mice in each group were challenged intranasally with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under anesthesia. Mixture of tiletamine hydrochloride (20 mg/kg) (United States Pharmacopeia, Maryland, U.S.A.), zolazepam hydrochloride (20 mg/kg) (United States Pharmacopeia), and xylazine (20 mg/kg) (Bayer HelthCare, Osaka, Japan) was injected intraperitoneally into mice for anesthesia.

Inactivated Dk/Hok/49/98 vaccines were also injected twice intraperitoneally into 4-week-old female BALB/c mice. Two weeks later, the vaccine was again intraperitoneally injected into the mice. One week after the second vaccination, 10 mice in each group were challenged intranasally with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under anesthesia.

On day 3 post-infection, five mice in each group were sacrificed and the lungs were homogenized to make a 10% (w/v) suspension with minimal essential medium (Nissui, Tokyo, Japan) with antibiotics (penicillin G potassium, streptomycin sulfate, gentamicin sulfate, and nystatin) and 0.5% Bovine Serum Albumin Fraction V (Roche, Basel, Switzerland). The virus titers of the supernatants of the lung tissue homogenates were calculated in 10-day-old embryonated chicken eggs and expressed as the EID_{50}/g of tissue.

In neutralization (NT) tests, titers were determined as the reciprocal of that maximum antibody dilution that completely prevented cytopathic effect caused by 100 plaque forming units of virus using MDCK cells.

RESULTS

Phylogenetic analysis of the HA genes of H9N2 influenza viruses: The HA genes of 22 H9N2 viruses were sequenced and phylogenetically analyzed by the neighbor-joining method. All of the

131 HA genes were classified into the Eurasian lineage, and further classified into the Korean (n=11),
132 Y280 (n=7), and G1 (n=4) sublineages (Fig. 1). The H9 viruses of the Korean and Y280
133 sublineages were isolated from water birds, poultry, pigs, and humans in east Asian countries, and
134 those of the G1 sublineage were isolated from poultry in west Asian countries (Fig. 1).

135 *Antigenicity of the H9N2 influenza viruses:* H9N2 influenza viruses were antigenically analyzed
136 by HI test (Table 2). Antisera against H9N2 viruses of the Y280 sublineage reacted slightly with
137 H9N2 viruses of the G1 and Korean sublineages. Antisera against H9N2 viruses of the G1
138 sublineage reacted more with H9N2 viruses of the Y280 sublineages than those of the Korean
139 sublineage. On the other hand, antisera against H9N2 viruses of the Korean sublineage reacted
140 with H9N2 viruses of all sublineages. This result suggested that the H9N2 vaccine strain should be
141 selected from the viruses of the Korean sublineage.

142 *Selection of H9N2 vaccine strain:* To select an H9N2 vaccine strain, four H9N2 viruses,
143 Dk/Hok/49/98, A/duck/Hokkaido/13/2000 (H9N2) (Dk/Hok/13/00), A/duck/Hokkaido/9/1999
144 (H9N2) (Dk/Hok/9/99), and A/duck/Hokkaido/26/1999 (H9N2) (Dk/Hok/26/99), were selected from
145 11 isolates of the Korean sublineage, and their replication and pathogenicity in embryonated chicken
146 eggs were assessed. HA titers of Dk/Hok/49/98, Dk/Hok/13/00, Dk/Hok/9/99, and Dk/Hok/26/99
147 were 512, 512, 256, and 128, respectively. Virus titers were $10^{9.7}$, $10^{8.3}$, $10^{8.3}$, and $10^{7.3}$ EID_{50}/ml,
148 respectively, indicating that Dk/Hok/49/98 replicated efficiently in 10-day-old embryonated chicken
149 eggs. Pathogenicity of Dk/Hok/49/98 in the embryonated chicken eggs was determined by mean
150 death time and that of Dk/Hok/49/98 was 91.8 h, indicating that Dk/Hok/49/98 had low
151 pathogenicity in chicken embryos. This virus was selected as a candidate H9N2 vaccine strain.

152 *Protective efficacy of the test vaccine in mice against H9N2 virus challenge:* To assess the
153 efficacy of the vaccine against H9N2 virus infection, HK/1073/99 was intranasally inoculated into
154 mice that had previously been vaccinated once with inactivated HK/1073/99 or Dk/Hok/49/98.

