



Title	Studies on the development of vaccine and molecular basis of pathogenicity of avian influenza viruses for chicken
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Citation	北海道大学. 博士(獣医学) 甲第9485号
Issue Date	2010-03-25
DOI	10.14943/doctoral.k9485
Doc URL	http://hdl.handle.net/2115/42815
Type	theses (doctoral)
File Information	soda_thesis.pdf



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**Studies on the development of vaccine and molecular basis
of pathogenicity of avian influenza viruses for chicken**

(鳥インフルエンザウイルスのワクチン開発
および病原性の分子基盤に関する研究)

Kosuke Soda

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Preface

Avian influenza is caused by an infection with genus *Influenzavirus A* of family *Orthomyxoviridae* [32]. Influenza A viruses infect a large variety of birds and mammals including humans. Influenza A viruses of each of the sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes (H1-H16 and N1-N9, respectively) have been isolated from water birds such as migratory ducks. Ducks are infected with the viruses through water-borne in the lakes and marshes where they nest in summer in the northern territories such as Siberia, Canada, and Alaska. The viruses replicate in the columnar epithelial cells which form crypts in the colon of ducks without showing clinical signs and are shed in the fecal materials [38]. In autumn, the ducks leave their nesting lakes to the south for migration. It is known that the pathogenicity of influenza viruses for chickens ranges from asymptomatic to systemic infections with low to high mortality. Highly pathogenic avian influenza (HPAI) viruses are selected when the avirulent viruses of ducks transmit to chickens through domestic water birds such as ducks and geese and terrestrial birds such as quails and turkeys, and passaged among chicken population [36]. HA subtypes of HPAI viruses are restricted to H5 and H7, although not all viruses of these subtypes cause HPAI.

In 1997, outbreaks of HPAI caused by H5N1 viruses occurred in three chicken

farms in Hong Kong and over 6,500 chickens died. This outbreak impacted not only to the poultry industry but to public health since 18 people were infected with the viruses and six died. More than 1.2 million chickens were culled as countermeasures and the H5N1 virus was eradicated [8]. HPAI outbreaks caused by H7 influenza viruses have been reported in Italy, the Netherlands, Pakistan, Canada, and North Korea in these 12 years and caused serious economic losses [1]. In the Netherlands, 86 people who handled affected poultry and three of their family were infected with H7N7 influenza viruses, showing inapparent to mild symptoms [14, 40]. In Japan, HPAI outbreaks caused by H5N1 viruses occurred in Yamaguchi and Kyoto prefectures in 2004 and then reoccurred in Miyazaki and Okayama prefectures in 2007 [48]. All of these outbreaks were well controlled by early diagnosis, movement restriction, and culling of all of the chickens in the affected farm, stamping-out policy, which is the fundamental measure for the control of HPAI. Meanwhile, it is suggested that when outbreaks spread to broad area, being uncontrollable, ring vaccination should be an optional measure to reduce the virus concentration and to suppress the spread of viruses [53]. Since commercial vaccines have been prepared from viruses belonging to the North American lineage in the USA and Mexico, they may be less effective for the control of current HPAI in Asia. Therefore, a vaccine which is prepared from the viruses antigenically similar to viruses circulating in Asia at present has been awaited.

In chapter I in the present thesis, establishment of proper seed strains for the preparation of vaccine against H5 and H7 influenza viruses is described. These candidate strains for vaccine were generated from the parental viruses isolated from natural reservoir, ducks by genetic reassortment. The reason why the author selected an HA gene provider of the H5 vaccine strains from the isolates from natural reservoirs is explained by antigenic and genetic analyses in Chapter II. The potency of the vaccine against HPAI caused by the H5 virus was also assessed using a mouse model in this chapter.

Since 1990s, strains of H9N2 avian influenza virus have caused outbreaks in poultry, resulting in serious economic losses in Asia and the Middle East [15, 16, 18, 19, 41-43, 45, 80]. In 2001 and 2002, H9N2 viruses were isolated from chicken meat and bone marrow imported from China at Yokohama Animal Quarantine Station in Japan [47]. Although these H9N2 strains are avirulent for SPF chickens and none of them have multiple basic amino acids at the cleavage site of the HA as observed in those of H5 or H7 HPAI viruses, co-infections of the H9N2 viruses with some bacteria exacerbated the disease [39]. It is important for controlling H9 virus infections in poultry hereafter to assess whether the H9N2 virus is capable of becoming pathogenic for chicken like H5 and H7 viruses. In the present study, a pair of di-basic amino acid residues was introduced into the HA cleavage site of the non-pathogenic H9 viruses and

its potential to become highly pathogenic to chickens was examined and concluded in chapter III. The results will help to understand why HA subtypes of HPAI viruses are restricted to H5 and H7.

Chapter I

Establishment of vaccine strains of H5 and H7 influenza viruses

Introduction

Highly pathogenic avian influenza (HPAI) caused by H5 and H7 influenza viruses have occurred worldwide, leading to serious economic losses in poultry industries. In Japan, there had been no outbreak since 1925 when HPAI caused by a H7N7 virus occurred in Chiba prefecture [69]. In the beginning of 2004, HPAI outbreaks caused by H5N1 viruses occurred at chicken farms in Yamaguchi and Kyoto prefectures [25, 48]. H5N1 infections then reoccurred on poultry farms in Miyazaki and Okayama prefectures in 2007. The depopulation of chickens in the relevant farms and appropriate control measures successfully prevented the spread of HPAI.

Standard countermeasures for the control of HPAI outbreaks are the testing and culling of affected chickens. However, when outbreaks spread to a broad area, being uncontrollable, ring vaccination would be an optional measure to reduce the concentration of the virus and, hence, suppress its spread [5]. In response to the 2004

outbreaks in Japan, commercial vaccines were urgently imported from Mexico as stockpiles. However, these vaccines have been suspected that it might be inadequate to control current outbreaks in Asia because they have been produced from H5N2 viruses of North American lineage. A domestic vaccine which has a similar antigenicity to viruses circulating in Asia at present has been awaited for stable production and provision in the case of a nation-wide outbreak which cannot be controlled.

The aim of the present study is to establish proper seed strains for vaccine manufacturing against the H5 and H7 influenza viruses in Asia. Here, it is reported that the characteristics of non-pathogenic H5N1 and H7N7 viruses generated from parental viruses isolated from ducks, a natural host of influenza A viruses, by genetic reassortment.

Materials and Methods

Viruses

All the parental viruses for generating vaccine candidate strains in this study were isolated in our laboratory from fecal samples of migratory ducks in Mongolia and Japan [36]. The viruses were propagated in ten-day-old embryonated chicken eggs for 48 hours at 35°C and infectious allantoic fluids were used for generating of reassortant viruses.

Generation of genetic reassortant viruses

To generate the H5N1 and H7N7 reassortant virus, parental viruses were mixed and inoculated into the allantoic cavities of embryonated chicken eggs. After incubation at 35°C for 24 h or 48 h, the allantoic fluids were collected, mixed with chicken antisera (α -A/Duck/Hong Kong/301/78 (H7N2) for the selection of A/duck/Hokkaido/Vac-1/04 (H5N1), α -A/turkey/England/73 (H7N3) and α -A/turkey/Massachusetts/3740/65 (H6N2) for A/duck/Hokkaido/Vac-3/07 (H5N1), and α -A/duck/Hokkaido/13/00 (H9N2) for A/duck/Hokkaido/Vac-2/04 (H7N7)) and incubated at room temperature for one hour prior to plaque cloning of the viruses in MDCK cells to neutralize the viruses which have undesired hemagglutinin (HA) and/or neuraminidase (NA) (e.g., H7HA and

N2NA in generation of A/duck/Hokkaido/Vac-1/04 (H5N1)). Cloned viruses were subtyped using hemagglutinin-inhibition (HI) [64] and neuraminidase-inhibition (NI) tests [3].

Plaque assay

Purification of viruses by plaque selection method was performed as described previously [21]. Briefly, confluent MDCK cells were incubated one hour at 35°C with ten-fold dilutions of virus. The cells were then washed and overlaid with MEM (Nissui Pharmaceutical, Tokyo, Japan) containing 0.7% Bacto-agar (Difco, Sparks, MD, U.S.A) and 0.5 µg/ml of trypsin and incubated at 35°C. After three days, plaques were picked to be further purified.

Sequencing

Viral RNAs were extracted from infectious allantoic fluids using a commercial kit (TRI LS reagent, Sigma-Aldrich, St. Louis, MO, U.S.A) and reverse-transcribed with the Uni12 primer [23] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A). Viral complementary DNA was amplified with the polymerase chain reaction (PCR) with the RNA LA PCR Kit Ver. 1.1 (Takara, Shiga, Japan) and a PTC-200 thermal cycler (Biorad, Alfred Novel Drive Hercules, CA, U.S.A). The primers used for

RT-PCR in the present study were gene-specific primer sets. Nucleotide sequences were determined from these RT-PCR products using a CEQ2000 automated DNA sequencer (Beckman Coulter, Fullerton, CA, U.S.A) according to the Dye Terminator Cycle Sequence Chemistry Protocol (Beckman Coulter). Sequence data were analysed using GENETYX Version 7.0 (Genetyx, Tokyo, Japan).

Pathogenicity tests

Mean death times (MDTs) of chicken embryos were determined according to the previous report [74]. Nine-day-old embryonated chicken eggs were inoculated with ten-fold dilutions of the viruses. Eggs were examined every eight hours by candling; the integrity of the circulatory system and movement of the embryo were the criteria for viability. Any embryos which were dead at 24 hour post-inoculation were discarded as nonspecific deaths and did not figure in the calculation of MDT. The MDT was calculated as the mean time in hours for the minimum dose lethal to kill the embryos. The intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) tests were performed as described elsewhere [2, 6]: each infectious allantoic fluid was inoculated into groups of ten one-day-old chicks and eight six-week-old chickens, via the intracerebral and intravenous route, respectively. In both studies, the birds were observed for clinical symptoms and mortality at 24-hour intervals for a period of eight

days for the ICPI test and ten days for the IVPI test. The ICPI and IVPI values were calculated as described [2].

Viral growth kinetics

One hundred and ten thousands or $10^{1.5}$ and $10^{3.5}$ 50% egg infectious dose (EID₅₀) for A/duck/Hokkaido/Vac-1/04 (H5N1) and A/duck/Hokkaido/Vac-3/07 (H5N1) or A/duck/Hokkaido/Vac-2/04 (H7N7) were inoculated into ten-day-old embryonated chicken eggs, respectively. The allantoic fluids were harvested at 34°C (35°C for A/duck/Hokkaido/Vac-3/07 (H5N1)) to determine viral titers at different time points (0, 12, 24, 48, and 72 hours). The EID₅₀ and hemagglutination titer of each virus present in the allantoic fluid was determined.

Results

Generation of H5N1 strains of Eurasian lineage

Until now, non-pathogenic H5N1 viruses of Eurasian lineage have been rarely isolated from natural hosts [67]. To establish H5N1 vaccine candidate strains, A/duck/Hokkaido/Vac-1/04 (H5N1) [Vac-1/04 (H5N1)] and A/duck/Hokkaido/Vac-3/07 (H5N1) [Vac-3/07 (H5N1)] were generated by the standard genetic reassortment procedure from non-pathogenic viruses of Eurasian lineage derived from water birds and origins of the internal proteins were determined (Fig. 1A and 1B). The PB2, PB1, PA, HA, NP, and M gene segments of Vac-1 (H5N1) were derived from A/duck/Mongolia/54/01 (H5N2), and the NA and NS gene segments, from A/duck/Mongolia/47/01 (H7N1) (Fig.1A). The HA and NS gene segments of Vac-3 (H5N1) were derived from A/duck/Hokkaido/101/04 (H5N3), while the other segments were from A/duck/Hokkaido/262/04 (H6N1) (Fig. 1B).

