FULL PAPER *Virology*

An H9N2 Influenza Virus Vaccine Prepared from a Non-Pathogenic Isolate from a Migratory Duck Confers Protective Immunity in Mice against Challenge with an H9N2 Virus Isolated from a Girl in Hong Kong

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ABSTRACT. H9N2 influenza viruses circulate in wild birds and poultry in Eurasian countries, and have been isolated from pigs and humans in China. H9N2 viruses isolated from birds, pigs and humans have been classified into three sublineages based on antigenic and genetic features. Chicken antisera to H9N2 viruses of the Korean sublineage reacted with viruses of different sublineages by the hemagglutination-inhibition test. A test vaccine prepared from a non-pathogenic A/duck/Hokkaido/49/1998 (H9N2) strain of the Korean sublineage, obtained from our influenza virus library, induced immunity in mice to reduce the impact of disease caused by the challenge with A/Hong Kong/1073/1999 (H9N2), which is of a different sublineage. The present results indicate that an inactivated whole virus vaccine prepared from a non-pathogenic influenza virus from the library could be used as an emergency vaccine during the early stage of a pandemic caused by H9N2 infection.
KEY WORDS: antigenicity, H9N2 influenza virus, vaccine.

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Avian influenza viruses of various subtypes are circulating in poultry worldwide [1, 18, 19, 21, 29, 38]. In particular, H9N2 influenza virus is prevailing poultry populations in Eurasian countries [9–11, 24]. 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 [6, 32]. The wide spread of H9N2 virus have been greatly concerned not only in the poultry industry but also for public health [6, 38]. The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into G1, Y280, and Korean sublineages [10]. H9N2 viruses do not substantially cause severe disease in poultry, but co-infection with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, or attenuated coronavirus vaccine exacerbates the disease [13, 22]. H9N2 viruses were also isolated from domestic pigs in China [38] and Korea, and from humans with febrile respiratory illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4, 5, 23, 31]. It has therefore been postulated that H9N2 virus has the potential to cause pandemic influenza in humans.

In the present study, as the preparedness for pandemic influenza, H9 virus strains from the influenza virus library in our laboratory [19] were analyzed antigenically and phylogenetically to select a strain suitable for a vaccine. A/duck/Hokkaido/49/1998 (H9N2) was selected and an inactivated whole virus vaccine was prepared. The efficacy of the vaccine against challenge with A/Hong Kong/1073/1999 (H9N2) was assessed in mice.

MATERIALS AND METHODS

Viruses: A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2), A/quail/Hong Kong/G1/1997 (H9N2), A/chicken/Hong Kong/FY20/1999 (H9N2), A/silkie chicken/Hong Kong/SF43/1999 (H9N2), and A/quail/Hong Kong/A17/1999 (H9N2) were provided by Dr. K. F. Shortridge (The University of Hong Kong, China). A/ostrich/South Africa/9508103/1995 (H9N2) and A/chicken/Pakistan/2/1999 (H9N2) were provided by Dr. I. H. Brown (Animal Health and Veterinary Laboratories Agency, Weybridge, U.K.). A/Hong Kong/1073/1999 (H9N2) (HK/1073/99), which was isolated from a 4-year-old girl in Hong Kong in 1999 [23], was provided by Dr. A. J. Hay (MRC National Institute for Medical Research, U.K.). H9N2 influenza virus strains isolated from birds and mammals, and A/duck/Hokkaido/49/1998 (H9N2) (Dk/Hok/49/98) [25] are listed in Table 1. The viruses were grown in 10-day-old embryonated chicken eggs and infectious allantoic fluids were stored at −80°C until use.

Phylogenetic analysis: Viral RNAs were extracted from the allantoic fluids of chicken embryos infected with viruses using TRIzol LS Reagent (Invitrogen, Carlsbad, CA, U.S.A.) and reverse-transcribed using the Uni12 primer [14] and M-MLV reverse transcriptase (Invitrogen). The cDNA was amplified by using the Takara Ex Taq (Takara Bio, Inc., Shiga, Japan). The first cycle of the amplifica-

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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.

tion program consisted of a 5 min period at 94°C and was followed by 30 cycles with the following conditions; 98°C for 10 sec, 55°C for 30 sec, and 72°C for 1 min. The last cycle was done at 72°C for 10 min. Polymerase chain reaction amplification of the viral genes was performed using a PTC-200 thermal cycler (BIO-RAD, Hercules, CA, U.S.A.). The primers used for HA gene amplification were H9-101F (5'-GGCCACCAGTCAACAAACTC-3') [24] and H9-1341R (5'-GTTTACATTCGCATCATGCTC-3'). Direct sequencing of the HA gene was performed using a CEQ 2000XL autosequencer (Beckman Coulter, Fullerton, CA, U.S.A.). For phylogenetic analysis, sequence data obtained for the genes together with those from public databases were analyzed using the neighbor-joining method [33] using MEGA 5.0 software (http://www.megasoftware.net/).