155 Immunogenicity of the inactivated vaccine was assessed by NT test, and virus titers in the lungs
156 were measured to assess protective immunity induced by the vaccine (Table 3). Serum antibodies
157 were detected in mice injected with 50, 10, and 2 μg protein of HK/1073/99 vaccine. The virus
158 titers in the lungs were $<10^{1.5}$-$10^{3.7}$ EID_{50}/g in mice injected with 50 or 10 μg protein of HK/1073/99
159 vaccine, and $10^{4.7}$-$10^{6.8}$ EID_{50}/g in the 2 and 0.4 μg vaccine groups, and in the PBS control group
160 (Table 3). A reduction in body weight was observed in mice injected with 10, 2, and 0.4 μg protein,
161 and in the control group from day 2 post-infection, reaching up to 10% body weight loss at days 3-4
162 post-infection, compared with in the mice that received 50 μg of protein (Fig. 2A).
163 We also tested the efficacy of vaccination with Dk/Hok/49/98 on protection against subsequent
164 intranasal infection with HK/1073/99. Serum antibodies were slightly detected in mice injected
165 with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein (Table 3). The virus titers in the lungs
166 of mice injected with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein were $10^{4.3}$-$10^{5.3}$
167 EID_{50}/g. In the mice injected with 2 and 0.4 μg protein, the virus titers in the lungs of mice were
168 similar to those of non-vaccinated control mice (Table 3). Although, a reduction in body weight
169 was observed in mice at all doses of the Dk/Hok/49/98 vaccine, slight significant difference was
170 observed in mice injecteded with 50 μg protein, compared with in mice injected with PBS (Fig. 2B).
171 In the mice injected twice with Dk/Hok/49/98 vaccine on days 0 and 14, serum antibodies
172 were detected in mice in the 50, 10, and 2 μg groups at one week after the second injection (Table 4).
173 The virus titers in the lungs were $<10^{1.5}$-$10^{3.8}$ EID_{50}/g in mice injected with 50, 10, and 2 μg protein,
174 and $10^{5.3}$-$10^{6.5}$ EID_{50}/g in the other vaccinated mice (Table 4). A reduction in body weight was
175 observed in mice injected with 2 and 0.4 μg protein, and in control group, reaching up to 10%
176 weight loss from days 4-6 post-infection, compared with in mice injected with 50 and 10 μg protein
177 (Fig. 3). These results suggest that the repeat administration of the test vaccine confers immunity,
178 and prevents body weight loss and decreases virus replication, after infection of mice with H9

179 influenza virus.

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183 **DISCUSSION**

184 H9N2 viruses of each of the three sublineages, G1, Y280, and Korean, were recently isolated
185 from wildbirds and poultry worldwide [3, 8, 27]. H9N2 viruses were also isolated from pigs and
186 humans in China [4-5, 37] and Korea, suggesting that these viruses have the potential to cause
187 pandemic influenza in humans. H9N2 viruses isolated from pigs in China and Korea were
188 classified into the Y280 and Korean sublineages, while H9N2 viruses isolated from humans in China
189 was classified into the G1 and Y280 sublineages [4-5, 23, 31]. It is suggested that H9N2 viruses
190 isolated from pigs and humans are antigenically distinct among viruses of the Korean, Y280, and G1
191 sublineages [5, 23, 31, 37]. Therefore, it is important that any H9N2 influenza virus vaccine to be
192 used for pandemic influenza can broadly cross-react with antisera against all sublineage viruses. In
193 the present study, Dk/Hok/49/98 was selected from the Korean sublineage, since antisera to the virus
194 cross-reacted with all sublineages virus. Furthermore, Dk/Hok/49/98 replicated efficiently in
195 embryonated chicken eggs and was non-pathogenic in chicken embryos. Recently, H9N2 viruses
196 were isolated from pigs and humans in China [4-5, 23, 31], it is necessary to analyze the antigenicity
197 of these H9 isolates and evaluate the efficacy of test vaccine against them. Taken together, it is
198 important to carry out surveillance of avian influenza consecutively and to analyze the isolates
199 antigenically and phylogenetically.

200 In the present study, it was suggested that the test whole particle vaccine has the potency
201 against challenge with H9N2 virus of different sublineage in mice. It was already reported that
202 whole particle vaccine induced strong immune responses and H5N1 whole particle vaccine induced

protective immunity against antigenically distinct challenge virus [12, 26]. Although the efficacy of the test vaccine observed slightly in mice vaccinated once due to the antigenic difference between Dk/Hok/49/98 and HK/1073/99, it was clear that the vaccine induced protective immunity in mice injected twice, indicating the usefulness for the preparedness of the pandemic.

The current cycle of seasonal influenza vaccine production requires detailed planning up to 6 months before vaccine manufacture [7]. In the case of influenza pandemic in 2009, it also took 5 months to have an H1N1 vaccine available [6, 36]. To prepare for the emergence of pandemic influenza in birds and mammals including humans, we have carried out global surveillance of avian influenza [15, 30, 33, 39]. Avian influenza viruses of 144 combinations of HA and NA subtypes have been stocked for use in vaccine and diagnosis. Since the viruses stocked in our influenza virus library were already assessed the pathogenicity and replication in embryonated chicken eggs, we can exclude those tests to select a vaccine strain and prepare a vaccine rapidly [16-17, 19]. The present results indicate that the inactivated whole virus vaccine prepared from an influenza virus from the library could be used as an emergency vaccine during the early stage of a pandemic caused by H9N2 influenza virus infection.

