Studies on the ecology and mechanism of interspecies transmission of avian influenza viruses

鳥インフルエンザウイルスの生態および異種宿主間伝播機構に関する研究

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Abbreviations

3′SLacNAc:	3′sialyllactosamine (Siaα2,3Galβ1,4GlcNAc)
3′SLeX:	sialyl Lewis X (Siaα2,3Galβ1,4(Fucα1,3)GlcNAc)
6′SLac:	6′sialyllactose (Siaα2,6Galβ1,4Glc)
BSA:	bovine serum albumin
BSL:	biosafety level
DAPI:	4′,6-Diamino-2-phenylindole, dihydrochloride
D-MEM:	Dulbecco's Modified Eagle's Medium
d.p.i.:	days post inoculation
EID_{50}:	50% egg infectious dose
FITC:	fluorescein isothiocyanate
Fuc:	fucose
Gal:	galactose
GalNAc:	*N*-acetylgalactosamine
Glc:	glucose
GlcNAc:	*N*-acetylglucosamine
HA:	hemagglutinin
HI:	hemagglutination-inhibition
HPAIV:	highly pathogenic avian influenza virus
HRP:	horse radish peroxidase
LacNAc:	lactosamine (Galβ1,4GlcNAc)
LPAIV:	low pathogenic avian influenza virus
MAA:	*Maackia amurensis* agglutinin
MAb:	monoclonal antibody
MDCK cells:	Madin–Darby canine kidney cells
MEM:	minimum essential medium
ML:	maximum-likelihood

M.O.I.:	multiplicity of infection
NA:	neuraminidase
NI:	neuraminidase-inhibition
PAA:	polyacrylamide
PBS:	phosphate buffered saline
PFU:	plaque forming unit
rHA:	recombinant hemagglutinin
RT-PCR:	reverse transcription polymerase chain reaction
SE:	standard error
Sia:	sialic acid (*N*-acetylneuraminic acid or *N*-glycolyl neuraminic acid)
SNA:	*Sambucus nigra* agglutinin
SPF:	specific pathogen free
TMB:	3,3',5,5'-tetramethylbenzidine
TRITC:	tetramethylrhodamine

Notes

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

1. **Hiono T, Ohkawara A, Ogasawara K, Okamatsu M, Tamura T, Chu DH, Suzuki M, Kuribayashi S, Shichinohe S, Takada A, Ogawa H, Yoshida R, Miyamoto H, Nao N, Furuyama W, Maruyama J, Eguchi N, Ulziibat G, Enkhbold B, Shatar M, Jargalsaikhan T, Byambadorj S, Damdinjav B, Sakoda Y, Kida H.** 2015. Genetic and antigenic characterization of H5 and H7 influenza viruses isolated from migratory water birds in Hokkaido, Japan and Mongolia from 2010 to 2014. Virus Genes **51:**57-68.

2. **Hiono T, Okamatsu M, Nishihara S, Takase-Yoden S, Sakoda Y, Kida H.** 2014. A chicken influenza virus recognizes fucosylated α2,3 sialoglycan receptors on the epithelial cells lining the upper respiratory tracts of chickens. Virology **456-457:**131-138.

3. **Hiono T, Okamatsu M, Igarashi M, McBride R, de Vries RP, Peng W, Paulson JC, Sakoda Y, Kida H.** Amino acid residues at positions 222 and 227 of the hemagglutinin together with the neuraminidase determine binding of H5 avian influenza viruses to sialyl Lewis X. Arch Virol *in press.*

Preface

Influenza A viruses belong to genus *Influenzavirus A* of family *Orthomyxoviridae*. Influenza A virus is an enveloped virus that contains two envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA), both of which recognize sialosides [1]. On the basis of the antigenic structure of HA and NA, influenza A viruses are classified into 16 HA subtypes and 9 NA subtypes. HA is a viral lectin, which specifically binds to terminal sialic acid (Sia) linked to penultimate galactose (Gal) with an α2,3 or 2,6 linkage and is responsible for the attachment of virions to host cell surface receptors [2]. The other envelope glycoprotein of influenza A viruses, NA, cleaves terminal Sia from penultimate Gal, which is essential for virus release from host cells and also facilitates cell attachment by destroying "decoy" receptors [3-5].

Influenza A viruses are zoonotic pathogens that are widely distributed among mammalian hosts such as humans, pigs and horses, as well as avian species such as chickens, ducks, many other poultry and wild birds [6, 7]. Influenza A viruses of each of the known subtypes have been isolated from water birds, especially from migratory ducks. In addition, influenza virus genome-like RNAs of a distinct lineage were recently detected in bats [8, 9]. Ducks are infected with influenza viruses by waterborne transmission at their nesting lakes close to the Arctic Circle in Siberia, Alaska, and Canada during their breeding season in the summer. These viruses replicate in columnar epithelial cells forming crypts in the colon, and are excreted in feces [10]. It has been suggested that the viruses are preserved in frozen lake water in the winter after the ducks leave for migration to the south [11]. Thus, the migratory ducks are the natural hosts for influenza A viruses.

It is known that the pathogenicity of influenza A viruses for chickens ranges from asymptomatic to systemic infections with low to high mortality. Since late 2003,

H5N1 highly pathogenic avian influenza viruses (HPAIVs) have seriously affected poultry in Eurasia and Africa. HPAIVs are generated when non-pathogenic viruses circulating among water birds are transmitted to chickens via domestic water and terrestrial birds, where they acquire pathogenicity in chickens via multiple infection and replication in the chicken population [12]. After 2005, H5N1 HPAIVs have been isolated from dead migratory water birds on the way back to their nesting lakes in Siberia in the spring. The pathogenicity of HPAIVs to migratory ducks varies depending on the virus strain [13-15]; in general, HPAIVs are less pathogenic to ducks compared with chickens [16]. Indeed, infected ducks shed viruses without showing any clinical signs [10]. Thus, it has been widely postulated that migratory ducks play crucial roles in the widespread dissemination of HPAIVs in poultry by carrying viruses along with their migrations.

In the previous study, intensive surveillance of avian influenza was conducted in Hokkaido, Japan since 1996 and Mongolia since 2001 in autumn [14, 17-20]. Japan and Mongolia are located on the migration route of wild birds that fly from their northern territory in Siberia to the south. Accordingly, surveillance of avian influenza of migratory water birds in these areas in autumn is effective to monitor viruses that are maintained in the nesting lakes in Siberia and spread southward with their migration. In the present study, surveillance of avian influenza of migratory water birds was conducted from 2010 to 2014. The results of antigenic and genetic analyses on the isolates of H5 and H7 subtypes were described in Chapter I. Genetic diversity and antigenic stability of these viruses are also discussed. The present results should provide a better understanding of the ecology of non-pathogenic avian influenza viruses circulating in migratory water birds.

Mammalian cells are covered with dense layers of glycolipids, glycoproteins, glycophospholipid anchors, and proteoglycans, which are called glycocalyx [21]. Sias

are typically outmost components of the glycan and are often target of pathogen attachment. Due to its high expression level on outer membrane and variety of linkages to the underlying sugar chain, it has been widely accepted that Sias play physiologically important roles. Influenza viruses recognize sialosides on host epithelial cells as their receptors and their glycan-binding specificities are the key determinant of host range; avian influenza viruses preferentially bind α2,3 sialosides, whereas human influenza viruses prefer α2,6 sialosides [22, 23]. This specificity is also consistent with receptor distribution in host tissues; α2,3 sialosides are predominantly detected on epithelial cells of the duck intestine [24, 25], whereas α2,6 sialosides are predominantly detected on epithelial cells of the human trachea [26]. Previous studies showed that chickens were rarely infected directly with non-pathogenic avian influenza viruses isolated from ducks, even though most influenza viruses isolated from chickens recognize α2,3 sialosides as the receptors, similarly to the viruses of duck origin [22, 27-29]. In Chapter II, distribution of α2,3 sialosides on epithelial cells of the tissues of ducks and chickens are compared. The results demonstrate that sialyl Lewis X (3´SLeX), which is a fucosylated α2,3 sialoside was predominantly detected on epithelial cells of the chicken trachea, whereas this glycan structure was not found in the duck intestinal tract. Receptor-binding specificity of duck and chicken influenza viruses to fucosylated and non-fucosylated α2,3 sialosides is also analyzed. The molecular basis on the interaction between fucosylated α2,3 sialosides and influenza viruses is discussed in Chapter III. The findings obtained in the present studies should contribute to the better understanding of the interspecies transmission of avian influenza viruses and expand knowledge on receptor specificity of influenza viruses.

Chapter I

Genetic and antigenic characterization of H5 and H7 influenza viruses isolated from migratory water birds in Hokkaido, Japan and Mongolia from 2010 to 2014

Introduction

Since late 2003, H5N1 HPAIVs have seriously affected poultry in Eurasia and Africa. After 2005, H5N1 HPAIVs have been isolated from dead migratory water birds on the way back to their nesting lakes in Siberia in the spring [14, 30-31]. In 2010, two H5N1 HPAIVs were isolated from fecal samples of ducks on the flyway of migration from Siberia to the south in Hokkaido [32]. In addition, during 2013–2014, outbreaks caused by H5N8 HPAIVs in poultry were reported in China, South Korea, Japan, Germany, the Netherlands, the United Kingdom, Italy, the United States of America, Taiwan, and Hungary [33]. These viruses were reassortants between H5 HPAIVs and viruses of water birds [34, 35]. It is of great concern whether these HPAIVs circulate among wild water birds in their northern nesting lakes in the summer. In addition, after the first case of human infection by the H7N9 influenza virus in March 2013, influenza viruses of the H7N9 subtype have been continuously detected in poultry in China [36, 37]. It is possible that these H7N9 viruses are transmitted to the wild bird population, which could result in the wide dissemination of this virus strain. Accordingly, continued monitoring for the H5 and H7 influenza viruses in the wild bird population is essential for the control of avian influenza in poultry.

Japan and Mongolia are located on the migration route of wild birds that fly from their northern territory in Siberia to the south. Accordingly, surveillance of avian influenza of migratory water birds in these areas in autumn is effective to monitor viruses that are maintained in the nesting lakes in Siberia and spread southward with their migration. In the present study, to monitor avian influenza viruses of the H5 and H7 subtypes in the wild water bird population, fecal samples of migratory water birds in

Hokkaido, Japan and Mongolia were collected in autumn from 2010 to 2014, and virus isolates were genetically and antigenically analyzed.

Materials and Methods

Isolation and identification of viruses

A total of 8,103 fecal samples were collected from migratory water birds in Sapporo (Ohno pond, 43°07’N, 141°34’E) and Wakkanai (Lake Ohnuma, 45°39’N, 141°77’E) in Hokkaido, Japan as well as in the Arkhangai province (Ugii nuur, 47°76’N, 102°74’E; Doitiin suagaan nuur, 47°37’N, 102°31’E; Durru tsagaan nuur, 49°00’N, 101°12’E; Tsaggaan nuur, 48°23’N, 102°35’E; Alagzegstei nuur, 47°37’N, 102°32’E; Ulzitte village, 48°04’N, 102°39’E) and the Bulgan province (Khunt nuur 48°25’N, 102°34’E; Khunt rashaan nuur, 48°27’N, 102°32’E; Sharga nuur, 48°55’N, 101°56’E) in Mongolia from 2010 to 2014. Each sample was mixed with transport medium as described previously [18] and inoculated into the allantoic cavities of 10-day-old chicken embryos. The subtypes of influenza viruses were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests with antisera to the reference influenza virus strains.

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluid of chicken embryos infected with viral isolates by TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA) and reverse-transcribed with the Uni12 primer [38] and M-MLV Reverse Transcriptase (Life Technologies). The full-length HA gene segment was amplified by polymerase chain reaction with gene-specific primer sets [38]. Direct sequencing of each gene segment was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and an auto-sequencer 3500 Genetic Analyzer (Life Technologies).

Sequencing data were analyzed using GENETYX® Network version 12 (GENETYX, Tokyo, Japan). The nucleotide sequences were phylogenetically analyzed based on H5 or H7 HA genes of influenza viruses by the maximum-likelihood (ML) method with Tamura-Nei model and bootstrap analysis (n=1,000) using MEGA 5.0 software (http://www.megasoftware.net/) with default parameters. Nucleotide sequences of 13 H5 and 19 H7 HA genes were compared with those of reference sequences. For reference sequences, H5 and H7 HA genes of viruses recently isolated in Asia and Europe as well as other classical reference strains were selected and obtained from GenBank/EMBL/DDBJ and Global Initiative on Sharing Avian Influenza Data (GISAID).

Antigenic analysis

The antigenic properties of representative isolates were determined by the cross HI test using chicken polyclonal antisera and the fluorescent antibody method with monoclonal antibodies (MAbs) against H5 or H7 HAs [39-41]. Preparation of chicken polyclonal antisera against representative influenza virus strains and HI tests were performed as described previously [42, 43]. For the fluorescent antibody assays, MDCK cells infected with representative influenza viruses were fixed with cold 100% acetone 8 h post-inoculation. The reactivity patterns of the MAbs with viruses were investigated by the immunofluorescence with a FITC-conjugated goat IgG to mouse IgG (ICN Biomedicals, Aurora, OH, USA). Fluorescence was visualized with an Axiovert 200 inverted microscope (Carl Zeiss, Oberkochen, Germany).

Results

Isolation of influenza A viruses from fecal samples of migratory water birds

A total of 350 viruses were identified from 8,103 fecal samples of migratory water birds (Table 1). Positive rates of influenza virus isolation were 4.0% in Sapporo, 1.9% in Wakkanai, and 5.6% in Mongolia. The subtypes of each isolate are indicated in Table 1. In total, 13 H5, including two HPAIVs [32] and 19 H7 influenza A viruses were isolated (Table 2).

Genetic analysis of H5 avian influenza viruses

Full-length sequences of the HA genes of the H5 isolates were determined. The deduced amino acid sequences of the HA cleavage site of the H5 isolates except A/duck/Hokkaido/WZ83/2010 (H5N1) and A/duck/Hokkaido/WZ101/2010 (H5N1), are RETR/GLF ("/" indicates HA1/HA2 cleavage site), the typical cleavage site motif of H5 low pathogenic avian influenza viruses (LPAIVs). The amino acid residues at positions 190, 225, 226, 227 and 228 (H3 numbering is used throughout [44]), which are well-established amino acid positions related to receptor specificity of influenza viruses, of these viruses are E, G, Q, S, and G, respectively; all of them are of the avian type motif [45].

The HA genes of the H5 isolates were phylogenetically analyzed by the ML method along with those of other strains containing recent isolates of HPAIVs and LPAIVs (Fig. 1). H5 HA genes were phylogenetically divided into two lineages: Eurasian and North American. Viruses in the Eurasian lineage clustered into three

Table 1. Influenza viruses isolated from migratory water birds in the surveillance in autumn between 2010 to 2014

Location	Subtypes of influenza viruses isolated in following years				
	2010	2011	2012	2013	2014
Sapporo, Japan	H3N8 (2)[a] H5N2 (1) H6N1 (2) H7N7 (10)	H3N8 (1) H4N6 (4)	H6N1 (1) H11N3 (2)	H2N1 (8) H12N2 (2)	H1N1 (1) H3N8 (3) H5N2 (1) H8N2 (1) H8N4 (5)
Wakanai, Japan	H2N3 (1) H3N8 (1) H5N1 (2)[b] H8N4 (2)	H7N7 (2)	H3N8 (1) H4N2 (2) H4N6 (1) H6N1 (7) H6N2 (2) H12N1 (1) H13N2 (2)	H3N8 (1) H4N2 (1) H4N6 (6) H6N5 (1) H7N2 (5) H16N3 (1)	H4N6 (2) H5N2 (1) H5N3 (2) H11N2 (1)
Mongolia, Arkhangai	H3N6 (5) H3N8 (3) H4N6 (3) H7N9 (1) H10N8 (2)	H3N6 (1) H3N8 (10) H4N3 (1) H4N6 (1) H5N3 (2) H8N4 (1) H10N7 (1)	H3N6 (1) H3N8 (3) H4N6 (1) H7N7 (1)	H1N1 (4) H1N3 (2) H3N1 (5) H3N5 (1) H3N8 (20) H4N1 (3) H4N5 (1) H4N6 (9) H6N1 (3) H6N2 (1) H6N5 (1) H12N5 (1)	H2N2 (1) H3N3 (1) H3N6 (3) H3N8 (43) H4N1 (1) H4N6 (15) H4N8 (7) H5N2 (1) H5N3 (1) H5N7 (2) H6N6 (5) H8N4 (1) H8N8 (1) H10N3 (2) H10N6 (2) H10N7 (2) H10N8 (2) H10N9 (2)
Mongolia, Bulgan	H1N1 (1) H3N3 (1) H3N6 (1) H3N8 (11) H4N6 (6) H10N8 (2)	H3N8 (9) H4N8 (1) H8N4 (1)	H3N8 (11) H4N6 (1) H4N8 (1) H8N4 (1)	H1N1 (1) H3N8 (5) H6N1 (1) H10N3 (1)	H3N6 (2) H3N8 (21) H4N6 (2) H6N6 (1)

[a] Number of isolates of each antigenic subtype is shown in parenthesis.

[b] Kajihara et al., 2011 [32].

H5 and H7 viruses are underlined.

Table 2. H5 and H7 influenza viruses isolated from migratory water birds in the surveillance in autumn between 2010 to 2014

HA subtypes	Years	Locations	Names	Acccession Number
H5	2010	Sapporo, Japan	A/duck/Hokkaido/101/2010 (H5N2)	LC018988
		Wakkanai, Japan	A/duck/Hokkaido/WZ83/2010 (H5N1)[a]	AB612901
			A/duck/Hokkaido/WZ101/2010 (H5N1)[a]	AB612909
	2011	Mongolia, Arkhangai	A/duck/Mongolia/194/2011 (H5N3)	AB677936
			A/duck/Mongolia/195/2011 (H5N3)	AB677937
	2014	Sapporo, Japan	A/duck/Hokkaido/166/2014 (H5N2)	LC018989
		Wakkanai, Japan	A/duck/Hokkaido/W240/2014 (H5N3)	LC018990
			A/duck/Hokkaido/W280/2014 (H5N3)	LC018991
			A/duck/Hokkaido/WZ20/2014 (H5N2)	LC011446
		Mongolia, Arkhangai	A/duck/Mongolia/107/2014 (H5N7)	LC011447
			A/duck/Mongolia/211/2014 (H5N7)	LC011482
			A/duck/Mongolia/256/2014 (H5N2)	LC011483
			A/duck/Mongolia/334/2014 (H5N3)	LC011484
H7	2010	Sapporo, Japan	A/duck/Hokkaido/1/2010 (H7N7)	AB622425
			A/duck/Hokkaido/3/2010 (H7N7)	LC018972
			A/duck/Hokkaido/4/2010 (H7N7)	LC018973
			A/duck/Hokkaido/5/2010 (H7N7)	LC018974
			A/duck/Hokkaido/6/2010 (H7N7)	LC018975
			A/duck/Hokkaido/10/2010 (H7N7)	LC018976
			A/duck/Hokkaido/14/2010 (H7N7)	LC018977
			A/duck/Hokkaido/45/2010 (H7N7)	LC018978
			A/duck/Hokkaido/47/2010 (H7N7)	LC018979
			A/duck/Hokkaido/50/2010 (H7N7)	LC018980
		Mongolia, Arkhangai	A/duck/Mongolia/129/2010 (H7N9)	AB828686
	2011	Wakkanai, Japan	A/duck/Hokkaido/W62/2011 (H7N7)	AB698072
			A/duck/Hokkaido/W63/2011 (H7N7)	AB698073
	2012	Mongolia, Arkhangai	A/duck/Mongolia/47/2012 (H7N7)	AB755793
	2013	Wakkanai, Japan	A/duck/Hokkaido/W19/2013 (H7N2)	LC018981
			A/duck/Hokkaido/W20/2013 (H7N2)	LC018982
			A/duck/Hokkaido/W57/2013 (H7N2)	LC018983
			A/duck/Hokkaido/WZ15/2013 (H7N2)	LC018984
			A/duck/Hokkaido/WZ77/2013 (H7N2)	LC018985

[a] Kajihara et al., 2011 [32].

