SUSCEPTIBILITY OF OPENBILL STORKS (*ANASTOMUS OSCITANS*) TO HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS SUBTYPE H5N1

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Abstract. This investigation detailed the clinical disease, gross and histologic lesions in juvenile openbill storks (*Anastomus oscitans*) intranasally inoculated with an avian influenza virus, A/chicken/Thailand/vsmu-3 (H5N1), which is highly pathogenic for chickens. High morbidity and mortality were observed in openbill storks inoculated with HPAI H5N1 virus. Gross lesions from infected birds were congestion and brain hemorrhage (10/20), pericardial effusions, pericarditis and focal necrosis of the cardiac muscle (2/20), pulmonary edema and pulmonary necrosis, serosanguineous fluid in the bronchis (16/20), liver congestion (6/20), bursitis (5/20), subcutaneous hemorrhages (2/20) and pinpoint proventiculus hemorrhage (2/20). Real time RT-PCR demonstrated the presence of viral RNA in organs associated with the lesions: brain, trachea, lungs, liver, spleen and intestines. Similar to viral genome detection, virus was also isolated from these vital organs. Antibodies to influenza virus detected with a hemagglutination inhibition test, were found only in the openbill storks who died 8 days post-inoculation.

Keywords: openbill storks, avian influenza virus, H5N1, susceptibility, HPAI

INTRODUCTION

Influenza is a common, important respiratory disease among pigs and birds throughout the world and has important public health implications. Influenza viruses are enveloped, single-stranded, negative-sense RNA viruses in the family Orthomyxoviridae. Influenza A viruses among aquatic birds (wild birds) have been proposed as the ancestors of all influenza A virus subtypes (Horimoto and Kawaoka, 2001). Currently, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified (Fouchier *et al*, 2005). Avian influenza (AI) viruses preferentially infect cells lining the intestinal tract of birds and are excreted in high concentrations in their feces. While AI viruses are generally nonpathogenic in wild birds, they sometimes cause significant mor-

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bidity and mortality upon transmission to other species (Webster *et al*, 1992; Nicholson *et al*, 2003).

From December 2003 to April 2005, highly pathogenic avian influenza (HPAI) H5N1 viruses caused outbreaks of disease among domestic poultry in nine Asian countries (OIE, 2011; WHO, 2006). The AI viruses that caused outbreaks among poultry and humans in Thailand, Indonesia and Vietnam were genotype Z (Li *et al*, 2004). There were several sporadic outbreaks of H5N1 HPAI among poultry in Thailand between 2004-2008 (Suwannakarn *et al,* 2009; Souris *et al,* 2010). The outbreaks in 2004 affected more than 60 out of 73 provinces resulting in the culling of over 62 million chickens (Tiensin *et al,* 2005). Outbreaks of H5N1 AI have shown strong seasonality. During 2007-2008, most of the outbreaks were in the Yom-Nan River basin in the southern part of the northern region of the country. Sequences of these viral isolates were identified as clade 1, genotype Z, and were closely related to viruses from previous years in the central region of the country (Chaichoune *et al,* 2009).

The Asian Openbill Stork or *Anastomus oscitans,* is a common species widely distributed throughout Asia. Its habitat includes freshwater marshes, rice paddy fields and cultivation ditches. Most of these bird migrate from Thailand to Brahmaputa and the Ganges Deltas of Bangladesh during the wet season (Kahl, 1971; Lekagul and Round, 1991). During the 2004-2007 outbreaks in Thailand, the positive detection rate for H5N1 in wild birds, including openbill storks, was 1.0% (Siengsanan *et al*, 2009). The peak annual prevalence was found in 2004, the first year of the outbreak in Southeast Asia. During the first outbreak in 2004, more than 500 openbill storks in the Bung Boraphet non-hunting area, Nakhon Sawan Province, Thailand, were infected with H5N1 HPAI virus and died (Siengsanan *et al*, 2009; Suksatu *et al,* 2009). The role of openbill storks in HPAI outbreaks was intensely discussed in relation to the co-habitation of wild waterfowl and free-grazing ducks offering high contact rates for viral transmission. Openbill storks are likely to be susceptible hosts for H5N1 HPAI virus and may play a role in the poultry outbreaks that occurred in those areas where the habitat is shared by aquatic birds. The knowledge gap in the natural history of H5N1 AI infection among openbill storks limit our understanding of the role that these birds might play in the epidemiology of H5N1 HPAI virus. The aims of this study were to determine the concentration of H5N1 virus required to cause infection or death in openbill storks, and describe the clinical signs and pathological findings associated with H5N1 API infection in this species.