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361 **FIGURE LEGENDS**
362 Fig. 1. Phylogenetic tree of the HA genes of H9N2 influenza viruses. Nucleotides 163-1,048
363 (886 bases) of the HA genes were used for the analysis. Horizontal distances are proportional to
364 the minimum number of nucleotide differences required to join nodes and sequences. Numbers at
365 the nodes indicate confidence levels in a bootstrap analysis with 1,000 replicates. Viruses stocked
366 in our laboratory are highlighted in gray. Representative viruses in each sublineage are underlined.
367 Abbreviations: Sw, swine; Ck, chicken; SCk, silky chicken; Dk, duck; Osr, ostrich; Qa, quail; Ty,
368 turkey; HK, Hong Kong; Hb, Hebei; S.Af, South Africa; Hok, Hokkaido; Pak, Pakistan; and Wis,
369 Wisconsin.
370

371 Fig. 2. Changes in body weight in mice vaccinated once following challenge with HK/1073/99.
372 Five vaccinated mice of each group injected with HK/1073/99 vaccine (A), and with Dk/Hok/49/98
373 (B) were inoculated intranasally with HK/1073/99 and body weight was monitored for 14 days.
374 Data are shown as mean body weight changes in each group with corresponding standard deviation
375 (S.D.). Asterisks indicate that body weights were not significantly ($p<0.05$) decreased than PBS
376 injected group.
377
378 Fig. 3. Changes in body weight in mice vaccinated twice with Dk/Hok/49/98 vaccine following
379 challenge with HK/1073/99. Five mice from each Dk/Hok/49/98 vaccine group were inoculated
380 intranasally with HK/1073/99 and body weight was monitored for 14 days. Data are shown as
381 mean body weight changes in each group with corresponding S.D. Asterisks indicate that body
382 weights were not significantly ($p<0.05$) decreased than PBS injected group.

Table 1. H9N2 viruses used in this study

Sublineage	Virus	HA gene[a)]
Y280	A/chicken/Hebei/1/1996	AF536693
	A/duck/Hong Kong/Y280/1997	AF156376
	A/chicken/Hong Kong/G9/1997	AF156373
	A/duck/Hong Kong/W213/1998	AB432938
	A/swine/Hong Kong/10/1998	AF222811
	A/chicken/Hong Kong/FY20/1999	AF222611
	A/silkie chicken/Hong Kong/SF43/1999	AF186268
Korean	A/ostrich/South Africa/9508103/1995	AF218102
	A/duck/Hokkaido/31/1997	AB125927
	A/duck/Hokkaido/49/1998	AB125928
	A/duck/Hokkaido/9/1999	AB125929
	A/duck/Hokkaido/26/1999	AB125930
	A/duck/Hokkaido/13/2000	AB125931
	A/duck/Hokkaido/HY57/2005	AB455035
	A/duck/Mongolia/564/2003	AB538969[b)]
	A/duck/Hokkaido/294/2006	AB538967[b)]
	A/duck/Hokkaido/W299/2006	AB538968[b)]
	A/duck/Hokkaido/238/2008	AB485600[b)]
G1	A/quail/Hong Kong/G1/1997	AF156378
	A/Hong Kong/1073/1999	AJ404626
	A/chicken/Pakistan/2/1999	AJ291392
	A/quail/Hong Kong/A17/1999	AF222606
North American	A/turkey/Wisconsin/1/1966	D90305

[a)] GenBank/EMBL/DDBJ Accession No.
[b)] The HA gene sequence was submitted to the GenBank/EMBL/DDBJ databases in this study.