The nucleotide sequences of the HA gene in the present study have been registered with GenBank/EMBL/DDBJ.

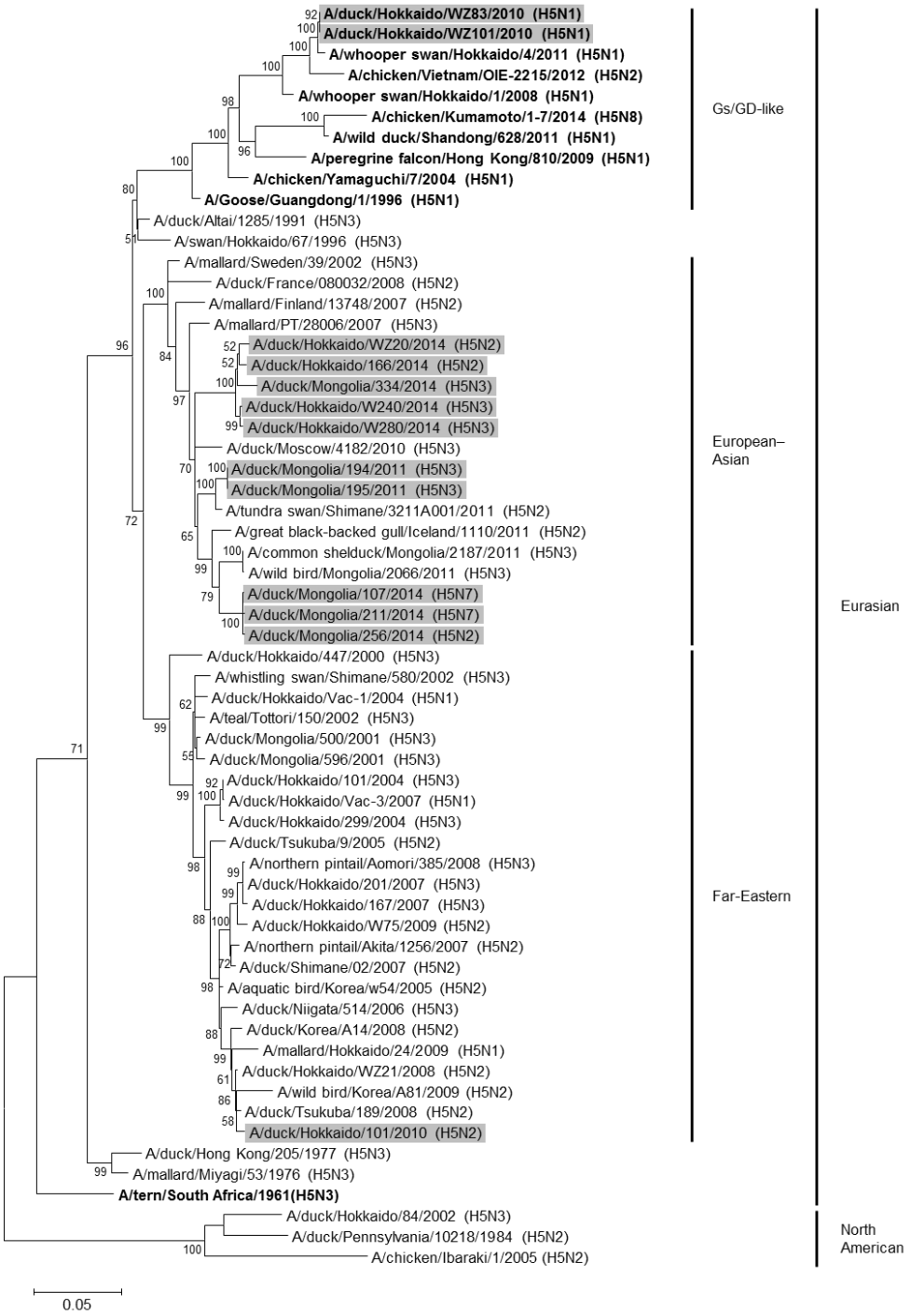


Fig. 1. Phylogenetic tree for the H5 HA genes of influenza viruses.

Full-length of the HA genes of all 13 viruses of the H5 subtype were analyzed by the maximum likelihood (ML) method along with that of reference strains using MEGA 5.0 software (http://www.megasoftware.net/). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray. HPAIVs are indicated in bold.

different sublineages: A/goose/Guangdong/1/1996 (H5N1) (Gs/GD)-like, to which recent HPAIVs circulating in Asia belong, Far-Eastern, and European–Asian. The HA gene of A/duck/Hokkaido/101/2010 (H5N2) was classified into the Far-eastern sublineage, whereas the other H5 LPAIVs isolated in the present study clustered into the European–Asian sublineage.

Antigenic analysis of H5 avian influenza viruses

Representative strains of the H5 isolates were antigenically analyzed by the cross HI test (Table 3). A/duck/Hokkaido/101/2010 (H5N2) and A/duck/Mongolia/194/2011 (H5N3) were antigenically closely related to A/mallard/Hokkaido/24/2009 (H5N1), which is previously shown to be antigenically closely related to other viruses circulating among wild water birds [20]. On the other hand, the antigenicity of these viruses was different from that of Gs/GD-like HPAIVs of genetic clade 2.3.2.1 recently isolated in Asia. Interestingly, the H5 viruses isolated in the present study reacted with the antiserum against A/chicken/Kumamoto/1-7/2014 (H5N8) of the clade 2.3.4.4 virus in Gs/GD-like sublineage at high titer compared with that of the homologous strain. On the other hand, A/chicken/Kumamoto/1-7/2014 (H5N8) reacted with the antiserum against A/mallard/Hokkaido/24/2009 (H5N1) at significantly lower titer compared with that of the homologous titer.

Representative strains of the H5 isolates were antigenically analyzed using a panel of MAbs recognizing six antigenic sites on the HA protein of A/duck/Pennsylvania/10218/84 (H5N2) [41] (Table 4). Each of the MAbs bound to LPAIVs, while most of the antibodies did not bind to Gs/GD-like HPAIVs recently

Table 3. Cross HI test of H5 influenza viruses with polyclonal antibodies

Lineage	Clade[a]	Viruses	HI titer of the antisera						
			Eurasian						North American
				Gs/GD-like					
			Mal/Hok/ 24/09	Ws/Hok/ 1/08	Pf/HK/ 810/09	Ck/Km/ 1-7/14	Ck/Yam/ 7/04	Tn/SA/ 61	Ck/Ibr/ 1/05
Eurasian									
Far-Eastern	-	A/duck/Hokkaido/101/2010 (H5N2)	2,560	160	80	1,280	2,560	2,560	1,280
	-	A/mallard/Hokkaido/24/2009 (H5N1)	1,280	80	40	2,560	1,280	2,560	1,280
European–Asian	-	A/duck/Mongolia/194/2011 (H5N3)	1,280	160	80	1,280	5,120	2,560	2,560
Gs/GD-like	2.3.2.1	**A/whooper swan/Hokkaido/1/2008 (H5N1)**	40	640	40	80	320	80	<20
	2.3.4	**A/peregrine falcon/Hong Kong/810/2009 (H5N1)**	20	20	1,280	20	40	<20	<20
	2.3.4.4	**A/chicken/Kumamoto/1-7/2014 (H5N8)**	20	<20	160	640	20	40	<20
	2.5	**A/chicken/Yamaguchi/7/2004 (H5N1)**	320	320	80	80	5,120	1,280	320
-[b]	-	**A/tern/South Africa/1961 (H5N3)**	640	20	20	640	1,280	2,560	320
North American	-	A/chicken/Ibaraki/1/2005 (H5N2)	320	20	<20	<20	1,280	320	20,480

[a] Genetic clades for Gs/GD-like sublineage viruses are according to the definition of WHO/OIE/FAO H5N1 Evolution Working Group [46].

[b] A/tern/South Africa/1961 (H5N3) is not classified into any of sublineages.

Viruses isolated in this study are highlighted in gray.

HPAIVs are shown in bold.

Homologous titers are underlined.

Mal, Mallard; Ws, Whooper swan; Pf, Peregrine falcon; Ck, Chicken; Tn, Tern.

Hok, Hokkaido; HK, Hong Kong; Km, Kumamoto; Yam, Yamaguchi; SA, South Africa; Ibr, Ibaraki.

Table 4. Antigenic analysis of H5 influenza viruses with monoclonal antibodies

Lineage	Clade[a]	Viruses	Monoclonal antibody[b]						
			I	II	III	IV		V	VI
			D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2
Eurasian									
Far-Eastern	-	A/duck/Hokkaido/101/2010 (H5N2)	+	+	+	+	+	+	+
	-	A/mallard/Hokkaido/24/2009 (H5N1)	+	+	+	+	+	+	+
European–Asian	-	A/duck/Mongolia/194/2011 (H5N3)	+	+	+	+	+	+	+
	-	A/duck/Hokkaido/W240/2014 (H5N3)	+	+	+	+	+	+	+
	-	A/duck/Mongolia/107/2014 (H5N7)	+	+	+	+	+	+	+
Gs/GD-like	2.3.2.1	**A/whooper swan/Hokkaido/1/2008 (H5N1)**	+	-	-	-	-	-	-
	2.3.4	**A/peregrine falcon/Hong Kong/810/2009 (H5N1)**	+	-	-	-	-	-	-
	2.3.4.4	**A/chicken/Kumamoto/1-7/2014 (H5N8)**	-	-	-	-	-	+	-
	2.5	**A/chicken/Yamaguchi/7/2004 (H5N1)**	-	+	+	+	+	-	+
-[c]	-	**A/tern/South Africa/1961 (H5N3)**	+	-	+	-	-	+	+
North American	-	A/chicken/Ibaraki/1/2005 (H5N2)	-	-	-	-	-	-	-

[a] Genetic clades for Gs/GD-like sublineage viruses are according to the definition of WHO/OIE/FAO H5N1 Evolution Working Group [46].

[b] Monoclonal antibodies to the HA of A/duck/Pensylvania/10218/1984 (H5N2) [41].

[c] A/tern/South Africa/1961 (H5N3) is not classified into any of sublineages.

Viruses isolated in this study are highlighted in gray.

HPAIVs are shown in bold.

circulating in Asia.

Genetic analysis of H7 avian influenza viruses

Full-length sequences of the HA genes of the H7 isolates were determined. The deduced amino acid sequences of the HA cleavage site of all H7 isolates tested were PKGR/GLF, indicating a low pathogenicity to chickens. The amino acid residue at position 226 was identified as Q in all the HA proteins of the isolates, in contrast to the L residue at amino acid position 226 of the HA of H7N9 influenza viruses from recent human isolates [47].

The HA genes of H7 isolates were phylogenetically analyzed by the ML method along with those of other reference strains of HPAIVs and LPAIVs (Fig. 2). H7 HA genes were phylogenetically divided into five lineages: Eurasian, Historical European, Australian, Equine, and North American. Viruses in the Eurasian lineage clustered into three sublineages: Old-Eurasian, Far-Eastern, and European–Asian. All viruses isolated in Sapporo in 2010 were found to belong to the Far-Eastern sublineage, whereas viruses isolated in Wakkanai in 2011, Mongolia in 2012, and Wakkanai in 2013 belonged to the European–Asian sublineage. Chinese H7N9 viruses were also classified into the Far-Eastern sublineage; however, these viruses and viruses isolated from wild water birds formed a different cluster.

Antigenic analysis of H7 avian influenza viruses

Representative strains of the H7 isolates were antigenically analyzed by the cross HI test (Table 5). HI titers of A/duck/Hokkaido/1/2010 (H7N7) and A/duck/Hokkaido/W19/2013 (H7N2) to each of the antisera against viruses in the

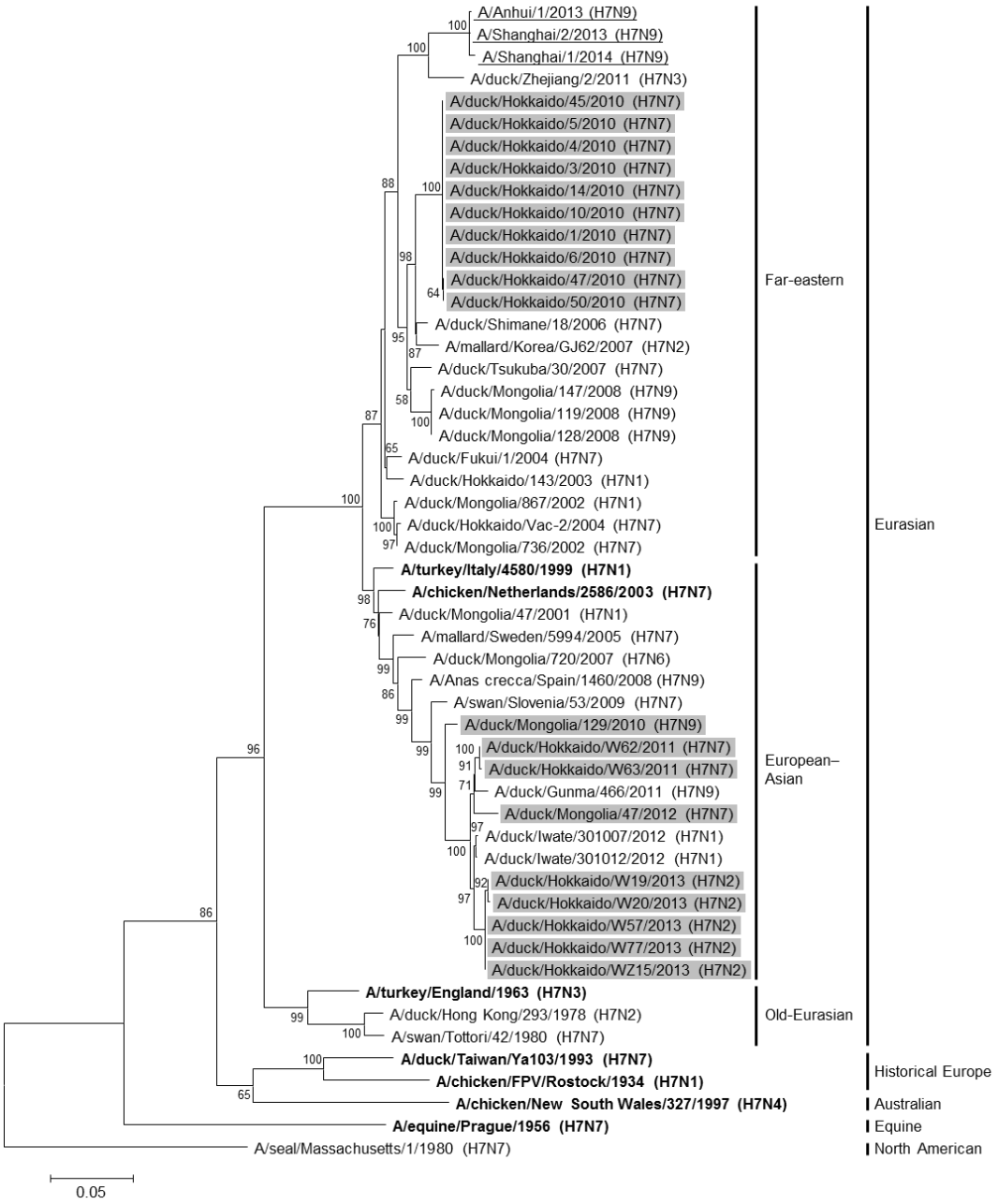


Fig. 2. Phylogenetic tree for the H7 HA genes of influenza viruses.
Full-length of the HA genes of all 19 viruses of the H7 subtype were analyzed by the maximum likelihood (ML) method along with that of reference strains using MEGA 5.0 software. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The virus isolated in this study is highlighted in gray. HPAIVs are indicated in bold. Chinese H7N9 viruses are underlined.

Table 5. Cross HI test of H7 influenza viruses with polyclonal antibodies

Lineage	Viruses	HI titers of the antisera							
		Eurasian					Historical Europe	Australian	North American
		Far-Eastern			European-Asian				
		Dk/Hok/ Vac-2/04	Anhui/ 1/13	Ck/NK/ 7916/05	Dk/Hok/ W19/13	Ty/Italy/ 4580/99	Dk/Tw/ Ya103/93	Ck/NSW/ 327/97	Sl/Mass/ 1/80
Eurasian									
Far-Eastern	A/duck/Hokkaido/1/2010 (H7N7)	5,120	1,280	2,560	10,240	1,280	640	5,120	320
	A/duck/Hokkaido/Vac-2/2004 (H7N7)	20,480	2,560	5,120	20,480	2,560	1,280	10,240	1,280
	A/Anhui/1/2013 (H7N9)	5,120	2,560	2,560	5,120	1,280	320	5,120	320
	A/chicken/North Korea/7916/2005 (H7N7)	640	1,280	1,280	5,120	1,280	320	2,560	160
European–Asian	A/duck/Hokkaido/W19/2013 (H7N2)	5,120	1,280	1,280	2,560	1,280	320	2,560	320
	A/turkey/Italy/4580/1999 (H7N1)	160	80	320	320	1,280	80	320	80
Historical Europe	**A/duck/Taiwan/Ya103/1993 (H7N7)**	160	160	320	320	80	2,560	160	40
Australian	**A/chicken/New South Wales/327/1997 (H7N2)**	1,280	640	1,280	5,120	1,280	320	5,120	320
North American	A/seal/Massachusetts/1/1980 (H7N7)	20,480	2,560	10,240	10,240	2,560	320	10,240	2,560

Viruses isolated in this study are highlighted in gray.

HPAIVs are shown in bold.

Homologous titers are underlined.

Dk, Duck; Ck, Chicken; Ty, Turkey; Sl, Seal.

Hok, Hokkaido; NK, North Korea; Tw, Taiwan; NSW, New South Wales; Mass, Massachusetts.

Far-Eastern sublineage were comparable. In addition, A/duck/Hokkaido/1/2010 (H7N7) reacted with the antiserum against A/duck/Hokkaido/W19/2013 (H7N2) at high titer compared with that of the homologous strain. These results indicate that these two strains are antigenically closely related despite their genetic variations.

Representative strains of the H7 isolates were antigenically analyzed using a panel of MAbs recognizing four antigenic sites on the HA protein of A/seal/Massachusetts/1/1980 (H7N7) and two antigenic sites on the HA protein of A/duck/Hokkaido/Vac-2/2004 (H7N7) [39, 40] (Table 6). Antigenic sites I, II, and V were well conserved in all H7 influenza viruses except A/duck/Taiwan/Ya103/1993 (H7N7) in the Historical European lineage. Epitopes in antigenic sites III and IV are known to be relatively variable even in viruses circulating in wild water birds; however, they were conserved in viruses isolated in the present study.

Table 6. Antigenic analysis of H7 influenza viruses with monoclonal antibodies

Lineage	Viruses	Monoclonal antibody[a]								
		I		II		III	IV	V		
		55/2	58/6	129/3	253/1	8/4	81/6	187/1	213/2	224/4
Eurasian										
Far-Eastern	A/duck/Hokkaido/1/2010 (H7N7)	+	+	+	+	+	+	+	+	+
	A/duck/Hokkaido/Vac-2/2004 (H7N7)	+	+	+	+	-	-	+	+	+
	A/Anhui/1/2013 (H7N9)	+	+	+	+	-	-	+	+	+
	A/chicken/North Korea/7916/2005 (H7N7)	+	+	+	+	+	+	+	+	+
European–Asian	A/duck/Mongolia/129/2010 (H7N9)	+	+	+	+	+	+	+	+	+
	A/duck/Hokkaido/W63/2011 (H7N7)	+	+	+	+	+	+	+	+	+
	A/duck/Mongolia/47/2012 (H7N7)	+	+	+	+	+	+	+	+	+
	A/duck/Hokkaido/W19/2013 (H7N2)	+	+	+	+	+	+	+	+	+
	A/turkey/Italy/4580/1999 (H7N1)	+	+	+	+	+	+	+	+	+
Historical Europe	**A/duck/Taiwan/Ya103/1993 (H7N7)**	-	+	-	-	-	-	-	-	-
Australian	**A/chicken/New South Wales/327/1997 (H7N2)**	+	+	+	+	+	+	+	+	+
North American	A/seal/Massachusetts/1/1980 (H7N7)	+	+	+	+	+	+	+	+	+

[a] Monoclonal antibodies to the HA of A/seal/Massachusetts/1/1980 (H7N7) or A/duck/Hokkaido/Vac-2/2004 (H7N7) [39, 40].