MATERIALS AND METHODS

Openbill storks

Twenty juvenile (2-month old) openbill storks were captured from wetlands in central Thailand by authorized officers of the Department of Wildlife and National Park Conservation, Ministry of Natural Resources and Environment, Thailand. The birds were trained to accept feeding of their natural food, apple snails (*Pila ampullacea*) or golden apple snails (*Pomacea canaliculata*), which were stored at -20°C. Frozen snails were thawed before feeding. The birds were adapted into a poultry isolator (model: CH12ISOL, Allentown Caging Equipment, Allentown, NJ) for at least 2 weeks prior to the experiments. H5N1-specific antibodies in the sera of these openbill storks were tested with

a hemagglutination inhibition test and micro-neutralization test. The birds had access to food and water *ad libitum*. Care was provided as required by the Institutional Animal Care and Use Committee based on the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Virus

The avian influenza A/chicken/Thailand/vsmu-3/2004 virus was isolated from a dead chicken obtained during the first outbreak in Thailand. All the 8 viral genomic segments were sequenced (GenBank accession no. EF593099 - EF593106). The virus was propagated in Madin-Darby canine kidney cells (MDCK cells, NBL-2) using methods described by a WHO manual (WHO, 2002). Viral pathogenicity was characterized by an intravenous pathogenicity index (IVPI) test according to an OIE manual (OIE, 2004). Briefly, 16 HA units of virus were injected into ten 6-week old chickens by intravenous route. The chickens were examined at 24-hour intervals for 10 days. At each observation, the inoculated chickens were given a score as follows: 0=normal, 1=sick, 2=paralysis and 3=death. The IVPI number was the mean score per chicken per observation over the 10-day period. The IVPI test was conducted at an animal-biosafety level 3 facility.

Experimental design

Openbill storks were divided into 6 treatment groups with 2-4 birds in each group. Each group was intranasally inoculated with 1 of 6 different viral dosages of influenza A/chicken/Thailand/vsmu-3/2004. Prior to inoculation, blood was collected from each of the birds for hemagglutination inhibition and micro-neutralizing tests for A/chicken/Thailand/vsmu-3/2004 to determine whether any of the birds possessed antibodies to avian influenza (AI) virus. All the birds tested negative. The serologically negative birds were inoculated intranasally with 0.5 ml of virus-culture supernatant containing 10 to 10^7 $TCID_{50}$ (50% tissue culture infective dosed) of A/chicken/Thailand/vsmu-3/2004 HPAI. Nasopharyngeal and coacal swab specimens were collected from inoculated chickens daily for 4 weeks or their death. A real-time polymerase chain reaction (RT-PCR) test and virus isolation were used for virus detection. Blood was drawn from each inoculated openbill stork every 3-4 days using a 24-guage needle from the right jugular vein. Within 8 hours of death, an autopsy and gross morphological examination were performed and tissues samples were collected and preserved in 10% neutral buffered formalin. After fixation, the tissues were processed and embedded in paraffin. Five millimeter sections were cut and then stained with hematoxylin and eosin. This study with animals was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC), Mahidol University (Approval No. MUVS-2009-40).

Virus isolation

Tracheal and cloacal swab specimens were inoculated onto MDCK cells. Viral growth was detected by hemmagglutination (HA) assay following WHO recommendations (WHO, 2002). Briefly, two-fold serial dilutions of culture supernatant were made in 50 l of phosphate-buffered saline (PBS) and placed into 96-well U-bottom plates. In each well, 50 l of 0.5% (v/v) chicken erythrocytes in PBS was then added. The plates were kept at 4ºC for 1 hour, after which the HA patterns were read and the HA titers were determined from the last dilution showing complete

hemagglutination. Viral RNA was extracted from cell-culture supernatant using a viral RNA extraction kit (Qiagen, Valencia, CA). RT-PCR was used to confirm virus isolation. All experiments with H5N1 virus were performed at a Biosafety Level 3 facility.

