Antigenic and genetic analyses of avian influenza viruses isolated in Japan

（日本で分離された鳥インフルエンザウイルスの
抗原性と遺伝子の解析）

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Contents

Abbreviations

AIV	: avian influenza virus
AMOE	: anhydromannitol-octadecenoate-ether
CID_{50}	: 50% chicken infective dose
CK	: chicken kidney
CLD_{50}	: 50% chicken lethal dose
CNS	: central nervous system
DMEM	: Dulbecco's Modified Eagle's Medium
EID_{50}	: 50% egg infective dose
ELISA	: Enzyme-linked immunosorbent assay
HA	: hemagglutinin
HI	: hemagglutination inhibition
HPAIV	: highly pathogenic avian influenza virus
LPAIV	: low pathogenicity avian influenza virus
MAb	: monoclonal antibody
MDCK	: Madin-Darby Canine Kidney
NA	: neuraminidase
NI	: neuraminidase inhibition
OIE	: world organisation of animal health
p.c.	: post challenge
p.i.	: post inoculation
PCR	: polymerase chain reaction
RT	: reverse transcription
SPF	: specific pathogen free
$TCID_{50}$	: 50% tissue culture infective dose

Preface

Influenza A viruses have been classified into subtypes according to their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes and nine NA subtypes are now recognized (1). Wild water birds, predominantly ducks, geese, and shorebirds are the natural reservoir of all subtypes of influenza A viruses (2, 3). Avian influenza viruses (AIVs) sporadically infect to poultry, resulting in clinical manifestations ranging from asymptomatic infection, decline in egg production, and mild respiratory disease to death.

Influenza A viruses infecting chickens are categorized into two pathotypes based on their virulence to chickens; namely, low pathogenicity avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV). Among the 16 HA subtypes of AIV, HPAIV has been associated with only a small proportion of the H5 or H7 subtypes (4). The HA molecules of HPAIVs differ from those of LPAIVs in that they possess multiple basic amino acids at the carboxyl terminus of HA1. This series of basic amino acids at the cleavage site is a motif that is recognized by ubiquitous intracellular proteases, allowing systemic infection with high mortality in poultry (5). In addition, some LPAIVs of the H5 and H7 subtypes can mutate to HPAIVs, and several mechanisms involved in the emergence of HPAIV from LPAIV precursor have been documented. It was shown that a LPAIV evolved into an HPAIV that caused a severe outbreak in Pennsylvania in 1983, by removing a carbohydrate side chain in the vicinity of the cleavage site (6). The causative agents of Mexican outbreaks from 1994 to 1995 were derived from a LPAIV that had accumulated a number of basic residues at the cleavage site (7), as were the viruses that caused outbreaks in British Columbia (8) and Chile (9).

Since 2003, the H5N1 HPAIVs have spread in 63 countries, in Asia, Europe and Africa. Japan and some countries where the introduction of H5N1 virus infection occurred in the poultry flocks were successful in rapid eradication of the infection by aggressive stamping-out policy (10, 11). However, the virus is still persisting in Asian and Northern African Countries.

Since the 1990s, outbreaks of H9N2 influenza virus infections in poultry have caused great economic losses in many countries in Asia and the Central East (12-16). The causal H9N2 virus strains, however, did not reproduce severe disease signs in specific pathogen free (SPF) chickens. Co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus* and *Haemophilus paragallinarum* or attenuated coronavirus vaccine strains with H9N2 virus exacerbates the disease (15, 17-19).

The present thesis is composed of three parts. In part 1 and 2, characterization of H5N2 LPAIVs isolated from chickens in 2005 and H5N1 HPAIVs isolated from whooper swans in 2008 in Japan are reported, respectively. In part 3, antigenic analysis of the HA of H9N2 influenza viruses is described.

Part 1

Characterization of H5N2 influenza A viruses isolated from chickens in Japan in 2005

Introduction

At the end of May 2005, an H5N2 influenza virus was isolated for the first time from chickens in Japan. HPAIV of the same subtype have caused three large outbreaks in poultry: in Pennsylvania in 1983 (6, 20), in Mexico from 1994 to 1995 (7, 21), and in Italy from 1997 to 1998 (22, 23). The H5N2 viruses became endemic in Central America in its low-pathogenicity form in the decade after 1994, despite years of eradication programs in combination with vaccination (24, 25).

On 23 May 2005, a hemagglutinating agent was isolated at a commercial laboratory using embryonated chicken eggs after inoculation with pooled tracheal homogenates from laying chickens that had shown a decline in egg production. The farm was located in Ibaraki Prefecture, 30 km east of Tokyo. Initially, infectious bronchitis virus was suspected; however, the virus could not be identified by the laboratory. It was subsequently turned over to the National Institute of Animal Health, Japan on 25 June. On the following day, the virus was identified as H5N2 influenza A virus by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays. The virus was designated A/chicken/Ibaraki/1/2005 (Ck/Ibaraki/1/05) (H5N2). Active surveillance was undertaken with virus isolation and serum antibody detection in all farms located within a 5 km radius around the affected farm as well as those with epidemiological relations. Movement control was implemented on those farms for 21 days.

By January 2006, 40 laying farms in Ibaraki and one in Saitama Prefecture were found to be affected by H5N2 viruses. Sixteen H5N2 viruses were isolated from chickens in nine of these farms. By April 2006, 5.78 million chickens had been killed as a countermeasure against the outbreaks. The Ibaraki prefectural governor declared the end of the H5N2 outbreak on 23 June 2006, 1 year after the first detection. Here, we describe the H5N2 LPAI outbreaks in Ibaraki and Saitama Prefectures in Japan

during the period May 2005 to June 2006. Comprehensive studies, including genetic and antigenic characterization as well as experimental infection with the H5N2 virus isolates, were carried out to understand the nature of the causative agent of these outbreaks.

Materials and Methods

Viruses. Ck/Ibaraki/1/05 (H5N2) was isolated from embryonated eggs inoculated with tracheal homogenates from chickens at a commercial laboratory, and was subsequently submitted to the National Institute of Animal Health, Japan for further analyses. The rest of the isolates from the outbreak were isolated from tracheal swab pools of chickens at the livestock hygiene service center of Ibaraki Prefecture. A/chicken/Yokohama/aq-55/2001 (Ck/Yokohama/aq-55/01) (H9N2) was used as a virus that efficiently infects chickens (19). The viruses used in the present study were propagated at 37°C for 2 days in the allantoic cavity of 10-day-old embryonated eggs. Harvested viruses were stored at −80°C until use.

Cells. Chicken kidney (CK) cells were prepared from the kidneys of 4 to 8-week-old chickens and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum and antibiotics.

Sequence and phylogenetic analysis. To determine the genetic relationships between these isolates and others, all eight gene segments of the isolates were sequenced. Viral RNA was extracted from virus-containing allantoic fluid using a commercial kit (Trizol LS Reagent; Invitrogen, MD, USA). After reverse transcription with Superscript II (Invitrogen, MD, USA) using random 9-mers, cDNAs were amplified by polymerase chain reaction (PCR) with ExTaq (Takara, Shiga, Japan). PCR amplification of the coding regions of the viral gene segments was performed with gene-specific primer sets. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and used as a template for sequencing with the Big Dye Terminator sequencing kit, version 3.1 (Applied Biosystems, CA, USA). Reactions were analyzed

on an ABI Prism 3100 genetic analyzer (Applied Biosystems, CA, USA). The nucleotide sequences obtained were assembled using Sequencher, version 4.2 (Hitachi Software Engineering, Tokyo, Japan). The sequence data were analyzed using version 6.1 of the sequence analysis software package GENETYX-WIN (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis of sequence data was performed by ClustalW running under Bioedit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the neighbor-joining method (26) with 1000 bootstraps by Molecular Evolutionary Genetics Analysis (MEGA, version 2.1) software (27). MEGA was also used to calculate the exact distance from node to node within branches of the phylogenetic trees. The nucleotide sequences obtained in this study are available from GenBank/EMBL/DDBJ under accession numbers AB261850 to AB261857, AB275420 to AB275434 and AB275649 to AB275663.

Serological tests of the isolates. HI test was performed as described by Sever et al (28). The antigenic relationships among H5 subtype influenza viruses were determined in HI tests using polyclonal chicken antiserum and a panel of monoclonal antibodies (MAbs) to the HA of A/Viet Nam/1203/04 (H5N1) and A/chicken/Pennsylvania/1370/83 (H5N2). These MAbs were kindly provided by Dr R. G. Webster, St Jude Children's Research Hospital, Memphis, TN, USA.

Experimental infection of chickens. The pathogenicity of Ck/Ibaraki/1/05 (H5N2) to chickens was determined according to the manual of diagnostic tests established by world organisation for animal health (OIE) (29). Briefly, 4-week-old SPF chickens were used in the present study. Eight of the chickens were inoculated intravenously with 0.2 mL of a 1/10 dilution of infective allantoic fluid. The chickens were observed for clinical signs or death at 24-hour intervals for 10 days.

For the evaluation of virus replication, antibody response, and transmissibility of Ck/Ibaraki/1/05 (H5N2), an experimental infection study was carried out using 6-week-old SPF chickens. Groups of chickens were inoculated intranasally with virus at a dose of $10^{5.7}$of 50% egg infective dose (EID_{50}). The chickens were sacrificed for virus isolation from the tissues at 3, 5, and 7 days post infection (p.i.). Brain, trachea,

lung, spleen, liver, pancreas, kidney, rectum, and muscle were obtained aseptically and homogenized using MULTI-BEADS SHOCKER (YASUI KIKAI, Osaka, Japan) to yield suspensions of 10% in DMEM containing antibiotics. After centrifugation, the supernatants were stored at -80°C until use. Virus titers were calculated by the method of Reed and Muench (30) and expressed as EID_{50} per gram of tissue. Tracheal and cloacal swabs were obtained 3, 5, 7, and 10 days p.i. Swab samples were diluted in 1 ml of the medium. Swab samples were titrated in a similar manner and expressed as EID_{50} per ml. Antibody response against the virus was confirmed by HI antibody responses at 14 days p.i.

In the transmission experiments, two groups of uninfected birds were placed in direct and indirect contact with inoculated chickens at 1 day p.i. Eight uninfected chickens were transferred into a cage with four infected chickens at 1 day p.i. to allow direct contact. The other group of four uninfected chickens was placed in an area separated from another four infected chickens by 10 cm and two nets. In each experiment, the chickens were inoculated as described above. Virus was isolated only from the drinking water and feces in the cages, to avoid transmission of virus to uninfected chickens by the handling of birds during sample collection. Infection with the virus was thus determined by antibody responses at 14 and 21 days p.i.

To determine the mean chicken infective dose (CID_{50}), 4-week-old SPF chickens were infected intranasally with 100 μl of serial tenfold dilutions of virus and then observed for 14 days. Infection with the virus was also determined by antibody responses at 14 days p.i.

Histopathological examination. Brain, larynx, trachea, lung, liver spleen, pancreas, kidney, large intestine, and muscle of infected chickens were collected and fixed in 10% neutral buffered formalin. Samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. In addition, immunohistochemistry was performed to examine the distribution of AIV antigens by the immuno-enzyme polymer method using a histofine simple stain MAX PO (M) kit (Nichirei Inc., Tokyo, Japan). A mouse MAb specific for the type A influenza matrix protein (diluted 1:500; clone GA2B; Oxford Biotechnology Ltd, UK) was used as the primary antibody.

Temperature sensitivity of virus replication. CK cells were infected with Ck/Ibaraki/ 1/05 (H5N2) or Ck/Yokohama/aq-55/01 (H9N2) using tenfold dilutions of virus, and propagated for 72 h at different temperatures (33, 37, 40, and 42°C). Virus titers were expressed as the mean log10 reduction of the mean tissue culture infective dose ($TCID_{50}$) compared with the $TCID_{50}$ at 37°C.

Results

Genetic analysis of isolated viruses. To elucidate the genetic relationships with the known viruses, all eight gene segments of Ck/Ibaraki/1/05 (H5N2) were sequenced and searched for similarities to the public database. It was found that the Japanese isolate was closely related (93.7-98.0% similarity) to the H5N2 LPAIVs prevalent in Central America such as A/chicken/Guatemala/45511-3/00 (Ck/Guatemala/00) (H5N2).

