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
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 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.

| 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 (H2N2)      | LC042002      |

Bold: Viruses indicated in the phylogenetic tree (Fig. 1).
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. Titters 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/ml acetylated trypsin (Sigma-Aldrich, St. Louis, MO, U.S.A.). Titters 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/ml 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 lineage included viruses isolated in Asia, Europe, and Alaska, while the North American lineage included viruses mainly isolated in North America. Viruses belonging to the Eurasian lineage 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
(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.

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.

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 (H2N2), 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 human H2N2 viruses, A/Singapore/1/1957 (H2N2), and A/duck/Hokkaido/17/2001 (H2N3), and A/duck/Hokkaido/96/2001 (H2N3), reacted with H2N3 viruses recently circulating among birds. Antisera against avian viruses belonging to the Eurasian lineage showed cross-reactivity with the North American lineage 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 with either A/duck/Hokkaido/162/2013 (H2N1) or A/swine/Missouri/2124514/2006 (H2N3) were low (Table 3).
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 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 TCID50 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 (H2N2) 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 help in preparing vaccines for both a future pandemic and allow for rapid vaccine preparation.

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