Study on the pandemic influenza vaccines prepared from avian influenza viruses

（鳥インフルエンザウイルスを用いたパンデミックインフルエンザワクチンの研究）

Mizuho Suzuki

CONTENTS

Abbreviations

BCA:	bicinchoninic acid
BPL:	beta-propiolactone
BSA:	bovine serum albumin
ELISA:	enzyme-linked immunosorbent assay
HA:	hemagglutinin
HAU:	hemagglutination unit
HI:	hemagglutination-inhibition
HRP:	horseradish peroxidase
MDCK cells:	Madin–Darby canine kidney cells
MEM:	minimum essential medium
ML:	maximum-likelihood
NA:	neuraminidase
PB2:	polymerase basic protein 2
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
SAS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
$TCID_{50}$:	50% tissue culture infectious dose
TMB:	3,3',5,5'-tetramethylbenzidine

Notes

Contents of the present thesis were published in the following articles.

1. **Suzuki M, Okamatsu M, Hiono T, Matsuno K, Sakoda Y.** 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. J Vet Med Sci 79, 1815-1821, 2017.

2. **Suzuki M, Okamatsu M, Hiono T, Matsuno K, Sakoda Y.** Potency of an inactivated influenza vaccine prepared from A/duck/Mongolia/245/2015 (H10N3) against H10 influenza virus infection in a mouse model. Jpn J Vet Res, *In press*.

Preface

Influenza A viruses belong to the genus *Influenzavirus A* of the family *Orthomyxoviridae* [44]. Influenza A viruses are further divided based on the antigenic properties of their two envelope glycoproteins into 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes. Although influenza A viruses are zoonotic pathogens that are widely distributed among mammalian hosts such as humans, pigs, and horses, and avian species such as chickens and ducks, the wild migratory aquatic birds are considered as natural reservoirs for influenza A viruses [24, 31, 72]. Influenza A viruses of all known subtypes (H1–H16 HA and N1–N9 NA) have been isolated from waterfowls [12, 34, 45]. Ducks are infected with influenza viruses by water-borne transmission at their nesting lakes close to the Arctic Circle in Siberia, Alaska, and Canada during their breeding season in summer [31].

Pandemics influenza are considered to arise when influenza viruses with HA and/or NA which are antigenically novel to human population are introduced into the population and the viruses acquire the ability of efficient human-to-human transmission [73]. Pigs have been proposed as the intermediate hosts where reassortment or adaptation of avian influenza viruses to humans may occur [25, 41]. Therefore, obtaining comprehensive knowledges of influenza viruses circulating among natural reservoirs and monitoring the transmission of the viruses to humans or other mammals is important for an early response to a pandemic. Historically, at least four confirmed pandemic influenza caused by H1N1, H2N2, and H3N2 influenza viruses have been reported. Genetic analyses suggested that the pandemic influenza resulted from the emergence of reassortant influenza viruses of which HA or NA gene had been replaced with gene segments derived from avian influenza viruses [61, 62, 73]. The pandemic H1N1 influenza viruses found in 2009 arose by the reassotantment of viruses in pigs

[50]. Avian influenza viruses of H5N1 and H7N9 subtypes have infected hundreds of people since their emergence in 1996 and 2013, respectively, although the viruses have failed to acquire human-to-human transmissibility [9, 10, 14, 67]. Furthermore, there have been case reports of human infections with H5N6, H6N1, H7N2, H7N3, H7N7, H9N2, H10N7, and H10N8 influenza viruses [3, 4, 11, 33, 40, 54, 53, 55, 79]. Among avian influenza viruses of these HA subtypes, H5, H6, H7, and H9 influenza viruses were frequently isolated from poultry and caused severe economic damages [1, 7, 17, 18]. Thus, genetic and antigenic characters of these viruses, and pandemic or human adaptation potential of these viruses are well studied [13, 69, 71]. In contrast, little has been reported on the genetic and antigenic characters of H2 and H10 influenza viruses; therefore, the present study focused on the H2 and H10 influenza viruses.

H2N2 influenza viruses caused pandemic known as Asian flu starting in 1957 and reported more than one million deaths around the world were reported. H2 influenza viruses have not been detected in mammals since 1968 except for H2N3 influenza viruses isolated from pigs in 2006 in Missouri, USA [43]. Therefore, the transmission of avian H2 influenza viruses to humans may result in a pandemic because of the lack of acquired immunity against H2 influenza viruses in the current human population [64]. Previous studies reported that H2 influenza viruses were occasionally isolated from wild birds in independent surveillance studies and few are isolated from poultry. However, comprehensive studies on genetic diversity of H2 influenza viruses in wild birds and antigenic characterization of them are limited, particularly among viruses isolated in Asian countries.

H10N8 influenza viruses were detected in humans in 2013–2014, and the infection source was considered as poultry in live bird markets in China [3, 4, 81]. This included the first fatal cases of H10 influenza virus infection in humans. H10 influenza viruses are continuously isolated from wild birds and poultry around the world. However,

studies on genetic properties of H10 influenza viruses currently circulating among wild birds are limited. Furthermore, antigenic characterization of H10 influenza viruses recently isolated from birds have not been conducted. Collectively, although H2 and H10 viruses were consequently isolated from birds, information regarding the genetic and antigenic properties of H2 and H10 influenza viruses circulating in both poultry and wild birds is limited.

Vaccination is considered as the most effective control measure for influenza in humans. In previous studies, live attenuated vaccines against influenza caused by H2 influenza viruses were studied as preparation for a future pandemic [5, 6]. In addition, the efficacy of 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) was indicated in a mouse model [37]. In these previous studies, viruses isolated in North America were involved, thus, supportive insights for the efficacy of vaccine prepared from viruses recently isolated from birds in Asia are required. An H10 influenza vaccine was experimentally generated from human H10N8 influenza viruses as an inactivated vaccine [74]. However, studies on vaccines which broadly effective against avian H10 influenza viruses recently circulating among birds have not been done. Therefore, genetic and antigenic characterization of H2 and H10 influenza viruses and development of appropriate vaccines are essential to prepare for a future pandemic caused by H2 and H10 influenza viruses.

In the previous studies, intensive surveillance of avian influenza was conducted 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 [20, 24, 77]. Japan and Mongolia are located on the migration route of wild birds that fly from their northern

territory in Siberia to the south. Accordingly, surveillance of avian influenza in migratory water birds in these areas is effective to monitor viruses maintained in wild waterfowls. Both H2 and H10 influenza viruses were isolated from fecal samples of wild birds in the surveillance studies. All viruses isolated in the surveillance study are stored in the influenza virus library in Hokkaido University (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, H5, H6, H7, and H9 influenza viruses in mouse models and infections with H5 and H7 viruses in macaque models [8, 26, 27, 48, 49, 51].

