18. Hessel A, Schwendinger M, Holzer GW, *et al*.: Vectors based on modified vaccinia ankara expressing influenza H5N1 hemagglutinin induce substantial cross-clade protection immunity. PLoS ONE 2011;6:e16247.

19. Kreijtz JHCM, Suezer Y, van Amerongen G, *et al*.: Recombinant modified vaccinia virus ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. JID 2007;195:1598-1606.

20. Kreijtz JHCM, Suezer Y, de Mutsert G, *et al*.: Recombinant modified vaccinia virus ankara expressing the hemagglutinin gene confers protection against homologous and heterologous H5N1 influenza virus infections in macaques. JID 2009;199:405-413.

21. Mayrhofer J, Coulibaly S, Hessel A, *et al*.: Non replication vaccinia virus vectors expressing the H5 influenza virus hemagglutinin produced in modified vero cells induce robust protection. J Virol 2009;83:5192-5203.

22. Poon LLM, Leung YHC, Nicholls JM, *et al*.: Vaccinia virus-based multivalent H5N1 avian influenza vaccines adjuvanted with IL-15 confer sterile cross-clade protection in mice. J Immunol 2009;182:3063-3071.

23. Nefkens I, Garcia J, Ling CS, *et al*.: Hemagglutinin pseudotyped lentiviral particles: characterization of a new method for avian H5N1 influenza sero-diagnosis. J Clin Virol 2007;39:27-33.

24. Nwe N, He Q, Damrongwatanapokin S, *et al*.: Expression of hemagglutinin protein from the avian influenza virus H5N1 in a baculovirus/insect cell system significantly enhanced by suspension culture. BMC Microbiol 2006;6:1-7.

25. Lin YJ, Deng MC, Wu SH, *et al*.: Baculovirus-derived hemagglutinin vaccine protects chickens from lethal homologous virus H5N1 challenge. J Vet Med Sci 2008;70:1147-1152.

26. Lin S, Huang M, Tsou P, Huang L, Chong P and Wu S: Recombinant trimeric HA protein immunogenicity of H5N1 avian influenza viruses and their combined use with inactivated or adenovirus vaccines. PLoS ONE 2011;6:e20052.

27. Shoji Y, Bi H, Musiychuk K, *et al*.: Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. Vaccine 2009;27:1087-1092.

28. Kalthoff D, Giritch A, Geisler K, *et al*.: Immunization with plant-expressed hemagglutinin protects chickens from lethal highly pathogenic avian influenza virus H5N1 challenge infection. J Virol 2010;84:12002-12010.

29. Shehata AA, Fiebig P, Sultan H, Hafez M and Liebert UG: Development of a recombinant ELISA using yeast (*Pichia pastoris*)-expressed polypeptides for detection of antibodies against avian influenza A subtype H5. J Virol Methods 2012;180:18-25.

30. Biesova Z, Miller MA, Schneerson R, *et al*.: Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. Vaccine 2009;27:6234-6238.

31. Panicali D, Davis SW, Weinberg RL and Paoletti E: Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. Proc Natl Acad Sci USA 1983;80:5364-5368.

32. Smith GL, Murphy BR, and Moss B: Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc Natl Acad Sci USA 1983;80:7155-7159.

33. Smith GL, Levin JZ, Palese P and Moss B: Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology 1987;160:336-345.

34. Itamura S, Morikawa Y, Shida H, Nerome K and Oya A: Biological and immunological characterization of influenza virus haemagglutinin expressed from the haemagglutinin locus of vaccinia virus. J Gen Virol 1990;71:1293-1301.

35. Andrew ME, Coupar BEH, Ada GL and Boyle DB: Cell-mediated immune responses to influenza virus antigens expressed by vaccinia virus recombinants. Microb Pathog 1986;1:443-452.

36. Endo A, Itamura S, Iinuma H, *et al.*: Homotypic and heterotypic protection against influenza virus infection in mice by recombinant vaccinia virus expressing the haemagglutinin or nucleoprotein gene of influenza virus. J Gen Virol 1991;72:699-703.

37. Hoffmann E, Stech J, Guan Y, Webster RG and Perez DR: Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 2001;146:2275-2289.

38. Sambrook J, Fritsch EF and Maniatis T: Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.

39. World Health Organization: WHO manual on animal influenza diagnosis and surveillance. WHO/CDS/CSR/NCS/2002.5. http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf.

40. Lerdsamran H, Pittayawonganon C, Pooruk P, *et al.*: Serological response to the 2009 pandemic influenza A (H1N1) virus for disease diagnosis and estimating the infection rate in Thai population. PLoS ONE 2012;6:e16164.

41. Vongsakul M, Kasisith J, Noisumdaeng P and Puthavathana P: The difference in IL-1β, MIP-1α, IL-8 and IL-18 production between the infection of PMA activated U937 cells with recombinant vaccinia viruses inserted 2004 H5N1 influenza HA genes and NS genes. Asian Pac J Allergy Immunol 2011;29:349-56.

42. Elliot AJ, Steinhauer DA, Daniels RS and Oxford JS: Functional and antigenic analyses of the 1918 influenza virus haemagglutinin using a recombinant vaccinia virus expression system. Virus Res 2006;122:11-19.

43. Shen S, Mahadevappa G, Oh HJ, *et al.*: Comparing the antibody responses against recombinant hemagglutinin proteins of avian influenza A (H5N1) virus expressed in insect cells and bacteria. J Med Virol 2008;80:1972-1983.

44. Igarashi M, Ito K, Kida H and Takada A: Genetically destined potentials for *N*-linked glycosylation of influenza virus hemagglutinin. Virology 2008;376:323-329.

45. Kuroda K, Geyer H, Geyer R, Doerfler W and Klenk H: The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. Virology 1990;174:418-429.

46. Kuroda K, Veit M and Klenk H: Retarded processing of influenza virus hemagglutinin in insect cells. Virology 1991;180:159-165.

47. Wagner R, Geyer H, Geyer R and Klenk H: *N*-acetyl-β-glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. J Virol 1996;70:4103-4109.

48. Claas EC, Osterhaus AD, van Beek R, *et al.*: Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472-477.

49. Yamada S, Suzuki Y, Suzuki T, *et al.*: Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 2006;444:378-382.

50. Ubol S, Suksatu A, Modhiran N, *et al.*: Intra-host diversities of the receptor-binding domain of stork faeces-derived avian H5N1 viruses and its significance as predicted by molecular dynamic simulation. J Gen Virol 2011;92:307-314.

51. Soundararaja V, Zheng S, Patel N, *et al.*: Networks link antigenic and receptor-binding sites of influenza hemagglutinin: mechanistic insight into fitter strain propagation. Sci Rep 2011;200:1-7.

52. Cai Z, Ducatez MF, Yang J, *et al.*: Identifying antigenicity-associated sites in highly pathogenic H5N1 influenza virus hemagglutinin by using sparse learning. J Mol Biol 2012;422:145-155.

53. Kitphati R, Pooruk P, Lerdsamram H, *et al.*: Kinetics and longevity of antibody response to influenza A H5N1 virus infection in humans. Clin Vaccine Immunol 2009;16:978-981.

54. Steel J, Lowen AC, Wang TT, *et al.*: Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio 2010;1:e00018-10.

55. Trosby M, van den Brink E, Jongeneelen M, *et al.*: Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM^+ memory B cells. PLoS ONE 2008;3:e3942.

56. Corti D, Suguitan AL Jr, Pinna D, *et al.*: Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J Clin Invest 2010;120:1663-1673.

TABLE 1. Immunological reactivity of recombinant H5 HA with human sera

Subject no.	Age (years)	Blood collection	Antibody to			NT antibody to		
			rBV-H5 HA		rVac-H5 HA	H5N1	H3N2	H1N1
			Lot no. 1[a]	Lot no. 3[b]				
H5N1 survivors								
1	32	1y 6m after disease onset	HA1, HA2	HA0, HA1, HA2	HA0, HA1, HA2	160	ND	ND
2	29	2y 8m after disease onset	HA1, HA2	HA0, HA1, HA2	HA0, HA1, HA2	160	ND	ND
3	7	11m after disease onset	HA1, HA2	HA0, HA1, HA2	HA0, HA1, HA2	80	ND	ND
H3N2 patients								
1	24	Acute bl.	HA2	HA2	HA2	<5	160	>2560
		Conv. bl.	HA2	HA2	HA2	<5	>2560	>2560
2	24	Acute bl.	No	HA2	HA2	<5	80	320
		Conv. bl.	HA2	HA2	HA2	<5	320	320
3	27	Acute bl.	No	HA2	HA2	<5	80	160
		Conv. bl.	No	HA2	HA2	<5	320	160
4	27	Acute bl.	No	HA2	No	<5	160	80
		Conv. bl.	No	HA2	No	<5	1280	80
5	49	Conv. bl.	HA2	HA2	HA2	<5	640	320
6	41	Conv. bl.	No	HA2	HA2	<5	640	1280
7	25	Conv. bl.	No	HA2	No	<5	640	320
8	23	Conv. bl.	No	HA2	No	<5	640	320
Healthy subjects								
1	56	Single bl.	No	HA2	HA2	<5	1280	40
2	41	Single bl.	No	HA2	HA2	<5	20	1280
3	25	Single bl.	No	HA2	No	<5	1280	640
4	24	Single bl.	No	HA2	No	<5	20	640
5	39	Single bl.	No	HA2	No	<5	160	640
6	27	Single bl.	No	HA2	No	<5	80	2560
7	27	Single bl.	No	HA2	No	<5	80	640
8	28	Single bl.	No	HA2	No	<5	160	160
9	25	Single bl.	No	HA2	No	<5	160	640

[a], Protein Sciences lot no. 45-05034RA-2 and [b], BEI Resources lot no. 59137402. ND, not done.

Figure legend

FIGURE 1. Specificity of a goat antiserum (A); and a mouse monoclonal antibody (B) against H5 HA as characterized by various lots of recombinant H5 HA expressed in baculovirus-insect cell system (rBV-H5 HA) and by our recombinant H5 HA expressed by vaccinia virus vector (rVac-H5 HA). The WB banding patterns of rBV-H5 HA are variable by lots and sources. Collective results from 3 lots of rBV-H5 HA demonstrate that the goat antiserum is specific to HA0, HA1 and HA2 domains with MW of 72, 45 and 25 kDa, respectively; whereas the mouse monoclonal antibody is specific only to HA0 and HA1 domains. Moreover, the MWs of HA proteins expressed by the vaccinia virus vector are higher than those expressed by the baculovirus-insect cell system, i.e., they are 75, 55 and 27 kDa for HA0, HA1 and HA2 domains, respectively.

FIGURE 2. Expression and localization of H5 HA protein in TK^- cells infected with recombinant vaccinia virus (rVac-H5 HA) as demonstrated by IFA using goat anti-H5 HA antiserum: TK^- cells infected with rVac-pSC11 virus as the negative control (A); and rVac-H5 HA virus infected TK^- cells (B).

FIGURE 3. Comparison on the Western blot banding patterns between the recombinant H5 HA protein produced in TK^- cells infected with rVac-H5 HA virus and the wild-type H5 HA produced in MDCK cells infected with H5N1 KAN-1 virus. Three bands of the same MWs for HA0, HA1 and HA2 domains are demonstrated by goat anti-H5 antiserum (A); and 2 bands for HA0 and HA1 domains are demonstrated by mouse anti-H5 monoclonal antibody (B). The result indicates similar biological processes including glycosylation and proteolytic cleavage in these 2 vertebrate cell sources used for H5 HA protein production.

FIGURE 4. Immunogenicity of rVac-H5 HA protein in the induction of antibody response in BALB/c mice. Pooled sera from mice immunized with lysates of TK^- cells infected with rVac-H5 HA virus are used to stain the recombinant H5 HA produced in the baculovirus-insect cell system (rBV-H5 HA lot no.3) and the wild-type H5 HA produced in the KAN-1 virus infected MDCK cells by WB assay. The result shows that 3 forms of HA proteins: HA0, HA1 and HA2 proteins are expressed and immunogenic in the mouse model.

FIGURE 5. WB assay for antibody to H5 HA in human sera using the recombinant H5 HA expressed by baculovirus vector (rBV-H5 HA lot no. 1) and vaccinia virus vector as the test antigens. The results demonstrate 3 reactive bands of antibodies directed to HA0, HA1 and HA2 domains in H5N1 survivor (A); and one reactive band of heterosubtypic antibody to HA2 domain in H5N1-uninfected subject (B).

