at 1.5 dpc. Viral antigen was found in tissues from all dead inoculated quails after 1.5 dpi and dead contact quails after 1.5 dpc. Viral antigen was commonly observed in the brain of dead inoculated quails (73.6%) and dead contact quails (71.4%). Infected cells were observed in all parts of the brain, including cerebrum, brain stem and cerebellum. Neurons, Purkinje cells and glia cells were also infected (Fig 2). In pancreas, heart and liver, viral antigen was also found in the pancreatic acinar cells around necrotic foci, cardiomyocytes and hepatocytes (Table 2). Viral antigen distribution of the inoculated and contact quails in groups 1, 2 and 3 was similar. No viral antigen was detected in tissues of the negative control group.

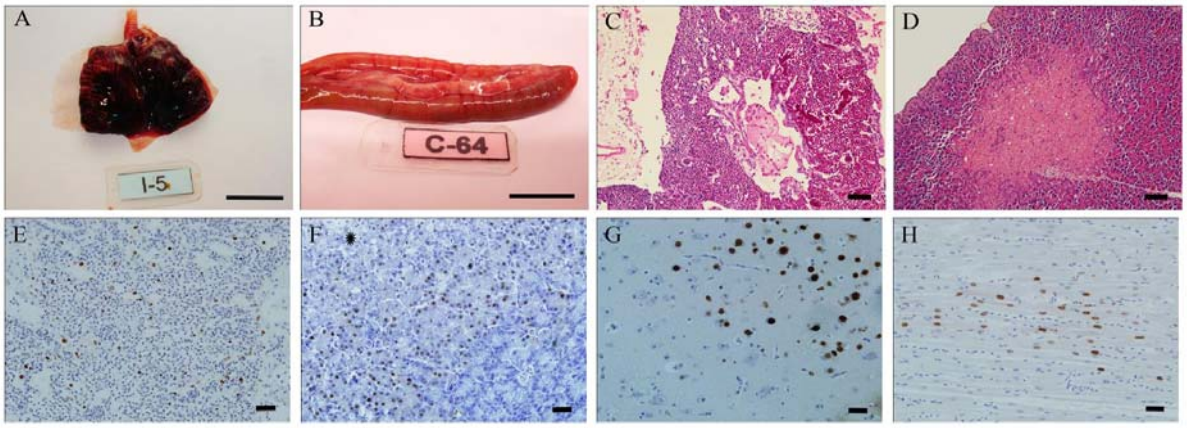

Figure 2 Gross lesions (scale bars = 1 cm), histopathological lesions (scale bars = 50 µm), and IHC staining (scale bars = 20 µm) of quails inoculated with HPAI-H5N1 (CU-K2) virus. (A) Gross lesion; lung, severe congestion, pulmonary edema and acute pneumonia, (B) Gross lesion; pancreas, multifocal necrosis, (C) Histopathological lesion; lung, severe acute interstitial pneumonia with infiltration of inflammatory cells, severe congestion and edema, (D) Histopathological lesion; pancreas, focal necrosis of pancreatic acinar cells, (E) IHC staining; lung, positive viral antigen in the nucleus of various cell types, (F) IHC staining; pancreas, positive viral antigen in the pancreatic acinar cells around necrotic foci (asterisk), (G) IHC staining; cerebrum, positive viral antigen in nucleus of neurons and glia cells, and (H) IHC staining; heart, positive viral antigen in the nucleus of cardiomyocytes.