The present thesis consists of two chapters; in Chapter I, genetic and antigenic properties of H2 influenza viruses isolated from wild birds in Hokkaido and Mongolia were analyzed. Genetic diversity and antigenic similarity of avian H2 influenza viruses were revealed. In addition, as an effective control measure for a future pandemic caused by H2 influenza viruses, the efficacy of an inactivated whole virus particle vaccine prepared from avian H2 influenza viruses in the influenza virus library was evaluated against a challenge with swine H2N3 influenza viruses in a mouse model. In Chapter II, genetic and antigenic properties of H10 influenza viruses recently isolated from wild birds in Hokkaido and Mongolia were analyzed. Animal experiments using mice were also conducted to examine the pathogenicity of avian H10 influenza viruses. Moreover, the efficacy of an inactivated whole virus particle vaccine against H10 influenza viruses was evaluated in a mouse model to prepare for a future H10 influenza virus infection.

Chapter I

Genetic and antigenic characterization of H2 influenza viruses and evaluation of the 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

Introduction

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 birds and sporadically isolated from domestic birds around the world [16, 32, 34, 56, 76, 77]. In addition, H2N3 influenza viruses were isolated from pigs in 2006 in Missouri, USA [43]. 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 [64]. Therefore, vaccines against H2 influenza viruses are needed to prepare for a future human pandemic [46].

The HA genes of H2 influenza viruses are phylogenetically divided into North American and Eurasian lineages [61]. 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. Antigenic cross-reactivity of an avian H2 influenza virus, A/black duck/New Jersey/1580/1978 (H2N3), with H2 influenza viruses isolated from humans and birds before 1991 was investigated [15, 29]. The H2N3 influenza viruses isolated from pigs show antigenic cross-reactivity with North American and Eurasian H2 avian influenza viruses [28]. 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 birds

in Asia is greatly needed.

Surveillance studies of avian influenza in wild waterfowls conducted by Hokkaido University started from 1996 revealed that H2 influenza viruses are continuously circulating among wild birds and isolated from fecal samples of wild ducks collected in Hokkaido and Mongolia even in recent years [20, 24, 77]. The H2 influenza viruses isolated in the surveillance study are stored in an influenza virus library (http://virusdb.czc.hokudai.ac.jp/).

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 [5, 6]. 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 a 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, USA. A/Singapore/1/1957 (H2N2) and A/duck/Hong Kong/278/1978 (H2N9) were generously provided by Dr. Kennedy F. Shortridge, the University of Hong Kong, Hong Kong SAR. A/pintail/Shimane/1086/1981 (H2N3) was generously provided by Dr. Koichi Otsuki, Tottori University, Japan. A/duck/Alaska/5111/1994 (H2N3), A/duck/Hokkaido/95/2001 (H2N2), A/duck/Hokkaido/162/2013 (H2N1), and other H2 influenza viruses stored in the library were isolated from fecal samples of migratory ducks in the surveillance study by Hokkaido University (Table 1) [20, 24]. 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 the titration of viral infectivity in serum-free MEM supplemented with antibiotics and acetylated trypsin (Sigma-Aldrich, St. Louis, MO, USA).

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluids of embryonated chicken eggs using TRIzol™ LS Reagent (Life Technologies,Carlsbad, CA, USA) and reverse-transcribed with Uni 12 primer (5'-AGCAAAAGCAGG-3') and M-MLV Reverse Transcriptase (Life Technologies) [21]. The full-length HA gene segment was amplified by polymerase chain reaction (PCR) using *Ex Taq* Polymerase® (TaKaRa Bio, Shiga,

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)

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 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 the eight representative strains of H2 viruses. Twenty-five μl of 8 hemagglutination units (HAU) of the test virus was added to 25 μl of 2-fold dilutions of each antiserum in pH 7.4 phosphatep-buffered saline (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. HI titers were expressed as the reciprocal of the highest serum dilution showing complete inhibition of hemagglutination.

Virus purification

A/duck/Hokkaido/95/2001 (H2N2), A/duck/Hokkaido/162/2013 (H2N1), A/swine/Missouri/2124514/2006 (H2N3), and A/Puerto Rico/8/1934 (H1N1) were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs respectively 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*. [30]. Briefly, allantoic fluids were ultracentrifuged and the

resulting pellets were layered onto a 10–50% sucrose density gradient and ultracentrifuged. The fractions containing viruses were collected based on the sucrose concentration, hemagglutination titer, and protein concentration. Then, the whole virus particles were pelleted from the sucrose fractions by ultracentrifugation and suspended in a small volume of PBS. The total protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA).

Effects of inactivation on purified A/duck/Hokkaido/95/2001 (H2N2) in vitro

In order to assess the effects of inactivation conditions on vaccine candidate strain, A/duck/Hokkaido/95/2001 (H2N2) of which antiserum showed broad cross-reactivity in the antigenic analysis and efficient propagation in chicken embryonated eggs, the small volume of the purified virus was inactivated by the incubation in 0.1, 0.2, or 0.5% formalin at 4 °C for 7 days. The virus was also inactivated by the incubation in 0.1% beta-propiolactone (BPL) at 37℃ for 2hr or 10 min. Virus inactivation was confirmed by the inoculation of the formalin/BPL-treated samples into embryonated chicken eggs. HA titer of each inactivated viruses and non-treated virus were determined to estimate the effects of inactivation reagents. In addition, the reactivity of antisera against A/duck/Hokkaido/95/2001 (H2N2), A/duck/Hokkaido/162/2013 (H2N1), and A/Singapore/1/1957 (H2N2) to the inactivated viruses were analyzed by HI assay as described above. Furthermore, the reactivity of a mouse anti-HA monoclonal antibody against A/duck/Hokkaido/95/2001 (H2N2), 95-2-1, to the inactivated virus were assumed by enzyme-linked immunosorbent assay (ELISA).

ELISA

The reactivity of the monoclonal antibody to the inactivated virus was analyzed by ELISA. One hundred μg/ml fetuin from fetal calf serum (Sigma-Aldrich) per well were

used to coat a Thermo Scientific™ CovaLink™ LockWell module C8 (Thermo Fisher Scientific). Each well was blocked with 2% bovine serum albumin (BSA) at room temperature for 1 hr. After washing with PBS containing 0.05% Tween20, the purified influenza viruses suspension (256, 128, 64, and 32 HAU in PBS) was added to each well, and the plates were incubated at 4 °C for 12 h. After washing, the mouse anti-HA monoclonal antibody 95-2-1, were added to each well, and the plates were incubated at 4 °C for 2 hr. The wells were then washed and incubated with goat anti-mouse IgG-horse radish peroxidase (HRP) conjugate (Bio-Rad, Hercules, CA, USA) at 4 °C for 2 hr. After washing, 100 µl of the substrate solution including 0.5 mM 3,3',5,5'-tetramethylbenzidine (TMB) and 0.04% H_2O_2, was added to each well. After incubation at room temperature for 10 min, the reactions were stopped using 50 µl of 2N H_2SO_4, and absorbance at 450/630 nm was measured using a MULTISCAN JX (Thermo Fisher Scientific).

