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Potency of an inactivated influenza vaccine prepared from A/duck/Mongolia/245/2015 (H10N3) against H10 influenza virus infection in a mouse model

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Abstract

The H10N8 influenza virus became a threat to public health when cases of fatal infections were identified in China in 2013 and 2014. Thus, genetic and antigenic characterization of H10 influenza viruses and development of an appropriate vaccine are essential to prepare for a future pandemic by H10 influenza viruses. However, current information regarding these properties of H10 influenza viruses circulating in birds is limited. In this study, genetic analysis of H10 influenza viruses revealed that the viruses recently circulating in wild birds in East Asia are genetically close to human H10N8 influenza viruses. Furthermore, the antigenicity of H10 influenza viruses was stable among the viruses circulating in birds. An inactivated vaccine was prepared from A/duck/Mongolia/245/2015 (H10N3), which is genetically and antigenically close to the human H10 influenza viruses. The vaccine induced sufficient neutralizing antibodies against homologous and heterologous viruses in mice. The inactivated vaccine induced protective immunity sufficient to reduce the impact of challenges with A/duck/Hokkaido/W87/2007 (H10N2), which is pathogenic strain in mice. This study demonstrates that the inactivated whole virus particle vaccine prepared from viruses isolated from wild birds would be useful against a future pandemic influenza by H10 influenza viruses.

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Introduction

In 2013 and 2014, H10N8 influenza viruses were transmitted to humans from birds, and epidemiological studies strongly suggested that the infection source was poultry in live bird markets[2,3]. This included the first fatal cases of H10 influenza virus infection in humans, although several sporadic and mild cases had reported in 2004 and 2010[2,39]. At present, the H10N8 influenza viruses and novel reassortant H10 influenza viruses derived from the human H10N8 influenza viruses are circulating in poultry in Asian countries[9,18,19,35,38]. These facts suggest that avian H10 influenza viruses may transmit to human populations in the future. Therefore, genetic and antigenic characterization of H10 influenza viruses is crucial to prepare for a future pandemic. However, information regarding the antigenicity of H10 influenza viruses circulating in both poultry and wild birds is limited[31]. Furthermore, few studies have reported on vaccine preparation for pandemic influenza by H10 influenza viruses[17,24,31,34].

Since 1996, we have conducted intensive surveillance of avian influenza in wild waterfowl in Alaska, Mongolia, and Hokkaido, Japan to monitor viruses that are maintained in the nesting lakes in northern territories such as Alaska and Siberia and spread southward along with the autumn migration of the birds[6,10,36]. H10 influenza viruses were isolated from fecal samples of wild birds in the surveillance study. All viruses isolated in the surveillance study are stored in our influenza virus library (http://virusdb.czc.hokudai.ac.jp/). Previous studies revealed that whole virus particle vaccines prepared from the library induce effective immunity against infections with H1, H2, H5, H6, H7, and H9 influenza viruses in mice and macaque models[4,11,12,20,21,22,32].

In the present study, genetic and antigenic properties of H10 influenza viruses isolated from wild water birds in Hokkaido and Mongolia were analyzed. In addition, the immunogenicity and protective efficacy of an inactivated whole virus particle vaccine against influenza caused by H10 influenza viruses prepared from viruses in our library was demonstrated in mice.

Materials and Methods

Viruses and cells: Influenza virus A/chicken/Germany/N/1949 (H10N7) was kindly provided by Dr. Robert G. Webster, St. Jude Children's Research Hospital, USA. A/duck/Hong Kong/786/1979 (H10N3) was generously provided by Dr. Kennedy F. Shortridge, the University of Hong Kong, Hong Kong SAR. A/duck/Shimane/45/1997 (H10N7) was kindly provided by Dr. Koichi Otsuki, Tottori University, Japan. A/duck/Hokkaido/18/2000 (H10N4), A/duck/Hokkaido/W87/2007 (H10N2), A/duck/Mongolia/97/2014 (H10N6), A/duck/Mongolia/66/2015 (H10N2), A/duck/Mongolia/709/2015 (H10N7), A/duck/Mongolia/245/2015 (H10N3), and A/duck/Alaska/658/1991 (H10N7) were isolated from fecal samples of wild water birds collected in our surveillance study[6,10,36]. A/duck/Vietnam/OIE-0483/2012 (H10N7) was isolated from a poultry duck in a live bird market in Vietnam[21]. All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hr, and the infectious allantoic fluids were stored at −80°C until use. Madin-Darby canine kidney (MDCK) cells were grown in minimum essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% inactivated calf serum and antibiotics, and used for titration of viral infectivity in serum-free MEM supplemented with antibiotics and acetylated trypsin (Sigma-Aldrich, MO, USA).