Real-time RT-PCR

RT-PCR was used to detect the influenza virus in the tracheal/cloacal swab specimens and tissues from the dead birds. Autopsied tissues from vital organs were prepared for RNA extraction using the RNAesay kit (Qiagen, Valencia, CA). Tissue samples were cut to approximately 30 mg in weight to determine the number of viral RNA copies per milligram of tissue. Real-time Taqman RT-PCR was used to detect hemagglutinin of H5N1 viruses using H5 specific primers (H5F: 5′-ACGTATGACTACCCGCAGTATTCAG-3′ and H5R: 5′-AGACCAGCTACCATGATTGC-3′) and an H5 specific probe (5′ FAM-TATACAGCTACCATGATTGC-TAMRA 3′) following a method modified from Spackman *et al* (2002). The RT step was conducted for 30 minutes at 50°C and 15 minutes at 95°C. Two step PCR cycling was used as follows: 40 cycles at 94°C for 15 seconds and 60°C for 20 seconds. The reactions was performed with the Artus3000 real-time PCR machine (Rotorgene™, Australia). Fluorescence data were acquired at the end of each annealing step. The number of copies of the HA gene was calculated by comparing with a standard concentration curve resulting from a 10-fold dilution of standard known concentration of H5 cDNA (Genekam Biotecnology, Duisburg, Germany).

Micro-neutralization test

An enzyme-linked immunosorbent assay (ELISA) based microneutralization test (microNT) was conducted to detect NT antibodies. The test protocol followed World Health Organization (WHO) recommendations with a small modification (WHO, 2002; Kitphati *et al*, 2009). The tested sera were heat inactivated at 56°C for 30 minutes and then two-fold diluted with MEM maintenance medium (Promega, Madison, WI) starting from a dilution of 1:5 to 1:2,560. The assay was performed by mixing 60 l of diluted serum with 60 l of the virus suspension at a concentration of 200 TCID50 and incubated at 37°C for 2 hours. One hundred microliters of the mixture was transferred onto an MDCK cell monolayer and further incubated at 37°C for 18 to 20 hours. In order to verify the amount of virus inoculum, back-titrations of the virus were conducted at doses of 0.1, 1, 10 and 100 $TCID_{50}$ and included in the assay, along with a positive control serum sample and a cell culture control. The test reaction was run in duplicate. Viral nucleoprotein produced in the infected MDCK cells was detected with on indirect ELISA using mouse monoclonal antibody to influenza A virus nucleoprotein (Chemicon International, Temecula, CA) as the primary antibody and goat anti-mouse immunoglobulin (Ig) conjugated with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) as the second antibody. The tetramethylbenzidine (TMB) peroxidase substrate system (Kirkegaard and Perry, Gaithersburg, MD) was used as the chromogenic substrate. The colored product was read for optical density (OD) at dual wavelengths of 450 and 630 nm. The corrected OD of the tested serum was obtained after subtracting the cell control OD from the original OD value. The corrected OD of the virus control at a working concentration of 100 $TCID_{50}$ was also obtained in the same manner. A positive microNT result was obtained when the test serum

yielded a ≥50% reduction in the corrected OD compared to the control. The antibody titer was defined as the reciprocal value of the highest serum dilution that gave ≥50% neutralization of 100 $TCID_{50}$ of the test virus (Kitphati *et al*, 2009).

Hemagglutination (HA) and hemagglutination inhibition (HI) assays

These assays were performed as described previously using 0.5% goose erythrocytes (WHO, 2002; Louisirirotchanakul *et al*, 2007). All virus samples were sonicated briefly for 5 minutes before use. For the hemagglutination assay, 50 l of a twofold serial dilution of virus in PBS were incubated at 4ºC for 1 hour with an equal volume of goose erythrocytes. The reciprocal of the highest virus dilution showing hemagglutination was noted as the endpoint titer, namely one hemagglutination unit (HAU)/25 l. For HI assays, 25 l of a twofold serial dilution of the tested serum in PBS was incubated at room temperature for 1 hour with an equal volume of 4 HAU of the virus, followed by the addition of 50 l of goose erythrocytes. The reciprocal of the highest dilution of the antibody showing no hemagglutination was noted as the HI titer (WHO, 2002).