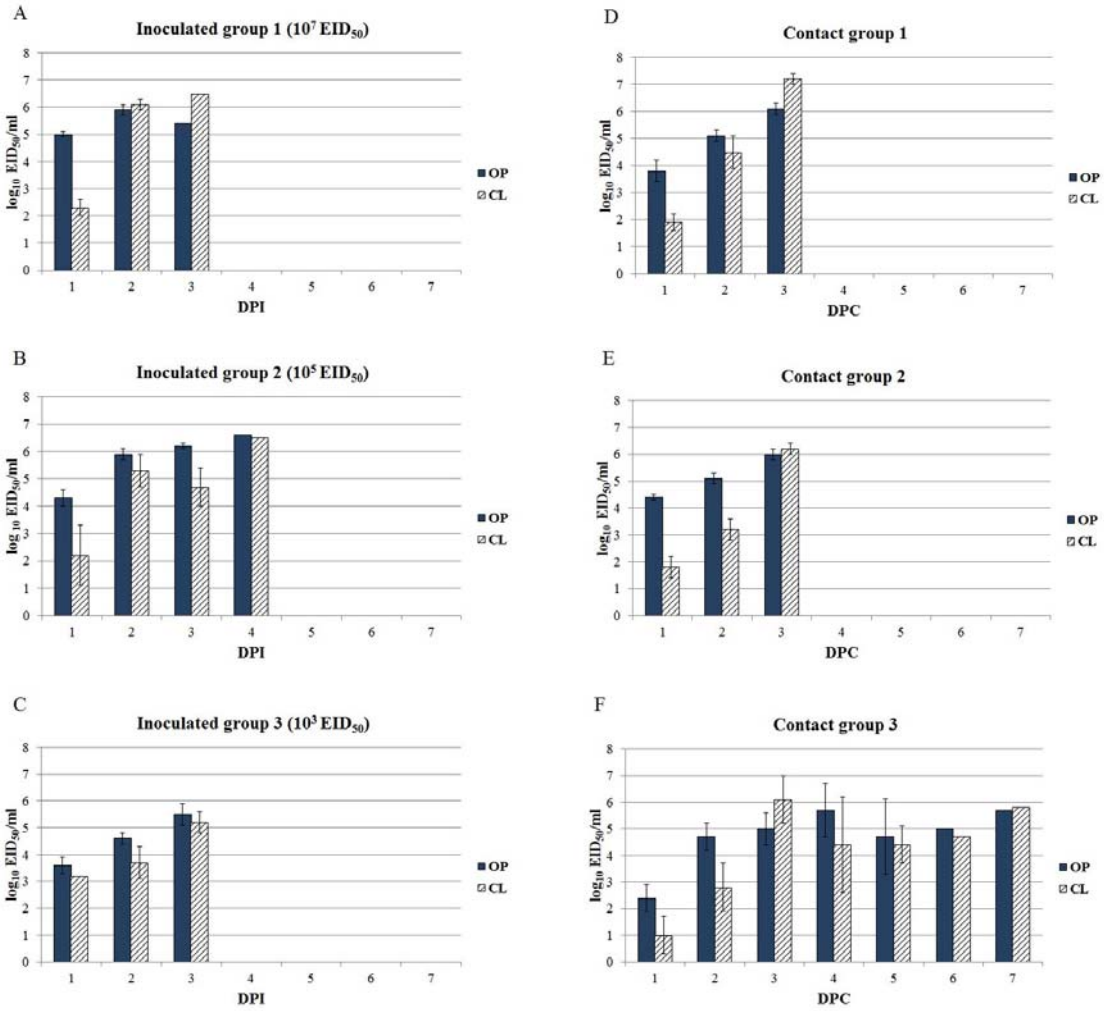

Figure 3 Mean virus titers (log_{10} EID_{50}) of HPAI-H5N1 CU-K2 infected quails in inoculated and contact groups. A: Inoculated group 1 with 10^7 EID_{50}, B: Inoculated group 2 with 10^5 EID_{50}, C: Inoculated group 3 with 10^3 EID_{50}, D: Contact group 1, E: Contact group 2, and F: Contact group 3.

Table 2 Immunohistochemical staining of visceral organs of HPAI-H5N1 (CU-K2) infected quails

Organ	Predominant cell types	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	Globlet cells, epithelial cells	+**	±	±	±	+	±
Lung	Mononuclear cells, alveolar macrophages, bronchial epithelial cells	++	++	++	++	++	++
Heart	Cardiomyocytes	++	++	++	+++	+++	++
Brain	Neurons, Purkinje cells, glial cells, ependymal cells, endothelial cells	+++	+++	+++	+++	+++	+++
Intestine	Intestinal epithelial cells	+	+	+	+	+	+
Pancreas	Pancreatic acinar cells	+	++	++	++	+++	+++
Liver	Hepatocytes, endothelial cells	+++	+++	++	++	+++	++
Kidney	Glomerular endothelial cells, renal tubular cells	+	++	+	+	++	+
Spleen	Endothelial cells, mononuclear cells	++	++	+	+	++	+
Oviduct	Epithelial cells, myocytes	++	++	+	++	+++	++
Ovary	Germinal epithelial cells, follicular epithelial cells, thecal cells	+	±	±	±	±	±
Bursa	Mononuclear cells	±	±	±	±	±	±