Phylogenetic analysis of the HA gene, along with those of A/duck/Tsukuba/ 394/2005 (H5N3) and A/duck/Tsukuba/419/2005 (H5N3), was also carried out (Fig. 1). These H5N3 duck strains were isolated from fecal samples during surveillance of migratory ducks at Kasumigaura Lake in Ibaraki Prefecture in winter 2004-2005. The HA gene of the Ck/Ibaraki/1/05 virus clustered with genotype B of the Mexican H5N2 lineage (Lee et al., 2004). In contrast, the duck viruses isolated earlier in the same year from the natural reservoir belonged to the Eurasian lineage.

The nucleotide and amino acid similarities of the HA genes (complete sequence) among the 16 chicken isolates were 97.4-99.9% and 95.8-100%, respectively. The deduced amino acid sequence at the cleavage site of the isolates was RETR, which is characteristic of avirulent H5 viruses.

The number of mutations from a putative ancestor of all the viruses isolated during the outbreaks and in the days after first sample was collected were plotted on a distribution chart with regard to the HA and NA genes (Fig. 2). The slope of the approximated line for the HA gene was eight times larger than that for the NA gene ($p<0.01$). The point of intersection of each line with the x-axis represents a putative date on which an ancestor may have been introduced. From analysis of the HA gene,

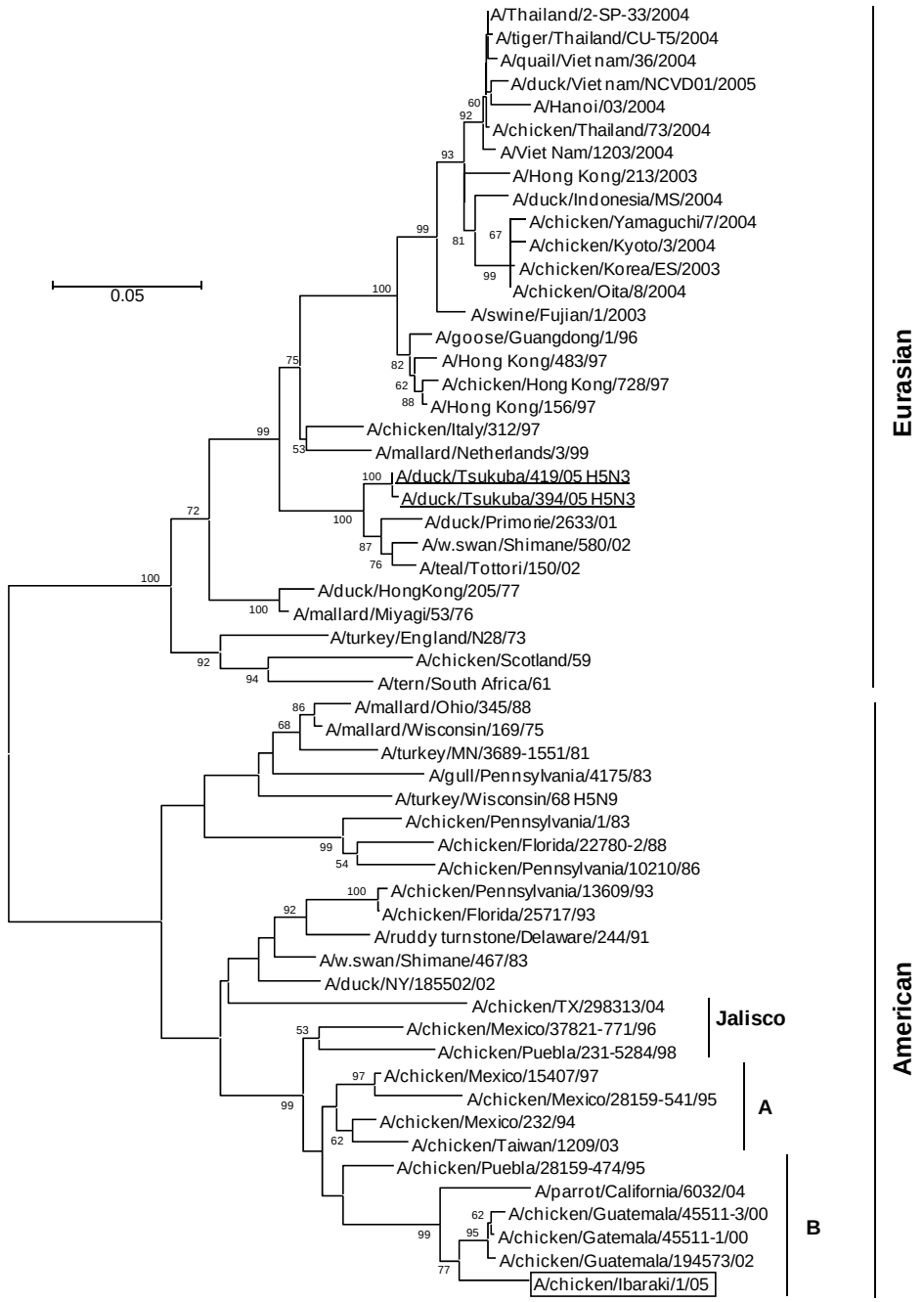

Fig. 1. Phylogenetic trees of the H5 HA gene of influenza A viruses.
A/chicken/Ibaraki/1/05 (H5N2) is shown in box. H5N3 viruses isolated in 2005 in Japan are underlined. Nucleotides 165-1046 (880 bases) of H5 HA were subjected to phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value. Classification of sub-lineages in American lineage (Jalisco, A, and B) was followed by Lee et al. (24).

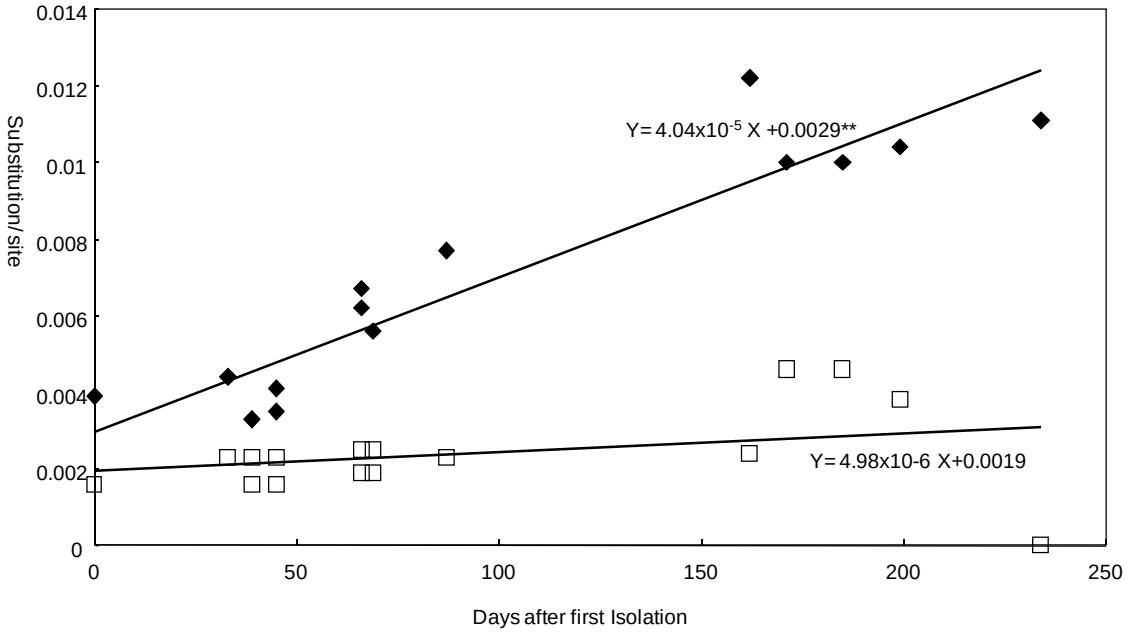

Fig. 2 Distribution chart showing mutation rates and days after first isolation of Ibaraki isolates.

Substitutions of the HA encoding region (♦) and NA encoding region (□) of each isolate were plotted with the days after first isolation. **: $p<0.01$.

the date of origin was estimated as 86.3 to 15.6 days before the first isolation (95% CI). Similar analysis of the NA gene did not provide a statistically significant estimation. These inconsistent results with the two surface glycoproteins meant that we could not estimate the date of origin with statistical significance.

To examine the molecular characteristics of the virus in detail, the amino acid sequences of the viral proteins were analyzed. A potential glycosylation site at position 84 (Asn) in the HA1 protein was lost (Ser) in Ck/Ibaraki/5/05 (H5N2). Amino acid changes at position 140 in Ck/Ibaraki/14/05 (H5N2), Ck/Ibaraki/15/05 (H5N2), and Ck/Ibaraki/17/06 (H5N2), and at 185 in Ck/Ibaraki/13/05 (H5N2), were previously found in H5 escape mutants (31). Mutations were also found in the vicinity of the antigenic site previously reported, at positions 151, 154, and 155 in Ck/Ibaraki/14/05 (H5N2) and Ck/Ibaraki/15/05 (H5N2), and at position 181 in Ck/Ibaraki/3/05 (H5N2), Ck/Ibaraki/13/05 (H5N2), and Ck/Ibaraki/17/06 (H5N2). These data suggest that antigenic variation might have occurred among the Ibaraki isolates. The residues forming the pocket of the HA receptor-binding site (32) were conserved among the isolates.

All of the Ibaraki isolates had a 20-residue deletion in the stalk region of the NA protein that was observed frequently among viruses isolated from chickens in North and Central America. The N2 neuraminidase of the isolates did not show any mutations at Glu119Val, Arg292Lys, or Asn294Ser, which are known to confer resistance to neuraminidase inhibitors (33-36). However, in the M2 protein of the isolates, there were amino acid substitutions at Ser31Asn, which confers resistance to amantadine (37, 38). Many of the Mexican linage viruses isolated in recent years, including Ck/Guatemala/00 (H5N2), had the same substitutions (data not shown).

Antigenic characterization. The antigenicity of HA was characterized to determine whether antigenic variation, suggested from the genetic analysis of the HA protein, had occurred among the viruses isolated from May 2005 to January 2006. Nine representative viruses from each farm were analyzed (Table 1). Fourfold or more decreased reactivity was found with these representatives against polyclonal sera raised against A/whistling swan/Shimane/580/02 (H5N3) and A/chicken/Yamaguchi/7/04 (H5N1), eightfold or more decreased reactivity against A/tern/South Africa/61 (H5N3),

Table 1. Antigenic characterization of the HA of Ck/Ibaraki/05 (H5N2) influenza viruses [a]

	Polyclonal antiserum (hyper-imuune)				Post-infection serum	Monoclonal antibodies [b]								
Virus	S580	Yamag	TnSA	TyOnt	Ck/Ibaraki/1	VN04-2	VN04-3	VN04-10	VN04-12	VN04-15	CP24	CP25	CP46	CP58
Swan/Shimane/580/02	5120	1280	2560	40	20	12800	6400	3200	800	<	1600	400	6400	400
Ck/Suphanburi/1/04	1280	320	1280	80	<20	12800	12800	3200	400	<	200	200	<	400
Ck/Yamaguchi/7/04	2560	2560	1280	320	<20	800	12800	25600	6400	25600	<	<	<	<
Tn/South Africa/61	2560	1280	5120	40	20	6400	25600	<	800	<	<	<	12800	<
Ty/Ontario/7732/66	1280	1280	1280	2560	<20	400	400	<	<	<	<	<	<	<
Ck/Pen/1370/83	2560	640	640	40	80	12800	6400	<	800	<	6400	25600	<	3200
Ck/Pen/21525/83	2560	640	1280	320	40	12800	12800	400	800	<	12800	25600	12800	25600
Dk/Tsukuba/419/05	5120	1280	1280	20	20	12800	6400	1600	1600	<	6400	400	3200	<
Ck/Ibaraki/1/05	1280	640	320	80	320	< [c]	<	<	<	<	<	25600	<	200
Ck/Ibaraki/2/05	1280	640	320	80	640	<	<	<	<	400	<	25600	<	<
Ck/Ibaraki/3/05	640	320	160	40	320	<	<	<	<	<	<	200	<	<
Ck/Ibaraki/5/05	640	320	160	80	320	<	<	<	<	200	<	25600	<	<
Ck/Ibaraki/9/05	1280	640	320	80	640	<	<	<	<	<	<	25600	<	<
Ck/Ibaraki/10/05	1280	640	320	80	640	<	<	<	<	<	<	51200	<	<
Ck/Ibaraki/12/05	1280	320	320	80	320	<	<	<	<	<	<	25600	<	<
Ck/Ibaraki/13/05	640	320	160	80	160	<	<	<	<	<	<	200	<	<
Ck/Ibaraki/14/05	1280	640	640	160	640	<	<	<	<	<	<	800	<	<

Homologous titer of the antiserum is underlined.