RESULTS

With the IVPI test most chickens that received 16HA units of virus died on the first day after inoculation; one chick developed abnormal neurological signs and and died on day 2. The IVPI of this virus was calculated to be 2.98. The virus used in this study, A/Chicken/Thailand/vsmu-3/2004(H5N1), was considered to be a highly pathogenic. The viral cleavage site of the hemagglutinin (HA_0) precursor protein contained the polybasic amino acid Q_{338}RERRRKKRG$_{347}$ (Genbank no. EF593102).

Based on the HI and micro-NT tests, none of the openbill storks used in the current study had preexisting antibodies to H5N1 virus or were excreting virus at the start of the experiment. Non-inoculated birds did not exhibit any evidence of infection during the course of the study, including viral shedding, clinical signs of disease, expression of viral antigen in the tissues or seroconversion.

All the openbill storks inoculated with low (10, 10^2 $TCID_{50}$), medium (10^3, 10^4 $TCID_{50}$) and high (10^5, 10^6 $TCID_{50}$) doses died. The ID50 and LD50 calculations could not be determined because of this. The mean survival times for the storks in the 10, 10^2, 10^3, 10^4,10^5 and 10^6 $TCID_{50}$ dose groups were 4.5, 4.3, 4, 6.5, 4.6 and 4.3 days post-inoculation (DPI), respectively. The overall average survival time for all 6 groups was 4.8 days (range: 2-8 days). The average onset of clinical signs was 4.1 DPI (range: 2-7 DPI). Most openbill storks had no clinical signs or a short duration of illness prior to death (average duration of sickness: 0.8 days; range: 0-3 days) (Table 1). After the incubation period, 7 birds (35%) had non-specific clinical signs of illness varying in severity: lethargy, weakness, diarrhea, apnea, nasal discharge, head tremors and seizures. Two inoculated birds (10%) had acute paralysis prior to death.

All the inoculated birds in the current study shed virus in various amounts. Tracheal and cloacal swabs collected 1-8 DPI, showed were positive for virus with both RT-PCR and viral culture. Most birds started to shed virus 1-2 DPI and continued to shed virus until death (Table 2). The minimum concentration of virus shed from a treacheal swab was $2.27x10^3$ copies/ml from a specimen taken 1 DPI and the maximum concentration was $5.11x10^9$ copies/ml at the time of animal's death 4

Table 1
Virus detection in oropharyngeal and cloacal swabs by real-time RT-PCR and virus isolation.

Dosage of virus inoculum (TCID50)	Bird code	Virus isolation and real-time RT-PCR detection															
		DPI of cloacal swabs								DPI of tracheal swabs							
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
10	O-23	–/–	+/–	+/+	+/+	L	L	L	L	–/–	+/+	+/+	+/+	L	L	L	L
	O-24	–/–	+/+	+/+	L	L	L	L	L	–/–	+/+	+/+	L	L	L	L	L
	O-25	–/–	+/–	+/+	+/+	L	L	L	L	–/–	+/–	+/+	+/+	L	L	L	L
	O-26	+/–	+/+	+/+	L	L	L	L	L	–/–	+/+	+/+	L	L	L	L	L
10^2	O-19	+/–	+/–	+/+	L	L	L	L	L	+/–	+/+	+/+	L	L	L	L	L
	O-20	–/–	+/+	+/+	L	L	L	L	L	+/+	+/+	+/+	L	L	L	L	L
	O-21	+/–	+/+	+/+	L	L	L	L	L	+/+	+/+	+/+	L	L	L	L	L
	O-22	–/–	+/+	+/+	+/+	L	L	L	L	+/–	+/+	+/+	+/+	L	L	L	L
10^3	O-16	–/–	+/+	–/+	L	L	L	L	L	–/–	+/–	+/+	L	L	L	L	L
	O-17	+/–	L	L	L	L	L	L	L	–/–	L	L	L	L	L	L	L
	O-18	–/–	+/+	–/+	–/+	+/+	L	L	L	–/–	+/–	+/+	+/+	+/+	L	L	L
10^4	O-14	–/–	+/–	++	++	++	++	++	++	–/–	–/–	++	++	++	++	++	++
	O-15	–/–	+/–	++	++	++	L	L	L	–/–	–/–	++	++	++	L	L	L
10^5	O-27	–/–	–/–	–/–	–/–	+	++	++	L	–/–	–/–	–/–	–/–	+	+/+	+/+	L
	O-28	–/–	–/–	++	++	++	L	L	L	–/–	–/–	–/+	+/+	+/+	L	L	L
	O-29	–/–	–/+	++	L	L	L	L	L	–/–	–/+	+/+	L	L	L	L	L
	O-30	–/–	–/–	++	++	++	++	L	L	–/–	–/–	–/+	+/+	+/+	+/+	L	L
10^6	O-3	+/+	+/–	+/+	+/+	+/+	L	L	L	+/+	+/–	+/+	+/+	+/+	L	L	L
	O-4	–/–	+/+	+/+	+/+	L	L	L	L	+/+	+/+	+/+	+/+	L	L	L	L
	O-1	–/+	+/+	ND	+/+	L	L	L	L	–/+	+/+	ND	+/+	L	L	L	L