* n : 5 in group 1 inoculated, n = 7 in other groups
**- : none; ± : rare; + : infrequence; ++ : common; +++ : widespread

Virus shedding: As for virus shedding, the inoculated quails in groups 1, 2 and 3 ($10^{7.0}$, $10^{5.0}$ and $10^{3.0}$ EID_{50}) shed virus via oropharynx and cloaca since 1 dpi until death (Fig 3). At 1 and 2 dpi, virus titers in oropharyngeal swabs of the inoculated quails in group 3 were statistically significant lower than in groups 1 and 2. Similarly, at 2 dpi, virus titers in cloacal swabs of the inoculated quails in group 3 were statistically significant lower than in groups 1 and 2. It was noted that, at 1 dpi, virus titers in oropharyngeal swabs of the inoculated quails in all groups were statistically significant higher than those in cloacal swabs and a significantly higher number of the inoculated quails shed virus from the oropharynx than from the cloaca. The virus was not detected by real-time RT-PCR in oropharyngeal and cloacal swab of quails in the negative control group from 1 dpi until the end of the experiment.

Similar to the inoculated quails, the contact quails in groups 1, 2 and 3 shed virus via the oropharynx and cloaca since 1 dpc until death. At 1 and 2 dpc, virus titers in oropharyngeal swabs of contact quails in group 3 were statistically significant lower than in groups 1 and 2. At 1 and 2 dpc, virus titers in cloacal swabs of the contact quails in group 3 were statistically significant lower than in group 1. In addition, at 1 dpc, the number of contact quails in group 3 shedding virus from the oropharynx was statistically significant lower than in groups 1 and 2. It was noted that, at 1 dpc, virus titers in oropharyngeal swabs of these contact quails in all groups were statistically significant higher than those in cloacal swabs and a significant higher number of contact quails shed virus from the oropharynx than from the cloaca. At 2 dpc, virus titers in oropharyngeal swabs of the contact quails in group 2 were statistically significant higher than virus titers in cloacal swabs.

Discussion

Studies on pathogenesis and transmission of HPAI-H5N1 infections in animal models are important since pathobiological features and transmission vary among virus strains and host species. Previous studies have reported significant differences in HPAI-H5N1 virulence among strains of viruses, different bird species and different breeds (Perkins and Swayne, 2001; Perkins and Swayne, 2003; Brown et al., 2009; Saito et al., 2009). Moreover, different inoculation doses of HPAI-H5N1 virus in each host species may affect pathobiological features (Middleton et al., 2007; Brown et al., 2009; Saito et al., 2009; Spekreijse et al., 2011).

In this study, the Thai HPAI-H5N1 (CU-K2) virus was proved as highly pathogenic virus in Japanese quails. The CU-K2 virus with $10^{3.0}$, $10^{5.0}$ and $10^{7.0}$ EID_{50} caused 100% mortality within 3.5 days post inoculation and 6.5 days post contact. Similar results have been reported in previous studies challenged with A/chicken/Suphanburi/1/2004, A/duck/Ang thong/72/2004, and A/quail/Angthong/71/2004 (Saito et al., 2009), A/chicken/Korea/IS/06 (Jeong et al., 2009), A/chicken/Hong Kong/220/97 (Perkins and Swayne, 2001), and A/duck/Guangdong/383/ 2008 (Sun et al., 2011).

Quails inoculated with higher doses of virus tend to have earlier onset of clinical signs. The result correlates with previous reports on ducks (Middleton et al., 2007). Thus, onset of clinical signs may depend on inoculation doses. Gross lesions of Thai HPAI-H5N1 (CU-K2) virus infection in quails were severe lung congestion and pancreatic necrosis. Histopathological lesions were prominent in lung, intestine and pancreas. Histopathological lesions in the lung (100%) and pancreas (75.0%) of dead quails were observed more often than in quails inoculated with A/chicken/Hong Kong/220/97 (92% and 55%) (Perkins and Swayne, 2001) even though mortality rates were comparable. 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 (72.5%) was detected more often than in naturally infected Japanese quails (46%) (Antarasena et al., 2006). Viral infection was confirmed in all tissues with IHC assay. Viral antigen was detected predominantly in brain

(Uno et al., 2013). Thus, brain is an important organ for HPAI-H5N1 diagnosis in Japanese quails similar to European quails (Bertran et al., 2013). It was noted that viral antigen could not be found in tissues of all inoculated and contact quails within 1.5 dpi and dpc. Thus, the IHC assay may not be a suitable test for HPAI-H5N1 diagnosis in quails during the early stage. However, gross lesions, histopathological lesions and distribution of virus were similar among groups, thus different inoculation doses do not correlate with lesions and viral antigen distribution.