Sequencing and phylogenetic analysis: Viral RNA was extracted from the allantoic fluids of embryonated chicken eggs using TRIzol™ LS Reagent (Life Technologies, CA, USA) and reverse-transcribed with Uni12 primer (5'-AGCAAA AGCAGG-3') and M-MLV Reverse Transcriptase

(Life Technologies)[7]. The full-length PB2 and HA gene segments were amplified by polymerase chain reaction (PCR) using *Ex Taq* Polymerase® (TaKaRa Bio, Shiga, Japan) and gene-specific primer sets. Direct sequencing of each gene segment was performed using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a 3500 Genetic Analyzer (Life Technologies). Sequencing data were analyzed and aligned using ClustalW in GENETYX® Network version 12 (Genetyx Co., Tokyo, Japan). The nucleotide sequences were phylogenetically analyzed by the maximum-likelihood (ML) method using MEGA 6.0 software (http://www.megasoftware.net/). Sequence data for H10 HA genes were compared with reference sequences selected and obtained from GenBank/EMBL/DDBJ. Nucleotide identity was determined using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/).

Antigenic analysis: To analyze the antigenic properties of H10 influenza viruses, the hemagglutination inhibition (HI) test was performed using hyper-immunized chicken antisera against A/duck/Mongolia/245/2015 (H10N3), A/chicken/Germany/N/1949 (H10N7), A/duck/Vietnam/OIE-0483/2012 (H10N7), and A/duck/Alaska/658/1991 (H10N7). Twenty-five µl of 8 hemagglutination (HA) units of the test virus was added to 25 µl of 2-fold dilutions of each antiserum in PBS and incubated at room temperature for 30 min. Thereafter, 50 µl of 0.5% chicken red blood cells in PBS was added and incubated at room temperature for another 30 min. Hemagglutination inhibition (HI) titers were expressed as the reciprocal of the highest serum dilution showing complete inhibition of hemagglutination.

Pathogenicity of H10 influenza viruses in mice: To analyze the pathogenicity of H10 infuenza viruses in mice, A/duck/Alaska/658/1991 (H10N7), A/duck/Hokkaido/W87/2007 (H10N2), A/duck/Hokkaido/18/2000 (H10N4), A/duck/Mongolia/66/2015 (H10N2), A/duck/Mongolia/709/2015

(H10N7), or A/duck/Mongolia/245/2015 (H10N3) were intranasally inoculated into two 7-week-old female BALB/c mice (Japan SLC Inc., Shizuoka, Japan) under anesthesia. Titers of inoculated viruses were $10^{5.5}$, $10^{5.3}$, $10^{5.8}$, $10^{5.8}$, $10^{5.5}$, and $10^{5.8}$ times the 50% tissue culture infectious dose ($TCID_{50}$)/30 µl, respectively. The mice were observed for clinical signs of infection and body weight was measured daily for 14 days.

Vaccine preparation: A vaccine strain, A/duck/Mongolia/245/2015 (H10N3), was inoculated 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 were purified by differential centrifugation and sedimentation through a sucrose gradient modified from Kida *et al.* (1982)[13]. Briefly, allantoic fluids were ultracentrifuged, and the resulting pellets were layered onto a 10%–50% sucrose density gradient and ultracentrifuged. The fractions containing the intact virus particles were collected based on the sucrose concentration, HA titer, and protein concentration. Then, the whole virus particles were pelleted from the sucrose fractions by ultracentrifugation and suspended in PBS. The purified viruses were inactivated by incubation in 0.1% formalin at 4°C for 7 days. Virus inactivation was confirmed by inoculation of the formalin-treated samples into embryonated chicken eggs. The purified and inactivated virus was used as a whole virus particle vaccine. The total protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). In addition, each viral protein in the vaccine was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the relative amounts of the HA protein were estimated as a ratio of the HA protein in the total protein using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Potency test of the vaccine in mice: In order to evaluate the immunogenicity and protective efficacy of the vaccine strain *in vivo,* experiments

in mouse model were conducted. A whole inactivated vaccine of A/duck/Mongolia/245/2015 (H10N3) (10, 50, and 100 μg total protein/vaccine, containing 2.5, 5.1, and 25.4 μg HA protein, respectively) was injected subcutaneously into ten 4-week-old female BALB/c mice (Japan SLC) and PBS was injected into the control mice. Serum samples were collected from each mouse 21 days after the vaccination. The vaccine (10, 50, and 100 μg total protein/vaccine, containing 2.5, 5.1, and 25.4 μg HA protein respectively) was also subcutaneously injected twice into ten mice with a 2-week interval. Fourteen days after the first vaccination, each mouse was injected again at the same dose as the first injection. After another 2-week interval, serum samples were collected from each mouse, and all of the mice were challenged with $10^{5.0}$ $TCID_{50}$/30 μl of A/duck/Hokkaido/W87/2007 (H10N2) intranasally under anesthesia. The neutralizing antibody titers of the mice sera against homologous viruses and A/duck/Hokkaido/W87/2007 (H10N2) were determined by serum neutralization tests using MDCK cells. Three days after the challenge, five mice from each group were sacrificed, and their lungs were collected. Titers of recovered viruses from the lung homogenates were measured using MDCK cells. The other five mice from each group were observed for clinical signs of infection, and their body weight was measured for 14 days. The statistical significance of weight loss and virus titers in the mice lungs were calculated by Student's t-test, and $P < 0.05$ was considered significant.

