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Characterization of a non-pathogenic H5N1 influenza virus isolated from a migratory duck flying from Siberia in Hokkaido, Japan, in October 2009

Naoki Yamamoto[1], Yoshihiro Sakoda[1], Masayuki Motoshima[1], Fumi Yoshino[1], Kosuke Soda[1], Masatoshi Okamatsu[1], Hiroshi Kida[1,2,3*]

Abstract

Background: Infection with H5N1 highly pathogenic avian influenza viruses (HPAIVs) of domestic poultry and wild birds has spread to more than 60 countries in Eurasia and Africa. It is concerned that HPAIVs may be perpetuated in the lakes in Siberia where migratory water birds nest in summer. To monitor whether HPAIVs circulate in migratory water birds, intensive surveillance of avian influenza has been performed in Mongolia and Japan in autumn each year. Until 2008, there had not been any H5N1 viruses isolated from migratory water birds that flew from their nesting lakes in Siberia. In autumn 2009, A/mallard/Hokkaido/24/09 (H5N1) (Mal/Hok/24/09) was isolated from a fecal sample of a mallard (*Anas platyrhynchos*) that flew from Siberia to Hokkaido, Japan. The isolate was assessed for pathogenicity in chickens, domestic ducks, and quails and analyzed antigenically and phylogenetically.

Results: No clinical signs were observed in chickens inoculated intravenously with Mal/Hok/24/09 (H5N1). There was no viral replication in chickens inoculated intranasally with the isolate. None of the domestic ducks and quails inoculated intranasally with the isolate showed any clinical signs. There were no multiple basic amino acid residues at the cleavage site of the hemagglutinin (HA) of the isolate. Each gene of Mal/Hok/24/09 (H5N1) is phylogenetically closely related to that of influenza viruses isolated from migratory water birds that flew from their nesting lakes in autumn. Additionally, the antigenicity of the HA of the isolate was similar to that of the viruses isolated from migratory water birds in Hokkaido that flew from their northern territory in autumn and different from those of HPAIVs isolated from birds found dead in China, Mongolia, and Japan on the way back to their northern territory in spring.

Conclusion: Mal/Hok/24/09 (H5N1) is a non-pathogenic avian influenza virus for chickens, domestic ducks, and quails, and is antigenically and genetically distinct from the H5N1 HPAIVs prevailing in birds in Eurasia and Africa. H5 viruses with the HA gene of HPAIV had not been isolated from migratory water birds in the surveillance until 2009, indicating that H5N1 HPAIVs had not become dominant in their nesting lakes in Siberia until 2009.

Background

Influenza viruses widely distribute in birds and mammals including humans. Viruses of each of the known hemagglutinin (HA) and neuraminidase (NA) subtypes (H1-H16 and N1-N9, respectively) have been isolated from migratory water birds. Ducks are orally infected with influenza viruses by waterborne transmission at their nesting lakes in Siberia, Alaska, and Canada close to the Arctic Circle during their breeding season, in summer [1]. These viruses replicate in the columnar epithelial cells forming crypts in the colon, and are excreted in feces [2]. The viruses are preserved in frozen lake water in winter after the ducks leave for migration to the south [3]. Nesting lakes for migratory ducks, thus, serve as influenza virus gene pools in nature.

Since late 2003, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have seriously affected poultry in

* Correspondence: kida@vetmed.hokudai.ac.jp
[1]Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
Full list of author information is available at the end of the article

Eurasia and Africa. Non-pathogenic avian influenza viruses (NPAIVs) circulating in waterfowl transmit to terrestrial birds such as quails and turkeys through domestic water birds such as ducks and geese in live bird markets. Then HPAIVs are generated during multiple transmission of low pathogenic H5 or H7 viruses in chicken population [1]. After 2005, H5N1 HPAIVs have been isolated from dead migratory water birds in China, Mongolia, Russia, and Japan on the way back to their nesting lakes in Siberia in spring [4-8]. It is a serious concern that HPAIVs may be perpetuated in the lakes where migratory water birds nest in summer, and that those migratory water birds may then bring HPAIVs to the south in autumn.

Since Japan and Mongolia are located on the flyways of migratory water birds that flew from their nesting lakes in Siberia to the south [1,9-11], intensive surveillance of avian influenza has been performed in autumn in Hokkaido, Japan, and Mongolia every year since 1996. The subtypes and the numbers of isolates in the surveillance in autumn between 1996 and 2009 have been reported [6,11-13]. A total of 634 viruses including 17 H5 viruses were isolated from fecal samples of migratory water birds in the surveillance (Tables 1 and 2). Until 2008, H5N1 virus had not been isolated from those of migratory water birds. In autumn 2009, an H5N1 virus, A/mallard/Hokkaido/24/09 (H5N1) (Mal/Hok/24/09), was isolated from the fecal sample of a mallard (*Anas platyrhynchos*) in Hokkaido, Japan. Pathogenicity of the isolate for chickens, domestic ducks, and quails was assessed by experimental infection studies, and the isolate was phylogenetically and antigenically analyzed.

Materials and methods

Isolation and identification of viruses

A total of 711 fecal samples were collected from migratory water birds at lakeside of Ono Pond on the campus of Hokkaido University, Sapporo and Lake Ohnuma in Wakkanai, Hokkaido, Japan, between September and November 2009. Each sample was mixed with Minimum Essential Medium (Nissui) containing antibiotics and inoculated into the allantoic cavities of ten-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 [14].

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluid of chicken embryos infected with the isolates by TRIzol LS Reagent (Invitrogen) and reverse-transcribed with the Uni12 primer [15] and SuperScript Reverse Transcriptase III (Invitrogen) or M-MLV Reverse Transcriptase (Invitrogen). The full-length of each gene segment was amplified by polymerase chain reaction with gene-specific primer sets [15]. Direct sequencing of each gene segment was performed using an auto-sequencer CEQ 2000XL (Beckman Coulter) or 3500 Genetic Analyzer (Applied Biosystems).

The nucleotide sequences were phylogenically analyzed based on those of the H5 HA and N1 NA genes of influenza viruses by the neighbor-joining method [3,16]. Sequence data of the viral genes were compared with those from GenBank/EMBL/DDBJ.