[a] HI antibody titers were shown. [b] Monoclonal antibodies were used at starting dilution 1:200.

[c] <: HI antibody titer of the monoclonal antibody was less than 200

Abbreviations: S580, Swan/Shimane/580/02; Yamag, Ck/Yamaguchi/7/04; TnSA, Tern/South Africa/61; TnOnt, Turkey/Ontario/7732/66.

and sixteen-fold or more against A/turkey/Ontario/7732/66 (H5N9). With these antisera along with post-infection serum against Ck/Ibaraki/1/05 (H5N2), no antigenic differences were observed among the Ibaraki isolates despite the results of the genetic analysis. A panel of MAbs against the HAs of A/chicken/Pennsylvania/1370/83 (H5N2) and A/Vet Nam/1203/04 (H5N1) were used for further characterization. Only CP25 MAb appeared to react with all of the Ibaraki isolates, though it reacted relatively poorly with Ck/Ibaraki/3/05 (H5N2), Ck/Ibaraki/13/05 (H5N2), and Ck/Ibaraki/14/05 (H5N2). These results indicate no major antigenic variation among the Ibaraki isolates examined, though minor differences recognized only by CP25 may have existed.

Pathogenicity of Ck/Ibaraki/1/05 (H5N2) in chickens. To determine the pathogenicity of the index isolate, Ck/Ibaraki/1/05 (H5N2), in chickens, it was inoculated intravenously into eight SPF chickens following the guidelines of the OIE (29). These chickens showed neither clinical symptoms nor mortality up to 10 days after inoculation, indicating that the isolate was avirulent in chickens. This result corresponded well with the amino acid sequence of the cleavage site of the HA protein (RETR), as well as the field observations of no clinical signs and no mortality on the farms.

Replication and transmission of Ck/Ibaraki/1/05 (H5N2) in chickens. Replication ability and transmissibility among chickens are major factors that influence the magnitude of outbreaks. Ck/Ibaraki/1/05 (H5N2) was inoculated intranasally into 10 SPF chickens. On day 3 after inoculation, only tracheal swabs (20%, 2 of 10) from inoculated chickens yielded the virus (Table 2). On day 5, all of the tracheal swabs and three of 10 cloacal swabs yielded the virus. On day 7, the virus was isolated only from one of 10 cloacal swabs. The virus was cleared by 10 days after infection.

Dissemination of virus in the brain, trachea, lung, spleen, pancreas, kidney, rectum, and muscle of inoculated chickens was examined on days 3, 5, and 7 after intranasal inoculation (Table 3). Virus was recovered from the trachea of one chicken at 3 and 5 days post-inoculation (p.i.). Virus was recovered from the lungs of two of three chickens at 5 and 7 days p.i., respectively. It was intriguing that the virus isolation rates

Table 2. Replication of A/chicken/Ibaraki/1/05 (H5N2) in chickens.

	Virus isolation [a]			
Swabs	Day 3	Day 5	Day 7	Day 10
Trachea	2/10 (3.5,2.7)	10/10 (3.7 ± 0.67)	0/10	0/10
Cloaca	0/10	3/10 (2.8 ± 0.76)	1/10 (1.0)	0/10

[a] Number of chickens with viruses/number of tested (Virus titer: $\log_{10}$ EID_{50}/ml ± S.D.)

Table 3. Replication of A/chicken/Ibaraki/1/05 (H5N2) in chicken organs

	Virus isolation from chickens (n=3)		
Organ	Day 3	Day 5	Day 7
Trachea	1/3 (6.5) [a]	0/3	0/3
Lung	0/3	1/3 (4.0)	2/3 (3.7, 4.3)
Pancreas	0/3	0/3	1/3 (6.5)
Kidney	0/3	1/3 (7.5)	1/3 (6.0)
Colon	1/3 (4.7)	2/3 (2.0, 5.5)	1/3 (4.5)

[a] Number of chickens with viruses/ number of tested (Virus titer: $\log_{10}$ EID_{50}/g).

from trachea and lung were relatively low considering that virus was recovered from all tracheal swabs at 5 day p.i. in the previous experiment shown in Table 3. Specimens from the rectum yielded viruses at 3, 5, and 7 days p.i. Virus was isolated from the pancreas and kidneys at 7 days p.i. and 5 and 7 days p.i., respectively. No virus was detected in brain, spleen, or muscle at any given time.

Ck/Ibaraki/1/05 (H5N2) did not produce any obvious gross lesions in the chickens. Limited histological lesions were observed predominantly within the respiratory tract. In the larynges, sloughed epithelial cells, loss of cilia, vacuolar degeneration, and necrosis were observed (Fig 3A). These lesions were observed at 3 days (100%) and 5 days (67%) p.i. There was a strong parity between viral antigens and histological lesions (Fig. 3B). Histological lesions and viral antigens were also observed in the pancreas, kidney, and large intestine, but in a limited number of chickens.

To determine whether the H5N2 virus can be transmitted efficiently to other chickens, two groups of four chickens were inoculated by the intranasal route (Table 4). One day after inoculation, one group was placed in direct contact with eight non-treated chickens and the other in indirect contact with four non-treated chickens as described in the materials and methods. Infection with the virus was determined by antibody responses at 14 days after inoculation. The drinking water and feces were also subjected to virus isolation.

At 5 and 7 days p.i., virus was isolated from drinking water in both conditions, and virus was also recovered from the feces in the direct-contact group at 5 day p.i. Viral transmissions from inoculated to non-treated chickens in both the direct- and the indirect-contact groups were confirmed by antibody responses at day 14 p.i. These results clearly showed that the virus can be easily transmitted even without direct contact.

The CID_{50} of the virus was calculated as $10^{2.5}$ EID_{50} by the result that the HI antibodies were detected from all chickens inoculated with 10^3 EID_{50} of the virus but not from chickens with 10^2 EID_{50} of the virus. These data demonstrate that only a small number of viruses were required to infect the chickens.

Temperature sensitivity of Ck/Ibaraki/1/05 (H5N2). Virus isolation and immunohistochemistry

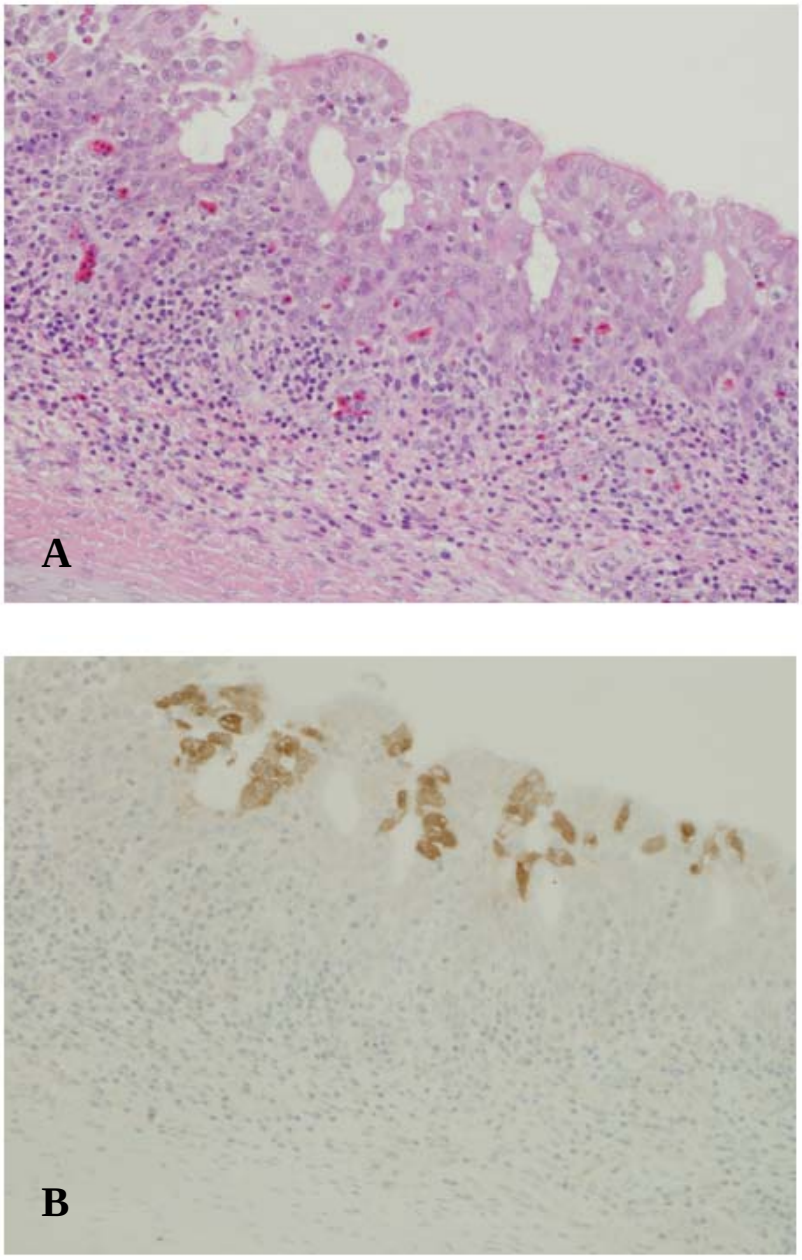

Fig.3 Larynges of 6-week-old chicken at 3 days after intranasal inoculation with Ck/Ibaraki/1/05. (A) Hematoxylin and eosin stain. (B) Immunohistochemistry using anti-M monoclonal antibody.

Table 4. Virus transmission study with A/chicken/Ibaraki/1/05 (H5N2)

	Virus isolation from water and feces [a]				Antibody titer [b]
	Day3	Day5	Day 7	Day10	Day14
Inoculated	− [c] / −	3.0/2.0	2.5/−	− / −	4/4 (8.3 ± 1.0)
Direct contact					8/8 (8.1 ± 0.6)
Inoculated	NT [d]	NT	NT	NT	4/4 (8.3 ± 1.0)
Indirect contact	− / −	2.0/ −	2.0/ −	− / −	4/4 (6.8 ± 0.5)

Virus isolation was performed from water reservoir and feces in same cage (direct contact) or in contact group cage (indirect contact).

[a] Virus titer ($\log_{10}$ EID_{50}/ml) in water / in feces.

[b] Number of positive samples/ number of tested (HI titer: $\log_2$ HI titer ± S.D.)

[c] −: less than 2.0

[d] NT: not tested.

of the tissues of infected chickens showed that Ck/Ibaraki/1/05 (H5N2) replicates more efficiently in the upper respiratory tract than in the lower tract. Since an influence of temperature on in vivo replication of the virus was suspected, growth of Ck/Ibaraki/1/05 (H5N2) at different temperatures was analyzed using primary CK cells in vitro. For comparison, Ck/Yokohama/aq-55/01 (H9N2), which replicates well in chicken trachea and lung (19), was also analyzed (Fig. 4). No significant reduction of virus growth was observed when both viruses were cultured at 40°C. The log10 reduction at 33°C was 0.37±0.17 for Ck/Ibaraki/1/05 (H5N2) and 0.56±0 for Ck/Yokohama/aq-55/01 (H9N2). That at 42°C was 1.37±0.17 for Ck/Ibaraki/1/05 (H5N2) and 1.31±0.25 for Ck/Yokohama/aq-55/01 (H9N2), indicating no difference in temperature sensitivity among those two viruses. Thus, the higher temperature in the lower respiratory tract may not be a factor restricting replication of the Ibaraki strain in the lung.