–/–, negative by both real-time RT-PCR and virus isolation; +/–, positive by real-time RT-PCR but negative by virus isolation; –/+ , negative by real-time RT-PCR but positive by virus isolation; +/+, positive by both real-time RT-PCR and virus isolation.
L, lethal; ND, not done; DPI , days post-inoculation.

Table 2
Severity of microscopic lesions in openbill storks inoculated intranasally with 10^5TCID$_{50}$ of A/chicken/Thailand/vsmu-3/04(H5N1) highly pathogenic avian influenza virus.

Tissue	Necropsy findings	Microscopic lesions
Brain	Congestion and hemorrhage (10/20)	Lymphoplasmacytic perivascular encephalitis
Heart	Pericardial effusion, pericaditis, focal necrosis (2/20)	Lymphoplasmacytic myocarditis, myocardial necrosis
Lungs and air sacs	Pulmonary edema and necrosis, serosanguineous fluid in bronchus (16/20)	Variable infiltrates of heterophils, lymphocytic air sacculitis
Liver	Congestion, hepatitis (6/20)	Focal hepatitis
Bursa of farbicious	Bursitis, serous effusion in bursa (5/20)	Lymphoid depletion
Skeletal muscle and integument	Subcutaneous hemorrhages (2/20)	Degeneration and necrosis of myocytes
Kidneys	Swelling (5/20)	Lymphocytic heterophilic nephritis
Alimentary tract	Pinpoint hemorrhages in the mucosa of the proventiculus (2/20)	Necrosis of acinar cells in the pancreas, focal necrosis in the proventiculus

DPI. The minimum concentration of virus shed from a cloacal swab was $2.55x10^3$ copies/ml 2 DPI and the maximum concentration shed was $7.13x10^8$ copies/ml 4 DPI. There were no significant differences in the minimum concentration of virus shed from the trachea and cloacae but the maximum virus shed from the trachea was significantly greater than the cloaca.

All the serum samples taken during the observation period were negative on the HI and NT tests except for one bird 8 DPI; that bird had a HI titer of 1:640 and died on 8 DPI. None of the openbill storks survived longer than 8 days. The most consistent gross pathological findings were pulmonary edema, congestion and necrosis with serosanguineous fluid in the bronchi. Other gross lesions included subcutaneous ecchymotic or pethechial hemorrhages, serous fluid surrounding the heart, liver and abdomen, pleural effusions, pericaditis with focal necrosis in the myocardium, pin-point hemorrhages in the proventicular mucosa, congestion and hemorrhages in the cerebrum and cerebellum. Other lesions included bursitis, liver congestion, swelling of kidneys, tracheal congestion and hemorrhages and fibrin adherence to the internal organs and peritoneum (Table 2).

Common microscopic findings included severe pulmonary edema, lymphoplasmacytic myocarditis, myocardial necrosis, necrosis in the cerebrum and cerebellum, lymphoplasmacytic perivascular encephalitis and lymphocytic air sacculitis. Other lesions included lym-