Experimental infection of chickens, domestic ducks, and quails with Mal/Hok/24/09 (H5N1)

To determine the intravenous pathogenicity index (IVPI), 0.2 ml of the 1:10 dilution of infectious allantoic fluid of embryonated eggs was inoculated intravenously into ten seven-week-old chickens (White Leghorn). The score for IVPI was calculated according to the manual of World Organisation for Animal Health (OIE) [17].

To assess the intranasal pathogenicity for poultry, Mal/Hok/24/09 (H5N1) of $10^{6.0}$ 50% egg infectious dose (EID_{50}) was inoculated intranasally into eight four-week-old chickens (Boris Brown), domestic ducks (Chelly Valley), and quails (Japanese Quail). Four of eight birds were euthanized three days post-inoculation (dpi), and the trachea and cloaca swabs, brain, trachea, lungs, kidneys, and colon were collected aseptically for virus recovery. The birds were observed daily for disease signs for 14 days after inoculation. Sera were collected from them on the day of inoculation and 14 dpi to test for antibodies against H5N1 virus. The swabs and tissue homogenates were inoculated into ten-day-old chicken embryos and the infectivity titers of virus were calculated and expressed as the EID_{50} per milliliter of swab or gram of tissue samples. Sera were examined for the presence of antibodies against H5N1 virus by enzyme-linked immunosorbent assay (ELISA) [18]. The purified A/duck/Hokkaido/Vac-1/04 (H5N1) generated from H5N2 and H7N1 viruses isolated from migratory water birds by genetic reassortment in embryonated chicken eggs [19] was used as antigen for ELISA. Each of the birds was housed in a self-contained isolator unit (Tokiwa Kagaku) at a BSL-3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Antigenic analysis

The antigenic properties of H5 viruses, A/duck/Hokkaido/WZ21/08 (H5N2), A/duck/Hokkaido/WZ75/09 (H5N2), Mal/Hok/24/09 (H5N1), A/whooper swan/Hokkaido/1/08 (H5N1), and A/peregrine falcon/Hong Kong/810/09 (H5N1), were determined by the fluorescent antibody method with monoclonal antibodies (MAbs) against H5 HA produced previously [20]. MDCK cells infected with H5 influenza viruses were fixed with cold 100% acetone for eight hours post-inoculation. The reactivity

Table 1 Influenza viruses isolated from fecal samples of migratory water birds in autumn between 1996 and 2009

Locations	Subtypes of influenza viruses isolated in following years													
	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Sapporo, Japan	NP[a]	NP	NP	NP	NP	H1N1 (9) H3N6 (1) H4N5 (1) H4N6 (1)	H3N8 (3) H5N3 (1) H11N9 (3)	H3N8 (11) H6N8 (2) H7N1 (18) H8N4 (1)	H1N1 (1) H3N8 (1) H4N2 (7) H5N3 (3) H6N1 (5) H6N8 (2) H10N5 (7)	H3N2 (1) H6N2 (4) H8N4 (2)	H3N8 (5) H4N6 (1) H6N2 (1) H9N2 (1) H11N9 (3)	H3N8 (2) H4N6 (2) H5N3 (2) H8N4 (2) H12N5 (1)	H3N2 (1) H3N6 (3) H4N6 (9) H7N7 (1) H9N5 (1) H10N7 (11)	H1N3 (1) H1N5 (1) H4N6 (5) H5N1 (1) H6N1 (4) H6N8 (2) H11N9 (3) H12N5 (1)
Wakkanai, Japan	H1N1 (1)[b] H3N8 (1) H5N3 (3) H6N1 (1)	H1N1 (1) H6N1 (2) H9N2 (1) H11N9 (1)	H6N2 (1) H9N2 (1)	H2N2 (1) H3N8 (2) H6N2 (4) H9N2 (2)	H4N6 (1) H5N3 (2) H6N2 (2) H8N4 (1) H9N2 (1) H10N4 (12)	H2N2 (1) H2N3 (4) H3N8 (6) H6N2 (4) H12N5 (2)	NP	NP	H4N6 (6) H6N2 (12) H6N8 (2) H7N7 (13) H8N4 (1) H10N6 (1) H11N9 (1) H12N5 (1)	H2N5 (1) H3N8 (3) H6N1 (1) H6N2 (3)	H3N6 (2) H3N8 (1) H4N9 (3) H6N1 (4) H6N5 (1) H9N2 (1) H10N8 (1) H11N9 (11) H13N6 (2)	H1N1 (1) H3N8 (1) H4N6 (2) H8N4 (1) H10N2 (1) H10N7 (1)	H4N6 (2) H5N2 (1) H6N1 (4) H6N2 (1) H6N5 (1) H6N8 (1) H6N9 (1) H9N9 (1) H10N9 (2) H11N9 (2)	H5N2 (1)
Mongolia	NP	NP	NP	NP	NP	H1N1 (1) H3N2 (1) H3N6 (3) H3N8 (11) H4N2 (1) H4N6 (12) H5N2 (1) H5N3 (2) H7N1 (1) H10N3 (4)	H1N1 (3) H3N6 (20) H3N8 (55) H4N6 (12) H4N7 (1) H4N8 (1) H7N1 (1) H7N7 (9) H8N4 (5) H10N7 (1) H12N5 (1)	H1N1 (1) H2N3 (1) H3N6 (6) H3N8 (28) H4N2 (1) H4N6 (25) H9N2 (1) H10N5 (5)	NP	H3N2 (1) H3N6 (2) H3N8 (10) H4N6 (6) H8N4 (1) H10N3 (11) H10N7 (1)	H2N2 (1) H3N8 (8) H4N6 (9)	H3N8 (14) H4N3 (1) H7N6 (1) H7N7 (4)	H3N6 (3) H3N8 (23) H4N6 (8) H4N8 (3) H7N9 (3)	H1N8 (1) H3N8 (2) H4N6 (3) H8N4 (3)

Surveillance data were referred from Okazaki *et al.* [11], Manzoor *et al.* [12], Sakoda *et al.* [6], and Asmah *et al.*[13].

[a] Surveillance did not be performed.