Immunogenicity of inactivated purified A/duck/Hokkaido/95/2001 (H2N2) in mice

Each inactivated viruses of A/duck/Hokkaido/162/2013 (H2N1) or A/swine/Missouri/2124514/2006 (H2N3) (100 µg total protein/virus) was injected subcutaneously into three 4-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan). Inactivated A/Puerto Rico/8/1934 (H1N1) was injected into three positive control mice. Serum samples were collected from each mouse at 21 days after the inoculation. The serum neutralizing antibody titers of mice against homologous viruses were determined by serum neutralization tests using MDCK cells.

Vaccine preparation

The selected vaccine strain, A/duck/Hokkaido/162/2013 (H2N1), and the challenge strain, A/swine/Missouri/2124514/2006 (H2N3), were purified and inactivated by the

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). 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 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 µg and 21.5 µg HA protein respectively) was injected subcutaneously into ten 4-week-old female BALB/c mice (Japan SLC) and PBS was injected into the ten control mice. Serum samples were collected from each mouse at 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 µg and 21.5 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 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, 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 for 14 days. The serum neutralizing antibody titers of mice 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 homogenates of mouse lungs 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). The virus 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.* [60]. Briefly, test sera and 100 $TCID_{50}$ of viruses 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 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, 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 the phylogenetic analysis, the H2 HA genes were classified into Eurasian and North American lineages as described in the previous study [64]. 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 four 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.

Antigenic analysis of H2 influenza viruses

Eight H2 influenza virus strains representatives of each genetic cluster were selected and antigenically analyzed by HI test (Table 2). Antisera against the avian H2

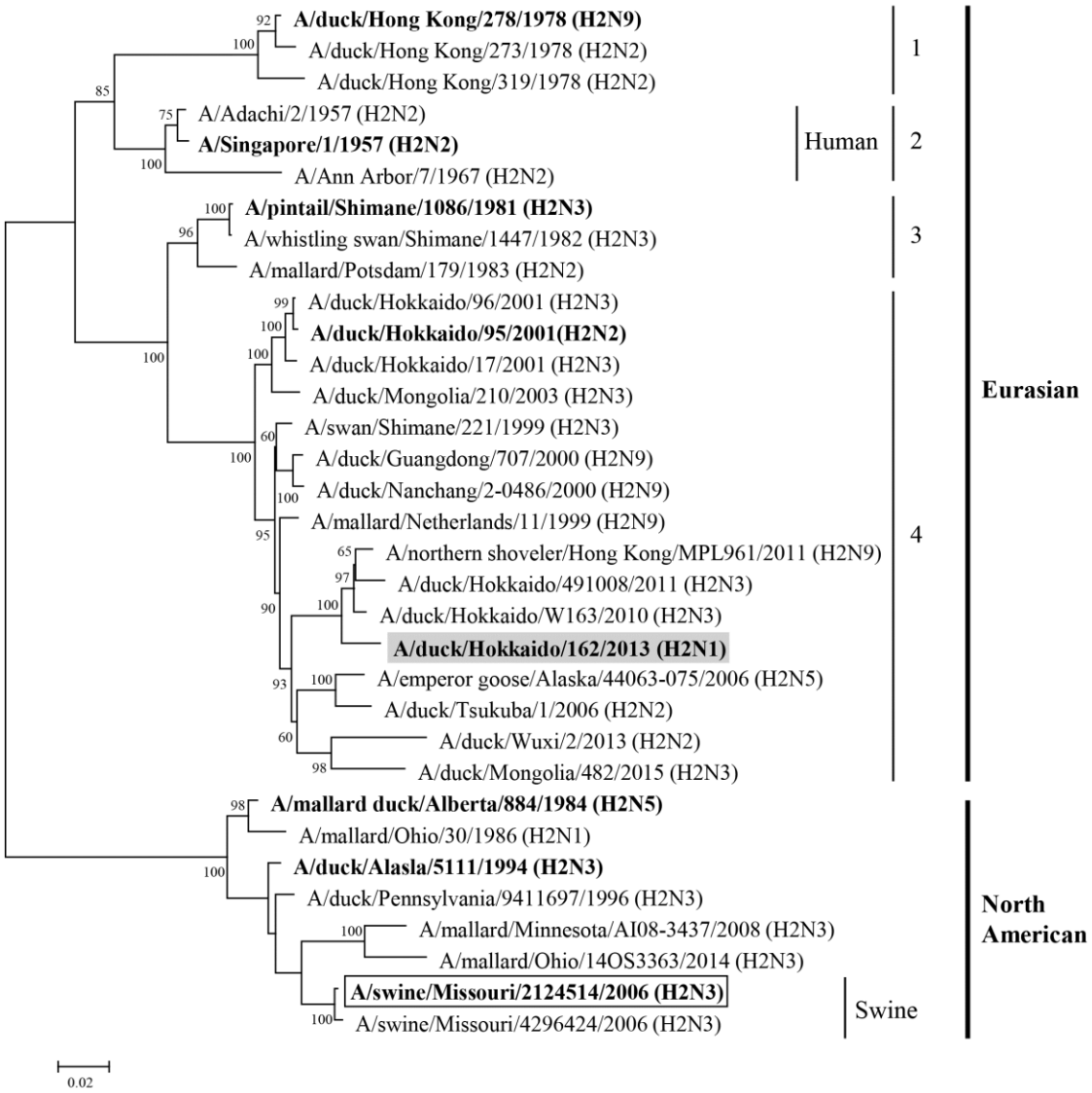

Fig. 1 Phylogenetic tree of H2 HA genes of influenza viruses Full-length nucleotide sequences of the HA gene were used for phylogenetic analysis using ML. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node indicate the confidence level in bootstrap analysis with 1,000 replications. The representative viruses of each cluster 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 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/01	Dk/Hok/13	Mal/ALB/84	Dk/AK/94	Swine/MO/06
Eurasian	1	Dk/HK/78 (H2N9)	320	2,560	640	2,560	320	640	2,560	640
	2 (Human)	Sing/57 (H2N2)	640	5,120	1,280	2,560	640	2,560	2,560	640
	3	Pin/SMN/81 (H2N3)	40	2,560	320	640	160	320	1,280	320
	4	Dk/Hok/01 (H2N2)	80	2,560	640	2,560	320	1,280	640	320
	4	Dk/Hok/13 (H2N1)	320	2,560	640	1,280	640	1,280	1,280	640
North American		Mal/ALB/84 (H2N5)	320	1,280	640	1,280	320	1,280	2,560	1,280
		Dk/AK/94 (H2N3)	320	2,560	320	1,280	320	640	2,560	1,280
	(Swine)	Swine/MO/06 (H2N3)	640	2,560	1,280	2,560	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/01, A/duck/Hokkaido/95/2001 (H2N2); 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)

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.

Effects of inactivation on purified A/duck/Hokkaido/95/2001 (H2N2) in vitro

In comparison with inactivation reagents and conditions, formalin inactivation of A/duck/Hokkaido/95/2001 (H2N2) seemed not to affect HA titers and reactivity of antisera to the viruses analyzed by HI test (Table 3). In contrast, HA titers of the viruses inactivated with 0.1% BPL decreased in each incubation period. HI titer of each antisera against the viruses inactivated using BPL was similar to the HI titers against the viruses inactivated using formalin. Reactivity of the monoclonal antibody against A/duck/Hokkaido/95/2001 (H2N2) to the viruses which were inactivated using formalin were dose dependent manner of the formalin, but that was lower in the case of BPL (Fig. 2).

