（**Original article**）

Antigenic structure of the hemagglutinin of H9N2 influenza viruses

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Summary

The hemagglutinins (HAs) of H9 influenza viruses isolated from birds and mammals of different species were antigenically and genetically analyzed. Antigenic variants were selected from A/swine/Hong Kong/10/98 (H9N2) and A/duck/Hokkaido/13/00 (H9N2) in the presence of monoclonal antibodies (MAbs), respectively. Based on the reactivity patterns of these mutants with a panel of MAbs, at least 5 non-overlapping antigenic sites were defined using 8 MAbs which recognized 7 distinct epitopes on the H9 HA molecule. Based on the reactivity patterns with the panel of monoclonal antibodies, 21 H9N2 virus strains isolated from birds and mammals were divided into 7 antigenically distinct groups. The present findings indicate that it is important to monitor the antigenic variation in H9 influenza viruses. The panel of MAbs in the present study, thus, should be useful for detailed antigenic analysis of the H9 HAs for epidemiological studies, the selection of vaccine strains, and diagnosis.

Keywords

H9N2, hemagglutinin, influenza virus, monoclonal antibody

Introduction

Since the 1990s, outbreaks of H9N2 influenza virus infections in poultry have caused great economic losses in many countries in Asia and the Central East [5-7, 9, 19]. The causal H9N2 virus strains, however, did not reproduce severe disease signs in specific pathogen free (SPF) chickens. Co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus* and *Haemophilus paragallinarum* or attenuated coronavirus vaccine strains with H9N2 virus exacerbate the disease [1, 9, 10, 15]. The hemagglutinins (HAs) of H9N2 virus isolates in Asia were antigenically and genetically grouped into three distinct lineages, represented by the viruses A/quail/Hong Kong/G1/97 (H9N2) (G1), A/duck/Hong Kong/Y280/97 (H9N2) (Y280), and A/duck/Hong Kong/Y439/97 (H9N2) (Y439) [5]. Many of the H9N2 viruses isolated from chickens, ducks [6, 7, 17], and pigs in southern China [3, 22, 29] belonged to the Y280 llineage. Antigenic and genetic analyses of H9N2 viruses in poultry revealed that the Y280 lineage designated as the A/chicken/Beijing/1/94-like group was further divided into two subgroups and triple or quadruple reassotants with gene segments of G1-like, Y280-like, and H5N1-like viruses in Hong Kong in 2001 continued to prevail in southern China [30, 31]. It was also assumed that Y280-like variant viruses originated from those circulating in poultry in Asian countries over 10 years.

These H9N2 influenza viruses had receptor-binding specificity to terminal sialic acid with α2-6 Gal linkage found on human cells [8, 21]. Actually, H9N2 viruses of G1 and Y280 lineage have been isolated from humans [18, 23]. Thus, in addition to H5N1 and H7N7, H9N2 viruses are possible canditates that have potential to cause pandemics in humans.

The HA of influenza virus is the major target for immune responses due to its role in mediating attachment to and penetration into host cells. The antigenic structure of HAs of H1, H2, H3, H5, and H9 subtypes of influenza A virus has been investigated by antigenic mapping and sequence analysis [2, 11, 12, 24, 26, 28]. Antigenic mapping of the H1 HA, A/PR/8/34 (H1N1), indicates five immunodominant antigenic sites designated Sa, Sb, Ca1, Ca2 and Cb [4] and is comparable to that of H3 subtype virus, A/Hong Kong/1968 (H3N2), designated A, B, C, D and E [28]. The analysis of H9 antigenic variants defined at least two antigenic sites on the H9 HA molecule [12]. In the present study, antigenic and genetic analyses of the HA of H9N2 viruses were performed using a panel of monoclonal antibodies (MAbs).

Materials and methods

Viruses

Influenza virus A/swine/Hong Kong/10/98 (H9N2) (Sw/HK/98), A/duck/Hokkaido/13/00 (H9N2) (Dk/Hok/00) and other H9 viruses isolated from birds and mammals of different species were grown in 10-day-old embryonated chicken eggs and were stored at -80^{o}C until used (Table 1). The viruses were purified by differential centrifugation and sedimentation through a sucrose gradient [13].

Monoclonal Antibodies

Monoclonal antibodies (MAbs) against Sw/HK/98 (H9N2) and Dk/Hok/00 (H9N2) were prepared as described previously [14]. Briefly, BALB/c mice were immunized with the purified virus. Spleen cells of the mice were fused with myeloma cells and hybridoma cells secreting specific MAbs were selected. Each of the hybridoma cells was inoculated to mice intraperitoneally and ascitic fluids containing MAbs were used as MAb. Isotypes of MAbs were determined using Mouse Monoclonal Antibody Isotyping Reagents (Sigma, MO, U.S.A).