Table 2. The cross-reactivity of H9N2 viruses with antisera by HI test

Sublineage	Virus[b)]	Antisera[a)]						
		Y280			Korean		G1	North American
		Ck/HK/G9/97	Dk/HK/Y280/97	Dk/HK/W213/98	Dk/Hok/49/98	Dk/Hok/13/00	Qa/HK/G1/97	Ty/Wis/1/66
Y280	Ck/Hb/1/96	10,240	10,240	2,560	2,560	2,560	2,560	40
	Ck/HK/G9/97	40,960	10,240	2,560	1,280	2,560	2,560	40
	Dk/HK/Y280/97	20,480	20,480	2,560	2,560	2,560	5,120	320
	Sw/HK/10/98	2,560	10,240	640	640	1,280	1,280	40
	Dk/HK/W213/98	40,960	20,480	2,560	1,280	2,560	2,560	80
	Ck/HK/FY20/99	10,240	10,240	2,560	2,560	2,560	5,120	160
Korean	Dk/Hok/31/97	640	640	640	1,280	2,560	160	640
	Dk/Hok/49/98	320	320	160	2,560	2,560	160	640
	Dk/Hok/9/99	640	640	160	2,560	2,560	80	320
	Dk/Hok/26/99	320	640	160	2,560	2,560	40	640
	Dk/Hok/13/00	640	640	160	1,280	2,560	80	640
	Dk/Mon/564/03	320	320	160	1,280	2,560	160	320
	Dk/Hok/HY57/05	640	320	320	1,280	2,560	80	320
	Dk/Hok/W299/06	640	320	640	1,280	2,560	80	640
	Dk/Hok/238/08	640	640	640	1,280	2,560	80	640
G1	Qa/HK/G1/97	640	1,280	320	1,280	1,280	5,120	320
	Ck/Pak/2/99	1,280	1,280	640	640	640	640	80
	HK/1073/99	1,280	320	160	1,280	80	1,280	320
North American	Ty/Wis/1/66	80	20	20	320	320	<20	640

[a)] Homologous reactions are underlined.

[b)] This panel showed the representative strains of each sublineage.

Table 3. Neutralizing antibody titers before challenge and virus titers of the lungs after challenge in mice vaccinated once

Vaccine	Dose of vaccine	NT titer to		Virus titer[a)] ($logEID_{50}/g$)
		HK/1073/99	Dk/Hok/49/98	
HK/1073/99	50 μg	320, 320, 160, 320, 640	ND	<1.5, <1.5, <1.5, <1.5, <1.5
	10 μg	80, 20, 80, 80, 40	ND	1.8, 3.7, 2.5, 2.3, 3.0
	2 μg	40, 20, 20, 20, 20	ND	4.7, 5.5, 5.5, 5.3, 6.0
	0.4 μg	<10, <10, <10, <10, <10	ND	5.7, 6.0, 5.0, 5.8, 5.5
	PBS	<10, <10, <10, <10, <10	ND	6.5, 6.2, 6.8, 6.5, 6.8
Dk/Hok/49/98	50 μg	40, 40, 40, 20, 40	160, 80, 80, 80, 80	4.3, 4.8, 4.5, 4.5, 4.8
	10 μg	<10, <10, <10, <10, <10	80, 80, 80, 80, 20	4.8, 5.0, 5.2, 4.7, 5.3
	2 μg	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	6.5, 5.8, 6.0, 6.3, 6.0
	0.4 μg	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	6.3, 5.8, 6.0, 6.5, 6.3
	PBS	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	6.2, 6.0, 6.5, 5.8, 6.5

Each of vaccine was injected intraperitoneally with 10 mice. Serum samples were collected 3 weeks after the vaccination. Mice were challenged with $10^{6.0}$ EID_{50} of HK/1073/99 intranasally.

[a)] The lung samples were collected at 3 d.p.c. and virus titers were measured.

Table 4. Neutralizing antibody titers before challenge and virus titers of the lungs after challenge in mice vaccinated twice

Vaccine	Dose of vaccine	NT titer to		Virus titer[a] ($logEID_{50}/g$)
		HK/1073/99	Dk/Hok/49/98	
Dk/Hok/49/98	50 μg	320, 320, 320, 160, 160	1,280, 640, 640, 320, 640	<1.5, <1.5, <1.5, <1.5, <1.5
	10 μg	40, <10, 20, 20, 40	320, 160, 320, 160, 160	2.0, 2.5, 2.0, 2.3, 2.5
	2 μg	<10, <10, <10, <10, <10	80, 160, 80, 80, 80	3.8, 3.5, 3.8, 3.5, 3.3
	0.4 μg	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	5.3, 5.8, 5.8, 6.0, 5.5
	PBS	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	5.8, 6.5, 6.0, 6.3, 5.8

Each of vaccine was injected intraperitoneally twice with 10 mice. Serum samples were collected 2 weeks after the second vaccination. Mice were challenged with $10^{6.0}$ EID_{50} of HK/1073/99 intranasally.

[a] The lung samples were collected at 3 d.p.c. and virus titers were measured.