Immunogenicity of inactivated purified A/duck/Hokkaido/95/2001 (H2N2) in mice

Based on the results of the comparison with the inactivation condition, A/duck/Hokkaido/95/2001 (H2N2), A/swine/Missouri/2124514/2006 (H2N3) or A/Puerto Rico/8/1934 (H1N1) were inactivated by incubation in 0.1% formalin. Immunogenicity of the inactivated A/duck/Hokkaido/95/2001 (H2N2) were evaluated in mice. Neutralizing antibody titers of sera collected from mice immunized with A/duck/Hokkaido/95/2001 (H2N2) were clearly lower than that of either A/swine/Missouri/2124514/2006 (H2N3) or A/Puerto Rico/8/1934 (H1N1) (Table 4).

Table 3 HA titers of inactivated A/duck/Hokkaido/95/2001 (H2N2) and reactivity of hyper immune chicken antisera to the inactivated viruses

Reagents	Concentration (%)	Incubation period	HA titers	HI titers of the antisera		
				Dk/Hok/01	Dk/Hok/13	Sing/57
Formalin	0.1	1 week	204,800	2,560	640	2,560
	0.2	1 week	204,800	2,560	1,280	2,560
	0.5	1 week	102,400	2,560	1,280	2,560
BPL	0.1	2 hr	25,600	2,560	640	1,280
	0.1	10 min	25,600	2,560	640	1,280
Not treated	-	-	409,600	2,560	640	1,280

Abbreviations: BPL, Beta-propiolactone; Dk/Hok/01, A/duck/Hokkaido/95/2001 (H2N2); Dk/Hok/13, A/duck/Hokkaido/162/2013 (H2N1); Sing/57, A/Singapore/1/1957 (H2N2)

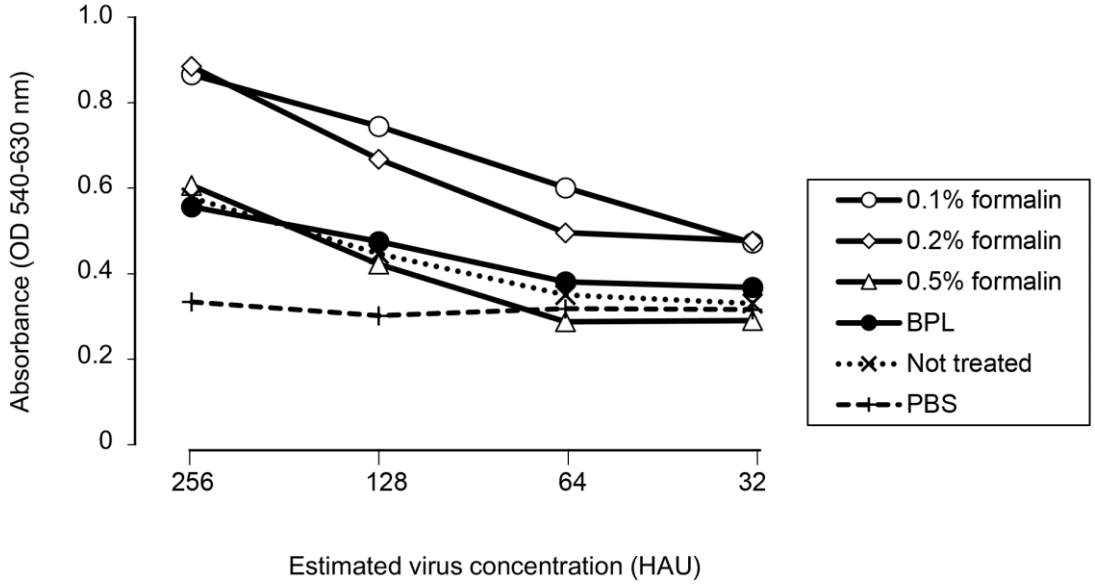

Fig. 2 Reactivity of a monoclonal antibody to the inactivated viruses Reactivity of monoclonal antibody against A/duck/Hokkaido/95/2001 (H2N2), 95-2-1, to the inactivated viruses binding to fetuin on the surface of plates were analyzed by ELISA. Small volume of purified A/duck/Hokkaido/95/2001 (H2N1) was inactivated using 0.1, 0.2, and 0.5% formalin at 4 °C for 1 week or using 0.1% BPL at 37 °C for 2 hr, respectively. Each inactivated viruses were diluted and the virus concentration were estimated as HA units. The data are presented as mean absorbance (540–630 nm) of duplicated experiments.

Table 4 Serum neutralizing antibody titers of mice injected with inactivated viruses

Viruses	Dose[a] (μg)	Neutralizing titer[b]		
A/duck/Hokkaido/95/2001 (H2N2)	100	20	40	80
A/duck/Hokkaido/162/2013 (H2N1)	100	160	80	80
A/swine/Missouri/2124514/2006 (H2N3)	100	160	320	160
A/Puerto Rico/8/1934 (H1N1)	100	160	320	160

[a] Total protein quantity measured by BCA assay

[b] Antibody titers against homologous viruses

Potency test of the vaccine against H2 influenza virus in mice

Besides antiserum against A/duck/Hokkaido/95/2001 (H2N2), antisera against the other viruses tested in the present study also showed cross-reactivity with viruses belonging to the other genetic groups based on the results of antigenic analysis. Thus, the most recent isolate at the beginning of this study, A/duck/Hokkaido/162/2013 (H2N1), was assumed to be a vaccine candidate 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 5). In contrast, the neutralizing antibody titers of sera collected from mice injected twice with either vaccines were reached up to 1:320 against the homologous virus (Table 6). The serum neutralizing antibody titers of the 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 5). 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 in spite of individual differences in neutralizing antibody titers (Table 6). 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 inoculated with $10^{5.0}$ $TCID_{50}$ of the swine H2N3 virus in this study survived during observation period and showed no overt clinical signs including body weight loss.

Table 5 Serum neutralizing antibody titers of mice injected once with vaccines and virus recovery from mouse 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 6 Serum neutralizing antibody titers of mice injected twice with vaccines and virus recovery from mouse 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)

Discussion

Vaccination is the most effective control measure for human pandemic influenza and the preparation of vaccines for future H2 influenza pandemics is necessary [46]. The results of this study 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 a future human pandemic. Antiserum against A/duck/Hokkaido/162/2013 (H2N1) showed broad cross-reactivity, and thus the virus 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 two 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 [2, 22, 51]. 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 a mouse model [37].

Findings in this study supported the effectiveness of inactivated whole virus particle vaccine against H2 influenza because avian H2 influenza viruses currently circulating among birds are also effective. The inactivated vaccine prepared from A/duck/Hokkaido/162/2013 (H2N1) required two 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.