Serological tests

The hemagglutination-inhibition (HI) test was performed by the standard method [25]. In the neutralization test (NT), titers were determined as the reciprocals of maximum antibody dilution which prevents the cytopathic effect of 100 $TCID_{50}$ of viruses using Madin-Darby Canine Kidney (MDCK) cells. Enzyme-linked immunosorbent assay (ELISA) was performed as described previously [14].

Selection of antigenic variants

Antigenic variants were selected and the frequencies of antigenic variants were determined as described

previously [14]. Briefly, the virus was incubated with excess antibody for 1 hour at room temperature, and the mixture was inoculated into 10-day-old embryonated chicken eggs. The yielded viruses were detected by HA test after 48-hour incubation at 35°C and cloned by limiting dilution in embryonated eggs.

Sequence analysis of virus genes

Viral RNAs were isolated from virus-containing allantoic fluid with Trizol LS reagent (Invitrogen, PO, U.S.A). HA genes were sequenced after viral RNA extraction and reverse transcription- polymerase chain reaction (RT-PCR) according to Liu et al. [20]. Other Sequence data were assembled and translated to the amino acid sequence by gene analyzing software GENETYX-WIN version 6.1.0 (Genetyx Corporation, Tokyo, Japan). The positions of amino acid substitution on the HA molecule were analyzed on the 3-dimensional structure obtained from the Protein Databank (PDB accession number, 1JSD) with the RasMol 2.7.3 program.

Results

Antigenic mapping of the H9 HA molecule

Six anti-HA monoclonal antibodies (MAbs) against A/swine/Hong Kong/10/98 (H9N2) (Sw/HK/98) and two against A/duck/Hokkaido/13/00 (H9N2) (Dk/Hok/00) were selected as representatives of 50 MAbs obtained in total. Nucleotide sequences of the HA gene of the antigenic variants selected in the presence of each of the MAbs were determined. A single amino acid substitution was found in the deduced amino acid sequence of the

HA of each of the antigenic variants (Table 2). A panel of MAbs recognizing 8 amino acid positions was established on the basis of the positions of amino acid substitution in the H9 HA molecule. The position of amino acid substitution at residue 72 (Gly→Glu) on the HA of Sw/L6/2, a mutant selected from Sw/HK/98 in the presence of MAb L6/2, was located in the vicinity of the bottom of the globular head domain of the H9 HA molecule in the proposed antigenic site E in the H3 subtype HA (Fig. 1A). Antigenic variant, SwG6/5 had an amino acid substitution at residue 127 (Ser→Asp) at the 'overlapping site' of Site I and Site II, that was reported by Kaverin et al. [12]. SwN4/2 had an amino acid substitution at residue 148 (Asn→Asp) on Site I. Substitution at residues 182 (Thr→Ile) and 183 (Asn→Asp) was located on Site II. An amino acid substitution of SwG12/1 at residue 212 (Leu→His) was found in the vicinity of the trimeric interface of the globular domains of the HA1. The Dk/Hok/00 antigenic variants DkD370/4 and DkD272/6 had amino acid substitutions at residues 98 (Leu→Gln) and 131 (Lys→Asn) (137 in H3 HA numbering), respectively.

The reactivity patterns of antigenic variants with the panel of MAbs showed that the MAbs of Sw/HK/98 and Dk/Hok/00 were divided into 7 groups (Table 3). Out of 7 groups, 5 groups of MAbs (group 1, 2, 5, 6 and 7) recognized independent epitopes, while the other 2 groups recognized antigenically overlapped epitopes.

The mutants SwG6/5 and SwL7/7 did not react with MAb D272/6 in addition to the mutant DkD272/6. The mutants SwG6/5 weakly reacted with MAb N4/2 compared with the parental virus. Therefore, at least five distinct antigenic sites were defined on the H9 HA using the present 8 MAbs.

Sequence analysis of the H9 HA molecule

H9N2 influenza viruses have been prevalent in poultry over the last decades. Within the period, many H9N2 viruses were isolated and confirmed that the viruses had undergone antigenic variation. Sequence analysis of the H9 HA genes of 133 H9N2 influenza virus strains isolated between 1994 and 2005 showed that 28 positions of which amino acid substitutions frequently occurred on H9 HA molecule were found (Table 4 and Fig. 1B). Twenty-two amino acid substitutions were found on the HA molecule and two substitutions (103 and 269) were inside of the molecule, whereas the others were found at the HA cleavage site. The positions of amino acid substitution on the HA molecule on the 3-dimensional structure revealed that at least 4 conformational antigenic sites were located on the H9 HA.

