Epidemiological studies of avian influenza

(鳥インフルエンザの疫学的研究)

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Contents

Chapter II

A vaccine prepared from a non-pathogenic H5N1 influenza virus strain from the influenza virus library conferred protective immunity to chickens against the challenge with antigenically drifted highly pathogenic avian influenza virus

Preface

Avian influenza is caused by infection with type A influenza viruses belonging to *Orthomyxoviridae* family. Type A influenza virus is an enveloped pleomorphic particle with eight separate gene segments of negative-sense single-stranded RNA in the form of ribonucleoprotein complex inside. Each gene segments carries its own polymerase complex consisting of polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA) proteins which along with nucleoprotein (NP), matrix protein 1 (M1) and non-structural protein 2 (NS2) controls virus replication [21]. The surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA), are antigenically divided into H1-H16 and N1-N9 subtypes, respectively [8, 43].

On the basis of their pathogenicity for chicken, they are divided into non-pathogenic, low pathogenic or highly pathogenic avian influenza viruses (HPAIVs) [1, 18, 20]. It has been demonstrated that non-pathogenic avian influenza viruses (NPAIVs) of subtypes H5 and H7 originated from migratory ducks were introduced into poultry and acquired pathogenicity for chickens during multiple replications in chickens, and some of the H5N2 and H7N7 viruses have occasionally caused HPAI outbreaks [27].

Influenza A viruses widely distributed in birds and mammals including humans. Wild water birds are the natural reservoir of influenza viruses [18, 20, 44]. Among those, viruses of each of the known HA and NA subtypes (H1-H15 and N1-N9, respectively) other than H16 which have been isolated from migratory ducks (the

H16N3 virus has only been isolated once, from a seagull in 2005, [32]). Each of the past pandemic strains emerged through the genetic reassortment between the viruses of avian and human origins in the cells lining upper respiratory tracts of pigs [15, 19].

Ecological studies have revealed that a vast influenza virus gene pool for avian and mammalian influenza exists in migratory ducks and their nesting lake water and that influenza is a typical zoonosis [15]. Each of the known subtypes of influenza A viruses perpetuates among migratory ducks and their nesting lake water in nature [13, 31]. Influenza viruses have been isolated from freshly deposited faecal materials and from the lake water, indicating that migratory ducks have an efficient way to transmit viruses, *i*.e., via faecal material in the water supply. Experimental infection studies have established that influenza viruses preferentially replicate in the columnar epithelial cells forming crypts in the colon of ducks, causing no disease signs, and are excreted in high concentrations in the faecal materials [20]. Ducks are orally infected with influenza viruses by waterborne transmission at their nesting lakes in Siberia, Alaska and Canada around the Arctic Circle during their breeding season in summer [20].

Since H5N1 HPAIV was first detected in 1996 at a goose farm in Guangdong Province in China [36, 45], this infection then has spread in poultry of many countries in Eurasia and Africa. Since the H5N1 virus infections have become endemic in poultry farms in some countries and caused accidental transmission to humans, H5N1 viruses are recognized as one of the candidates for the next pandemic [10, 22, 33]. The widespread of H5N1 HPAIV in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the transmission of viruses to wild bird populations.

Since HPAIVs have been detected in migratory ducks found dead in China, Mongolia and other Eurasian countries in spring in 2005- 2010, it is, therefore, a serious concern that these HPAIVs may perpetuate in the lakes where migratory water birds nest in summer in Siberia. In the previous studies [15, 25], influenza A viruses of different subtypes were isolated from water of the lakes where migratory water birds nest in summer, even in autumn when wild water birds had left for the south for migration, suggesting that influenza A viruses are preserved in frozen lake water each year while the wild water birds are absent [13, 31].

In Chapter I of the present thesis, the results obtained from virological surveillance of avian influenza that has been carried out in the lakes in Hokkaido, Japan, where ducks congregate on their migration path from Siberia to the south in autumn are described. The information should be useful for better understanding of the ecology of influenza viruses and as the preparedness for future pandemics.

Geographical separation of host species has shaped the influenza gene pool into largely independently evolving Eurasian and American lineages [28]. Influenza virus isolates from faecal samples of ducks in their nesting lakes in Siberia phylogenetically belong to Eurasian lineage and closely correlate to those from birds, pigs and horses in Asia. It was also noted that these isolates closely correlated to the H5N1 influenza viruses isolated from chickens and humans [31]. Phylogenetic analysis of the HA of H5 influenza virus isolates from ducks in Japan revealed a close relationship with those of H5N1 influenza viruses from Hong Kong, southern China, Thailand, and Viet Nam indicating that the H5HA of these viruses originated from influenza viruses maintained in migratory ducks nesting in Siberia [13, 15]. In

addition, reassortment between viruses of Eurasian and North American lineages has been found in wild water bird populations, indicating that these two geographically segregated lineages represent mixing populations of viruses [6, 23, 25]. Nevertheless, these results indicate that the precursor genes of pandemic influenza viruses are perpetuated in water in the lakes where ducks nest in the northern territories.

Multicomponent control strategy has been recommended for the control of avian influenza outbreaks in poultry, such as maintenance of adequate bio-security in farms and industrial units [24], implementation of coordinated regional or national surveillance and diagnostic programs, stamping out of all infected poultry through culling and vaccination of uninfected flocks. Although stamping out of all infected poultry is the golden standard for the control and eradication of HPAIV, yet development of inactivated vaccine that match the antigenic properties of prevalent variant viruses is valuable. Vaccine strains should be antigenically closely related to the pandemic strain, non-pathogenic for humans and chicken embryos, of high yield in chicken embryos, and immediately prepared when the causative virus is characterized [15].

To provide seed vaccine strains, the Laboratory of Microbiology, Graduate School of Veterinary Medicine, Hokkaido University, designated as the OIE Reference Laboratory for Animal Influenza directed by Professor Hiroshi Kida has established a library of vaccine strain candidates consisting of isolates from wild water birds [15]. It has been demonstrated that the virus strains isolated from natural hosts in the library are useful for the preparedness for future pandemics [37].

Previous studies [12, 30, 35], have demonstrated that inactivated avian influenza vaccines prepared from influenza viruses from the library were potent enough against the challenge with HPAIVs. The vaccine strain A/duck/Hokkaido/Vac-1/2004 (H5N1) confers clinical protection and reduction of virus shedding against the challenge with HPAIV [31]. Many available commercial vaccines have been prepared from viruses of the North American lineage and these may be effective to control influenza caused by the viruses of the American lineage but not for the current HPAI outbreaks caused by infection with viruses of Eurasian lineage [37].

Therefore, in Chapter II, it is described that the efficacy of the vaccine prepared from a non-pathogenic influenza virus strain of the Eurasian lineage from the influenza virus library was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1), which is more antigenically closely related to the challenge virus strain A/duck/Hokkaido/1/2008 (H5N1), in chickens.

Chapter 1

Virological surveillance and phylogenetic analysis of the PB2 genes of influenza viruses isolated from wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan, in autumn

Introduction

Ecological studies have revealed that a vast influenza virus gene pool for avian and mammalian influenza exists in migratory ducks [15]. Each of the sixteen HA and nine NA subtypes of influenza A viruses are perpetuated among migratory water birds and their nesting lake water in nature [8, 13, 31, 43]. Transmission of H5 or H7 influenza viruses to domestic birds and especially in chickens may result in the emergence of HPAIV [27].