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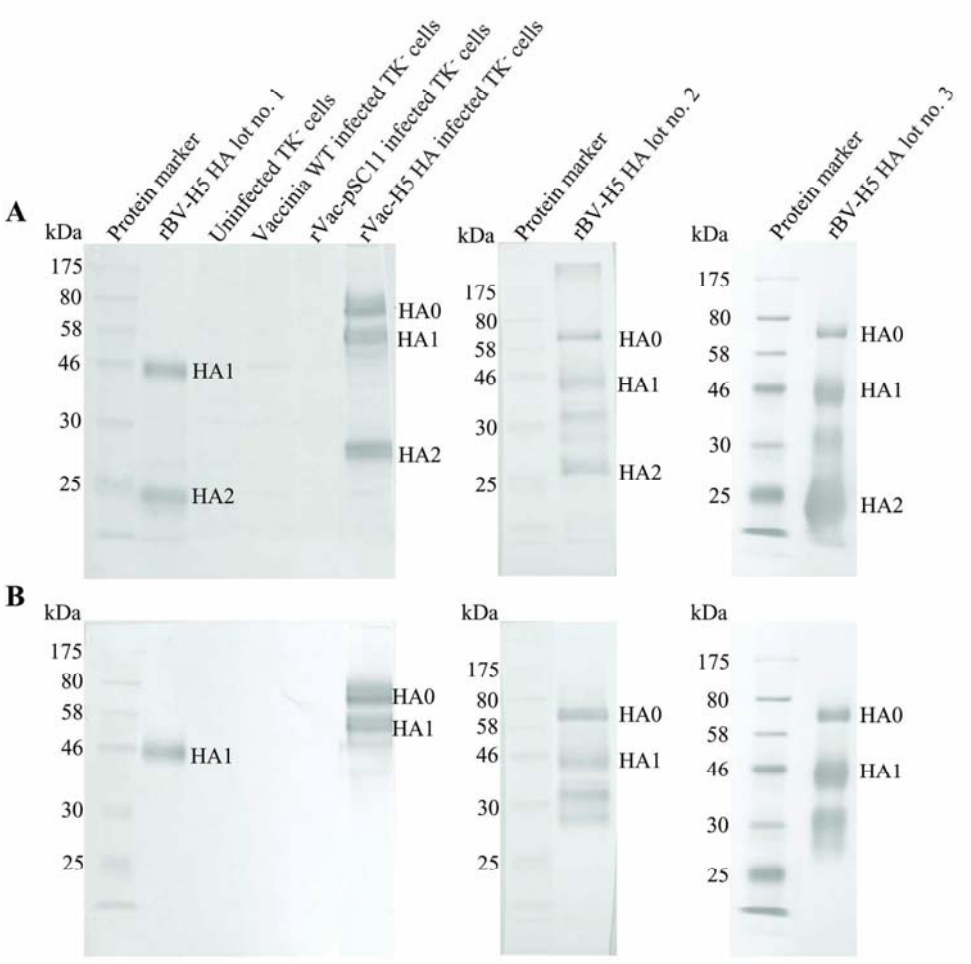

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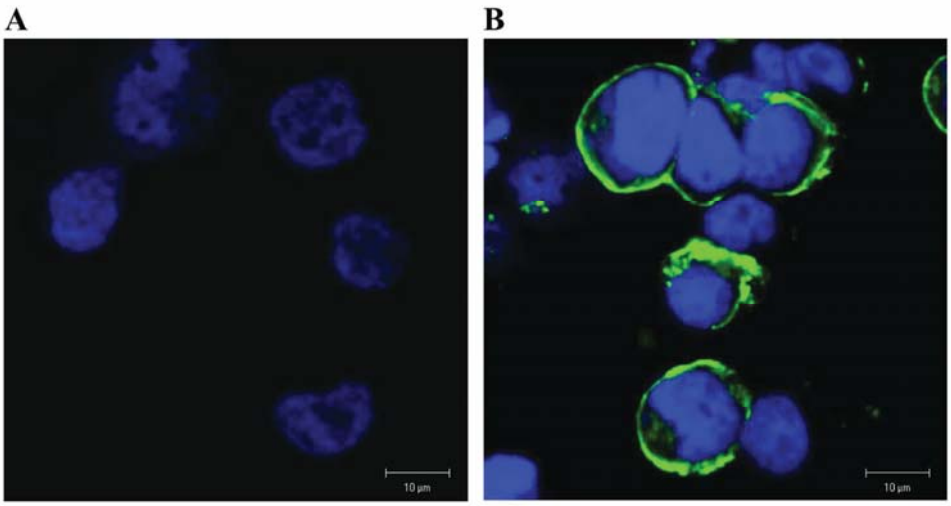

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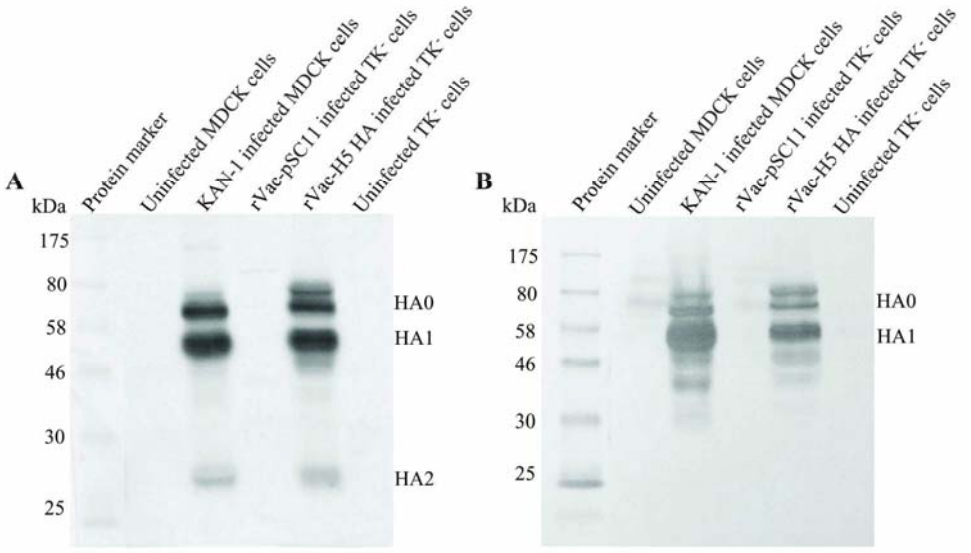

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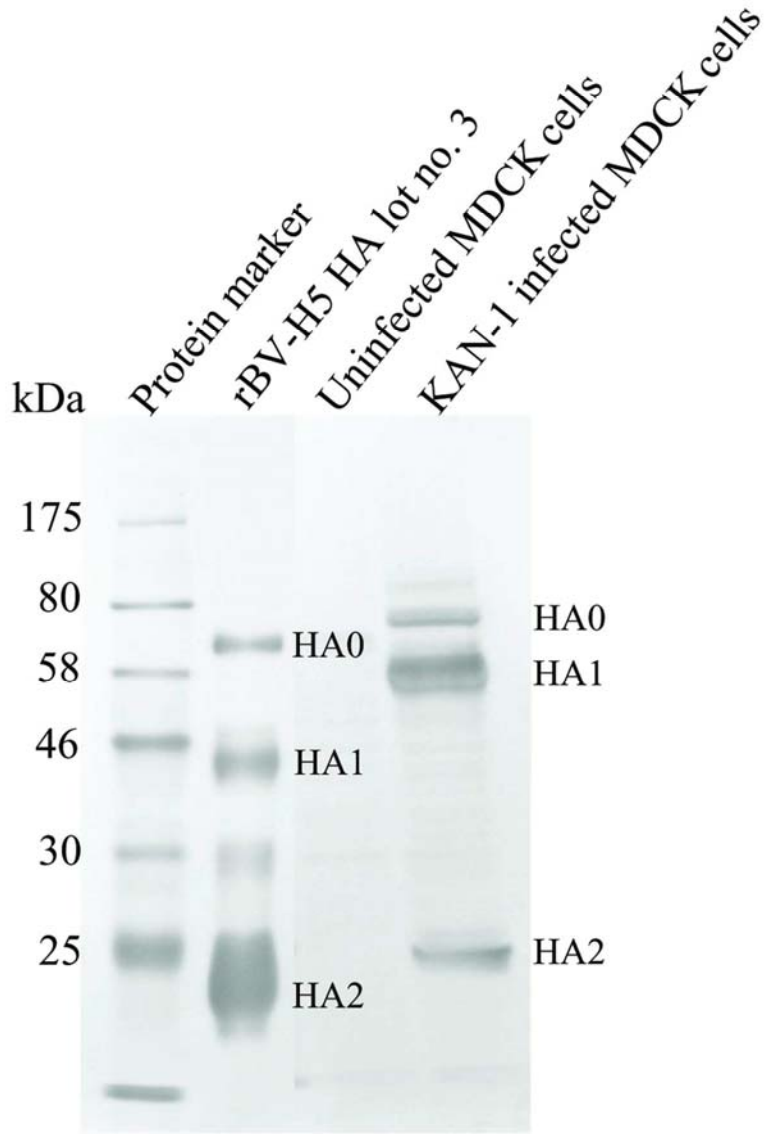

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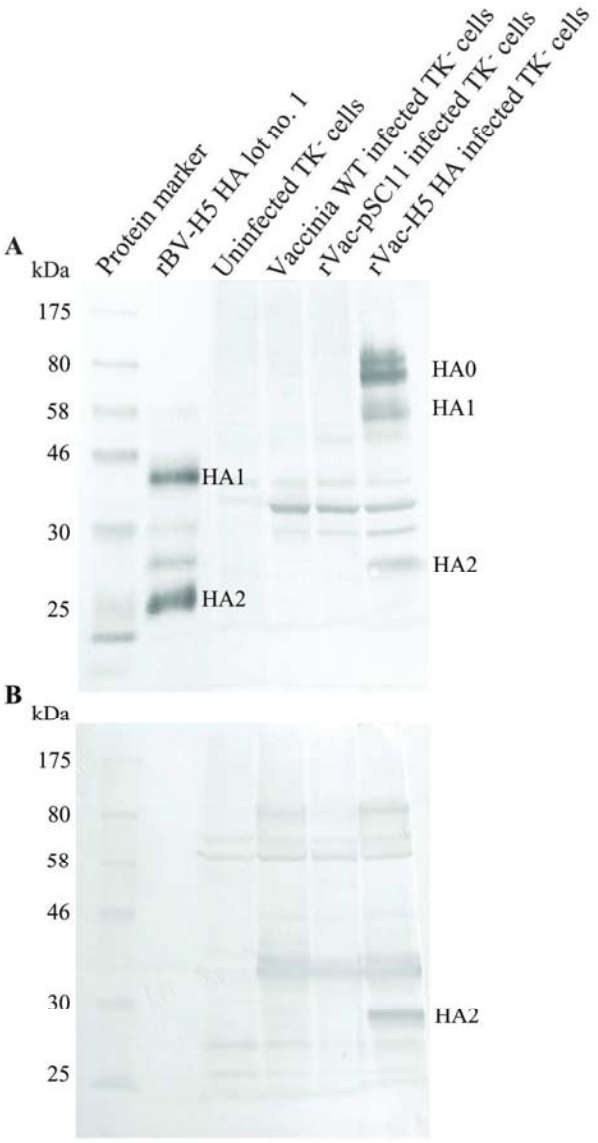

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Electron micrographs of highly and low pathogenic avian influenza viruses

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Short running title: Morphology of influenza virus particles

Background: The outbreak of highly pathogenic avian influenza (HPAI) H5N1 virus was first reported in Thailand in 2004. Up to present, electron micrographs demonstrating the morphology of HPAI H5N1 virus particle are quite limited.

Objective: To demonstrate the morphology of free particles of human influenza viruses, HPAI H5N1 viruses and low pathogenic avian influenza (LPAI) viruses as well as the H5N1 structural components in the infected cells. In addition, the amino acid substitutions affecting the virus morphology was also investigated.

Methods: Electron micrographs of the negative stained virus particles and the positive stained thin sections of the HPAI H5N1 virus infected cells were visualized under a transmission electron microscope (TEM). The M1 and M2 amino acid sequences were retrieved from GenBank.

Results: Morphologically, the free influenza virus particles appeared in 3 forms: spherical, regular and irregular rods, and long filamentous particles, were demonstrated. However, spherical form was the most predominant morphological type and took account of more than 80% of the virus populations examined. In addition, the step of viral entry and exit including incomplete particles in the infected MDCK cells were found. ~~mainly spherical.~~

Conclusion: Of all virus isolates studied, we demonstrated that the spherical particles were the major -population observed regardless of virus subtypes, host of origin, virus virulence, passage history and amino acid substitutions in M1and M2 proteins.