Virus titration: Ten-fold dilutions of virus samples or mice lung homogenates were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for 1 hr. Unbound viruses were removed and the cells were washed with PBS. The cells were subsequently overlaid with MEM containing 5 μg/ml acetylated trypsin. The virus titers were determined as the product of the reciprocal value of the highest virus dilution showing 50% of the cytopathic effects following

72 hr incubation and expressed as $TCID_{50}$.

Serum neutralization test: Serum neutralizing antibody titers were measured according to the method of Sakabe *et al.*[27]. Briefly, test sera and 100 $TCID_{50}$ of A/duck/Hokkaido/W87/2007 (H10N2) or the vaccine strain virus were mixed and incubated for 1 hr at room temperature. The mixture was inoculated onto MDCK cells and incubated at 35°C for 1 hr. Unbound viruses were removed, and the cells were washed with PBS. The cells were subsequently incubated in MEM containing 5 μg/ml acetylated trypsin (Sigma-Aldrich). Cytopathic effects were observed following 72 hr incubation, and the neutralizing antibody titers were determined as the reciprocal of the serum dilution yielding 50% inhibition of the cytopathic effects.

Ethics statement: All animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 16-0105), and all experiments were performed according to the guidelines of this committee.

Results

Genetic analysis of H10 influenza viruses

Nucleotide sequences of HA genes of H10 influenza viruses in the influenza virus library were determined and phylogenetically analyzed along with reference sequences available in the public database (Table 1, Fig. 1). Based on the results of the phylogenetic analysis, the H10 HA genes were classified into Eurasian and North American lineages as described in previous studies[18,41]. The Eurasian lineage viruses were isolated in Asian, European, African countries and also in Canada. Viruses isolated from birds in recent years grouped separately from Old-Eurasian H10 influenza viruses, represented by A/chicken/Germany/N/1949 (H10N7) and A/duck/

Table 1. Accession numbers of the HA and the PB2 gene sequences submitted to the GenBank/EMBL/DDBJ in this study

Viruses	Accession No.
A/duck/Hokkaido/131/2008 (H10N7)	LC337290, AB569451*
A/duck/Mongolia/97/2014 (H10N6)	LC337289, LC342073
A/duck/Mongolia/66/2015 (H10N2)	LC108117, LC108120
A/duck/Mongolia/709/2015 (H10N7)	LC121433, LC121436
A/duck/Mongolia/245/2015 (H10N3)	LC121345, LC121348