The present study suggested that vaccine strains should be selected based on the results of *in vivo* experiments as well as antigenicity or growth efficiency of viruses *in vitro*. Formalin-inactivated A/duck/Hokkaido/95/2001 (H2N2) did not induced sufficient amount of neutralizing antibodies in mice against the homologous strain after immunization. The possible reason has not been revealed when HA titers of the inactivated viruses, reactivity of the hyper-immunized antisera or the monoclonal antibody to the viruses were compared in this study, although differences in reactivity of the monoclonal antibody might be attributed to binding affinity of the viruses to fetuin. In this study, inactivation efficiency of BPL did not evaluated in a mouse model. Thus, further studies are needed in order to comparing immunogenicity between viruses inactivated using either formalin or BPL in animal models.

An influenza virus library have been established for storing various influenza viruses for use as seeds for vaccines in Hokkaido University. Influenza viruses of 144 combinations including 16 HA and 9 NA subtypes isolated from animals or generated in the laboratory have been stored in the library. 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 [8, 26, 27, 48, 49, 51]. 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. 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 [20, 77]. Thus, the library is updated each season, providing specimens from which novel information regarding the antigenicity of H2 influenza viruses circulating in wild birds in East Asian countries might be gain.

In further studies, monitoring of the 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.

Brief summery

H2N2 influenza virus caused a pandemic starting in 1957 but has not been detected in humans since 1968. Thus, most people are immunologically naïve 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, genetic and antigenic characterization of H2 influenza viruses recently isolated from wild birds in Asian countries were conducted. Furthermore, a vaccine against H2 influenza was prepared as an effective control measure against a future human pandemic. Antiserum against A/duck/Hokkaido/162/2013 (H2N1) showed broad cross-reactivity, therefore the virus was selected as a vaccine candidate strain from H2 influenza viruses recently isolated from wild birds. 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 a challenge 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.

Chapter II

Genetic and antigenic characterization of H10 influenza viruses and evaluation of the potency of an inactivated influenza vaccine prepared from A/duck/Mongolia/245/2015 (H10N3) against H10 influenza virus infection in a mouse model

Introduction

In 2013 and 2014, H10N8 influenza viruses were detected from humans, and epidemiological studies strongly suggested that the infection source was poultry in live bird markets [3, 4, 81]. This included the first fatal cases of H10 influenza virus infection in humans, although several sporadic and mild cases had been reported in 2004 and 2010 [3, 81]. 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 [23, 42, 47, 75 80]. 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 [68]. Furthermore, few studies have been reported on vaccine preparation for pandemic influenza by H10 influenza viruses [35, 39, 57, 68, 74].

Surveillance studies of avian influenza in wild waterfowls conducted by Hokkaido University started from 1996 revealed that H10 influenza viruses are continuously circulating among wild birds and isolated from fecal samples of wild ducks collected in Hokkaido and Mongolia even in recent years [20, 24, 77]. The H10 influenza viruses isolated in the surveillance study are stored in the 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 [8, 26, 27, 48, 49, 51].

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 the 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 the surveillance study conducted by Hokkaido University [20, 24, 77]. A/duck/Vietnam/OIE-0483/2012 (H10N7) was isolated from a poultry duck in a live bird market in Vietnam [51]. 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. MDCK cells were grown in MEM (Nissui Pharmaceutical Co.) 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).

Sequencing and phylogenetic analysis

The full-length nucleotide sequences of polymerase basic protein 2 (PB2) and HA gene segments were determined as described in Chapter I. In brief, nucleotide

sequencing of PCR fragments from viral RNA 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.). The nucleotide sequences were phylogenetically analyzed by the 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 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 HAU 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. 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 influenza 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.) under anesthesia. Titers of inoculated viruses were $10^{5.5}$, $10^{5.3}$, $10^{5.8}$, $10^{5.8}$, $10^{5.5}$, and $10^{5.8}$ $TCID_{50}$/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 as described in Chapter I. The purified viruses were inactivated by incubation in 0.1% formalin at 4 °C for 7 days. The purified and inactivated virus was used as a whole virus particle vaccine. The total protein concentration was measured by BCA assay and relative amounts of the HA protein were estimated as described in Chapter I.

Potency test of the vaccine in mice

In order to evaluate the immunogenicity and protective efficacy of the vaccine strain *in vivo,* experiments in a 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, 12.7, 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 ten control mice. Serum samples were collected from each mouse at 21 days after the vaccination. The vaccine (10, 50, and 100 µg total protein/vaccine, containing 2.5, 12.7,

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 serum neutralizing antibody titers of mice 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 body weight was measured daily 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

Virus titers were measured as described in Chapter I. In brief, ten-fold dilutions of virus samples or mice lung homogenates were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C with MEM containing 5 μg/ml acetylated trypsin. The virus titers were determined and expressed as $TCID_{50}$.

Serum neutralization test

Serum neutralizing antibody titers were measured as described in Chapter I. Briefly, test sera and 100 $TCID_{50}$ of A/duck/Hokkaido/W87/2007 (H10N2) or the vaccine strain virus were mixed and inoculated onto MDCK cells and incubated at 35 °C with

MEM containing 5 μg/ml acetylated trypsin. 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 7, Fig. 3). 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 [42, 83]. 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/Hong Kong/786/1979 (H10N3). A/duck/Mongolia/245/2015 (H10N3) isolated from a fecal sample of wild duck 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 [80]. 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 were analyzed by HI tests (Table 8).

Table 7 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)	LC342073, LC337289
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)