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***Key words*:** electron micrographs, virus morphology, influenza virus, highly pathogenic avian influenza H5N1 virus, low pathogenic avian influenza virus

Introduction

Influenza A virus particle comprises a 8 segmented RNA genome surrounded by nucleocapsid and the outermost envelope which is underlined with matrix protein and covered with two kinds of surface glycoprotein projections: hemagglutinin (H) and neuraminidase (N) [1]. Influenza virus particles are pleomorphic and vary in size. The visualized morphology under an electron microscope was classified into three forms: the spherical forms with the diameter at range of 80 to 120 nm, the rod forms at size of 120-300 nm, and the filamentous forms which are normally longer than 300 nm [1-4].

Collective information from previous works suggested that morphology of influenza virus particles were influenced by two factors: type of host in which the viruses were propagated - either cell culture or embryonated egg, and virus passage at investigation - either early or late passage [5-6]. It was reported that the filamentous form predominated among the virus population isolated from chick embryo or cell culture at early passages; alternatively, the laboratory-adapted strains were predominantly spherical [5]. On the other hand, it had been reported that both filamentous and spherical forms appeared simultaneously in tissue culture [2, 6]. Moreover, it had been suggested that spherical form was the final product from segmentation of filamentous particles [5]. Subsequent observation showed that polarized cell type and integrity of the actins microfilament network were important for the formation of filamentous particles [6]. On the other hand, it had been suggested that morphologic features of influenza viruses were under genetic control, not a result of host induced modification [7-11]. It had been suggested that filament forming ability of the viruses was genetically stable under control of multiple genes: *H*, *N*, *M*) (matrix) and *NP* (nucleoprotein) genes [8, 12]. Using reverse genetic technique to analyze M1 amino acid sequences, Elleman and Barclay [9] suggested that 3 residues: Ala41, Arg95 and Ala218 might contribute to filamentous morphology; whereas the residues Arg95 and Glu204 were

proposed by Bourmakina et al. [10]. In addition, Rossman et al. [11] suggested that Ser71, Met72 and Arg73 in M2 cytoplasmic tail may contribute to the formation of filamentous morphology.

On the basis of H and N antigens, influenza A viruses are further divided into 17 H and 9 N subtypes [13-16]. The first 16 subtypes have been found in aquatic birds; and most of them are low pathogenic, except for some isolates in H5 and H7 subtypes that are considered to be highly pathogenic avian influenza (HPAI) viruses [1, 13-15]. The 17th subtype was detected in bats by molecular techniques, but the attempts to isolate the virus did not success [16]. On the other hand, influenza in human population is attributed only to H1N1, H1N2, H2N2 (which existed from 1957 to 1968) and H3N2 subtypes [17-18]. Nevertheless, a cross species barrier of avian viruses to humans had been documented [19]. Transmission of HPAI H5N1 viruses from poultry to humans were first reported in 1997 and the disease subsided within that year [ref]. The re-emergence of HPAI H5N1 outbreaks since 2003 is even more catastrophic. It globally spreads and involved more human cases with higher fatality rate of about 60% [ref].

Electron micrographs of influenza virus particles had been demonstrated by groups of investigators for longer than 50 years ago, and those studies were mostly confined to human viruses [2-4]. Novel influenza subtypes have been discovered time to time, assuming that morphology of these influenza subtypes are similar to what had been previously shown. Up to present, electron micrographs demonstrating the morphology of avian influenza virus particle, especially HPAI H5N1 viruses are quite limited. Eventually, the viral morphology is one piece of basic information for characterizing a newly virus. With the higher resolution of the electron microscope and the higher quality of reagents and equipment now supported, it is interesting to characterize the morphology of H5N1 virus in terms of: 1) comparative morphology with human influenza viruses and LPAI viruses; 2) the viral structures during

replication in MDCK cells; and 3) the amino acid residues in M1 and M2 proteins that might affect the virus morphology.

Materials and methods

The study viruses

The virus isolates employed in this study were derived from either human or poultry origin. List of the viruses studied comprised 9 HPAI H5N1 isolates (4 from humans and 5 from avian), 4 LPAI isolates and 2 human influenza isolates. These viruses were isolated and propagated in Madin-Darby canine kidney (MDCK) cells or in 9 days-old chick embryonated eggs as shown in **Table 1**. MDCK cells were grown in Eagle's minimal essential medium (EMEM) (Gibco, U.S.A.) containing 10% fetal bovine serum (FBS) (Hyclone, U.S.A.), 200 U/ml penicillin, 20 µg/ml gentamycin and 1 mg/ml fungizone. Upon virus propagation, cells were maintained in EMEM supplemented with antibiotics, fungizone and 1.5 µg/ml trypsin tosyl phenylalanyl chloromethyl ketone (TPCK, Sigma, St. Louis, MO), and no FBS supplement. These virus isolates were stored at -80°C until tested. The experiments on H5N1 viruses were performed in the biosafety laboratory level 3 (BSL-3) in the Faculty of Medicine Siriraj Hospital and the Faculty of Veterinary Science, Mahidol University.

Negative staining of the free virus particles

In order to destroy the viral infectivity, the virus particles in the culture supernatants or allantoic fluids were treated with 2% glutaraldehyde before spinning by high speed centrifugation at 18,000x for 90 minutes. The viral pellets were negatively stained with phosphotungstic acid (PTA), and morphology of the stained virus particles were visualized under a TEM (JEM-1230, JEOL Company, Tokyo, Japan) [20]. Briefly, 10 µl of the virus suspension was absorbed on a grid (SPI supplies, 200 meshes, West Chester, U.S.A.) coated

with formvar-carbon for 1 minute; the excess virus suspension was drained off, followed by UV irradiation of both sides of the grid for 5 minutes. A drop of 1.6% PTA, pH 7.0 is then applied for 1 minute, and the excess fluid was removed prior to examining under a TEM.

Staining of HPAI H5N1 virus infected MDCK cells

MDCK cell monolayer was inoculated with the test virus, A/Thailand/1(KAN-1)/2004 (H5N1), at multiplicity of infection of 1 for 24 hours at 37°C in a CO_2 incubator. The infected cells were pelleted before processing and embedding. Thin sections of the virus infected cells and the uninfected cell control were positively stained and examined under a TEM as previously described [21]. The cell pellets were pre-fixed with 4% glutaraldehyde in PBS for 30 minutes at 4°C, and followed by 3 washes with Millonig's phosphate buffer and post-fixed with 2% phosphate buffered osmium tetroxide for 30 minutes at room temperature, and lastly by two washes in distilled water. Then, the cells were stained with 2% uranyl acetate aqueous solution for 20 minutes at room temperature as described after the protocol for rapid tissue processing [22]. The stained sample was dehydrated in an order of steps as follows: 70% ethyl alcohol for 90 seconds with 2 changes, 80% ethyl alcohol for 90 seconds with 2 changes, 90% ethyl alcohol for 3 minutes with 2 changes, 95% ethyl alcohol for 3 minutes with 3 changes, absolute ethanol for 3 minutes with 3 changes, and, lastly, with propylene oxide for 3 minutes with 3 changes. Then, the stained sample was infiltrated with the 50:50 mixture of propylene oxide and epoxy resin for 30 minutes at 37°C. Subsequentlyt, the mixture solution was replaced with epoxy resin solution and further incubated for 2 hours at 37°C. Finally, the sample was embedded in polypropylene capsules and polymerized in hot air oven at 70°C overnight followed by sectioning with ultra-microtome. The ultra-thin section was mounted onto a copper support grid (SPI supplies, 200 meshes, West Chester, U.S.A.) prior to nuclear staining with uranyl acetate for 30 minutes and followed by

cytoplasmic staining with lead citrate for 15 minutes. The stained grid was examined under a TEM.

Genetic characterization of the study viruses

Our study analyzed the amino acid residues that might affect the virus morphology after those described by Elleman and Barclay [9] and Bourmakina and Garcia-Sastre [10] for M1; and by Rossman et al. for M2 [11]. The M1 and M2 amino acid sequences of our study viruses together with the reference filamentous or spherical type particles were retrieved from the GenBank database (**Table 1**). The amino acid sequences were aligned using the programs BioEdit version 7.0.9.0 [23]. The HPAI H5N1 viruses in this study belong to clade 1, except A/Laos/Nongkhai 1/2007 which belongs to clade 2.3.4.

Result

Electron microscopy of influenza virus particles

Electron micrographs of HPAI H5N1 virus particles originated from humans and avian are demonstrated in **Figures 1** and **2**, respectively; and those of human H1N1 and H3N2 viruses as well as LPAI H3N8, H5N3 and H7N1 viruses are shown in **Figures 3** and **4**, respectively. These virus isolates comprised 3 morphological types of particles: spherical at diameter of 80-120 nm, regular and irregular rods at length between 120-300 nm and filaments at length longer than 300 nm. Nevertheless, majority of them were spherical particles. The multilayered-coil structure suggestive of the helical ribonucleocapsids could be seen inside a virus particle in this study **(Figure 3a).**

Using electron micrographs at magnification of 50,000x and/or 80,000x, numbers of virus particles with different morphological types in a virus isolate was enumerated. In order to avoid an invalid data as too low number of the particles was present in the stained samples,

the results were excluded when the counting number was lower than 80. Therefore, the enumeration was successful with 3 HPAI H5N1 virus isolates and one human H1N1 isolate which demonstrated that approximately 80-90% of the virus populations observed were spherical, and the remaining was the mixed population between rod and filamentous particles **(Table 2).** Unfortunately, numbers of the viral particles for LPAI viruses and human viruses were less than 30 particles; therefore, the counting numbers obtained were excluded from percentage calculation. Nevertheless, it was clearly seen that most of particles were spherical. Collectively, we demonstrated that majority of all influenza viruses in this study were spherical, while filamentous particles were the minority.

Electron microscopy of influenza virus infected MDCK cells

H5N1 virus infected MDCK cells as well as the uninfected cell control was examined using positive staining technique. Morphology of the uninfected MDCK cell surface appendages might be mislead for their appearance as the filamentous like particle (**Figure 5a**). The infected cell with chromatin condense suggesting of cell necrosis (**Figure 5b**), as well as invagination of cell membrane to endocytose a virus particle (**Figure 5c**), budding virus particles (**Figures 5d** and **5e**) and the released virus particles (**Figure 5f**) were visualized. Spherical particle was the predominant form of HPAI H5N1 viruses in all stages of replication in MDCK cells.

Genetic characterization of the study viruses

Our study analyzed the amino acid residues that might influence the formation of filamentous or spherical particle according to previous groups of investigators, i.e., the residues Ala41, Arg95 and Ala218 in M1 for filamentous particle formation and the residues …. and … for spherical particle formation were followed after Elleman and Barclay [9]; the

residues Arg95 and Glu204 in M1 for filamentous particle formation were followed after Bourmakina and Garcia-Sastre [10]; and the residues Ala71, Ala72 and Ala73 in M2 cytoplasmic tail for filamentous particle formation were followed after Rossman et al. The analyses demonstrated the presence of all amino acid residues that were suggestive of filamentous morphology in almost of our virus isolates which contained mainly the spherical type particles **(Table 3).** Therefore, our study suggested that there was no amino acid residue unique for certain morphological type of the viral particles.

Discussion

Based on electron microscopy, this study demonstrated 3 morphological types of the virus particles: spherical, rod, and filamentous forms in all of the influenza viruses studied, irrespective of their human or avian host of origin and of their low or high pathogenicity. However, the spherical particles were the predominant population, whereas filamentous particles were the minor populations which took account for approximately 4-16.5%. A previous group of investigator [5] reported that the long filamentous form was predominantly found in early passages of the viruses grown in embryonated eggs; and the filamentous form could turn to be the spherical form after a few to 4-7 sub-passages. Unfortunately, we did not have an opportunity to study the virus isolates at earlier passages due to an inadequate numbers of the virus particles for electron microscopic examination. Nevertheless, almost of our virus isolates were younger than 8 sub-passages. On the other hand, our finding was supported by the electron microscopic study on HPAI H5N1 viruses of the 1997 outbreak in Hong Kong, in which the spherical virions were predominant among the mixed virus population after few subpassages in embryonated eggs, i.e., 65% for the viruses isolated from chicken and 84% for those isolated from humans [24].

Refer to the genetic control of the virus morphology, we analyzed 5 amino acid residues (Ala41, Arg95, Glu204 and Ala218) in M1 and 3 amino acid residues (Ser71, Met72 and Arg73) in M2 cytoplasmic tail that were predictive of filamentous feature together with 4 amino acid residues (Val41 and Lys95 ……) in M1 that were suggestive of spherical morphology based on previous reports [9,… We demonstrated that Ala41, Arg95, Glu204, Thr218, Ser71, Met72 and Arg73 were found in almost of our viruses which contained mainly the spherical particles, irrespective of the virus subtypes, host of origins and the virus virulence. Therefore,our study suggest that the morphology of influenza virus particles is less likely under control of the viral genetics. Unfortunately, we have no opportunity to explore if it is influenced by the number of virus passages.