Since 2003, H5N1 HPAIVs have spread to 63 countries and seriously affected poultry in Eurasia and Africa. Well over billion birds have died from the infection or been killed for the control purposes. A HPAIV is generated when a non-pathogenic virus brought in by migratory birds from their nesting lakes in the north is transmitted to chickens via domestic ducks, geese, quails, turkeys and acquires pathogenicity for chickens. During over-wintering, some migratory birds were conversely infected with HPAIV H5N1 from poultry and have been found dead at lakes in the Eurasia in April to May on the way back to their nesting lakes in northern territories. It was found that each of the viruses isolated from these birds were genetically closely related to those of the isolates from poultry in China [4, 15, 33]. Thus, HPAIV strains currently circulating in poultry have returned to migratory water birds and spread worldwide [15].

Since it is of a great concern that these H5N1 viruses may perpetuate in the lakes in Siberia where migratory water birds nest in summer, virological surveillance

and phylogenetic analysis of influenza viruses have been carried out in autumn when these birds flew to Hokkaido, Japan in 2008-2009.

It is known that the PB2 protein is a component of the viral polymerase complex that plays an important role in virus replication [10, 22, 38], and is a determinant of host range and pathogenicity of influenza viruses [36, 39]. Therefore, PB2 genes of influenza viruses isolated from migratory ducks have been phylogenetically analyzed in the present study.

Materials and methods

Sample collection and virus isolation

A total of 1,626 faecal samples of wild water birds were collected in autumn in 2008-2009 at Lake Ohnuma, Wakkanai, and Ohno pond, Hokkaido University, Sapporo, Japan. The faecal samples collected were kept in chilled containers and transported to the laboratory. Virus isolation and subtyping were performed as previously described [17]. One virus of each of the HA and NA combinations was selected randomly by year of isolation among those isolated between 2000 and 2009 for genetic analyses (Table 1).

RNA extraction, RT-PCR, and nucleotide sequencing

RNA extraction and RT-PCR were conducted as previously described [25]. Partial-length PB2 genes were amplified using PB2 gene-specific primer set PB2-625F (5`-CAT GTA TGC TAC CAT CAA GGG-3`), and the universal primer Ba-PB2-2341R [11]. The PCR products were separated by 0.8% agarose gel electrophoresis and purified using the MiniEluteTM Gel Extraction Kit (Qiagen, USA) as recommended by the manufacturer. The purified products were used as templates in sequencing reactions using a BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). DNA sequences were assembled and edited using the program Genetyx ATGC (2008 Genetyx Corp.). The

accession numbers of PB2 genes sequenced in this study are available from DDBJ/EMBL/GenBank under accession numbers given in Table 2.

Phylogenetic analysis of the PB2 genes

Phylogenetic analysis was conducted using PB2 gene sequences of 36 representative strains from a total of 57 that were sequenced. Published sequences used in this study for phylogenetic comparison were obtained using BLAST homology searches from the influenza sequence database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The PB2 gene tree was generated using the Neighbor Joining (NJ) bootstrap method (1,000 replicates) implemented in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0) [41]. The evolutionary distances were calculated by the Maximum Composite Likelihood method [42]. Of those, 36 sequence data were phylogenetically analyzed. A phylogenetic tree was constructed on the basis of the partial nucleotide sequences of the PB2 genes (positions 1425-2192) of viruses isolated from wild water birds in Hokkaido in 2000 to 2009 (Fig. 1).

Results

Influenza A viruses isolated from faecal samples of wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

In the surveillance of avian influenza conducted in Hokkaido in autumn 2008-2009, 62 influenza viruses have been isolated from a total of 1,626 faecal samples. The HA (H1, H3-H7, H9-H12) and NA (N1-N3, N5-N9) subtypes of the isolates were identified. Twenty one HA and NA subtype combinations were detected in the present study (Table 1). In the surveillance studies in 2000-2009 performed by the laboratory, no H5N1 HPAIV was isolated from wild water birds that flew from their nesting lakes in Siberia to Hokkaido, Japan in autumn [25].

Sequencing and phylogenetic analysis of the PB2 genes of influenza virus isolates from migratory birds

Randomly selected 57 isolates out of 283 avian influenza viruses isolated in the surveillance studies in 2000-2009 were sequenced.

Phylogenetic tree of the PB2 genes was divided into American and Eurasian lineages. Duan *et al*. [6] showed that Eurasian lineage could be further divided into early and contemporary sublineages. The results of the phylogenetic analysis of the PB2 genes of the isolates in the present study, the PB2 genes of all of the 36 strains belonged to the Eurasian lineage and were grouped (bootstrap values more than 85) into contemporary

sublineages I and II.

The majority of the PB2 genes clustered in different groups of sublineage I. They either clustered together or showed close relation to the PB2 genes of influenza viruses isolated from domestic and wild birds in China, Russia, Australia and Korea. Among the viruses examined joined in sublineage I, three viruses A/duck/Hokkaido/69/2000 (H5N3), A/duck/Hokkaido/447/2000 (H5N3), and A/duck/Hokkaido/1005/2001 (H3N6), phylogenetically clustered with the PB2 genes of A/Hong Kong/486/1997 (H5N1), A/Hong Kong/485/1997 (H5N1), and A/Hong Kong/1074/1999 (H9N2) influenza viruses isolated from humans in Hong Kong in 1997 and 1999, respectively. This finding supports the results of a previous study indicating that the precursor genes for viruses with human pandemic potential are perpetuated in migratory ducks that originated from northern nesting lakes [31]. It was also noted that some of the strains characterized in this study, A/duck/Hokkaido/WZ76/2008 (H6N2), A/duck/Hokkaido/W76/2008 (H6N9) and A/duck/Hokkaido/69/2008 (H4N6) were closely related to strains, A/mallard/Korea/gH170/2007 (H7N7) and A/magpie/Korea/YJDI74/2007 (H7N7) isolated from domestic birds in Korea. Some viruses that fell in sublineage I were phylogenetically closely related to an isolate obtained from pintails in Alaska, virus strain A/northern pintail/Alaska/44204-108/06 (H3N1). Novel reassortant H5N1 HPAIV, A/chicken/Laos/P0130/2007 (H5N1) isolated from Laos [2] also belonged to this sublineage but was most closely related to a virus isolated from a migratory bird in Korea, virus strain A/shorebird/Korea/S6/2006 (H1N2).

The Eurasian sublineage II consisted of only one group (Fig 1). The H5N1 HPAIVs isolated from wild birds in China, Europe, and Japan belonged to this sublineage but none of the isolates tested in this study are closely related to these H5N1 HPAIVs.