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

Table 2 H5 viruses isolated from migratory water birds in the surveillance in autumn between 1996 and 2009

Years	Locations	Names	Subtypes
1996	Wakkanai, Japan	Swan/Hokkaido/4/96	H5N3
		Swan/Hokkaido/51/96	H5N3
		Swan/Hokkaido/67/96	H5N3
2000	Wakkanai, Japan	Dk/Hokkaido/447/00	H5N3
		Dk/Hokkaido/69/00	H5N3
2001	Mongolia	Dk/Mongolia/54/01	H5N2
		Dk/Mongolia/500/01	H5N3
		Dk/Mongolia/596/01	H5N3
2002	Sapporo, Japan	Dk/Hokkaido/84/02	H5N3
2004	Sapporo, Japan	Dk/Hokkaido/101/04	H5N3
		Dk/Hokkaido/193/04	H5N3
		Dk/Hokkaido/299/04	H5N3
2007	Sapporo, Japan	Dk/Hokkaido/167/07	H5N3
		Dk/Hokkaido/201/07	H5N3
2008	Wakkanai, Japan	Dk/Hokkaido/WZ21/08	H5N2
2009	Wakkanai, Japan	Dk/Hokkaido/W75/09	H5N2
	Sapporo, Japan	Mal/Hokkaido/24/09	H5N1

Abbreviations: Dk (Duck), Mal (Mallard).

patterns of the MAbs to H5 viruses were investigated by the immunofluorescent method with a FITC-conjugated goat IgG to mouse IgG (ICN Biomedicals). Fluorescence was visualized with the Axiovert 200 (Carl Zeiss).

Results

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

In 2009, a total of 19 viruses were isolated from 711 fecal samples of migratory water birds. Those were 1 H1N3, 1 H1N5, 5 H4N6, 1 H5N1, 1 H5N2, 4 H6N1, 2 H6N8, 3 H11N9, and 1 H12N5 viruses. In our previous surveillance until 2008, H5N1 virus had not been isolated (Table 1) [6,11-13]. In autumn 2009, Mal/Hok/24/09 (H5N1) was isolated from a fecal sample of a mallard in Hokkaido, Japan.

Pathogenicity of Mal/Hok/24/09 (H5N1) in chickens, domestic ducks, and quails

The pathogenicity of Mal/Hok/24/09 (H5N1) was evaluated by IVPI test using chickens. None of the ten birds intravenously inoculated with Mal/Hok/24/09 (H5N1) showed clinical signs during ten days of observation (IVPI = 0.00). None of the chickens, domestic ducks, and quails intranasally inoculated with $10^{6.0}$ EID_{50} of Mal/Hok/24/09 (H5N1) showed clinical signs during 14 days of observation (Table 3). The virus was not recovered from the tracheal and cloacal swabs and tissues of chickens intranasally inoculated with Mal/Hok/24/09 (H5N1) on three dpi, and there were no antibodies to H5N1 virus detected by ELISA on 14 dpi (Table 3), indicating that chickens were not infected with the isolate. Although virus was not recovered from the swabs and tissues of domestic ducks inoculated with the virus on three dpi, antibodies against H5N1 virus were detected in the sera of the birds, indicating that domestic ducks were infected with the isolate. Viruses of $10^{3.3}$ and $10^{3.6}$ EID_{50}/ml were recovered from tracheal swabs of two of four quails inoculated with the virus on three dpi, respectively. Antibodies against H5N1 virus were detected in the sera of the birds on 14 dpi. These findings indicate that quails are susceptible to infection with the isolate.

Table 3 Virus recovery from birds experimentally inoculated with A/mallard/Hokkaido/24/09 (H5N1)

Birds	No. of Birds	Days[a] p.i.	Clinical signs	Virus recovery[d]							Antibody[e] response
				Swabs (log EID_{50}/ml)		Tissues (log EID_{50}/g)					
				Trachea	Cloaca	Brain	Trachea	Lungs	Kidneys	Colon	
Chickens	1 - 4	3	-[b]	<	<	<	<	<	<	<	NT
	5 - 8	14	-	NT[c]	NT	NT	NT	NT	NT	NT	< 40[f]
Domestic ducks	1 - 4	3	-	<	<	<	<	<	<	<	NT
	5 - 8	14	-	NT	NT	NT	NT	NT	NT	NT	1,600[f]
Quails	1	3	-	3.3	<	<	<	<	<	<	NT
	2	3	-	3.6	<	<	<	<	<	<	NT
	3 - 4	3	-	<	<	<	<	<	<	<	NT
	5 - 8	14	-	NT	NT	NT	NT	NT	NT	NT	800[f]

[a] All birds were sacrificed.

[b] -: Birds did not show any clinical signs during observation days.

[c] NT: Not tested.

[d] Digit: Virus titers. <: Virus titer was less than 0.8 log EID_{50}/ml swab or 1.5 log EID_{50}/g tissue.

[e] ELISA titers on 14 dpi.

[f] ELISA titers of three birds were equal.

Genetic analysis of Mal/Hok/24/09 (H5N1)

Each gene of Mal/Hok/24/09 (H5N1) was phylogenetically analyzed. The HA and NA genes of Mal/Hok/24/09 (H5N1) were classified into the Eurasian lineage, and were different from HA and NA genes of H5N1 HPAIVs, respectively (Figure 1). In addition, the other six genes of Mal/Hok/24/09 (H5N1) were not closely related to those of HPAIVs, but related to those of NPAIVs isolated from migratory water birds (data not shown). The eight segments of Mal/Hok/24/09 (H5N1) were analyzed by the Basic Local Alignment Search Tool (BLAST) available from the DDBJ/EMBL/GenBank (Table 4). It was found that all genes of Mal/Hok/24/09 (H5N1) were derived from those of the viruses circulating in water birds in nature. M gene of the virus was classified into North American lineage, and the other genes were classified into Eurasian lineage (Table 4), indicating that genetic reassortment occurs between the viruses whose genes classified into North American and Eurasian lineages. The amino acid sequence of the HA cleavage site of Mal/Hok/24/09 (H5N1) was RETR/GLF, and insertion or substitution of multiple basic amino acids found in the HAs of HPAIVs [21] was not observed.

Antigenic analysis of the HA of Mal/Hok/24/09 (H5N1)

The HA of Mal/Hok/24/09 (H5N1) was antigenically analyzed using a panel of MAbs recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/84 (H5N2) [20]. Each of the MAb bound to the antigen of Mal/Hok/24/09 (H5N1) as well as those of the other non-pathogenic H5 viruses, and few MAbs bound to the antigen of H5N1 HPAIVs recently isolated in Mongolia, Japan, and Hong Kong (Table 5), indicating that the HA of Mal/Hok/24/09 (H5N1) is antigenically closely related to the H5 HA of the viruses circulating in migratory water bird.