Bruce et al. [26] demonstrated that TEM failed to demonstrate the morphogenesis of the long filament viruses extruding from the infected cells present in the positively stained thin section, whereas it was successful by the scanning electron microscopy (SEM). In this study, TEM was employed to demonstrate the virion at entry and exit from the infected cells. The multilayered-coil structure of a virus similar to what reported by Clader et al. [27] was visulaized. In addition, the appearance of surface pilli on the uninfected MDCK cell membrane which some time may be misinterpreted as filamentous particles [3] could be identified by the high resolution and magnification of TEM together with professional experience.

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1U19CI000399-01 from Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC. PR was from Srinakharinwirot University. The authors would like to express their sincere thanks to Mekkla Thompson and Steve Durako, Westat, U.S.A. for their kind coordination, and also to Dr. Dianne Gross and Dr. Jacquerine Katz, CDC for their kind supervision of the project. There is no conflict of interest that might influence the outcome of this study.

References

1. Wright PF, Webster RG. Orthomyxoviruses. In: Knipe GM, Howley PM, editors. Fields virology. Philadelphia: Lippincott Williams & Wilkins; 2001. p.1533-79.
2. Wyckoff RW. Formation of the particles of influenza virus. J Immunol. 1953;70:187-96.
3. Morgan C, Roee HM, Moore DH. Structure and development of viruses observed in the electron microscope. J Exp Med. 1956;104:171-82.
4. Murphy JS, Bang FB. Observations with the electron microscope on cells of the chick chorio-allantoic membrane infected with influenza virus. J Exp Med. 1952;95:259-68.
5. Choppin PW, Murphy JS, Tamm I. Studies of two kinds of virus particles which comprise influenza A virus strains. J Exp Med. 1960;112:945-52.
6. Roberts PC, Compans RW. Host cell dependence of viral morphology. Proc Natl Acad Sci U S A. 1998;95:5746-51.
7. Roberts PC, Roberts PC, Lamb RA, Compans RW. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virol. 1998: 240:127-37
8. Smirnov YA, Kuznetsova MA, Kaverin NV. The genetic aspects of influenza virus filamentous particle formation. Arch Virol. 1991;118:279-84.

9. Elleman CJ, Barclay WS. The M1 matrix protein controls the filamentous phenotype of influenza A virus. Virol. 2004;32:144-53.
10. Bourmakina SV, Garcia-Sastre A. Reverse genetics studies on the filamentous morphology of influenza A virus. J Gen Virol. 2003;84:517-27.
11. Rossman JA, Jing X, Leser GP, Balannik V, Pinto LH, Lamb RA. Influenza virus M2 ion channel protein is necessary for filamentous virion formation. J Virol. 2010;84:5078-88.
12. Jin H, Leser GP, Zhang J, Lamb RA. Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. EMBO J. 1997; 16:1236-47.
13. Fouchier RA, Osterhaus AD, Brown IH. Animal influenza virus surveillance. Vaccine. 2003;21:1754-57.
14. Avian influenza. World Organisation for Animal Health (OIE) [online]. 2009 [cited 2009 Aug 25], Available from: http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf
15. Peiris JSM, de Jong MD, Guan Y. Avian Influenza virus (H5N1): a threat to human health. Clin Microbiol Rev. 2007;20:243-67.
16. Tong S, Li Y, Rivailler P, Conrardy C, Alvarez Castillo DA, Chen L-M, et at. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci USA. 2012;109:4269-74
17. World Health Organization: Influenza update. Http//www.who.int/influenza/surveillance_monitoring/updates/latest_update_GIP_surveillance/en/index.html.
18. World Health Organization: Influenza A (H1N2) viruses (update). Wkly Epidemiol Rec. 77;77-80.
19. Neumann G, Kawaoka Y. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis. 2006;12:881-6.

20. Hazelton PR, Gelderblom HR. Electron microscopy for rapid diagnosis of infectious agents in emergent situation. Emerg Infect Dis. 2003;9:294-303.
21. Thakerngpol K, Stitnimankarn T. The role of electron microscopy in viral diagnosis. Siriraj Hosp Gaz. 2003;55:459-62.
22. Rowden G, Lewis MG. Experience with a three-hour electron microscopy service. J Clin Path. 1974;25:505-10.
23. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. Nucleic Acids Symp Ser. 1999;4:95-8.
24. Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, et al. Characterization of Avian H5N1 Influenza Viruses from Poultry in Hong Kong. Virol. 1998;252:331-42.
25. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature. 2009;460:1021-5.
26. Bruce EA, Digard P, Stuart AD. The Rab11 pathway is required for influenza A virus budding and filament formation. J Virol. 2010;84:5848-59.
27. Calder LJ, Wasilewski S, Berriman JA, Rosenthal PB. Structural organization of a filamentous influenza A virus. Proc Natl Acad Sci. 2010;197:10685-90.
28. Zhang C, Yang Y, Zhou X, Liu X, Song H, He Y, et al. Highly pathogenic avian influenza A virus H5N1 NS1 protein induces caspase-dependent apoptosis in human alveolar basal epithelial cells. Virol J. 2010;7:51-6.

Figure legends

Figure 1. Electron micrographs of HPAI H5N1 isolates from humans: A/Thailand/676(NYK)/2005 (**a**); A/Laos/Nong khai 1/2007 (**b**); A/Thailand/1(KAN-1)/2004 (**c**, **d**); and A/Thailand/NBL 1/2006 (**e**, **f**). Note pleomorphic morphology of spherical and filamentous particles.

Figure 2. Electron micrographs of HPAI H5N1 isolates from animals: A/Chicken/Thailand/ICRC-VS143/2007 (**a**); A/Chicken/Thailand/ICRC-VS195/2008 (**b**); A/Chicken/Thailand/ICRC-VS213/2007 (**c**); and A/Duck/Thailand/ICRC-VS629/2008 strains (**d**).

Figure 3. Electron micrographs of human influenza viruses: A/New Caledonia/20/1999 (H1N1)-like virus (**a**, **b**) w; and A/Fujian/411/2002 (H3N2)-like virus (**c**, **d**). The multilayered-coil structure of a virus is demonstrated in spherical (**a**) and rod (**d**) particles.

Figure 4. Electron micrographs of LPAI isolates from animals: A/Aquatic bird/Hong Kong/D125/2002 (H1N1) (**a**); A/Duck/Shantou/1883/2001 (H3N8) (**b**); A/Duck/Jiangxi/6151/2003 (H5N3) (**c**); and A/Ostrich/Zimbabwe/222/1996 (H7N1) (**d**).

Figure 5. Electron micrographs of H5N1 infected MDCK cells: MDCK cell control (**a**); cytopathic change of H5N1 infected cell (**b**); H5N1 virus [A/Thailand/1(KAN-1)/2004] at entry (**c**); virus budding from infected cell (**d**); virus releasing from the infected cells (**e**); and free virions (**f**).

Figure 6. Amino acid sequence alignment of influenza M1 protein. The programs BioEdit version 7.0.9.0 were used to produce the alignment. Boxes indicate the positions of amino acid substitution.

Figure 7. Amino acid sequence alignment of influenza M2 protein. The programs BioEdit version 7.0.9.0 were used to produce the alignment. Boxes indicate the positions of amino acid substitution.

Table 1 The study viruses and GenBank accession numbers of influenza M1 and M2 amino acid sequences.

Virus name	Source*	Passage history**	GenBank accession No.	
			M1 protein	M2 protein
H5N1 influenza viruses*				
A/Thailand/1(KAN-1)/2004	MU	LLC-MK2/ MDCK 6	AAV35110	AAV35111
A/Thailand/676(NYK)/2005	MU	MDCK 5	ABC72651	ABC72652
A/Laos/Nong khai 1/2007	MU	MDCK 4	ACA64012	ACA64013
A/Thailand/NBL 1/2006	MU	MDCK 4	ACU46646	ACU46647
A/Chicken/Thailand/ICRC-VS143/2007	MU	MDCK 3/Egg 1	ABW89592	ABW89593
A/Chicken/Thailand/ICRC-VS195/2008	MU	Egg 4	ACE73587	ACE73588
A/Chicken/Thailand/ICRC-VS 213/2007	MU	MDCK 3/Egg 1	ACF36779	ACF36780
A/Duck/Thailand/ICRC-VS629/2008	MU	Egg 3		
A/Chicken/Thailand/ICRC-VS1069/2008	MU	Egg 2		
Human influenza viruses				
A/New Caledonia/20/99 (H1N1)-like virus (Siriraj07/2000)	MU	MDCK 8	ABF21304	ABF21305
A/Fujian/411/02 (H3N2)-like virus (Siriraj 03/2004)	MU	MDCK 8	-	ABB71834
Low pathogenic avian influenza (LPAI) viruses				
A/Aquatic bird/Hong Kong/D125/2002 (H1N1)	SJ	Egg 1/MDCK 3		
A/Duck/Shan tou/1883/2001 (H3N8)	SJ	Egg X/MDCK 2		
A/Duck/Jiangxi/6151/2003 (H5N3)	SJ	Egg 4	ABA12315	ABA12316
A/Ostrich/Zimbabwe/222/1996 (H7N1)	SJ	Egg 2		
Reference strains				
A/Udorn/1972(H3N2)	-	-	ABD79033	ABD79034
A/FW/1/1950(H1N1)	-	-	CAA30888	CAA30889
A/Port Chalmers/1/1973-mouse adapted(H3N2)	-	-	CAA30886	CAA30887

*MU= Mahidol University; SJ =St. Jude Children's Research Hospital

**All H5N1 viruses belong to clade 1 except A/Laos/Nong khai 1/07 which belongs to clade 2; Egg X = unknown passage in egg

Table 2 Morphological types of HPAI H5N1 and H1N1 viruses

Morphological type	Spherical forms	Rod and filamentous forms	Total
A/Thailand/1 (KAN-1) /2004 (H5N1)	79 (90.8%)	8 (9.2%)	87 (100%)
A/Thailand/676(NYK)/2005 (H5N1)	223 (95.7%)	10 (4.3%)	233 (100%)
A/Laos/ Nongkhai/1 /2007 (H5N1)	147 (83.5%)	29 (16.5%)	176 (100%)
A/New Caledonia/20/99 (H1N1)-like virus (Siriraj07/2000)	113 (90.6%)	12 (9.4%)	125 (100%)

Table 3. Panel of amino acid residues that might affect influenza virus morphology in M1 and M2 protein

Virus name*	Amino acid residues in*						
	M1				M2		
	41	95	204	218	71	72	73
A/Udorn/301/1972 (H3N2) [filament]	A	R	E	A/V	S	M	R
A/Victoria/3/1975(H3N2) [filament]	A	R	E	A			
A/WSN/1933 (H1N1) [spherical]	V	K	D	T			
A/PR8/1934 (H1N1)) [spherical]	V	K	E	T			
A/Port Chalmers/1/1973-mouse adapted (H3N2) [spherical]	V	R	E	A	S	M	R
A/Thailand/1(KAN-1)/2004 (H5N1)	A	R	E	T	S	M	R
A/Thailand/676(NYK)/2005 (H5N1)	A	R	E	T	S	M	R
A/Laos/Nong khai 1/2007 (H5N1)	A	R	E	T	S	M	R
A/Thailand/NBL 1/2006 (H5N1)	A	R	E	T	S	M	R
A/Chicken/Thailand/ICRC-VS143/2007 (H5N1)	A	R	E	T	S	M	R
A/Chicken/Thailand/ICRC-VS195/2008 (H5N1)	A	K	E	T	S	M	R
A/Chicken/Thailand/ICRC-VS 213/2007(H5N1)	A	R	E	T	S	M	R
A/New Caledonia/20/99 (H1N1)-like virus (Siriraj07/2000)	A	R	E	A	S	M	R
A/Duck/Jiangxi/6151/2003 (H5N3)	-	-	E	T	S	M	R

*Position of amino acid residues at M1: 41, 95, 218 (Elleman and Barclay. Virol 2004) and 204 (Bourmakina and Garcia-Sastre. J Gen Virol 2003); and at M2: 71,72 and 73 (Rossman JA, et al. J Virol 2010)