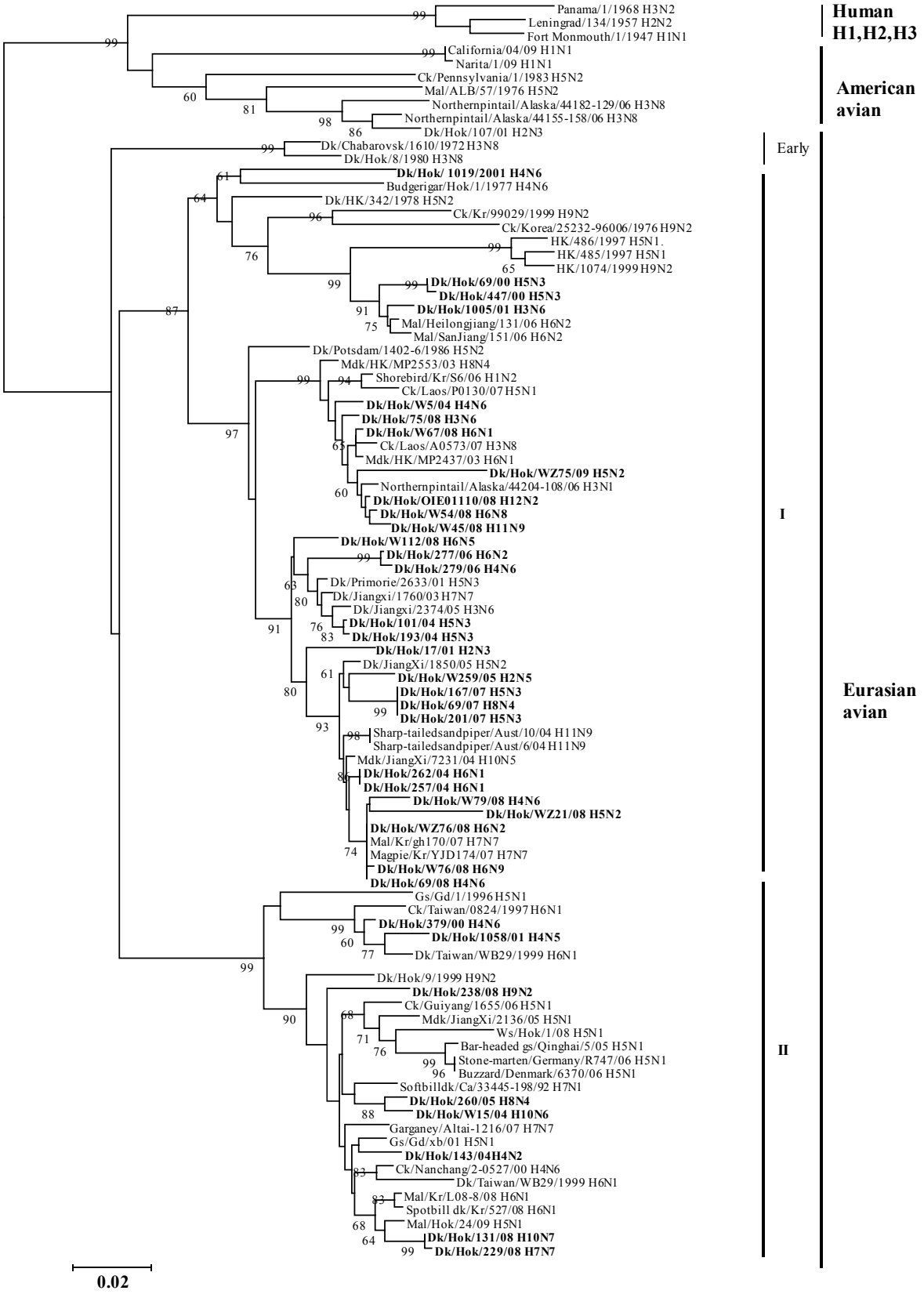
Panama/1/1968 H3N2
Leningrad/134/1957 H2N2
Fort Monmouth/1/1947 H1N1
California/04/09 H1N1
Narita/1/09 H1N1
Ck/Pennsylvania/1/1983 H5N2
Mal/ALB/57/1976 H5N2
Northernpintail/Alaska/44182-129/06 H3N8
Northernpintail/Alaska/44155-158/06 H3N8
Dk/Hok/107/01 H2N3
Dk/Chabarovsk/1610/1972 H3N8
Dk/Hok/8/1980 H3N8
Dk/Hok/ 1019/2001 H4N6
Budgerigar/Hok/1/1977 H4N6
Dk/HK/342/1978 H5N2
Ck/Kr/99029/1999 H9N2
Ck/Korea/25232-96006/1976 H9N2
HK/486/1997 H5N1.
HK/485/1997 H5N1
HK/1074/1999 H9N2
Dk/Hok/69/00 H5N3
Dk/Hok/447/00 H5N3
Dk/Hok/1005/01 H3N6
Mal/Heilongjiang/131/06 H6N2
Mal/SanJiang/151/06 H6N2
Dk/Potsdam/1402-6/1986 H5N2
Mdk/HK/MP2553/03 H8N4
Shorebird/Kr/S6/06 H1N2
Ck/Laos/P0130/07 H5N1
Dk/Hok/W5/04 H4N6
Dk/Hok/75/08 H3N6
Dk/Hok/W67/08 H6N1
Ck/Laos/A0573/07 H3N8
Mdk/HK/MP2437/03 H6N1
Dk/Hok/WZ75/09 H5N2
Northernpintail/Alaska/44204-108/06 H3N1
Dk/Hok/OIE01110/08 H12N2
Dk/Hok/W54/08 H6N8
Dk/Hok/W45/08 H11N9
Dk/Hok/W112/08 H6N5
Dk/Hok/277/06 H6N2
Dk/Hok/279/06 H4N6
Dk/Primorie/2633/01 H5N3
Dk/Jiangxi/1760/03 H7N7
Dk/Jiangxi/2374/05 H3N6
Dk/Hok/101/04 H5N3
Dk/Hok/193/04 H5N3
Dk/Hok/17/01 H2N3
Dk/JiangXi/1850/05 H5N2
Dk/Hok/W259/05 H2N5
Dk/Hok/167/07 H5N3
Dk/Hok/69/07 H8N4
Dk/Hok/201/07 H5N3
Sharp-tailedsandpiper/Aust/10/04 H11N9
Sharp-tailedsandpiper/Aust/6/04 H11N9
Mdk/JiangXi/7231/04 H10N5
Dk/Hok/262/04 H6N1
Dk/Hok/257/04 H6N1
Dk/Hok/W79/08 H4N6
Dk/Hok/WZ21/08 H5N2
Dk/Hok/WZ76/08 H6N2
Mal/Kr/gh170/07 H7N7
Magpie/Kr/YJD174/07 H7N7
Dk/Hok/W76/08 H6N9
Dk/Hok/69/08 H4N6
Gs/Gd/1/1996 H5N1
Ck/Taiwan/0824/1997 H6N1
Dk/Hok/379/00 H4N6
Dk/Hok/1058/01 H4N5
Dk/Taiwan/WB29/1999 H6N1
Dk/Hok/9/1999 H9N2
Dk/Hok/238/08 H9N2
Ck/Guiyang/1655/06 H5N1
Mdk/JiangXi/2136/05 H5N1
Ws/Hok/1/08 H5N1
Bar-headed gs/Qinghai/5/05 H5N1
Stone-marten/Germany/R747/06 H5N1
Buzzard/Denmark/6370/06 H5N1
Softbilldk/Ca/33445-198/92 H7N1
Dk/Hok/260/05 H8N4
Dk/Hok/W15/04 H10N6
Garganey/Altai-1216/07 H7N7
Gs/Gd/xb/01 H5N1
Dk/Hok/143/04H4N2
Ck/Nanchang/2-0527/00 H4N6
Dk/Taiwan/WB29/1999 H6N1
Mal/Kr/L08-8/08 H6N1
Spotbill dk/Kr/527/08 H6N1
Mal/Hok/24/09 H5N1
Dk/Hok/131/08 H10N7
Dk/Hok/229/08 H7N7
0.02
Human H1,H2,H3
American avian
Early
I
Eurasian avian
II

Fig. 1. Phylogenetic tree of influenza A virus PB2 genes

The phylogenetic tree was constructed using neighbour joining (NJ) method (1,000 replicates). For construction of this tree, 36 representative strains from a total of 57 that were sequenced. PB2 gene sequences each comprising 767 nucleotides (positions 1425-2192) were analyzed. This figure showing complete phylogram of avian influenza virus lineages with overall lineage of these isolates were of Eurasian avian and divided further into 2 distinct contemporary sublineages I and II. Bootstrap values below 60 are not shown. The strains sequenced in this study are indicated in bold.