*Accession No. of PB2 was given in our previous study (Samad *et al.*, Jpn J Vet Res, 2011)
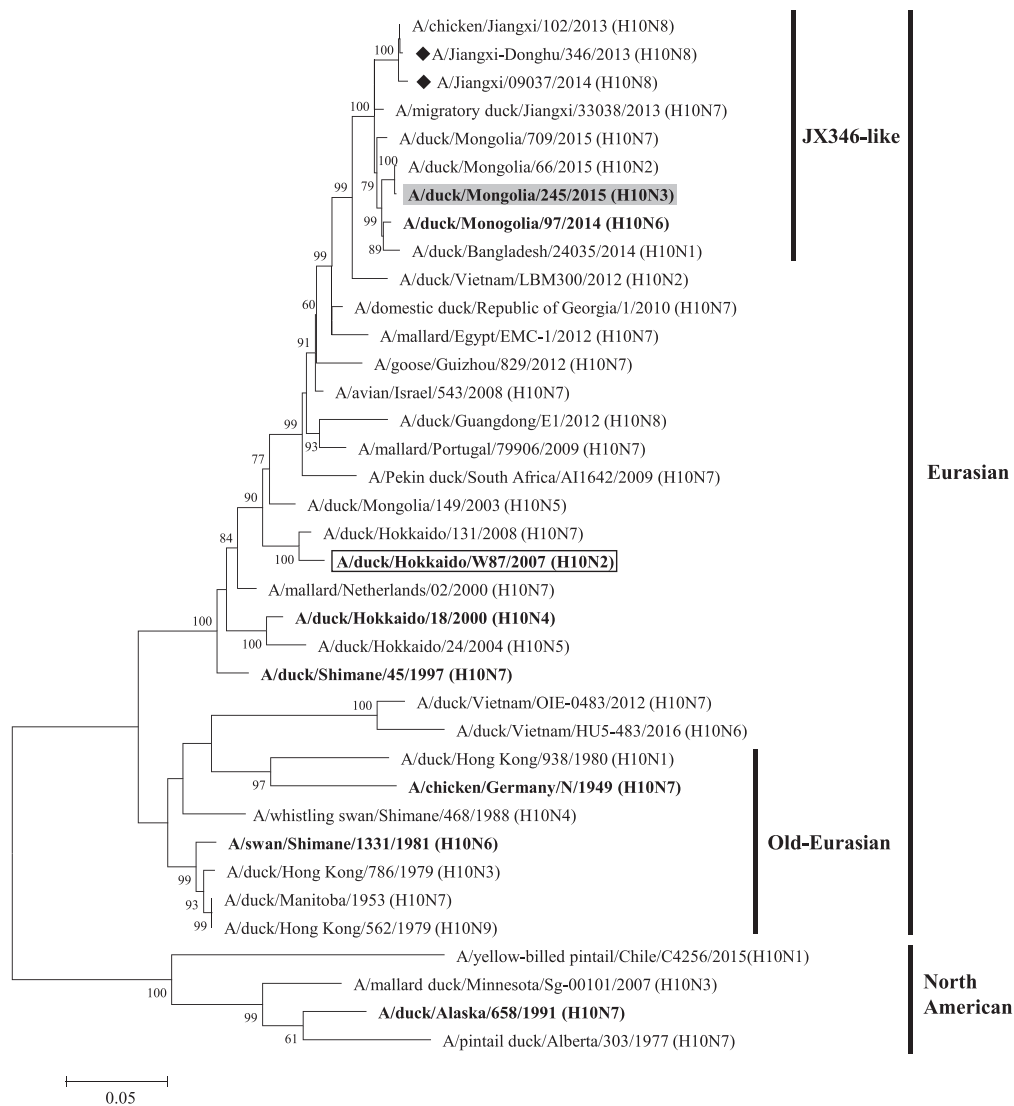
Fig. 1. Phylogenetic tree of H10 HA genes of influenza viruses. Full-length nucleotide sequences of the HA gene were used for phylogenetic analysis using maximum likelihood (ML). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node indicate the confidence level in bootstrap analysis with 1,000 replications. The viruses marked with black diamonds were isolated from human patients. The viruses used for antigenic analysis are shown in bold. The vaccine strain is highlighted in gray, and the challenge strain is enclosed in a box.

Table 2. The cross-reactivity of H10 viruses with chicken antisera to representative strains of each lineage in HI test

Linages	Viruses	HI titers of the antisera			
		Dk/Mon/245	Dk/VN/OIE	Ck/Ger/N	Dk/AK/658
Eurasian	A/duck/Mongolia/245/2015 (H10N3)	<u>1,280</u>	2,560	640	10,240
	A/duck/Mongolia/97/2014 (H10N6)	640	1,280	640	2,560
	A/duck/Hokkaido/W87/2007 (H10N2)	1,280	2,560	640	10,240
	A/duck/Hokkaido/18/2000 (H10N4)	2,560	2,560	1,280	20,480
	A/duck/Shimane/45/1997 (H10N7)	640	2,560	640	5,120
	A/duck/Vietnam/OIE-0483/2012 (H10N7)	1,280	<u>2,560</u>	1,280	5,120
	A/chicken/Germany/N/1949 (H10N7)	640	2,560	<u>2,560</u>	5,120
	A/duck/Hong Kong/786/1979 (H10N3)	320	320	160	1,280
North American	A/duck/Alaska/658/1991 (H10N7)	1,280	2,560	640	<u>5,120</u>

Homologous titers are underlined.
Abbreviations: Dk/Mon/245, A/duck/Mongolia/245/2015 (H10N3); Dk/VN/OIE,
A/duck/Vietnam/OIE-0483/2012 (H10N7); Ck/Ger/N, A/chicken/Germany/N/1949 (H10N7);
Dk/AK/658, A/duck/Alaska/658/1991 (H10N7)

Hong Kong/786/1979 (H10N3). A/duck/Mongolia/245/2015 (H10N3) isolated from a fecal sample of wild duck in Alag zegstei nuur in Mongolia was genetically close to human H10N8 influenza viruses, represented by A/Jiangxi-Donghu/346/2013 (H10N8), as well as viruses isolated from poultry in China, which were classified as JX346-like H10 influenza viruses[38]. A/duck/Vietnam/OIE-0483/2012 (H10N7) and A/duck/Vietnam/HU5-483/2016 (H10N6) were genetically close to the Old-Eurasian H10 influenza viruses. Viruses showing highest identity analyzed using NCBI BLAST with these viruses isolated in Vietnam were A/duck/Hong Kong/562/1979 (H10N9) and A/whistling swan/Shimane/468/1988 (H10N4) respectively, and the identity of the nucleotide sequences of two viruses was 95.6%.