**Abbreviations of amino acids: A= Ala; D= Asp; E =Glu; I= Ile; K =Lys; M = Met; R= Arg; S= Ser; T= Thr; V= Val

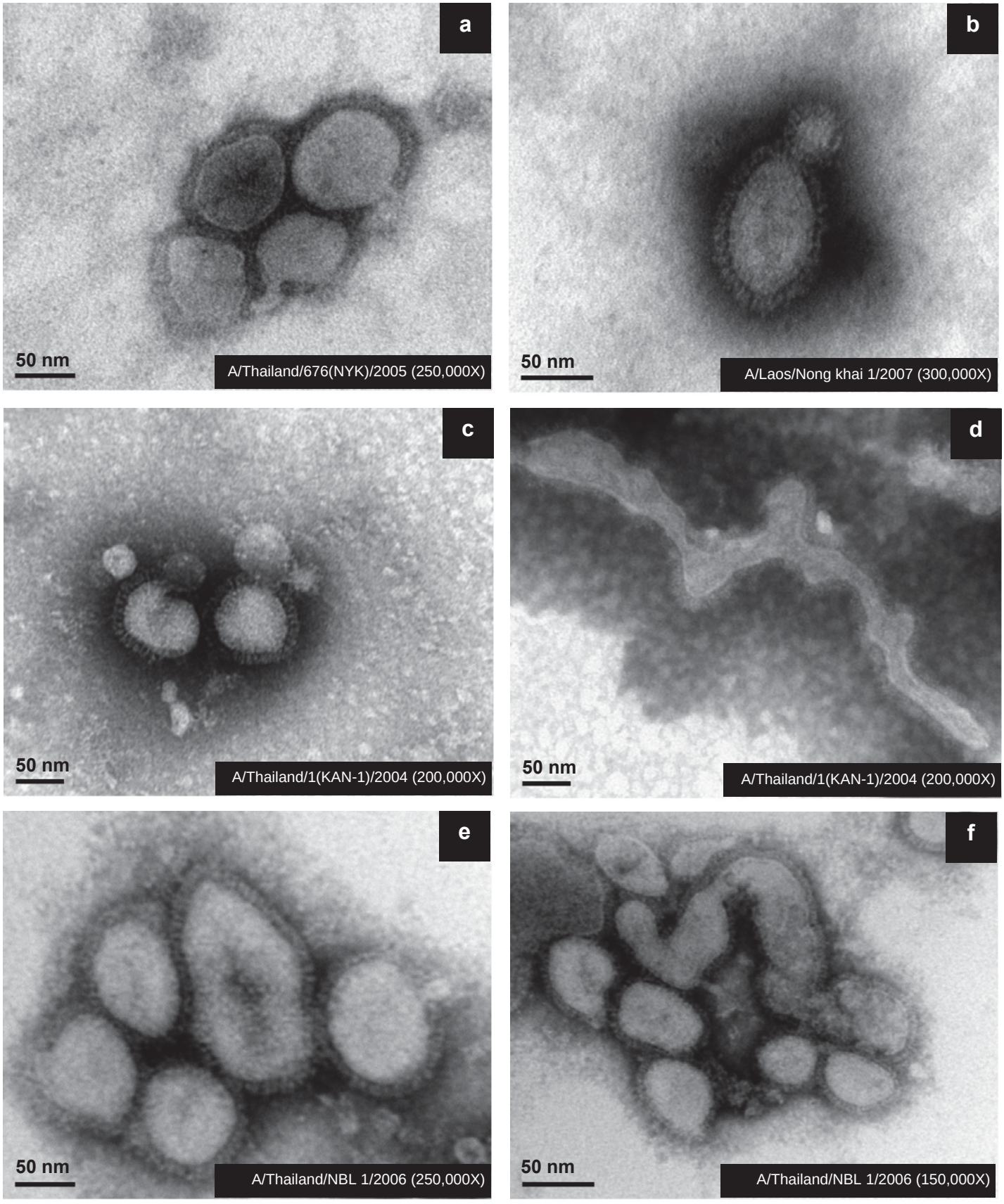

a
50 nm
A/Thailand/676(NYK)/2005 (250,000X)
b
50 nm
A/Laos/Nong khai 1/2007 (300,000X)
c
50 nm
A/Thailand/1(KAN-1)/2004 (200,000X)
d
50 nm
A/Thailand/1(KAN-1)/2004 (200,000X)
e
50 nm
A/Thailand/NBL 1/2006 (250,000X)
f
50 nm
A/Thailand/NBL 1/2006 (150,000X)

Figure 1.

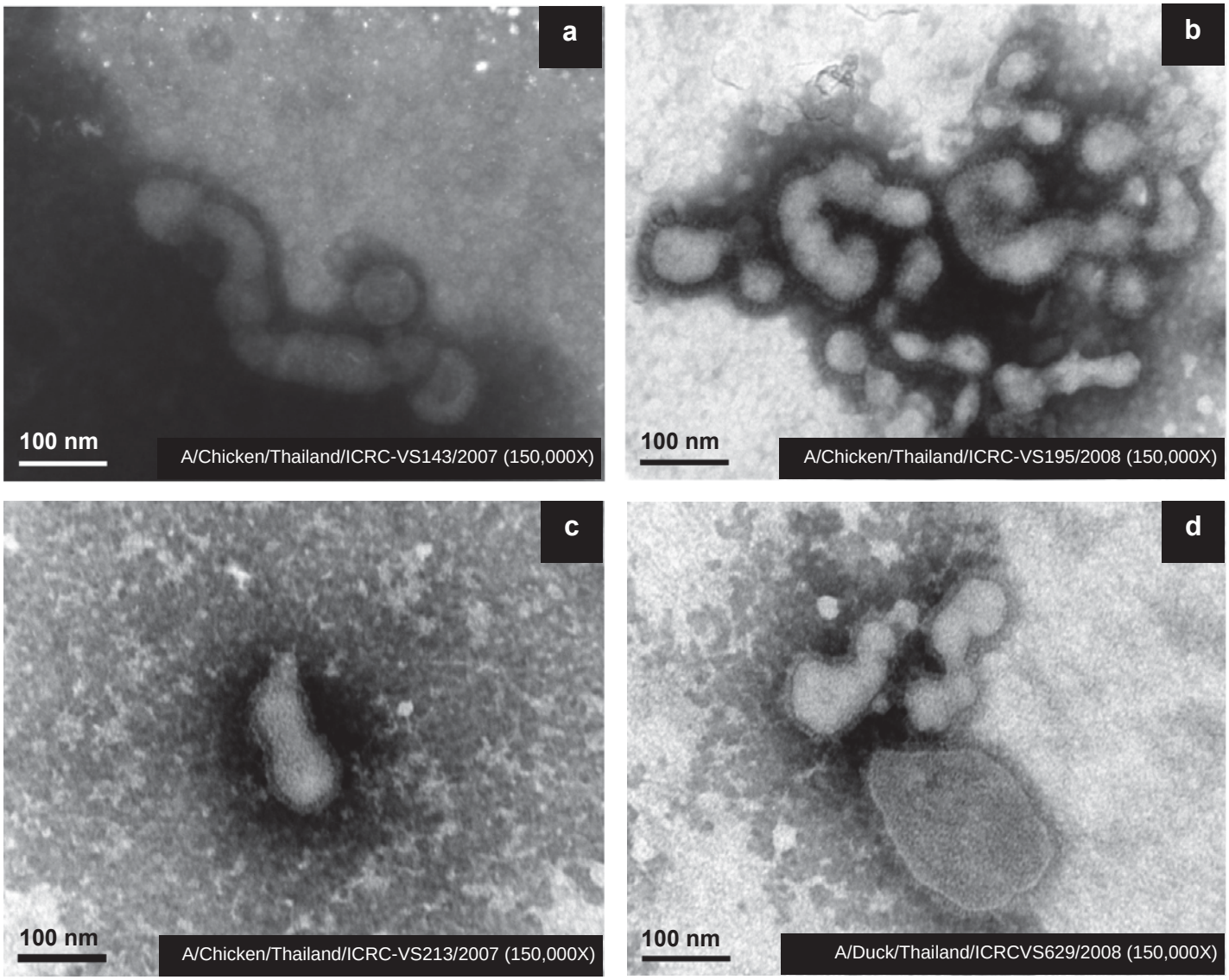

a
100 nm
A/Chicken/Thailand/ICRC-VS143/2007 (150,000X)
b
100 nm
A/Chicken/Thailand/ICRC-VS195/2008 (150,000X)
c
100 nm
A/Chicken/Thailand/ICRC-VS213/2007 (150,000X)
d
100 nm
A/Duck/Thailand/ICRCVS629/2008 (150,000X)

Figure 2.

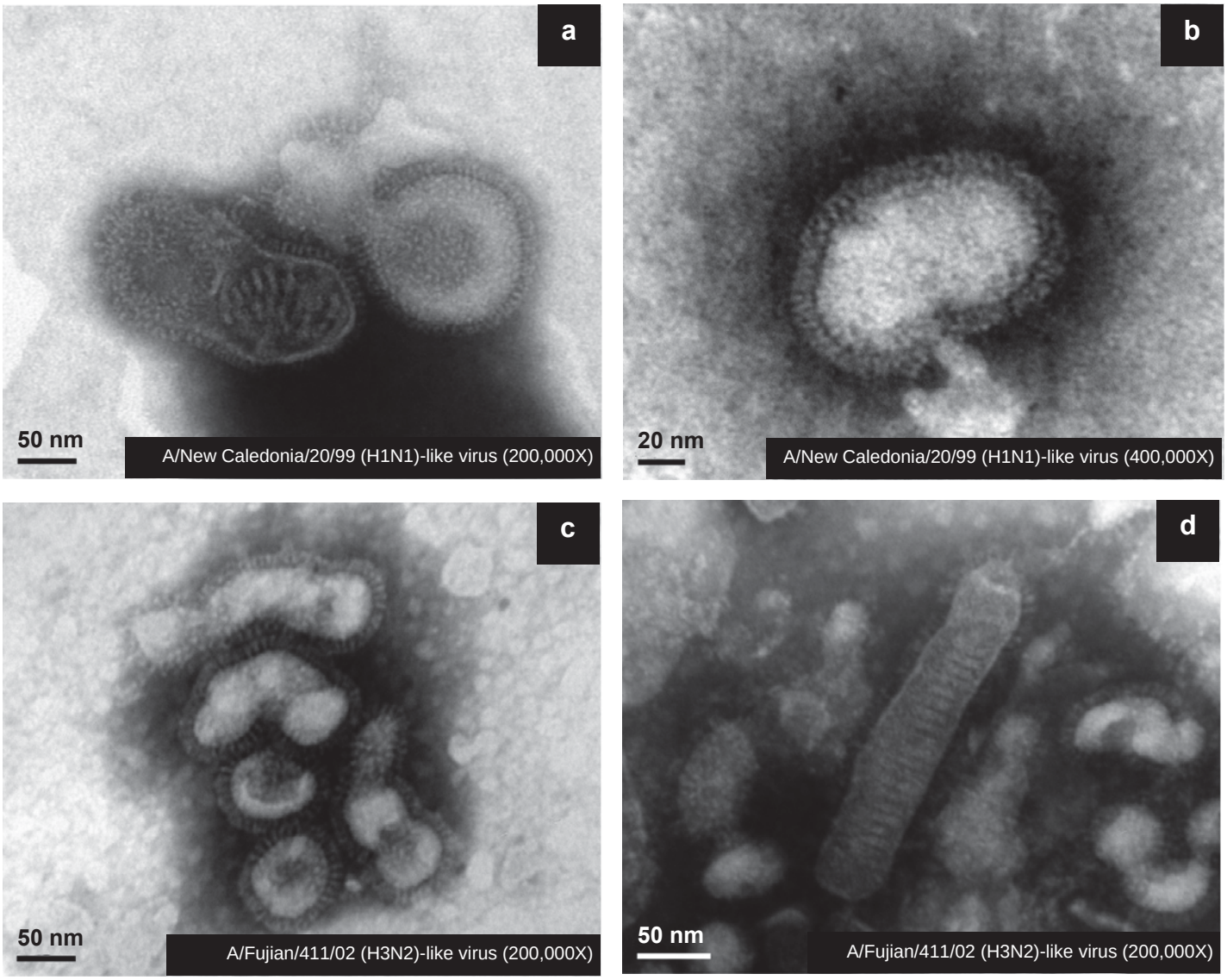

a
50 nm
A/New Caledonia/20/99 (H1N1)-like virus (200,000X)
b
20 nm
A/New Caledonia/20/99 (H1N1)-like virus (400,000X)
c
50 nm
A/Fujian/411/02 (H3N2)-like virus (200,000X)
d
50 nm
A/Fujian/411/02 (H3N2)-like virus (200,000X)

Figure 3.