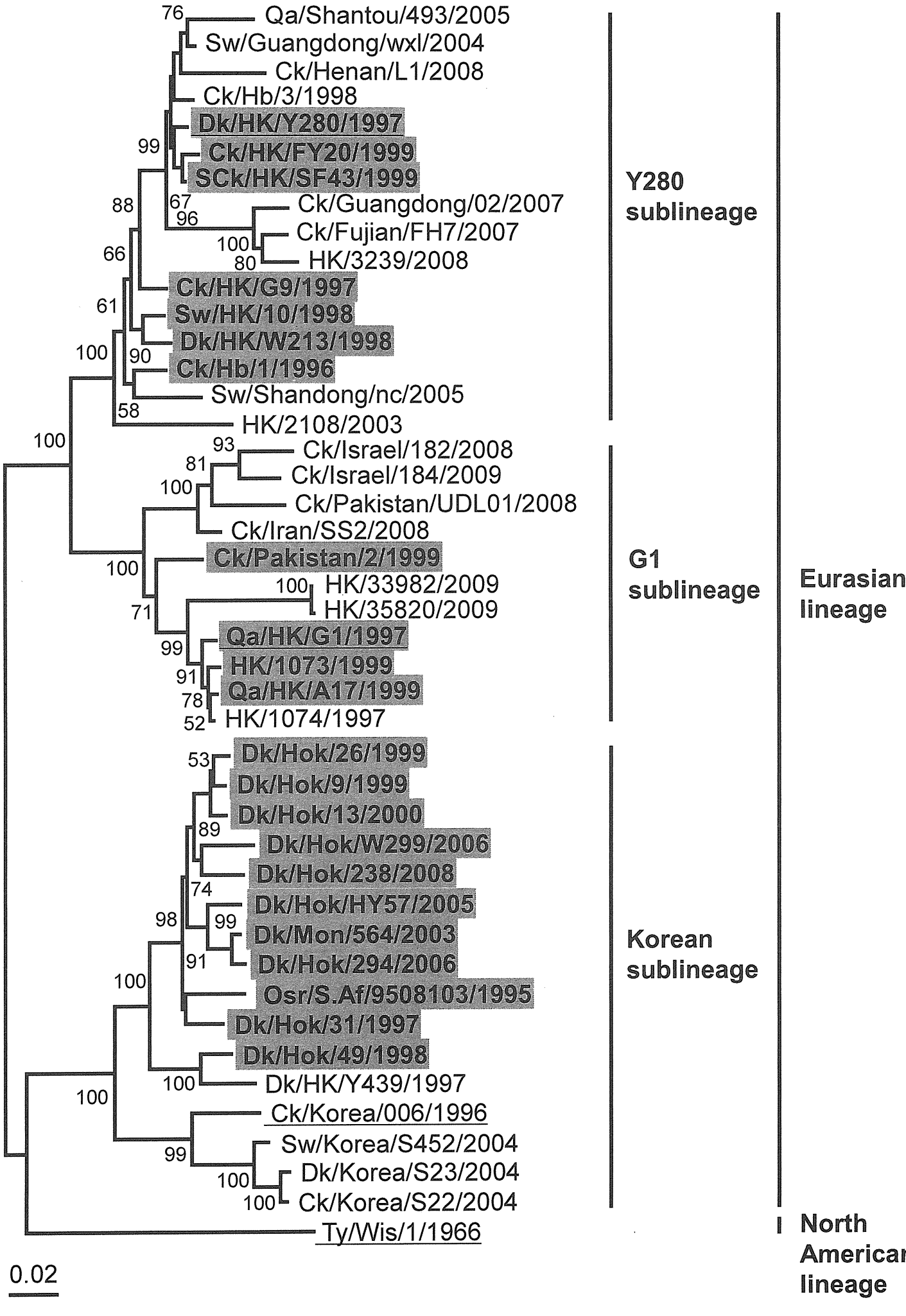