In this study, the quails shed the virus via oropharynx and cloaca at 1 dpi and 1 dpc similar to previously report (Uno et al., 2013). These results indicated that the incubation period of CU-K2 virus in quails was relatively short and the virus was able to spread rapidly. Interestingly, the virus was isolated from oropharyngeal swabs more frequently than cloacal swabs especially at 1 dpi and 1 dpc. Moreover, the virus titers in oropharyngeal swabs were statistically significant higher than those in the cloacal swabs. Similar findings were observed in previous studies (Makarova et al., 2003; Jeong et al., 2009; Uno et al., 2013). The result suggested that transmission of CU-K2 virus in quails was more pronounced via the oral-oral than the fecal-oral route. Thus, the oropharyngeal swab is a better choice for sample collection in HPAI-H5N1 surveillance in quails.

Our experiments demonstrated that different inoculation doses correlated with pathobiological features including incubation period, mean death time and virus shedding of CU-K2 virus in quails. In previous reports, HPAI-H5N1 viruses with similar genetic composition have demonstrated varied incubation period and MDT (Saito et al., 2009). However, our findings indicated that incubation period and MDT correlated with not only the virus strain, but also the infectious doses. In this study, all quails inoculated with 10^3, 10^5 and 10^7 EID_{50} of CU-K2 virus became ill and died. This finding suggested that the amounts of virus were efficient enough to infect and cause death. In addition, all contact quails became ill and died after contact. This finding suggested that viral particles shed by inoculated quails were sufficient to induce infection in contact quails (Spekreijse et al., 2011). However, the contact quails in 10^3 EID_{50} group developed clinical signs and died significantly later than the contact quails in the 10^7 EID_{50} group. Our result demonstrated that the higher the dosage, the shorter the mean death time (negative correlation). This finding correlated well with previous reports on chickens and ducks reporting that the incubation period and death time decreased significantly with increasing doses (Middleton et al., 2007; Saito et al., 2009). On the other hand, a positive correlation was observed between inoculation doses and virus shedding. With higher dosage of CU-K2 virus, more pronounced virus shedding via oropharynx and cloaca was observed in quails. The result was consistent with previous reports on ducks that the amount of virus shedding correlated with inoculation doses (Middleton et al., 2007).

In conclusion, our study demonstrated that the Thai HPAI-H5N1 (CU-K2) produced highly pathogenic infection in Japanese quails. The different inoculation doses caused similar mortality, histopathological lesions and virus distribution in tissues. On the other hand, our study demonstrated that the inoculation doses correlated with MDT and virus shedding. Since virus shedding was commonly observed in oropharyngeal swabs, it is recommended to apply oropharyngeal swabs for active influenza surveillance programs in quails. In addition, viral antigen was commonly observed in brain, thus, it is recommended to apply brain for passive influenza surveillance programs in quails.

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References

Amonsin A, Choatrakol C, Lapkuntod J, Tantilertcharoen R, Thanawongnuwech R, Suradhat S, Suwannakarn K, Theamboonlers A, Poovorawan Y 2008. Influenza virus (H5N1) in live bird markets and food markets, Thailand. Emerg Infect Dis. 14: 1739-1742.

Antarasena C, Sirimujalin R, Prommuang P, Blacksell SD, Promkuntod N 2006. Tissue tropism of a Thailand strain of high-pathogenicity avian influenza virus (H5N1) in tissues of naturally infected native chickens (*Gallus Gallus*), Japanese quail (*Coturnix Coturnix Japonica*) and ducks (*Anas Spp.*). Avian Pathol. 35: 250-253.

Bertran K, Dolz R, Busquets N, Gamino V, Vergara-Alert J, Chaves A, Ramis A, Abad XF, Hofle U, Majo N 2013. Pathobiology and transmission of highly and low Pathogenic avian influenza viruses in European quail (*Coturnix C. Coturnix*). Vet Res. 44: 23.

Brown JD, Stallknecht DE, Berghaus RD, Swayne DE 2009. Infectious and lethal doses of H5N1 highly pathogenic avian influenza virus for house sparrows (*Passer Domesticus*) and rock pigeons (*Columbia Livia*). J Vet Diagn Invest. 21: 437-445.