Discussion

Efforts to monitor avian influenza in migratory water birds have increased worldwide in recent years due to concern that migratory water birds may disseminate HPAIVs. Intensive surveillance of avian influenza has been conducted every autumn in Hokkaido, Japan, and

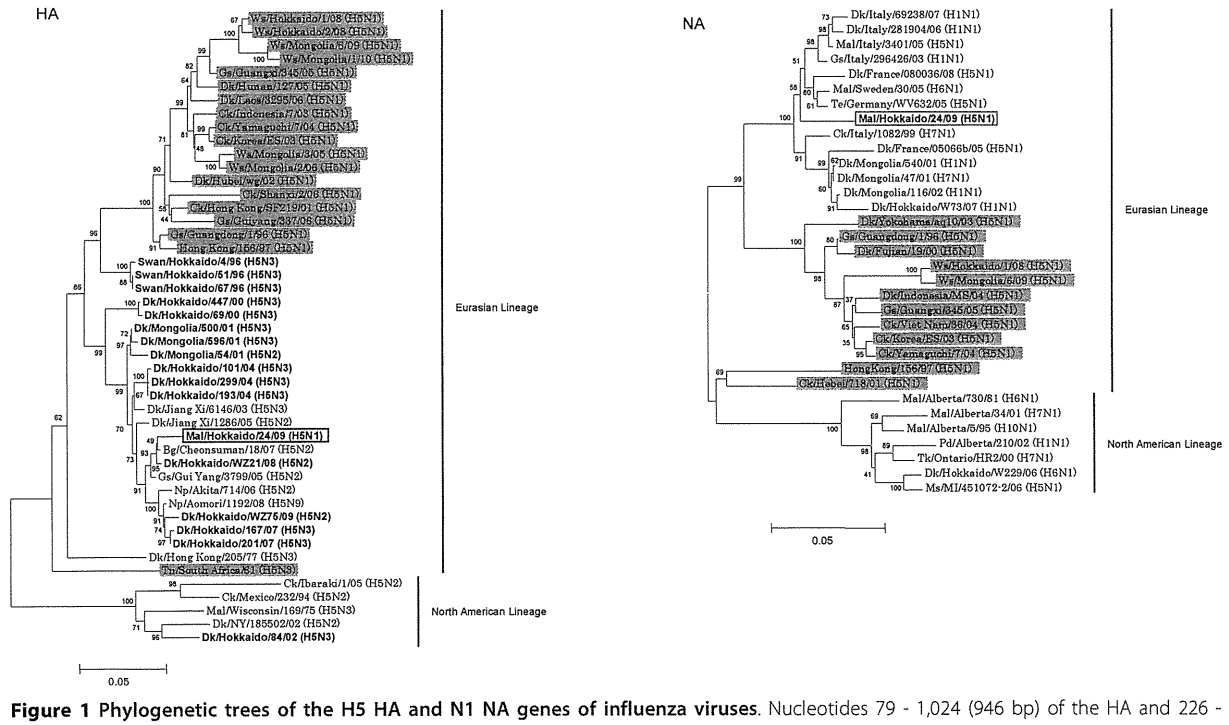

Figure 1 Phylogenetic trees of the H5 HA and N1 NA genes of influenza viruses. Nucleotides 79 - 1,024 (946 bp) of the HA and 226 - 1,098 (873 bp) of the NA were used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. HPAIVs are highlighted in gray. Mal/Hok/24/09 (H5N1) is shown in the enclosed square. H5 viruses isolated from migratory water birds in the surveillance in autumn between 1996 and 2009 are denoted in bold. Abbreviations: Ws (Whooper swan), Gs (Goose), Dk (Duck), Ck (Chicken), Mal (Mallard), Bg (Bean goose), Np (Northern pintail), Tn (Tern), Gu (Gull), Te (Teal), Ms (Mute swan), Pd (Pintail duck), Tk (Turkey).

Table 4 Characterization of the genes of A/mallard/Hokkaido/24/09 (H5N1)

Gene segments[a]	Region of examined nucleotides	Viruses with highest homology		Homologies (%)	Lineages
		Name[b]	Accession numbers		
PB2	14-2293	Sbd/Korea/619/08 (H6N2)	GQ414790	98	Eurasian
PB1	9-2269	Sbd/Korea/540/08 (H6N1)	GQ414822	98	Eurasian
PA	1-2200	Dk/Shiga/8/04 (H4N6)	AB304146	98	Eurasian
HA	79-1726	Bg/Cheonsuman/18/07 (H5N2)	FJ767718	98	Eurasian
NP	31-1527	Mal/SanJiang/151/06 (H6N2)	EF592496	99	Eurasian
NA	1-1422	Gs/Italy/296426/03 (H1N1)	FJ432780	97	Eurasian
M	1-983	Mal/Minnesota/153/98 (H9N2)	GU051519	98	North American
NS	1-838	Gu/Astrakhan/1846/98 (H13N6)	GU052231	98	Eurasian

[a] GenBank accession number of each gene of Mal/Hokkaido/24/09 (H5N1): PB2 [AB530989], PB1 [AB530990], PA [AB530991], HA [AB530992], NP [AB530993], NA [AB530994], M [AB530995], and NS [AB530996].

[b] Abbreviations: Sbd (Spot-billed duck), Dk (Duck), Bg (Bean goose), Mal (Mallard), Gs (Goose), Gu (Gull).

Mongolia. As shown in Table 1, H5N1 virus had not been isolated from migratory water birds in the surveillance until 2008. In autumn 2009, Mal/Hok/24/09 (H5N1) was isolated from a fecal sample of a mallard that flew from the northern territory in Siberia to Hokkaido, Japan. In the present study, the H5N1 isolate was examined for pathogenicity in chickens, domestic ducks, and quails. Based on the results of IVPI test, the isolate was designated a NPAIV. Chickens were not susceptible to infection with Mal/Hok/24/09 (H5N1) (Table 3). Domestic ducks and quails were infected with the isolate but did not show clinical signs. These findings indicate

Table 5 Reactivity of H5 viruses with MAbs against HA of A/duck/Pennsylvania/10218/84 (H5N2)