Antigenic analysis of H10 influenza viruses

The antigenicity of H10 influenza viruses isolated in our surveillance study, including A/duck/Alaska/658/1991 (H10N7) belonging to the North American lineage, A/duck/Shimane/45/1997 (H10N7), and A/duck/Vietnam/OIE-0483/2012 (H10N7) were analyzed by HI tests (Table 2). Antiserum against A/duck/Mongolia/245/2015 (H10N3) reacted with avian H10 influenza viruses isolated in recent years in Asia but not

with A/duck/Hong Kong/786/1979 (H10N3) within 2-fold difference compared with HI titers of homologous combination. Antiserum against A/duck/Mongolia/245/2015 (H10N3) also cross-reacted with the virus belonging to the North American lineage. Antiserum against A/duck/Vietnam/OIE-0483/2012 (H10N7) broadly reacted with H10 influenza viruses except for A/duck/Hong Kong/786/1979 (H10N3) similar to the antiserum against A/duck/Mongolia/245/2015 (H10N3). Antiserum against A/duck/Alaska/658/1991 (H10N7) showed cross-reactivity with viruses belonging to the Eurasian lineage. Antiserum against A/chicken/Germany/N/1949 (H10N7) did not react well with viruses recently isolated from birds, and this result is consistent with a previous study[31].

Pathogenicity of avian H10 influenza viruses in mice

To analyze the pathogenicity of H10 infuenza viruses in mice, six strains of avian H10 influenza viruses were inoculated into two naïve mice respectively and observed their clinical signs for 14 days. Significant body weight loss (a decrease of 25% during the observation period as compared with that of before the challenge) was observed in mice inoculated with A/duck/
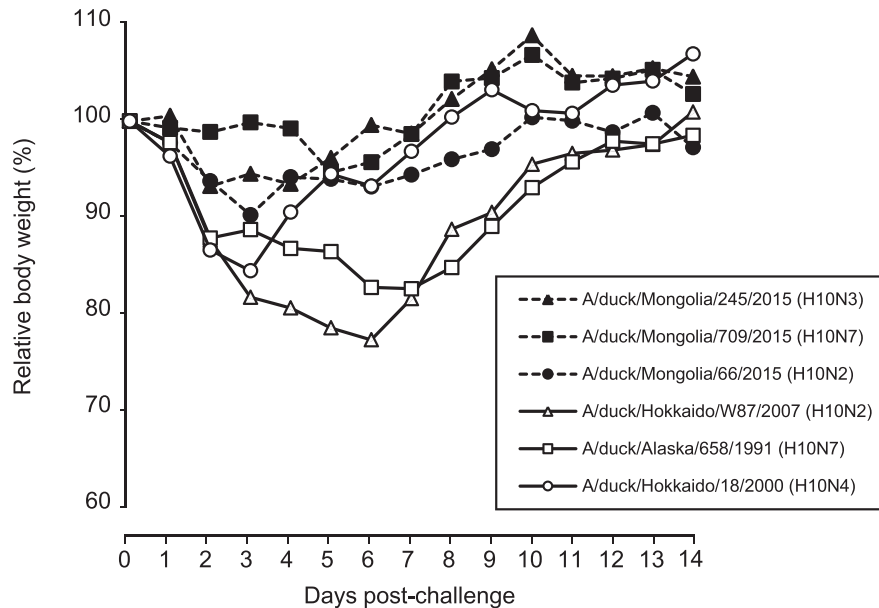
Fig. 2. Changes in the body weight of mice inoculated with avian H10 influenza viruses. Body weight was monitored for 14 days. Each strain was inoculated into two mice.

Hokkaido/W87/2007 (H10N2), A/duck/Alaska/658/1991 (H10N7), and A/duck/Hokkaido/18/2000 (H10N4) (Fig. 2). On the other hand, a relatively mild reduction of body weight was seen in the group of mice inoculated with A/duck/Mongolia/245/2015 (H10N3), A/duck/Mongolia/709/2015 (H10N7), and A/duck/Mongolia/66/2015 (H10N2). The amino acids sequences of PB2 and HA of the strains in Fig. 2 and A/Jiangxi-Donghu/346/2013 (H10N8) were compared (Table 3). Common amino acid sequences were observed at position 340 of the PB2, and 46, 326, and 506 of the HA of A/duck/Hokkaido/W87/2007 (H10N2), A/duck/Alaska/658/1991 (H10N7), and A/duck/Hokkaido/18/2000 (H10N4), which significantly reduced body weight of mice. No viruses had lysine (K) at the position 627 on the PB2 except for the human H10N8 influenza virus.