Bublot M, Le Gros F-X, Nieddu D, Pritchard N, Mickle TR, Swayne DE 2007. Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian influenza isolate from a chicken in Thailand. Avian Dis. 51: 332-337.

Horm SV, Sorn S, Allal L, Buchy P 2013. Influenza A (H5N1) virus surveillance at live poultry markets, Cambodia, 2011. Emerg Infect Dis. 19: 305-308.

Jeong OM, Kim MC, Kim MJ, Kang HM, Kim HR, Kim YJ, Joh SJ, Kwon JH, Lee YJ 2009. Experimental infection of chickens, ducks and quails with the highly pathogenic H5N1 avian influenza virus. J Vet Sci. 10: 53-60.

Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR 2003. Replication and transmission of influenza viruses in Japanese quail. Virology. 310: 8-15.

Middleton D, Bingham J, Selleck P, Lowther S, Gleeson L, Lehrbach P, Robinson S, Rodenberg J, Kumar M, Andrew M 2007. Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. Virology. 359: 66-71.

OIE 2012. Avian Influenza. In manual of dignostic tests and vaccines for terrestrial animals (chapter 234) (Paris, World Organisation for Animal Health).

Perkins LE, Swayne DE 2001. Pathobiology of A/Chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven Gallinaceous species. Vet Pathol. 38: 149-164.

Perkins LE, Swayne DE 2003. Varied pathogenicity of a Hong Kong-origin H5N1 avian influenza virus in four Passerine species and Budgerigars. Vet Pathol. 40: 14-24.

Reed LJ, Muench H 1938. A simple method of estimating fifty percent endpoints. Am J Hyg. 27: 493-497.

Saito T, Watanabe C, Takemae N, Chaisingh A, Uchida Y, Buranathai C, Suzuki H, Okamatsu M, Imada T, Parchariyanon S, Traiwanatam N, Yamaguchi S 2009. Pathogenicity of highly pathogenic avian influenza viruses of H5N1 subtype isolated in Thailand for different poultry species. Vet Microbiol. 133: 65-74.

Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 40: 3256-3260.

Spekreijse D, Bouma A, Stegeman JA, Koch G, de Jong MC 2011. The effect of inoculation dose of a highly pathogenic avian influenza virus strain H5N1 on the infectiousness of chickens. Vet Microbiol. 147: 59-66.

Sun H, Jiao P, Jia B, Xu C, Wei L, Shan F, Luo K, Xin C, Zhang K, Liao M 2011. Pathogenicity in quails and mice of H5N1 highly pathogenic avian influenza viruses isolated from ducks. Vet Microbiol. 152: 258-265.

Suwannakarn K, Amonsin A, Sasipreeyajan J, Kitikoon P, Tantilertcharoen R, Parchariyanon S, Chaisingh A, Nuansrichay B, Songserm T, Theamboonlers A, Poovorawan Y 2009. Molecular evolution of H5N1 in Thailand between 2004 and 2008. Infect Genet Evol. 9: 896-902.

Thontiravong A, Wannaratana S, Tantilertcharoen R, Prakairungnamthip D, Tuanudom R, Sasipreeyajan J, Pakpinyo S, Amonsin A, Kitikoon P, Oraveerakul K 2012. Comparative study of pandemic (H1N1) 2009, Swine H1N1, and Avian H3N2 influenza viral infections in quails. J Vet Sci. 13: 395-403.

Tiensin T, Chaitaweesub P, Songserm T, Chaisingh A, Hoonsuwan W, Buranathai C, Parakamawongsa T, Premashthira S, Amonsin A, Gilbert M, Nielen M, Stegeman A 2005. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg Infect Dis. 11: 1664-1672.

Uno Y, Usui T, Soda K, Fujimoto Y, Takeuchi T, Ito H, Ito T, Yamaguchi T 2013. The pathogenicity and host immune response associated with H5N1 highly pathogenic avian influenza virus in quail. J Vet Med Sci. 75: 451-457.

Viseshakul N, Thanawongnuwech R, Amonsin A, Suradhat S, Payungporn S, Keawchareon J, Oraveerakul K, Wongyanin P, Plitkul S, Theamboonlers A, Poovorawan Y 2004. The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. Virology. 328: 169-176.