Table 1. Influenza viruses isolated from faecal samples of free-flying water birds 2008-2009

Subtypes of influenza viruses isolated in the following year	
2008	2009
H3N2 (1)[a]	H1N3 (1)
H3N6 (3)	H1N5 (1)
H4N6 (11)	H4N6 (5)
H5N2 (1)	H5N1 (1)
H6N1 (4)	H5N2 (1)
H6N2 (1)	H6N1 (4)
H6N5 (1)	H6N8 (2)
H6N8 (1)	H11N9 (3)
H6N9 (1)	H12N5 (1)
H7N7 (1)	
H9N5 (1)	
H9N9 (1)	
H10N9 (2)	
H10N7 (11)	
H11N9 (2)	
H12N2 (1)	

[a]Number of isolates are shown in parenthesis.

Table 2. PB2 genes analyzed

Virus strain	Subtype	Accession number	Virus strain	Subtype	Accession number
A/duck/Hokkaido/379/00	H4N6	AB478622	A/duck/Hokkaido/277/06	H6N2	AB478604
A/duck/Hokkaido/69/00	H5N3	AB300036	A/duck/Hokkaido/W162/06	H6N5	AB478618
A/duck/Hokkaido/18//00	H10N4	AB282876	A/duck/Hokkaido/W299/06	H9N2	AB478621
A/duck/Hokkaido/1169/01	H1N1	AB478607	A/duck/Hokkaido/W95/06	H10N8	AB569460
A/duck/Hokkaido/95/01	H2N2	AY422042	A/duck/Hokkaido/W73/07	H1N1	AB478614
A/duck/Hokkaido/17/01	H2N3	AY422040	A/duck/Hokkaido/W282/07	H4N6	AB478623
A/duck/Hokkaido/86/01	H2N3	AY422041	A/duck/Hokkaido/167/07	H5N3	AB378679
A/duck/Hokkaido/1005/01	H3N6	AB478606	A/duck/Hokkaido/201/07	H5N3	AB378687
A/duck/Hokkaido/56/01	H3N8	AB478611	A/duck/Hokkaido/69/07	H8N4	AB569464
A/duck/Hokkaido/1058/01	H4N5	AB569458	A/duck/Hokkaido/75/08	H3N6	AB569452
A/duck/Hokkaido/1019/01	H4N6	AB569457	A/duck/Hokkaido/W79/08	H4N6	AB569462
A/duck/Hokkaido/24/02	H11N9	AB478596	A/duck/Hokkaido/69/08	H4N6	AB569448
A/duck/Hokkaido/83/04	H1N1	AB478598	A/duck/Hokkaido/WZ21/08	H5N2	AB569454
A/duck/Hokkaido/18/04	H3N8	AB478595	A/duck/Hokkaido/W67/08	H6N1	AB569588
A/duck/Hokkaido/ 143/04	H4N2	AB569459	A/duck/Hokkaido/WZ76/08	H6N2	AB569453
A/duck/Hokkaido/W5/04	H4N6	AB569461	A/duck/Hokkaido/W112/08	H6N5	AB569466
A/duck/Hokkaido/193/04	H5N3	AB299377	A/duck/Hokkaido/W54/08	H6N8	AB569449
A/duck/Hokkaido/257/04	H6N1	AB478601	A/duck/Hokkaido/W76/08	H6N9	AB569465
A/duck/Hokkaido/W109/04	H6N2	AB478616	A/duck/Hokkaido/ 229/08	H7N7	AB569456
A/duck/Hokkaido/W12/04	H6N2	AB478609	A/duck/Hokkaido/ 238/08	H9N2	AB569467
A/duck/Hokkaido/W59/04	H8N4	AB478612	A/duck/Hokkaido/131/08	H10N7	AB569451
A/duck/Hokkaido/89/04	H10N5	AB478599	A/duck/Hokkaido/WZ16/08	H10N9	AB569463
A/duck/Hokkaido/W259/05	H2N5	AB478620	A/duck/Hokkaido/W45/08	H11N9	AB569455
A/duck/Hokkaido/12/05	H3N2	AB478594	A/ws/Hokkaido/OIE110/08	H12N2	AB569450
A/duck/Hokkaido/W70/05	H3N8	AB478613	A/duck/Hokkaido/75/09	H5N2	AB569468
A/duck/Hokkaido/W268/05	H6N1	AB478603			
A/duck/Hokkaido/260/05	H8N4	AB478602			
A/duck/Hokkaido/279/06	H4N6	AB478605			
A/duck/Hokkaido/W206/06	H6N1	AB478619			

Discussion

Rapid world wide spread of HPAIV to 63 countries in Eurasia and Africa with H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in spring 2005, 2006, 2009 and 2010 raises concern that they may perpetuate in the northern nesting lakes in Siberia in summer. Since it was found that these H5N1 HPAIVs genetically closely related to those influenza viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan, intensive surveillance of avian influenza in migratory water birds should be continued. In 2008-2009 avian influenza surveillance, we isolated 62 influenza viruses from faecal samples collected from migratory ducks that flew from their northern nesting lakes to Hokkaido, Japan in autumn. Influenza viruses of different subtypes have been isolated from these wild water birds. Twenty one combinations of the HA and NA subtypes of influenza viruses were detected. No H5N1 HPAIV was found during the surveillance period, indicating that the H5N1 HPAIV has not been perpetuated, at least dominantly in wild water birds that nest in northern territory in summer. The present findings are in agreement with previous study [25] showing that the H5N1 HPAIV has not persisted yet in wild water birds that nest in Siberia in summer.

The phylogenetic analyses in the present study revealed that none of the PB2 gene sequences of influenza viruses tested closely related to HPAIV and none belonged to the American lineage. Although influenza A viruses of the H5N1 subtype were not detected in wild water birds in Hokkaido during the surveillance period, phylogenetic analysis

showed that some viruses examined in this study clustered with those of the H5N1 and H9N2 viruses isolated from humans in Hong Kong in 1997 and 1999, respectively [39]. This finding is in agreement with earlier report that indicated that precursor genes of pandemic influenza viruses are perpetuated in ducks nesting in Siberia [31]. These data further suggest that influenza virus genes similar to those that contributed to the emergence of the H5N1 virus in Hong Kong may still be circulating in migratory ducks that fly to Hokkaido from their nesting lakes in northern territory.

In addition, previous studies conducted in the Laboratory of Microbiology, School of Veterinary Medicine, Hokkaido University found some internal protein genes (PB2, PA, and M) of influenza viruses isolated from migratory birds in Hokkaido which phylogenetically clustered with those of influenza viruses of the American lineage [23, 25], indicating that inter-regional transmission of influenza virus genes does occur between the American and Eurasian gene pools among viruses obtained in Hokkaido. The grouping together of the PB2 gene of an influenza virus isolated from a pintail *(Anas acuta)* in Alaska with those of some viruses examined presently testifies to this phenomenon. The pintail (*Anas acuta*) species has been implicated in the inter-hemispheric transmission of influenza viruses between the American and Eurasian gene pools [14].