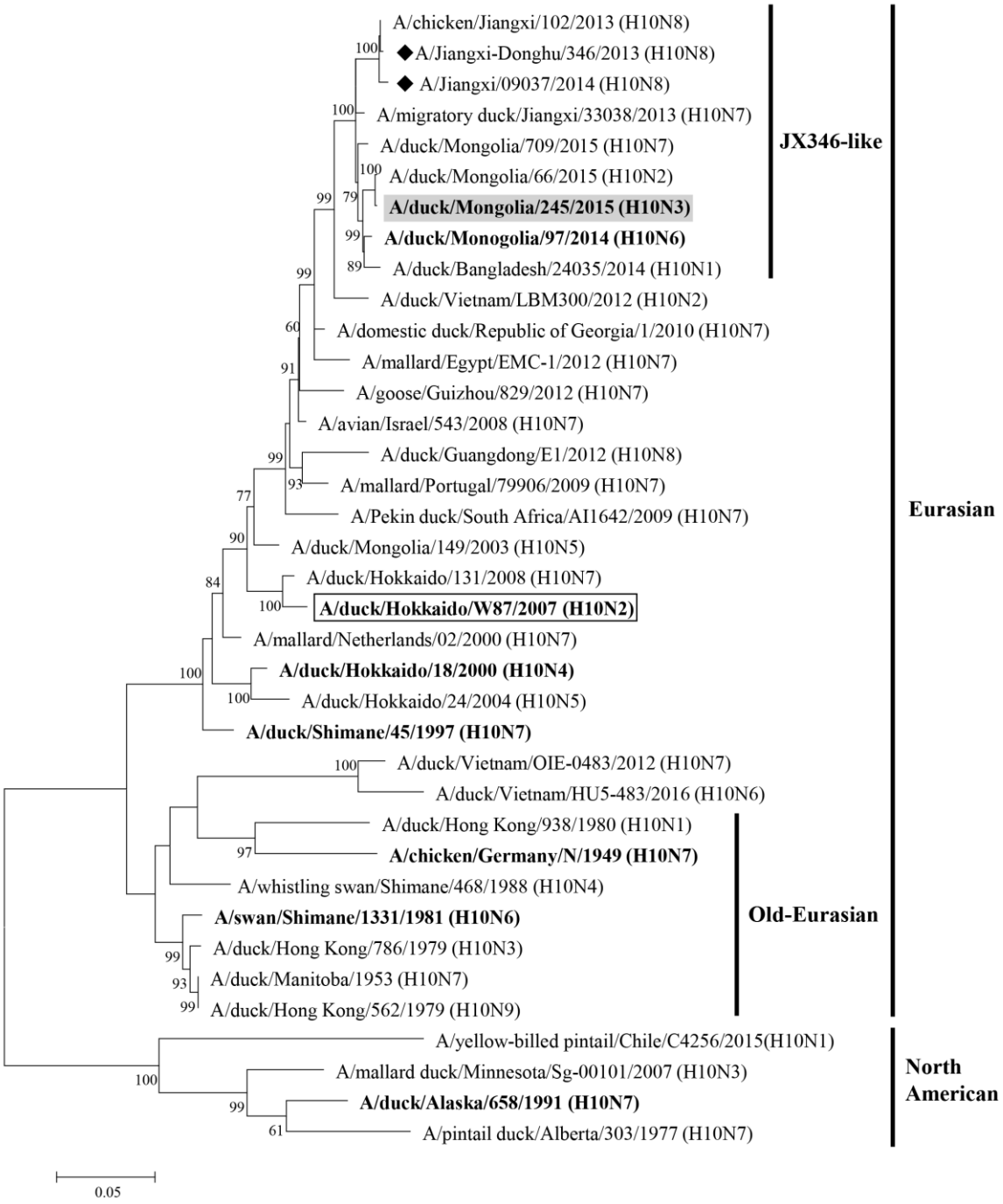

Fig. 3 Phylogenetic tree of H10 HA genes of influenza viruses Full-length nucleotide sequences of the HA gene were used for phylogenetic analysis using ML. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node 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 8 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)	1,280	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	2,560	1,280	5,120
	A/chicken/Germany/N/1949 (H10N7)	640	2,560	2,560	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	5,120

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)

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 [68].

Pathogenicity of avian H10 influenza viruses in mice

To analyze the pathogenicity of H10 influenza viruses in mice, six strains of avian H10 influenza viruses were inoculated into two naïve mice, respectively, and clinical signs were observed 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/Hokkaido/W87/2007 (H10N2), A/duck/Alaska/658/1991 (H10N7), and A/duck/Hokkaido/18/2000 (H10N4) (Fig. 4, white triangle, square, and circle, respectively). On the other hand, a relatively mild reduction of body weight was seen in the group of the mice inoculated with A/duck/Mongolia/245/2015 (H10N3), A/duck/Mongolia/709/2015 (H10N7), and A/duck/Mongolia/66/2015 (H10N2) (Fig. 4, black triangle, square, and circle, respectively). The amino acids sequences of PB2 and HA of the strains in Fig. 3 and

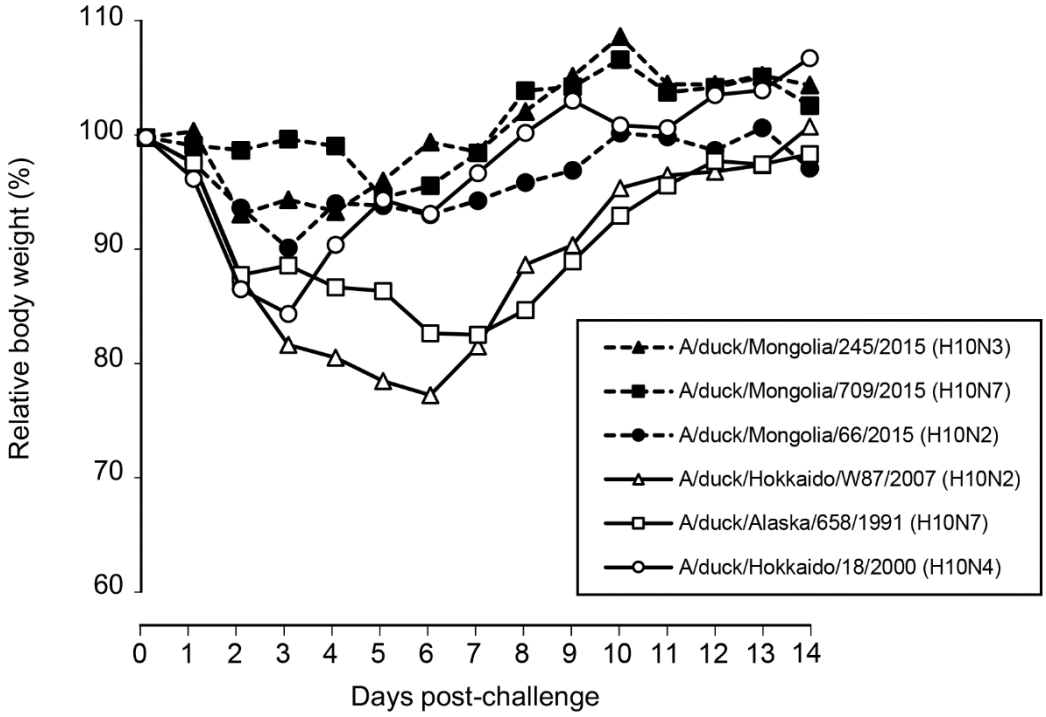

Fig. 4 Changes in the body weight of mice inoculated with avian H10 influenza viruses Body weight was monitored for 14 days. Each strain was inoculated into two mice. Mice with significant body weight loss (a decrease of 25% during the observation period as compared with that of before the challenge) were indicated using white triangles, squares, and circles. Mice with mild body weight change were indicated using black triangles, squares, and circles.

A/Jiangxi-Donghu/346/2013 (H10N8) were compared (Table 9). Among amino acid sequences 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 (Table 9, gray highlighted), common amino acid sequences were observed at position 340 of the PB2, and 46, 326, and 506 of the HA. No viruses except for the human H10N8 influenza virus had lysine (K) at the position 627 of the PB2 which is known for the most important marker for mammalian adaptation of avian influenza viruses and leads to enhanced polymerase activity. Aspartic acid (D) at the position 701 of the PB2 was conserved among viruses compared in this study. Amino acid residues with asparagine (N) at 701 of the PB2 were also considered to be one of the most important marker for mammalian adaptation of avian influenza viruses.