Antigenic analysis: Antigenic characterization of H9N2 influenza viruses was done by hemagglutination-inhibition (HI) test [35]. Hyperimmunized chicken antisera against seven H9N2 viruses were prepared [20]. Briefly, the sera were serially two-fold diluted with phosphate buffered saline (PBS) in 96-well microplates. The diluted sera were mixed with 8 hemagglutinin units of virus antigen and incubated at room temperature for 30 min. Chicken red blood cells (0.5%) were added to the antigen-serum dilution mixtures and incubated at room temperature for 30 min. HI titers were expressed as reciprocals of the highest serum dilutions that showed complete HI.

Virus replication and pathogenicity in embryonated chicken eggs: Viruses were inoculated into 10-day-old embryonated chicken eggs and incubated for 48 hr at 35°C. HA titers and 50% egg infectious dose (EID_{50}) were measured every 12 hr post-inoculation. Pathogenicity of Dk/Hok/49/98 against embryonated chicken eggs was evaluated by the mean death time as described Abenes *et al.* [2].

Vaccine preparation: Dk/Hok/49/98 and HK/1073/99 were injected into the allantoic cavities of 10-day-old embryonated chicken eggs and propagated at 35°C for 48 hr. The viruses in the allantoic fluids (512 HA for Dk/Hok/49/98 and 1,024 HA for HK/1073/99) were purified by differential centrifugation and sedimentation through a sucrose gradient [14]. The protein concentration was measured using the BCA Protein Assay Reagent (Thermo Fisher Scientific K. K., Waltham, MA, U.S.A.). The purified virus was inactivated with 0.1% formalin at 4°C for 7 days. The HA content was standardized as described [28]. Proteins of purified viruses were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% gel (BIO-RAD) and stained with Coomassie brilliant blue. The gel image was captured and analyzed by LumiVisionPRO (AISIN, Aichi, Japan), and the ratio of HA protein to total protein was calculated. On the basis of this method, concentration of HA protein was 14.7 μg in 50 μg of vaccine.

Challenge with HK/1073/99 into mice vaccinated once or twice: Inactivated Dk/Hok/49/98 or HK/1073/99 vaccines were injected once intraperitoneally into 4-week-old female BALB/c mice (Japan SLC, Inc., Shizuoka, Japan). PBS was injected into control mice. Three weeks later, 10 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, Rockville, MA, 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 HA genes were classified into the Eurasian lineage, and further classified into the Korean (n=11), Y280 (n=7), and G1 (n=4) sublineages (Fig. 1). The H9 viruses of the Korean and Y280 sublineages were isolated from water birds, poultry, pigs, and humans in East Asian countries, and those of the G1 sublineage were isolated from poultry in west Asian countries (Fig. 1).

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

Selection of H9N2 vaccine strain: To select an H9N2 vaccine strain, four H9N2 viruses, Dk/Hok/49/98, A/duck/Hokkaido/13/2000 (H9N2) (Dk/Hok/13/00), A/duck/Hokkaido/9/1999 (H9N2) (Dk/Hok/9/99), and A/duck/Hokkaido/26/1999 (H9N2) (Dk/Hok/26/99), were selected from 11 isolates of the Korean sublineage, and their replication and pathogenicity in embryonated chicken eggs were assessed. HA titers of Dk/Hok/49/98, Dk/Hok/13/00, Dk/Hok/9/99, and Dk/Hok/26/99 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}/m*l*, respectively, indicating that Dk/Hok/49/98 replicated efficiently in 10-day-old embryonated chicken eggs. Pathogenicity of Dk/Hok/49/98 in the embryonated chicken eggs was determined by mean death time and that of Dk/Hok/49/98 was 91.8 hr, indicating that Dk/Hok/49/98 had low pathogenicity in chicken embryos. This virus was selected as a candidate H9N2 vaccine strain.