Viruses isolated in this study are highlighted in gray.

HPAIVs are shown in bold.

Discussion

Surveillance of avian influenza in migratory water birds was conducted in the present study. Before 2010, HPAIVs were identified from dead migratory water birds that were on the way back to their northern nesting lakes from the south. In 2010, two HPAIVs were isolated form fecal samples of migratory water birds in autumn in Wakkanai, Hokkaido, Japan [32], suggesting that these HPAIVs could have been kept in the wild water bird population in their northern territory during the spring–summer period. On the other hand, no HPAIVs were identified from fecal samples of migratory water birds in autumn in the subsequent 4 years, indicating that these HPAIVs temporarily invaded the northern nesting lake but may not have been dominantly transmitted.

Phylogenetic analysis of the HA genes of the H7 isolates revealed that viruses circulating among wild water birds were distinct from H7N9 viruses isolated from humans (Fig. 2). These findings indicate that H7N9 influenza viruses have not directly returned into wild water birds. In addition, no H7 isolate had an L residue in the amino acid position at 226 of the HA protein, which was found in the recent human isolates of H7N9 influenza viruses. Although ducks are less susceptible to these Chinese H7N9 viruses compared with chickens, they can be experimentally infected and are capable of shedding the viruses [48]. The observation that H7N9 influenza viruses are still circulating among poultry in China could result in the widespread dissemination of these viruses if they invade the wild bird population. Accordingly, continued monitoring of H7 viruses among wild water birds should be important.

Although homology between the HA gene of A/duck/Hokkaido/1/2010 (H7N7), a representative strain of the H7 Far-Eastern sublineage, and A/duck/Hokkaido/W19/2013 (H7N2), a representative strain of the H7 European–Asian sublineage, is relatively low (91%), the amino acid sequences of the HA proteins are highly conserved (97%). This pattern was similarly observed between A/duck/Hokkaido/101/2010 (H5N2) of the H5 Far-Eastern sublineage and A/duck/Mongolia/194/2011 (H5N3) of the H5 European–Asian sublineage (91% homology in nucleotide; 98% in amino acid). These findings indicate that most of the nucleotide substitutions are synonymous substitution; thus, these HAs are evolutionarily stable at the protein level by accumulating random point substitutions in the nucleotide sequences [49]. In addition, antigenic analysis of the H5 and H7 influenza viruses indicated that antigenicity of influenza viruses circulating in wild water birds is highly stable within these subtypes despite their nucleotide sequence variations (Table 3-6). Ducks are known to produce two different isoforms of IgY antibodies. Truncated IgY, which lacks Fc region from full-length IgY, is incapable of participating in Fc receptor mediated immune response and also HI (reviewed in [50]). Previous study on the comparative experimental infection of ducks and chickens showed that ducks pre-exposed with an LPAIV were susceptible for homosubtypic reinfection with LPAIVs while chickens were not [51]. These facts suggest that immune responses are poor in ducks infected with non-pathogenic avian influenza viruses [10]. Accordingly, non-pathogenic avian influenza viruses are circulating among wild ducks under the relatively reduced selective pressure of antibodies; thus, they are antigenically stable [52]. On the other hand, the antigenicity of the H5 viruses circulating among wild water birds was different from that of HPAIVs of clade 2.3.2.1 and only partially related to the A/chicken/Kumamoto/1-7/2014 (H5N8)

of the clade 2.3.4.4 virus. It is interesting that the antiserum against A/chicken/Kumamoto/1-7/2014 (H5N8) cross-reacted with the H5 viruses isolated in the present study (Table 3). The present results could not explain this unusual reactivity pattern of the antiserum against A/chicken/Kumamoto/1-7/2014 (H5N8). Nevertheless, reactivity pattern of A/chicken/Kumamoto/1-7/2014 (H5N8) against each of the antisera and MAbs obviously indicates that this virus is antigenically distinct from the other representative H5 influenza virus strains (Table 3, 4). These H5 HPAIVs of clade 2.3.4.4 are also antigenically distinct from a clade 2.3.4 virus used for Re-5 vaccine, which is applied in China [53]. It is generally speculated that these antigenic variants are circulating among poultry under antibody selective pressure induced by vaccination of domestic fowls, which accelerates antigenic variation. To clarify the mechanism of the emergence of these antigenically drifted viruses, further effort should be conducted.

The HA gene of A/swan/Hokkaido/67/1996 (H5N3), which was also isolated in the previous surveillance in Hokkaido, was relatively genetically close to that of Gs/GD-like viruses (Fig. 1). H5 influenza viruses isolated in the early to middle 1990s in East Asia are thought to be progenitors of Gs/GD-like viruses [54]. Interestingly, H5 HA genes of viruses isolated in the present study were phylogenetically distinct from that of these progenitor strains. On the other hand, amino acid sequence similarity between the HA proteins of A/swan/Hokkaido/67/1996 (H5N3) and A/duck/Hokkaido/101/2010 (H5N2) is highly conserved (98%), whereas that between A/swan/Hokkaido/67/1996 (H5N3) and A/duck/Hokkaido/WZ83/2010 (H5N1) is much lower (88%). Thus, from 1996 to 2010, the HA of Gs/GD-like viruses have been rapidly evolved compared with that of LPAIVs.

The antigenic property of A/Anhui/1/2013 (H7N9) of the H7N9 Chinese lineage was closely related to that of viruses isolated in this study. A previous study suggested that a prototype vaccine prepared from A/duck/Mongolia/119/2008 (H7N9) of the Far-Eastern sublineage strain was effective against the challenge with A/Anhui/1/2013 (H7N9) in mice [55]. Because of the lack of immunological pressure by vaccination in poultry, the antigenic drift of H7N9 viruses was less significant compared with that of H5 Gs/GD-like HPAIVs (Table 3-6); thus, virus isolates from migratory water birds can be applied to a pre-pandemic vaccine.

In the present surveillance of avian influenza in wild water birds, HPAIVs and H7N9 viruses of the Chinese lineage were not isolated. On the other hand, invasion of these viruses to wild birds may result in adaptation of these viruses to wild bird species, leading to wide dissemination across the world. In 2014, HPAIVs of the H5N8 subtype were endemic in China and South Korea and also outbreaks were reported in Japan, Germany, the Netherlands, the United Kingdom, Italy, the United States of America, Taiwan, and Hungary [33]. It is possible that these H5 HPAIVs were reintroduced into wild water birds and transferred into domestic poultry during their migration to the south. Consequently, further efforts on the eradication of HPAIV infections in poultry and monitoring influenza viruses circulating among wild birds are important for the control of avian influenza and preparedness for a pandemic influenza.

Summary

Migratory water birds are the natural reservoir of influenza A viruses. H5 and H7 influenza viruses are isolated over the world and also circulate among poultry in Asia. In 2010, two H5N1 highly pathogenic avian influenza viruses (HPAIVs) were isolated from fecal samples of water birds on the flyway of migration from Siberia, Russia to the south in Hokkaido, Japan. H7N9 viruses are sporadically isolated from humans and circulate in poultry in China. To monitor whether these viruses have spread in the wild bird population, virological surveillance of avian influenza in migratory water birds was conducted in autumn from 2010 to 2014. A total of 8,103 fecal samples from migratory water birds were collected in Japan and Mongolia and 350 influenza viruses, including 13 H5 and 19 H7 influenza viruses, were isolated. A phylogenetic analysis revealed that all isolates are genetically closely related to viruses circulating among wild water birds. The results of the antigenic analysis indicated that the antigenicity of viruses in wild water birds is highly stable despite their nucleotide sequence variations but is distinct from that of HPAIVs recently isolated in Asia. The present results demonstrate that HPAIVs and Chinese H7N9 viruses were not predominantly circulating in migratory water birds; however continued monitoring of H5 and H7 influenza viruses both in domestic and wild birds is recommended for the control of avian influenza.

Chapter II

A chicken influenza virus recognizes fucosylated α2,3 sialoside receptors on epithelial cells lining the upper respiratory tracts of chickens

Introduction

The binding specificities of the HA of influenza viruses to sialosides vary depending on the host species from which the virus was isolated: avian influenza viruses preferentially bind to α2,3 sialosides, whereas human influenza viruses prefer α2,6 sialosides [22, 23]. In addition, these specificities are consistent with receptor distributions in the host tissues: epithelial cells of the duck intestine predominantly express α2,3 sialisides [24, 25], whereas epithelial cells of the human trachea predominantly express α2,6 sialosides [26]. Two lectins, MAA recognizing carbohydrate structures of Siaα2,3Galβ1,4GlcNAc or Siaα2,3Galβ1,3GalNAc [56, 57], and SNA recognizing Siaα2,6Gal/GalNAc [58] have been used for analyzing the distribution of α2,3 or α2,6 sialoside receptors in the host tissues [24, 25]. However, these lectins do not bind some of α2,3 or α2,6 sialoside, e.g., MAA do not recognize carbohydrate structures of Siaα2,3Galβ1,3GlcNAc or Siaα2,3Galβ1,3Glc [59]. Therefore, further analysis on the distribution of α2,3 or α2,6 sialosides in the host tissues is required for the understanding of host specificity of avian influenza viruses.

Previous studies showed that chickens were rarely infected directly with viruses isolated from ducks, even though most influenza viruses isolated from chickens recognize α2,3 sialosides as the receptors, similarly to the viruses of duck origin [22, 27-29]. Until now, inconsistent results regarding the distribution of sialoside recepors in epithelial cells of the chicken trachea have been reported [60-62]. Despite such a receptor specificity of chicken influenza viruses, a report demonstrated that α2,6 sialosides predominantly exist on the surface of epithelial cells lining the trachea of chickens [60]. Gambaryan et al. reported that influenza viruses isolated from

terrestrial birds, including chickens, preferentially bind to 6-O-sulfo 3´SLeX [Siaα2,3Galβ1,4(Fucα1,3)(6HSO_3)GlcNAc], to which viruses from ducks do not bind [63]. This result suggests importance of modifications in α2,3 sialosides such as fucosylation or sulfation of antepenultimate GlcNAc for the recognition of receptors by influenza viruses. Recent developments in technology for glycoanalysis enable us to analyze the binding of influenza viruses including chicken influenza viruses to vast numbers of glycans [64, 65]. However, the effects of the structure of glycans other than the linkage of terminal Sia and penultimate Gal on the receptor recognition of influenza viruses have not been fully understood. Detailed analyses of distribution of glycans on the human bronchial epithelial cells and chicken erythrocytes revealed diversity of sialoside receptors on the surface of the host cells [66, 67]. While, distribution of sialosides which are not recognized by MAA or SNA lectins in the chicken respiratory tracts are not known. Consequently, the relationship between glycan-binding specificity of influenza viruses isolated from chickens and the distribution of virus receptors in chickens is not well understood, although it is necessary for the comprehensive understanding of the mechanisms of interspecies transmission of avian influenza viruses form ducks to chickens. To clarify the virus receptor of chicken influenza viruses, the present study focused on the fucosylation of α2,3 sialosides. Furthermore, the distribution of the virus receptor in the host tissues and binding specificity of the chicken influenza virus strain A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR) to these receptors were analyzed.

Materials and Methods

Viruses and cells

An LPAIV, Ck/IBR [68], was kindly provided by National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan. Influenza virus, A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG), was isolated from fecal samples of migratory ducks in Mongolia in 2001 [14]. The viruses were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h, and the infectious allantoic fluids were used as virus stocks. MDCK cells were maintained in MEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mg/ml L-glutamine, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 8 μg/ml gentamicin and 10% calf serum. Human embryonic kidney 293T cells were maintained in D-MEM (Life technologies) supplemented with 0.3 mg/ml L-glutamine, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 8 μg/ml gentamicin and 10% fetal bovine serum.

Reverse genetics

Eight genes from Ck/IBR and Dk/MNG each were cloned to produce viruses by reverse genetics as described previously [38, 69, 70]. Ck/IBR, Dk/MNG, and rgMNG/IBR-HA (the HA gene from Ck/IBR and the other gene segments from Dk/MNG) were generated and used in the following experiments.

Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were removed, and the cells

were washed with PBS. The cells were then overlaid with MEM containing 0.7% Bacto-agar (Life technologies) and 5 μg/ml trypsin acetylated (Sigma Aldrich, St. Louis, MO, USA). After 48 h of incubation at 35 °C, cells were stained with 0.005% neutral red. After 24 h, visible plaques were counted.

Experimental infection of chickens

Chickens (*Gallus gallus*, Boris Brown) were obtained from Hokkaido Chuo Shukeijou, Hokkaido, Japan. Four-week-old SPF chickens were infected with $10^{5.0}$ PFU of viruses intranasally. On 3 days post inoculation (d.p.i.), oral and cloacal swabs of the chickens were collected. On 5 d.p.i., chickens were euthanized and oral and cloacal swabs, larynx, trachea, lungs, kidney and colon were collected. To prepare a 10% suspension with MEM, tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Infectivity titers of swabs and tissue samples were calculated by plaque assay. All infected chickens were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All animal experiments were performed according to the guidelines of the institutional animal care and use committee of Hokkaido University (approval number; 11-0087).

Solid-phase direct binding assay

Receptor binding specificity of the viruses was assessed using a solid-phase direct binding assay with sialylglycopolymers 3′sialyllactosamine (3′SLacNAc)-PAA, 3′SLeX-PAA, and 6′sialyllactose (6′SLac)-PAA (Cosmo Bio Co., Ltd., Tokyo, Japan) as described previously [71] (Fig. 3). These were approximately 30-kDa polymers

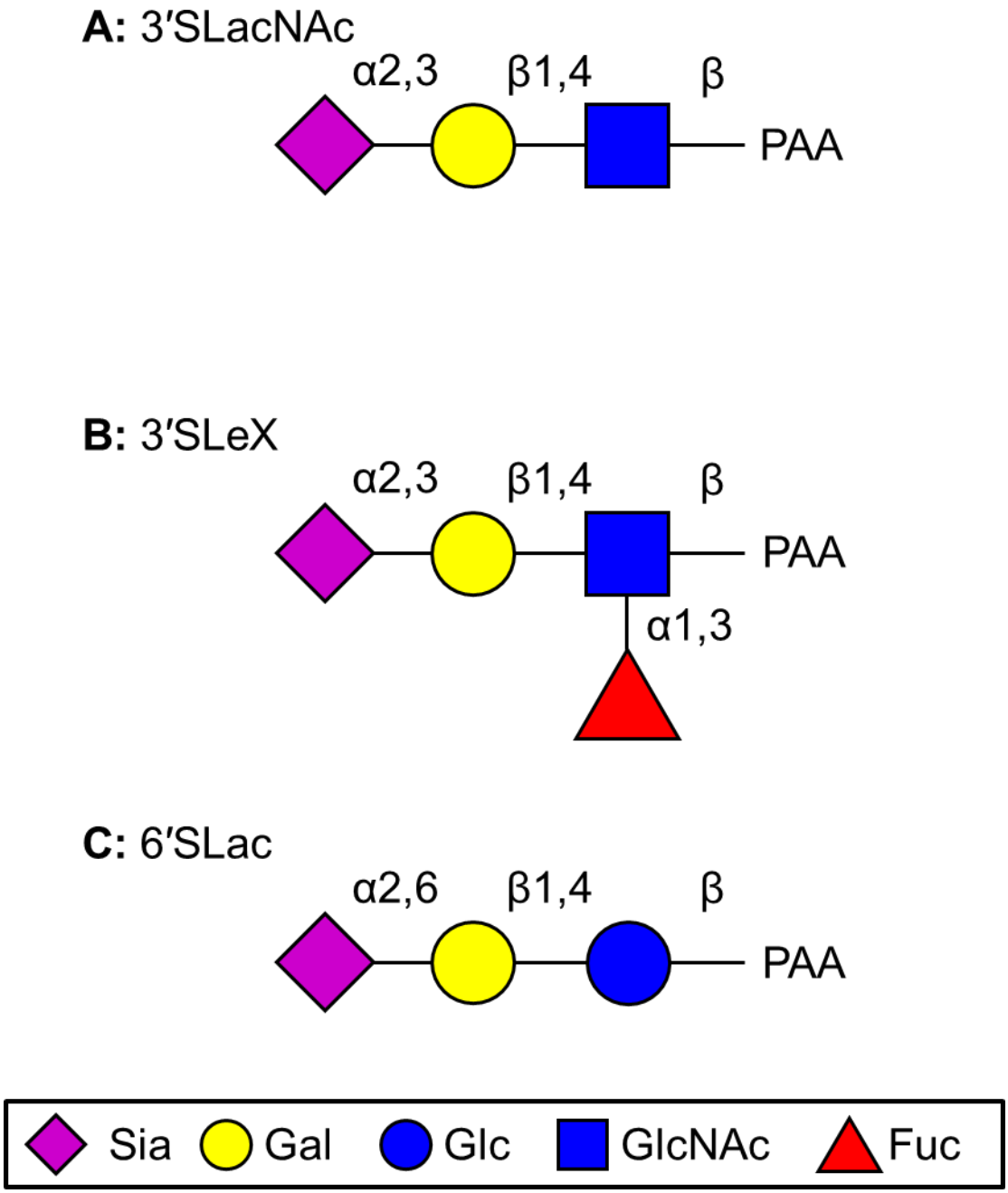


Fig. 3. Carbohydrate structures of sialylglycopolymers used in this study.
Each symbol indicates sialic acid (Sia, purple diamonds), galactose (Gal, yellow circles), glucose (Glc, blue circles), N-acetylglucosamine (GlcNAc, blue squares), and fucose (Fuc, red triangles), respectively. PAA indicates polyacrylamide.

containing 20% mol sugar linked to a polyacrylamide backbone. Each sialylglycopolymer was serially diluted and added to each well of a Universal-BIND™, 96 well polystyrene strip well microplate (Corning, Corning, NY, USA). Each well was blocked with 1% BSA at room temperature for 1 h. After washing with PBS containing 0.05% Tween 20 (PBST), a solution containing influenza viruses (16 HA/50 μl in PBS) was added to each well and the plates were incubated at 4 °C for 12 h. After washing, mouse anti-HA monoclonal antibodies [H3/2; previously established using A/duck/Pennsylvania/10218/1984 (H5N2)] were added to each well and the plates were incubated at 4 °C for 2 h. The wells were then washed and incubated with goat anti-mouse IgG-HRP conjugate (Bio-Rad, Hercules, CA, USA) at 4 °C for 2 h. After washing, 100 μl of the substrates including 0.5 mM TMB and 0.04% H_2O_2, were added to each well. After incubation at room temperature for 10 min, the reactions were stopped using 50 μl of 2N H_2SO_4, and absorbance at 450/630 nm was measured using a MULTISCAN JX (Thermo Fisher Scientific Inc, Waltham, MA, USA).