	Viruses[a]	Clades	Monoclonal antibodies					
			D101/1 (88[b])	A310/39 (145)	64/1 (157)	B9/5 (168)	B59/5 (169)	25/2 (205)
NPAIVs	Dk/Pennsylvania/10218/84 (H5N2)	-[c]	+	+	+	+	+	+
	Swan/Hokkaido/4/96 (H5N3)	-	+	+	+	+	+	+
	Swan/Hokkaido/51/96 (H5N3)	-	+	+	+	+	+	+
	Swan/Hokkaido/67/96 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/447/00 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/69/00 (H5N3)	-	+	+	+	+	+	+
	Dk/Mongolia/54/01 (H5N2)	-	+	+	+	+	+	+
	Dk/Mongolia/500/01 (H5N3)	-	+	+	+	+	+	+
	Dk/Mongolia/596/01 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/84/02 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/101/04 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/193/04 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/299/04 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/167/07 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/201/07 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/WZ21/08 (H5N2)	-	+	+	+	+	+	+
	Dk/Hokkaido/WZ75/09 (H5N2)	-	+	+	+	+	+	+
	Mal/Hokkaido/24/09 (H5N1)	-	+	+	+	+	+	+
HPAIVs	Ws/Mongolia/3/05 (H5N1)	2.2	+	-	+	+	-	+
	Ws/Hokkaido/1/08 (H5N1)	2.3.2	+	-	-	-	-	-
	Pf/Hong Kong/810/09 (H5N1)	2.3.4	-	-	-	-	-	-

The results, except Dk/Hokkaido/WZ21/08 (H5N2), Dk/Hokkaido/WZ75/09 (H5N2), Mal/Hokkaido/24/09 (H5N1), Ws/Hokkaido/1/08 (H5N1), and Pf/Hong Kong/810/09 (H5N1), were referred from previous report [20].

[a] Abbreviations: Dk (Duck), Mal (Mallard), Ws (Whooper swan), Pf (Peregrine falcon).

[b] Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies [20].

[c] Dashes (-) indicate classical HA gene which is not classified into the clades 0 - 9.

that the isolate is non-pathogenic in chickens, domestic ducks, and quails. Phylogenetic analyses demonstrated that Mal/Hok/24/09 (H5N1) was distinguished from H5N1 HPAIVs that are prevailing in birds in Eurasia and Africa. Antigenic comparisons of the HAs of H5 viruses indicated that the antigenicity of the HA of Mal/Hok/24/09 (H5N1) is closely related with the H5 NPAIVs circulating in nature (Table 5).

After 1996, H5N1 HPAIVs with both HA and NA genes of A/goose/Guangdong/1/96 (H5N1) have spread to Eurasia and Africa [22]. After 2005, H5N1 HPAIVs were isolated from dead migratory water birds in China, Mongolia, Russia, and Japan in spring [4-8], suggesting that the birds were infected with HPAIVs in the south during the spring and died on the way back to the northern territories. In the surveillance studies of avian influenza in autumn since 1996, H5 viruses with the HA gene of A/goose/Guangdong/1/96 (H5N1) had not been isolated from migratory water birds that flew from Siberia to Japan and Mongolia (Figure 1) indicating that H5N1 HPAIVs had not become dominant in their nesting lakes in Siberia until 2009.

On 14th October, 2010, H5N1 HPAIVs were isolated from migratory water birds that flew from Siberia to Japan (under publication). Then, H5N1 HPAIVs have been isolated from migratory water birds and poultry in other places in Japan.

For the control of HPAIV infection in birds and mammals, early detection of the viruses and stamping out to contain the viruses in the domestic poultry are essential.

Conclusion

In autumn 2009, Mal/Hok/24/09 (H5N1) was isolated from a fecal sample of a mallard. Mal/Hok/24/09 (H5N1) is a NPAIV for chickens, domestic ducks, and quails, and is antigenically and genetically distinct from H5N1 HPAIVs that are prevailing in birds in Eurasia and Africa. Phylogenetic analysis of the HA genes revealed that H5 viruses with the HA gene of HPAIV had not been isolated from migratory water birds in the surveillance until 2009. These findings indicate that H5N1 HPAIVs had not become dominant in their nesting lakes in Siberia until 2009.

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Author details

[1]Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan. [2]Research Center for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, Hokkaido 001-0020, Japan. [3]Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan.

Authors' contributions

NY carried out the animal experiments and the antigenic and phylogenetic analyses, and drafted the manuscript. MM and FY collected the fecal samples and carried out the viral isolation and identification of subtypes. MO carried out the IVPI test. KS participated in the antigenic analysis. YS and HK participated in coordination of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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FULL PAPER *Virology*

A Low Pathogenic H5N2 Influenza Virus Isolated in Taiwan Acquired High Pathogenicity by Consecutive Passages in Chickens

Kosuke SODA[1], Ming-Chu CHENG[2,3], Hiromi YOSHIDA[1], Mayumi ENDO[1], Shu-Hwae LEE[2], Masatoshi OKAMATSU[1], Yoshihiro SAKODA[1], Ching-Ho WANG[3] and Hiroshi KIDA[1,4,5]*

[1]*Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo, Hokkaido 060–0818, Japan,* [2]*Animal Health Research Institute, Council of Agriculture, 376 Chung-Cheng Road, Tansui, Taipei 25158,* [3]*Department of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan,* [4]*Research Center for Zoonosis Control, Hokkaido University, Kita-20 Nishi-10, Kita-ku, Sapporo, Hokkaido 001–0020 and* [5]*SORST, Japan Science and Technology Agency (JST), 4–1–8 Honcho Kawaguchi, Saitama 331–0012, Japan*

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ABSTRACT. H5N2 viruses were isolated from cloacal swab samples of apparently healthy chickens in Taiwan in 2003 and 2008 during surveillance of avian influenza. Each of the viruses was eradicated by stamping out. The official diagnosis report indicated that the Intravenous Pathogenicity Indexes (IVPIs) of the isolates were 0.00 and 0.89, respectively, indicating that these were low pathogenic strains, although the hemagglutinin of the strain isolated in 2008 (Taiwan08) had multibasic amino acid residues at the cleavage site (PQRKKR/G). In the present study, these H5N2 viruses were assessed for their intravenous and intranasal pathogenicity for chickens. It was examined whether Taiwan08 acquires pathogenicity through consecutive passages in chickens. Intravenous pathogenicity of Taiwan08 depended upon the age of the chickens used for the IVPI test; all of the eight-week-old chickens intravenously inoculated with Taiwan08 showed clinical signs but survived for ten days post inoculation (IVPI=0.68), whereas all the six-week-old chickens died (IVPI=1.86). Taiwan08-P8, which were passaged in chickens for eight times, killed all the eight-week-old chickens (IVPI=2.36). The four-week-old chickens died after intranasal inoculation of Taiwan08-P8, indicating that Taiwan08 must have become highly pathogenic during circulation in chicken flocks. These results emphasize the importance of a stamping out policy for avian influenza even if the IVPI of the causal virus is low.