Potency test of the vaccine against H10 influenza virus in mice

Antiserum against 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. Based on these genetic and antigenic analyses, and this virus was selected as a vaccine candidate strain. An inactivated vaccine was prepared and used to immunize 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 viruses, A/duck/Mongolia/245/2015 (H10N3) and A/duck/Hokkaido/W87/2007 (H10N2), respectively (Table 10). The serum neutralizing antibody titers of the mice injected twice with 100 μg of the vaccine reached 1:320 to both homologous and heterologous

Table 9 Comparison of the amino acid sequences of the PB2 and the HA among H10 influenza viruses inoculated into mice

Viruses	Deduced amino acids of 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	·	·	·	·	·	·	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 of 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	·	·	·	S	·	·	·	·	V	A	·	S	·	·	·	A	·	E	·	·
A/duck/Alaska/658/1991 (H10N7)	H	R	A	K	S	·	D	K	·	Y	·	D	S	·	·	·	D	·	·	S	A	V	·	·	·
A/duck/Hokkaido/18/2000 (H10N4)	R	·	·	·	S	·	·	·	S	·	·	·	·	·	I	·	S	·	D	·	A	I	·	L	·
A/Jiangxi-Donghu/346/2013 (H10N8)	·	·	·	·	·	I	·	·	K	·	M	·	·	·	·	V	·	R	·	·	A	·	·	·	R

[b] H3 numbering

Viruses	Deduced amino acids of 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 10 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)

viruses (Table 11). The virus titers in the lungs of the mice injected twice with the vaccine were suppressed in a 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. 5). 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 11 Serum neutralizing antibody titers of mice injected twice with vaccines and virus recovery from mouse 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)

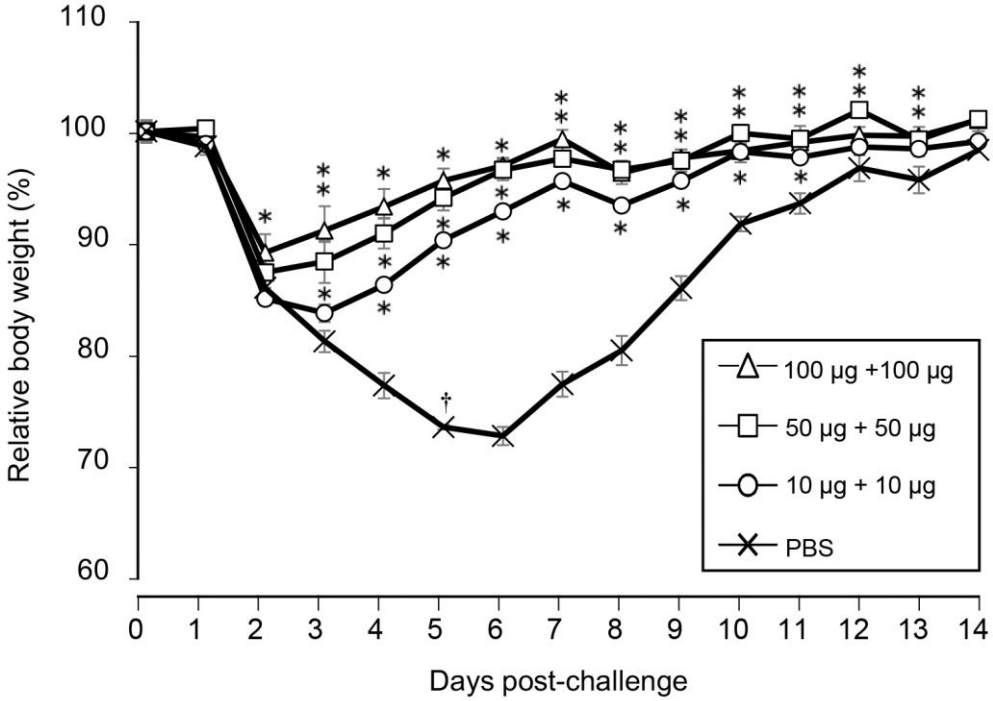

Fig. 5　Changes in the body weight of mice subcutaneously vaccinated twice with the A/duck/Mongolia/245/2015 (H10N3) following a challenge with A/duck/Hokkaido/W87/2007 (H10N2)　Body weight was monitored for 14 days. Data are shown as the mean body weight ± standard error.　Asterisks indicate that body weights of the vaccinated groups recovered significantly more than the PBS-injected group ($P < 0.05$).　†: Mice died.

Discussion

During the 2013–2014 winter season in China, H10 influenza viruses were isolated from humans, and two fatal cases were reported [4, 81]. Thus, preparation for future pandemics caused by H10 influenza viruses is greatly needed. Vaccination is one of the effective control measures 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 [42]. Therefore, the vaccine prepared from A/duck/Mongolia/245/2015 (H10N3) may be potentially useful for the control of a future pandemic influenza caused by H10 influenza viruses. In addition, inactivated whole virus particle influenza vaccines are more effective than split influenza vaccines [2, 22, 51]. 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 the protective efficacy in a mouse model [75]. The present study also provide an insight that the inactivated whole virus particle vaccine prepared from avian H10 influenza viruses is also effective in mice. Furthermore, the findings in this study 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 an RNA vaccine or other novel techniques reported in some studies using human H10 influenza viruses [39, 57].

Genetic analysis also revealed that H10 influenza viruses isolated from fecal samples of wild birds in Hokkaido and Mongolia are close to the human H10N8 influenza viruses, and the antigenicity of these viruses is similar to each other. Interestingly, A/duck/Vietnam/OIE-0483/2012 (H10N7) and A/duck/Vietnam/HU5-483/2016 (H10N6) isolated from domestic ducks 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 [52]. 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 analyses of H10 influenza viruses should be continued.

In the PB2 protein of human H10N8 influenza virus, the amino acid residue at 627 is a mixture of glutamic acid (E) and K [4]. A previous study suggested that E627K in the PB2 is a key to the adaptation of avian H10 influenza viruses to mammals [58]. In mammals, this mutation and D701N of the PB2 leads to enhanced polymerase activity, resulting in host adaptation and enhanced virulence [19, 36, 38, 63, 65, 66]. Amino acid mutation PB2-E627K effects on polymerase activity and bonding of the polymerase to the viral RNA in mammalian cells, and D701N considered to be affecting the nuclear localization pattern of the PB2. 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 [4, 80]. 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 [59, 70, 78]. 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 avian H10 influenza viruses analyzed in this study. In spite of 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 the pathogenicity in mice were not understood. It has been reported that the combination of amino acid substitutions in the NA and PB2 contribute to the mouse adaptation of H10 influenza viruses [82]. 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, findings of the present study suggest that intensive global surveillance of H10 influenza viruses both in poultry and wild birds should be continued to monitor the introduction of H10 influenza viruses 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.

Brief summery

The H10N8 influenza virus became a threat to public health when cases of human 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 similar to each other among the viruses circulating in wild 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 a challenge with A/duck/Hokkaido/W87/2007 (H10N2), which is a pathogenic strain in mice. This study demonstrated 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.