Glycan-binding specificity of MAA lectin and the anti-3′SLeX antibody (clone name: KM93, Kyowa Hakko KIRIN Co., Ltd., Tokyo, Japan) were assessed by solid-phase direct binding assay as described above with modifications. Briefly, each sialylglycopolymer was serially diluted and add to each well of Universal-BINDTM, 96 well polystyrene strip well microplate (Corning). Each well was blocked with BSA for 1 h. After washing 5 times with PBST, a solution containing FITC-labeled MAA (1:200 dilution; EY Laboratory, San Mateo, CA, USA; catalogue number: F-7801-2) or KM93 antibody (1:500 dilution) was added to each well and the plates were incubated at room temperature for 1 h. After washing 10 times with PBST, sheep anti-FITC-HRP conjugate (SouthernBiotech, Birmingham, AL, USA; for FITC-labeled

MAA) or goat anti-mouse IgM(μ)-HRP conjugate (American Qualex, San Clemente, CA, USA; for KM93 antibody) was added to each well and the plates were incubated at room temperature for 1 h. After washing 10 times with PBST, 100 μl of premixed substrate including 0.5 mM TMB and 0.04% H_2O_2 was added each well. After incubation at room temperature for 5 min, the reactions were stopped with 50 μl of 2N H_2SO_4, and absorbance at 450/630 nm was measured.

Detection of sialoside receptors in chickens and ducks

Chickens (Boris Brown) were obtained from Hokkaido Chuo Shukeijou, Hokkaido, Japan. Ducks (*Anas platyrhynchos* var. *domesticus*, Cherry Valley) were obtained from Takikawa Shinseien, Hokkaido, Japan. The animals were euthanized, and their larynxes, tracheas and colons were collected. Tissue samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen in cold hexane. Sections of each tissue (8 μm) were cut with a cryostat (CM1100; Leica Microsystems, Wetzlar, Germany), air dried, and fixed for 5 min in cold acetone. Before staining, the sections were washed 3 times with PBS, and blocked with 1% BSA in PBS. Confluent monolayers of MDCK or MDCK-FUT cells seeded on 8-well chamber slides (SCS-008; Matsunami, Osaka, Japan) were washed once with PBS, and fixed for 5 min in cold acetone. Before staining, the sections were washed 3 times with PBS, and blocked with 1% BSA in PBS. To detect fucosylated and non-fucosylated α2,3 sialosides, tissue sections and cells were incubated with 250 μl of the mixture of TRITC-labeled MAA (1:100 dilution; EY Laboratories catalogue number: R-7801-2) and anti-3′SLeX antibody (KM93, 1:250 dilution) at room temperature for 1 h. After being washed 3 times with PBS, the sections and cells were

incubated with 250 μl of FITC-labeled anti-mouse IgM antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. After being washed three times with PBS, sections and cells were counterstained with DAPI (Dojindo Molecular Technologies, Kumamoto, Japan). The tissue sections and cells were observed under a fluorescence microscope (Axiovert 200; Carl Zeiss).

Phylogenetic analysis of α1,3/4 fucosyltransferase genes of human and chicken

Nucleotide sequences of FUT3 (GenBank accession number: NM_000149.3), FUT4 (NM_002033.3), FUT5 (NM_002034.2), FUT6 (NM_000150.2), FUT7 (NM_004479.3), FUT9 (NM_006581.3) of human fucosyltransferase genes and cFUT3/5/6 (XM_004948742.1), cFUT4 (NM_001031487.1), cFUT7 (NM_001031476.1) and cFUT9 (NM_001079502.1) of chicken fucosyltransferase genes were obtained from GenBank/EMBL/DDBJ. These sequences were analyzed by the ML method using MEGA 5.0 software (http://www.megasoftware.net). The reliability of phylogenetic inference at each branch node was estimated by the boot strap method with 1,000 replications.

Detection of fucosyltransferase mRNA in the chicken trachea

Chickens were euthanized and their tracheas were collected. The tracheas were washed 3 times with PBS and treated with Liberase DH Research Grade (500 μg/ml; Roche, Basel, Switzerland) in PBS for 30 min at 37 °C to collect epithelial cells. The suspension of the collected epithelial cells was centrifuged at 3,000 g for 5 min and washed twice with PBS. Total RNA of the cell pellet was extracted using a TRI-zol Reagent (Life technologies) and reverse transcribed with Oligo(dT)20 Primer (Life

technologies) and M-MLV Reverse Transcriptase (Life technologies). PCR amplification of a target sequence was performed with primers 5′-CACCTCGAGAAAACTATGAG-3′ and 5′-TTATACAAACCACTTAGACAG-3′ for chicken cFUT3/5/6 gene, primers 5′-GGCCTTCGAGAACTCCCAGC-3′ and 5′-TCAGCTCTCAAACCAGCCGGC-3′ for chicken FUT4 gene, and primers 5′-CCCACGACATCCAAGTCCAA-3′ and 5′-CTAGGAGTTGAACCAGCTCTG-3′ for chicken FUT7 gene. PCR were performed as follows: 95 °C for 5 min, then, 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 40 sec, and finally, 72 °C for 5 min. The PCR products were separated using electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under Printgraph AE-6932 (ATTO, Tokyo, Japan).

Establishment of MDCK cells exogenously introduced a chicken fucosyltransferase gene

The 5′ half of the chicken cFUT3/5/6 gene was amplified with KOD -Plus- Neo (Toyobo, Osaka, Japan) using primers 5′-ATGCAGCCATCACTCAGTGC-3′ and 5′-CCATCTTGGTCTTGGGTGGG-3′. The 3′ half of the chicken cFUT3/5/6 gene was amplified using primers 5′-CCAAGTCATTCTCCAAACTTA-3′ and 5′-TTATACAAACCACTTAGACAG-3′. PCR products were independently cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and designated as cFUT3/5/6-5′half-pGEM-T and cFUT3/5/6-3′half-pGEM-T, respectively. cFUT3/5/6-5′half-pGEM-T and cFUT3/5/6-3′half-pGEM-T were digested with *Sac* II (Toyobo) at 37 °C for 2 h, following the digestion with *Bpu*1102 I (Takara, Shiga, Japan) at 37 °C for 11 h. The complete open reading frame of the chicken cFUT3/5/6

gene was constructed in pGEM-T Easy Vector by ligation of the digested fragments using DNA Ligation Kit <Mighty Mix> (Takara), and designated as cFUT3/5/6-pGEM-T. cFUT3/5/6-pGEM-T was digested with EcoRI (Toyobo) at 37 °C for 2 h. Then, DNA fragments coding the entire chicken cFUT3/5/6 gene were cloned into pCXN2 mammalian expression vector [72, 73] and designated cFUT3/5/6-pCXN2. MDCK cells were transfected with 1 μg of cFUT3/5/6-pCXN2 or empty pCXN2 vectors using Lipofectamine 2000 (Life technologies) and maintained in a culture medium supplemented with 500 μg/ml of G418 sulfate (Calbiochem, La Jolla, CA, USA). To establish cloned MDCK cells which stably expressing cFUT3/5/6 of chickens, MDCK-FUT cells, cFUT3/5/6-pCXN2 transfected cells were passaged 7 times and then, G418-resistant clones were isolated and transferred to 24-well plates. Each clone was passaged 3 additionally times in the presence of 500 μg/ml G418 sulfate and was frozen in aliquots. MDCK-FUT clone A32 and clone C33 cells were established from the cells batches independently transfected with cFUT3/5/6-pCXN2. To obtain control MDCK cells which are resistant to G418, MDCK-Empty cells, MDCK cells transfected with empty pCXN2 vectors were passaged 7 times in the presence of 500 μg/ml G418 sulfate and were frozen in aliquots. MDCK-FUT and MDCK-Empty cells were maintained in the presence of 500 μg/ml G418 sulfate, except when they were used for viral infection. The cells in these experiments were used at passage levels from 3-10 starting from the frozen cell stock.

Virus growth in MDCK and MDCK-FUT cells

Confluent monolayers of MDCK, MDCK-FUT clone A32, clone C33, and MDCK-Empty cells seeded on 6-well plates were infected with Ck/IBR at the M.O.I of

0.1. Four, 8, 12, and 24 hours post infection, cell supernatants were harvested. Infectivity titers of the supernatants were measured using 10-day-old chicken embryonated eggs. Titers were calculated by the method of Reed and Muench, and expressed as 50% egg infectious dose (EID_{50}) per milliliter [74].

Results

Experimental infection of chickens with an influenza virus of chicken or duck origin

In order to confirm whether Ck/IBR replicates in chickens, $10^{5.0}$ PFU of Ck/IBR was intranasally inoculated into 4-week-old SPF chickens (Table 7). An influenza virus of wild duck origin, Dk/MNG, was also inoculated into chickens. Viruses were recovered from the respiratory tracts of the chickens inoculated with Ck/IBR, whereas no virus was recovered from chickens inoculated with Dk/MNG. In order to investigate whether HA protein of influenza viruses, which plays roles of virus attachment and entry to host cells, is responsible for the efficient replication of Ck/IBR in chickens, rgMNG/IBR-HA, having only the HA gene derived from Ck/IBR and the others from Dk/MNG, was generated and intranasally inoculated into chickens. As well as Ck/IBR, rgMNG/IBR-HA replicated in the upper respiratory tracts of the chickens. The results indicate that the HA is the major determinant for efficient growth of Ck/IBR in chickens.

Binding specificities of Ck/IBR and Dk/MNG to fucosylated and non-fucosylated α2,3 sialosides

The results of experimental infection of chickens indicate that virus attachment to host receptors by the HA is responsible for the efficient growth of Ck/IBR in chickens. Therefore, binding specificities of Ck/IBR and Dk/MNG to sialoside receptors were investigated using a solid-phase direct binding assay. Both Ck/IBR and Dk/MNG specifically bound to α2,3 sialosides, but not to 6′SLac, α2,6 sialosides (Fig. 4A and B).

Table 7. Virus recovery from the chickens intranasally inoculated with each virus

Viruses	Chicken Number	Swabs (log PFU/ml)				Tissue samples (log PFU/g, 5 dpi)				
		Oral		Cloacal						
		3 dpi	5 dpi	3 dpi	5 dpi	Larynx	Trachea	Lung	Kidney	Colon
Ck/IBR	1	2.9	2.3	-	-	4.0	-	-	-	-
	2	3.0	2.0	-	-	3.0	-	-	-	-
	3	-*	1.7	-	-	-	-	-	-	-
Dk/MNG	4	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-
rgMNG/IBR-HA	7	2.4	1.5	-	-	-	-	-	-	-
	8	2.2	1.3	-	-	4.1	2.7	-	-	-
	9	2.0	2.0	-	-	3.0	2.6	-	-	-

*Dash (-) indicates <1.0 for swabs and <2.0 for tissue samples.

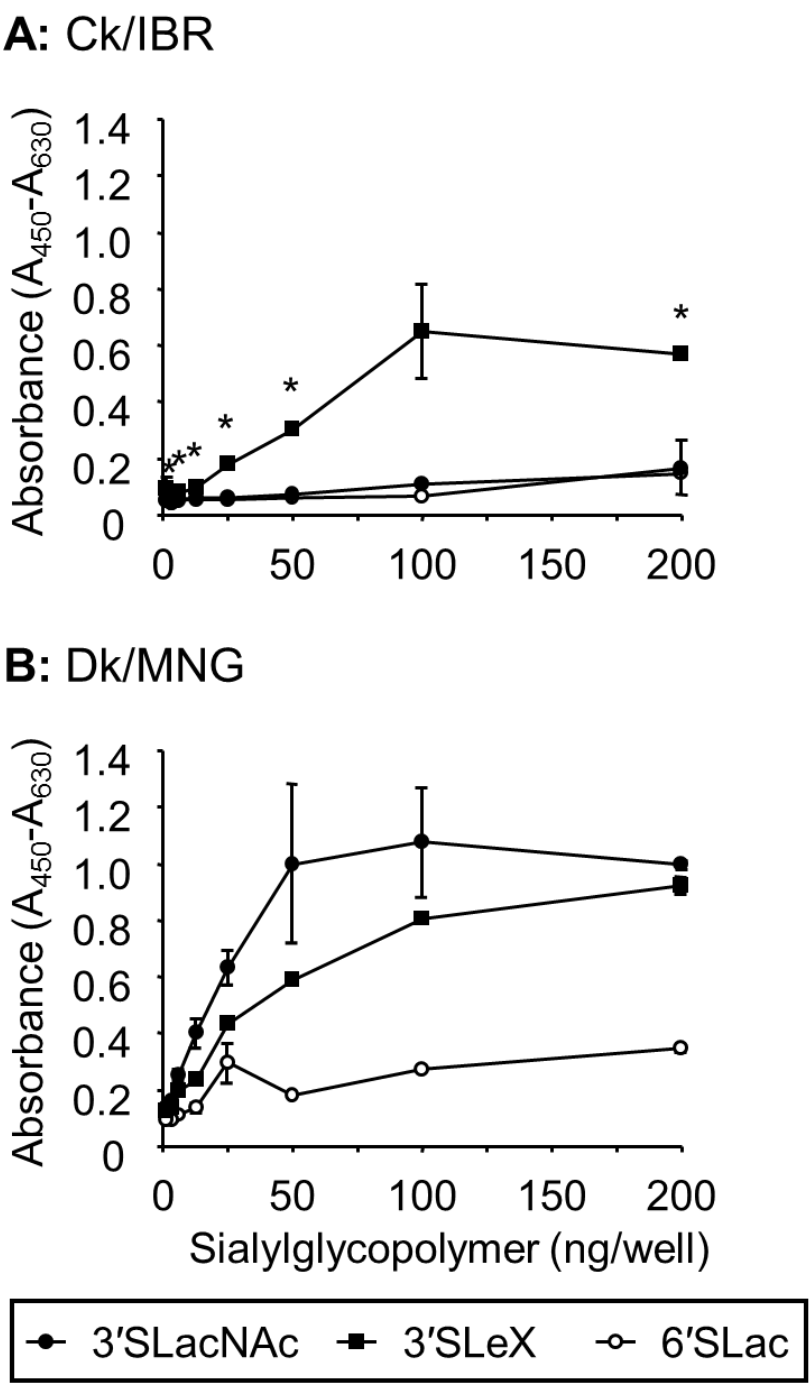


Fig. 4. Receptor specificities of Ck/IBR and Dk/MNG.
Binding of Ck/IBR (A) and Dk/MNG (B) to 3′SLacNAc (closed circles), 3′SLeX (closed squares), or 6′SLac (open circles) covalently linked polyacrylamide chain was investigated using solid-phase direct binding assays. The data are presented as mean±S.E. of triplicate experiments. *p<0.05 in the comparison of 3′SLacNAc and 3′SLeX.

Notably, Ck/IBR bound to 3´SLeX, which is a fucosylated α2,3sialoside, whereas binding towards 3′SLacNAc, which is a non-fucosylated α2,3 sialoside, was weak.

Fucosylated SAα2,3Gal glycans are expressed on epithelial cells of the upper respiratory tracts of chickens

MAA lectin and an anti-3´SLeX monoclonal antibody, KM93, were subjected to a solid-phase direct binding assay to confirm their binding specificity to sialosides. MAA lectin did not bind to 3´SLeX, which is fucosylated α2,3 sialoside, but did to 3′SLacNAc, which is non-fucosylated α2,3 sialoside (Fig. 5A) In contrast, KM93 bound to 3´SLeX, but did not bind to 3′SLacNAc (Fig. 5B).

The distribution of fucosylated and non-fucosylated α2,3 sialoside receptors on epithelial cells lining the upper respiratory tracts of chickens and colon of ducks were examined using MAA lectin and KM93. On epithelial cells of the chicken larynx and trachea, non-fucoslated α2,3 sialosides were not detected using MAA lectin, whereas fucosylated α2,3 sialosides were abundantly detected by KM93 (Fig. 6A). Non-fucosylated α2,3 sialosides were predominantly detected on the epithelial cells of the duck colon using MAA lectin as reported previously [25], whereas fucosylated α2,3 sialosides were not detected (Fig. 6B).

Fucosylation of α2,3 sialosides recognized by KM93 is catalyzed by α1,3/4 fucosyltransferase. So far, 6 fucosyltransferases, fucosyltransferase 3, 4, 5, 6, 7, and 9 are known as α1,3/4 fucosyltransferases in humans [75]. On the other hand, enzymatic function of fucosyltransferases of chickens is totally unknown. Then, the coding regions of each fucosylltransferase gene of human and chicken was phylogenetically analyzed (Fig. 7A). In chickens, 3 glycosyltransferase genes, cFUT4, cFUT7, and

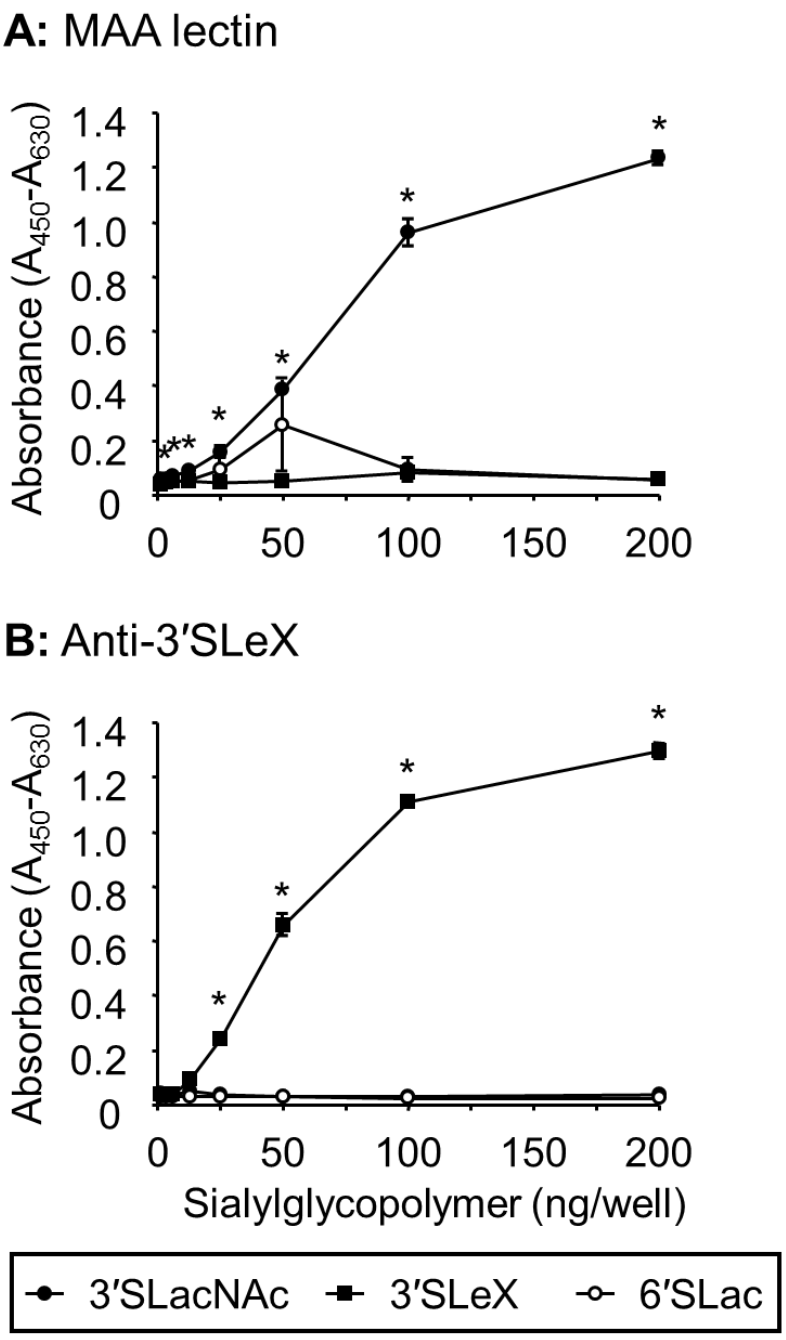


Fig. 5. Recognition of glycans by MAA lectin and the KM93 anti-3′SLeX antibody.

Binding of MAA lectin (A) and the KM93 anti-sLeX antibody (B) to 3′SLacNAc (closed circles), 3′SLeX (closed squares), or 6′SLac (open circles) covalently linked polyacrylamide chain was investigated using solid-phase direct binding assays. The data are presented as mean±S.E. of triplicate experiments. *p<0.05 in the comparison of 3′SLacNAc and 3′SLeX.