KEY WORDS: chicken, H5N2, influenza virus, passage, pathogenicity.

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Influenza A viruses of each of the known subtypes (H1 to H16 and N1 to N9) are circulating in waterbirds, especially in migratory ducks [4]. A previous study showed that chickens were not directly infected with viruses from waterbirds [10]. Low pathogenic avian influenza viruses (LPAIVs) capable of being transmitted to chickens have emerged through domestic waterbirds such as ducks and geese and terrestrial birds such as quails and turkeys. LPAIVs may become highly pathogenic to chickens after more than six months of multiple passages in chicken populations [7, 8, 21]. The hemagglutinins (HAs) of highly pathogenic avian influenza viruses (HPAIVs) have multibasic amino acid residues at their cleavage site [19]. This structure permits ubiquitous proteases, such as furin and PC6, that recognize multiple basic amino acids to cleave the HA, leading to systemic infection. By contrast, HAs of LPAIVs are cleaved only by trypsin-like proteases that are expressed in the cells of the respiratory or intestinal tracts, so the viruses cause localized infections, resulting in mild or subclinical diseases. It is presently believed that only strains with H5 or H7 subtype HAs become HPAIVs during extensive infections in chicken populations [9].

H5N2 HPAIVs have caused three large outbreaks in poultry: in Pennsylvania in 1983 [1, 10], in Mexico from 1994 to 1995 [5, 7] and in Italy from 1997 to 1998 [1, 3]. H5N2 LPAIVs have become endemic in Central America since 1994, despite eradication programs in combination with vaccination [11, 13]. LPAIVs, A/chicken/Taiwan/1209/2003 (H5N2) (Taiwan03) and A/chicken/Taiwan/K703-1/2008 (H5N2) (Taiwan08), were isolated from apparently healthy chickens during routine surveillance in Taiwan [2]. At the end of May 2005, an LPAIV, A/chicken/Ibaraki/1/2005 (H5N2) (Ibaraki05), was isolated for the first time from chicken in Japan [17]. Genetic analyses of the eight segments of these H5N2 isolates revealed that although Ibaraki05 was closely related to the H5N2 LPAIVs prevalent in Central America [16], Taiwan03 and Taiwan08 were reassortants whose HA and NA gene segments belonged to the American lineage, and the other six genes belonged to the Eurasian lineage [2], indicating that multiple passages in the poultry population, possibly with genetic reassortment events, resulted in introduction of some gene segments from other endemic viruses, such as H6N1 viruses [12], in Taiwan. One or two basic amino acid substitutions were found in the HA cleavage sites of Taiwan03 and Taiwan08, respectively [2]. The intravenous pathogenicity index (IVPI) of Taiwan08 was 0.89, indicating that the virus was in the process of acquiring high pathogenicity in chickens. In the present study, these H5N2 virus isolates in Taiwan were assessed for antigenicity and intravenous/

* CORRESPONDENCE TO: KIDA, H., Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo, Hokkaido 060–0818, Japan.
e-mail: kida@vetmed.hokudai.ac.jp

intranasal pathogenicity for chickens. Furthermore, the potential of Taiwan08 to acquire further pathogenicity through passage in a chicken population was experimentally investigated.

MATERIALS AND METHODS

Viruses: Taiwan03 and Taiwan08 were isolated from cloacal swab samples of apparently healthy chickens in Taiwan [2]. A/duck/Hokkaido/WZ21/2008 (H5N2) and A/duck/Hokkaido/WZ75/2009 (H5N2) were isolated from fecal samples of ducks migrating to the South. A/chicken/Ibaraki/1/2005 (H5N2) [17] was kindly provided by the National Institute of Animal Health (Tsukuba, Ibaraki, Japan). Viruses were propagated in ten-day-old embryonated chicken eggs for 48 hr at 35°C.

Antigenic analyses: Antigenic specificity of H5 influenza viruses was assessed by a fluorescent antibody method with monoclonal antibodies (MAbs) recognizing H5 HA epitopes and by a neutralization test using polyclonal chicken antiserum raised against A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac1) including a water-in-oil adjuvant provided by Kyoto Biken Laboratories, Inc., (Uji, Kyoto, Japan). The experiments were carried out as previously described in the literature [20].

Consecutive passages in the air sacs of chicks and in chickens: Two hundreds microliters of Taiwan08 was inoculated into the caudal thoracic air sacs of three 3-day-old chicks. The chicks were sacrificed, and their lungs and brains were aseptically collected at three days postinoculation (d.p.i.). The tissue samples were homogenized by a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) to prepare a 10% suspension with Minimum Essential Medium (Nissui Pharmaceutical, Tokyo, Japan). Consecutive passages in the air sacs of three 3- to 7-day-old chicks were performed with 200 μl of a pooled tissue suspension of infected organs four times. Brain samples were used as the inoculum when both samples (lungs and brains) tested positive for the virus. Four-week-old (4w) chickens (Boris Brown, Hokuren Central Breeding Farm, Hokkaido, Japan) were used for further passaging study. Three chickens were intranasally inoculated with 100 μl of allantoic fluid containing the viruses at $10^{6.3}$ 50% egg infectious dose (EID_{50}; $10^{5.7}$ for Taiwan08-P6). The brains were collected from the dead chickens, and their suspensions, the inoculum for the next passage, were prepared as above. The passaged viruses were propagated in the allantoic cavities of ten-day-old embryonated chicken eggs.

Sequencing: Viral RNAs of each passaged Taiwan08 were extracted from infectious allantoic fluids using a commercial kit (TRI LS reagent, Sigma-Aldrich, St. Louis, MO, U.S.A.) and reverse transcribed with the Uni12 primer [6] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). PCR-based amplification of the full genomes of the eight gene segments was performed with universal primer sets [6]. Nucleotide sequences were determined from these RT-PCR products using a CEQ 2000XL automated DNA sequencer (Beckman Coulter, Fullerton, CA, U.S.A.) according to the Dye Terminator Cycle Sequencing Chemistry Protocol (Beckman Coulter). Sequence data were analysed using GENETYX version 10 (Genetyx Corporation, Tokyo, Japan).