Discussion

In this report, it is clearly shown that the H5N2 viruses isolated in the Ibaraki Prefecture in Japan were genetically and antigenically related to each other, suggesting that they arose from a common ancestor. Phylogenetic analysis showed that these viruses were closely related to viruses isolated in Central America, particularly those belonging to the B sublineage of the Mexican lineage (24). The H5N2 viruses isolated in Japan had features similar to those of the Mexican viruses; that is, a deletion in the NA stalk (39), and an M2 protein resistant to amantadine. During winter 2004-2005, before the H5N2 outbreaks in Ibaraki and Saitama, we isolated viruses from fecal samples from wild aquatic birds in Ibaraki Prefecture. Two strains of the H5 subtype, designated A/duck/Tsukuba/394/2005 (H5N3) and A/duck/Tsukuba/419/2005 (H5N3), were isolated. However, genetic analysis showed that these viruses were unrelated to the H5N2 viruses isolated in Ibaraki Prefecture. It was difficult to infer a putative direct ancestor of the Ibaraki isolates from the sequence analysis coupled with the phylogenetic analysis. A more extensive sequence database would help to elucidate the direct ancestor of the causative agent of an outbreak, but this is not always feasible since the direct ancestor

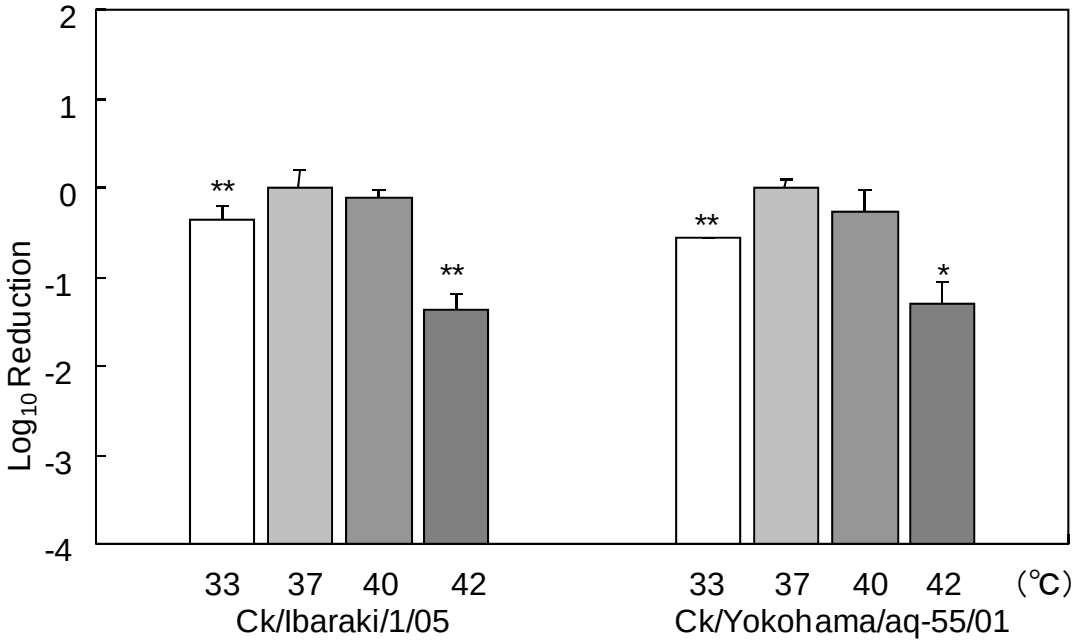

Fig. 4 Temperature sensitivity of Ck/Ibaraki/1/05 and Ck/Yokohama/aq-55/01 in chicken kidney (CK) cells. Infected cells were incubated at 33°C, 37°C, 40°C, and 42°C. The results are expressed as the log10 reduction compared with the mean titer at 37°C (0 reduction) and as mean±SD. *: $p<0.05$, **: $p<0.01$.

may not have been isolated and sequenced before the outbreak.

Current Japanese guidelines on HPAI infection state that affected poultry should be subject to control measures if an HPAIV or LPAIV of H5 or H7 subtype is isolated. The OIE terrestrial animal health code designates both HPAI and LPAIVs of H5 or H7 subtype as notifiable AIV. LPAIVs of the H5 and H7 subtypes have been shown to change into highly pathogenic forms in the field as well as in a laboratory setting (6, 40-43). It appears that some low-pathogenicity viruses of the H5 and H7 subtypes can undergo genetic changes, one of which is an acquisition of multiple basic amino acid insertions at the HA cleavage site, after circulating in poultry for an extended period of time. There was no scientific evidence that allowed us to predict whether or not the Ibaraki isolates would turn into a highly pathogenic form. Under these circumstances, control measures against the Ibaraki outbreaks were undertaken as if the causative agents were AIVs of highly pathogenic form, which is scientifically reasonable and justifiable. Without the measures taken on this occasion, the Ibaraki strains might have become highly pathogenic, with deleterious consequences for the poultry industry in Japan.

Ck/Ibaraki/1/05 (H5N2) was shown to be transmitted efficiently even without direct contact in an isolator. Fecal-oral transmission rather than aerosol transmission is the major of infection route of AIVs (44-46). However, we demonstrated transmission in indirect-contact chickens, which cannot be fully explained by ordinary fecal-oral transmission. Although the two groups were separated by 10 cm with two stainless nets, they were in the same isolator. Under such conditions, the route of transmission is likely to be dust-borne or droplet-borne. Dust could have arisen from contaminated feces and droplets from the respiratory secretions of infected chickens. We showed that Ck/Ibaraki/1/05 (H5N2) infects chickens as efficiently as do some HPAI viruses, since the CID_{50} of Ck/Ibaraki/1/05 (H5N2) by the intranasal route was $10^{2.5}$ EID_{50} while the chicken lethal (=infectous) doses (CLD_{50}) of A/chicken/Yamaguchi/7/2004 (H5N1), A/Hong Kong/156/1997 (H5N1) and A/Hong Kong 213/2003 (H5N1) were reported as $10^{2.5}$ EID_{50}, $10^{2.3}$ and $10^{3.2}$ plaque-forming units, respectively (47-49). Because of this low CID_{50}, the virus might be transmitted easily by the dust-borne and/or droplet-borne route.

Human influenza A viruses can replicate well in the upper respiratory tract rather

than in lower respiratory tract because of temperature-dependent multiplication of virus (50). In this study, though virus was isolated efficiently from tracheal swabs of chickens inoculated with Ck/Ibaraki/1/05 (H5N2), it was not isolated as efficiently from the trachea or lung. Histopathological and immunohistochemical studies revealed that the virus did not replicate predominantly in the lower respiratory tract. We therefore hypothesized that, because of temperature-dependent multiplication, the Ibaraki viruses could not replicate well in the lower respiratory tract where the temperature is relatively high. Although Ck/Ibaraki/1/05 (H5N2) showed temperature-dependent multiplication in vitro as expected, Ck/Yokohama/aq-55/01 (H9N2), which replicates well in both trachea and lung, showed a similar replication profile, suggesting that temperature alone may not be a key factor restricting the replication of Ck/Ibaraki/1/05 (H5N2) in the lung. Other factors, such as receptor recognition of the virus, need to be explored.

In conclusion, the virus isolated in this outbreak was 1) efficiently replicated in the respiratory tract without clinical signs and 2) easily transmitted by dust and/or droplets. Phylogenetic analysis showed that a putative ancestor strain may have arisen from endemic H5N2 viruses circulating in poultry in Central America over the previous decade. The results suggested that the H5N2 LPAIVs isolated in Japan were highly adapted to chickens.

Brief Summary

At the end of May 2005, a LPAIV of subtype H5N2 was isolated for the first time from chickens in Japan. Through active and epidemiological surveillance, 16 H5N2 viruses were isolated. Antigenic analysis revealed antigenic similarity of these isolates. Phylogenetic analysis showed that they originated from a common ancestor and clustered with the H5N2 strains prevalent in Central America that have been circulating since 1994. Experimental infection of chickens with the index isolate (A/chicken/Ibaraki/1/2005 (H5N2)) demonstrated that this virus replicated efficiently in the respiratory tract without clinical signs and infected to chickens with $10^{2.5}$ 50% chicken infective dose as low as HPAIV, thus dust-borne and/or droplet-borne transmission was considered as a possible mode of transmission. These results suggested that the H5N2 LPAIVs isolated in Japan were highly adapted to chickens.

Part 2

Characterization of H5N1 highly pathogenic avian influenza viruses isolated from dead whooper swans (*Cygnus cygnus*) found in northern Japan in 2008

Introduction

Thousands of migratory birds of several species died due to H5N1 HPAIV infection at Qinghai Lake in China in 2005 (51, 52). Viruses similar to the Qinghai-virus spread to Asia, Europe and Africa (53-55). The responses to infection with the H5N1 HPAIV vary in different wild water birds. The ducks inoculated with HPAIV survived and showed neurological signs with the replication of the virus in the brain (56-58). On the other hand, highly susceptible species such as swans (*Cygnus* spp.) show high mortality by infection with HPAIV (59), rising concern that migratory birds may transmit HPAIVs to poultry and even to humans.

The long-term endemic of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries. A broadly cross-protective vaccine for antigenic variants of H5N1 virus may be a useful option as a tool for the control of avian influenza (29). Previously, we developed avian influenza vaccine prepared from non-pathogenic avian influenza viruses isolated from migratory ducks (60). The vaccine conferred protective immunity to suppress the manifestation of disease signs and reduction of virus shed in chickens and monkeys (*Cynomolgus macaques*) against H5N1 viruses isolated 2004 and 2005 (61, 62).

In this study, a whooper swan found dead at Saroma lakeside was pathologically examined since there are few reports on the pathology of swans naturally infected with H5N1 HPAIV (63, 64). Furthermore, the H5N1 virus isolate was compared genetically and antigenically with the isolates from swans found dead in Japan in 2008 (65, 66). An inactivated avian influenza vaccine prepared from A/duck/Hokkaido/Vac-1/2004 (Dk/Hok/Vac-1/04) (H5N1) was also assessed for its potency to suppress the manifestation of disease signs.

Materials and Methods

Viruses. A/whooper swan/Hokkaido/1/2008 (Ws/Hok/1/08) (H5N1) and A/whooper swan/Hokkaido/2/2008 (Ws/Hok/2/08) (H5N1) were isolated from trachea of whooper swans found dead at the Notsuke Peninsula, and at the Lake Saroma, respectively, in Hokkaido Prefecture, Japan. All viruses used in this study were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 hours and stored -80°C until use.

Sequencing and phylogenetic analysis. Viral RNAs were extracted with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) from allantoic fluids. Nucleotide sequences of all eight gene segments were determined after RT-PCR as described previously (60). The sequence data were analyzed using GENETYX ver. 9.1 (GENETYX corporation, Tokyo, Japan). Phylogenetic analysis of HA gene was performed by BioEdit ver. 7.0 and MEGA 4 by the neighbor-joining method with 1000 bootstraps. The nucleotide sequences obtained in this study are available from GenBank/ EMBL/ DDBJ under accession numbers AB436547-AB436554, AB436899 - AB436906.

Intravenous pathogenicity test in chickens. The intravenous pathogenicity test to chickens for influenza viruses were carried out according to the OIE standard method (29). Briefly, 1/10 dilutions of infectious allantoic fluids containing the viruses were intravenously inoculated into eight 7-week-old chickens. These chickens were housed for 10 days in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan) at a BSL 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Histopathology and immunohistochemistry. An adult male whooper swan found dead at the Saloma lakeside on 5th May, 2008 was pathologically examined. The tissues of swan were fixed in 10% formalin in PBS (pH 7.2). Paraffin-embedded

sections were processed for hematoxylin and eosin staining and immunohistochemistry. For the detection of influenza virus antigens in the tissues, the sections were incubated with rabbit anti-A/whistling swan/Shimane/499/1983 (H5N3) hyper-immune serum at a 1:1000 dilution. Bound antibodies were detected by the peroxidase-labeled streptoavidin–biotin method (Histofine SAB-PO rabbit kit: Nichirei, Tokyo, Japan).

Antigenic analysis of the viruses. HI test was performed as described by Sever (28). A panel of monoclonal antibodies to H5 HA of A/duck/Pennsylvania/10218/1984 (H5N2) was used as previously (60, 67). Hyper-immune antisera against Ws/Hok/1/08 (H5N1), Dk/Hok/Vac-1/04 (H5N1) and A/tern/South Africa/1961 (H5N3) were prepared according to Kida and Yanagawa (68).

Potency test of vaccine efficacy in chickens against Ws/Hok/1/08. The inactivated avian influenza virus Dk/Hok/Vac-1/04 (H5N1) vaccine was intramuscularly inoculated to chickens as described previously (61, 69). Briefly, Dk/Hok/Vac-1/04 (H5N1) was inactivated with formalin and mixed with oil adjuvant containing anhydromannitol-octadecenoate-ether (AMOE). Eleven four-week-old chickens were intramuscularly immunized and 3 weeks later, were inoculated intranasally with a 100 CLD_{50} of Ws/Hok/1/08. Clinical signs were monitored for 14 days post-challenge (p.c.) and chickens were sacrificed on day 2 and 4 p.c. When chickens died or were sacrificed, tracheal and cloacal swabs and their tissues (trachea, lung, kidney, and colon) were collected. Virus titers were measured by EID_{50}.