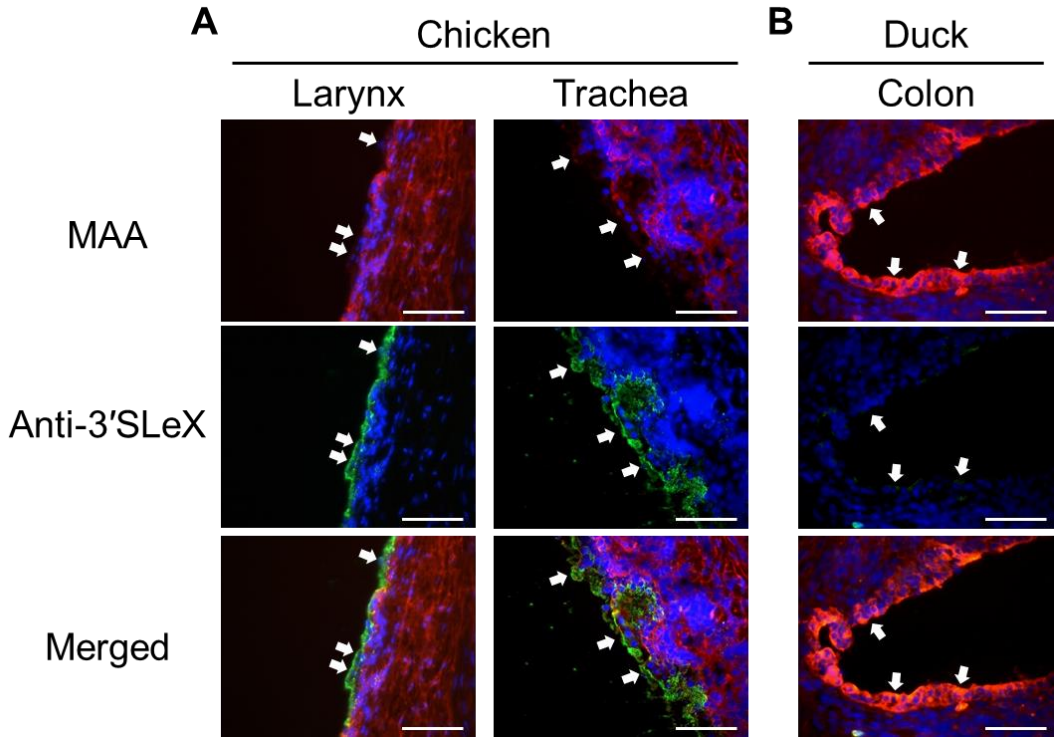


Fig. 6. Detection of fucosylated α2,3 sialosides in the host tissues.
Non-fucosylated α2,3 sialosides were detected using TRITC-labeled MAA (red), and fucosylated α2,3 sialosides were detected using an anti-3′SLeX antibody (KM93: green) by immunofluorescence assay in the chicken larynx and trachea (A) or the duck colon (B). Nuclei were stained with DAPI (blue). Original magnification was ×200. Arrows indicate epithelial cells. Scale bars are 100 μm.

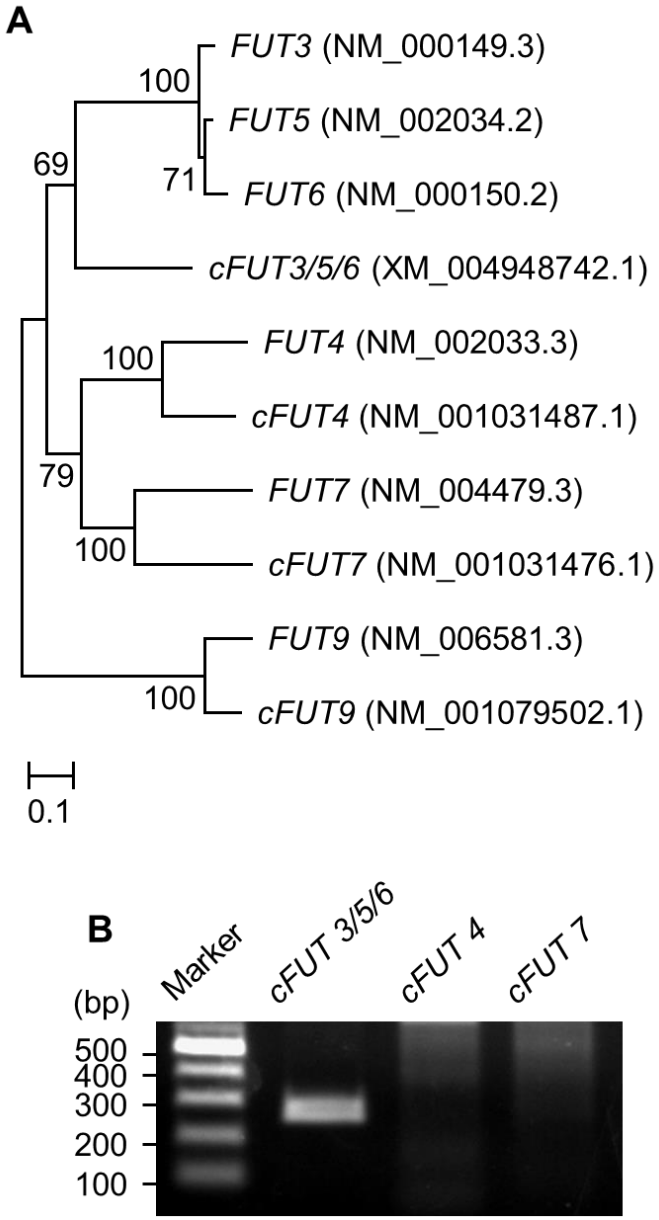


Fig. 7. Expression of α1,3/4 fucosyltransferase genes in epithelial cells of the chicken trachea.

α1,3/4 fucosyltransferase genes of human and chicken were phylogenetically analyzed (A). Complete coding regions of each fucosyltransferase gene were used for ML phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at each node indicate the confidence level in bootstrap analysis with 1,000 replications. Chicken α1,3/4 fucosyltransferase genes were detected by RT-PCR (B). Predicted PCR products are 266 (cFUT3/5/6), 361 (cFUT4), and 381 (cFUT7) base pairs (bp), respectively. Specific PCR products were detected in the cFUT3/5/6 gene.

cFUT9 genes were predicted as orthologs of human fucosyltransferase genes, FUT4, FUT7, and FUT9, respectively. A fucosyltransferase gene of chicken was identified as an ortholog of human FUT3, FUT5 and FUT6 genes, and designated as cFUT3/5/6 gene. Among these glycosyltransferases, human fucosyltransferase 9 does not catalyze fucosylation of sialosides; thereby human fucosyltransferase 3, 4, 5, 6, and 7 are involved in the synthesis of 3´SLeX [75]. In order to clarify whether these fucosyltransferase genes are expressed in epithelial cells of the chicken trachea, mRNA expression of the cFUT3/5/6, cFUT4, and cFUT7 genes were investigated by RT-PCR (Fig. 7B). Of these 3 fucosyltransferase genes, mRNA of cFUT3/5/6 gene was detected, indicating that α2,3 sialoside receptors existing on epithelial cells lining the trachea of chickens can be fucosylated by chicken fucosyltransferase 3/5/6.

Accordingly, these results demonstrate that fucosylated α2,3 sialosides are expressed on epithelial cells of the upper respiratory tracts of chickens.

The effect of fucosylation of SAα2,3Gal receptor on the growth of the influenza virus of chicken origin

Glycan-binding specificity of Ck/IBR indicated that fucosylation of α2,3 sialoside receptors influences the attachment of Ck/IBR to the cell surface (Fig. 4A). In order to clarify whether fucosylation of α2,3 sialoside receptors enhance the growth of Ck/IBR, chicken cFUT3/5/6 gene was transfected into MDCK cells to establish MDCK-FUT cells, which stably express fucosylated α2,3 sialosides on their cell surfaces. Two independent clones of MDCK-FUT cells were obtained, and they were designated as MDCK-FUT clone A32 or clone C33 cells respectively. Fig. 8 shows the distribution of fucosylated and non-fucosylated α2,3 sialosides on parental MDCK (A),

MDCK-FUT clone A32 (B), clone C33 (C) cells, and MDCK cells which were transfected with empty pCXN2 vectors (MDCK-Empty cells; D). On parental MDCK cells, non-fucosylated α2,3 sialosides were detected using MAA, whereas not fucosylated α2,3 sialosides. On the other hand, exogenously introduced chicken fucosyltransferase catalyzed fucosylation of α2,3 sialosides, thereby, fucosylated α2,3 sialosides were abundantly detected on MDCK-FUT cells of each clone. The receptor distribution on MDCK-Empty cells was similar to that of parental MDCK cells.

Then, growth potential of Ck/IBR was compared in MDCK and MDCK-FUT cells (Fig. 9). MDCK, MDCK-FUT clone A32, clone C33, and MDCK-Empty cells were infected with Ck/IBR at the M.O.I of 0.1. Virus growth of Ck/IBR in MDCK-FUT cells of each clone was significantly higher than that in parental MDCK cells or MDCK-Empty cells. At 24 h post infection, virus titers in MDCK-FUT cells of each clone were 5- to 13-fold higher than those of MDCK or MDCK-Empty cells. Accordingly, fucosylation of α2,3 sialoside receptors enhanced virus growth of Ck/IBR.

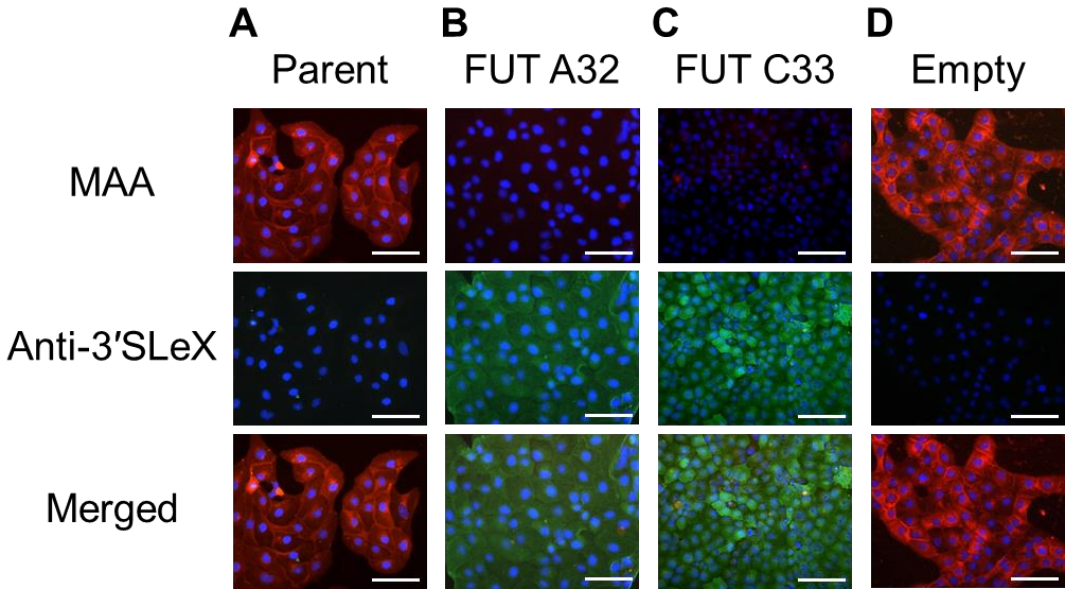


Fig. 8. Receptor distribution on MDCK and MDCK-FUT cells.
Non-fucosylated α2,3 sialosides were detected using TRITC-labeled MAA (red), and fucosylated α2,3 sialosides were detected using an anti-3′SLeX antibody (KM93: green) by immunofluorescence assay on parental MDCK cells (A), MDCK-FUT cells clone A32 (B), clone C33 (C), and MDCK-Empty cells (D). Nuclei were stained with DAPI (blue). Original magnification was ×200. Scale bars are 100 μm.

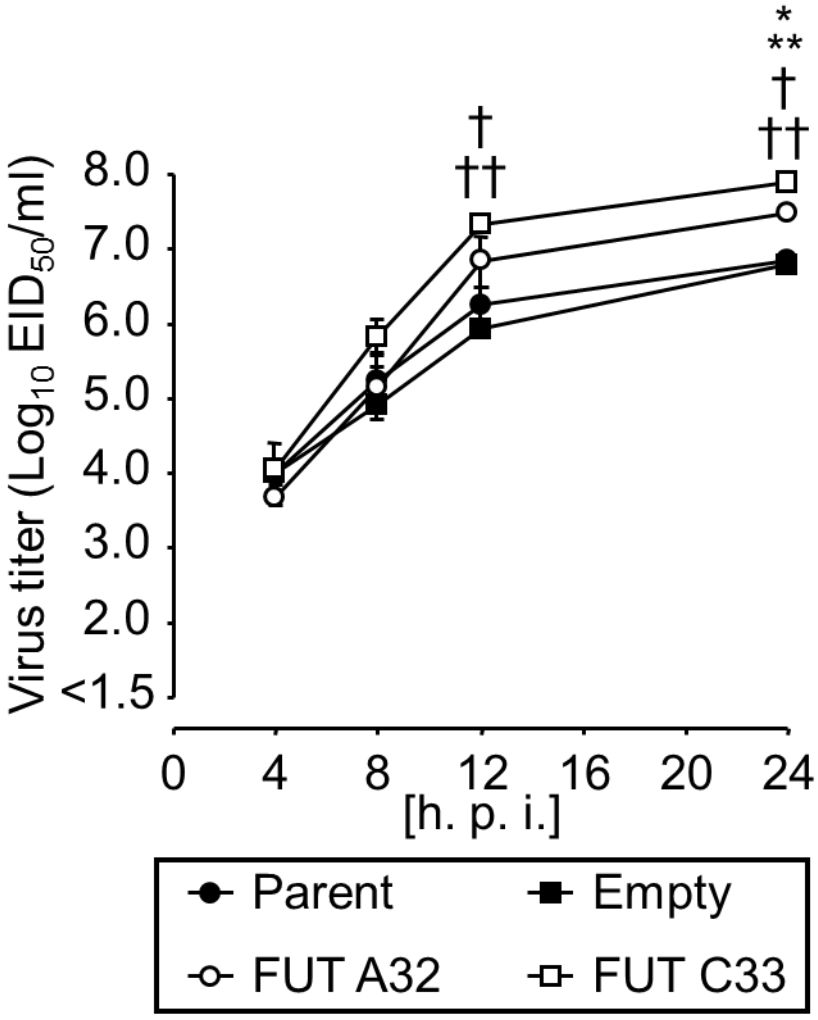


Fig. 9. Virus growth of Ck/IBR in MDCK or MDCK-FUT cells.

MDCK cells (closed circles), MDCK-FUT clone A32 cells (open circles), clone C33 cells (open squares), and MDCK-Empty cells (closed squares) were infected with Ck/IBR at M.O.I. of 0.1. The data are presented as mean ± SE of independent 3 experiments. *p<0.05 between MDCK-FUT clone A32 and parental MDCK cells, **p<0.05 between MDCK-FUT clone A32 and MDCK-Empty cells, †p<0.05 between MDCK-FUT clone C33 and parental MDCK cells, and ††p<0.05 between MDCK-FUT clone C33 and MDCK-Empty cells.

Discussion

The relationship between receptor specificity of the viruses and distribution of virus receptors in the host tissues is major determinants of host range and tissue tropism of the viruses. Understanding the mechanisms of interspecies transmission of influenza viruses is essential for the control of avian and mammalian including human influenza. It is, thus, important to clarify the mechanisms of recognition of receptors by influenza viruses.

So far, distribution of α2,3/2,6 sialosides detected by MAA or SNA lectin in the tissues of several mammals and birds have been reported [24-26, 60, 76]; however MAA is reported not to bind all α2,3 sialosides [57]. This is the first report that fucosylated α2,3 sialosides, which are not recognized by MAA lectin, were detected in epithelial cells of the upper respiratory tracts of chickens (Fig. 6A). Some of the previous reports suggested that α2,6 sialosides are predominantly present on epithelial cells of the chicken trachea [60], though influenza viruses isolated from chickens preferentially bind to α2,3 sialosides [27]. Thus, these findings have important implications for the understanding of receptor usage of chicken influenza viruses.

In the present study, it was demonstrated that carbohydrate structures other than the linkage of terminal Sia and penultimate Gal affect the receptor recognition of influenza viruses (Fig. 9). It was reported that sulfation or fucosylation of antepenultimate GlcNAc in α2,3 sialosides affects the binding affinity of HA to these glycans [63, 77]. However, effects of these modifications of α2,3 sialosides on virus growth were not clarified. Ck/IBR grew better in MDCK-FUT cells than in MDCK cells, suggesting that Ck/IBR prefer fucosylated α2,3 sialosides as virus receptors (Fig. 9). On the other

hand, Ck/IBR also replicated in parental MDCK cells, which do not express fucosylated α2,3 sialosides on their cell surface (Fig. 9). One explanation is that Ck/IBR utilized receptors which are not detected by MAA lectin or KM93. Meanwhile, the glycome of MDCK cells is not known. The glycome analysis on the host cells as well as comprehensive analysis on the receptor binding of influenza viruses should provide the better understanding of the receptor recognition and host range of influenza viruses.

Ck/IBR and rgMNG/IBR-HA, which possesses the HA derived from Ck/IBR, efficiently replicated in chickens (Table 7). In the present study, rgIBR/MNG-HA, having only HA gene derived from Dk/MNG and the others from Ck/IBR was also generated. Virus was not recovered form chickens intranasally inoculated with this virus, similar to Dk/MNG, the donor of the HA (data not shown). These results indicate that the HA of Ck/IBR is a necessary and sufficient factor for influenza viruses to replicate in chickens. Although Dk/MNG bound to 3´SLeX, which are expressed on epithelial cells of the upper respiratory tracts of chickens, Dk/MNG did not replicate in chickens. Thereby, further analysis on the receptor recognition of Dk/MNG was conducted in Chapter III.

Previous studies of glycan microarray screenings of avian influenza viruses indicated that some of the influenza viruses isolated from chickens bound to fucosylated α2,3 sialosides and some did not [64, 65]. The present results indicate that fucosylation of α2,3 sialoside receptor is the key factor determining the receptor binding of Ck/IBR. However, many other factors in the topology of α2,3 sialosides may be involved in the binding of chicken influenza viruses. The present study showed that fucosylated α2,3 sialosides on epithelial cells lining the upper respiratory tracts of chickens (Fig. 6A). Due to the lack of information about distribution of α2,3 sialoside

receptors which are not detected by MAA lectin, on the surface of host cells, the effects of the structure of glycans other than the linkage of terminal Sia and penultimate Gal on the receptor recognition of influenza viruses have not been fully understood. Analyses of the receptor binding to glycans which exist on the surface of host cells is the base for understanding the host range of influenza viruses. To understand the molecular mechanisms of interspecies transmission of influenza viruses, it is necessary to clarify the carbohydrate structures of the receptors which influenza viruses recognize. The present study indicates that a chicken influenza virus, Ck/IBR, recognizes fucosylated α2,3 sialosides as virus receptors (Fig. 9). The present results will contribute to elucidate the mechanisms of the host range and receptor specificity of influenza viruses.

Summary

Influenza viruses recognize sialoglycans as receptors. Although viruses isolated form chickens preferentially bind to α2,3 sialosides as do those of ducks, chickens were not experimentally infected with viruses isolated from ducks. A chicken influenza virus, A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR) bound to fucosylated α2,3 sialosides, whereas the binding towards non-fucosylated α2,3 sialosides was weak. On epithelial cells of the upper respiratory tracts of chickens, fucosylated α2,3 sialosides were detected, but not non-fucosylated α2,3 sialosides. The growth of Ck/IBR in MDCK cells expressing fucosylated α2,3 sialosides by exgenously introduced chicken fucosyltransferase was significantly higher than that in the parental MDCK cells. The present results indicate that fucosylated α2,3 sialosides existing on epithelial cells lining the upper respiratory tracts of chickens are critical for recognition by Ck/IBR.