Intensive surveillance of avian influenza conducted in Hokkaido in autumn in 2008-2009, has demonstrated that no HPAIVs were isolated from wild water birds flying from their nesting lakes in Siberia, indicating that the HPAIV has not yet dominantly perpetuated in the lakes where they nest in summer. This finding is

strongly supported by the intensive surveillance of influenza virus in 19 years (1991-2009) (Fig. 2) in migratory birds from their northern nesting lakes that flew to the south in autumn where 22,744 samples collected, from those 795 influenza viruses were isolated and no HPAIV were detected. Surveillance of avian influenza in Hokkaido in October 2010, has detected two H5N1 HPAIVs (Fig. 3). On the basis of this finding, it was warned to strengthen surveillance of avian influenza in domestic poultry and wild birds. Then, due to the strengthened surveillance, in November 2010 in Shimane, chickens were found to be positive for infection with the H5N1 HPAIV, and then the H5N1 HPAIVs were detected in wild birds in Toyama, Tottori and Kagoshima prefectures. Due to the early detection of infections and proper control measures, each of the infections was well controlled.

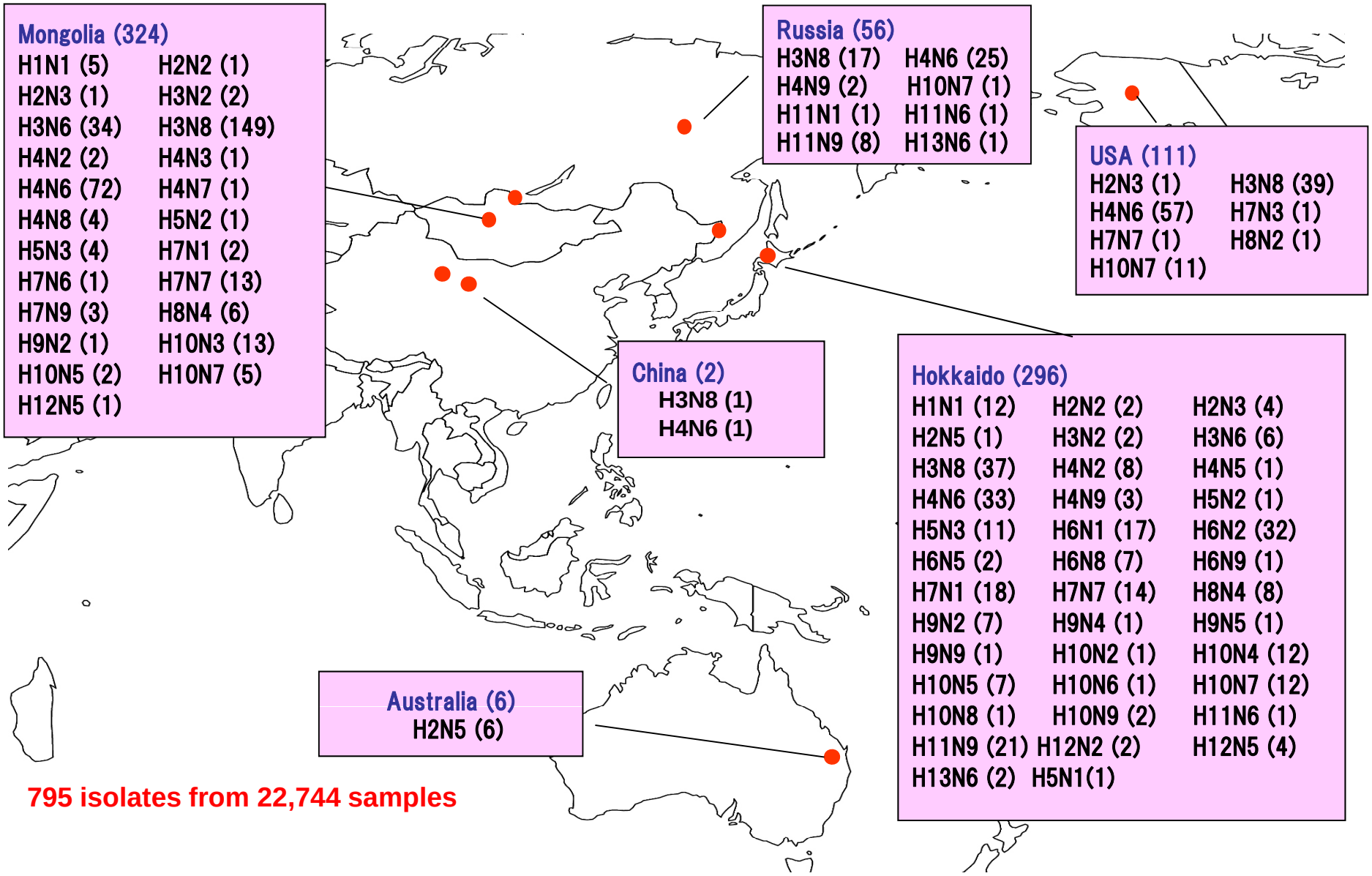

Fig. 2. Surveillance of avian influenza (1991～2009)