The complete nucleotide sequences of Vac-1 (H5N1) and Vac-3 (H5N1) obtained in the present study have been registered in GenBank/EMBL/DDBJ. (Accession numbers: AB259709-AB259716, and AB355926-AB355933)

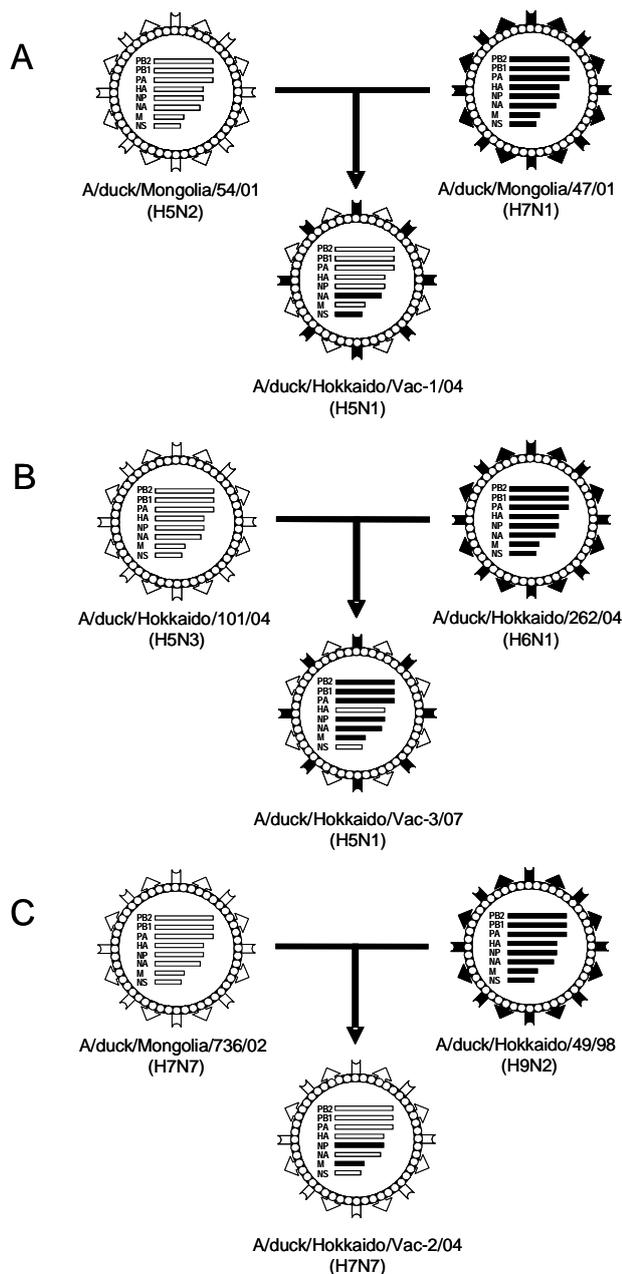


Fig. 1. Establishment of candidate strains for a vaccine against H5 and H7 highly pathogenic avian influenza viruses by genetic reassortment. Vac-1/04 (H5N1), Vac-3/07 (H5N1), and Vac-2/04 (H7N7) were generated from two non-pathogenic avian influenza viruses isolated from migratory ducks. The origin of viral segments of reassortant viruses is indicated with white and black bands. (A); Vac-1/04 (H5N1) was a reassortant generated with A/duck/Mongolia/54/01 (H5N2) as the HA gene provider and A/duck/Mongolia/47/01 (H7N1) as the NA gene provider. (B); Vac-3/07 (H5N1) was generated with A/duck/Hokkaido/101/04 (H5N3) as the HA gene provider and A/duck/Hokkaido/262/04 (H6N1) as the NA gene provider (C); Vac-2/04 (H7N7) was generated with Mon/736 (H7N7) as the HA and NA gene provider and Hok/49 (H9N2) to give high growth potential in chicken embryos.

Generation of an H7N7 virus with good growth potential

Dozens of non-pathogenic H7N7 viruses have been isolated from fecal samples of water birds during our surveillance studies since 2001. Based on the results of phylogenetic and antigenic analyses, A/duck/Mongolia/736/02 (H7N7) [Mon/736 (H7N7)] was selected as a vaccine candidate. However, the growth potential of Mon/736 (H7N7) in chicken embryonated eggs was poor in terms of the HA titer and yield of viral protein. To improve the growth potential of Mon/736 (H7N7), A/duck/Hokkaido/Vac-2/04 (H7N7) [Vac-2/04 (H7N7)] was generated as a genetic reassortant virus between Mon/736 (H7N7) and A/duck/Hokkaido/49/98 (H9N2) [Hok/49 (H9N2)], which possesses good growth potential in chicken embryos (Fig. 1C). The PB2, PB1, PA, HA, NA, and NS gene segments of Vac-2/04 (H7N7) were derived from Mon/736 (H7N7), and the NP and M genes from Hok/49 (H9N2) (Fig. 1C).

The complete nucleotide sequences of Vac-2 (H7N7) obtained in the present study have been registered in GenBank/EMBL/DDBJ. (Accession numbers: AB243417-AB243424).

Pathogenicity of vaccine candidate strains

The pathogenicity of vaccine candidates for chickens, chicks, and chicken embryos was evaluated by measuring the IVPI, ICPI, and MDT. All of the chickens inoculated

intravenously with Vac-1 (H5N1), Vac-3 (H5N1), or Vac-2 (H7N7) survived for ten days without showing any clinical signs, and IVPI values were 0.00 (Table 1). The pathogenicity of Vac-1 (H5N1) and Vac-2 (H7N7) for chicks was very low, the ICPI being 0.10 and 0.20, respectively. Half of the chicks inoculated intracerebrally with Vac-3 (H5N1) died on two to eight days post-inoculation (ICPI, 0.58), indicating a relatively high pathogenicity for chicks as compared with the other two candidate strains. The MDT of Vac-1 (H5N1), Vac-3 (H5N1), and Vac-2 (H7N7) was 64, 73, and 81 hours, respectively.

Growth kinetics of vaccine candidate strains in chicken embryos

The growth kinetics of vaccine candidate strains in chicken embryos was determined. As shown in Table 2, all the candidates replicated well, and reached peak titers from 48 hours post-inoculation. There was no significant difference in peak titers regardless of the dose of each vaccine candidate.

Table 1. Pathogenicity of vaccine candidate strains for chickens and chicken embryos

Viruses	Amino acid sequence of HA cleavage site	IVPI ^{a)}	ICPI ^{b)}	MDT ^{c)} (hr)
Vac-1/04 (H5N1)	PQRETR/G	0.00	0.10	64
Vac-3/07 (H5N1)	PQRETR/G	0.00	0.58	73
Vac-2/04 (H7N7)	PEIPKGR/G	0.00	0.20	81

^{a)} Intravenous pathogenicity index

^{b)} Intracerebral pathogenicity index

^{c)} Mean death time

Table 2. Growth kinetics of vaccine candidate strains in chicken embryos ^{a)}

Incubation time (hr)	Vac-1/04 (H5N1)				Vac-3/07 (H5N1)				Vac-2/04 (H7N7)			
	10 ^{2.0b)}				10 ^{2.0}				10 ^{4.0}			
	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer
0	≤0.5	<2	≤0.5	<2	≤0.5	<2	≤0.5	<2	≤0.5	<2	≤0.5	<2
12	3.7	<2	4.5	<2	2.3	<2	5.5	<2	3.5	<2	5.1	<2
24	8.7	32	9.5	256	8.3	512	8.4	1024	7.9	4	8.7	64
48	9.3	256	9.5	512	9.3	2048	9.5	2048	9.5	512	9.1	512
72	9.1	512	9.3	256	9.5	2048	9.5	2048	9.1	512	9.3	512

^{a)} The virus titers (logEID₅₀/ml) and HA titers (HAU/ml) were determined from allantoic fluids of chicken embryos inoculated with Vac-1/04 (H5N1), Vac-3/07 (H5N1), and Vac-2/04 (H7N7), respectively.

^{b)} Dose of inoculum given to chicken embryo (EID₅₀/egg)

Discussion

Antigenicity, pathogenicity, and yield in chicken embryos are the most important for vaccine strains. The vaccine strains in this study meet these requirements, suggesting that they are suitable for the production of vaccines.

To obtain non-pathogenic H5N1 strains for vaccines, Vac-1 (H5N1) and Vac-3 (H5N1) were generated by genetic reassortment. These two strains showed low pathogenicity in chickens and chicken embryos (Table 1). Additionally, they grew well in chicken embryos after 48 hours post-inoculation, relatively shorter than their MDT (Table 2), indicating that they should be useful as potential vaccine candidate strains. The vaccine for chickens by using Vac-1 (H5N1) had been produced in collaboration with vaccine producers and its protective effect was already assessed [26].

Vac-2 (H7N7) was obtained as a vaccine candidate strain against H7N7 viruses (Fig. 1C). Mon/736 (H7N7), providing the HA and NA gene of Vac-2 (H7N7), showed poor growth potential in chicken embryos (16HA, data not shown). To solve this problem, a genetic reassortant was generated using Hok/49 (H9N2), which has high growth potential in eggs. The NP and M gene segments of Vac-2 (H7N7) were derived from Hok/49 (H9N2), indicating that the NP and/or M gene segments were responsible for the growth. To determine the factors responsible for growth potential of Vac-2

(H7N7) in chicken embryos, further investigation is needed.

Brief summary

To establish vaccine strains of H5 and H7 influenza viruses, A/duck/Hokkaido/Vac-1/04 (H5N1) [Vac-1/04 (H5N1)], A/duck/Hokkaido/Vac-3/07 (H5N1) [Vac-3/07 (H5N1)], and A/duck/Hokkaido/Vac-2/04 (H7N7) [Vac-2/04 (H7N7)] were generated from non-pathogenic avian influenza viruses isolated from migratory ducks. Vac-1/04 (H5N1) and Vac-3/07 (H5N1) were generated by genetic reassortment between H5N2 or H5N3 viruses as HA gene providers and H7N1 or H6N1 viruses as NA gene providers. Vac-2/04 (H7N7) was a genetic reassortant obtained using H7N7 and H9N2 viruses to confer high growth character of the H9N2 virus in chicken embryos. The results of sequence analyses and experimental infections revealed that these H5N1 and H7N7 reassortant viruses were non-pathogenic in chickens and embryos, and had good growth potential in chicken embryos. These viruses should be useful to develop vaccines against H5 and H7 highly pathogenic avian influenza viruses.

Chapter II

Antigenic and genetic analyses of H5 influenza viruses isolated from water birds for the use of vaccine

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 strains have occurred in many countries, leading to serious economic losses in the poultry industry. In addition, the H5N1 HPAI virus returned to the natural host, migratory water birds, and spread to Asia, Central East, Europe, and Africa [7, 44, 78]. These incidents have increased the possibility of further spread of HPAI viruses in poultry and transmission to humans. In Japan, there had been no outbreak since 1925 when HPAI caused by H7N7 virus occurred in Chiba prefecture [69]. In January 2004, HPAI outbreaks caused by H5N1 virus occurred in Yamaguchi, Oita, and Kyoto prefectures in Japan [25, 48]. H5N1 virus infection then reoccurred in chicken farms in Miyazaki and Okayama prefectures in January 2007. The depopulation of chickens in the relevant farms and control measures successfully prevented the spread of the HPAI. The source of infection is unclear although it was revealed that the H5N1 virus isolates from the

affected chickens in Japan in 2004 were phylogenetically identical to HPAI viruses isolated in Guangdong Province of China in 2003 [48]. It was also suggested that the H5N1 viruses isolated in 2007 outbreaks in Japan showed close phylogenetic relationship with the similarity to the viruses isolated from water birds in Qinghai lake, Mongolia, Korea, and even in Nigeria during 2005-2007.

As described above, standard measures for the control of HPAI outbreaks are testing and culling of all of the chickens in the farm. But it is, in addition, suggested by OIE that when outbreaks spread to broad area, being uncontrollable, ring vaccination would be an optional measure in addition to stamping out to reduce the virus concentration and, hence, to suppress the spread of viruses [5].

It has been established that influenza viruses perpetuate between ducks and water of the lakes where they nest in summer [29, 75]. Viruses of 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified in avian species [13]. Viruses have been highly conserved in water birds antigenically and genetically [35]. Phylogenetic analysis revealed that each of the influenza A viruses of birds and mammalian hosts including humans is originated from water bird reservoirs [75]. Thus, continuous surveillance of avian influenza is essential for the preparedness against the emergence of HPAI and human pandemics. To provide information on the precursor genes of future pandemic influenza viruses, it has been conducted that global surveillance of avian

influenza since 1977 in Alaska, Australia, China, Japan, Mongolia, Siberia, and Taiwan [25, 29, 37, 54]. Virus isolates from birds in the surveillance have been stored in the influenza virus strain library in our laboratory for vaccine and diagnostic use [25].

The aim of the present study is to assess applicability of the virus strain library for vaccine preparation. Here, it is reported that the usefulness of the library by choosing a H5N1 virus strain for vaccine preparation as a model.

Materials and methods

Viruses

A total of 10,549 fecal samples were collected from water birds in 1996-2007 in Siberia, Mongolia, China, Australia and Japan. The samples were kept cool, transported to Hokkaido University, and stored at 4°C or frozen at -80 °C until assayed. Each sample was inoculated into the allantoic cavities of ten-day-old embryonated chicken eggs. Subtype identification of influenza virus isolates was done by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests using a series of standard antisera to the reference strains of influenza viruses [37].

A/duck/Pennsylvania/10218/1984 (H5N2) [51] was kindly provided by Dr. R. G. Webster, St. Jude Children's Research Hospital (Memphis, Tennessee, U.S.A). A/Hong Kong/156/1997 (H5N1) and A/Hong Kong/483/1997 (H5N1) [68] were obtained from Dr. K. F. Shortridge, University of Hong Kong (Hong Kong, Special Administrative Region, China). A/duck/Yokohama/aq-10/2003 (H5N1) [25] was received from Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan). A/chicken/Yamaguchi/7/2004 (H5N1) [48] and A/chicken/Ibaraki/1/2005 (H5N2) were obtained from the National Institute of Animal Health (Tsukuba, Ibaraki, Japan). A/Viet Nam/1194/2004 (H5N1) [Vietnam/1194] [50] was received from Dr. Y. Kawaoka,

University of Tokyo (Tokyo, Japan). A/duck/Hokkaido/Vac-1/2004 (H5N1) [Vac-1/04 (H5N1)] [65] was generated in our laboratory by the standard genetic reassortment procedure [25] as described below.

Generation of genetic reassortant virus

To generate the H5N1 reassortant virus, parental viruses, A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) were mixed and inoculated into the allantoic cavity of ten-day-old chicken embryos. After incubation at 35°C for 48 h of the chicken embryos the allantoic fluids were collected, mixed with chicken antiserum raised against A/duck/Hong Kong/301/1978 (H7N2), and incubated at 37°C for one hour prior to plaque cloning of the viruses in MDCK cells. Cloned viruses were subtyped by HI and NI tests.

Phylogenetic analysis

To evaluate the genetic relationships among H5 influenza virus strains, nucleotide sequences of the HA genes (position 54-1012) were determined and compared with those of H5 viruses from NCBI database (<http://www.ncbi.nlm.nih.gov/>). Viral RNA was extracted from the allantoic fluid of chicken embryos infected with viruses by using a commercial kit (TRIzol LS Reagent, Invitrogen, Carlsbad, CA, U.S.A) and

reverse-transcribed with the Uni12 primer [23] and M-MLV Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) -based amplification of the coding regions of the HA genes was performed with gene-specific primer sets. The following primers were designed on the basis of published nucleotide sequences of influenza virus HA genes; forward primer (BmHA-1) 5'-TATTCGTCTCAGGGAGCAAAAGCAGGGG-3' [23] and reverse primer (H5-1695R) 5'-CGATCCATTGGAGCACATCC-3'. Direct sequencing of the HA gene was performed by using an autosequencer CEQ2000 (Beckman Coulter, Fullerton, CA, U.S.A). The nucleotide sequences were proofread using the GENETYX Version 7.0 (Genetyx, Tokyo, Japan). For phylogenetic analysis, sequence data of the genes together with those from GenBank were analysed by the neighbor-joining method [60]. The 959 bp fragments of the HA of each isolate were aligned using the CLUSTAL X version 1.83 program [71]. The transition/transversion rates were calculated using the PUZZLE 5.2 program [62]. Bootstrapping values were calculated using the modules SEQBOOT (random number seed: 123; 1,000 replicates), DNADIST (distance estimation: maximum likelihood; analysis of 1,000 data sets), NEIGHBOR (Neighbor-joining method; random number seed:99; analysis of 1,000 data sets) and CONSENSE from the PHYLIP package, version 3.67 [12]. The phylogenetic trees were computed with DNADIST and NEIGHBOR modules with the same parameters as above. For

visualization of the trees, TREEVIEW version 1.6.6 was utilized [55].

The nucleotide sequences of H5 isolates obtained in the present study have been registered in GenBank (Accession numbers: AB233320, AB241614, AB241616-AB241626, AB284068-AB284073, AB298276, AB299162, AB299181, AB299377-AB299378, AB299802-AB299832, AB300036-AB300050, AB300223-AB300050, AB300223-AB300235, AB300434-AB300441, AB301913-AB301917, AB302086, AB378682, and AB378690) .

Monoclonal antibodies

Monoclonal antibodies (MAbs) to the HA of A/duck/Pennsylvania/10218/1984 (H5N2) were prepared [34]. Briefly, a BALB/c mouse (CLEA Japan Inc, Tokyo, Japan) was immunized with two intraperitoneal injections of purified influenza virus (100 µg protein each) two weeks apart. Two weeks after the second injection, the same antigen (50 µg) was intravenously administered, and three days later, the spleen cells were fused with myeloma SP2/0-Ag14 cells. Hybridoma cells producing antibodies specific to the HA were selected on the basis of the result of immunoprecipitation and enzyme-linked immunosorbent assay (ELISA) as described [34]. The hybridoma cells were then cloned in soft agar (Bacto agar, Difco, Sparks, MD, U.S.A) and grown as ascites in BALB/c mice. To select the escape mutants, a 1:10 dilution of ascites containing MAbs was

mixed with an equal volume of serial ten-fold dilutions of the parental viruses. After incubation for 60 min at room temperature, the mixture was inoculated into the allantoic cavities of ten-day-old embryonated chicken eggs. Virus that grew in the presence of MAbs was cloned by plaque formation in MDCK cells and its nucleotide sequence was determined and compared with of wild type strain.

Antigenic analyses

Antigenic specificity of H5 influenza viruses was assessed by fluorescent antibody method with MAbs and neutralization test. MDCK cells infected with each of 24 H5 influenza viruses were fixed with 100% acetone at eight hour post-inoculation. The reactivity patterns of the MAbs to H5 strains were investigated by immunofluorescent method with a FITC-conjugated goat IgG to mouse IgG (ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A). For neutralization test, polyclonal chicken antiserum raised against Vac-1/04 (H5N1) including a water-in-oil adjuvant provided by Kyoto Biken Laboratories, Inc., (Uji, Kyoto, Japan) and 100 TCID₅₀ of test viruses were mixed and incubated for one hour at room temperature. This mixture was inoculated onto MDCK cells in 96-well tissue culture plates and incubated for one hour at 35°C. Then the cells were washed with PBS and incubated in MEM (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 5 µg/ml trypsin (SIGMA-ALDRICH, Inc., St. Louis, MO, U.S.A)

without serum for two days at 35°C. The cytopathic effect was observed and neutralization titers were expressed as reciprocals of the highest dilution of serum sample that showed complete neutralization.

Immunization and challenge of mice

To assess the potency of Vac-1/04 (H5N1) as a H5N1 vaccine strain, the test whole virus vaccine was prepared as described previously [70]. Five 4-week-old female C57BL/6 mice (CLEA Japan Inc, Tokyo, Japan) were injected subcutaneously with 100, 20, 4, and 0.8 µg proteins of inactivated Vac-1/04 (H5N1) whole virus vaccine, respectively. Two weeks later, the mice were boosted by subcutaneous injection with the vaccine. Control mice were injected with PBS as well. One week after the second vaccination, five mice in each group were challenged intranasally with 30 µl of 100 MLD₅₀ (50% mouse lethal dose) of Vietnam/1194 under anesthesia. Challenge study was carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a biosafety level (BSL) 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Serum samples were obtained from mice before challenge. Serum samples were pooled by each group and examined by HI test.

Results

Isolation of influenza viruses from fecal samples of water birds

During 1996-2007, 524 influenza virus strains have been isolated from 10,549 fecal samples of migratory ducks and swans that flew from their nesting lakes in Siberia to Mongolia, China, and Japan in autumn each year. Fourteen of them were H5 influenza viruses shown in Table 3.

Generation of H5N1 genetic reassortant virus

To prepare non-pathogenic H5N1 influenza viruses for vaccine production, H5N1 genetic reassortants were generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) isolated from migratory ducks, and origin of the internal protein genes was determined. PB2, PB1, PA, HA, NP, and M gene segments of generated H5N1 virus, Vac-1/04 (H5N1), were derived from A/duck/Mongolia/54/2001 (H5N2) which was one of the non-pathogenic strain isolated from a water bird in this study (Table 3), and NA and NS gene segments from A/duck/Mongolia/47/2001 (H7N1).

Table 3.

H5 avian influenza virus isolates examined in this study

Isolates	Year of isolation	Amino acid sequence at the cleavage site	Pathotype
Ohnuma Pond, Wakkanai, Hokkaido, Japan			
Swan/Hokkaido/4/1996 (H5N3)	October, 1996	RETR/G	non-pathogenic
Swan/Hokkaido/51/1996 (H5N3)	October, 1996	RETR/G	non-pathogenic
Swan/Hokkaido/67/1996 (H5N3)	October, 1996	RETR/G	non-pathogenic
Duck/Hokkaido/69/2000 (H5N3)	October, 2000	RETR/G	non-pathogenic
Duck/Hokkaido/447/2000 (H5N3)	October, 2000	RETR/G	non-pathogenic
Ohno Pond, Sapporo, Hokkaido, Japan			
Duck/Hokkaido/84/2002 (H5N3)	September, 2002	KETR/G	non-pathogenic
Duck/Hokkaido/101/2004 (H5N3)	October, 2004	RETR/G	non-pathogenic
Duck/Hokkaido/193/2004 (H5N3)	October, 2004	RETR/G	non-pathogenic
Duck/Hokkaido/299/2004 (H5N3)	November, 2004	RETR/G	non-pathogenic
Duck/Hokkaido/167/2007 (H5N3)	October, 2007	RETR/G	non-pathogenic
Duck/Hokkaido/201/2007 (H5N3)	November, 2007	RETR/G	non-pathogenic
Ugii nuur, Arkhangai, Mongolia			
Duck/Mongolia/54/2001 (H5N2)	September, 2001	RETR/G	non-pathogenic
Duck/Mongolia/500/2001 (H5N3)	September, 2001	RETR/G	non-pathogenic
Duck/Mongolia/596/2001 (H5N3)	September, 2001	RETR/G	non-pathogenic

Phylogenic analysis of the H5 isolates

The HA genes of the 14 H5 isolates were sequenced and analysed by the neighbor-joining method along with those of other H5 strains containing HPAI viruses presently circulating in Asia. The amino acid sequence at the cleavage site of the HA was deduced from the nucleotide sequence of the corresponding gene of each of the isolates. As shown in Table 3, HAs of all isolates had RETR or KETR sequences at the cleavage sites, which are typically found in the HA of viruses non-pathogenic for chicken. The HA of 13 out of 14 isolates were of the Eurasian type and the HA of A/duck/Hokkaido/84/2002 (H5N3) [Dk/Hok/84/02] was North American type by phylogenetic analysis (Fig. 2). Sequence analysis of Dk/Hok/84/02 revealed that the seven other gene segments were classified into Eurasian lineage (data not shown). The 13 isolates of Eurasian type constituted a different cluster from that of HPAI viruses isolated in Asia.

Antigenic comparison of H5 influenza virus isolates

For the antigenic analysis of H5 influenza viruses, a panel of MAbs with neutralizing activities toward the HA of A/duck/Pennsylvania/10218/1984 (H5N2) was prepared (Table 4). By a sequence analysis of the HA genes of escape mutants selected in the presence of these seven MAbs, the epitopes recognized by these MAbs were

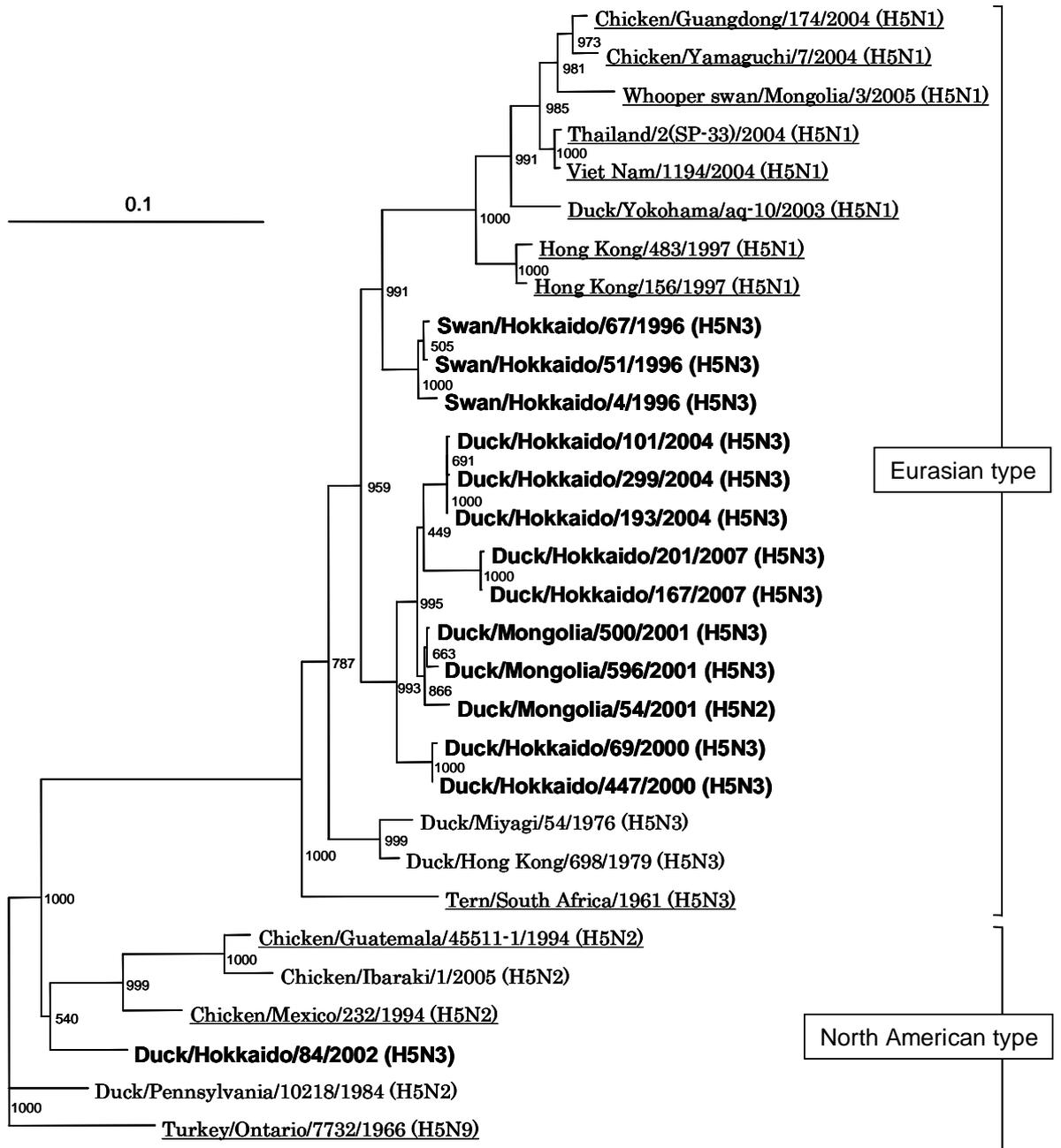


Fig. 2. Phylogenetic tree of the HA genes of H5 influenza viruses. Nucleotides 54-1032 (979 bases) of the H5 HA genes were used for the analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. Viruses isolated in this study are in bold. HPAI viruses are underlined.

Table 4.

Antigenic analyses of H5 influenza viruses

Viruses	Monoclonal antibodies ^{a)}						Polyclonal antibodies ^{c)}	
	I (88 ^{b)})	II (145)	III (157)	IV (168)		V (169)	VI (205)	α -Vac-1/04 (H5N1)
	D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2	
LPAIV								
Duck/Pennsylvania/10218/1984 (H5N2)	+	+	+	+	+	+	+	512
Swan/Hokkaido/4/1996 (H5N3)	+	+	+	+	+	+	+	128
Swan/Hokkaido/51/1996 (H5N3)	+	+	+	+	+	+	+	256
Swan/Hokkaido/67/1996 (H5N3)	+	+	+	+	+	+	+	256
Duck/Hokkaido/69/2000 (H5N3)	+	+	+	+	+	+	+	128
Duck/Hokkaido/447/2000 (H5N3)	+	+	+	+	+	+	+	512
Duck/Mongolia/54/2001 (H5N2)	+	+	+	+	+	+	+	256
Duck/Mongolia/500/2001 (H5N3)	+	+	+	+	+	+	+	32
Duck/Mongolia/596/2001 (H5N3)	+	+	+	+	+	+	+	256
Duck/Hokkaido/84/2002 (H5N3)	+	+	+	+	+	+	+	512
Duck/Hokkaido/101/2004 (H5N3)	+	+	+	+	+	+	+	64
Duck/Hokkaido/193/2004 (H5N3)	+	+	+	+	+	+	+	128
Duck/Hokkaido/299/2004 (H5N3)	+	+	+	+	+	+	+	128
Chicken/Ibaraki/1/2005 (H5N2)	-	-	-	-	-	-	-	256
Duck/Hokkaido/167/2007 (H5N3)	+	+	+	+	+	+	+	1280
Duck/Hokkaido/201/2007 (H5N3)	+	+	+	+	+	+	+	80
Duck/Hokkaido/Vac-1/2004 (H5N1)	+	+	+	+	+	+	+	256
HPAIV								
Tern/South Africa/1961 (H5N3)	+	-	+	-	-	+	+	256
Hong Kong/156/1997 (H5N1)	-	+	+	+	+	+	+	64
Hong Kong/483/1997 (H5N1)	-	+	+	+	+	+	+	64
Duck/Yokohama/aq-10/2003 (H5N1)	-	-	+	+	+	+	+	256
Chicken/Yamaguchi/7/2004 (H5N1)	-	+	+	+	+	-	+	256
Viet Nam/1194/2004 (H5N1)	+	+	+	+	+	-	+	128
Whooper Swan/Mongolia/3/2005 (H5N1)	+	-	+	+	+	-	+	256

^{a)} Fluorescent antibody methods were performed with monoclonal antibodies to the HA of A/duck/Pennsylvania/10218/1984 (H5N2).

^{b)} Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies.

^{c)} Neutralizing antibody titers

mapped to the globular head of the H5 HA molecule (data not shown) and the MAbs were divided into six groups (Groups I–VI) accordingly. Reactivity of 24 H5 influenza virus strains with the panel of MAbs was analysed by the immunofluorescent assay. Although there was some difference in their patterns of reactivity with MAbs, all the isolates and HPAI viruses were neutralized by the polyclonal chicken antiserum raised against Vac-1/04 (H5N1) with a water-in-oil adjuvant (Table 4).

Protective effect of the test vaccine in mice against H5 HPAI virus challenge

The test inactivated vaccine was prepared from Vac-1/04 (H5N1). To assess the potency of the vaccine against H5 HPAI virus infection, mice vaccinated subcutaneously with inactivated Vac-1/04 (H5N1) were challenged intranasally with a lethal dose of Vietnam/1194 virus strain. Survival numbers of the mice after virus challenge are shown in Fig. 3. All the control mice died within nine days after challenge, while all of the mice immunized with 100 µg vaccine survived 14 days without showing any disease signs. Survival rate were correlated to the dose of vaccine. The mice died of virus challenge showed clinical signs including ruffled fur, inactivity, and depression from eight day post inoculation. The mean HI titers against vaccine strain and challenged strain of mice immunized with 100, 20, 4, and 0.8 µg protein before challenge were 1:128, 64, 32, and 8 (Vac-1/04), 1:16, 16, 8, and <8 (Vietnam/1194).

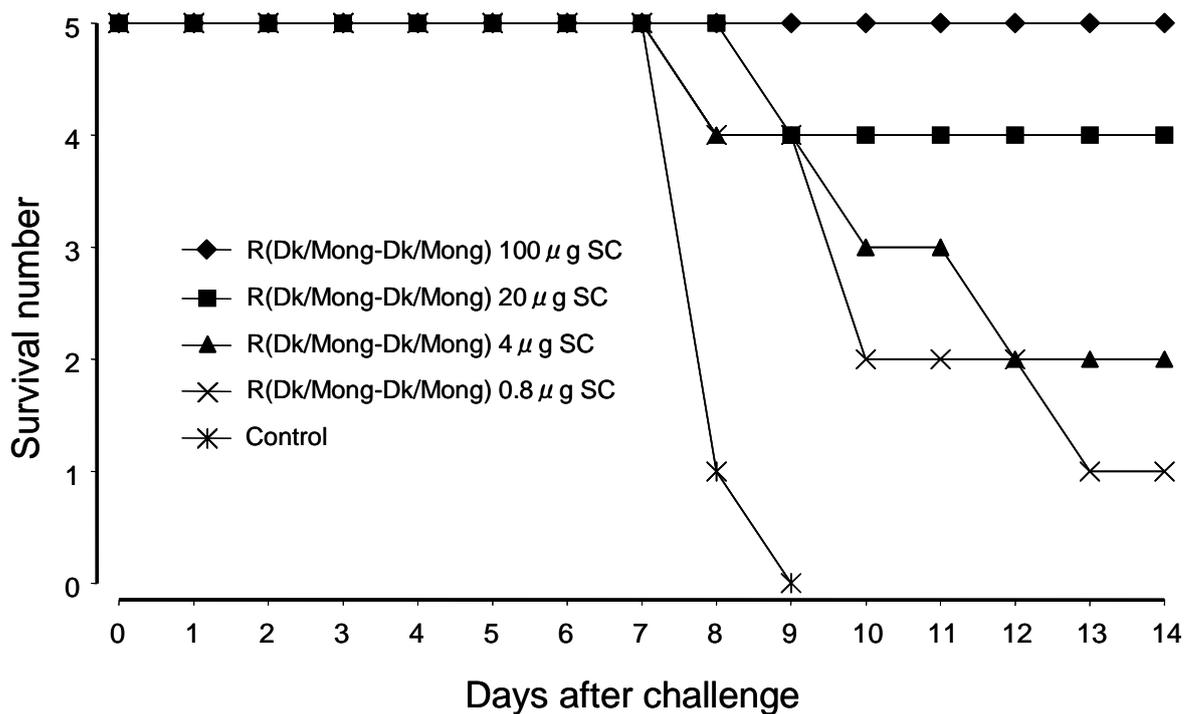


Fig. 3. Survival numbers of mice after challenge with Vietnam/1194. Five 4-week-old female C57BL/6 mice were vaccinated subcutaneously with inactivated Vac-1/04 (H5N1) virus particles. The mice were boosted two weeks later. One week after the second vaccination, five mice in each group were intranasally challenged with 100LD₅₀ of Vietnam/1194. PBS was inoculated subcutaneously into the control mice.

Discussion

The results of the phylogenetic analysis of the H5 HAs of 14 isolates revealed that 13 belong to the Eurasian lineage and the other one, Dk/Hok/84/02, to the North American lineage. Since other gene segments of Dk/Hok/84/02 were classified into Eurasian lineage, Dk/Hok/84/02 should be a reassortant virus whose HA gene was originated from a virus strain which was introduced into Eurasian area by migrating water birds from their nesting lakes in North America.

The pathogenicity of avian influenza virus has been shown to be associated with the presence of multiple basic amino acids at the cleavage site of the HA molecule [33]. As shown in Table 3, all the H5HAs of the isolates were non-pathogenic type, possessing only two basic amino acid residues at their cleavage sites. The homology in amino acid sequence of the HA1 among the 13 isolates of Eurasian lineage was over 95% (data not shown), indicating HA genes have been well conserved in natural hosts. A previous study already indicated that ddY mice immunized with a formalin-inactivated vaccine prepared from A/swan/Hokkaido/67/1996 (H5N3) [Swan/Hok/67/96], one of the isolates belonging to Eurasian lineage, survived a challenge with HPAI viruses, A/Hong Kong/156/1997 (H5N1) and A/Hong Kong/483/1997 (H5N1) [70]. Therefore, the 13 isolates of Eurasian type including

Swan/Hok/67/96 could be useful as potential vaccine strains for HPAI caused by H5 strains.

Antigenic analysis of H5 influenza viruses with the panel of MAbs to the HA molecule of A/duck/Pennsylvania/10218/1984 (H5N2) confirmed that H5 isolates from water birds and the HPAI viruses share epitopes at the globular heads of HAs though there were some differences in reactivity with MAbs (Table 4). In addition, all H5 HPAI viruses were neutralized by the chicken polyclonal antiserum to Vac-1/04 (H5N1). Thus, it was confirmed that there were little difference in antigenicity among the H5 influenza viruses.

HI titers of mice against Vac-1/04 (H5N1) before HPAI virus challenge were positively correlated with the dose of vaccine. Mice vaccinated subcutaneously with 100 µg of inactivated Vac-1/04 (H5N1) were protected from lethal infection with Vietnam/1194 (Fig. 2). The HI titer of the pooled serum of mice against Vac-1/04 (H5N1) was 1:128 before challenge. Thus, it was speculated that 1:128HI antibody titer was prerequisite for complete protection of mice from manifestation of disease signs. All seven H5 HPAI viruses tested in this study were neutralized by the chicken polyclonal antiserum to Vac-1/04 (H5N1), suggesting that the test vaccine could be protective to HPAI virus of the clade 1 viruses such as Vietnam/1194, but also to clades 2 and 3 viruses such as A/chicken/Yamaguchi/7/2004 (H5N1) and A/Hong

Kong/483/1997 (H5N1), respectively [77]. These findings support the notion that Vac-1/04 (H5N1) virus vaccine is potent against infection with H5N1 HPAI virus strains presently circulating in the world. The vaccine prepared from the present Vac-1/04 (H5N1) strain has been produced in collaboration with vaccine producers and its protective effect is under investigation.

None of the 16 HA and 9 NA subtypes can be ruled out as potential candidates causing future pandemic influenza. To provide vaccine strains for the seed of the vaccine, it is important to establish a library of vaccine strain candidates of isolates from water birds [25]. The present results demonstrates that the library of a panel of influenza virus strains isolated from natural hosts in the global surveillance of avian influenza is useful for preparedness for future pandemics.

Brief Summary

In order to prepare H5N1 influenza virus vaccine, the hemagglutinins (HAs) of 14 H5 virus isolates from water birds in Asia were antigenically and genetically analysed. Phylogenetic analysis of the H5 HA genes revealed that 13 isolates belong to Eurasian and the other one to North American lineages. Each of the deduced amino acid sequences of the HAs indicated a non-pathogenic profile. Antigenic analysis using a panel of monoclonal antibodies recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/1984 (H5N2) and chicken antiserum to an H5N1 reassortant strain generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), [65] showed that the HAs of highly pathogenic avian influenza (HPAI) viruses currently circulating in Asia were antigenically closely related with those of the present isolates from water birds. Mice subcutaneously injected with formalin inactivated Vac-1/04 (H5N1) were protected from challenge with 100 mouse lethal dose of A/Viet Nam/1194/2004 (H5N1). The present results support the notion that the H5 isolates and the reassortant H5N1 strain should be useful for vaccine preparation.

Chapter III

H9N2 avian influenza virus acquires high intravenous pathogenicity by the introduction of a pair of di-basic amino acid residues at the hemagglutinin cleavage site and consecutive passages in chickens

Introduction

Influenza A viruses of each of the known subtypes (H1 to H16 and N1 to N9) are circulating in water birds, especially in migratory ducks [13]. Chickens were not infected with viruses directly from water birds [39]. Low pathogenic avian influenza (LPAI) viruses capable of being transmitted to chickens have emerged through domestic waterbirds such as ducks and geese and terrestrial birds such as quails and turkeys. LPAI viruses can become highly pathogenic to chickens after more than six months of multiple passages in chicken population [24, 76]. The hemagglutinins (HAs) of highly pathogenic avian influenza (HPAI) viruses differ from those of LPAI viruses in that they have at least a pair of di-basic amino acid residues at their cleavage site [63]. This structure permits ubiquitous proteases such as furin and PC6 which recognize multiple

basic amino acids to cleave the HA, leading to systemic infection. By contrast, HAs of LPAI viruses are cleaved only by trypsin-like proteases which are expressed in the cells on the respiratory or intestinal tract, so the viruses only cause localized infections, resulting in mild or subclinical diseases. It is presently believed that only strains with H5 or H7 subtype HAs become HPAI viruses during extensive infections in chicken populations [28]. However, the reason why HPAI viruses are restricted to the H5 and H7 subtypes is not known although a model of the cleavage of the H5 HA and the notion that H5 HA is cleaved by furin have been proposed [9, 17].

Since the 1990s, strains of H9N2 avian influenza virus have caused outbreaks in poultry, resulting in serious economic losses in Asia and the Middle East [15, 16, 18, 19, 41-43, 45, 80]. The causal strains, however, are avirulent and none of them have multiple basic amino acids at the cleavage site of the HA [18, 45]. No specific-pathogen-free (SPF) chickens experimentally infected with H9N2 isolates from diseased chickens showed any clinical symptoms [49]. Co-infections of H9N2 viruses with bacteria such as *Staphylococcus aureus* and *Haemophilus paragallinarum* or with attenuated coronavirus vaccine exacerbated the disease [4, 19, 20, 39]. In addition, since H9N2 viruses have been isolated not only from domestic birds but also from pigs and humans, the H9 virus has the potential to cause a next pandemic in humans [16, 46, 56, 57, 59].

It is important for controlling H9 virus infections in poultry to assess whether the H9N2 virus is capable of becoming pathogenic like H5 and H7 viruses. Such information would also help to reveal how avian influenza viruses become highly pathogenic to chickens. In the present study, a pair of di-basic amino acid residues was introduced into the cleavage site of the H9 and H5 HAs of non-pathogenic viruses. These mutant H9 and H5 viruses were then serially passaged in the air sac of chicks and their pathogenicity was assessed by inoculating four-week-old chickens intravenously or intranasally.

Materials and Methods

Viruses

A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2) isolated from chicken meat imported from China upon quarantine was kindly provided by Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan) [47]. A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1) was generated by the standard genetic reassortment procedure from non-pathogenic viruses, A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) [26, 65, 66]. Viruses were propagated in ten-day-old embryonated chicken eggs for 48 hours at 35°C.

Reverse genetics

Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with the Y55 and Vac1 strains using a commercial kit (TRIzol LS Reagent, Sigma-Aldrich, St. Louis, MO, U.S.A) and reverse-transcribed with the Uni12 primer [10] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A). PCR-based amplification of the full genomes of the eight gene segments was performed with universal primer sets [23]. The PCR products were cloned into the vector pCR2.1-TOPO (Invitrogen) or pGEM-T Easy Vector (Promega, Mannheim, Germany).

After confirmatory sequencing, T-vector clones were digested with *BsmBI* and inserted into the vector pHW2000 [22]. Madin-Darby Canine Kidney (MDCK) cells and 293T cells were maintained in Minimum Essential Medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% calf serum and D-MEM (Invitrogen) containing 10% FBS, respectively. Before transfection, confluent 293T and MDCK cells in 75 cm² flasks were trypsinized, and 10% of each cell line was mixed in 12 ml of Opti-MEM I (Invitrogen); 2 ml of the suspension was seeded into each well of six-well tissue culture plates (Nunc Inc., Naperville, IL). The cocultured 293T and MDCK cells were used for the transfection. TransIT-293 (Panvera, Madison, WI) was used to transfect cells according to the manufacturer's directions. Briefly, two microliters of TransIT-293 per microgram of DNA was mixed, incubated at room temperature for 45 minutes, and added to the cells. The transfection mixture was replaced with Opti-MEM I after six hours of incubation at 37°C. Thirty hours later, Opti-MEM I containing one microgram per microliter of trypsin was added. At 48 to 72 hours post-transfection, the culture supernatant was collected and propagated in ten-day-old embryonated chicken eggs.

Site-directed-mutagenesis

To generate H9 and H5 mutant viruses with basic amino acid residue substitutions (sub) or insertions (ins) at the HA cleavage site, mutations were introduced into the HA

genes of the Y55 and Vac1 strains using a QuikChange II site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The mutant viruses, rgY55sub (H9N2), rgVac1sub (H5N1), and rgVac1ins (H5N1), were rescued by reverse genetics as described above, and the HA genes were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations. The amino acid sequence at the HA cleavage site of each mutant virus is shown in Table 5.

Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for one hour. Unbound viruses were removed by washing the cells with MEM. Cells were then overlaid with MEM containing 0.7% Bacto-agar (Difco, Sparks, MD) in the presence or absence of trypsin (5 µg/ml). After 48 hours of incubation at 35°C, cells were stained with 0.005% neutral red.

Consecutive passage in the air sac of chicks

The caudal thoracic air sacs of three 3-day-old chicks were inoculated with 200 µl of each of the mutant Y55 and Vac1 viruses. The chicks were sacrificed, and their lungs and brains were collected at three days post-inoculation. Serial passages in the air sacs

Table 5. Amino acid sequences at the HA cleavage sites of the viruses, replication in MDCK cells and acquisition of virulence during consecutive passages in the air sac of chicks

Virus	HA cleavage site sequence ^{a)}										Plaque formation (log PFU/ml)		Virulence (no. dead/no. sick/total no.) against		
	HA1										With trypsin	Without trypsin	3-day-old chick (air sac inoculation)	4-week-old chicken (intravenous inoculation)	
	-9	-8	-7	-6	-5	-4	-3	-2	-1	HA2 +1					
rgY55 (H9N2)	P	A	-	-	-	R	S	S	R	/	G	8.1	- ^{b)}	NT ^{c)}	0/0/8
rgY55sub (H9N2)	P	A	-	-	-	R	K	K	R	/	G	8.0	-	0/0/3	0/0/8
rgY55sub-P5 (H9N2)	P	A	-	-	-	R	K	K	R	/	G	7.6	-	0/0/4	0/0/8
rgY55sub-P6 (H9N2)	P	A	-	-	-	R	K	K	R	/	G	7.2	6.7	3/3/3	0/1/8
rgY55sub-P7 (H9N2)	P	A	-	-	-	R	K	K	R	/	G	7.8	7.5	3/3/3	0/5/8
rgY55sub-P8 (H9N2)	P	A	-	-	-	R	K	K	R	/	G	7.6	7.6	3/3/3	2/7/8 (4.0) ^{d)}
rgY55sub-P9 (H9N2)	P	A	-	-	-	R	K	K	R	/	G	7.2	6.9	3/3/3	1/8/8 (10.0)
rgY55sub-P10 (H9N2)	P	A	-	-	-	R	K	K	R	/	G	6.5	6.1	3/3/3	6/8/8 (1.8)
rgVac1 (H5N1)	P	Q	-	-	-	R	E	T	R	/	G	7.6	-	NT	0/0/8
rgVac1sub (H5N1)	P	R	-	-	-	R	K	K	R	/	G	7.6	7.8	0/0/3	0/1/8
rgVac1sub-P1 (H5N1)	P	R	-	-	R	R	K	K	R	/	G	6.8	6.8	3/3/3	3/5/8 (5.3)
rgVac1sub-P2 (H5N1)	P	R	-	-	R	R	K	K	R	/	G	6.4	6.5	6/6/6	8/8/8 (2.6)
rgVac1ins (H5N1)	P	Q	R	E	R	R	K	K	R	/	G	7.3	-	0/0/3	0/0/8
rgVac1ins-P1 (H5N1)	P	Q	R	E	R	R	K	K	R	/	G	7.8	7.1	3/3/3	6/7/8 (6.8)
rgVac1ins-P2 (H5N1)	P	Q	R	E	R	R	K	K	R	/	G	7.1	7.1	4/4/4	8/8/8 (4.4)

^{a)} The dashes are included to adjust the alignment. The slashes indicate the cleavage site.

^{b)} No plaques.

^{c)} Not tested.

^{d)} Mean time to death (days) is shown in parentheses.

of three to six 3-day-old chicks were performed with 200 µl of a pooled 10% tissue suspension of infected organs. Brain samples were used as the inoculum when both samples (lungs and brains) tested positive for the virus. Isolates were identified by their parental strain's name, mutation (substitution or insertion), and number of passages. For example, the designation rgY55sub-P10 (H9N2) indicates that the amino acids at the HA cleavage site of the Y55 virus were substituted with basic amino acids as shown in Table 5, then passaged ten times in the air sac. Passaged viruses were propagated in the allantoic cavities of ten-day-old embryonated chicken eggs for 48 hours at 35°C. The allantoic fluid was harvested and stored at -80°C.

Experimental infection of chickens with mutant virus strains

Four-week-old Boris Brown chickens were used to test the pathogenicity of the passaged viruses. Eight chickens were intravenously inoculated with 200 µl of each virus (1:10 diluted allantoic fluid), and examined for clinical signs at intervals of 24 hours over a period of ten days. Similarly, three chickens were infected intranasally with 100 µl of allantoic fluid containing each virus at a 50% egg infectious dose (EID₅₀) of 10^{6.5} and observed for 14 days. Specific antibodies against homologous viruses after 14 days of infection were detected in serum using the hemagglutination inhibition (HI) test and/or enzyme-linked immunosorbent assay (ELISA) as described previously [34]. To

study viral replication, each virus was inoculated into three chickens at an EID₅₀ of 10^{6.5}. The birds were euthenized three days post-challenge, and tissue and blood were collected aseptically. To make a 10% suspension with MEM, the tissue samples were homogenized using a Muti-Beads Shocker (Yasui Kikai, Osaka, Japan). These suspensions were serially diluted ten-fold with PBS and inoculated into ten-day-old embryonated eggs and incubated at 35°C for 48 hours. Viral titers were calculated by the method of Reed and Muench [58] and expressed as EID₅₀ per gram and milliliter of tissue and blood, respectively.

All experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The experiments were performed according to the guidelines of the institutional animal care and use committee of the graduate school.

Results

Generation and characteristics of mutant viruses

To investigate whether non-pathogenic H9 and H5 influenza viruses, A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2) and A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1), respectively, acquire pathogenicity by the introduction of a pair of di-basic amino acid residues at their HA cleavage site, rgY55sub (H9N2), rgVac1sub (H5N1) and rgVac1ins (H5N1) were generated by site-directed-mutagenesis and reverse genetics. Amino acid sequences at the HA cleavage sites of these mutant strains are shown in Table 5. A pair of di-basic amino acid residues is a *sine qua non* for the virus to become highly pathogenic to chickens, RKKR motif was, thus, introduced into the H9 HA cleavage site. The virus with the insertion at the H9 HA cleavage site was not rescued from plasmid-transfected cells (data not shown).

The RRKKR motif, which has been observed at the HA cleavage site of H5 HPAI viruses, was introduced into the H5 HA of Vac1. Both rgVac1sub (H5N1) and rgVac1ins (H5N1) showed growth similar to their parental viruses in MDCK cells. Only rgVac1sub (H5N1) replicated in the absence of trypsin. Chickens infected intravenously with these mutant viruses did not show any signs of disease except one chicken with rgVac1sub (H5N1), which was slightly depressed one day post-infection.

Consecutive passages of the viruses in the air sacs of chicks

The H9 and H5 mutant viruses were serially passaged in the air sac of chicks to assess their ability to acquire pathogenicity. Amino acids at the HA cleavage site, the formation of plaques in MDCK cells, and pathogenicity in three-day-old chicks and four-week-old chickens are shown in Table 5. RgY55sub (H9N2) replicated in MDCK cells in the absence of trypsin and killed all of the chicks after six consecutive passages. Two of the eight four-week-old chickens inoculated intravenously with rgY55sub-P8 (H9N2) died within five days. Finally, over 75% of the chickens infected intravenously with rgY55sub-P10 (H9N2) died, a rate of pathogenicity similar to that of highly pathogenic avian influenza viruses defined by the OIE [53].

RgVac1sub (H5N1) obtained an additional arginine at the HA cleavage site after one passage in the air sac of chicks. The passaged virus, rgVac1sub-P1 (H5N1), was pathogenic to both chicks and four-week-old chickens, and mortality increased after one more passage. RgVac1ins (H5N1) replicated in MDCK cells in the absence of trypsin and killed all of the chicks after one passage. RgVac1ins-P1 (H5N1) caused 75% mortality among four-week-old chickens, indicating that it acquired marked pathogenicity after only one passage. The lethal effect of rgVac1ins-P1 (H5N1) on chickens increased with one additional passage in the air sac of chicks, as in the case of rgVac1sub (H5N1).

Amino acid mutations during consecutive passages in the air sac of chicks

Whole genomes of the viruses passaged in the air sacs of chicks were determined and compared with the sequences of their parental viruses (Tables 6 and 7). Leu234 in the HA of rgY55sub (H9N2) was substituted with Gln during the initial passage. No other amino acid mutation was observed up to the fifth passage. Four amino acid substitutions were found in the HA, NA and M2 at the sixth passage. Finally, eight amino acid differences between rgY55sub (H9N2) and rgY55sub-P10 (H9N2) were found. On the passaging of rgVac1sub (H5N1) and rgVac1ins (H5N1), six and one amino acid substitution were observed, respectively. It is worth noting that one arginine was inserted at the HA cleavage site of rgVac1sub (H5N1) after one passage in chickens.

Pathogenicity of the viruses on intranasal infection

To examine whether the pathogenicity of each virus via the natural route of infection correlated with that by intravenous infection or not, three 4-week-old chickens were challenged intranasally with the viruses at an EID₅₀ of 10^{6.5} and observed for clinical signs until day 14 post-infection (Table 8). All chickens inoculated with rgY55sub-P10 (H9N2) or its parental viruses survived without showing any clinical symptoms, and serum antibody responses were detected in the HI test.

Table 6. Amino acid mutation during consecutive passages of rgY55sub (H9N2) in the air sac of chicks

	PB2	HA			NP	NA	M2	
	271 ^{a)}	29	234	357	391	54	195	51
P0	Thr	Asn	Leu	Ala	Asn	Trp	Thr	Ile
P1-P5	b)	.	Gln
P6	.	His	Gln	Asp	.	.	Ala	Thr
P7	.	His	Gln	Asp	Asn/Asp	.	Ala	Thr
P8	Thr/Ala ^{c)}	His	Gln	Asp	Asn/Asp	.	Ala	Thr
P9	Thr/Ala	His	Gln	Asp	Asn/Asp	Gly	Ala	Thr
P10	Ala	His	Gln	Asp	Asp	Gly	Ala	Thr

^{a)} Methionine encoded by the AUG start codon is defined as position 1.

^{b)} Periods indicate same amino acids as the parental virus.

^{c)} Amino acid quasispecies are observed.

Table 7. Amino acid mutation during consecutive passages of rgVac1 mutants in the air sac of chicks

Passage Number	rgVac1 sub (H5N1)						rgVac1 ins (H5N1)	
	PA		HA		M1	M2	HA	
	35 ^{a)}	65	308	338	101	45	157	465
P0	Leu	Ser	His	none	Arg	Arg	Ser	Asp
P1	Phe	.	Gln	Arg ^{b)}	.	His	Pro	Glu
P2	Phe	Tyr	Gln	Arg	Lys	His	Pro	.

^{a)} Methionine encoded by the AUG start codon is defined as position 1.

^{b)} Arginine is inserted at the HA cleavage site as shown in Table 1.

^{c)} Periods indicate same amino acids as the parental virus.

Table 8. Antibody response of chickens intranasally inoculated with the virus strains

Virus	Days p.i.	Disease signs	Antibody response	
			HI	ELISA
rgY55 (H9N2)	14d sacrificed	-	512	NT ^{a)}
	14d sacrificed	-	256	NT
	14d sacrificed	-	2,048	NT
rgY55sub (H9N2)	14d sacrificed	-	512	NT
	14d sacrificed	-	128	NT
	14d sacrificed	-	256	NT
rgY55sub-P10 (H9N2)	14d sacrificed	-	2,048	NT
	14d sacrificed	-	512	NT
	14d sacrificed	-	512	NT
rgVac1 (H5N1)	14d sacrificed	-	<2	<40
	14d sacrificed	-	<2	<40
	14d sacrificed	-	<2	<40
rgVac1sub (H5N1)	14d sacrificed	-	<2	<40
	14d sacrificed	-	<2	<40
	14d sacrificed	-	32	40,960
rgVac1sub-P2 (H5N1)	4d dead	+	NT	NT
	11d dead	+	NT	NT
	14d sacrificed	-	<2	<40
rgVac1ins (H5N1)	14d sacrificed	-	<2	<40
	14d sacrificed	-	<2	<40
	14d sacrificed	-	128	655,360
rgVac1ins-P2 (H5N1)	8d dead	+	NT	NT
	11d dead	+	NT	NT
	14d sacrificed	-	16	163,840

^{a)} Not tested.

One chicken inoculated with rgVac1sub (H5N1) or rgVac1ins (H5N1) showed seroconversion after 14 days although no chickens were infected with rgVac1 (H5N1). RgVac1sub-P2 (H5N1) and rgVac1ins-P2 (H5N1) were highly pathogenic as in the intravenous experiment, killing two of three chickens by day 11 post-inoculation.

Replication of the viruses intranasally inoculated in chickens

To investigate the correlation between acquired virulence and tissue tropism, viral titers in tissue and blood samples from four-week-old chickens intranasally inoculated with each virus at three days post infection were determined (Table 9). RgY55 (H9N2) and rgVac1 (H5N1) were scarcely recovered, and the mutant strains before passage showed broader tissue tropism than the parental viruses. None of the chickens inoculated with rgY55sub-P10 (H9N2) showed any signs of disease, and viruses were recovered from all samples except the brain and blood. One chicken inoculated with rgVac1sub-P2 (H5N1) showed clinical signs such as depression, and viruses were recovered from virtually all of the organs and blood samples. Two of three chickens inoculated with rgVac1ins-P2 (H5N1) showed symptoms, and one died two days post inoculation. The viruses were recovered from almost all samples of the two chickens showing signs of disease.

Table 9. Recovery of viruses from chickens inoculated intranasally

Virus	Days p.i.	Disease signs	Virus recovery (log EID ₅₀ /g)							
			Brain	Trachea	Lung	Liver	Kidney	Colon	Blood ^{b)}	
rgY55 (H9N2)	3d sacrificed	-	^{a)}	-	-	-	-	-	-	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
	3d sacrificed	-	-	1.7 \geq	-	-	-	-	-	-
rgY55sub (H9N2)	3d sacrificed	-	-	5.7	6.7	2.7	-	-	-	-
	3d sacrificed	-	-	5.5	-	-	-	-	-	-
	3d sacrificed	-	-	6.5	2.7	-	2.5	-	-	-
rgY55sub-P10 (H9N2)	3d sacrificed	-	-	3.3	6.0	2.5	4.5	4.5	-	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
	3d sacrificed	-	-	-	3.7	-	4.3	3.3	-	-
rgVac1 (H5N1)	3d sacrificed	-	-	-	-	-	-	-	-	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
rgVac1sub (H5N1)	3d sacrificed	-	-	-	-	-	-	-	-	-
	3d sacrificed	-	2.7	-	2.5	-	-	2.0 \geq	-	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
rgVac1sub-P2 (H5N1)	3d sacrificed	-	-	-	-	-	-	-	-	-
	3d sacrificed	+	5.0	3.8	5.0	5.2	7.5	5.2	4.3	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
rgVac1ins (H5N1)	3d sacrificed	-	2.6 \geq	3.5	3.3	-	2.7	3.0	1.6 \geq	-
	3d sacrificed	-	-	-	3.7	3.5	3.0	2.5	2.8	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
rgVac1ins-P2 (H5N1)	3d sacrificed	+	3.8	3.7	3.0	2.0 \geq	4.7	2.0 \geq	-	-
	3d sacrificed	-	-	-	-	-	-	-	-	-
	2d dead	+	3.5	3.4	4.7	3.7	4.8	4.7	NT ^{c)}	-

^{a)} 1.5 \geq (0.5 \geq for blood samples)

^{b)} log EID₅₀/ml

^{c)} Not tested.

Discussion

In the present study, it was demonstrated that the H9 influenza virus acquired intravenous pathogenicity after a pair of di-basic amino acid residues was introduced into the HA cleavage site and passaging in chicks. RgY55sub-P10 (H9N2) killed 75% of chickens infected intravenously, a pathogenicity comparable to that of HPAI viruses (Table 5). However, chickens inoculated intranasally with rgY55sub-P10 (H9N2) did not show any clinical signs of disease (Table 8). These results are consistent with a previous study in chickens that found some H10 influenza viruses did not show intranasal pathogenicity although their intravenous pathogenicity index was over 1.2, a criterion for classification as an HPAI virus according to the European Union [79]. Amino acid mutations during consecutive passages in the air sac of chicks (Table 6) are considered to be responsible for the acquisition of intravenous pathogenicity, and their effects on the functions of viral proteins need to be clarified further. Here the author focus on two substitutions at positions 29 and 234 of the H9 HA molecule. It has been reported that residue 226, based on the H3 HA numbering (234 in the present study), in the H9 HA is related to cell tropism and receptor specificity [73]. Strain Y55 (H9N2) originally had a leucine at this position, and the change to glutamine with serial passages in the air sac of chicks indicates that the mutation was involved in viral

adaptation for chicken. One of the asparagine-linked (N-linked) glycosylation sites on the HA of rgY55 (H9N2) lost a carbohydrate attachment with the mutation at residue 29. The site was sterically in the vicinity of the HA cleavage site, suggesting that the deletion of the carbohydrate chain affected the susceptibility of the HA to the host protease [76]. This notion is also supported by the present finding that the rgY55sub viruses (H9N2) after six passages in the air sac of chicks replicated in MDCK cells in the absence of exogenous trypsin (Table 5). Ohuchi *et al.* [52] reported that the insertion of additional basic amino acids into the H3 HA cleavage site resulted in intracellular proteolytic cleavage. Therefore, cleavage-based activation by an ubiquitous protease is not restricted to the H5 and H7 HAs. The pathogenicity of rgY55sub-P10 (H9N2) was not increased by an additional five passages in the air sac of chick (data not shown).

RgVac1sub (H5N1) and rgVac1ins (H5N1) acquired marked intravenous and intranasal pathogenicity after a few passages (Tables 5 and 9). Ito *et al.* [27] reported that an avirulent H5 virus isolated from wild swans became highly pathogenic in chickens after 24 consecutive passages in the air sac, followed by five passages in chicken brain. The differences in time required for the viruses to become highly pathogenic between these studies depended on the amino acid motif at the HA cleavage site prior to passaging. One arginine was inserted into the HA cleavage site of rgVac1sub (H5N1) after only one passage in chickens (Table 5), suggesting that an

additional mutation was efficiently occurred under the conditions that in the presence of serial basic amino acid residues at the cleavage site. One third of the chickens inoculated intranasally with rgVac1sub-P2 (H5N1) or rgVac1ins-P2 (H5N1) survived 14 days (Table 8). Moreover, one of the birds inoculated with rgVac1sub-P2 (H5N1) was not infected. Because the Vac1 (H5N1) strain was generated from avirulent viruses of wild-duck origin [26, 65, 66], the H5 viruses passaged in the air sac did not completely adapt to chickens, and showed extensive intranasal pathogenicity only when a viral infection was accomplished. On the passage of rgVac1ins (H5N1), only one amino acid at position 157 of H5 HA was replaced (Table 7). The position was located in the vicinity of the receptor binding site on H5 HA molecule, suggesting that receptor specificity and/or antigenicity of the virus were altered by the mutation. Additional study using H5 viruses with intranasal pathogenicity and low infectivity should reveal the molecular determinants for the host range of influenza viruses.

The intranasal pathogenicity of the H9 and H5 mutants differed although these viruses similarly replicated in MDCK cells in the absence of trypsin, and killed chickens when inoculated intravenously (Tables 5 and 8). The viruses were recovered from the brain and blood of some chickens infected with rgVac1 mutants (H5N1), and morbidity was closely associated with viral titers in the brain (Table 9). On the other hand, no viruses were recovered from the brain of chickens infected with rgY55 mutants

(H9N2), explaining why rgY55sub-P10 (H9N2) did not show intranasal pathogenicity. All the viruses passaged in the air sac of chicks killed chicken embryos by 48 hours post allantoic inoculation (data not shown). RgVac1sub-P2 (H5N1) and rgVac1ins-P2 (H5N1) were more pathogenic to chicken embryos than rgY55sub-P10 (H9N2); the allantoic fluid obtained from the embryonated eggs inoculated with the passaged H5 viruses was turbid. It has been reported that infection of a highly pathogenic H7 virus were strictly confined to endothelial cells in chicken embryos or chickens [11, 72]. Therefore, it is suggested that endotheliotropism differed between the H9 and H5 passaged viruses and affected their intranasal pathogenicity. Taken together, it is assumed that rgVac1sub-P2 (H5N1) and rgVac1ins-P2 (H5N1) showed marked intranasal pathogenicity with high levels of viremia caused by replication in vascular endothelial cells, leading to invasion of the brain. In the intravenous experiment, rgY55sub-P10 (H9N2) easily reached systemic organs including the brain hematogenously, replicated through the cleavage of HA by an ubiquitous protease, and then exerted its pathogenicity. Further study including a pathological analysis is currently underway to test this hypothesis.

H9N2 viruses which have the motif PARSKR or PARSRR at their HA cleavage site have been isolated from turkeys, ostriches, and chickens in Israel and quails in China [80] although PARSSR motif has been found in most H9N2 isolates, indicating

that substitutions with basic amino acids occur in nature. If serine at the c-terminus of the HA1 of the H9 virus was substituted with lysine, the amino acid motif would be consistent with that of rgY55sub (H9N2) which acquired intravenous pathogenicity on consecutive passages in the air sac of chicks. LPAI caused by H9N2 strains in poultry is now creating serious economic losses [15, 16, 18, 19, 41-43, 45, 80], and its eradication is still difficult because of its low pathogenicity, frequently causing inapparent infections. The present study demonstrated that H9N2 viruses circulating in chicken flocks can acquire further intravenous pathogenicity. It is predicted that co-infections of rgY55sub-P10 (H9N2) with bacteria exacerbate not only intravenous pathogenicity but intranasal pathogenicity as shown in a previous study [39]. Therefore, continuous monitoring in poultry is important to prevent the emergence of more pathogenic H9 viruses.

Brief Summary

Strains of virus with only H5 or H7 hemagglutinins (HAs) have been isolated from sick or dead birds as highly pathogenic avian influenza (HPAI) viruses. The reason why HPAI viruses are restricted to the H5 and H7 subtypes is not known. In the present study, it was investigated whether a non-pathogenic virus with H9 HA, A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2), becomes pathogenic to chickens when a pair of di-basic amino acid residues are introduced at the cleavage site of its HA molecule. The mutant in which the basic amino acid residues substituted previously existing residues, rgY55sub (H9N2), replicated in Madin-Darby Canine Kidney cells in the absence of trypsin after six consecutive passages in the air sacs of chicks, and acquired intravenous pathogenicity after four additional passages. More than 75% of chickens inoculated intravenously with the passaged virus, rgY55sub-P10 (H9N2), died, a pathogenicity comparable to that of HPAI viruses defined by the OIE (World Organization for Animal Health). On the other hand, all chickens inoculated via the intranasal route survived without showing any clinical signs. An avirulent H5 strain, A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1), readily acquired intranasal pathogenicity after a pair of di-basic amino acid residues was artificially introduced into the cleavage site of the HA, followed by two passages by air sac inoculation. These results

demonstrate that H9 viruses have the potential to acquire intravenous pathogenicity like H5 viruses. The morbidity of rgY55sub-P10 (H9N2) via the respiratory infection was low, suggesting that the ability of the virus to replicate in the absence of trypsin-like proteases was not necessarily associated with the systemic infection in chickens.

Conclusion

Since 1997, outbreaks of highly pathogenic avian influenza caused by H5N1 viruses have occurred. The present study revealed that the antigenicities of H5HA of the isolates from water birds and the HPAI viruses were similar. Sakabe *et al.* [61] suggested that antigenicities of the H7HA of avian influenza viruses were also conserved. Based on these results, A/duck/Hokkaido/Vac-1/2004 (H5N1) [Vac-1/04 (H5N1)], A/duck/Hokkaido/Vac-3/2007 (H5N1), and A/duck/Hokkaido/Vac-2/2004 (H7N7) [Vac-2/04 (H7N7)] were generated from non-pathogenic avian influenza viruses isolated from migratory ducks as the vaccine strain candidates. Mice vaccinated subcutaneously with 100 µg of inactivated Vac-1/04 (H5N1) were protected from lethal infection with Vietnam/1194/2004 (H5N1). It was also reported that Vac-1/04 (H5N1) and Vac-2/04 (H7N7) were potent in giving protective immunity against HPAI virus challenge to chickens and cynomolgus macaques [26, 30, 31, 61]. These findings support the notion that non-pathogenic viruses isolated from water birds are useful for production of vaccine against infection with HPAI virus presently circulating in the world.

However, misuse of vaccine may lead to antigenic drift or shift of the HPAI viruses. Actually, HPAI viruses have been perpetuating and killing not only poultry but also

humans in some countries doing vaccination procedure without testing and culling, fundamental countermeasures against HPAI. Therefore, examining the cross reactivities between epidemic strains and vaccine strain candidates in the present study should be continued and it is recommended that all of the countries where HPAI occurs should make a effort for the eradication of HPAI.

In addition to HPAI caused by H5 or H7 viruses, avian influenza caused by low pathogenic H9N2 viruses have occurred in poultry, resulting in serious economic losses in Asia and the Middle East [15, 16, 18, 19, 41-43, 45, 80]. Its eradication is still difficult because of its low pathogenicity, frequently causing inapparent infection. It is important for the control of avian influenza to assess whether the H9N2 virus is capable of becoming pathogenic like H5 and H7 viruses. In the present study, H9 virus acquired high intravenous pathogenicity by introducing a pair of di-basic amino acid residues at the HA cleavage site as observed in H5 or H7 HPAI viruses and passaging in the air sac of chicks. Artificial mutation in H9HA cleavage site and amino acid mutations during consecutive passages in the air sac of chicks must have been involved in conferring high pathogenicity on Y55. On the other hand, chickens inoculated intranasally with rgY55sub-P10 (H9N2) did not show any clinical signs of disease while H5 viruses were readily acquired high intranasal pathogenicity. It is, thus, predicted that H5 viruses passaged in chicks exerted their intranasal pathogenicity with high levels of viremia

caused by replication in vascular endothelial cells, leading to invasion of the brain of chickens. Further study including a pathological analysis is needed to assess this hypothesis. The present study demonstrated that H9N2 viruses can acquire further intravenous pathogenicity. Co-infection of rg Y55sub-P10 (H9N2) with bacteria may exacerbate not only intravenous pathogenicity but intranasal pathogenicity as reported by Kishida *et al* [39].

Taken together, the results shown in this thesis suggest that continuous surveillance in migratory birds and poultry is important to predict and prevent the emergence of pathogenic viruses, and to stock the viruses as vaccine strains.

Acknowledgements

The author would like to acknowledge Prof. H. Kida, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University (Sapporo, Hokkaido, Japan), for his guidance and encouragement during the course of this work.

Great appreciation is extended to Prof. T. Umemura, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, and Prof. A. Takada, Department of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University.

The author is grateful to Dr. Y. Sakoda and Dr. M. Okamatsu, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, and Dr. H. Ozaki, Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University (Tottori, Japan), for their valuable advice, support and encouragement throughout the present study.

The author thanks to Dr. R. G. Webster, St. Jude Children's Research Hospital (Memphis, Tennessee, U.S.A), Dr. K. F. Shortridge, University of Hong Kong (Hong Kong, Special Administrative Region, China), Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan), Dr. Y. Kawaoka, University of Tokyo (Tokyo, Japan), and the National Institute of Animal Health (Tsukuba, Ibaraki, Japan) for providing the

valuable viruses used in this study.

The author heartily thanks to Dr. T. Sasaki, Avian Biologics Department, Kyoto Biken Laboratories, Inc. (Uji, Kyoto, Japan), Dr. R. Sakamoto, Division 2, Second Research Department, The Chemo-Sero-Therapeutic Research Institute (Kikuchi, Kumamoto, Japan), Dr. K. Saijo, Research Center for Biologicals, The Kitasato Institute (Kitamoto, Saitama, Japan), and Dr. J. Hagiwara, Research Department, Nippon Institute for Biological Science (Ome, Tokyo, Japan) for producing the inactivated avian influenza vaccine with high efficacy.

The author earnestly appreciates all colleagues of Laboratory of Microbiology, faculty of Veterinary Medicine, Hokkaido University for their invaluable help and friendships.

Finally, the author express his mourning for all of the animals sacrificed their precious lives for these studies.

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和文要旨

1997年以來、H5 または H7 ウイルスの感染に因る高病原性鳥インフルエンザ(HPAI)の発生が続いている。筆者はインフルエンザ A ウイルスの自然宿主である野生水禽に維持されている H5 または H7 ウイルスと HPAI ウイルス間でヘマグルチニン(HA)の抗原性が類似していることを示した。本結果に基づき、野生水禽から分離された非病原性鳥インフルエンザウイルスを元に、A/duck/Hokkaido/Vac-1/2004 (H5N1) [Vac-1/04 (H5N1)]、A/duck/Hokkaido/Vac-3/2007 (H5N1)、および A/duck/Hokkaido/Vac-2/2004 (H7N7) [Vac-2/04 (H7N7)]をワクチン候補株として作出した。ホルマリン不活化した Vac-1/04 (H5N1)を 100 μ g 皮下接種したマウスは、HPAI ウイルス Vietnam/1194/2004 (H5N1)の致死量の攻撃に耐過した。Vac-1/04 (H5N1)および Vac-2/04 (H7N7)によって試製したワクチンを接種したニワトリおよびサルは、HPAI ウイルスの攻撃に耐過した。以上の結果は野生水禽から分離した非病原性ウイルスが HPAI ウイルスに因る感染症に対するワクチン株として有用であることを示している。

一方で、ワクチンの濫用は HPAI ウイルスの見えない流行拡大を助長する恐れがある。ワクチン接種に依存し HPAI 対策の基本である摘発淘汰が疎かとなっている地域では、抗原変異ウイルスの出現を招くと共に、人の感染例が増加し

ている。本研究で確立したワクチン株と流行株間の交差反応性を調べると共に、世界各国が摘発淘汰を基本とした対策を行い、鳥インフルエンザを封じ込める必要がある。

H5 または H7 ウイルスに因る HPAI に加え、近年低病原性 H9N2 ウイルスによる鳥インフルエンザの発生がアジア・中近東で続いており、家禽に甚大な被害を及ぼしている。従って H9 ウイルスが H5 および H7 ウイルスのように家禽に対する高い病原性を獲得し得るかどうかを確認しておくことは防疫上重要である。本研究において、H9 ウイルスの HA 開裂部位に HPAI ウイルスに見られる塩基性アミノ酸の連続配列を人工的に導入し、さらにヒヨコの気嚢内で 10 代継代したウイルス、rgY55sub-P10 (H9N2) はニワトリに対して静脈内接種病原性を示した。HA 開裂部位に導入した塩基性アミノ酸、および継代によって起こったアミノ酸の置換がニワトリに対する高い病原性に関与するものと考えられる。rgY55sub-P10 (H9N2) は MDCK 細胞における増殖に外来性のトリプシン(ニワトリの呼吸器・腸管に局在)を必要としなかったが、本株を鼻腔内に接種したニワトリは H5 ウイルスを接種した場合と異なり全く症状を示さなかった。本結果はニワトリ体内のユビキタスなプロテアーゼによる HA の開裂活性化はウイルスの全身感染に必要なが、十分条件ではないことを示している。H5 ウイルスは H9 ウイルスに比べニワトリの血管内皮細胞で効率的に増殖してウイルス血症を引き起こし、さらに脳に侵入して高い病原性を発揮するものとする。

本研究では、H9N2 ウイルスが静脈内接種病原性を獲得し得ることを示した。

H9 ウイルスはニワトリに細菌と共感染すると、鼻腔内接種病原性が増強することが報告されている。HA 開裂部位に塩基性アミノ酸の置換変異を有するウイルスが実際に野外のニワトリから分離されており、このようなウイルスが鶏群内で感染を繰り返すことによって、静脈内接種病原性に加え鼻腔内接種病原性を獲得する恐れがあるので、監視を続ける必要がある。

本研究で得られた成績は野生水禽および家禽におけるインフルエンザサーベイランスが、① 環境中のウイルスの抗原性を把握する、② HPAI に対するワクチン株を得る、③ 新たな HPAI ウイルスの出現予測、のために重要であることを示している。