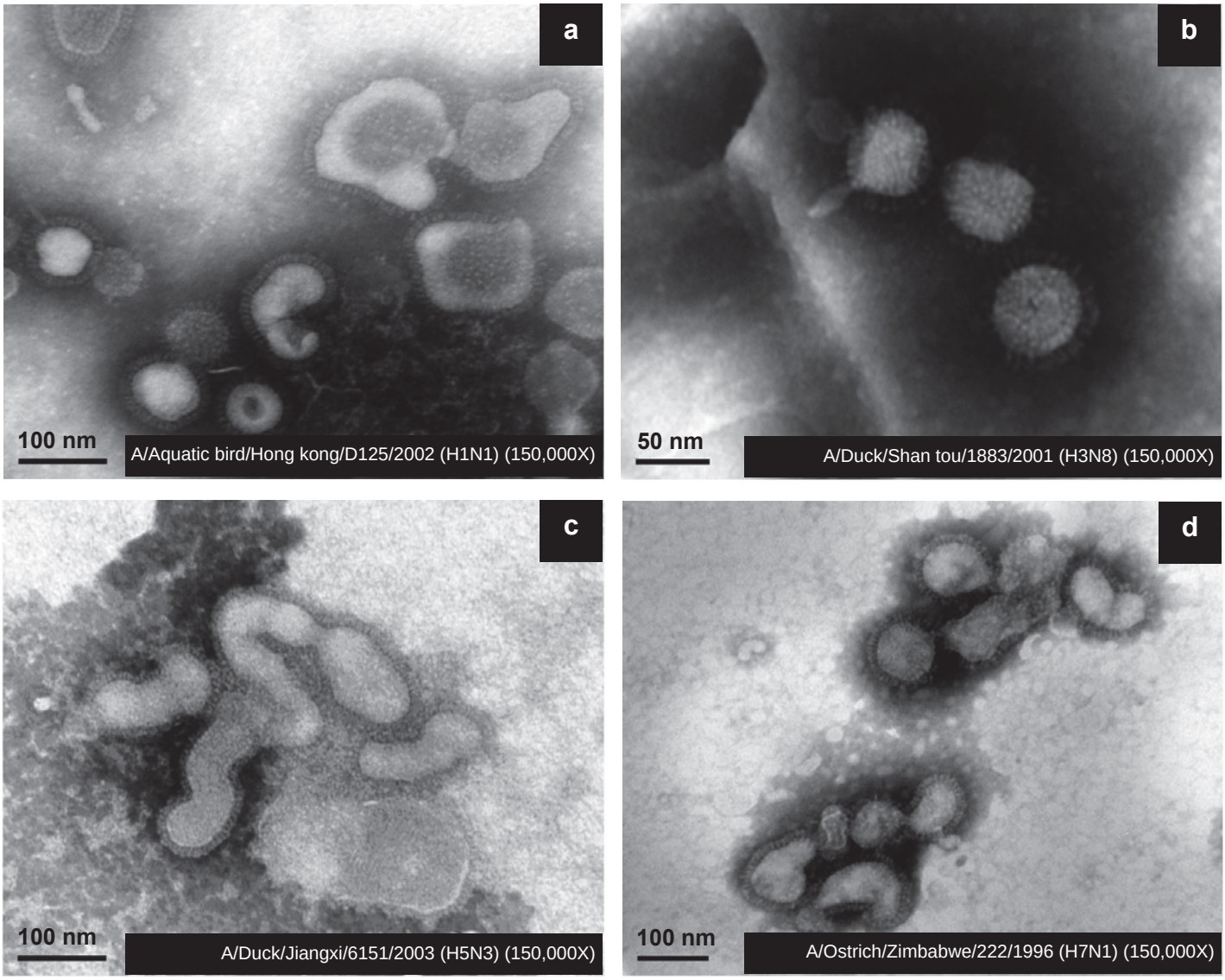

a
100 nm
A/Aquatic bird/Hong kong/D125/2002 (H1N1) (150,000X)
b
50 nm
A/Duck/Shan tou/1883/2001 (H3N8) (150,000X)
c
100 nm
A/Duck/Jiangxi/6151/2003 (H5N3) (150,000X)
d
100 nm
A/Ostrich/Zimbabwe/222/1996 (H7N1) (150,000X)

Figure 4.

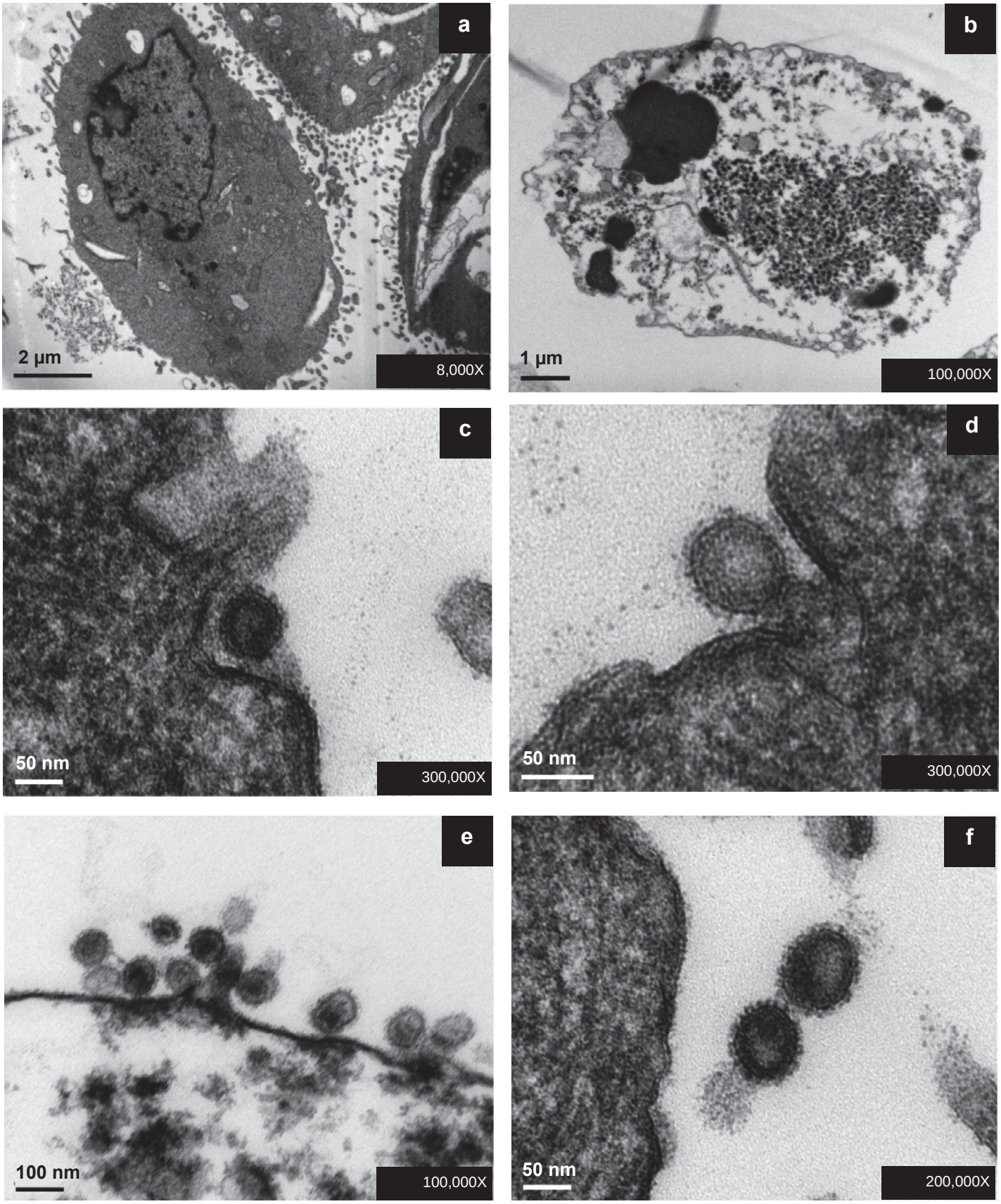
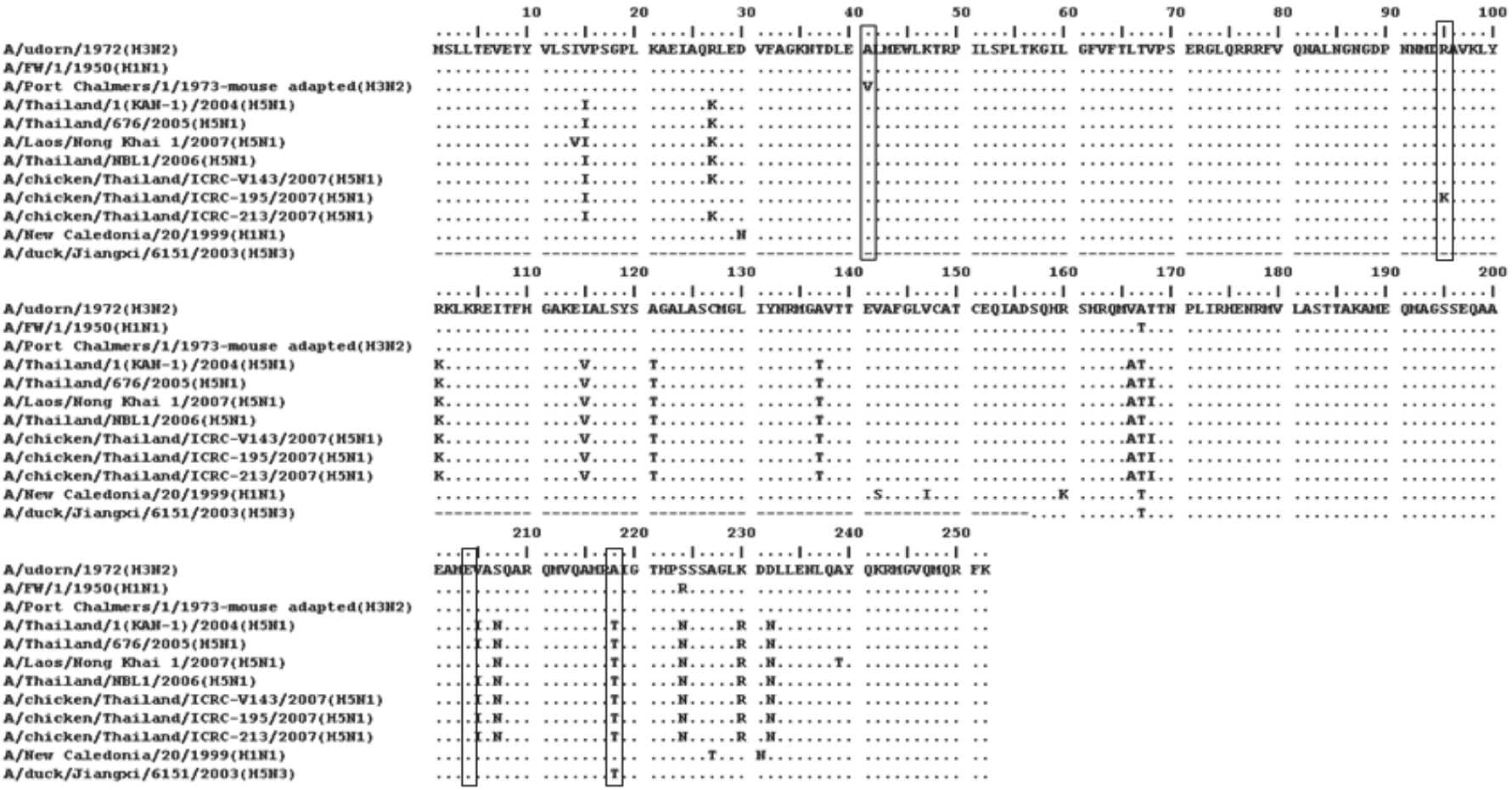

a
2 µm
8,000X
b
1 µm
100,000X
c
50 nm
300,000X
d
50 nm
300,000X
e
100 nm
100,000X
f
50 nm
200,000X

Figure 5.