Potency test of the vaccine against H10 influenza virus in mice

Based on the antigenic analysis, the H10 influenza virus A/duck/Mongolia/245/2015 (H10N3), which was genetically close to human H10N8 influenza viruses and recently isolated from wild birds, showed cross-reactivity with a broad range of H10 influenza viruses and was selected as a vaccine candidate strain. An inactivated vaccine was prepare and immunized into mice. Neutralizing antibody titers of sera collected from mice immunized once with A/duck/Mongolia/245/2015 (H10N3) reached at most 1:40 to both homologous and heterologous virus, A/duck/Hokkaido/W87/2007 (H10N2) (Table 4). In contrast, the neutralizing antibody titers of mouse sera injected twice with 100 μg of the vaccine reached 1:320 to both homologous virus and A/duck/Hokkaido/W87/2007 (H10N2) (Table 5). The virus titers in the lungs of the mice injected twice with the vaccine were suppressed in vaccine-dose dependent manner. All mice vaccinated twice with each dose of vaccine survived for 14 days but exhibited roughing fur, and loss of body weight for three days post-challenge and then recovered (Fig. 3). The body weight reduction was significantly suppressed when the vaccine antigen dosage was 100 or 50 μg. These results indicate that the A/duck/Mongolia/245/2015 (H10N3) vaccine induced immunity in mice sufficient to reduce the impact of a challenge with A/duck/Hokkaido/W87/2007 (H10N2).

Table 3. Comparison of the amino acid sequences among H10 influenza viruses inoculated into mice

Viruses	Deduced amino acids on the PB2 at the position[a]																						
	3	4	87	127	156	187	190	195	282	292	340	389	411	478	553	588	598	627	648	660	674	676	701
A/duck/Mongolia/245/2015 (H10N3)	R	I	D	H	S	K	K	D	E	I	K	R	I	V	I	A	T	E	L	K	T	T	D
A/duck/Mongolia/709/2015 (H10N7)	·	·	·	·	A	·	·	·	D	·	·	·	·	·	·	·	·	·	·	·	A	·	·
A/duck/Mongolia/66/2015 (H10N2)	·	·	·	·	A	R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·
A/duck/Hokkaido/W87/2007 (H10N2)	K	K	·	Y	A	·	·	·	·	·	R	·	·	·	V	·	·	·	·	R	A	I	·
A/duck/Alaska/658/1991 (H10N7)	·	·	·	·	A	·	·	·	·	·	R	·	I	I	·	·	·	·	·	·	A	·	·
A/duck/Hokkaido/18/2000 (H10N4)	·	·	·	·	A	·	R	·	·	V	R	·	·	·	·	·	·	·	·	·	A	·	·
A/Jiangxi-Donghu/346/2013 (H10N8)	·	E	·	·	A	·	·	G	·	V	R	K	V	·	·	V	V	E/K	V	·	A	M	·

[a]Numbering from start codon

Viruses	Deduced amino acids on the HA at the position[b]																								
	7	20	32	45	46	47	48	49	56	57	82	90	92	93	102	104	118	137	160	173	174	179	198	203	210
A/duck/Mongolia/245/2015 (H10N3)	K	A	Q	T	G	L	N	R	N	H	T	E	N	I	V	E	N	K	N	T	V	M	G	S	Q
A/duck/Mongolia/709/2015 (H10N7)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·
A/duck/Mongolia/66/2015 (H10N2)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A/duck/Hokkaido/W87/2007 (H10N2)	·	T	·	A	S	·	·	K	S	·	·	·	·	V	·	S	S	·	·	·	A	·	E	·	·
A/duck/Alaska/658/1991 (H10N7)	H	R	A	K	S	·	D	·	·	Y	·	D	S	·	A	·	D	·	·	S	A	V	·	·	·
A/duck/Hokkaido/18/2000 (H10N4)	R	·	·	·	S	E	·	·	S	·	·	·	·	·	·	·	S	·	D	·	A	I	·	L	·
A/Jiangxi-Donghu/346/2013 (H10N8)	·	·	·	·	·	I	·	·	K	·	M	·	·	·	·	·	·	R	·	·	A	·	·	·	R