Results

Pathological findings of the whooper swan. A whooper swan found dead beside Lake Saroma on 5th May, 2008, presented as well-nourished with sufficient body fat reserves. Gross lesions were not found except some hurts of its head and neck which may be due to the bite by wild animal. At necropsy, the swan showed diffuse severe congestive edema of the lungs with thickening of the pleura (Fig. 5). Echymotic hemorrhage was scattered in the pancreas and epicardium. A whooper swan found dead in Notsuke Peninsula on

24th April, 2008 was not pathologically examined since the swan already decomposed when it arrived.

The predominant histological lesions were found exclusively in the brain, pancreas and lungs. In the cerebrum and cerebellum, glial nodules were scattered with spongiform change of the neuropil and with necrosis of nerve and glial cells (Fig. 6A). Small necrotic foci of acinar cells were observed in the pancreas (Fig. 6C). Only a small number of heterophils and macrophages were infiltrated in the cerebral and pancreatic lesions. The lungs were severely congested with diffuse moderate edema of interlobular and peribronchial connective tissues. Small amount of fibrin and heterophils exuded into parabronchi and infundibula. By the immunohistochemical examination, influenza virus antigens were found in the brain, pancreas, lungs and trachea. In the cerebrum and cerebellum, nerve and glial cells within and around the glial nodules were stained positive by hyperimmune serum to A/whistling swan/Shimane/499/1983 (H5N3) (Fig. 6B). In the necrotic areas of the pancreas, some necrotic and degenerative acinar cells were stained positive (Fig. 6D). In the lungs and trachea, the antigen was detected in only a few respiratory and mucosal epithelial cells.

Pathogenicity of the isolates in chickens. Ws/Hok/1/08 (H5N1) and Ws/Hok/2/08 (H5N1) were inoculated intravenously into eight 7-week-old chickens, respectively. Within 2 days post-inoculation, all chickens died. This result was consistent with a prediction based on the amino acid sequence at the cleavage site of the HA protein of the isolates in Hokkaido were more than a pair of dibasic amino acid residues (PQRERRRKR/GLF).

Genetic analysis of virus isolates from dead whooper swans. To elucidate the genetic relationships of the isolates with other H5N1 influenza virus isolates, all eight gene segments were compared. It was revealed that the all gene segments of the isolates were closely related to each other and to the H5N1 HPAIVs isolated from whooper swans in Akita and Aomori Prefecture in 2008 (more than 99.0 similarity in all genes) (66, 70). It was also revealed that all isolates in Japan in 2008 closely related to those of the isolates in Korea in 2008 (personal communication). Phylogenetic analysis of the HA

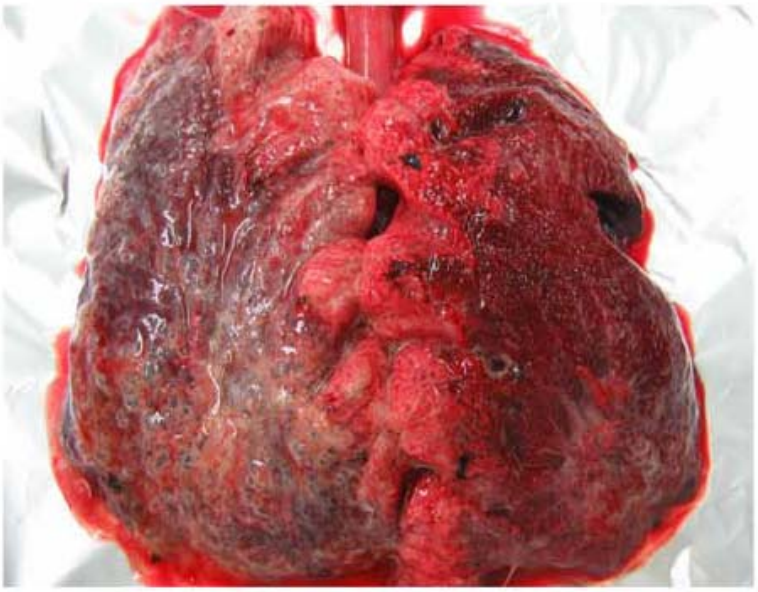

Fig. 5 Gross appearance of the lungs of whooper swan found dead beside Lake Saloma.

The lungs show diffuse congestive edema. The pleura is edematously thickened.

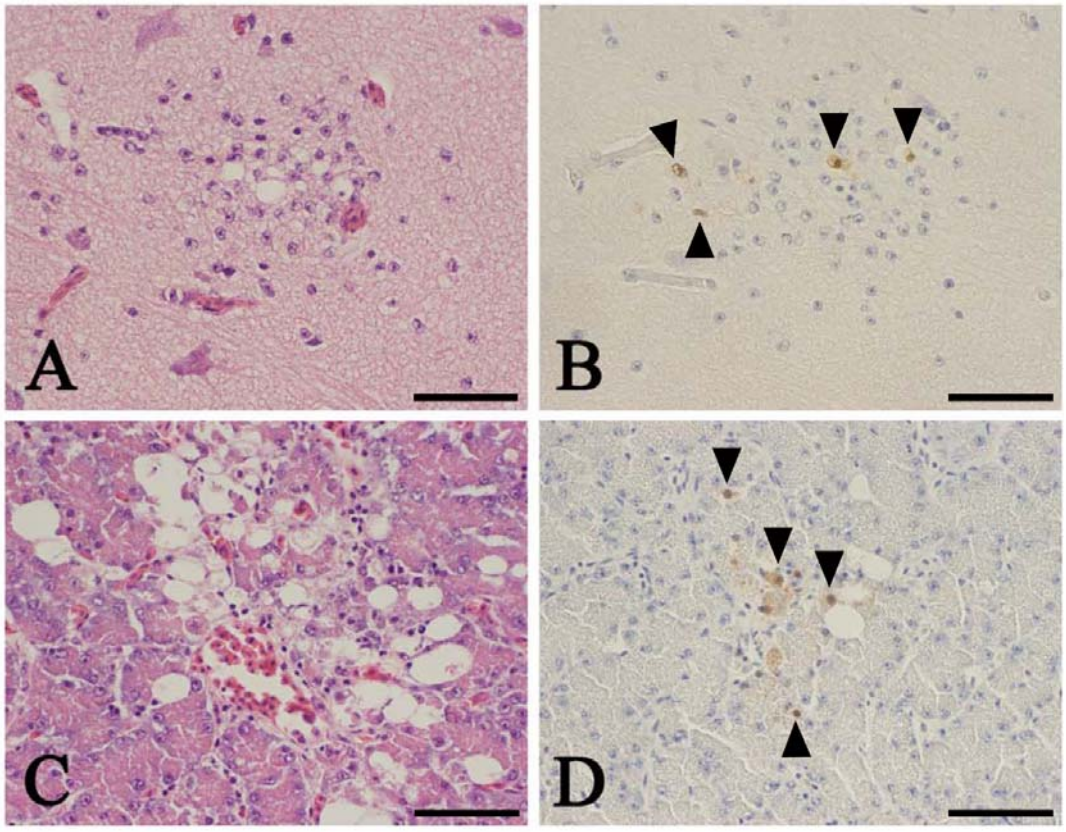

Fig. 6 Histpathological and immunohistochemical findings of the whooper swan. A) Glial nodule with spongiform changed of neuropile. Cerebrum, HE stain. B) Nerve and glial cells in the glial nodule are positively stained for influenza virus antigen (*arrowheads*). C) Focal necrosis of acinar cells. Pancreas, HE stain. D) Necrotic and degenerative acinar cells show positive staining for influenza virus antigen (*arrowheads*). Bars = 50 μm.

genes showed that these isolates belonged to Clade 2.3.2 and formed a unique branch with the isolates in Hong Kong in 2007-08 and those in Russia in 2008 (Fig. 7).

Antigenic characterization of the HA of the isolates. Ws/Hok/1/08 (H5N1) was identified as H5N1 by HI and NI tests. Antigenic analysis of the HA of the isolates with antisera to H5 of influenza viruses and monoclonal antibodies to the HA of A/duck/ Pennsylvania/10218/1984 (H5N2) was performed by HI test. The antigenicity of the HA of the isolates in 2008 were similar to each other but different from these of Dk/Hok/Vac-1/04 (H5N1), which is the reassortant virus generated from the isolates from fecal samples of wild ducks, and H5N1 HPAIVs isolated from chickens and whooper swans in Asia (Table 5).

Potency of the vaccine against the isolates in chickens. Ws/Hok/1/08 (H5N1) was selected as the challenge strain for the vaccine potency test since the isolates from whooper swans were genetically and antigenically identical. Eleven chickens intra-muscularly inoculated with the vaccine prepared from Dk/Hok/Vac-1/04 (H5N1) and 3 non-vaccinated chickens were challenged intranasally with Ws/Hok/1/08 (H5N1) on 3 weeks after vaccination. The HI titers of the sera of the vaccinated chickens were 1:128-512 and 1:4-8 with the vaccine strain and with the isolate, respectively. All vaccinated chickens survived without showing any disease signs after challenge, whereas all of the control chickens died within 2 days p.c. Viruses were not recovered from the swabs of any of the vaccinated chickens after challenge. Low titers of infectious virus were recovered from the trachea, lungs, kidneys, and colon of three of the four vaccinated birds on day 2 p.c. (Table 6).

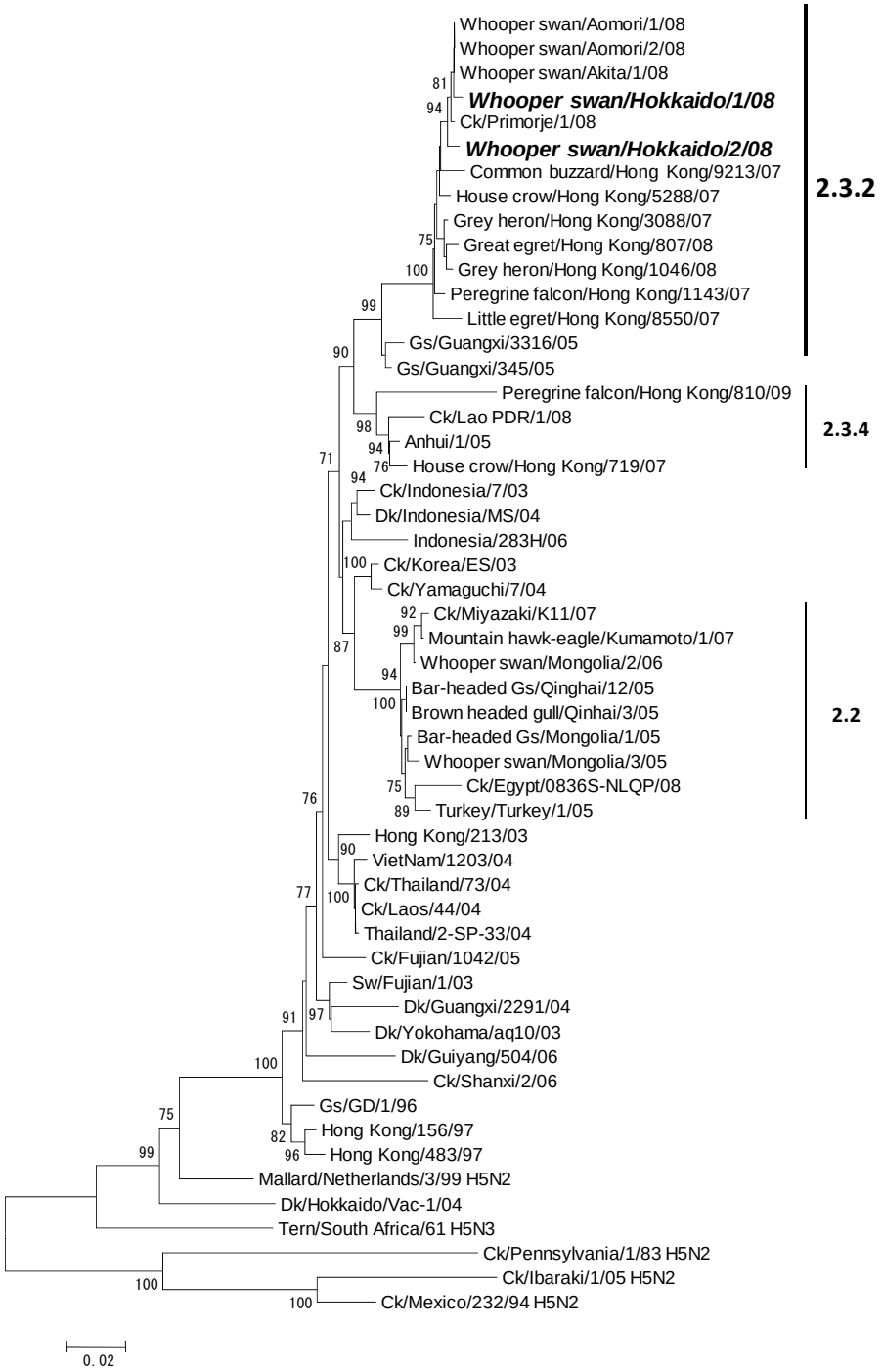

Fig. 7 Phylogenetic tree of the HA genes of H5 influenza viruses.