A/udorn/1972(H3N2)
A/FW/1/1950(H1N1)
A/Port Chalmers/1/1973-mouse adapted(H3N2)
A/Thailand/1(KAN-1)/2004(H5N1)
A/Thailand/676/2005(H5N1)
A/Laos/Nong Khai 1/2007(H5N1)
A/Thailand/NBL1/2006(H5N1)
A/chicken/Thailand/ICRC-V143/2007(H5N1)
A/chicken/Thailand/ICRC-195/2007(H5N1)
A/chicken/Thailand/ICRC-213/2007(H5N1)
A/New Caledonia/20/1999(H1N1)
A/duck/Jiangxi/6151/2003(H5N3)
MSLLTEVETY VLSIVPSGPL KAEIAQRLED VFAGKNTDLE ALMEWLKTRP ILSPLTKGIL GFVFTLTVPS ERGLQRRRFV QNALNGNGDP NNMDRAVKLY
RKLKREITFH GAKEIALSYS AGALASCMGL IYNRMGAVTT EVAFGLVCAT CEQIADSQHR SHRQMVATTN PLIRHENRMV LASTTAKAME QMAGSSEQAA
EAMEVASQAR QNVQAMRAIG THPSSSAGLK DDLLENLQAY QKRMGVQMQR FK

1

2

3 **Figure 6.**

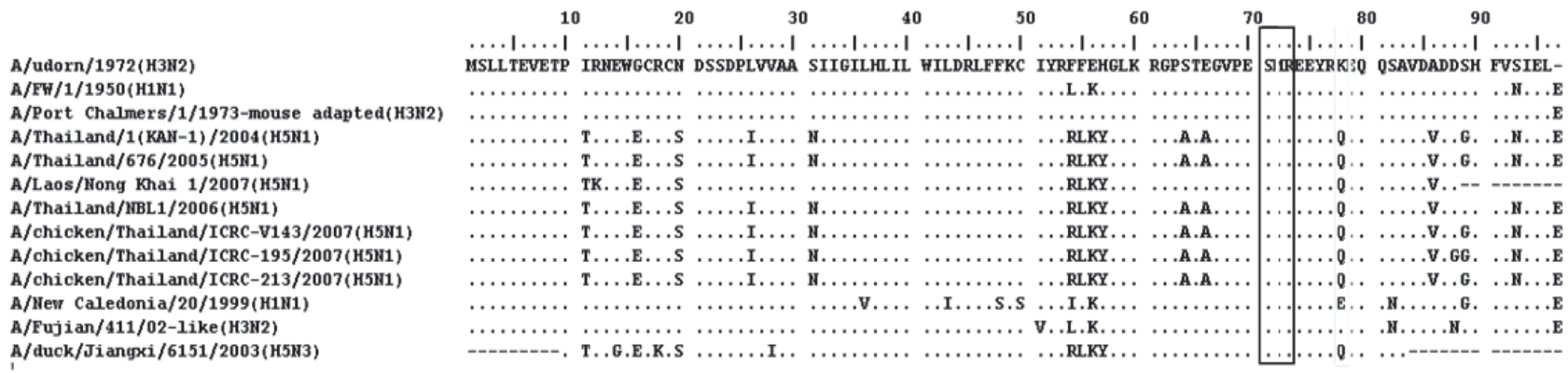

10 20 30 40 50 60 70 80 90
A/udorn/1972(H3N2) MSLLTEVETP IRNEWGCRCN DSSDPLVVAA SIIGILHLIL WILDRLFFKC IYRFFEHGLK RGPSTEGVPE SMREEYRKEQ QSAVDADDSH FVSIEL-
A/FW/1/1950(H1N1)
A/Port Chalmers/1/1973-mouse adapted(H3N2)
A/Thailand/1(KAN-1)/2004(H5N1)
A/Thailand/676/2005(H5N1)
A/Laos/Nong Khai 1/2007(H5N1)
A/Thailand/NBL1/2006(H5N1)
A/chicken/Thailand/ICRC-V143/2007(H5N1)
A/chicken/Thailand/ICRC-195/2007(H5N1)
A/chicken/Thailand/ICRC-213/2007(H5N1)
A/New Caledonia/20/1999(H1N1)
A/Fujian/411/02-like(H3N2)
A/duck/Jiangxi/6151/2003(H5N3)

Figure 7.

PLoS ONE

Satellite tracking on the flyways of Brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus

--Manuscript Draft--

Manuscript Number:	PONE-D-12-17685
Article Type:	Research Article
Full Title:	Satellite tracking on the flyways of Brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus
Short Title:	Potential role of Brown-headed gulls on AI spread
Corresponding Author:	Pilaipan Puthavathana Mahidol University Bangkoknoi, Bangkok THAILAND
Keywords:	Highly pathogenic avian influenza H5N1 virus; Brown-headed gulls; satellite telemetry; migratory flyways
Abstract:	Brown-headed gulls (Larus brunnicephalus), winter visitors of Thailand, were tracked by satellite telemetry during 2008-2011 for investigating their roles in the highly pathogenic avian influenza (HPAI) H5N1 virus spread. Eight gulls negative for influenza virus infection were marked with solar-powered satellite platform transmitters, their movements were monitored by the Argos satellite tracking system, and locations were mapped. Five gulls completed their migratory cycles, which spanned 7 countries (China, Bangladesh, India, Myanmar, Thailand, Cambodia, and Vietnam) affected by the HPAI H5N1 virus. Gulls migrated from their breeding grounds in China to stay overwinter in Thailand and Cambodia; while Bangladesh, India, Myanmar, and Vietnam were the places of stopovers during migration. Gulls traveled an average distance of about 2400 km between Thailand and China and spent 1-2 weeks on migration. Although AI surveillance among gulls was conducted at the study site, no AI virus was isolated and no H5N1 viral genome or specific antibody was detected in the 75 gulls tested, but 6.6% of blood samples were positive for pan-influenza A antibody. No AI outbreaks were reported in areas along flyways of gulls in Thailand during the study period. Distance and duration of migration, tolerability of the captive gulls to survive the HPAI H5N1 virus challenge and days at viral shedding after the virus challenging suggested that the Brown-headed gull could be a potential species for AI spread, especially among neighboring countries. This is the only study that demonstrated flyways which links between China and Southeast Asia, the epicenter of H5N1 AI outbreak.
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Cover letter

I would like to submit the article entitled “Satellite tracking on the flyways of Brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus” for publishing in PLoS ONE. Using satellite telemetry technique, we demonstrated the migratory routes of Brown-headed gulls that encompassed 7 countries affected by H5N1 avian influenza outbreaks: Thailand, Cambodia, Vietnam, Myanmar, India, Bangladesh, and China. It is also the only study that revealed the flyways which linked between China and Southeast Asian countries, the epizootic regions of H5N1avian influenza. Distance and duration of migration, tolerability of the captive gulls to survive the HPAI H5N1 virus challenge and days at viral shedding after the virus challenging suggested that the Brown-headed gull may be a prime candidate species for AI spread, especially among nearby countries.

We are looking forward for your kind consideration.

Title

Satellite tracking on the flyways of Brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus

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Abstract

Brown-headed gulls (*Larus brunnicephalus*), winter visitors of Thailand, were tracked by satellite telemetry during 2008-2011 for investigating their roles in the highly pathogenic avian influenza (HPAI) H5N1 virus spread. Eight gulls negative for influenza virus infection were marked with solar-powered satellite platform transmitters, their movements were monitored by the Argos satellite tracking system, and locations were mapped. Five gulls completed their migratory cycles, which spanned 7 countries (China, Bangladesh, India, Myanmar, Thailand, Cambodia, and Vietnam) affected by the HPAI H5N1 virus. Gulls migrated from their breeding grounds in China to stay overwinter in Thailand and Cambodia; while Bangladesh, India, Myanmar, and Vietnam were the places of stopovers during migration. Gulls traveled an average distance of about 2400 km between Thailand and China and spent 1-2 weeks on migration. Although AI surveillance among gulls was conducted at the study site, no AI virus was isolated and no H5N1 viral genome or specific antibody was detected in the 75 gulls tested, but 6.6% of blood samples were positive for pan-influenza A antibody. No AI outbreaks were reported in areas along flyways of gulls in Thailand during the study period. Distance and duration of migration, tolerability of the captive gulls to survive the HPAI H5N1 virus challenge and days at viral shedding after the virus challenging suggested that the Brown-headed gull could be a potential species for AI spread, especially among neighboring countries. This is the only study that demonstrated flyways which links between China and Southeast Asia, the epicenter of H5N1 AI outbreak.

Introduction

To date, 16 hemagglutinin (H) and nine neuraminidase (N) subtypes of influenza A viruses have been identified. All the H and N subtypes have been isolated from wild aquatic birds, particularly from orders *Anseriformes* (ducks, geese, and swans) and *Charadriiformes* (gulls, terns, and shorebirds). Therefore, aquatic birds are widely accepted as the main natural reservoirs of influenza A viruses. Influenza viruses isolated from these birds are the mostly avirulent, low pathogenic avian influenza (LPAI) viruses [1-3].

Only some members of the H5 and H7 subtypes are highly pathogenic [1, 3]. The HPAI H5N1 virus was the first avian virus known to cross the species barriers to infect humans and was recognized as the most virulent subtype. In the first identified occurrence of H5N1 HPAI infection in humans in Hong Kong in 1997, 18 humans were infected and 6 died (fatality rate 33.3%) [4]. The HPAI H5N1 virus re-emerged in Hong Kong in 2001, twice in 2002, and subsequently in 2003. The viruses isolated in Hong Kong before 2002 were pathogenic in gallinaceous birds but not domestic or wild waterfowl. Death of aquatic birds from the HPAI H5N1 virus was first recognized in the 2002 outbreak [5]. The resurging HPAI H5N1 strain is highly virulent in both avian and humans, with a fatality rate of approximately 60% being reported in infected humans [6, 7]. In January 2004, an outbreak of H5N1 HPAI was reported in poultry and humans in Thailand. The virus initially isolated in Thailand belonged to clade 1; while clade 2.3.4 virus was introduced into northeast Thailand in 2006 [8, 9]. However, all of the virus isolates in central Thailand still remained in clade 1. There were 25 cases reported in humans, with 17 deaths (fatality rate 68%). No cases of human infection have occurred since August 2006, but there were some AIV outbreaks in poultry until 2008 [6, 7].

Based on virological data and satellite telemetry studies, many groups of investigators have linked migratory birds with H5N1 HPAI spread [10-17]. The first evidence that supported this claim was an outbreak of genotype z, clade 2 H5N1 viruses that occurred in wild bird populations at Qinghai Lake, western China during late April until June 2005 causing death of more than six thousand birds. The main victim infected were Bar-headed geese (*Anser indicus*) which accounted for more than 50% of the deaths, whereas Brown-headed gulls were the second most species affected [10, 11]. Subsequently, H5N1 HPAI viruses similar to those in the Qinghai area were also isolated in other Asian countries, Europe, the Middle East and some African countries [3]. H5N1 viruses were likely transmitted along the flyways of birds that shared habitats, wintering sites, breeding areas or

stopovers [3, 15]. In addition, the results from surveillance for H5N1 HPAI in migratory and wildlife birds in 14 provinces of China from 2004 to 2007 showed that highest infection rates (4.37%) occurred in mallards; while it was 2.39% for Brown-headed gulls [18]. Birds in the Qinghai province had the highest rates of infection, possibly because there are many lake areas in Qinghai, which are natural habitats and breeding ground of numerous bird species [11, 18]. Investigation of the outbreaks and phylogenetic analysis suggested that the migrating birds in Qinghai got infected with H5N1 HPAI viruses through spillover from domestic poultry [11].

Satellite telemetry has been conducted by several groups of investigators to study the potential role of migrants in H5N1 HPAI spread [13-16]. With this technique, flyways of migrants can be illustrated with high accuracy; and the linkage between bird locations and geographical areas of AI outbreaks can be determined. Spatial analysis of distance of migration, duration of asymptomatic infection and duration of viral shedding strongly supported potential role of migratory birds on H5N1 HPAI spread. However, those satellite telemetry studies explored the flyways which spanned Africa, Europe and East Asia, while none involved flyways to Southeast Asia which was the epicenter of the outbreaks.

The Brown-headed gull (*Larus brunnicephalus*, Family *Laridae*) is the most common bird species to annually visit Thailand, staying from October to May [19-22]. According to the Department of National Parks, Wildlife and Plant Conservation of Thailand, there are 2 large flocks of Brown-headed gulls in Thailand. The larger flock consists of approximately 5,000 birds foraging around the inner gulf of Thailand mainly at mangrove mudflats in the Bang Poo Rest and Rehabilitation Center of the Royal Thai Army, Samut Prakan province (Figure 1). Our group at the Faculty of Veterinary Science, Mahidol University isolated HPAI H5N1 virus from 4 of 153 (2.6 %) apparently healthy birds in this flock between 2005 and 2008 (unpublished data), which indicates that this flock may play a role in the spread of the HPAI virus along its flyways. However, the entire migratory route of Brown headed gulls remains unknown. In order to determine the potential role of this bird species on AI spread along its migratory path, satellite telemetry was employed to track the flyways of Brown-headed gulls during 2008-2011. We searched for linkage between bird locations and occurrence of reported H5N1 HPAI outbreak while the birds overwintered in Thailand. Status of H5N1 HPAI infection in the flock was also determined. Gulls in captivity were challenged with H5N1 HPAI virus in order to determine their tolerability to virus infection. The result demonstrated that the infected gulls with virus shedding survived long enough to spread the virus along their migratory routes.

Results

Migratory routes and flight distances of Brown headed gulls

Eight Brown-headed gulls employed in the satellite telemetry study were captured from the flock at the Bang Poo study site, Samut Prakarn province, Thailand (Figure 1). They were of adult age, negative for influenza virus infection (see material and method), physiological healthy and energetic. Tagging the birds with satellite transmitters was performed in two consecutive years. The first Brown-headed gull was tagged in March 2008; and the other 7 gulls were tagged between February and March 2009. Flyways of these gulls were monitored until the satellite signals were lost. Demographic data and duration of tracking of each bird are shown in Table 1. Two gulls (I.Ds. 74795 and 88216) completed one migratory cycle, 3 gulls (I.Ds. 88215, 91416 and 91417) completed 2 cycles (Table 2); while 3 gulls (I.Ds. 88217, 88218 and 91418) were lost in Thailand within one month after tagging (Table 1). Flyways of the 5 marked gulls including their habitats in different countries are as shown in Tables 3, and individual flyway of each gull was described in details in Supplementary data.