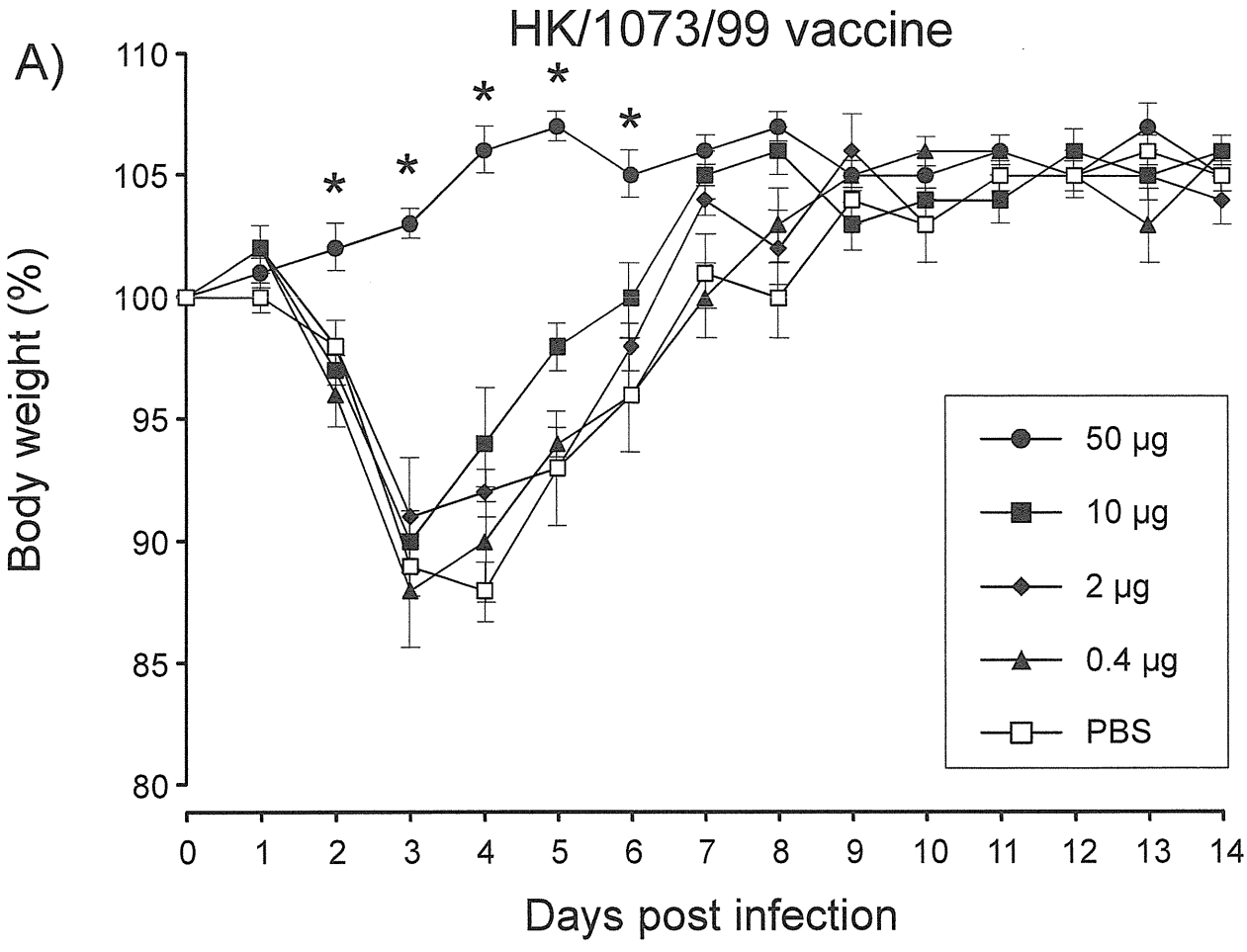