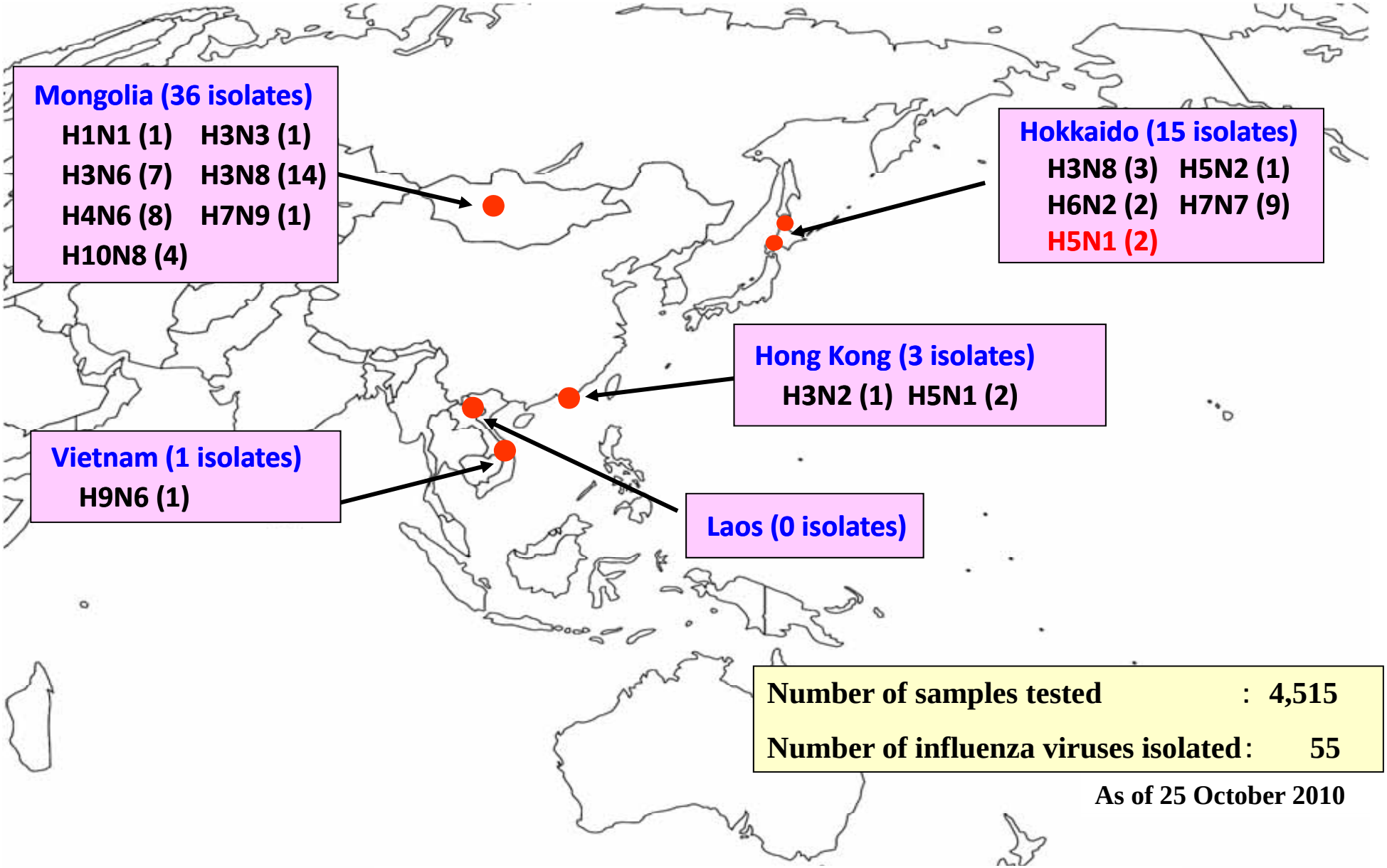

Fig. 3. Surveillance of avian influenza in 2010

Brief summary

Recent introduction of H5N1 HPAIV in wild birds from poultry in Eurasia signalled the possibility that this virus may perpetuate in nature. Surveillance of avian influenza especially in migratory birds, therefore, has been conducted to provide information on the viruses brought by them to Hokkaido, Japan, from their nesting lakes in Siberia in autumn. During 2008-2009, 62 influenza viruses of 21 different combinations of HA and NA subtypes were isolated. Up to September 2010, no HPAIV has been found, indicating that H5N1 HPAIV has not perpetuated at least dominantly in the lakes where ducks nest in summer in Siberia. The PB2 genes of 57 influenza viruses out of 283 influenza viruses isolated in Hokkaido in 2000-2009 were phylogenetically analyzed. None of the genes showed close relation to those of H5N1 HPAIVs that were detected in wild birds found dead in Eurasia on the way back to their northern territory in spring.

Chapter II

A vaccine prepared from a non-pathogenic H5N1 influenza virus strain from the influenza virus library conferred protective immunity to chickens against the challenge with antigenically drifted highly pathogenic avian influenza virus

Introduction

H5N1 highly pathogenic avian influenza virus (HPAIV) is causing panzootic outbreaks in poultry in Eurasia and Africa, posing serious concern for public health as well as live stock industry. The outbreaks of avian influenza caused by H5N1 HPAIVs spread to 63 countries and well over billion birds have died or been killed. In 15 countries human cases have been reported [15]. In addition, the H5N1 HPAIVs had returned to migratory birds, spread to Eurasia and Africa [5, 15]. Since each of the HA genes of pandemic influenza viruses has been originated from avian influenza viruses [44], it is now in a potential pandemic threat. H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in April to May in 2005, 2006, 2009 and 2010 were genetically closely related to H5N1 viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan [5, 15, 34]. Viruses similar to those have spread world-wide and it is therefore, a serious concern that these HPAIVs may perpetuate in the lakes in Siberia where they nest in summer and that those birds may bring HPAIVs to the south in autumn.

Early disease detection, movement restriction and stamping-out are the standard measures for the control of HPAI in poultry and found to be successful in rapid eradication of the HPAIV infection [7]. Vaccination is a limited application when stamping-out is not effective enough to control the disease [3]. Vaccination may be an optional tool to reduce virus load in the environment. Many commercial vaccines have been prepared from viruses of the North American lineage. These vaccines may be

less effective for the control of current HPAI outbreaks caused by the infection with viruses of the Eurasian lineage in Asia [12, 37]. Inactivated influenza vaccines for the control of the current avian influenza in Asia, therefore, had better to be prepared from an H5N1 virus strain belonging to the Eurasian lineage.

The OIE Reference Laboratory for HPAI at Hokkaido University has established the library of influenza viruses of all hemagglutinin (HA) and neuraminidase (NA) subtypes and their genes [15, 16]. The previous study [37] has demonstrated that the library of a panel of influenza virus strains isolated from natural hosts is useful for the preparedness for future pandemics. These influenza virus strains are stored in the library and have been used for the purpose of vaccine production and diagnosis.

Prolonged endemics of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries [30]. Previous studies showed that avian influenza vaccine prepared from non-pathogenic avian influenza viruses from the library conferred protective immunity against the challenge virus of antigenically similar [12, 30]. Ideally, vaccine strains that are antigenically and genetically closely related to the circulating variant strain and induce immunity against these antigenically drifted viruses are preferable. In the present study, the efficacy of the vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05 (H5N1) by reverse genetics against the challenge with antigenically drifted HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1) in chickens.

Materials and methods

Viruses

Influenza viruses, A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/08), A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) and A/whooper swan/Mongolia/3/2005 (H5N1) (Ws/Mon/05) and mutant A/whooper swan/Mongolia/3/2005 (H5N1) (ΔRRRRK rg-Mon/05), of clade 2.3.2, classical and clade 2.2 respectively were used. All viruses used in this study have been propagated and characterized antigenically and genetically as described [30]. All viruses were propagated in 10-day-old embryonated chicken eggs at 35ºC for 30 to 48 hrs and stored at -80ºC until use.

Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were used for vaccine preparation. A non-pathogenic avian influenza Dk/Vac-1/04 virus from the library, was generated as a reassortant virus between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) [12]. Ws/Mon/05 virus isolated from a whooper swan (*Cygnus cygnus)* found dead in Lake Khunt nuur, Mongolia [34] was genetically modified by reverse genetics with site-directed mutagenesis to generate a mutant ΔRRRRK rg-Mon/05 strain.

Meanwhile, Ws/Hok/08 (H5N1) virus that was isolated from whooper swan found dead at Notsuke Peninsular, in Hokkaido, Japan in May on their way back to their nesting lakes in northern territories [30] was used as the challenge virus.

Preparation of a genetically modified H5N1 HPAIV

Ws/Mon/05 was genetically modified by reverse genetics with site-directed mutagenesis as described [26]. Briefly, the amino acid RRRRK at the cleavage site of the HA were deleted and replaced with amino acid T by site-directed mutagenesis. The T mutation was introduced into the HA of Ws/Mon/05 cloned into the pHW2000 expression vector, using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the desired mutations was confirmed by sequencing the full length of the cloned HA genes. The mutant virus was designated ΔRRRRK rg-Mon/05 and confirmed to be non-pathogenic to chickens.

Intravenous pathogenicity (IVPI) of ΔRRRRK rg-Mon/05 in chickens

The intravenous pathogenicity index (IVPI) of ΔRRRRK rg-Mon/05 was 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 6-week-old chickens. The birds were observed for their disease manifestation at intervals of 24 hours in a 10 day period and each bird was scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead, at each observation. The IVPI was the mean score per bird per observation by the 10 days post inoculation.

To determine the amount of viral shedding from organs groups of six 4-week-old chickens were inoculated with the ΔRRRRK rg-Mon/3/05. Briefly, each of six 4-week-old chickens was inoculated with 0.1ml of the infectious allantoic fluids by intranasal route. The birds were observed for clinical signs in 14 days. Three days post inoculation,

each chicken from inoculated groups were culled and tissues (trachea, colon and kidneys) and swabs (cloacal and tracheal) samples were collected aseptically. To make a 10% suspension with MEM, the collected tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai). These suspension were serially 10-fold diluted with PBS and were inoculated into 10-day-old embryonated chicken eggs and incubated at 35ºC for 48 hours. Virus titres were calculated as previously described [12] and expressed as EID_{50} per gram and ml of tissue and swab, respectively. Serum samples were examined for the present of antibodies against the ΔRRRRK rg-Mon/3/05 by HI test. Chickens were observed for 14 days.