Nucleotide sequences of the HA genes of H5 influenza viruses isolated in the present study (shown in bold italic) and the sequence information of other related viruses were cited from the public database. 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 boot strap analysis with 1,000 replications. Abbreviations: Ck, Chicken; Gs, Goose; Dk, Duck; Qa, Quail; Sw, Swine.

Table 5. Antigenic property of influenza viruses isolated in Japan in 2008

Viruses	Clade	Polyclonal antiserum (hyper-immune)			Monoclonal antibodies [a]		
		Ws/Hok/1	Dk/Vac-1	Tn/SA	A310/39	64/2	25/2
Whooper swan/Hokkaido/1/08 (H5N1)	2.3.2	**1280**	40	40	<	<	<
Whooper swan/Hokkaido/2/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Whooper swan/Akita/1/08 (H5N1)	2.3.2	1280	20	40	<	<	<
Whooper swan/Akita/2/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Viet Nam/1194/04 (H5N1)	1	80	160	160	40	1280	80
Whooper swan/Mongolia/3/05 (H5N1)	2.2	320	320	160	<	320	320
Whooper swan/Mongolia/2/06 (H5N1)	2.2	80	320	160	<	1280	80
Chicken/Yamaguchi/7/04 (H5N1)	2.5	320	640	320	40	640	160
Duck/Hokkaido/Vac-1/04 (H5N1)	Classical	40	**640**	160	320	320	40
Tern/South Africa/61 (H5N3)	Classical	40	320	**640**	<	160	20
Chicken/Ibaraki/1/05 (H5N2)	American	20	80	40	<	<	<

Homologous titer of the antiserum is underlined. <; The HI titer was lower than 1:20.

[a] Monoclonal antibodies against Dk/Pennsylvania/84 (H5N2).

Abbreviations: Dk/Vac-1, Duck/Hokkaido/Vac-1/04 (H5N1); Tn/SA, Tern/South Africa/61 (H5N3).

Table 6. Antibody titers and virus recovery in chickens

	Days p.c.	HI titer (0 dpc)		HI titer (14 dpc)		Virus recovery				
		Dk/Vac-1/04	Ws/Hok/1	Dk/Vac-1/04	Ws/Hok/1	Swabs (log EID_{50}/ml)		Tissues (log EID_{50}/g)		
						Trachea	Cloaca	Trachea	Lung	Kidney
Vaccinated	2	256	8	NT	NT	−	−	2.0	2.5	2.5
chickens	2	256	4	NT	NT	−	−	1.8	−	−
	2	256	8	NT	NT	−	−	−	−	2.5
	2	512	8	NT	NT	−	−	−	−	−
	4	128	4	NT	NT	−	−	−	3.3	−
	4	256	4	NT	NT	−	−	−	−	−
	4	256	8	NT	NT	−	−	−	−	−
	14	256	4	512	64	NT	NT	NT	NT	NT
	14	256	4	512	64	NT	NT	NT	NT	NT
	14	256	8	512	64	NT	NT	NT	NT	NT
	14	128	4	1024	128	NT	NT	NT	NT	NT
Non-vaccinated	2†	<2	<2	NT	NT	4.5	4.3	8.5	7.5	7.3
chickens	2†	<2	<2	NT	NT	6.3	4.8	9.3	7.8	7.3
	2†	<2	<2	NT	NT	6.8	4.5	7.8	7.5	9.5

− :The titer of the virus recovery lower than 0.5 (swabs) or 1.5 (tissues).

NT: Not tested †: Chicken died

Discussion

In this study, genetic analysis of the H5N1 viruses isolated from the swans in Hokkaido in Japan revealed that those are Clade 2.3.2. The results also indicate that whooper swans were infected with the HPAIV though water born transmission somewhere in a lake or pond where feral water birds, who were infected with HPAIV probably in Southern China, congregated on the way back to the north in spring. Although the viruses belonging to the Clade 2.2 Qinghai-like viruses had been spread in Asia, Europe and Africa by wild water birds (53-55, 71), the present results indicate that the viruses belonging to Clade 2.3.2, different from Qinghai-like viruses, also spread by wild water birds. Actually, the case reports of infections of wild birds with H5N1 HPAIV belonging to Clade 2.3.2 and 2.3.4 are increasing since 2008 (58, 72).

High mortality in wild water birds infected with HPAIV had not been recognized before 2005. However, swans and geese are apparently the most affected species by infection with the recent H5N1 virus strains (59, 64, 73). In this study, pathological changes of dead whooper swan with HPAIV confined to the central nervous system (CNS), pancreas and lungs. Inflammatory reaction of the wild water birds infected with H5N1 HPAIV was limited. The present findings indicate that the whooper swan died due to severe congestive edema of the lungs at the early stage of systemic infection with HPAIV. Neither myocardial necrosis nor influenza virus antigen was found in the heart of the swan. These findings coincide with those of the gross lesions of mute swans and whooper swans which were multifocal pancreatic necrosis and hemorrhage and lung edema during the outbreak in Germany in 2006 (59).

In poultry population in Asia, antigenic variants of H5N1 HPAIV have been selected, indicating that these wild birds were infected with the H5N1 viruses prevailing in domestic poultry (74). Antigenic analysis revealed that the isolates were different from the virus isolates from poultry and wild water birds in Japan, Mongolia, and China including the vaccine strain that we previously generated. It is suggested that the antigenicity of H5N1 HPAIVs have been further drifted during circulation in the chicken population since 2007. Based on this notion, the chickens inoculated with the vaccine we previously developed were challenged with the present HPAIV isolate. In the

challenge study to vaccinated chickens, higher titer of the challenge viruses were recovered from various tissues of the chickens than those from birds challenged with A/chicken/Yamaguchi/7/2004 (H5N1) strain in the previous study, although all of the vaccinated chickens were survived for 14 days after the challenge with Ws/Hok/1/08 (H5N1). This may be influenced by antigenic difference between the vaccine strain and the challenge virus. In poultry, avian influenza viruses have not been under constant immunological selection pressure induced by vaccines. Since vaccine use for poultry has increased in these days in several countries, antigenic variation could occur in H5N1 HPAIV as did H5N2 viruses in the1990s in Mexico (24). It is suggested that if the vaccine was used for the control of HPAI in future in addition to stamping-out policy, the antigenically matched vaccine strain which was generated by reverse genetics should be more effective. The important things to control HPAI, continuing surveillance to understand the influenza virus infection in birds and mammals and preparation for the diagnosis of influenza virus infection such as technical training, making the antiserum, and sharing the information are essential.

Brief Summary

In April and May 2008, whooper swans (*Cygnus cygnus*) were found dead in Hokkaido in Japan. In this study, an adult whooper swan found dead at Saroma lakeside was pathologically examined and the H5N1 influenza virus isolates were genetically and antigenically analyzed. Pathological findings indicate that the swan died due to severe congestive edema in the lungs. Phylogenetic analysis of the HA genes of the isolates revealed that these are the progeny viruses of the isolates from poultry and wild birds in China, Russia, Korea, and Hong Kong. Antigenic analyses indicated that the viruses are different from the H5N1 viruses isolated from wild birds and poultry before 2007. The chickens vaccinated with A/duck/Hokkaido/Vac-1/2004 (H5N1) survived 14 days after challenge with A/whooper swan/Hokkaido/1/2008 (H5N1), although small amount of the challenge virus was recovered from the tissues of the birds. These findings indicate that the H5N1 highly pathogenic avian influenza virus are circulating in wild birds in addition to domestic poultry in Asia and showing antigenic variation that may be due to vaccination.

Part 3

Antigenic structure of the hemagglutinin of H9N2 influenza viruses

Introduction

The HAs of H9N2 virus isolates in Asia were antigenically and genetically grouped into three distinct lineages, represented by the viruses A/quail/Hong Kong/G1/1997 (H9N2) (G1), A/duck/Hong Kong/Y280/1997 (H9N2) (Y280), and A/duck/Hong Kong/Y439/1997 (H9N2) (Y439) (12). Many of the H9N2 viruses isolated from chickens, ducks (13, 14), and pigs in southern China (75-77) belonged to the Y280 lineage. Antigenic and genetic analyses of H9N2 viruses in poultry revealed that the Y280 lineage designated as the A/chicken/Beijing/1/1994-like group was further divided into two subgroups and triple or quadruple reassotants with gene segments of G1-like, Y280-like, and H5N1-like viruses in Hong Kong in 2001 continued to prevail in southern China (78, 79). It was also assumed that Y280-like variant viruses originated from those circulating in poultry in Asian countries over 10 years. These H9N2 influenza viruses had receptor-binding specificity to terminal sialic acid with α2-6 Gal linkage found on human cells (80, 81). Actually, H9N2 viruses of G1 and Y280 lineage have been isolated from humans (82, 83). Thus, in addition to H5N1 and H7N7, H9N2 viruses are possible candidates that have potential to cause pandemics in humans.

The HA of influenza virus is the major target for immune responses due to its role in mediating attachment to and penetration into host cells. The antigenic structure of HAs of H1, H2, H3, H5, and H9 subtypes of influenza A virus has been investigated by antigenic mapping and sequence analysis (31, 84-88). Antigenic mapping of the H1 HA, A/PR/819/34 (H1N1), indicates five immunodominant antigenic sites designated Sa, Sb, Ca1, Ca2 and Cb (89) and is comparable to that of H3 subtype virus, A/Hong Kong/1968 (H3N2), designated A, B, C, D and E (88). The analysis of H9 antigenic variants defined at least two antigenic sites on the H9 HA molecule (85). In the present study, antigenic and genetic analyses of the HA of H9N2 viruses were performed using a panel of MAbs.

Materials and Methods

Viruses. Influenza virus A/swine/Hong Kong/10/1998 (Sw/HK/98) (H9N2), A/duck /Hokkaido/13/2000 (Dk/Hok/00) (H9N2) and other H9 viruses isolated from birds and mammals of different species were grown in 10-day-old embryonated chicken eggs and were stored at -80°C until used (Table 7). The viruses were purified by differential centrifugation and sedimentation through a sucrose gradient (68).

Monoclonal Antibodies. MAbs against Sw/HK/98 (H9N2) and Dk/Hok/00 (H9N2) were prepared as described previously (90). Briefly, BALB/c mice were immunized with the purified virus. Spleen cells of the mice were fused with myeloma cells and hybridoma cells secreting specific MAbs were selected. Each of the hybridoma cells was inoculated to mice intraperitoneally and ascitic fluids containing MAbs were used as MAb. Isotypes of MAbs were determined using Mouse Monoclonal Antibody Isotyping Reagents (Sigma, MO, U.S.A).

Serological tests. The HI test was performed by the standard method (28). In the neutralization test, titers were determined as the reciprocals of maximum antibody dilution which prevents the cytopathic effect of 100 $TCID_{50}$ of viruses using Madin-Darby Canine Kidney (MDCK) cells. Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (90).

Selection of antigenic variants. Antigenic variants were selected and the frequencies of antigenic variants were determined as described previously (90). Briefly, the virus was incubated with excess antibody for 1 hour at room temperature, and the mixture was inoculated into 10-day-old embryonated chicken eggs. The yielded viruses were detected by HA test after 48-hour incubation at 35°C and cloned by limiting dilution in embryonated eggs.