Figure 1 Nomura *et al.*



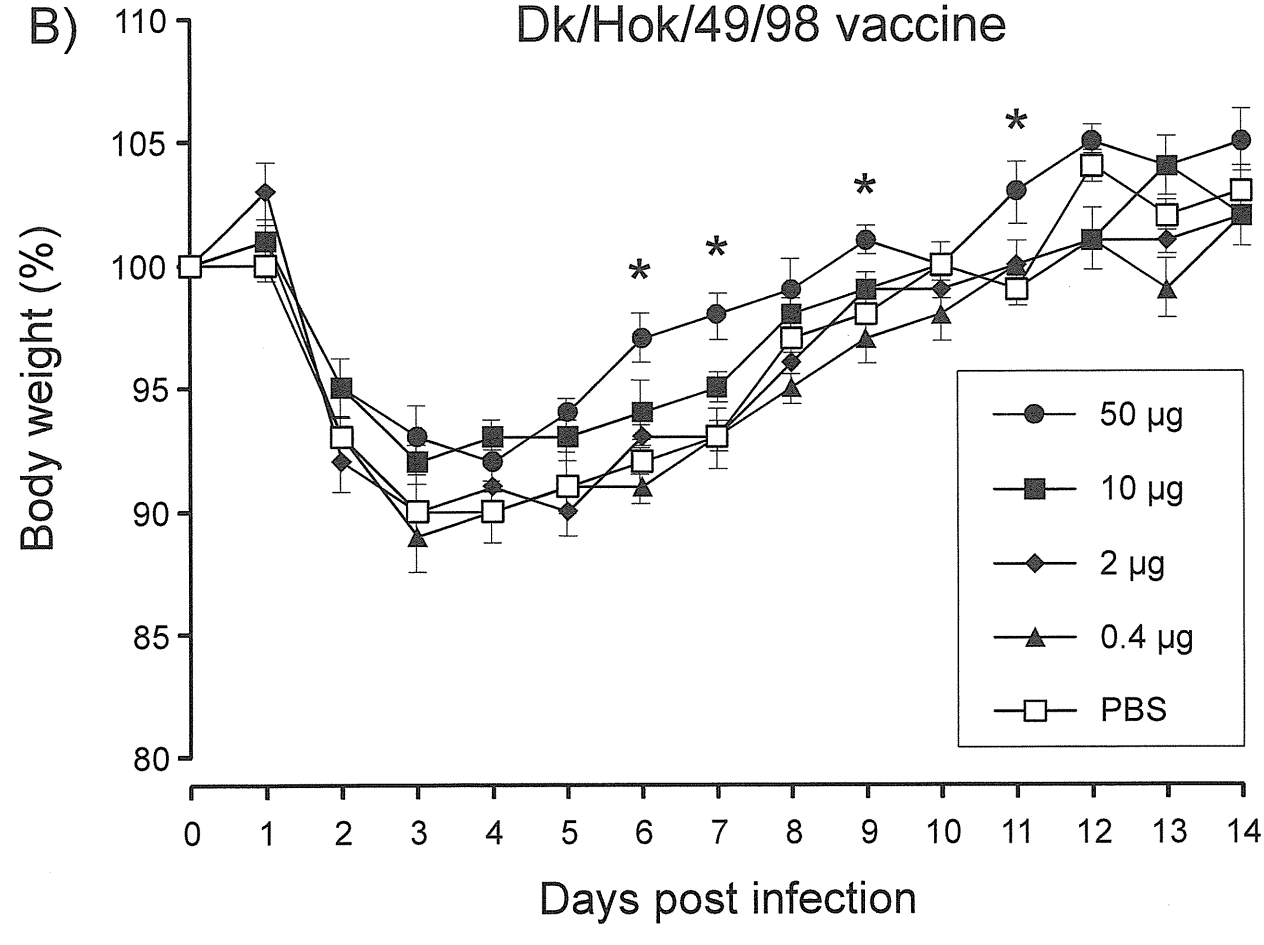


Figure 2 Nomura *et al.*

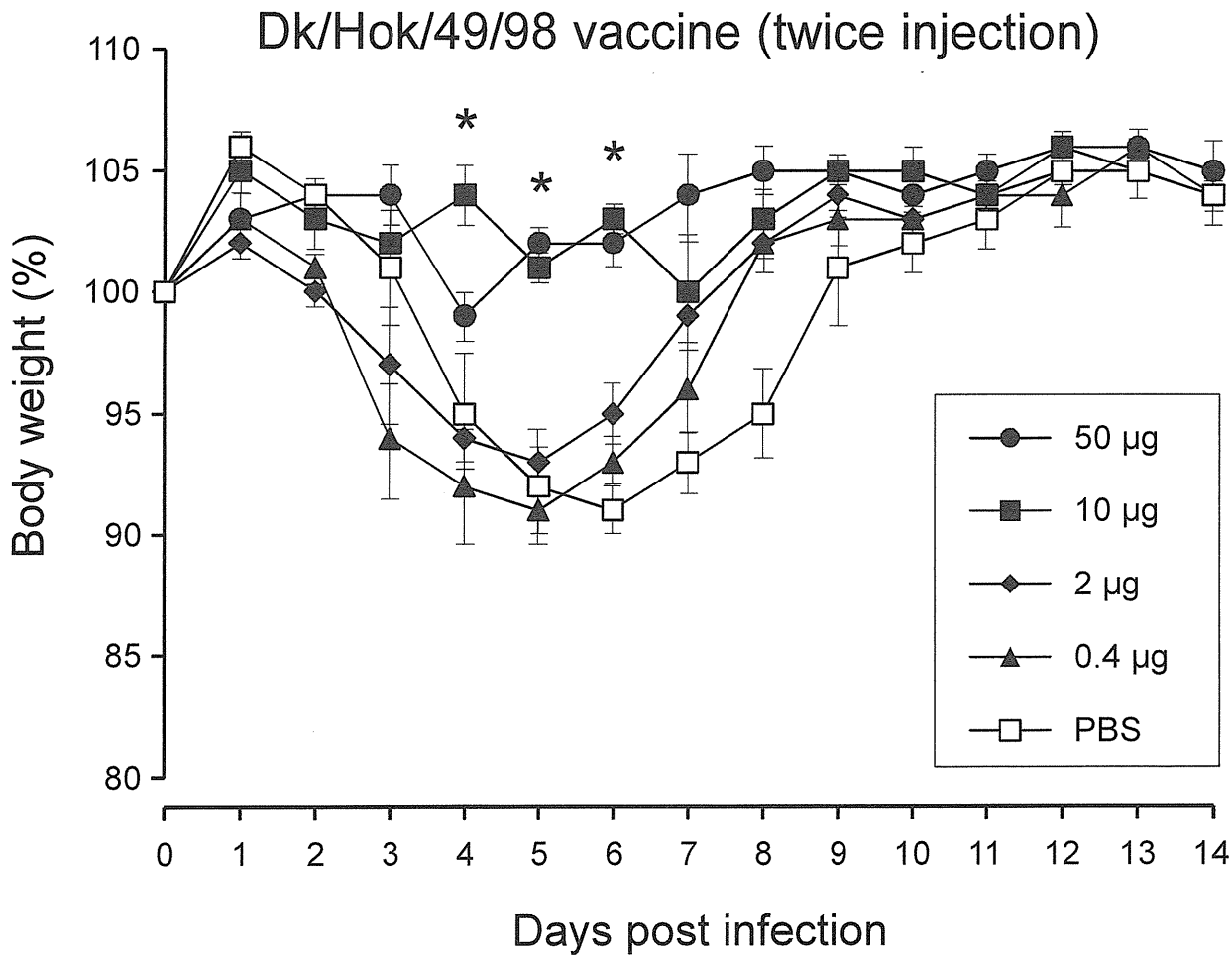


Figure 3 Nomura *et al.*

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ORIGINAL ARTICLE

Characterization of avian influenza viruses isolated from domestic ducks in Vietnam in 2009 and 2010

Naoki Nomura · Yoshihiro Sakoda · Mayumi Endo · Hiromi Yoshida · Naoki Yamamoto · Masatoshi Okamatsu · Kenji Sakurai · Nam Van Hoang · Long Van Nguyen · Huy Duc Chu · Tien Ngoc Tien · Hiroshi Kida

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Abstract In the surveillance of avian influenza in Vietnam, 26 H9N2, 1 H3N2, 1 H3N8, 7 H4N6, 3 H11N3, and 1 H11N9 viruses were isolated from tracheal and cloacal swab samples of 300 domestic ducks in April 2009, and 1 H9N6 virus from 300 bird samples in March 2010. Out of the 27 H9 virus isolates, the hemagglutinins of 18 strains were genetically classified as belonging to the sublineage G1, and the other nine belonged to the Korean sublineage. Phylogenetic analysis revealed that one of the 27 H9 viruses was a reassortant in which the PB2 gene belonged to the Korean sublineage and the other seven genes belonged to the G1 sublineage. Three representative H9N2 viruses were intranasally inoculated into ducks, chickens, pigs, and mice. On the basis of experimental infection studies, it was found that each of the three viruses readily infected pigs and replicated in their upper respiratory tracts, and they infected chickens with slight replication. Viruses were recovered from the lungs of mice inoculated with two of the three isolates. The present results reveal that H9 avian influenza viruses are prevailing and genetic reassortment occurs among domestic ducks in Vietnam. It is recommended that careful surveillance of swine influenza with H9 viruses should be performed to prepare for pandemic influenza.