All chickens in these experiments were housed for 10 and 14 days respectively in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo) at a BSL 3 facility at the Hokkaido University Research Center for Zoonosis Control, Japan. All animal experiments were conducted in accordance to guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Vaccine preparation

A virus suspension of Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4ºC respectively. Virus inactivation was confirmed by inoculation into embryonated chicken eggs. The inactivated Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 viruses suspension were diluted with phosphate-buffered saline (PBS) (pH = 7.2) to appropriate concentrations based on HA titres and adjuvanted as described [12, 34].

Potency test of vaccine efficacy in chickens against antigenically drifted strains Ws/Hok/08

The potency of the vaccines was evaluated by challenging chickens inoculated with vaccines prepared from Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 with antigenically drifted Ws/Hok/08. Thirty 4-week-old chickens were divided into three groups and the inactivated avian influenza virus Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 vaccines were intramuscularly inoculated to ten chickens respectively as described [12, 35]. PBS was inoculated in ten control chickens. Three weeks after vaccination, all chickens were challenged intranasally with a dose 10^3 50% chicken lethal dose (CLD_{50}) of Ws/Hok/08. Four chickens of each group were sacrificed on day 3 post-challenge and the remaining six chickens were observed clinically for 14 days. When chickens died or were sacrificed, tracheal and cloacal swabs and their tissues (trachea, kidney, colon) were collected. Virus infectivity titres were determined by plaque assays.

Plaque assays

Virus infectivity titres in swabs and tissue samples were determined by plaque assay as described [40]. Briefly, MDCK cells were grown in 6-well tissue culture plates, when monolayer became confluent, ten-fold serial dilutions of swab and tissue samples were prepared in minimal essential medium (MEM) and 200 ul of each dilution was added to each well. After 1 hour adsorption at 37□C the inoculums were removed and the cells were overlaid with Eagle's MEM containing 0.9% Bacto-Agar (Difco). After incubation for 24-48 hrs, second overlay containing neutral red

(0.005%) was made and plaques were enumerated after overnight incubation. The limit of virus detection was 10^3 plaque forming units (PFU)/g of tissues or ml of swabs.

Results

Antigenic relatedness among the challenge and vaccine strain viruses

Okamatsu et al., 2010 [30] has shown that antigenicities of the HA of the isolates in 2008 were different from the H5N1 viruses isolated from wild birds and poultry before 2007. These findings indicate that the challenge virus strain Ws/Hok/08 (H5N1) is antigenically different from Dk/Vac-1/04 (H5N1).

Pathogenicity of ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1)

None of the chickens inoculated intravenously with ΔRRRRK rg-Mon/05 showed clinical signs in the 10 days observation period. The IVPI value of the strain is 0 indicating that ΔRRRRK rg-Mon/05 is non-pathogenic for chickens.

No virus was recovered from organs of the chickens inoculated with ΔRRRRK rg-Mon/05 intranasally except one bird from which organ sample trachea showed 1.75 EID_{50}/g. HI titres of chicken serum after 14 days post inoculation was 8HI and 4HI respectively.

Potency of vaccines in chickens

Table 1 shows serum HI antibody titres in the vaccinated chickens. The HI antibody titres of the vaccinated chickens showed increase in the antibody titres after 3 weeks of vaccination. Chickens inoculated with the test vaccines were challenged with HPAIV, Ws/Hok/08 on 3 weeks post vaccination. Two weeks after challenge, approximately 2^4 fold of HI antibody titres were increased in all vaccinated chickens to the homologous and heterologous viruses indicating virus replication occurred. In addition, all vaccinated chickens survived after challenge throughout 14 observation days without showing any disease signs, whereas all of the control chickens died within 2 days post challenge (Fig. 1). Viruses were not recovered from swabs and tissue samples of any of the vaccinated chickens after challenge by plaque assay.

Table 1. Hemagglutination-inhibition antibody titres of the sera of chickens before (0 day) and after (14 days) challenge

Vaccine strain	Chicken Number	HI titres with the following antigens on the day post challenge					
		Vac-1/04 (H5N1)		Ws/Mon/05 (H5N1)		Ws/Hok/08 (H5N1)	
		0	14	0	14	0	14
Vac-1/04	1	640	-[a]	320	-	320	-
	2	1280	-	160	-	160	-
	3	1280	-	160	-	80	-
	4	5120	-	1280	-	80	-
	5	5120	10240	1280	5120	80	640
	6	640	2560	160	1280	160	320
	7	2560	20480	160	5120	40	2560
	8	640	2560	160	1280	80	640
	9	2560	5120	80	1280	40	640
	10	5120	20480	320	640	40	160
ΔRRRRK rg-Mon/05	11	40	-	80	-	80	-
	12	20	-	40	-	80	-
	13	80	-	640	-	320	-
	14	40	-	320	-	160	-
	15	320	1280	640	2560	640	1280
	16	1280	2560	1280	2560	160	640
	17	640	640	640	640	80	160
	18	1280	2560	1280	5120	320	1280
	19	640	1280	640	1280	320	640
	20	320	1280	640	1280	320	1280
PBS	21-30	<20	-	<20	-	<20	-