Antigenic analysis of the HAs of H9N2 virus isolates using a panel of MAbs

Reactivity of H9N2 influenza viruses isolated from birds and mammals of different species with a panel of MAbs to the HA of Sw/HK/98 and Dk/Hok/00 were analyzed (Table 5). On the basis of the reactivity patterns with the panel of MAbs, 21 H9N2 influenza virus strains were divided into 7 different groups. Viruses belonging to antigenic group I had all seven epitopes which each of MAbs recognized on the HA molecule. Viruses belonging to antigenic group II to VI had several epitopes and A/shorebird/Delaware/9/96 did not reacted with any MAbs. The HAs of the viruses belonging to the Y280 lineage used in the present study were antigenically conserved and different from those to other lineages.

Discussion

The aim of the present study was to characterize the antigenic structure of the HA molecule of H9N2 influenza viruses recently prevailing in poultry in Asia. The antigenic drift of H9N2 viruses has been detected by reactivity with polyclonal antisera [5, 30, 31]. In the present study, a panel of MAbs to the HA of H9N2 influenza viruses was prepared and used for antigenic comparison of these H9 influenza viruses.

Several subtypes of the HA have been analyzed by using a panel of MAbs [4, 11, 12, 16, 28]. In the present study, the reactivity patterns of antigenic variants with the panel of MAbs showed that the MAbs were

divided into 7 groups. Out of 7 groups, 5 groups of MAbs recognized independent epitopes. Two antigenic sites on the H9 HA molecules, the Site I (amino acid positions 129, 147, and 152) and Site II (amino acid positions 135, 183, and 216), were revealed [20]. According to the position of amino acid substitution of the HA of antigenic variant, MAb G6/5 recognizes the Site I and E2/3 and L7/7 recognizes the Site II, respectively. MAbs L6/2, G12/1 and D370/4 recognize epitopes in 3 antigenic sites distinct from the sites I and II. The mutants whose deduced amino acid changes were detected in Ser127Asn, Lys131Asn and Asn183Asp did not react with MAb D272/6, suggested that D272/6 recognized conformational overlapping epitopes. Weak reaction of antigenic variant selected in the presence of MAb G6/5 with N4/2 may due to the mutation Ser127Asn that forms glycosylation site. The panel of MAbs recognized 7 epitopes in the present study recognized at least 5 different antigenic sites in the vicinity of the receptor biding site.

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

escape mutants selected in the present study and in earlier publication [12]. It is suggested that at least 5 antigenic sites were located on the H9 HA molecule and the antigenic variation occurred in these sites after the long-term prevalent of H9N2 influenza viruses in poultry.

The H9N2 influenza viruses were divided into Eurasian and North American lineages, and the former was divided further into Y280, G1 and Y439 sub-lineages [5]. In the present study, it was revealed that reactivity patterns of H9N2 influenza viruses isolated from birds and animals of different species with the panel of MAbs showed that the HA of viruses belonging to the Y280 lineage was antigenically conserved and different from that of other lineages. The present results coincide with the findings that viruses belonging to the Y280 or G1 lineage are antigenically different from other lineages [5].

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

diagnosis kit [27].

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Figure legend

Fig. 1. Schematic representation of monomer structures of the H9 haemagglutinin molecule showing location of amino acid substitutions on HA1. Images were created with RasMol 2.7.3. A: Amino acid changes of escape mutants selected with monoclonal antibody against A/swine/Hong Kong/10/98 (H9N2) (red) and A/duck/Hokkaido/13/00 (H9N2) (blue). The positions of the oligosaccharide attachment sites are shown as green lines. Amino acid positions are designated by H9 numbering. B: Hyper-variable amino acid positions on HA of H9N2 viruses clustered in Y280 lineage isolated from 1994 to 2005.