Experimental infection of the chickens with each virus: The IVPI test was carried out according to the OIE (World Organisation for Animal Health) manual [15]. To reduce the number of birds used for the experiment, eight, not ten, chickens were applied to assess the intravenous pathogenicity of each virus. Each of eight 6- or 8-week-old (6 w or 8 w) chickens were intravenously inoculated with 0.2 m*l* of a 1/10 dilution of the infectious allantoic fluid. Each bird was observed for disease manifestation at intervals of 24 hr over a ten-day period and scored 0 if normal, 1 if sick, 2 if severely sick and 3 if dead. IVPI was the mean score per bird per observation over the ten-day period. Four-week-old chickens were used to test the intranasal pathogenicity of the viruses. Three chickens were intranasally inoculated with 100 μl of allantoic fluid containing each virus at $10^{6.3}$ EID_{50} ($10^{5.7}$ for Taiwan08-P8) and observed for 14 days. Specific antibodies against homologous viruses after 14 days of infection were detected in serum by a hemagglutinin inhibition (HI) test as described previously [22]. To study viral replication, each virus was inoculated into three chickens at $10^{6.3}EID_{50}$ ($10^{5.7}$ for Taiwan08-P8). The birds were euthanized three days postchallenge, and their tissues and blood were collected aseptically. Viral titers were calculated by the method of Reed and Muench [18] and expressed as the EID_{50} per gram and milliliter of tissue and blood, respectively.

All animal experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at the BSL-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The experiments were performed according to the guidelines of the institutional animal care and use committee of the Graduate School of Veterinary Medicine.

RESULTS

Antigenic analyses: Reactivity of H5 viruses with the panel of MAbs to H5 HA was analyzed by immunofluorescent assay and compared with our previous data [20]. The reactivity patterns of Taiwan03 and Taiwan08 with the panel of MAbs were similar to those of nonpathogenic H5 viruses isolated from waterbirds in nature (Table 1). Taiwan03 and Taiwan08 were neutralized by the polyclonal chicken antiserum raised against Vac1, as were other H5 viruses.

Intravenous pathogenicity of Taiwan03 and Taiwan08 in chickens: Intravenous pathogenicity of Taiwan03 and Taiwan08 was reconfirmed by an IVPI test using 6 w or 8 w chickens (Table 2). The 6 w chickens intravenously inoculated with Taiwan03 did not show any clinical signs and survived for ten d.p.i. (IVPI=0.00). Five of eight 8 w chickens inoculated with Taiwan08 showed severe disease signs at

Table 1. Antigenic analyses of H5 influenza viruses

Viruses	Monoclonal antibodies[a]							Polyclonal antibodies[c]
	I (88[b])	II (145)	III (157)	IV (168)		V (169)	VI (205)	α-Dk/Hok/
	D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2	Vac-1/2004 (H5N1)
H5N2 viruses isolated from chickens in Taiwan								
Chicken/Taiwan/1209/2003 (H5N2)	+	+	+	+	+	+	+	80
Chicken/Taiwan/A703–1/2008 (H5N2)	+	+	+	+	+	+	+	80
LPAI viruses								
Duck/Hokkaido/101/2004 (H5N3)[d]	+	+	+	+	+	+	+	64
Chicken/Ibaraki/1/2005 (H5N2)[d]	–	–	–	–	–	–	–	256
Duck/Hokkaido/WZ21/2008 (H5N2)	+	+	+	+	+	+	+	1,280
Duck/Hokkaido/WZ75/2009 (H5N2)	+	+	+	+	+	+	+	640
HPAI viruses								
Chicken/Yamaguchi/7/2004 (H5N1)[d]	–	+	+	+	+	–	+	256
Whooper swan/Mongolia/3/2005 (H5N1)[d]	+	–	+	+	+	–	+	256

a) Fluorescent antibody methods were performed with monoclonal antibodies to the HA of A/duck/Pennsylvania/10218/1984 (H5N2). b) Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies. c) Neutralizing antibody titers. d) Soda *et al.* [21].

Table 2. The intravenous pathogenicity of the viruses

Inoculated virus	Age	Clinical signs	Days post inoculation										IVPI
			1	2	3	4	5	6	7	8	9	10	
Taiwan03	6 w	Normal	8	8	8	8	8	8	8	8	8	8	0.00
		Sick	0	0	0	0	0	0	0	0	0	0	
		Severely sick	0	0	0	0	0	0	0	0	0	0	
		Dead	0	0	0	0	0	0	0	0	0	0	
Taiwan08	8 w	Normal	8	8	5	3	0	1	2	5	5	5	0.68
		Sick	0	0	3	3	3	3	3	3	1	3	
		Severely sick	0	0	0	2	5	4	3	0	2	0	
		Dead	0	0	0	0	0	0	0	0	0	0	
Taiwan08	6 w	Normal	7	2	0	0	0	0	0	0	0	0	1.86
		Sick	1	6	8	0	0	0	0	0	0	0	
		Severely sick	0	0	0	8	8	8	6	4	0	0	
		Dead	0	0	0	0	0	0	2	4	8	8	
Taiwan08-P4	8 w	Normal	8	4	1	1	1	0	0	1	1	1	1.85
		Sick	0	4	6	1	0	1	1	0	0	0	
		Severely sick	0	0	1	6	3	2	0	0	0	0	
		Dead	0	0	0	0	4	5	7	7	7	7	
Taiwan08-P8	8 w	Normal	8	0	0	0	0	0	0	0	0	0	2.36
		Sick	0	4	4	0	0	0	0	0	0	0	
		Severely sick	0	3	3	3	1	1	0	0	0	0	
		Dead	0	1	1	5	7	7	8	8	8	8	

five d.p.i., but all of them survived for ten days (IVPI=0.68). These results were in agreement with the official diagnostic results reported by the animal health authority in Taiwan to the OIE [2, 14] showing that Taiwan03 and Taiwan08 were LPAIV strains.

Since the 8 w chickens inoculated with Taiwan08 showed clinical signs, the intravenous pathogenicity of Taiwan08 for younger chickens was assessed. Taiwan08 showed high pathogenicity for 6 w chickens and killed all of the birds by nine d.p.i. The IVPI of Taiwan08 was 1.86, and so Taiwan08 was defined as an HPAIV [15]. It was, therefore, indicated that assessment of the pathogenicity of Taiwan08 could depend on the age of the chickens for the IVPI test.