Protective efficacy of the test vaccine in mice against H9N2 virus challenge: To assess the efficacy of the vaccine against H9N2 virus infection, HK/1073/99 was intranasally inoculated into mice that had previously been vaccinated once with inactivated HK/1073/99 or Dk/Hok/49/98. Immunogenicity of the inactivated vaccine was assessed by NT test, and virus titers in the lungs were measured to assess protective immunity induced by the vaccine (Table 3). Serum antibodies were detected in mice injected with 50, 10, and 2 μg protein of HK/1073/99 vaccine. The virus 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 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 (Table 3). A reduction in body weight was observed in mice injected with 10, 2, and 0.4 μg protein, and in the control group from day 2 post-infection, reaching up to 10% body weight loss at days 3–4 post-infection, compared with in the mice that received 50 μg of protein

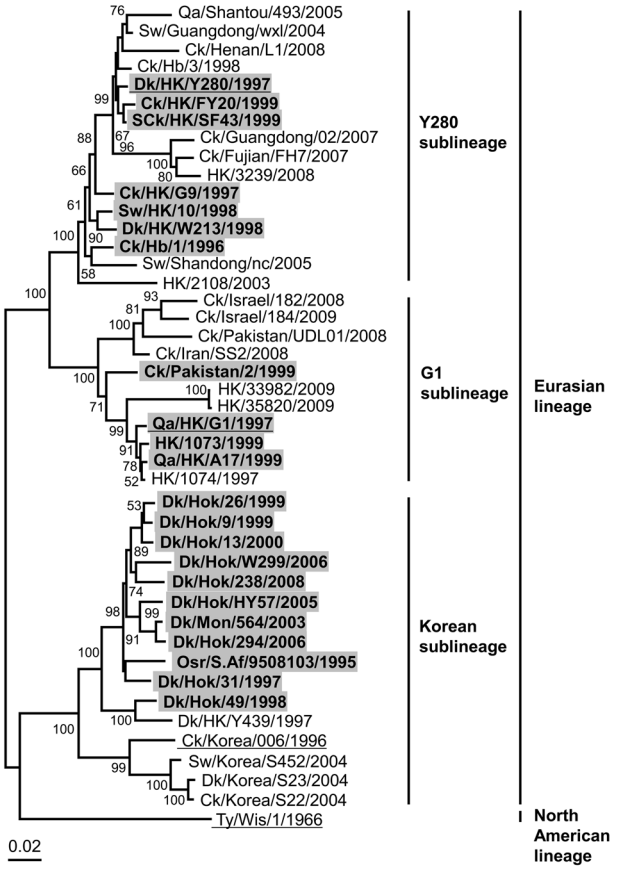

Fig. 1. Phylogenetic tree of the HA genes of H9N2 influenza viruses. Nucleotides 163–1,048 (886 bases) of the HA genes were used for the analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate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

(Fig. 2A).

We also tested the efficacy of vaccination with Dk/Hok/49/98 on protection against subsequent intranasal infection with HK/1073/99. Serum antibodies were slightly detected in mice injected with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein (Table 3). The virus titers in the lungs of mice injected with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein were $10^{4.3}$–$10^{5.3}$ EID_{50}/g. In the mice injected with 2 and 0.4 μg protein, the virus titers in the lungs of mice were similar to those of non-vaccinated control mice (Table 3). Although, a reduction in body weight was observed in mice at all doses of the Dk/Hok/49/98 vaccine, slight significant difference was observed in mice injecteded with 50 μg protein, compared with in mice injected with PBS (Fig. 2B).

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.

In the mice injected twice with Dk/Hok/49/98 vaccine on days 0 and 14, serum antibodies were detected in mice in the 50, 10, and 2 μg groups at one week after the second injection (Table 4). 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, and $10^{5.3}$–$10^{6.5}$ EID_{50}/g in the other vaccinated mice (Table 4). A reduction in body weight was observed in mice injected with 2 and 0.4 μg protein, and in control group, reaching up to 10% weight loss from days 4–6 post-infection, compared with in mice injected with 50 and 10 μg protein (Fig. 3). These results suggest that the repeat administration of the test vaccine confers immunity, and prevents body weight loss and decreases virus replication, after infection of mice with H9 influenza virus.

DISCUSSION

H9N2 viruses of each of the three sublineages, G1, Y280, and Korean, were recently isolated from wildbirds and poultry worldwide [3, 6, 27]. H9N2 viruses were also isolated from pigs and humans in China [4, 5, 37] and Korea, suggesting that these viruses have the potential to cause pandemic influenza in humans. H9N2 viruses isolated from pigs in China and Korea were classified into the Y280 and