Table 7. H9N2 viruses used in this study

Lineage	Viruses	HA gene[a]
Eurasian		
Y280	A/chicken/Hong Kong/G9/1997	AF156373
	A/chicken/Hebei/3/1998	AF536695
	A/chicken/Hong Kong/FY20/1999	AF222611
	A/duck/Hong Kong/Y280/1997	AF156376
	A/silkie chicken/Hong Kong/SF43/1999	AF186268
	A/swine/Hong Kong/10/1998	AF222811
Y439	A/ostrich/South Africa/9508103/1995	AF218102
	A/duck/Hokkaido/31/1997	AB125927
	A/duck/Hokkaido/49/1998	AB125928
	A/duck/Hokkaido/9/1999	AB125929
	A/duck/Hokkaido/26/1999	AB125930
	A/duck/Hokkaido/13/2000	AB125931
G1	A/quail/Hong Kong/G1/1997	AF156378
	A/quail/Hong Kong/A17/1999	AF222606
	A/chicken/Pakistan/2/1999	AJ291392
	A/Hong Kong/1073/1999	AJ404626
North American	A/turkey/Wisconsin/1/1966	D90305
	A/turkey/Minnesota/38391-6/1995	AF156387
	A/goose/Minnesota/5733-1/1980	AF156389
	A/quail/Arkansas/29209-1/1993	AF156388
	A/shorebird/Delaware/9/1996	AF156386

a：GenBank/ EMBL/ DDBJ Accession No.

Sequence analysis of virus genes. Viral RNAs were isolated from virus-containing allantoic fluid with Trizol LS reagent (Invitrogen, PO, U.S.A). HA genes were sequenced after viral RNA extraction and RT-PCR according to Liu et al. (91). Other Sequence data were assembled and translated to the amino acid sequence by gene analyzing software GENETYX-WIN version 6.1.0 (Genetyx Corporation, Tokyo, Japan). The positions of amino acid substitution on the HA molecule were analyzed on the 3-dimensional structure obtained from the Protein Databank (PDB accession number, 1JSD) with the RasMol 2.7.3 program.

Results

Antigenic mapping of the H9 HA molecule. Six anti-HA MAbs against Sw/HK/98 (H9N2) and two against Dk/Hok/00 (H9N2) were selected as representatives of 50 MAbs obtained in total. Nucleotide sequences of the HA gene of the antigenic variants selected in the presence of each of the MAbs were determined. A single amino acid substitution was found in the deduced amino acid sequence of the HA of each of the antigenic variants (Table 8). A panel of MAbs recognizing 8 amino acid positions was established on the basis of the positions of amino acid substitution in the H9 HA molecule. The position of amino acid substitution at residue 72 (Gly→Glu) on the HA of Sw/L6/2, a mutant selected from Sw/HK/98 (H9N2) in the presence of MAb L6/2, was located in the vicinity of the bottom of the globular head domain of the H9 HA molecule in the proposed antigenic site E in the H3 subtype HA (Fig. 8A). Antigenic variant, SwG6/5 had an amino acid substitution at residue 127 (Ser→Asp) at the 'overlapping site' of Site I and Site II, that was reported by Kaverin et al. (85). SwN4/2 had an amino acid substitution at residue 148 (Asn→Asp) on Site I. Substitution at residues 182 (Thr→ Ile) and 183 (Asn→Asp) was located on Site II. An amino acid substitution of SwG12/1 at residue 212 (Leu→His) was found in the vicinity of the trimeric interface of the globular domains of the HA1. The Dk/Hok/00 (H9N2) antigenic variants DkD370/4 and DkD272/6 had amino acid substitutions at residues 98 (Leu→Gln) and 131 (Lys→ Asn) (137 in H3 HA numbering), respectively.

Table 8. Biological properties of the monoclonal antibodies to H9 HA molecule

Viruses [a]	MAbs	Antibody titers			Isotype	Mutation of escape mutants			
						Nucleotide		Amino acid	
		ELISA [b]	HI	NT		Position [c]	Change	Position [d]	Change
Sw	L6/2	7.1	80	80	IgG2a	269	G→A	72	Gly→Glu
Sw	G6/5	6.5	25600	204800	IgG1	434	G→A	127	Ser→Asn
Sw	I4/5	5.9	640	10240	IgG1	434	G→A	127	Ser→Asn
Sw	M10/1	7.1	2560	5120	IgG1	434	G→A	127	Ser→Asn
Sw	119/6	6.2	2560	204800	IgG2a	434	G→A	127	Ser→Asn
Sw	N4/2	6.5	2560	20480	IgG1	496	A→G	148	Asn→Asp
Sw	E2/3	7.1	2560	5120	IgG2a	599	C→T	182	Thr→Ile
Sw	I7/5	6.5	640	2560	IgG2a	599	C→T	182	Thr→Ile
Sw	M7/1	7.1	2560	5120	IgG2a	601	A→G	183	Asn→Asp
Sw	L7/7	7.1	320	5120	IgG2a	601	A→G	183	Asn→Asp
Sw	G12/1	6.5	2560	10240	IgG2a	689	T→A	212	Leu→His
Dk	D370/4	5.9	5120	80	IgG3	347	T→A	98	Leu→Gln
Dk	D272/6	7.7	1280	10240	IgG1	447	G→A	131	Lys→Asn

a:M onoclonal antibodies against A/swine/Hong Kong/10/98 (H9N2) (Sw) or A/duck/Hokkaido/13/00 (H9N2) (Dk).
b: Titers are expressed as $\log_{10}$.
c,d: Positions of nucleotide and amino acid substitutions are numbered according to HA molecule of H9 subtype virus.

The reactivity patterns of antigenic variants with the panel of MAbs showed that the MAbs of Sw/HK/98 (H9N2) and Dk/Hok/00 (H9N2) were divided into 7 groups (Table 9). Out of 7 groups, 5 groups of MAbs (group 1, 2, 5, 6 and 7) recognized independent epitopes, while the other 2 groups recognized antigenically overlapped epitopes. The mutants SwG6/5 and SwL7/7 did not react with MAb D272/6 in addition to the mutant DkD272/6. The mutants SwG6/5 weakly reacted with MAb N4/2 compared with the parental virus. Therefore, at least five distinct antigenic sites were defined on the H9 HA using the present 8 MAbs.

Sequence analysis of the H9 HA molecule. H9N2 influenza viruses have been prevalent in poultry over the last decades. Within the period, many H9N2 viruses were isolated and confirmed that the viruses had undergone antigenic variation. Sequence analysis of the H9 HA genes of 133 H9N2 influenza virus strains isolated between 1994 and 2005 showed that 28 positions of which amino acid substitutions frequently occurred on H9 HA molecule were found (Table 10 and Fig. 8B). Twenty-two amino acid substitutions were found on the HA molecule and two substitutions (103 and 269) were inside of the molecule, whereas the others were found at the HA cleavage site. The positions of amino acid substitution on the HA molecule on the 3-dimensional structure revealed that at least 4 conformational antigenic sites were located on the H9 HA.

Antigenic analysis of the HAs of H9N2 virus isolates using a panel of MAbs. Reactivity of H9N2 influenza viruses isolated from birds and mammals of different species with a panel of MAbs to the HA of Sw/HK/98 (H9N2) and Dk/Hok/00 (H9N2) were analyzed (Table 11). On the basis of the reactivity patterns with the panel of MAbs, 21 H9N2 influenza virus strains were divided into 7 different groups. Viruses belonging to antigenic group I had all seven epitopes which each of MAbs recognized on the HA molecule. Viruses belonging to antigenic group II to VI had several epitopes and A/shorebird/Delaware/9/96 did not reacted with any MAbs. The HAs of the viruses belonging to the Y280 lineage used in the present study were antigenically conserved and

Table 9. Reactivity patterns of antigenic variants with monoclonal antibodies

			Antigenic variants selected from [a]								
			Sw/HK/98						Dk/Hok/00		
MAb Group	MAbs	Sw/HK/00	SwL6/2	SwG6/5	SwN4/2	SwE2/3	SwL7/7	SwG12/1	DkD272/6	DkD370/4	Dk/Hok/00
1	L6/2		–						–	–	–
2	G6/5			–					–	–	–
3	D272/6			–			–		–		
4	N4/2			<	–				–	–	–
5	E2/3					–	–		–	–	–
	L7/7					–	–		–	–	–
6	G12/1							–	–	–	–
7	D370/4									–	

[a] : Each of the monoclonal antibodies used in variant selection was titrated in ELISA with antigenic variant.

"–" indicates no binding and no entry indicates significant binding to the variant virus antigen.

"<" indicates weak binding compared to the parental virus (ELISA titer were decreased 32-64 times).

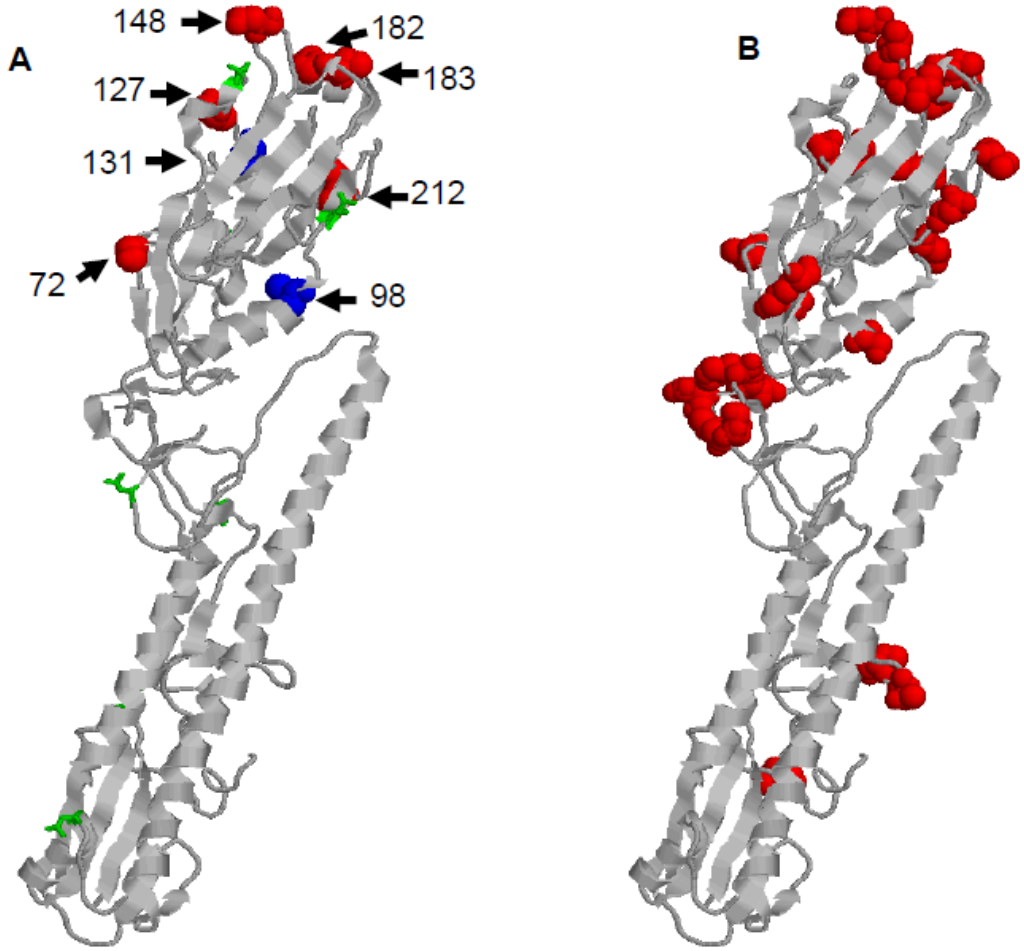

Fig. 8 Schematic representation of monomer structures of the H9 haemagglutinin molecule showing location of amino acid substitutions on HA1. Images were created with RasMol 2.7.3. A: Amino acid changes of escape mutants selected with monoclonal antibody against A/swine/Hong Kong/10/98 (H9N2) (red) and A/duck/Hokkaido/13/00 (H9N2) (blue). The positions of the oligosaccharide attachment sites are shown as green lines. Amino acid positions are designated by H9 numbering. B: Hyper-variable amino acid positions on HA of H9N2 viruses clustered in Y280 lineage isolated from 1994 to 2005.