Consecutive passage of Taiwan08 in the air sacs of chicks and in chickens: Taiwan08 was passaged in the air sacs of three- to seven-day-old chicks four times and subsequently in 4 w chickens four times to assess the potential of Taiwan08 to acquire further pathogenicity in chickens. Two of the three chicks died by air sac inoculation of Taiwan08 on three d.p.i. (Table 3). From the first passage (P1) onwards, the passaged viruses, Taiwan08-P1, P2 and P3, killed all of the chicks, and their time to death was gradually shortened. All of the chickens intranasally inoculated with Taiwan08-P4 showed clinical signs such as depression after six d.p.i., and one died on eight d.p.i. Shortened time to manifestation of disease and death and increased mortality

Table 3. Acquisition of virulence during consecutive passages in the air sacs of the chicks and in chickens

Inoculated animals	Passage number	Virulence (the no. of dead/sick/total)	Manifestation of disease (day)[a)]	Lethal time (day)
3-day-old chicks (air sac inoculation)	P0	2 / 3 / 3	2, 3, 3	3, 3
	P1	3 / 3 / 3	2, 2, 2	3, 3, 3
	P2	3 / 3 / 3	2, 2, 3	2, 3, 3
	P3	3 / 3 / 3	2, 2, 2	2, 2, 3
4-week-old chickens (intranasal inoculation)	P4	1 / 3 / 3	6, 6, 6	8
	P5	1 / 2 / 2	4, 4	7
	P6	2 / 3 / 3	3, 3, 3	6, 12
	P7	1 / 3 / 3	3, 6, 6	6
	P8	2 / 3 / 3	4, 4, 5	6, 6

a) The chicks or chickens that died are underlined.

Table 4. Amino acid mutation during consecutive passages of Taiwan08 in the air sacs of chicks

Passage	PB2	PA		HA	NA		M1		NS1
number	613[a)]	427	444	389	197	214	104	138	55
P0	Val	Asp	His	Gly	Thr	Ser	Arg	Val	Lys
P1	.[b)]	Glu	Asn	.	Ser	Asn	.	.	.
P2	.	Glu	Asn	.	Ser	Asn	.	.	.
P3	.	Glu	Asn	.	Ser	Asn	.	.	.
P4	.	Glu	Asn	.	Ser	Asn	.	.	.
P5	.	Glu	Asn	Arg	Ser	Asn	.	Val/Ile[c)]	Lys/Asn
P6	.	Glu	Asn	Arg	Ser	Asn	Asn	.	Asn
P7	.	Glu	Asn	Arg	Ser	Asn	Asn	.	Asn
P8	Val/Ile	Glu	Asn	Arg	Ser	Asn	Asn	.	Asn

a) Methionine encoded by the AUG start codon is defined as position 1. b) Periods indicate same amino acids as the parental virus. c) Amino acid quasispecies are observed.

rate were observed through the passage study in 4 w chickens. Eventually, two of the three chickens inoculated with Taiwan08-P8 showed clinical signs at four d.p.i. and then died two days later.

Amino acid changes during consecutive passages: Nucleotide sequences of the viruses passaged in chicks and chickens were determined and compared with that of parental Taiwan08 (Table 4). Four amino acid substitutions were found in the PA and NA at the initial passage. No other amino acid change was observed up to the fourth passage. During the passages of Taiwan08 in 4 w chickens, five amino acid substitutions were newly found in PB2, HA, M1 and NS1.

Intravenous pathogenicity of the passaged Taiwan08: The passaged viruses, Taiwan08-P4 and Taiwan08-P8, were assessed for intravenous pathogenicity for chickens by an IVPI test (Table 2). Intravenous pathogenicity increased as the number of passages increased. All of the 8 w chickens intravenously inoculated with Taiwan08-P4 showed clinical signs, and seven of them died by seven d.p.i. (IVPI=1.85). All of the chickens inoculated with Taiwan08-P8 died within seven days (IVPI=2.36).

Pathogenicity of the viruses on intranasal inoculation: To examine whether the pathogenicity of each virus via the natural route of infection correlated with that by intravenous inoculation, three 4 w chickens were challenged intranasally with each of the viruses at $10^{6.3}$ EID_{50} ($10^{5.7}$ for Taiwan08-P8) and observed for 14 days (Table 5). All chickens inoculated with Taiwan03 or Taiwan08 survived without showing any clinical signs, and serum antibody responses were detected in an HI test. One or two chickens inoculated with Taiwan08-P4 or Taiwan08-P8 died at eight or six d.p.i., respectively. The rest of them showed clinical signs and seroconversion and survived for 14 days. Each virus was detected in the systemic organs, except the blood, of the dead chickens.

To investigate the correlation between virulence and tissue tropism of the viruses, the virus titers at three d.p.i. in tissue and blood samples from 4 w chickens intranasally inoculated with each virus were determined (Table 5). Taiwan03 was scarcely recovered from the samples. Taiwan08 showed broader tissue tropism than Taiwan03, although the virus titers in the tissues were low. Taiwan08-P4 and Taiwan08-P8 were recovered from the colon and blood of the chickens in addition to the other tissues. These passaged viruses replicated well in each tissue as compared with the parental virus. Taiwan08-P8 showed 2-log higher titers than Taiwan08-P4 in the respiratory organs. Although Taiwan08 and the passaged viruses replicated in the systemic organs, no chickens inoculated with either virus showed any clinical signs by three d.p.i. It is worth noting that the virus titers in the brains of the dead chickens inocu-

Table 5. Virus recovery from the chickens intranasally inoculated with each virus strain

Inoculated virus	No. of chickens	Days p.i. (Health status)	Virus titer (log EID_{50}/g)							Antibody response (HI)
			Brain	Respiratory organs		Liver	Kidney	Colon	Blood[a]	
				Trachea	Lung					
Taiwan03	3	3 (sacrificed)	–, –, –[b]	–, –, –	–, –, ≤1.6	–, –, –	–, –, –	–, –, –	–, –, –	ND[c]
	3	14 (sacrificed)	ND	ND	ND	ND	ND	ND	ND	32, 32, 32
Taiwan08	3	3 (sacrificed)	–, ≤1.8, ≤2.5	≤1.8, ