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Virology

Potency of an inactivated influenza vaccine prepared from A/duck/Hokkaido/162/2013 (H2N1) against a challenge with A/swine/Missouri/2124514/2006 (H2N3) in mice

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ABSTRACT. H2N2 influenza virus caused a pandemic starting in 1957 but has not been detected in humans since 1968. Thus, most people are immunologically naive to viruses of the H2 subtype. In contrast, H2 influenza viruses are continually isolated from wild birds, and H2N3 viruses were isolated from pigs in 2006. H2 influenza viruses could cause a pandemic if re-introduced into humans. In the present study, a vaccine against H2 influenza was prepared as an effective control measure against a future human pandemic. A/duck/Hokkaido/162/2013 (H2N1), which showed broad antigenic cross-reactivity, was selected from the candidate H2 influenza viruses recently isolated from wild birds in Asian countries. Sufficient neutralizing antibodies against homologous and heterologous viruses were induced in mice after two subcutaneous injections of the inactivated whole virus particle vaccine. The inactivated vaccine induced protective immunity sufficient to reduce the impact of challenges with A/swine/Missouri/2124514/2006 (H2N3). This study demonstrates that the inactivated whole virus particle vaccine prepared from an influenza virus library would be useful against a future H2 influenza pandemic.

KEY WORDS: antigenicity, H2 influenza, pre-pandemic, vaccine

H2N2 influenza virus was the causative agent of an influenza pandemic known as Asian flu, which started in 1957. More than one million deaths were reported worldwide. However, H2 influenza viruses have not been detected in the human population since 1968 in replacement of another pandemic influenza caused by H3N2 influenza viruses. In contrast, H2 avian influenza viruses have been continuously circulating in wild aquatic and sporadically isolated from domestic birds around the world [6, 16, 17, 24, 28, 29]. In addition, H2N3 influenza viruses were isolated from pigs in 2006 in Missouri, U.S.A. [19]. These facts suggest that avian H2 influenza viruses may occasionally transmit to pigs and can be re-introduced into the human population in the future. Such an event could result in a pandemic because of the lack of acquired immunity against H2 influenza viruses in the current human population [27]. Therefore, vaccines against H2 influenza viruses are needed to prepare for a future human pandemic [20].

The HA genes of H2 influenza viruses are phylogenetically divided into North American and Eurasian lineages [26]. The H2N2 influenza viruses that caused Asian flu belong to the Eurasian lineage and the H2N3 influenza viruses that were isolated from pigs in 2006 belong to the North American lineage. The avian H2 influenza virus A/black duck/New Jersey/1580/1978 (H2N3) antigenically cross-reacts with H2 influenza viruses isolated from humans and birds before 1991 [5, 14]. The H2N3 influenza viruses isolated from pigs show antigenic cross-reactivity with North American and Eurasian H2 avian influenza viruses [13]. However, information regarding the H2 influenza viruses recently isolated in Asia, particularly on the antigenicity of such viruses is limited. To prepare in case of H2 influenza virus transmission to the human population from animals, the characterization of genetic and antigenic properties of recent isolates, including viruses recently isolated from wild bird in Asia is greatly needed. Since 1996, we have conducted intensive surveillance of avian influenza in wild waterfowl in Hokkaido, Japan and Mongolia to monitor viruses that are maintained in the nesting lakes in Siberia and spread southward along with their migration in autumn. We reported the isolation of influenza viruses of various subtypes including H2 influenza viruses [7, 10, 29]. All viruses isolated in the surveillance study are stored in our influenza virus library (http://virusdb.czc.hokudai.ac.jp/).

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

Viruses	Accession No.
A/pintail/Shimane/1086/1981 (H2N3)	LC042004
A/whistling swan/Shimane/1447/1982 (H2N3)	LC042007
A/duck/Alaska/5111/1994 (H2N3)	LC042003
A/swan/Shimane/221/1999 (H2N3)	LC042005
A/duck/Hokkaido/96/2001 (H2N3)	LC041992
A/duck/Mongolia/210/2003 (H2N3)	LC041993
A/duck/Hokkaido/W163/2010 (H2N3)	LC042006
A/duck/Hokkaido/491008/2011 (H2N3)	LC041994
A/duck/Hokkaido/162/2013 (H2N1)	LC041995
A/duck/Hokkaido/178/2013 (H2N1)	LC041996
A/duck/Hokkaido/179/2013 (H2N1)	LC041997
A/duck/Hokkaido/181/2013 (H2N1)	LC041998
A/duck/Hokkaido/183/2013 (H2N1)	LC041999
A/duck/Hokkaido/189/2013 (H2N1)	LC042000
A/duck/Hokkaido/203/2013 (H2N1)	LC042001
A/duck/Hokkaido/211/2013 (H2N1)	LC042002

Bold: Viruses indicated in the phylogenetic tree (Fig. 1).

Previous studies demonstrated that cold-adapted live vaccines generated by human and avian H2 influenza viruses induce effective immunity against challenge using parental strains in mouse and ferret models [2, 3]. However, studies on the preparation of inactivated vaccine against H2 influenza are still limited. The aim of the present study is to evaluate the efficacy of an inactivated whole virus particle vaccine prepared from viruses recently isolated from wild birds in Asia based on its antigenicity, immunogenicity, and protective effects against challenge with swine H2 influenza virus in mice.

MATERIALS AND METHODS

Viruses and cells

Influenza viruses, A/swine/Missouri/2124514/2006 (H2N3) and A/mallard/Alberta/884/1984 (H2N5), were kindly provided by Dr. Richard J. Webby and Dr. Robert G. Webster, St. Jude Children's Research Hospital, U.S.A. A/Singapore/1/1957 (H2N2) and A/duck/Hong Kong/278/1978 (H2N9) were kindly provided by Dr. Ken F. Shortridge, the University of Hong Kong, Hong Kong SAR. A/pintail/Shimane/1086/1981 (H2N3) was kindly provided by Dr. Koichi Otsuki, Tottori University, Japan. A/duck/Alaska/5111/1994 (H2N3), A/duck/Hokkaido/162/2013 (H2N1), and other H2 influenza viruses stored in our library (Table 1) were isolated from fecal samples of migratory ducks in our surveillance study [7, 10]. All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hr, and 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, Tokyo, Japan) supplemented with 10% inactivated calf serum and antibiotics and were used for titration of viral infectivity.

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluids of embryonated chicken eggs using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, U.S.A.) and reverse-transcribed with the Uni 12 primer (5′-AGCAAAAGCAGG-3′) and M-MLV Reverse Transcriptase (Life Technologies) [8]. The full-length HA gene segment was amplified by polymerase chain reaction (PCR) using Ex-Taq (TaKaRa, Shiga, Japan) and gene-specific primer sets [8]. Direct sequencing of each gene segment was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and an auto-sequencer 3500 Genetic Analyzer (Life Technologies). Sequencing data were analyzed and aligned using Clustal W using 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 H2 HA genes were compared with reference sequences selected and obtained from GenBank/EMBL/DDBJ.

Antigenic analysis

To analyze the antigenic properties of H2 influenza viruses, the hemagglutination inhibition (HI) test was performed using hyper-immunized chicken antisera against 7 representative strains of H2 viruses. Twenty-five microliters of 8 hemagglutination 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. After the incubation, 50 μl of 0.5% chicken red blood cells in PBS was added and incubated at room temperature for 30 min. HI titers were expressed as the reciprocal of the highest serum dilution showing complete inhibition of hemagglutination.

Vaccine preparation

The selected vaccine strain, A/duck/Hokkaido/162/2013 (H2N1), and the challenge strain, A/swine/Missouri/2124514/2006 (H2N3), were 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* [15]. Briefly, allantoic fluids were ultracentrifuged and pellets were layered onto 10 to 50% sucrose density gradient and ultracentrifuged. The fractions containing viruses were collected based on the sucrose concentration, hemagglutination titer, and protein concentration. Whole virus particles were pelleted from the sucrose fractions by ultracentrifugation and suspended in a small volume of 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 total protein concentration was measured using the BCA Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Each viral protein in the vaccine was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the relative amounts of the hemagglutinin (HA) protein were assumed as a ratio of the HA protein in the total protein using ImageJ (http://rsb.info.nih.gov/ij/index.html).

Potency test of vaccines in mice

Each whole inactivated vaccine of A/duck/Hokkaido/162/2013 (H2N1) or A/swine/Missouri/2124514/2006 (H2N3) (100 μg total protein/vaccine, containing 26.4 and 21.5 μg HA protein respectively) was injected subcutaneously into 10 4-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan). PBS was injected into control mice. Serum samples were collected from each mouse 21 days after the vaccination, and all mice were challenged with $10^{5.0}$ times the 50% tissue culture infectious dose ($TCID_{50}$) /30 μl of A/swine/Missouri/2124514/2006 (H2N3) intranasally under anesthesia. Each vaccine (100 μg total protein/vaccine, containing 26.4 and 21.5 μg HA protein respectively) was also subcutaneously injected twice into 10 mice with a 2-week interval. Fourteen days after the vaccination, serum samples were collected from each mouse and each vaccine was injected into the mice again at the same dose as 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/swine/Missouri/2124514/2006 (H2N3) intranasally under anesthesia. Three days after the challenge, 5 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 5 mice from each group were observed clinical signs for 14 days. The neutralizing antibody titers of mice sera against homologous viruses and A/swine/Missouri/2124514/2006 (H2N3) were determined by serum neutralization test using MDCK cells.

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/m*l* acetylated trypsin (Sigma-Aldrich, St. Louis, MO, U.S.A.). Titers were determined as the product of the reciprocal value of the highest virus dilution showing 50% of the cytopathic effects after 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* [25]. Briefly, test sera and 100 $TCID_{50}$ of A/swine/Missouri/2124514/2006 (H2N3) or 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/m*l* acetylated trypsin (Sigma-Aldrich). Cytopathic effects were observed after 72 hr incubation and neutralizing antibody titers were determined as the reciprocal of the serum dilution yielding 50% inhibition of the cytopathic effects.

Ethics statement

Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approved numbers: 13-0104, 15-0063), and all experiments were performed according to the guidelines of this committee.

RESULTS

Genetic analysis of H2 influenza viruses

Nucleotide sequences of HA genes of the H2 viruses in the influenza virus library were determined and phylogenetically analyzed along with reference sequences available in the public database (Table 1 and Fig. 1). Nucleotide sequences of viruses isolated in Hokkaido in 2013 showed high similarity (99.7–100%) and A/duck/Hokkaido/162/2013 (H2N1) was selected as a representative strain. Based on the results of phylogenetic analysis, the H2 HA genes were classified into Eurasian and North American lineages as the previous study described [26]. The Eurasian linage included viruses isolated in Asia, Europe, and Alaska, while the North American linage included viruses mainly isolated in North America. Viruses belonging to the Eurasian linage were further divided into 4 clusters. Viruses in cluster 1 were avian influenza viruses isolated before the 1980's. Human H2N2 influenza viruses formed a single cluster, cluster 2. This study revealed that avian H2 influenza viruses isolated in Japan in the 1980's

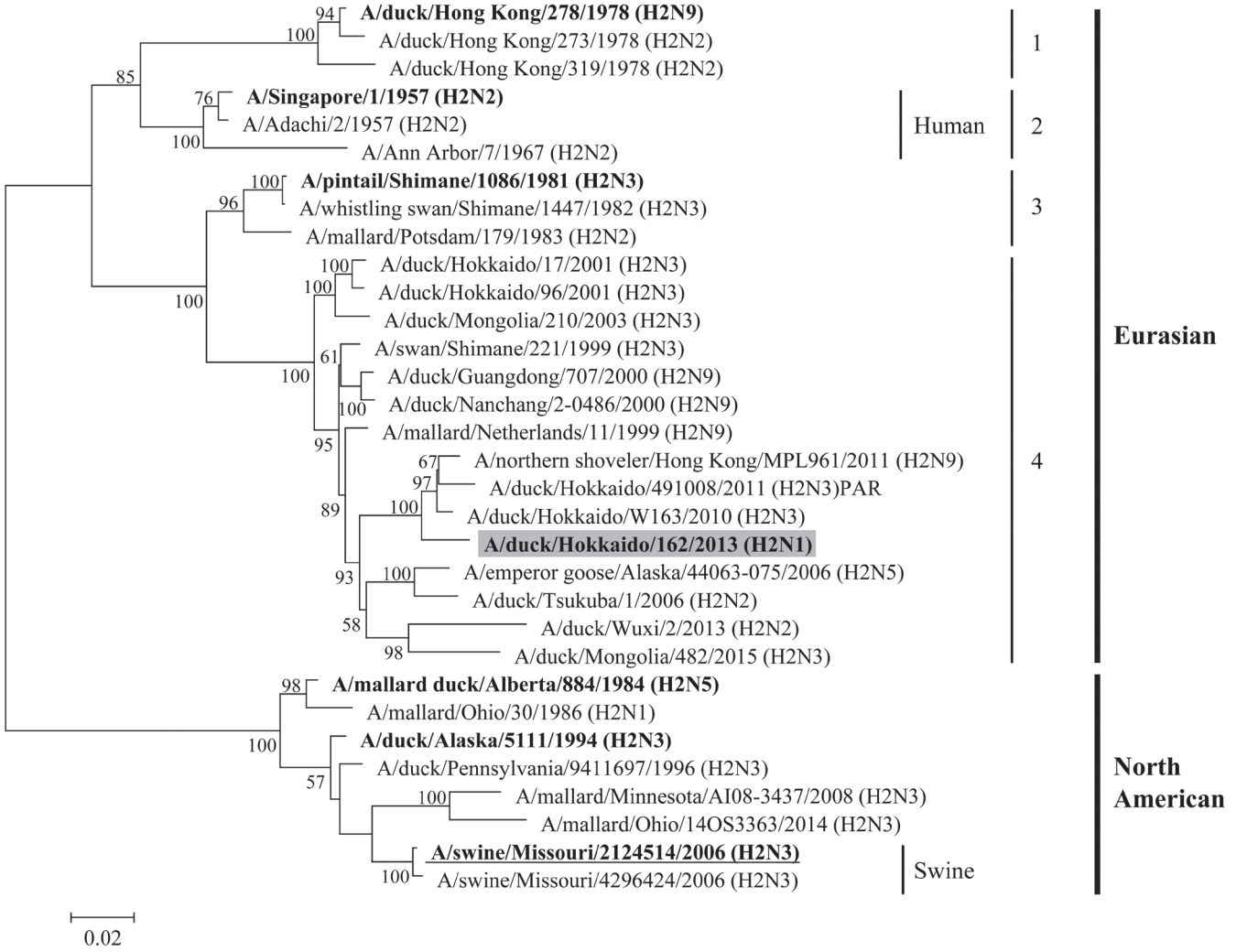

Fig. 1. Phylogenetic tree of H2 HA genes of influenza viruses. Full-length nucleotide sequences of the HA gene were used for phylogenetic analysis using the ML method. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node indicate the confidence level in bootstrap analysis with 1,000 replications. The representative viruses of each cluster are shown in bold. The vaccine strain is highlighted and the challenge strain is underlined.

(represented by A/pintail/Shimane/1086/1981 (H2N3) in the phylogenetic tree) belonged to cluster 3, along with European isolates around the same period. Recent isolates from avian species in European and Asian countries formed cluster 4. These results clearly demonstrate that H2 influenza viruses recently circulating among birds are genetically distant from human H2N2 viruses. Swine H2N3 viruses belong to the North American lineage and no avian viruses recently isolated in the East Asia region are genetically close to the swine H2N3 viruses.

Antigenic analysis of H2 influenza viruses

Seven H2 influenza virus strains representatives of each genetic cluster were selected and antigenically analyzed by HI test (Table 2). Antisera against the avian H2 influenza viruses, A/duck/Hong Kong/278/1978 (H2N9), A/pintail/Shimane/1086/1981 (H2N3), A/duck/Hokkaido/162/2013 (H2N1), A/mallard/Alberta/884/1984 (H2N5), and A/duck/Alaska/5111/1994 (H2N3), reacted with the human H2N2 influenza virus, A/Singapore/1/1957 (H2N2). Antisera against viruses belonging to the Eurasian lineage showed cross-reactivity with the North American linage, and *vice versa*. All antisera reacted with the swine H2N3 virus at HI titers similar to those of the homologous viruses.

Potency test of the vaccine against H2 influenza virus in mice

Based on the results of antigenic analysis, all viruses tested in the present study showed cross-reactivity with viruses belonging to the other genetic groups. Thus, the most recent isolate at the beginning of this study, A/duck/Hokkaido/162/2013 (H2N1), was assumed to be vaccine candidates and used in following examinations. Neutralizing antibody titers of sera collected from mice immunized once with either A/duck/Hokkaido/162/2013 (H2N1) or A/swine/Missouri/2124514/2006 (H2N3) were low (Table 3).

Table 2. The cross-reactivity of H2 influenza viruses with chicken antisera to representative strains of each lineage in HI test

Lineages	Clusters	Viruses	HI titers of the antisera						
			Dk/HK/78	Sing/57	Pin/SMN/81	Dk/Hok/13	Mal/ALB/84	Dk/AK/94	Swine/MO/06
Eurasian	1	Dk/HK/78 (H2N9)	320	2,560	640	320	640	2,560	640
	2 (Human)	Sing/57 (H2N2)	640	5,120	1,280	640	2,560	2,560	640
	3	Pin/SMN/81 (H2N3)	40	2,560	320	160	320	1,280	320
	4	Dk/Hok/13 (H2N1)	320	2,560	640	640	1,280	1,280	640
North American		Mal/ALB/84 (H2N5)	320	1,280	640	320	1,280	2,560	1,280
		Dk/AK/94 (H2N3)	320	2,560	320	320	640	2,560	1,280
	(Swine)	Swine/MO/06 (H2N3)	640	2,560	1,280	640	5,120	2,560	2,560

Homologous titers are underlined. Abbreviations: Dk/HK/78, A/duck/Hong Kong/278/1978 (H2N9); Sing/57, A/Singapore/1/1957 (H2N2); Pin/SMN/81, A/pintail/Shimane/1086/1981 (H2N3); Dk/Hok/13, A/duck/Hokkaido/162/2013 (H2N1); Mal/ALB/84, A/mallard/Alberta/884/1984 (H2N5); Dk/AK/94, A/duck/Alaska/5111/1994 (H2N3); Swine/MO/06, A/swine/Missouri/2124514/2006 (H2N3).

Table 3. Neutralizing antibody titers of mice sera injected once with vaccines and virus recovery from mice lungs after challenge with A/swine/Missouri/2124514/2006 (H2N3)

Vaccine strain	Neutralizing titer of sera at 21 days post immunization against										Virus titer in lungs at 3 days post challenge (log_{10} $TCID_{50}$/g)				
	Dk/Hok/13 (H2N1)					Swine/MO/06 (H2N3)									
Dk/Hok/13 (H2N1)[a)]	<40	<40	40	<40	80	<40	<40	<40	<40	80	≤3.0	4.3	≤2.8	≤3.3	≤3.0
Swine/MO/06 (H2N3)[b)]	-	-	-	-	-	80	<40	40	40	160	≤2.8	≤2.8	≤3.0	≤2.8	≤2.8
PBS	-	-	-	-	-	<40	<40	<40	<40	<40	6.6	7.0	5.8	6.3	6.0

-: Not tested, a) A/duck/Hokkaido/162/2013 (H2N1), b) A/swine/Missouri/2124514/2006 (H2N3).

Table 4. Neutralizing antibody titers of mice sera injected twice with vaccines and virus recovery from mice lungs after challenge with A/swine/Missouri/2124514/2006 (H2N3)

Vaccine strain	Neutralizing titer of sera at 28 days post immunization against										Virus titer in lungs at 3 days post challenge (log_{10} $TCID_{50}$/g)				
	Dk/Hok/13 (H2N1)					Swine/MO/06 (H2N3)									
Dk/Hok/13 (H2N1)[a)]	160	80	80	320	320	40	80	160	40	80	<	<	<	<	<
Swine/MO/06 (H2N3)[b)]	-	-	-	-	-	160	320	320	160	320	<	<	<	<	<
PBS	-	-	-	-	-	<40	<40	<40	<40	<40	6.5	6.3	6.5	6.0	5.7

-: Not tested, <: Not detected, a) A/duck/Hokkaido/162/2013 (H2N1), b) A/swine/Missouri/2124514/2006 (H2N3).

In contrast, the neutralizing antibody titers of mice sera injected twice with either vaccines were reached up to 1:320 against the homologous virus (Table 4). The neutralizing antibody titers of sera from mice vaccinated twice with A/duck/Hokkaido/162/2013 (H2N1) against A/swine/Missouri/2124514/2006 (H2N3) were 1:40–1:160. The virus titers in the lungs of the mice immunized once with either A/duck/Hokkaido/162/2013 (H2N1) or A/swine/Missouri/2124514/2006 (H2N3) were restrained at a low level compared with the lungs of mice in non-vaccinated group (Table 3). The virus titers in the lungs of the mice injected twice with either A/duck/Hokkaido/162/2013 (H2N1) or A/swine/Missouri/2124514/2006 (H2N3) were below the detection limit despite individual differences in neutralizing antibody titers (Table 4). These results indicated the A/duck/Hokkaido/162/2013 (H2N1) vaccine induced immunity in mice sufficient to reduce the impacts of the challenge strain, A/swine/Missouri/2124514/2006 (H2N3), in the lungs comparable to that of the homologous vaccine strain, A/swine/Missouri/2124514/2006 (H2N3). All of the mice in this study inoculated with $10^{5.0}$ $TCID_{50}$ of the swine H2N3 virus survived during observation period and showed no overt clinical signs including body weight loss.

DISCUSSION

Vaccination is the most effective control measure for human pandemic influenza and the preparation of vaccines for future H2 influenza pandemics is necessary [20]. Our results demonstrated that an inactivated whole virus particle vaccine prepared from recent avian H2 influenza virus, A/duck/Hokkaido/162/2013 (H2N1), is effective for use in future human pandemics. A/duck/Hokkaido/162/2013 (H2N1) showed broad antigenic cross-reactivity and thus was selected as the vaccine candidate strain in this study. The inactivated vaccine prepared from A/duck/Hokkaido/162/2013 (H2N1) induced neutralizing antibodies against the homologous virus and A/swine/Missouri/2124514/2006 (H2N3) in mice after 2 subcutaneous injections. The inactivated vaccine was also sufficiently protective to reduce the impact of the challenge with A/swine/Missouri/2124514/2006 (H2N3) at a level comparable to that of the vaccine prepared from the homologous strain of the challenge virus.

Inactivated whole virus particle influenza vaccines are more effective than split influenza vaccines [1, 9, 20, 23]. Lenny *et al.* reported that monovalent or multivalent inactivated whole virus particle vaccines generated from A/Singapore/1/1957 (H2N2), A/duck/Hong Kong/319/1978 (H2N2), or A/swine/Missouri/2124514/2006 (H2N3) are effective against a challenge with one of the three viruses in mouse model [18].

Our findings supported the effectiveness of inactivated whole virus particle vaccine against H2 influenza because avian H2 influenza viruses currently circulating among birds are also effective. Our inactivated vaccine prepared from A/duck/Hokkaido/162/2013 (H2N1) required 2 rounds of vaccination to induce neutralizing antibodies in mice to A/swine/Missouri/2124514/2006 (H2N3); thus, the dosage of vaccine and the most effective administration strategy should be considered to improve the efficacy of this vaccine.

We have established an influenza virus library for storing various influenza viruses for use as seed for vaccines. Influenza viruses of 144 combinations including 16 HA and 9 neuraminidase subtypes isolated from animals or generated in our laboratory have been stored in the library. Our previous studies revealed that whole virus particle vaccines prepared from this library induce effective immunity against infections with H1, H5, H6, H7 and H9 influenza viruses in mice and macaque models [4, 11, 12, 21–23]. In the present study, the vaccine candidate strain against H2 influenza selected from the influenza library is shown to be potentially useful for a future H2 influenza pandemic. Our annual influenza surveillance in wild birds in Japan and Mongolia effectively monitors virus circulation in wild birds in East Asian countries and also provides a variety of influenza viruses [7, 29]. Thus, our library is updated each season, providing specimens from which we might gain novel information about the antigenicity of H2 influenza viruses circulating in wild birds in East Asian countries.

In further studies, monitoring of introduction of H2 influenza virus into pig population and emergence of mammalian adapted H2 influenza viruses is important for early response to a human pandemic. In addition, the continuous surveillance and antigenic analysis of H2 influenza viruses in both wild birds and poultry are necessary to prepare for a future pandemic and allow for rapid vaccine preparation.

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