Table 10. Amino acid variations in natural isolates

Amino acid position	Changed amino acids	Surface (S) or Inside (I)
20	A,I,T	S
22	N,S,T	S
45	D,G,N	S
48	H,P,R	S
53	D,K,N	S
69	I,P,Q	S
94	I,N,T	S
103	A,L,S,T	I
131[a]	K,R,S	S
135[b]	D,G,N	S
146	E,H,K,Q	S
148[a]	D,N,S	S
150	A,D,S,T	S
153	I,T,V	S
164	E,K,R	S
179[b]	A,N,T	S
180	A,E,G,T,V	S
182[a]	D,E,N	S
200	D,N,T	S
206	L,M,S,V	S
216[b]	L,M,Q	S
264	H,K,N,T,Y	S
265	G,K,N,R,S	S
267	D,N,S	S
269	A,T,V	I
316	A,S,T	cleavage site
317	G,K,R	cleavage site
318	A,L,S	cleavage site

Amino acid comparison was performed with 133 viruses clustered with Y280-lineage isolated from 1994 to 2005.

Positions were selected in which three or more amino acids were different.

Escape mutant has changed in this residue in this study (a) and Kaverin *et al*. (b) (81).

Table 11. Reactivity patterns of 21 H9N2 viruses with a panel of monoclonal antibodies

		Monoclonal antibodies[a]								
	MAb groups	1	2	3	4	5		6	7	
lineage	Viruses	L6/2	G6/5	D272/6	N4/2	E2/3	L7/7	G12/1	D370/4	Antigenic Group
Y280	**Sw/HK/10/98**	**+**[a]	**+**	**+**	**+**	**+**	**+**	**+**	**+**	I
	Ck/HK/G9/97	+	+	+	+	+	+	+	+	I
	Ck/HK/FY20/99	+	+	+	+	+	+	+	+	I
	Ck/Hb/3/98	+	+	+	+	+	+	+	+	I
	SCk/HK/SF43/99	+	+	+	+	+	+	+	+	I
	Dk/HK/Y280/97	+	+	+	+	+	+	+	+	I
Y439	Dk/Hok/31/97	+	-	+	-	-	-	-	+	II
	Dk/Hok/49/98	+	-	+	-	-	-	-	+	II
	Dk/Hok/13/00	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**+**	III
	Osr/S.Af/9508103/95	-	-	+	-	-	-	-	-	IV
	Dk/Hok/9/99	-	-	-	-	-	-	-	+	V
	Dk/Hok/26/99	-	-	-	-	-	-	-	+	V
G1	Ck/Pak/2/99	+	-	-	-	+	+	-	+	VI
	HK/1073/99	+	-	+	-	-	-	-	+	II
	Qa/HK/G1/97	-	-	-	-	-	-	-	+	V
	Qa/HK/A17/99	-	-	-	-	-	-	-	+	V
North America	Ty/Wis/1/66	-	-	+	-	-	-	-	+	III
	Ty/Min/38391-6/95	-	-	+	-	-	-	-	+	III
	Gs/Min/5733-1/80	-	-	+	-	-	-	-	+	III
	Qa/Ark/2920901/93	-	-	+	-	-	-	-	-	IV
	Sb/Del/9/96	-	-	-	-	-	-	-	-	VII

Sw: swine, Ck: chicken, SCk: silky chicken, Dk: duck, Osr: ostrich, Qa: quail, Ty: turkey, Gs: goose, Sb: shorebird, HK: Hong Kong, Hb: Heibei, S. Af: South Africa, Hok: Hokkaido, Pak: Pakistan, Wis: Wisconsin, Min: Minnesota, Alk: Alaska, Del: Delaware

[a] : ELISA titer, +□1: 10,000, -: ELISA titer ＜10,000. Homologous reactions are shown in bold.

different from those to other lineages.

Discussion

The aim of the present study was to characterize the antigenic structure of the HA molecule of H9N2 influenza viruses recently prevailing in poultry in Asia. The antigenic drift of H9N2 viruses has been detected by reactivity with polyclonal antisera (12, 78, 79). In the present study, a panel of MAbs to the HA of H9N2 influenza viruses was prepared and used for antigenic comparison of these H9 influenza viruses.

Several subtypes of the HA have been analyzed by using a panel of MAbs (31, 85, 88, 89, 92). In the present study, the reactivity patterns of antigenic variants with the panel of MAbs showed that the MAbs were divided into 7 groups. Out of 7 groups, 5 groups of MAbs recognized independent epitopes. Two antigenic sites on the H9 HA molecules, the Site I (amino acid positions 129, 147, and 152) and Site II (amino acid positions 135, 183, and 216), were revealed (85). According to the position of amino acid substitution of the HA of antigenic variant, MAb G6/5 recognizes the Site I and E2/3 and L7/7 recognizes the Site II, respectively. MAbs L6/2, G12/1 and D370/4 recognize epitopes in 3 antigenic sites distinct from the sites I and II. The mutants whose deduced amino acid changes were detected in Ser127Asn, Lys131Asn and Asn183Asp did not react with MAb D272/6, suggested that D272/6 recognized conformational overlapping epitopes. Weak reaction of antigenic variant selected in the presence of MAb G6/5 with N4/2 may due to the mutation Ser127Asn that forms glycosylation site. The panel of MAbs recognized 7 epitopes in the present study recognized at least 5 different antigenic sites in the vicinity of the receptor biding site.

The amino acid comparison of HA molecules of natural isolates belonging to Y280 lineage of H9N2 viruses showed 28 amino acid positions were more variable on the HA molecule. These positions located in the vicinity of the antigenic site known in H1 or H3 subtypes although there are some differences in 3-dimensional structure. In these positions, 6 amino acid substitutions were identical with the amino acid substitutions in the escape mutants selected in the present study and in earlier publication (85). It is

suggested that at least 5 antigenic sites were located on the H9 HA molecule and the antigenic variation occurred in these sites after the long-term prevalent of H9N2 influenza viruses in poultry.

The H9N2 influenza viruses were divided into Eurasian and North American lineages, and the former was divided further into Y280, G1 and Y439 sub-lineages (12). In the present study, it was revealed that reactivity patterns of H9N2 influenza viruses isolated from birds and animals of different species with the panel of MAbs showed that the HA of viruses belonging to the Y280 lineage was antigenically conserved and different from that of other lineages. The present results coincide with the findings that viruses belonging to the Y280 or G1 lineage are antigenically different from other lineages (12).

After long-term prevalence of H9N2 viruses in poultry, it is assumed that the antigenic variants were selected in the host immune pressure. And the vaccination also should lead the antigenic variation of the viruses. The panel of MAbs prepared in the present study should be useful for detailed antigenic analysis in the epidemiological study of currently circulating H9N2 viruses, especially the viruses belonging in Y280 lineage. Furthermore, the present panel of MAbs should be useful for the development of an HA subtype-specific diagnosis kit (67).

Brief Summary

The hemagglutinins (HAs) of H9 influenza viruses isolated from birds and mammals of different species were antigenically and genetically analyzed. Antigenic variants were selected from A/swine/Hong Kong/10/1998 (H9N2) and A/duck/Hokkaido/1320/00 (H9N2) in the presence of monoclonal antibodies (MAbs), respectively. Based on the reactivity patterns of these mutants with a panel of MAbs, at least 5 non-overlapping antigenic sites were defined using 8 MAbs which recognized 7 distinct epitopes on the H9 HA molecule. Based on the reactivity patterns with the panel of monoclonal antibodies, 21 H9N2 virus strains isolated from birds and mammals were divided into 7 antigenically distinct groups. The present findings indicate that it is important to monitor the antigenic variation in H9 influenza viruses. The panel of MAbs in the present study, thus, should be useful for detailed antigenic analysis of the H9 HAs for epidemiological studies, the selection of vaccine strains, and diagnosis.

Conclusion

H5 and H9 influenza virus isolates in Japan were genetically and antigenically characterized and the bird infected with the virus were pathologically examined.

In 2005, H5N2 influenza viruses were isolated form layer chickens in Ibaraki Prefecture, Japan and the Ibaraki prefectural governor declared the end of the H5N2 outbreak in June, 2006, 1 year after the first detection. Sixteen H5N2 viruses were isolated from 9 farms of the affected 41 farms. Phylogenetic and antigenic analysis of the isolates showed that these isolates were closely related to the H5N2 strains prevalent in Central America that have been circulating since 1994. Experimental infection of chickens with the index isolate (A/chicken/Ibaraki/1/2005 (H5N2)) demonstrated that this virus replicated efficiently in the respiratory tract without clinical signs. Pathological findings of the chickens indicated that the virus efficiently replicated in salivary epithelial cells. The virus was transmitted among the chickens in separated cage. This result indicated that the infection to chickens spread by droplet transmission in the outbreaks.

In 2008, H5N1 highly pathogenic avian influenza virus was isolated from whooper swans (*Cygnus cygnus*) found dead in Hokkaido, Japan. Pathological findings indicate that the swan died due to severe congestive edema in the lungs. Phylogenetic analysis of the HA genes of the isolates revealed that these are the progeny viruses of the isolates Clade 2.3.2 from poultry and wild birds in China, Russia, Korea, and Hong Kong. Antigenic analyses indicated that the viruses are different from the H5N1 viruses isolated from wild birds and poultry before 2007. The chickens vaccinated with A/duck/Hokkaido/Vac-1/2004 (H5N1) survived 14 days after challenge with the isolate, although small amount of the challenge virus was recovered from the tissues of the birds. These findings indicate that the H5N1 highly pathogenic avian influenza virus are circulating in wild birds in addition to domestic poultry in Asia and showing antigenic variation that may be due to vaccination.

Recently, H9N2 influenza viruses were isolated from poultry and mammals in Asian countries. The hemagglutinins (HAs) of H9 influenza viruses isolated from birds and mammals of different species were antigenically and genetically analyzed. Based

on the reactivity patterns with the panel of 8 monoclonal antibodies, 21 H9N2 virus strains isolated from birds and mammals were divided into 7 antigenically distinct groups. The present findings indicate that H9N2 viruses are genetically and antigenically varied.

The vaccinations to poultry in some countries resulted in antigenic variation of H5 and H9 influenza viruses isolated in Asia. These result indicate that the stamping-out policy is most important to control for avian influenza.

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Summary in Japanese (和文要旨)

日本で分離されたH5およびH9インフルエンザウイルスについて、遺伝子と抗原性を解析するとともに、宿主鳥の病理学的検索を実施した。

2005年、茨城県下で採卵鶏に鳥インフルエンザが発生し、流行が終息するまでに一年を要した。感染が確認された41農場のうち9農場の鶏から16株のH5N2ウイルスが分離された。分離株の抗原性および遺伝子解析の結果、いずれも1994年から中米の家禽で流行しているH5N2ウイルスと極めて近縁であることが明らかになった。分離株A/chicken/Ibaraki/1/2005 (H5N2)を実験感染させたニワトリは、臨床症状を示さなかったが、呼吸器からウイルスが回収された。病理検索の結果、唾液腺上皮細胞でウイルスが効率よく増殖していることが判った。また、本ウイルスは隔離ケージ間のニワトリに伝播したことから、飛沫により鶏群に感染が拡大したと推察された。

2008年、北海道のサロマ湖畔で発見された斃死オオハクチョウから、H5N1高病原性鳥インフルエンザウイルスを分離した。この斃死体の病理学的検索の結果、死因は重度の肺のうっ血性浮腫であった。分離ウイルス遺伝子の解析により、中国、ロシア、韓国および日本の秋田県、青森県で同年に分離されたウイルスと近縁であり、クレード2.3.2に属することが判った。分離ウイルスはこれまでに分離されたH5N1ウイルスの抗原性と大きく異なっていたため、国内備蓄Vac-1ワクチンの本ウイルスの攻撃に対するワクチンの防御免疫賦与効果を調べた。攻撃後、ワクチン接種ニワトリの組織の一部からウイルスが回収されたが、いずれの鳥も14日間症状を呈することなく生残した。すなわち、国内備蓄ワクチンは現在流行しているウイルスの感染に対しても、防御効果を示した。

近年アジアの家禽や哺乳動物からH9N2インフルエンザウイルスが分離されている。そこで、アジアの家禽に流行しているウイルスに近縁なA/swine/Hong Kong/10/1998（H9N2）および野生水禽から分離されたA/duck/Hokkaido/13/2000 (H9N2)に対するモノクローン抗体を作出し、当研究室のインフルエンザウイルスライブラリーに保管されている21株のH9ウイルスの抗原性を解析した。その結果、調べたH9N2ウイルスは抗原性と遺伝子が多様であることが分かった。

これらの鳥インフルエンザウイルスの抗原性が多様であることの理由として、一部の国で家禽にH5およびH9ウイルスワクチンが使用されていることが挙げられる。以上の成績は、家禽のインフルエンザは早期摘発淘汰を基本とする対策によって制圧されることを示している。