[b]H3 numbering

Viruses	Deduced amino acids on the HA at the position[b]																								
	226	227	228	236	261	270	272	273	276	284	291	310	311	312	315	326	328	339	385	472	476	489	490	495	506
A/duck/Mongolia/245/2015 (H10N3)	Q	S	G	L	I	D	P	I	N	R	R	N	K	R	M	L	G	I	I	A	S	S	Q	A	T
A/duck/Mongolia/709/2015 (H10N7)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A/duck/Mongolia/66/2015 (H10N2)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A/duck/Hokkaido/W87/2007 (H10N2)	·	·	·	·	V	·	·	·	·	·	R	·	·	·	·	I	·	·	·	·	·	·	·	·	K
A/duck/Alaska/658/1991 (H10N7)	·	·	·	V	R	G	L	V	D	K	K	S	·	·	L	I	E	·	·	K	N	T	·	·	K
A/duck/Hokkaido/18/2000 (H10N4)	·	·	·	·	·	E	·	·	D	·	K	·	·	K	·	I	·	·	·	·	·	·	H	S	K
A/Jiangxi-Donghu/346/2013 (H10N8)	·	·	·	·	·	·	·	·	·	·	·	·	R	·	·	·	·	L	V	·	·	·	·	·	·

[b]H3 numbering

Table 4. Serum neutralizing antibody titers of mice injected once with vaccines

Vaccine	Dose*	Neutralizing titer against 21 days post immunization against									
		Dk/Mon/245 (H10N3)					Dk/Hok/W87 (H10N2)				
Dk/Mon/245 (H10N3)	10	<40,	<40,	<40,	<40,	<40	<40,	<40,	<40,	<40,	<40
	50	<40,	<40,	40,	40,	40	<40,	<40,	<40,	40,	40
	100	<40,	40,	<40,	<40,	<40	<40,	40,	<40,	<40,	<40
PBS		<40,	<40,	<40,	<40,	<40	<40,	<40,	<40,	<40,	<40

* Total protein concentrations were measured using the BCA assay.
Abbreviations: Dk/Mon/245, A/duck/Mongolia/245/2015 (H10N3); Dk/Hok/W87, A/duck/Hokkaido/W87/2007 (H10N2)

Table 5. Serum neutralizing antibody titers of mice injected twice with vaccines and virus recovery from mice lungs

Vaccine	Dose*	Neutralizing titer against 28 days post immunization against										Virus titer in lungs at 3 days post challenge ($\log_{10}$ TCID$_{50}$/g)				
		Dk/Mon/245 (H10N3)					Dk/Hok/W87 (H10N2)									
Dk/Mon/245 (H10N3)	10	<40,	<40,	<40,	<40,	40	<40,	40,	40,	<40,	<40	3.8,	3.8,	3.8,	5.3,	4.6
	50	40,	80,	40,	160,	40	40,	80,	40,	40,	40	≤1.8,	3.1,	2.8,	2.8,	3.6
	100	160,	80,	80,	320,	320	160,	40,	320,	40,	80	2.8,	≤1.8,	≤1.8,	≤1.8,	≤1.8
PBS		<40,	<40,	<40,	<40,	<40	<40,	<40,	<40,	<40,	<40	4.8,	5.3,	6.3,	5.3,	4.3

* Total protein concentrations were measured using the BCA assay.
Abbreviations: Dk/Mon/245, A/duck/Mongolia/245/2015 (H10N3); Dk/Hok/W87, A/duck/Hokkaido/W87/2007 (H10N2)

Discussion

During the 2013–2014 winter season in China, H10 influenza viruses were transmitted to humans, and two fatal cases were reported[3,39]. Thus, preparation for future pandemics caused by H10 influenza viruses is greatly needed. Vaccination is one of the effective control measure for human influenza. The present study demonstrated that an inactivated whole virus particle vaccine prepared from A/duck/Mongolia/245/2015 (H10N3) induced neutralizing antibodies against the homologous virus and A/duck/Hokkaido/W87/2007 (H10N2) in mice after two subcutaneous injections. This inactivated virus vaccine is sufficient to reduce the impact of a challenge with A/duck/Hokkaido/W87/2007 (H10N2) in vaccinated mice. Genetic analysis revealed that H10 influenza viruses recently circulating in wild birds are genetically close to JX346-like H10N8 influenza viruses[18]. Therefore, the vaccine prepared from A/duck/Mongolia/245/2015 (H10N3) may be potentially useful

for a future pandemic influenza caused by H10 influenza viruses. In addition, inactivated whole virus particle influenza vaccines are more effective than split influenza vaccines[1,8,22]. An inactivated whole virus particle vaccine prepared from reassortant virus having HA and NA genes derived from human H10 influenza viruses on an A/Puerto Rico/8/34 (H1N1) backbone showed protective efficacy in mouse model[34]. The present study also provide insight that the inactivated whole virus particle vaccine prepared from avian H10 influenza viruses also effective in mice. Furthermore, our findings regarding antigenicity and immunogenicity of H10 influenza viruses isolated from wild birds in this study also can be applied not only to preparation of whole virus particle vaccines, but also to vaccine developments involving RNA vaccine or other novel techniques reported in some studies using human H10 influenza viruses[17,24].