Conclusion

Pandemic threat caused by H2 and H10 influenza viruses are existing. However, little is known on the genetic and antigenic properties of H2 and H10 influenza viruses recently circulating among wild birds which are considered as natural reservoirs. In addition, vaccine preparation against future pandemics caused by H2 and H10 influenza viruses are required as an effective control measure. In the present thesis, the author analyzed genetic and antigenic properties of H2 and H10 influenza viruses circulating in Asian countries and recently isolated from wild birds. Moreover, the efficacies of inactivated whole virus particle vaccines prepared from viruses isolated from wild birds were evaluated in a mouse model.

In Chapter I, genetic and antigenic properties of H2 influenza viruses circulating in wild birds in Hokkaido and Mongolia were analyzed. Antiserum against the H2 influenza virus recently isolated from the wild bird, A/duck/Hokkaido/162/2013 (H2N1), showed broad cross-reactivity, and thus the virus was selected as the vaccine candidate strain. 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 the challenge with A/swine/Missouri/2124514/2006 (H2N3).

In Chapter II, genetic properties of H10 influenza viruses were studied. It was found 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 similar to each other among the viruses circulating in wild birds. The inactivated vaccine was prepared from avian H10 influenza viruses isolated from a fecal

sample of wild duck. The vaccine induced protective immunity sufficient to reduce the impact of the challenges with pathogenic H10 influenza virus in mice.

This study demonstrates that the inactivated whole virus particle vaccines prepared from viruses isolated from wild birds would be useful against a future pandemic influenza caused by H2 and H10 influenza viruses. These findings provide supportive insights into preparation for future pandemics.

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Summary in Japanese（和文要旨）

A 型インフルエンザウイルスは人獣共通感染症であるインフルエンザの原因ウイルスである。A 型インフルエンザウイルスの自然宿主は野生水禽であり、野生水禽の間で維持されている鳥インフルエンザウイルスのうち、ブタを介してヒトの間で効率よく伝播するようになったウイルスが、ヒトでインフルエンザの流行を引き起こす。これまでに H1N1、H2N2、H3N2 亜型の A 型インフルエンザウイルスによる世界的大流行（パンデミック）が知られている。このうち H1N1 亜型および H3N2 亜型のウイルスのみが季節性インフルエンザの原因ウイルスとして現在もヒトの間で維持されている。さらに近年では、H5N1 亜型および H7N9 亜型の鳥インフルエンザウイルスが家禽からヒトへ直接感染したとみられる例が数多く報告されており、公衆衛生上の重大な問題となっている。そのため、ヒトにおけるインフルエンザの制御と流行予測、将来起こりうるヒトでの流行に備えたワクチンの準備には、野生水禽、家禽およびブタを含めた動物から分離されるウイルスの性状を調べることが必要である。

H2 亜型のウイルスは 1957 年にアジア風邪として知られるパンデミックを起こし、その後 1968 年まで季節性インフルエンザの原因ウイルスとしてヒトの間で維持された。しかし、1968 年以降ヒトからの分離報告はなく、現在では多くのヒトが H2 ウイルスに対する獲得免疫を有していない。そのため、現在野鳥の間で維持されている H2 亜型のウイルスがヒトに伝播した場合、パンデミックとなる可能性がある。また 2013 年から 2014 年にかけて、中国で H10N8 亜型のウイルスがヒトから分離され、2 例の死亡例が報告された。ヒトから分離された H10N8 亜型のウイルスと遺伝的に近縁なウイルスは、現在も中国の野鳥や家禽から継続的に分離されており、再びヒトへ伝播する可能性がある。これらの事実

にも関わらず、H2 亜型および H10 亜型のウイルスは、家禽や野鳥の間での流行状況や、その遺伝子と抗原性について包括的には調べられていない。また将来のヒトへの感染やパンデミックに対する備えとして、H2 亜型および H10 亜型のウイルスに対するワクチンの準備が望まれるが、先行研究は限られている。

本研究では、野鳥における H2 亜型および H10 亜型インフルエンザウイルスの流行状況を調べることを目的に、北海道とモンゴルで採取した野生水禽の糞便から分離したウイルス株を含め、H2 亜型および H10 亜型のウイルスの遺伝子と抗原性を調べた。さらにそれぞれの亜型のウイルスについて、将来のヒトでの流行に備えたワクチンの準備に資するため、不活化全粒子ワクチンを試製し、その有効性をマウスモデルで評価した。1 章では H2 亜型のウイルス、2 章では H10 亜型のウイルスについて報告した。

H2 亜型のウイルスの HA 遺伝子はユーラシア系統と北米系統に大別されるが、ユーラシア系統に属するウイルスの HA 遺伝子は、さらに 4 つのクラスターに分類され、遺伝的に多様であることを明らかにした。現在アジアで野鳥の間で循環している H2 ウイルスは、過去にアジア風邪を起こしたウイルスとは異なるクラスターに属した。一方、代表株に対する抗血清を用いた抗原性解析の結果、H2 亜型のウイルスの抗原性は、遺伝子の多様性によらず広く保存されていることがわかった。近年アジア地域で野鳥から分離された H2 亜型のウイルスのうち、マウスに対する免疫原性の高い A/duck/Hokkaido/162/2013 (H2N1) を用いて不活化全粒子ワクチンを試製した。このワクチンを接種したマウスでは、ワクチン株に対する中和抗体が誘導された。またこのマウスを 2006 年にアメリカでブタから分離された A/swine/Missouri/2124514/2006 (H2N3)で攻撃したところ、肺でのウイルス増殖が有意に抑制された。

H10 亜型のウイルスでは、ヒトから分離された H10N8 ウイルスと遺伝的に近縁な HA 遺伝子をもつウイルスが、近年アジア地域の野鳥や家禽から分離されていることを示した。これらのウイルスは、Old-Eurasian と新たに呼称した 1970 年代以前に野鳥や家禽から分離されたウイルスとは遺伝的に異なっていた。H10 亜型のウイルスの抗原性は、近年分離されたウイルスの間では広く保存されているが、Old-Eurasian に属するウイルスに対する抗血清は近年分離されたウイルスとの反応性が低いことを明らかにした。また、ヒトから分離された H10N8 ウイルスに遺伝的に近縁な株である野生のカモの糞便から分離した A/duck/Mongolia/245/2015 (H10N3)を用いて不活化全粒子ワクチンを試製した。このワクチンを接種したマウスでは、ワクチン株に対する中和抗体が誘導された。またこのワクチンを接種したマウスでは、マウスにおける病原性の高い株である A/duck/Hokkaido/W87/2007 (H10N2)での攻撃に対して、肺でのウイルス増殖を抑制し、体重減少の程度を緩和した。

以上から、インフルエンザウイルスライブラリーから試製した不活化全粒子ワクチンが、H2 および H10 亜型ウイルスの感染に対して有用であることが示唆された。本研究により得られた知見は、将来起こりうるインフルエンザのパンデミック対策に資することが期待される。