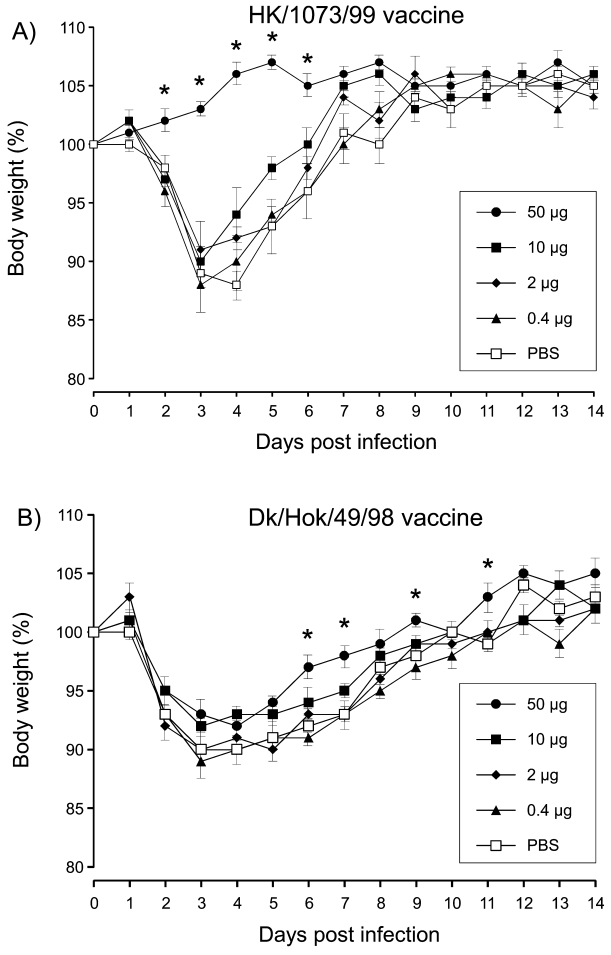

Fig. 2. Changes in body weight in mice vaccinated once following challenge with HK/1073/99. Five vaccinated mice of each group injected with HK/1073/99 vaccine (A), and with Dk/Hok/49/98 (B) were inoculated intranasally with HK/1073/99 and body weight was monitored for 14 days. Data are shown as mean body weight changes in each group with corresponding standard deviation (SD). Asterisks indicate that body weights were not significantly (P<0.05) decreased than PBS injected group.

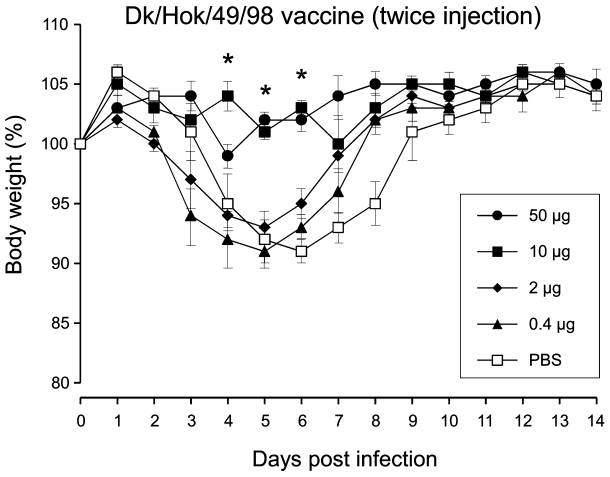

Fig. 3. Changes in body weight in mice vaccinated twice with Dk/Hok/49/98 vaccine following challenge with HK/1073/99. Five mice from each Dk/Hok/49/98 vaccine group were inoculated intranasally with HK/1073/99 and body weight was monitored for 14 days. Data are shown as mean body weight changes in each group with corresponding SD. Asterisks indicate that body weights were not significantly (P<0.05) decreased than PBS injected group.

Korean sublineages, while H9N2 viruses isolated from humans in China was classified into the G1 and Y280 sublineages [4, 5, 23, 31]. It is suggested that H9N2 viruses isolated from pigs and humans are antigenically distinct among viruses of the Korean, Y280, and G1 sublineages [5, 23, 31, 37]. Therefore, it is important that any H9N2 influenza virus vaccine to be used for pandemic influenza can broadly cross-react with antisera against all sublineage viruses. In the present study, Dk/Hok/49/98 was selected from the Korean sublineage, since antisera to the virus cross-reacted with all sublineages virus. Furthermore, Dk/Hok/49/98 replicated efficiently in embryonated chicken eggs and was non-pathogenic in chicken embryos. Recently, H9N2 viruses were isolated from pigs and humans in China [4, 5, 23, 31], it is necessary to analyze the antigenicity of these H9 isolates and evaluate the efficacy of test vaccine against

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.

them. Taken together, it is important to carry out surveillance of avian influenza consecutively and to analyze the isolates antigenically and phylogenetically.

In the present study, it was suggested that the test whole particle vaccine has the potency against challenge with H9N2 virus of different sublineage in mice. It was already reported that 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 [8, 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, 34, 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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