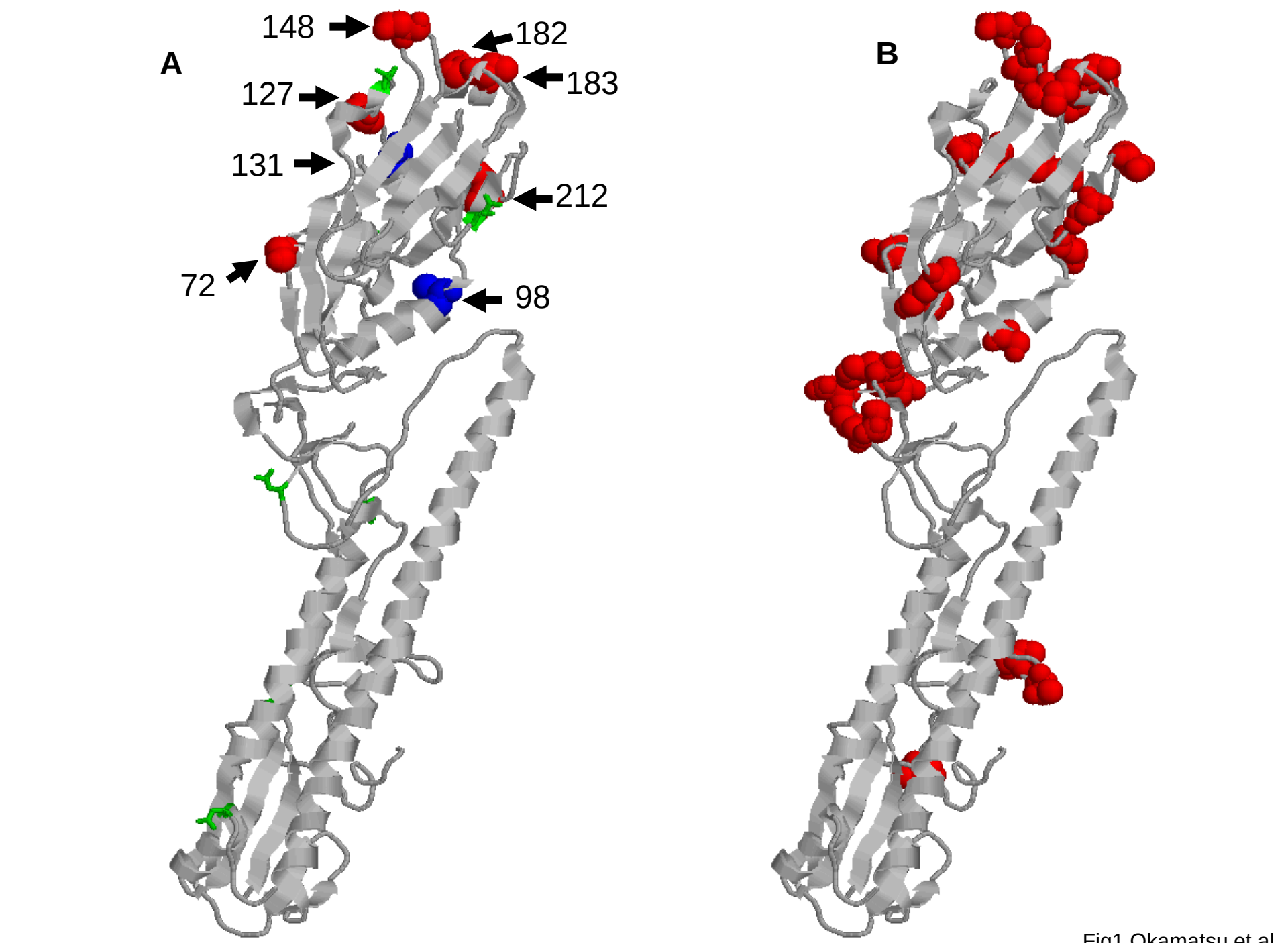

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Table 1 . H9N2 viruses used in this study

Lineage	Viruses	HA gene[a]
Eurasian		
Y280	A/chicken/Hong Kong/G9/97	AF156373
	A/chicken/Hebei/3/98	AF536695
	A/chicken/Hong Kong/FY20/99	AF222611
	A/duck/Hong Kong/Y280/97	AF156376
	A/silkie chicken/Hong Kong/SF43/99	AF186268
	A/swine/Hong Kong/10/98	AF222811
Y439	A/ostrich/South Africa/9508103/95	AF218102
	A/duck/Hokkaido/31/97	AB125927
	A/duck/Hokkaido/49/98	AB125928
	A/duck/Hokkaido/9/99	AB125929
	A/duck/Hokkaido/26/99	AB125930
	A/duck/Hokkaido/13/00	AB125931
G1	A/quail/Hong Kong/G1/97	AF156378
	A/quail/Hong Kong/A17/99	AF222606
	A/chicken/Pakistan/2/99	AJ291392
	A/Hong Kong/1073/99	AJ404626
North American	A/turkey/Wisconsin/1/66	D90305
	A/turkey/Minnesota/38391-6/95	AF156387
	A/goose/Minnesota/5733-1/80	AF156389
	A/quail/Arkansas/29209-1/93	AF156388
	A/shorebird/Delaware/9/96	AF156386

a：GenBank Accession No.

Table 2. Biological properties of the monoclonal antibodies to H9 HA molecule

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

[a] Monoclonal antibodies against A/swine/Hong Kong/10/98 (H9N2) (Sw) or A/duck/Hokkaido/13/00 (H9N2) (Dk).

[b] Titers are expressed as $\log_{10}$.

[c, d] Positions of nucleotide and amino acid substitutions are numbered according to HA molecule of H9 subtype virus.

[e] This amino acid change was predicted to acquire oligosaccharide chain.

Table 3. Reactivity patterns of antigenic variants with monoclonal antibodies

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

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

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

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

Table 4. Amino acid variations in natural isolates

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

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

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

Escape mutant has changed in this residue in this study (a) and Kaverin et al. (b) [12].

Table 5. Reactivity patterns of 21 H9N2 viruses with a panel of monoclonal antibodies

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

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

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