[a] : no samples tested

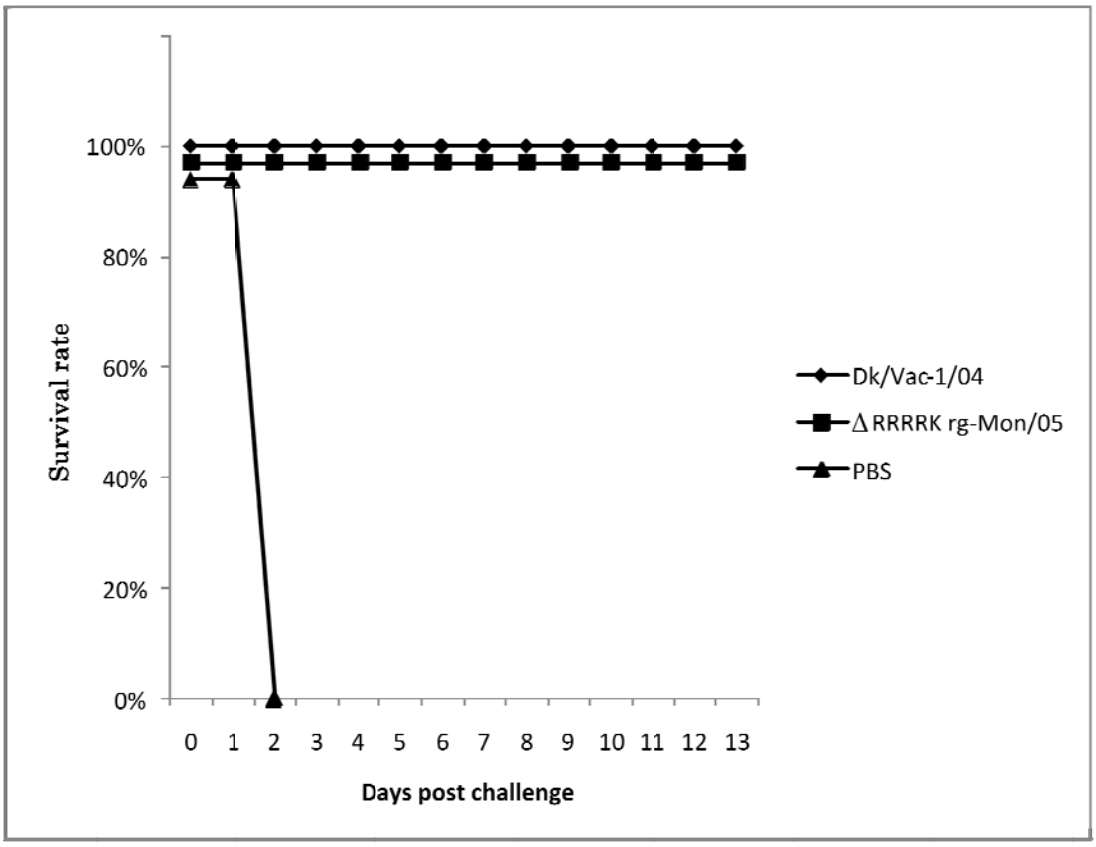

Fig.1. Survival of chickens after challenge with Ws/Hok/08

Ten 4-week-old chickens in each group were vaccinated with Dk/Vac-1/04, and ΔRRRRK rg-Mon/05 vaccines respectively. Three weeks after vaccination, chickens were challenged with Ws/Hok/08 virus. Control chickens (black triangle) were dead within 24 to 48 hrs after challenge. Vaccinated chickens were observed for 14 days.

Discussion

It is strongly recommended that stamping-out without misuse of vaccine is the best option for the eradication of HPAIV. Vaccination may be an optional tool in cases where the infection spreads widely [3]. Such a vaccine should ideally meet the following criteria: (i) safe for both hosts and the environment; (ii) economically feasible; and (iii) efficacious.

In the present study, both vaccine strains induced sufficient antibody response against the challenge with phylogenetically and antigenically different HPAIV. Previous studies [12, 30] have shown that Dk/Vac-1/04 vaccine protect chickens from clinical signs and induced antibodies against homologous and heterologous strains after challenge. In the present study, it was shown that vaccinated chickens with Dk/Vac-1/04 not only induced antibodies against homologous and heterologous strain but conferred protective immunity to chickens against the challenge of antigenically drifted HPAIV. These findings indicate that vaccine prepared from non-pathogenic avian influenza virus from the virus library is efficacious and protect chickens from HPAI. Furthermore, chickens even challenged with higher doses of viral challenge (10^3 CLD_{50}) did not show clinical signs indicating that the vaccines induced sufficient protective immunity in chickens to prevent clinical manifestations.

On the basis of the findings in the present study, inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain from the virus library conferred protective immunity against the challenge with antigenically drifted HPAIV.

The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05, which is more antigenically related to the challenge virus strain, in chickens. Therefore, it is proposed that vaccine strain selected from the non-pathogenic influenza virus library is efficacious and safe in protecting chickens from HPAI.

Brief summary

Inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library conferred protective immunity to chickens against the challenge of antigenically drifted HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1). The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1), which is more antigenically related to the challenge virus strain, in chickens.

Conclusion

In the present study, the author focused on the epidemiology of influenza viruses in the natural hosts, migratory ducks in order to better understand the ecology of influenza viruses in nature and on the usefulness of non-pathogenic influenza virus from the virus library as vaccine strains to confer protective immunity against H5N1 HPAIV.

Virological surveillance of avian influenza virus in the migratory birds that fly from their nesting lakes in Siberia to Hokkaido, Japan has been carried out every year in autumn since 2000. In the present study, no H5N1 HPAIV has been detected suggesting that H5N1 HPAIV have not dominantly perpetuated at their nesting lakes in Siberia until 2009. However, some of the isolates in the present study, having closely related to influenza viruses isolated in pintails which capable of flying long distances indicate that migratory birds played an important role in the spread of influenza viruses across their migratory routes. Recent positive cases of H5N1 HPAIV detected in domestic poultry and wild birds in Shimane, Toyama, Tottori, and Kagoshima prefectures respectively, highlighting the need to strengthen monitoring of influenza viruses in the natural hosts as well as other avian species and mammals. Due to the early detection of infections and proper control measures, each of the infections was well controlled.

The significance of using virus library for providing candidate vaccine strains was strengthened in the present study which showed that a vaccine prepared from a

non-pathogenic H5N1 influenza virus isolated from migratory ducks are equally effective to that prepared from a mutant H5N1 HPAIV in conferring protective immunity against antigenically drifted H5N1 HPAIV. Thus, for future preparation of a vaccine that could provide efficacious protective immunity to chickens, it is useful as an optional tool by selecting the virus strains from the influenza virus library.

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和文要旨

2005年以後、毎年春にユーラシアの各地で H5N1高病原性鳥インフルエンザウイルス（HPAIV）に感染し、斃死した野生水禽が見つかっている。南中国を含む東南アジアの越冬地でウイルスに感染し、北方圏の営巣湖沼に辿り着く前に斃死したものである。中には、シベリアの営巣湖沼までウイルスを持ち込む水禽もいるに違いない。もし、この HPAIV が、営巣湖沼に定着して優勢になれば、毎年秋に渡り鳥が HPAIV を運んでくる恐れがある。これをモニターするために、北海道大学 OIE Reference library for Animal Influenza では、毎年秋に北海道稚内市と札幌の湖沼にシベリアから飛来する渡り水禽の疫学調査を実施している。本研究では、2008～2009年に、21の異なる亜型から成る62株のインフルエンザウイルスが分離された。HPAIV は検出されなかった。1991 年から本研究を含め19年間、毎年秋にシベリアからモンゴルおよび北海道に飛来した野生水禽の糞便22,744検体から分離された795株のインフルエンザ A ウイルスの中に、HPAIV は検出されていない。この背景があったため、2010年10月14日に稚内市大沼に飛来したカモの糞便183検体から2株の H5N1 HPAIV が分離された際には、直ちに国内の野鳥と家禽の疫学検査の強化を提案し、11月に島根県で家禽および12月に富山、鳥取と鹿児島で野鳥のウイルス感染が早期に検出され、的確に対応することができた。

2000年から2009年までに北海道で分離されたウイルス452株の中から57株を選び、これらの PB2 遺伝子を解析した。2005年以来、春にユーラシアで斃死した野生水禽から分離された H5N1 HPAIV のそれらと近縁のものはなかった。

鳥インフルエンザ対策の基本は、感染の早期摘発と当該農場の全羽淘汰、周辺の家禽と生産物などの移動制限、ならびに管理衛生の徹底である。もし、発生・流行が拡大してこれらで対応することが困難な場合には、不活化ワクチンを使用することが選択肢の一つとして認められている。日本においては、斯かる不測の事態に備えて、家禽用 H5N1ウイルスワクチンが備蓄されている。アジアにおける H5N1 HPAIV の感染・流行が治まらず、抗原性が異なる変異ウイルスが次々に

選択されている現状の下で、著者は、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非病原性の A/duck/Hokkaido/Vac-1/2004 (H5N1)株で調製した備蓄ワクチンを接種したニワトリが、抗原変異 HPAIV、A/whooper swan/Hokkaido/2008 (H5N1)株の攻撃に耐えることを明らかにした。さらに、その免疫原性は、モンゴルで斃死したオオハクチョウから分離された、攻撃株の抗原性とより近縁な H5N1 HPAIV を reverse genetics と site-directed mutagenesis によって弱毒化した、RRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1)株で調製したワクチンのそれと同等であることを実証した。