The mean flying distance of the tagged gulls during migration was 230 km/day (167-295 km/day at 95% C.I.); whereas the local movements within a given site ranged from 1 to 28 km/day (1-12 km/day at 95% C.I.) (Table 2). In general, gulls traveled an estimated migratory distance of 2,400 km between China and Thailand with duration of migration of approximately 12 days in average (7-23 days at 95% C.I.). Although all gulls began their flight from the same study site in Thailand, their destinations in China were different. They also arrived and left Thailand at different time points. These results indicated that these Brown-headed gulls belong to different flocks while they were in China, and just gathered together in the same overwintering site in Thailand. Migratory routes of the 5 tracked gulls spanned 7 countries- Thailand, Myanmar, India, China, Bangladesh, Vietnam and Cambodia (Figure 2). Almost all tracked gulls, except gull I.D. 88216, stayed in Cambodia for approximately 30-40 days in a migratory season; and only the gull I.D. 91417 had traveled further to Vietnam. If seasonal habitat is defined as the places where gulls stayed for longer than 1 month, those places were mainly located in three countries: China, Thailand and Cambodia; while Myanmar, India, Bangladesh and Vietnam were considered to be the stopover countries. Moreover, the stopover places of individual gull in each country were different. They could be West Bengal or Assum in India, the Gulf of Martaban, Ayeyarwaddy

or Rakhine in Myanmar and Kien Giang in Vietnam. In the three gulls that were tracked for over two migratory cycles, the migratory routes were only slightly different between years.

Ecologically, seasonal habitats were always along the coast, wetlands or inland lakes (Figure 3). Birds stayed over the cold season for 5-6 months in Thailand and stayed in their breeding places in China for another 5-6 months. Duration that gulls stayed in each country is shown in Figure 4. Seasonal habitats in Thailand were mainly in three provinces: Samut Prakan, Samut Sakhon and Samut Songkhram, all of which were situated along the inner gulf of Thailand. In China, the breeding habitats were in Tibet, Qinghai and Xinjiang. However, gulls also spent their times at a third habitat in Siem Reap, Cambodia around Tonle Sap, the largest fresh water lake in Southeast Asia [23]. Although this flock of gulls stayed through winter along the inner gulf of Thailand, no AIV outbreak was reported in that area through the extensive national surveillance system.

Prevalence of avian influenza virus infection in gulls

A total of 75 birds were captured from the Bang Poo study site during 2008 to 2010. Throat and cloacal swabs as well as blood samples were collected for the investigation of influenza virus infection. No influenza virus was isolated and the viral genome was not detected in cloacal and throat swab samples. The H5N1 antibody was not detected in the HI and MicroNT assays. However, 6.6% of samples were positive for the pan-influenza A antibody by ELISA.

Virus challenge assay

Brown headed gulls kept in captivity were inoculated with HPAI H5N1virus in order to explore whether the infected birds could survive the infection, and therefore might be able to carry on migratory activity. Group of 3 gulls was inoculated with the H5N1 virus at the inoculum dose of 10, 10^3 or 10^4 tissue culture infective dose 50 (TCID50) per head (Table 4). The result demonstrated that all gulls inoculated with the high inoculum dose of 10^3 or 10^4 TCID50 began shedding the virus in cloacae and trachea after 1-2 days post inoculation (dpi.). These infected birds developed clinical symptoms, but they could tolerate the infection for 4-6 days before death. One of the three gulls inoculated with 10 TCID50 could shed the virus at 3 dpi. and died on day 7. The two gulls that survived the infection neither shed the virus nor developed any clinical symptom, of which one of them underwent seroconversion.

Discussions

The worldwide resurgence of H5N1 HPAI since 2003 has resulted in massive deaths in poultry and human deaths, especially in East and Southeast Asian countries. The global spread of H5N1 HPAI is likely mediated by migratory birds, trade in poultry, and possibly, trade in wild birds [24]. Integrated data and phylogenetic analyses of virus isolates obtained from different geographical locations suggest that the H5N1 spread throughout Asian and African countries involved both migratory birds and trade in poultry, whereas the spread in European countries involved migratory birds [24]. Human population, duck population (particularly, free grazing ducks) and intensity of the rice crop can increase the risk of propagation of H5N1 HPAI in Southeast Asian countries [25-26]. Density of the duck population can be a major risk factor for virus dispersal, especially when viral shedding is observed in asymptomatic infected ducks [27]. There is a close relationship between the density of free grazing ducks and the double or triple cultivation of rice in Thailand, because the falling rice grains left in the fields after harvesting are the source of low-cost food for ducks. Movements of free grazing ducks to post-harvested rice fields has been suggested as the major source of virus spread in Thailand during the initial waves of HPAI outbreaks. Nevertheless, duck movement has been later on prohibited. Natural infection in the other animal species such as tigers and leopards [28], cats [29], and dog [30] was mainly caused by ingestion of infected carcasses or chicken meat. However, most cases of H5N1 HPAI infection in humans in Thailand were mainly caused by exposure to sick or dead chicken.

It has been very difficult to trace how the H5N1 HPAI virus was introduced into Thailand because of the delay between the initial cases and the subsequent spread of the virus throughout of the country [31]. It has been hypothesized that the virus might have been introduced via migratory birds. HPAI usually occurs during low temperature months (October to February) which coincide with the time of arrival of wintering migratory birds [31]. In our study, Brown-headed gulls seem to be a prime candidate species for spreading avian influenza. They are long distant migrant and their flyways involved 7 countries, all of which had been affected by the H5N1 HPAI. Nevertheless, on the basis of an inhabiting time of 1 month or longer, only China, Thailand and Cambodia, were considered to be the gulls' seasonal habitats, whereas Myanmar, Bangladesh, India and Vietnam were stopover places. In our study, Brown-headed gulls spent their breeding time in the lake areas of Tibet, Qinghai and Xinjiang. All of these breeding sites are cold and high altitude lakes of varying salinity

[21]. They migrated to warmer places and stayed overwinter in mudflats along the inner gulf of Thailand and Tonle Sap Lake areas in Cambodia.

Our study illustrates complete migratory cycles of Brown-headed gulls. Gulls in this flock arrived and left Thailand asynchronously; their destinations on migration to China were different, suggesting that they belonged to different flocks while in China. Gulls from these flocks gathered together during the winter time in Thailand. The first group of gulls reached the Bang Poo study site in October and the last group left in May. The migration distance of about 2400 km between Thailand and China and the migration time at range of 5-23 days in our tracked Brown-headed gulls were more or less similar to those previously reported in wildfowl in which the movements of up to 2,900 km in 5-15 days were within the timeframe that was compatible with the preclinical symptom and virus dispersal [16].

We looked for a linkage between the virus from the Qinghai outbreaks and the H5N1 HPAI viruses isolated from Brown-headed gulls in Thailand in 2005 and 2008, but no linkage was found. Phylogenetic analysis showed that the viruses isolated from birds in Qinghai in 2005 belonged to clade 2.2 [10, 11], whereas the viruses isolated from gulls in Thailand between 2005 and 2008 belonged to clade 1 (unpublished data). Nevertheless, phylogenetic analysis showed a virological linkage between countries situated in the migratory corridor of this flock of Brown-headed gulls. The viruses causing outbreaks in Bangladesh belonged to the Qinghai like-lineage [32]; and the viruses causing the first epidemic wave in Thailand, Cambodia and Vietnam belonged to clade 1 [8, 33]. The clade 1 virus was originally isolated in Yunnan, southern China in 2002 and 2003, and probably spread to Vietnam by poultry trade across the shared border that is 600 km long [34]. Thereafter, the viruses spread from the North to the South of Vietnam. Interestingly, the H5N1 virus isolates in Thailand are closely related to the isolates in Vietnam [6]. It is also claimed that the clade 1 virus spread from Thailand to Cambodia [34]. Nevertheless, it remains unknown for how the clade 1 virus was introduced into Thailand. The firstly H5N1 HPAI outbreaks in Thailand occurred in central part, not at the border of the country. Interestingly, Vietnam, Thailand and Cambodia reported the first outbreak in the country at about the same period of time in 2004, i.e., on January 23 for Thailand, on January 8 for Vietnam, and January 24 for Cambodia [6]. Although there might be a lag time period for outbreak identification and report, it is implied that the H5N1 HPAI outbreaks occurred in these countries more or less about the same time. In addition, the time at occurrence of outbreaks in these 3 countries coincides with the overwintering period of Brown headed gulls.

Our study showed that Brown headed gulls kept in captivity were vulnerable to HPAI H5N1 virus. Nevertheless, gulls infected with the inoculum dose of 10^4 $TCID_{50}$ could survive for 4 days and those inoculated with 10^3 $TCID_{50}$ could survive for 5-6 days, and with viral shedding from trachea and cloacae which began after 1 dpi. In addition, a gull infected with the low inoculum dose of 10 TCID50 could survive for 7 days with viral shedding which began at 3 dpi. The results implies that the infected gulls may be able to complete migratory route between China and Thailand, or at least, at a shorter distant between nearby countries with virus spread along their flyways.

Although cross border trade may be the major route of H5N1 HPAI virus spread in Asian countries, it cannot be excluded that migratory birds might also play an additional role in the spread of the virus across countries or domestically along its flyways. In spite of the fact that Brown-headed gulls appear to be an ideal candidate for spreading the virus, no H5N1 virus including other influenza subtypes could be isolated, and no H5N1 antibody could be detected in our flock of Brown-headed gulls during the study period. Only anti-influenza antibody was detected by ELISA, indicating previous infection with some subtype of AIVs in these birds. Besides waterfowl, gulls and shorebirds also maintain an influenza gene pool in their species. Predominant AIV subtypes found in gulls are H13 and H16 [2].

The inability to detect the H5N1 virus, however, may simply be due to our small sample size collected from gulls as all migratory birds in Thailand are protected by law; and it may be also due to the absence of HPAI outbreaks during our investigation period of 2008-2011. There was only one outbreak occurred in poultry in Nakhon Sawan and Phichit provinces (upper part of central Thailand) in January 2008, and one in backyard poultry in Sukhothai province (lower north of the country in November 2008). No AI outbreak has been reported since then [6]. Nevertheless, this study is the only flyway monitoring that encompassed the epicenter of H5N1 HPAI in Southeast Asia to date; and it is also the longest flyway monitoring covering complete migratory seasons which has never been reported before.

Methods

Approvals and permissions

This study was approved by the Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University, and compiled with the Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals of the Office of

Laboratory Animal Welfare (OLAW No. A5731-01), U.S. Department of Health and Human Services. The study was carried out with permission of the Department of National Parks, Wildlife and Plant Conservation, Thailand, in accordance with the Wildlife Conservation Act of Thailand.

Study site

This study was conducted at Bang Poo, a recreation area located in the Samut Prakan province, Thailand, 37 km east of Bangkok. This 63 km^2 coastal area owned by the Royal Thai Army is a part of the inner gulf of Thailand and comprised mangrove and large mudflat habitats. Gulls gather together here to feed on an abundance number of clams that embedded in the muddy beaches. Bang Poo is an Important Bird Areas (IBA) with more than 135 water bird species being recorded, including 7 species of ducks, 50 species of waders, and 18 species of terns and gulls. Thirteen globally threatened species have been recorded in this area [22]. The Brown-headed gull is an important species in this wetland IBA [19-22].

Study birds

The Brown headed gull (*Larus brunnicephalus*) is a waterfowl belonging to the family *Laridae.* The common name for this species comes from the presence of brown color on the head of adult birds. These gulls normally feed on small fishes and crabs; nevertheless, they can adapt to a variety of available foods.

Bird capture and specimen collection

All migratory birds in Thailand are protected animals by law. Therefore, bird capture was performed by the authorized persons from the Department of National Park, Wildlife and Plant Conservation. Gulls were attracted by fried pork rind, and captured by a hand net. Approximately 10-20 birds were captured in each trap. Tracheal and cloacal swabs as well as blood samples were collected for laboratory investigations to determine the prevalence of AIV infection. The captured birds were also physically examined for general health conditions (body weight, body size) and the healthy, energetic adults that were negative for influenza viral infection, as screened by rapid antigen detection kit (Rapid H5 AIV Ag Test, Bionote, Inc., Gyeonggi-Do, South Korea) at the study site, were chosen for satellite telemetry tracking. Results of samples negative for the virus infection by antigen detection