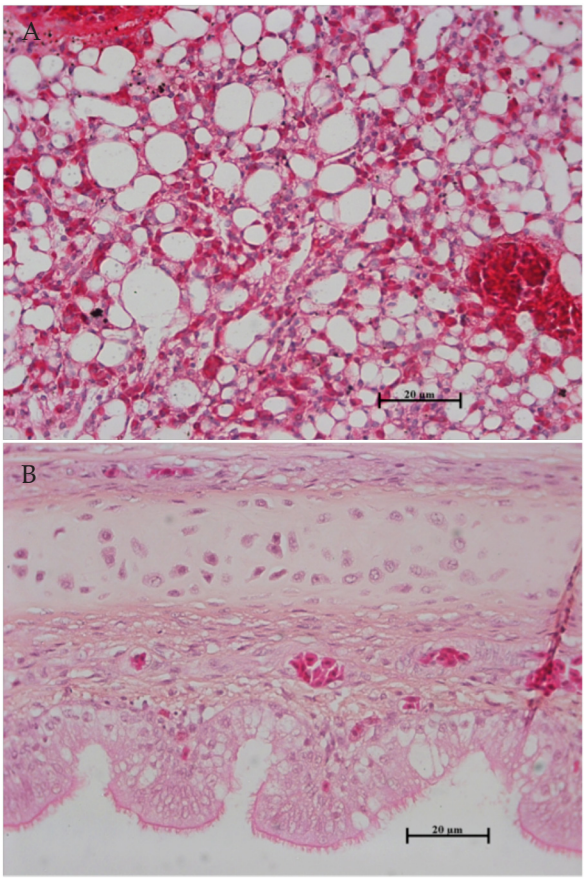

Fig 1–Microscopic pathogenic changes in the tissue of an openbill stork infected with A/chicken/Thailand/vsmu-3/04 (H5N1). (A) lung, heterophilic infiltrate and lymphocytic air sacculitis. (B) trachea; variable infiltrates of heterophils in epithelium of trachea.

phocytic and heterophilic nephritis, infiltration of heterophils in the epithelium of the trachea, degeneration and necrosis of myocytes in the skeleton muscles and necrosis of acinar cells in the pancreas.

Viral RNA was detectable in all the vital organs of all the infected birds. The majority of the birds had the highest amount of viral RNA in the upper and lower respiratory organs (trachea or lungs) (Fig 2) where we found severe pneumonitis with heterophilic cell infiltration in the pulmonary (Fig 1a) and tracheal (Fig 1b) tissue. The lowest concentrations of viral RNA were found in the liver and cardiac tissues (Fig 2).

DISCUSSION

Thailand experienced an H5N1 avian influenza virus epidemic during 2004-2008. How the virus entered the country is not known. One possible route was migratory birds. One of these birds is the Asian opened-billed stork. The involvement of these wild birds as carriers of HPAI viruses was the focus of debate in 2004 and 2005.Wild birds have been blamed for dissemination of HPAI in Asia, Europe and Africa since May 2005 (Chen *et al*, 2005). Openbill storks were suspected as vectors in Thailand because of finding infected and dead storks with H5N1 virus 2004 (BirdLife International, 2004; Melville and Shortridge, 2006). This was the reason why the Thai government initiated monitoring wild birds for HPAI virus. Viral genetic analysis showed multiple introductions of the HPAI virus into the openbill stork population in Thailand during 2004 and 2005; this was demonstrated by HA phylogenetic tree analysis of the HPVI H5N1 virus isolated from poultry and wild birds in Thailand (Uchida *et al*, 2008).

In our study, all the openbill storks inoculated with the H5N1 HPAI virus died within 8 days of inoculation. The sensitivity of these birds is similar to that of chickens inoculated with the H5N1 HPAI virus, which died within 96 hours of infection (Liu *et al*, 2007). In contrast, inoculated pigeons exhibited no signs of illness for 14 to 21 DPI (Liu *et al*, 2007).