Genetic analysis also revealed that H10 influenza viruses isolated from fecal samples of wild birds in Hokkaido and Mongolia are close to
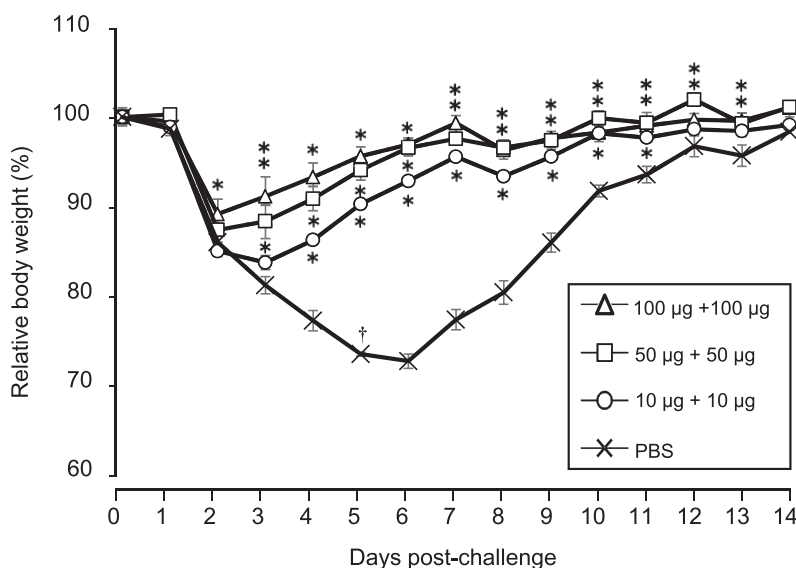
Fig. 3. Changes in the body weight of mice subcutaneously vaccinated twice with the A/duck/ Mongolia/245/2015 (H10N3) following a challenge with A/duck/Hokkaido/W87/2007 (H10N2). Body weight was monitored for 14 days. Data are shown as the mean body weight ± standard error. Asterisks indicate that body weights of the vaccinated groups recovered significantly more than the PBS-injected group ($P < 0.05$). †: Mice Died.

the human H10N8 influenza viruses, and the antigenicity of these viruses is stable in wild birds. Interestingly, A/duck/Vietnam/OIE-0483/ 2012 (H10N7) and A/duck/Vietnam/HU5-483/ 2016 (H10N6) isolated from a domestic duck in the live bird markets in Mekong Delta in southern Vietnam were genetically close to the Old-Eurasian H10 influenza viruses, but were not antigenically distinct from avian H10 influenza viruses recently isolated in Asian countries[23]. Data regarding the antigenicity of H10 influenza viruses isolated from humans or poultry in Asia is not yet available. Thus, studies on genetic and antigenic analysis of H10 influenza viruses must be continued.

In the PB2 protein of human H10N8 influenza virus, the amino acid residue at 627 is mixture of glutamic acid (E) and K[3]. A previous study suggested that E627K in the PB2 is key to mammal adaptation of avian H10 influenza viruses[25]. In mammals, this mutation and another residue at 701, from aspartic acid (D) to asparagine (N) in the PB2, leads to enhanced polymerase activity, resulting in host adaptation and enhanced virulence[5,15,16,28,29,30]. In the present

study, viruses with mutations at 627 or 701 in the PB2 were not identified among H10 influenza viruses isolated from wild ducks. Additionally, the human H10N8 influenza virus conserved amino acid motif of glutamine-serine-glycine (Q-S-G) at position 226-228 of the HA which is considered to be associated with receptor binding specificity of the virus to avian-type receptors[3,38]. In accordance with the amino acid sequences, the human H10N8 influenza virus shows a preference for avian-type receptors detected on epithelial cells of the upper respiratory tracts of ducks[26,33,37]. According to the result of this study, the amino acid motif of Q-S-G at position 226-228 of the HA was conserved among analyzed avian H10 influenza viruses. Despite these characteristics of amino acid sequences in the PB2 and the HA, some avian H10 influenza viruses showed severe pathogenicity in mice, but the crucial amino acid residues or motif for pathogenicity in mice were not understood. It has been reported that combination of amino acid substitutions in the NA and PB2 contribute to the mouse adaptation of H10 influenza viruses[40]. Therefore, further studies on amino acid differences involving eight

genes of H10 influenza viruses would clarify pathogenicity markers and pathogenic strains of H10 influenza viruses in mammals.

Taken together, our findings suggest that intensive global surveillance of H10 influenza viruses both in poultry and wild birds must be continued to monitor the introduction of H10 influenza virus into poultry. Furthermore, monitoring for the introduction of H10 influenza viruses to humans or other mammals, including pigs, is important for an early response to a pandemic.

Acknowledgments

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