Chapter III

Amino acid residues at positions 222 and 227 of the hemagglutinin together with the neuraminidase determine binding of H5 avian influenza viruses to sialyl Lewis X

Introduction

It was previously reported that influenza viruses isolated from terrestrial poultry prefer fucosylated α2,3 sialosides such as 3´SLeX or 6-O-sulfo-3´SLeX for the receptor [63]. In chapter II, it was demonstrated that 3´SLeX was predominantly detected on epithelial cells of the upper respiratory tract of chickens, which is the primary replication site of chicken-adapted influenza viruses. On the contrary, 3´SLeX was not detected on epithelial cells of duck colon, suggesting that α1,3 fucosylation of antepenultimate GlcNAc in α2,3 sialosides is a species barrier between ducks and chickens. Modifications on the antepenultimate GlcNAc of α2,3 sialosides are considered a key factor explaining differential susceptibility of ducks and chickens to influenza virus infection. A previous study on the structural basis of recognition of this particular glycan motif suggested that the fucose moiety of 3´SLeX is positioned close to the 220-loop of the HA [78]. Role of the 220-loop, particularly an amino acid residue at position 222, in recognition of α1,3 fucosylated sialosides have been previously discussed in H3, H5, and H7 HA [77, 79, 80]. However, effects of the particular amino acid motif in the 220-loop on the glycan-binding property of HA are totally unknown in H5 HA; thus, the detailed molecular basis of this interaction between H5 HA and α1,3 fucosylated sialosides should be analyzed.

The other envelope glycoprotein of influenza A virus, NA, cleaves terminal Sia from penultimate Gal, which is essential for virus release from host cells and also facilitates cell attachment by destroying “decoy” receptors [4, 5]. Previous studies suggested that the functional balance between HA (receptor binding) and NA (receptor destroying) contributes to pathogenicity and host range of influenza A viruses [81].

Nevertheless, the mechanism by which NA contributes to virus–glycan interaction has not been extensively studied because methods for the analyses on interaction of NA with modified α2,3 sialosides are still limited [82].

The present study identified that two amino acid residues located at positions 222 and 227 in the HA of H5 influenza A viruses have a crucial role in recognition of 3´SLeX. In addition, the present results demonstrate a contribution of NA to the binding of virions of an influenza A virus to 3´SLeX.

Materials and Methods

Viruses and cells

Preparation of virus stocks of A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR) and A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG) was described in Chapter II. MDCK cells and HEK 293T cells were maintained as described in Chapter II. HEK 293S GnTI−/− cells were maintained in pyruvate free D-MEM (Life Technologies) supplemented with 0.3 mg/ml L-glutamine, 10 U/ml penicillin G, 0.01 mg/ml streptomycin and 10% fetal bovine serum.

Reverse genetics

Eight genes from Ck/IBR and Dk/MNG each were cloned to produce viruses by reverse genetics as described in Chapter II. Amino acid substitutions in the HA of Ck/IBR and Dk/MNG, were generated by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. The mutant viruses were generated by reverse genetics. All eight segments of the genome were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

Expression of the recombinant HA (rHA)

The cDNAs of the HA genes of Ck/IBR, Dk/MNG and their mutants were cloned into the pCD5 expression vector as described previously [83]. The rHA proteins were expressed in HEK293S GnTI−/− cells [84] and purified from the cell culture supernatants as described previously [85].

Glycan microarray

The glycan microarray was carried out with 53 glycans focused on α2,3 sialosides which are the preferred receptors of avian influenza viruses. On the array slide, 10 non-sialylated glycans (glycans #1–10), 36 non-fucosylated α2,3 sialylated glycans (glycans #11–46) and 7 fucosylated and α2,3 sialylated glycans (glycans #47–53) were printed (Fig. 10). Most glycans used were reported previously [83, 86], except for a few with extended glycan chains that were synthesized using methods reported previously (#31-35, 39-42, 51) [87, 88]. The rHA was used at 50 μg/ml precomplexed with HRP-linked anti-Strep-tag mouse antibody and with Alexa 488-linked anti-mouse IgG (4:2:1 molar ratio) prior to incubation for 30 min on ice in 100 μl of PBS containing 0.05% Tween 20 (PBST) and incubated on the array surface in a humidified chamber for 90 min. Slides were subsequently washed by successive rinses with PBST, PBS and de-ionized H_2O. Washed arrays were dried by centrifugation and immediately scanned for fluorescence signals on a Perkin-Elmer ProScanArray Express confocal microarray scanner (Waltham, MA, USA). Fluorescence signal intensity was measured using Imagene (Biodiscovery, Hawthorne, CA, USA), and the mean intensity minus mean background was calculated and graphed using MS Excel. For each glycan, the mean signal intensity was calculated from six replicates. The highest and lowest signals of the six replicates were removed, and the remaining four replicates were used to calculate the mean signal and standard error (SE).

In silico prediction of the binding of the HA of Ck/IBR and 3´SLeX

The three-dimensional (3D) structure of the H5 HA of Ck/IBR was constructed on the basis of the HA crystal structure of A/Vietnam/1194/2004 (H5N1) (PDB code 2IBX)

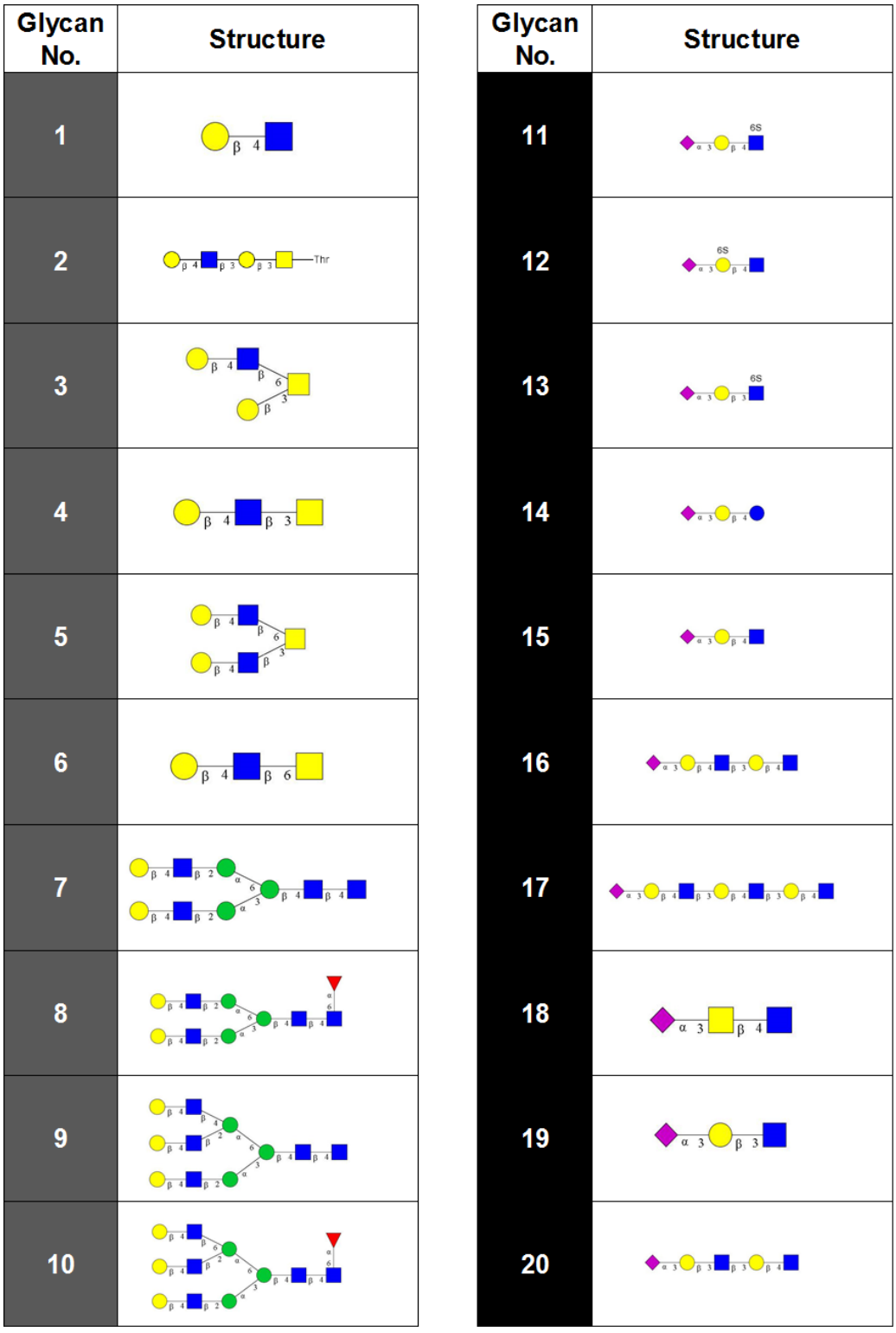


Fig. 10. Glycans imprinted on the microarray.

10 non-sialylated glycans (glycans #1–10, gray), 36 non-fucosylated α2,3 sialylated glycans (glycans #11–46, black) and 7 fucosylated and α2,3 sialylated glycans (glycans #47–53, white) were printed on the array.

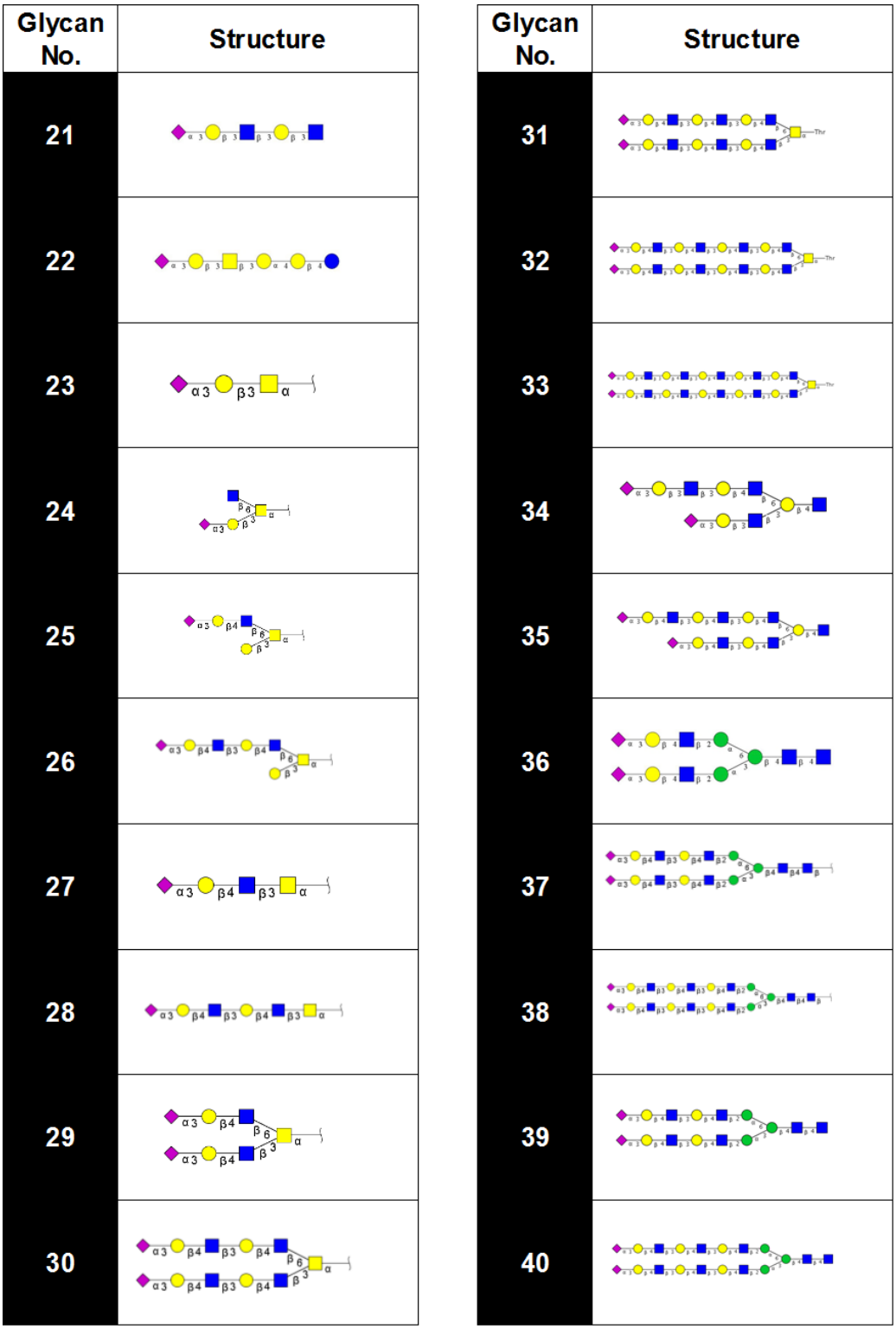
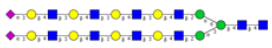
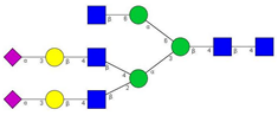
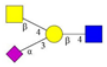
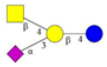
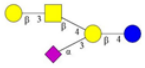
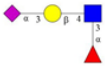
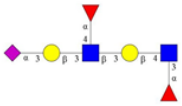
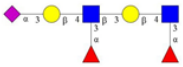
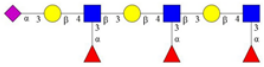


Fig. 10. Glycans imprinted on the microarray (continued).

10 non-sialylated glycans (glycans #1–10, gray), 36 non-fucosylated α2,3 sialylated glycans (glycans #11–46, black) and 7 fucosylated and α2,3 sialylated glycans (glycans #47–53, white) were printed on the array.

Glycan No.	Structure
41	
42	
43	
44	
45	
46	
47	
48	
49	
50	

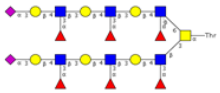
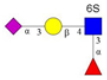
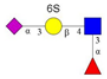
Glycan No.	Structure
51	
52	
53	

Fig. 10. Glycans imprinted on the microarray (continued).

10 non-sialylated glycans (glycans #1–10, gray), 36 non-fucosylated α2,3 sialylated glycans (glycans #11–46, black) and 7 fucosylated and α2,3 sialylated glycans (glycans #47–53, white) were printed on the array.

[89] as described previously [90]. A predicted 3D structure of 3´SLeX was downloaded from GLYCAM-Web (http://glycam.org/). To obtain the structure of HA of Ck/IBR bound to 3´SLeX, each structure was first superimposed on the crystal structure of the H2 HA in complex with 3´SLacNAc (PDB code 2WR3) [91], and coordinates of 2WR3 were then removed using Discovery Studio version 4.1 (Dassault Systemes Biovia, San Diego, CA, USA). This model structure was refined by energy minimization followed by a 5 ns of molecular dynamics with the AMBER 14 software (Conflex USA, San Diego, CA, USA). The ff99SB force field and GLYCAM06 force field were used for the HA and 3´SLeX, respectively. The energy minimization and molecular dynamics calculations were conducted in a similar way, as described previously [92]. The definition of hydrogen bonds is the following: distance between donor and acceptor is less than 3.5 Å, and angle between donor, hydrogen and acceptor is greater than 120°.

Solid-phase direct binding assay

The receptor binding specificity of viruses was assessed using a solid-phase direct binding assay with sialylglycopolymers 3´SLacNAc-PAA and 3´SLeX-PAA (Cosmo Bio Co., Ltd.) as described in Chapter II. The solid-phase direct binding assay was also performed in the presence of the NA inhibitor peramivir, which was a kind gift from Dr. Masanori Kobayashi of Shionogi & Co., Ltd. In this case, viruses were pre-incubated with either 2.5, 10 or 40 nM peramivir in PBS for 1 h on ice. The mixture was used as a virus containing solution.

A solid-phase direct binding assay was also conducted using rHA. Each sialylglycopolymer was serially diluted and added to each well of a Nunc Immobilizer Amino C8, 96 well strip well microplate (Thermo Fisher Scientific) and the plates were

incubated at 37 °C for 1 h. Each well was then blocked with PBST containing 2% BSA at room temperature for 3 h. The rHA was used at 5 μg/ml precomplexed with HRP-linked anti-Strep-tag mouse antibody (1:2000 dilution) and with goat anti-mouse IgG-HRP conjugate (1:1000 dilution) prior to incubation for 30 min on ice in PBST containing 0.5% BSA with or without 40 nM peramivir. After washing the plates, the complexes were added and incubated at room temperature for 1 h. After washing, 100 μl of the TMB substrates were added to each well. The reactions were stopped using 50 μl of 2N H_2SO_4, and absorbance at 450/630 nm was measured using a MULTISCAN JX.

Amino acid sequence comparison of the H5 HA

A total of 2,901 amino acid sequences of H5 HA were obtained from GenBank/EMBL/DDBJ. Sequences were aligned with mafft version 7.215 (http://mafft.cbrc.jp/alignment/software/). The amino acid sequences of highly pathogenic avian influenza viruses were removed according to the sequence of HA cleavage site. The remaining 631 sequences were further divided into viruses isolated from Anseriformes, non-chicken Galliformes or chickens according to the original host of each virus determined by the strain name.

Results

Computational analysis of the binding of the HA of a chicken influenza A virus to 3´SLeX

The binding model of an HA derived from a chicken influenza A virus, Ck/IBR to 3´SLeX was predicted using in silico analysis (Fig. 10). The fucose moiety of 3´SLeX is positioned close to two arginine (R) residues at positions 222 and 227 of the HA. The R residues at positions 222 and 227 are located within 3.5 Å of the C-2 and C-3 hydroxyl groups of the fucose, indicating potential hydrogen bonding. By comparing the amino acid sequence of Ck/IBR with that of a duck influenza virus, Dk/MNG, a lysine (K) residue at position 222 and a serine (S) residue at position 227 instead of arginines were observed. Accordingly, it was hypothesized that these amino acid substitutions contribute to differences in receptor binding specificity.

Glycan-binding specificity of the HA of Ck/IBR, Dk/MNG and their mutants

The rHAs of Ck/IBR and Dk/MNG were generated and subjected to glycan microarray analysis to evaluate their glycan-binding specificity (Fig. 12A and B). The rHA of Ck/IBR interacted with glycans #51 (3´SLeXTriLN-Core4) and #52 [Siaα2,3Galβ1,4(Fucα1,3)(6O-sulfo)GlcNAcβ-propyl-NH2] with high signal intensity. Although the rHA of Ck/IBR slightly interacted with non-fucosylated α2,3 sialosides, these signals were much weaker than those of glycans #51 and #52. No interaction was observed between the rHA of Dk/MNG and fucosylated α2,3 sialosides. Interestingly, most of the glycans, which were bound by the rHA of Dk/MNG, are biantennary glycans with multiple LacNAc repeats (e.g. #30-32 and #35). The results

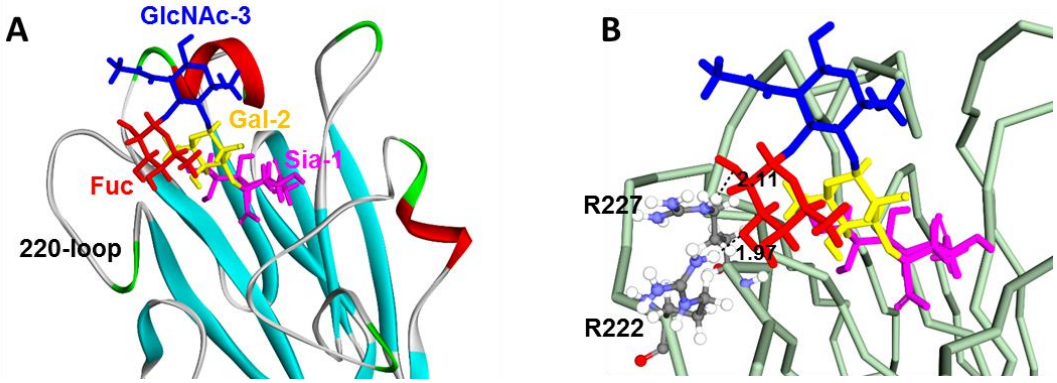


Fig. 11. Structure model of sialyl Lewis X (3´SLeX) bound to the HA of Ck/IBR, which was taken from snapshot at 5 ns.