Openbill storks are highly susceptible

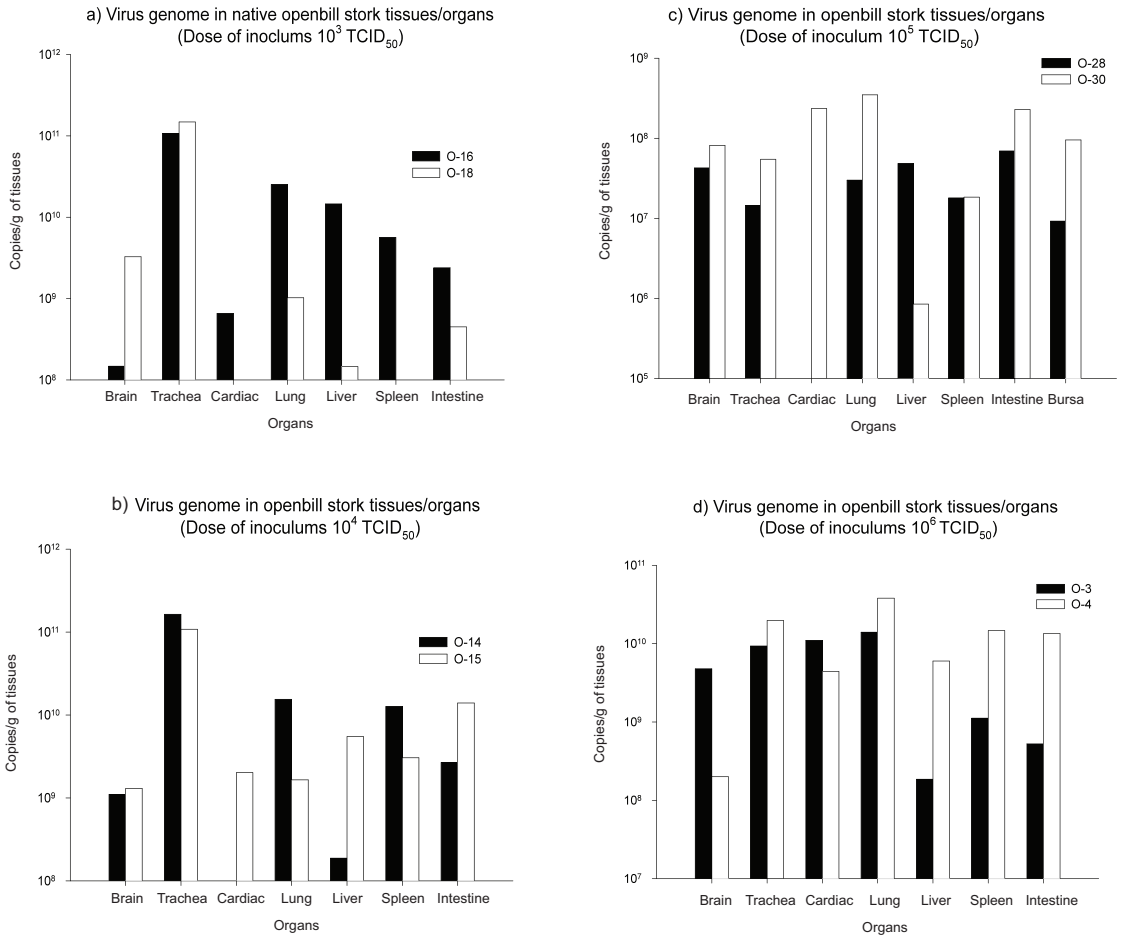

Fig 2–Schematics showed copy number of influenza virus per gram of tissue received from infected openbill storks. a) Tissue of openbill storks inoculated with 10^3 $TCID_{50}$ virus dosages. b) Tissue of openbill storks inoculated with 10^4 $TCID_{50}$ virus dosages. c) Tissue of openbill storks inoculated with 10^5 $TCID_{50}$ virus dosages. d) Tissue of openbill storks inoculated with 10^6 $TCID_{50}$ virus dosages.

to infection with the H5N1 HPAI virus and a relatively small concentration of this virus caused infection and death. Wood ducks and domestic chickens have also been found to be highly susceptible to the H5N1 HPAI virus (Perkins and Swayne, 2003; Brown *et al*, 2007). Our results are in agreement with the first outbreak in 2004, where large numbers of deaths occurred in openbill storks (BirdLife International, 2004). Outbreaks among openbill storks with HPAI usually occur at same time as outbreaks among domestic poultry (BirdLife International, 2004). The openbill storks likely contracted the virus from other infected aquatic poultry or wild birds.

Since none of the openbill storks survived it seems unlikely openbill storks serve as vectors of the virus over long distances. The infected openbill storks shed large amounts of virus for 2-6 DPI from

both the respiratory and intestinal tracts. Only a small amount of the virus was required to induce infection and death in openbill storks. These data suggest the openbill stork represents an effective indicator species for H5N1 HPAI virus monitoring.

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