N. Nomura · Y. Sakoda · M. Endo · H. Yoshida · N. Yamamoto · M. Okamatsu · H. Kida (✉)
Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060-0818, Japan
e-mail: kida@vetmed.hokudai.ac.jp

K. Sakurai
OIE Regional Representation for Asia and the Pacific, Food Science Building 5F, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

N. V. Hoang · L. V. Nguyen · H. D. Chu · T. N. Tien
Ministry of Agriculture and Rural Development, 15/78 Giaiphong, Phuongmai, Dongda, Hanoi, Vietnam

H. Kida
Research Center for Zoonosis Control, Hokkaido University, Kita 20 Nishi 10, Kita-ku, Sapporo 001-0020, Japan

H. Kida
Japan Science and Technology Agency Basic Research Programs, 4-1-8, Honcho Kawaguchi, Saitama 332-0012, Japan

Introduction

Avian influenza viruses of various subtypes are circulating in poultry in Asian countries [1, 15, 20, 30, 40]. In particular, H9N2 influenza virus is present in poultry in Eurasian countries [9–11, 25]. Since H9N2 viruses were isolated from quails in Hong Kong in 1988, they have become prevalent in live-bird markets and poultry farms in Asia [8, 34]. H9N2 virus infections have greatly affected not only the poultry industry but also public health [8, 40]. The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into G1, Y280, and Korean sublineages [10]. H9N2 viruses do not usually cause severe disease in poultry, but co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, or attenuated coronavirus vaccine exacerbates the disease [12, 21]. H9N2 viruses were also isolated from domestic pigs in China [39] and Korea, and from humans with febrile respiratory illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4, 7, 23, 33, 43]. Thus, it is postulated that H9N2 virus may cause pandemic influenza in humans.

In our laboratory, avian influenza has been surveyed in Japan, Alaska, Siberia, Mongolia, and Australia since 1977