The receptor binding site of the HA of Ck/IBR is shown in either a ribbon (A) or line (B) cartoon representation. In the glycan structure, Sia is shown in purple, Gal is shown in yellow, GlcNAc is shown in blue and Fuc is shown in red. In (B), dash lines indicate hydrogen bonds and the numbers indicate the predicted length of each hydrogen bond.

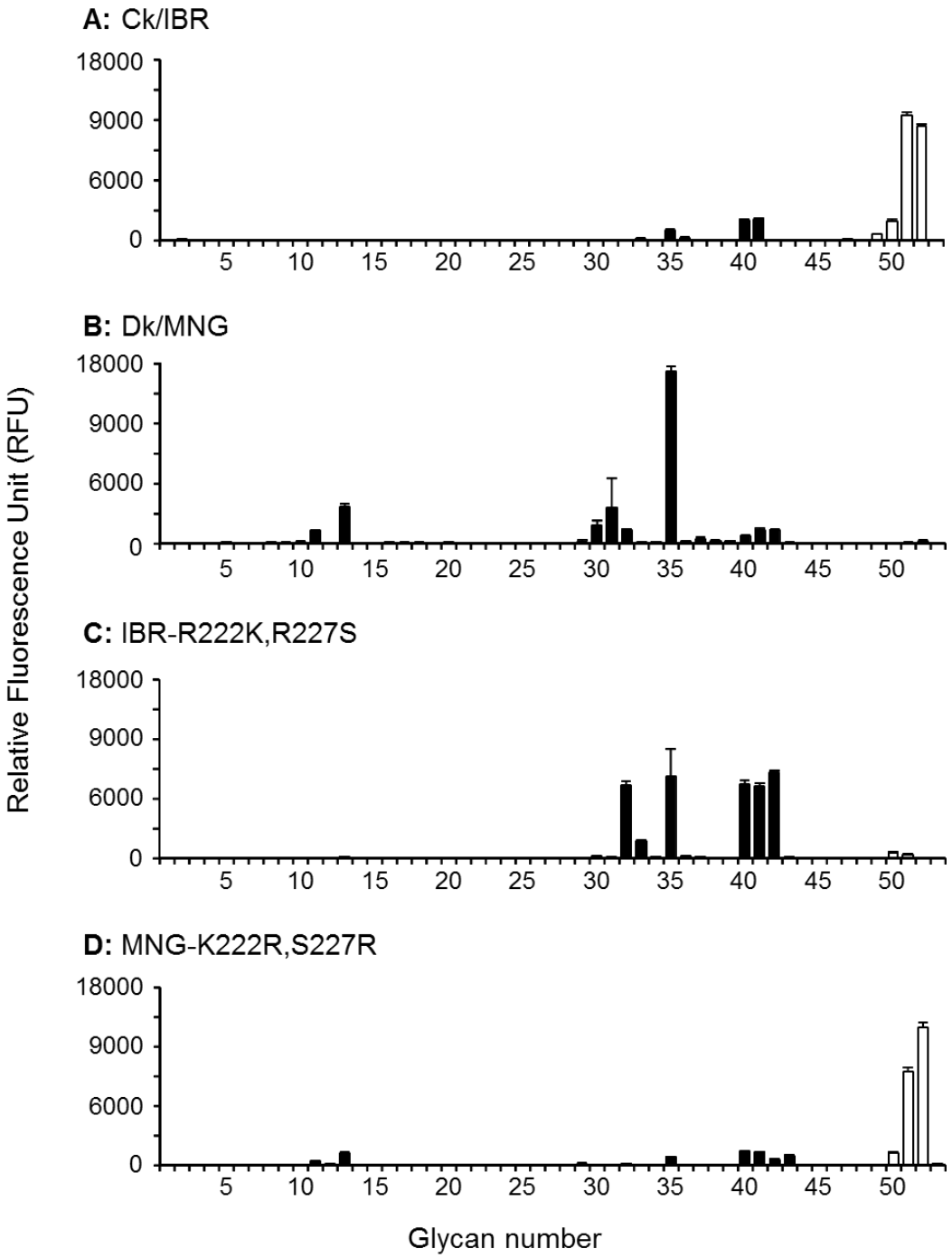


Fig. 12. Glycan-binding specificity of the rHAs.

The glycan-binding specificity of rHA of Ck/IBR (A), Dk/MNG (B), IBR-R222K,R227S (C) and MNG-K222R,S227R (D) was analyzed by glycan microarray. Non-sialylated controls are shown as grey bars (#1–10), non-fucosylated α2,3 sialylated glycans are presented as black bars (#11–46) and fucosylated and α2,3 sialylated glycans are shown in white bars (#47–53). Each bar represents the mean signal minus background for each glycan sample and error bars are the SE value.

indicate that the rHA of Ck/IBR selectively binds α1,3 fucosylated sialosides, whereas that of Dk/MNG selectively binds non-fucosylated α2,3 sialosides. To evaluate the contribution of amino acid residues at positions 222 and 227 of the HA to this binding specificity of rHAs, mutant rHAs of Ck/IBR and Dk/MNG, in which amino acid residues at positions 222 and 227 were altered (IBR-R222K,R227S and MNG-K222R,S227R respectively) were generated and these rHAs were subjected to glycan microarray analysis (Fig. 12C and D). The rHA of IBR-R222K,R227S interacted with non-fucosylated α2,3 sialosides, whereas no binding was observed with fucosylated α2,3 sialosides. The rHA of MNG-K222R,S227R interacted with glycans #51 and #52 with high specificity, whereas almost no binding was observed with non-fucosylated α2,3 sialosides. These results indicate that the glycan-binding specificity of rHA to fucosylated and non-fucosylated α2,3 sialosides is determined by the amino acid motifs at positions 222 and 227 of the HA.

Glycan-binding specificity of the virions of Ck/IBR, Dk/MNG and their mutants

In Chapter II, virions of Ck/IBR specifically bound to α1,3 fucosylated sialosides, 3´SLeX in a solid-phase direct binding assay; whereas those of Dk/MNG bound to both non-fucosylated α2,3 sialosides, 3´SLacNAc and 3´SLeX. To further elucidate the importance of the amino acid motifs at positions 222 and 227 of HA, mutant viruses, namely rgIBR/HA-R222K,R227S and rgMNG/HA-K222R,S227R were generated, and their receptor binding preferences were characterized by a solid-phase direct binding assay (Fig. 13). Consistent with the results in Chapter II, virions of Ck/IBR specifically bound to 3´SLeX, whereas those of Dk/MNG bound to both 3´SLacNAc and 3´SLeX. rgIBR/HA-222K,227S bound to both 3´SLacNAc and 3´SLeX,

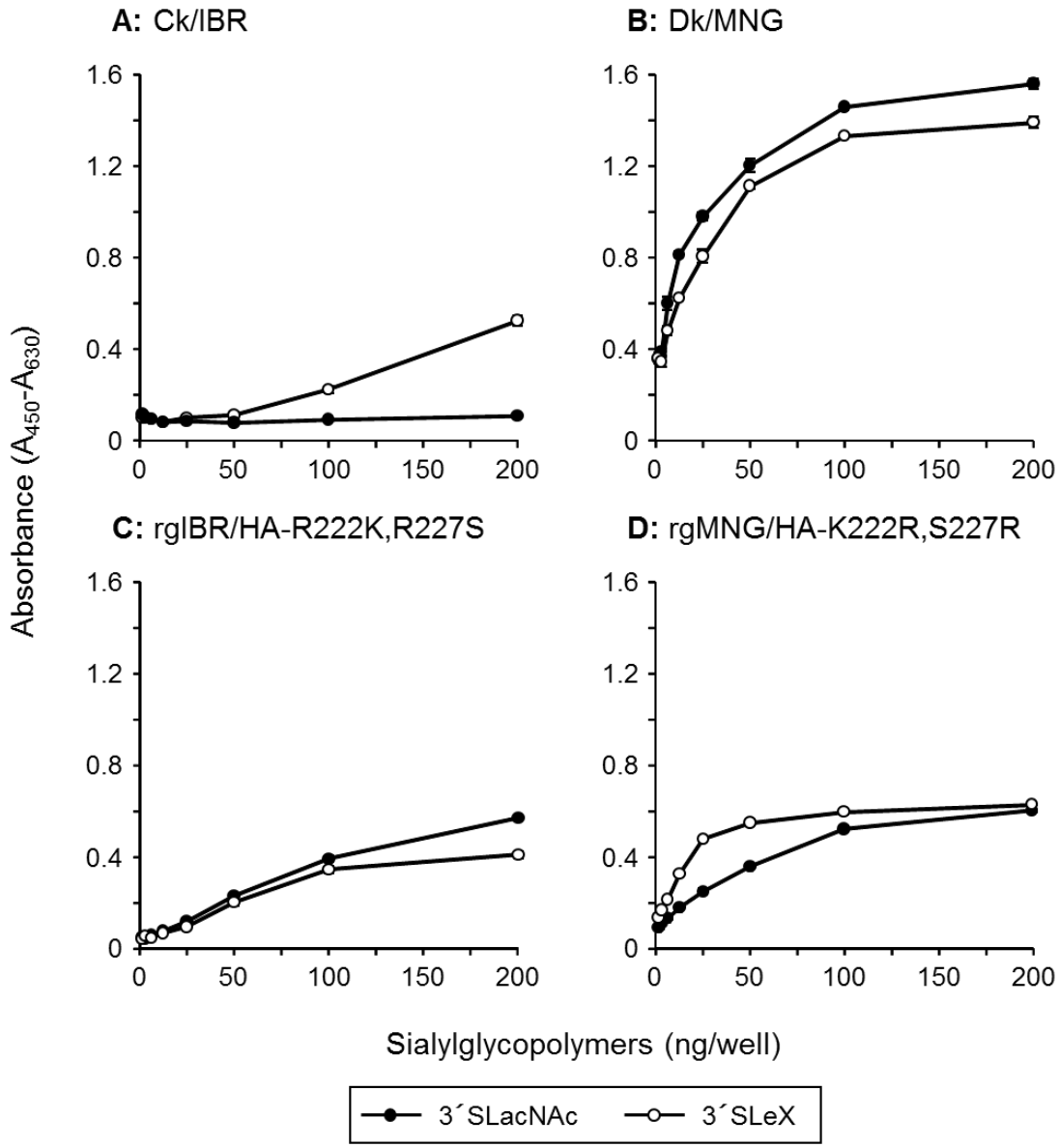


Fig. 13. Glycan-binding specificity of virions.

The glycan-binding specificity of Ck/IBR (A), Dk/MNG (B), rgIBR/HA-R222K,R227S (C) and rgMNG/HA-K222R,S227R (D) to 3′SLacNAc (closed circles) or 3′SLeX (open circles) covalently linked polyacrylamide chain was investigated using solid-phase direct binding assays. The data are presented as the mean ± SE of triplicate experiments.

indicating that R222K and R227S mutations facilitate binding of non-fucosylated glycans. rgMNG/HA-222R,227R also bound to both 3´SLacNAc and 3´SLeX; however, binding avidity to 3´SLacNAc was significantly lower than that to 3´SLeX.

Dk/MNG virions bind to fucosylated α2,3 sialosides through the neuraminidase

The rHA of Dk/MNG specifically bound to non-fucosylated α2,3 sialosides; whereas virions of Dk/MNG bound to both fucosylated and non-fucosylated sialosides (Fig. 12B and 13B). To investigate contribution of NA, which is the other Sia-interacting viral protein of influenza A viruses, to glycan-binding by influenza virus virions, a solid-phase direct binding assay was conducted in the presence of the NA inhibitor, peramivir (Fig. 14). Binding of Dk/MNG to 3´SLeX was diminished in the presence of 2.5 nM peramivir. Although binding to 3´SLacNAc was slightly affected in the presence of higher concentration of peramivir, almost no binding to 3´SLeX was observed in the presence of 10 or 40 nM peramivir. This indicates that the NA of Dk/MNG binds to fucosylated α2,3 sialosides. To confirm that peramivir specifically inhibit the glycan-binding by the NA and has no effect on the glycan-binding mediated by the HA of the HA in the assay, a solid-phase direct binding assay was conducted with the rHA of Dk/MNG in the presence of peramivir (Fig. 15). Consistent with the result of glycan microarray (Fig. 2B), the rHA of Dk/MNG preferentially bound 3´SLacNAc in the absence of peramivir (Fig. 15A). The binding of the rHA to 3´SLacNAc and 3´SLeX was similar in the presence of 40 nM peramivir, and no inhibition was observed (Fig. 15B). These results confirmed that peramivir has no effect on the interaction between sialosides and the rHA of Dk/MNG. Accordingly, the present result indicates that the NA of Dk/MNG binds to fucosylated α2,3 sialosides.

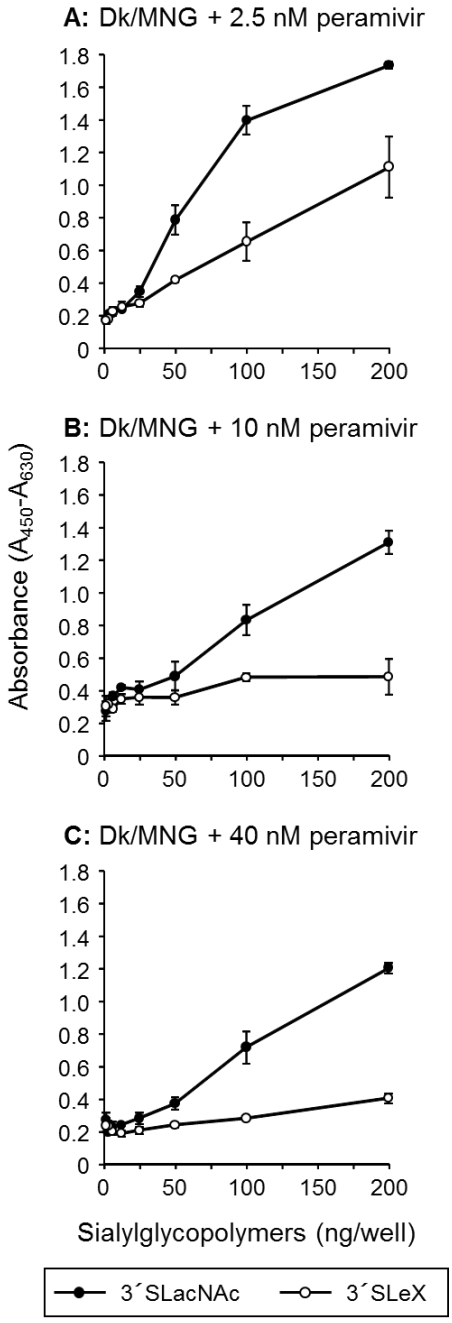


Fig. 14. Glycan-binding specificity of virions of Dk/MNG in the presence of peramivir.

The glycan-binding specificity of virions of Dk/MNG to 3′SLacNAc (closed circles) or 3′SLeX (open circles) covalently linked polyacrylamide chain was investigated in the presence of either 2.5 (A), 10 (B) or 40 (C) nM of a neuraminidase inhibitor peramivir. The data are presented as the mean ± SE of triplicate experiments.

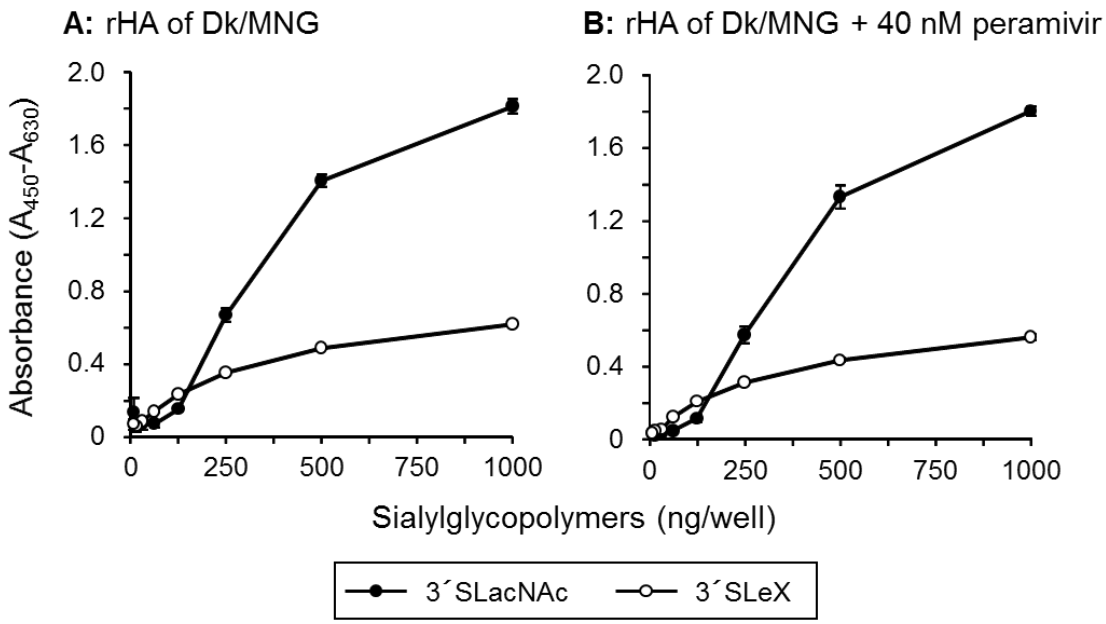


Fig. 15. Glycan-binding specificity of the rHA of Dk/MNG in the presence of peramivir.

The glycan-binding specificity of the rHA of Dk/MNG to 3′SLacNAc (closed circles) or 3′SLeX (open circles) covalently linked polyacrylamide chain was investigated in the absence (A) or presence (B) of a neuraminidase inhibitor peramivir. The data are presented as the mean ± SE of triplicate experiments.

Amino acid sequence comparison of HA of H5 avian influenza viruses

The amino acid motifs at positions 222 and 227 in HA of H5 avian influenza viruses isolated from Galliformes and Anseriformes were analyzed (Table 8). The majority of LPAIVs isolated from chickens have a glutamine (Q) residue at position 222 and an arginine (R) residue at position 227. On the other hand, the majority of viruses isolated from Anseriformes have a lysine (K) residue at position 222 and a serine (S) residue at position 227. Interestingly, most of the viruses isolated from non-chicken Galliformes have a K residue at position 222 and an S residue at position 227, which isthe same amino acid sequence present in isolates from Anseriformes.An R residue at position 222 as observed in Ck/IBR is a relatively rare motif in H5 avian influenza viruses. Consequently, mutant viruses, which possess same amino acid residues as consensus motif of chicken LPAIVs at positions 222 and 227 of the HA, namely rgIBR/HA-R222Q and rgMNG/HA-K222Q,S227R were generated. Glycan-binding analysis by solid-phase direct binding assays demonstrated that rgIBR/HA-R222Q specifically bound to 3´SLeX (Fig. 16A). Also, glycan-binding preference of rgMNG/HA-K222Q,S227R is similar to that of rgMNG/HA-222R,227R (Fig. 16B). These results indicate that Q and R residue at position 222 of the HA have similar effects on the glycan-binding property of HA in combination with R residue at position 227.

Table 8. Amino acid sequence comparison of 222/227 motifs in HA of H5 LPAIVs.

Host	Amino acid residue		Number of strains
	222	227	
Chicken	Q	R	70
(98 strains)	K	S	26
	R	S	1
	R	**R**	1
Anseriformes	**K**	**S**	471
(506 strains)	R	S	18
	Q	S	13
	E	R	2
	N	S	1
	R	R	1
Non-chicken Galliformes	K	S	25
(27 strains)	R	S	1
	Q	R	1

Amino acid sequences of H5 HA were obtained form GenBank/EMBL/DDBJ.
Amino acid motifs of Ck/IBR and Dk/MNG were shown in bold.

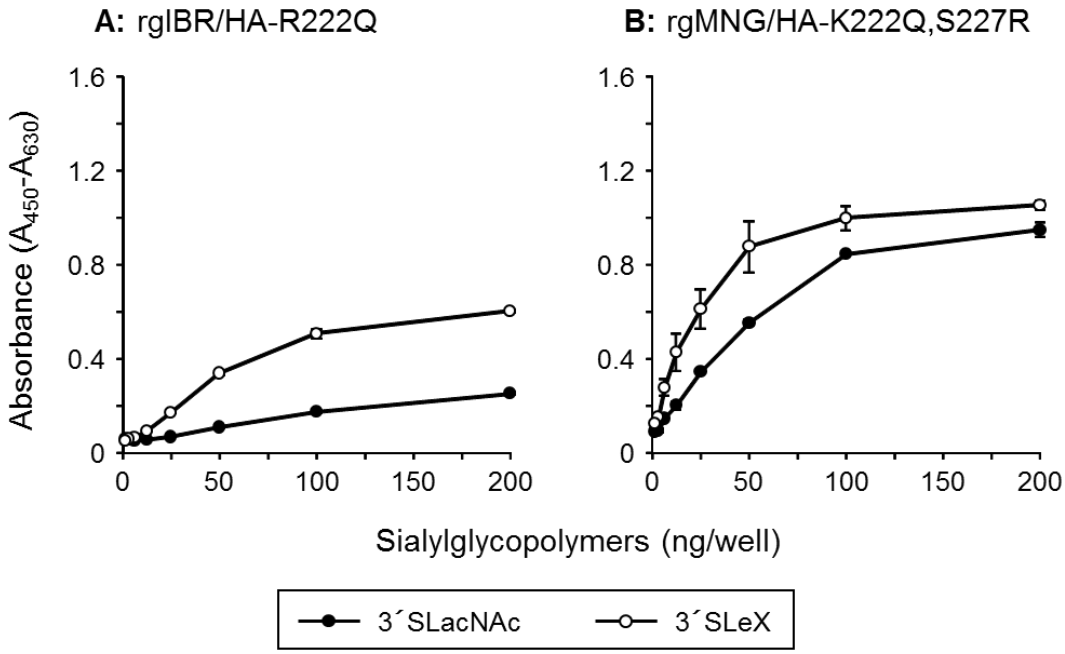


Fig. 16. Glycan-binding specificity of rgIBR/HA-R222Q and rgMNG/HA-K222Q,S227R.

Glycan-binding specificity of virions of rgIBR/HA-R222Q (A) and rgMNG/HA-K222Q,S227R (B) to 3′SLacNAc (closed circles) or 3′SLeX (open circles) covalently linked polyacrylamide chain was investigated using solid-phase direct binding assays. The data are presented as the mean ± SE of triplicate experiments.

Discussion

In Chapter II, it was shown that α2,3 sialosides expressed on epithelial cells of the chicken trachea are fucosylated. Although modification of α2,3 sialosides has a critical impact on the glycan-binding specificity of influenza viruses [63, 67], the interaction of these modified α2,3 sialosides with HA is not fully understood. In the present study, binding specificity of two H5 LPAIVs, Ck/IBR and Dk/MNG, to fucosylated or non-fucosylated α2,3 sialosides was analyzed. The present results reveal that R222 and R227 in the HA of Ck/IBR are located close to the α1,3 fucose linked to antepenultimate GlcNAc (Fig. 11). A previous structural analysis indicated that this fucose moiety is positioned close to K222 in the HA of a highly pathogenic avian influenza virus and that it de-stabilized the interaction of the HA with glycan [78]. Glycan microarray analysis of rHAs demonstrated that K222R substitution in a combination with S227R in the HA of Dk/MNG altered its glycan-binding specificity from non-fucosylated to fucosylated α2,3 sialosides (Fig. 12B and D). This suggests that these substitutions in the HA alter the interaction with fucose and stabilizes the binding.

Sequence comparison of HA of H5 influenza A viruses revealed that the amino acid motif K222 and S227 were highly conserved within LPAIVs circulating among migratory ducks (Table 8). Receptor binding analyses indicate that viruses with K222 and S227 specifically bound non-fucosylated α2,3 sialosides, whereas viruses with R222 and R227 as well as Q222 and R227 specifically bound fucosylated α2,3 sialosides (Fig. 13 and 15). These facts suggest that H5 LPAIVs isolated from chickens prefer fucosylated α2,3 sialosides and those isolated from ducks prefer

non-fucosylated α2,3 sialosides. Interestingly, viruses isolated from non-chicken Galliformes species, such as quails or turkeys have the same amino acid motif as LPAIVs isolated from Anseriformes. Influenza viruses circulating among migratory ducks are transmitted to chickens via terrestrial poultry such as quails or turkeys [7, 93]. These facts suggest that the acquisition of "chicken type receptor specificity" may not occur during adaptation in ducks or later in non-chicken terrestrial poultry, but occurs during the multiple replication and transmission events in chickens.

Glycan-binding analysis of virions and rHAs showd different glycan-binding preferences (Fig. 12 and 13). Interestingly, virions of Dk/MNG bound to both fucosylated α2,3 sialosides, 3´SLeX and non-fucosylated α2,3 sialosides, 3´SLacNAc, whereas the rHA of Dk/MNG bound only non-fucosylated α2,3 sialosides. A solid-phase direct binding assay in the presence of peramivir suggests that the NA contributes to receptor binding specificity of Dk/MNG virions to 3´SLeX (Fig. 14). A solid-phase direct binding assay using rHA confirmed that the rHA of Dk/MNG preferentially bound non-fucosylated α2,3 sialosides (Fig. 15A). The presence of peramivir had no effect on the glycan-binding of the rHA of Dk/MNG (Fig. 15B), indicating that peramivir specifically inhibited glycan-binding of the NA in the solid-phase direct binding assay using virions of Dk/MNG (Fig. 14). These results indicate that the glycan-binding specificity of virions of Dk/MNG results from additive effects of glycan-binding to 3´SLacNAc mediated by the HA and to 3´SLeX mediated by the NA, and thus, the binding to 3´SLeX was diminished by the NA inhibitor. NA of influenza viruses has sialidase activity, which means that NA also binds sialylated glycans [1, 3]. In the case of H7N9 influenza viruses recently isolated in China, it was proposed that the preferential cleavage of α2,3 sialosides by the NA along with intrinsic

weak binding to α2,6 sialosides by the HA exaggerated receptor binding preference of these viruses to α2,6 sialosides [83]. Thus, the NA modulates the receptor binding preference of influenza virus by cancelling certain receptor binding with its sialidase activity. The NA of Dk/MNG modulates receptor specificity via a mechanism which is distinct from the previous model, as the NA obviously promote binding to 3´SLeX (Fig. 2B and 3B). However, it is still unknown whether glycan binding by the NA is functional in that it would lead to infection. Thus, determining the receptor binding preferences of influenza viruses requires complementary assays using rHA and complete virions for the elucidation of fine receptor binding preferences of influenza viruses.

It should also be pointed out that rHA of Dk/MNG did not exhibit binding to glycan #15 (Fig. 12B), which has exactly same glycan structure with the non-fucosylated α2,3 sialoside used in solid-phase direct binding assay. The virions of Dk/MNG strongly bound this glycan structure in the solid-phase direct binding assay (Fig. 13B). In the case of solid-phase direct binding assay, glycans are polymerized with PAA. In addition, whole virus particles were employed in the solid-phase direct binding assay; thereby interaction of HA and glycans are multivalent in this assay. On the other hand, monomeric glycans were printed in the case of glycan microarray. These differences in the settings of each assay may lead the difference of the results in these two assays.

The present results clarify the molecular basis of the interaction between α1,3 fucosylated sialosides and HA of influenza viruses. α2,3 sialosides are structurally divergent; penultimate Gal and the antepenultimate GlcNAc can be sulfated, the linkage between Gal and GlcNAc can be a β1,3 or β1,4 linkage and the core structure of glycans

can be N-linked, O-linked or glycolipid. It was reported that presentation and internal complexity of glycan structure influenced their interaction with HA (See [88] and references therein). Indeed, the internal structure of glycans affected binding specificity of HA; the rHA of Dk/MNG preferred non-fucosylated α2,3 sialosides with multiple LacNAc repeats (Fig. 12B). Similarly, rHA of Ck/IBR bound to glycan #51 with high signal intensity, whereas signals to glycan #47, 49 and 50, which contain the same glycan sequence with glycan #51 on the non-redacting end, was much weaker (Fig. 12A). The importance of the internal glycan structure for influenza virus infection is not at all understood, even though glycan microarray data on the receptor binding specificity of influenza viruses should provide further implications on the relationship between glycan structure and glycan-binding specificity of influenza viruses. The present results demonstrat the significance of the structural diversity of sialosides on the interaction of influenza A viruses with host cell surface receptors.

Summary

Influenza viruses isolated from ducks are rarely able to infect chickens; it is therefore postulated that these viruses need to adapt in some way to be able to transmit to chickens in nature. In Chapter II, it was demonstrated that sialyl Lewis X (3´SLeX), which is fucosylated α2,3 sialoside, was predominantly detected on epithelial cells of the chicken trachea, whereas this glycan structure is not found in the duck intestinal tract. To clarify the mechanisms of the interspecies transmission of influenza viruses between ducks and chickens, the receptor specificity of low pathogenic avian influenza viruses isolated from these two species was compared. Glycan-binding analysis of the recombinant hemagglutinin (HA) of a chicken influenza virus, A/chicken/Ibaraki/1/2005 (H5N2), showed a binding preference to α1,3 fucosylated sialosides. On the other hand, the HA of a duck influenza virus, A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG), particularly bound to non-fucosylated α2,3 sialosides such as 3′-sialyllactosamine (3´SLacNAc). Computational analyses along with binding analyses of the mutant HAs demonstrated that this glycan-binding specificity of the HA was determined by amino acid residues at positions 222 and 227. Inconsistent with the glycan-binding specificity of the recombinant HA protein, virions of Dk/MNG bound to both 3´SLacNAc and 3´SLeX. Glycan-binding analysis in the presence of a neuraminidase (NA) inhibitor indicated that the NA conferred binding to 3´SLeX to virions of Dk/MNG. The present results clarify the molecular basis of the interaction between fucosylated α2,3 sialosides and influenza viruses.

Conclusion

Avian influenza is a highly contagious disease of poultry. Since late 2003, HPAIVs have seriously affected poultry in Eurasia and Africa. LPAIVs have also been prevailing poultry populations in Southeast Asia, potentially causing significant economic loss. Understanding ecology and mechanism of interspecies transmissions of avian influenza viruses is essential for the control of both highly and low pathogenic avian influenza in poultry.

Wild water birds play crucial roles in widespread dissemination of avian influenza viruses. In the present study, surveillance of avian influenza in wild birds was conducted from 2010 to 2014 in Hokkaido and Mongolia to monitor influenza viruses of H5 and H7 subtypes in wild water birds. The present results confirm that HPAIVs and H7N9 influenza viruses have not been dominantly prevailing among wild water birds. Genetic and antigenic analysis of H5 and H7 viruses isolated from wild water birds indicated that antigenicity of influenza viruses circulating in wild water birds is highly stable within these subtypes despite their diversity in nucleotide sequences of the HA gene. The present study elucidates genetic and antigenic feature of avian influenza viruses in wild water birds and demonstrate the importance of continued monitoring of avian influenza viruses of H5 and H7 subtypes both in wild and domestic birds.

In order to elucidate mechanism of interspecies transmission of avian influenza viruses from ducks to chickens, Chapter II focused on fucosylation of antepenultimate GlcNAc of α2,3 sialosides. The present results demonstrate that α1,3 fucosylated sialosides, 3´SLeX was predominantly detected on epithelial cells lining the upper

respiratory tract of chickens, whereas this glycan structure is not found in the duck intestinal tract. It was also indicated that Ck/IBR, which replicated in the upper respiratory tract of chickens preferentially utilize fucosylated α2,3 sialosides for virus infection.

In Chapter III, it was shown that amino acid residue positions 222 and 227 of HA determine glycan-binding specificity to fucosylated and non-fucosylated α2,3 sialosides. The amino acid sequence comparison of HA of H5 influenza A viruses implied that the majority of H5 LPAIVs isolated from chickens prefer fucosylated α2,3 sialosides and those isolated from ducks prefer non-fucosylated α2,3 sialosides. The present study demonstrates importance of α1,3 fucosylation in antepenultimate GlcNAc of α2,3 sialosides in interspecies transmission of H5 LPAIVs from ducks to chickens.

The present study provides a better understanding of ecology and interspecies transmission of avian influenza viruses. These results should contribute to the control of avian influenza in both wild and domestic birds.

Acknowledgements

The author would like to acknowledge Prof. Y. Sakoda, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University (Sapporo, Japan), and Prof. H. Kida, Research Center for Zoonosis Control, Hokkaido University for their guidance and encouragement during the course of this work.

Great appreciation is extended to Prof. M. Horiuchi, Laboratory of Veterinary Hygiene, Department of Applied Veterinary Science, Graduate School of Veterinary Medicine, Hokkaido University and Prof. A. Takada, Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University for their critical review of the manuscript.

The author is extremely grateful to Dr. M. Okamatsu and Dr. K. Matsuno, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University for their valuable advice, supports and encouragements throughout the present study.

The author heartily thanks Dr. RP. de Vries, Utrecht Institute for Pharmaceutical Sciences, Utrecht University (Utrecht, The Netherlands); Prof. JC. Paulson, Mr. R. McBride and Dr. P. Wenjie, Department of Cell and Molecular Biology, Chemical Physiology and Immunology and Microbial Science, The Scripps Research Institute (La Jolla, CA, USA); Prof. S. Takase-Yoden and Prof. S. Nishihara, Soka University (Hachioji, Japan); Dr. M. Igarashi Research Center for Zoonosis Control, Hokkaido University; Ms. A. Ohkawara and K. Ogasawara, Laboratory of Microbiology,

Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University for their excellent contribution and support in the present study.

The author thanks to Dr. T. Saito, National Institute of Animal Health (Tsukuba, Japan); Dr. M. Tashiro, National Institute of Infectious Diseases (Tokyo, Japan); Dr. LSM. Geraldine, Tai Lung Veterinary Laboratory (Hong Kong SAR, China); Dr. P. Selleck, Australian Animal Health Laboratory (Geelong, Australia); and Dr. I. Capua, Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) for providing the valuable viruses used in the present study; Ms. C. Yamamoto for the kind help in the sampling in Mongolia; Dr. M. Kobayashi and Mr. K. Taniguchi, Shionogi & Co., Ltd., (Osaka, Japan) for the kindest supports in the present experiments using peramivir.

The author earnestly appreciates all colleagues of Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University for their invaluable helps and supports.

The Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists, the Program for Leading Graduate Schools (F01) from JSPS supported this work.

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

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Summary in Japanese（和文要旨）

鳥インフルエンザは A 型インフルエンザウイルスに起因し、高い伝播性を特徴とする家禽のウイルス性疾病である。カモに代表される野生の水禽類からは、すべての亜型の A 型インフルエンザウイルスが分離される。すなわち、カモは A 型インフルエンザウイルスの自然宿主である。カモの有する非病原性の鳥インフルエンザウイルスが他の水生家禽や陸生家禽に伝播する過程を経ると、ニワトリへの感染性を獲得することがある。さらにウイルスが鶏群内で感染伝播を繰り返すと、HA の亜型が H5 または H7 のウイルスに限りニワトリに対して致死的な病原性を示す、高病原性鳥インフルエンザウイルスが出現することがある。現在、高病原性および低病原性鳥インフルエンザウイルスがユーラシアとアフリカの家禽に蔓延し、経済的な損害を与えている。本疾病のコントロールのためには、鳥インフルエンザウイルスの生態と異種宿主間伝播機構の解明が必須である。

2010 年北海道稚内市の大沼にて、北の営巣湖沼から南に渡ってきた野生水禽の糞便から 2 株の H5 亜型高病原性鳥インフルエンザウイルスが分離された。また 2013 年に初めて人から H7N9 亜型のインフルエンザウイルスが分離され、同様のウイルスが中国南部の家禽で流行している。家禽疾病の制御のために、これら家禽に被害を与える可能性がある H5 および H7 亜型鳥インフルエンザウイルスの野生水禽における浸潤状況を調べる必要がある。そこで本研究では、2010 年から 2014 年に野生水禽における鳥インフルエンザのサーベイランスを行った。8,103 の糞便を採取し、そこから 350 株のインフルエンザウイルスを分離した。H5 亜型ウイルスは 13 株、H7 亜型ウイルスは 19 株分離された。これらのウイルスは上述の 2 株の高病原性鳥インフルエンザウイルスを除き、すべてが野生

水禽で循環する非病原性鳥インフルエンザウイルスであった。このことから H5 亜型高病原性鳥インフルエンザウイルスや H7N9 亜型鳥インフルエンザウイルスが野生水禽の中で優勢に存続していないことが確認された。また、これらのウイルスの抗原性は、野生水禽で循環するウイルスのそれと同様であり、野生水禽の中ではウイルスの抗原性が安定であることが示された。

カモで循環する非病原性の鳥インフルエンザウイルスはニワトリに直接伝播することはない。すなわちニワトリとカモではインフルエンザウイルスに対する感受性が異なっているが、これを規定する因子については不明である。ニワトリから分離された低病原性鳥インフルエンザウイルス A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR) 株は、α2,3 シアロ糖鳥型レセプターの中でもフコシル化 α2,3 シアロ糖に優位に結合した。また、ニワトリの上部気道にはフコシル化 α2,3 シアロ糖が、カモの結腸にはフコシル化のない α2,3 シアロ糖が分布していた。さらに、犬腎臓由来株化細胞 (MDCK 細胞) においてフコース転移酵素を安定発現した MDCK-FUT 細胞を樹立した。この細胞と元の MDCK 細胞における Ck/IBR 株の増殖性を比較したところ、Ck/IBR 株は MDCK-FUT 細胞でより増えた。以上のことからニワトリから分離された Ck/IBR 株はニワトリの上部気道に発現するフコシル化 α2,3 シアロ糖を主要なレセプターとすることが示された。

Ck/IBR 株の HA とフコシル化 α2,3 シアロ糖の結合をコンピューターで予測したところ、フコシル化 α2,3 シアロ糖のフコース基はヘマグルチニン (HA) の 222 および 227 位にそれぞれ存在するアルギニン残基と水素結合を形成した。この部位のアミノ酸を Ck/IBR 株と、野生水禽の糞便より分離された A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG) 株で比較したところ、Ck/IBR 株では 222 位と 227 位に共にアルギニンを有しているのに対し、Dk/MNG 株では 222 位にリジン、227 位にセリンを有していた。そこで Ck/IBR 株と Dk/MNG 株の発

現 HA を構築し、その糖鎖結合特異性を調べた。その結果、Ck/IBR 株の HA はフコシル化 α2,3 シアロ糖と、Dk/MNG 株の HA はフコシル化のない α2,3 シアロ糖と特異的に結合した。また、この糖鎖結合特異性は HA の 222 および 227 位のアミノ酸配列が規定していた。以上の成績は H5 亜型鳥インフルエンザウイルスのフコシル化 α2,3 シアロ糖認識機構を明らかにしたものである。

以上、本研究により明らかになった知見は、鳥インフルエンザウイルスの生態と異種宿主間伝播機構のさらなる理解に貢献するものであり、家禽における高病原性および低病原性鳥インフルエンザの制御に資することが期待される。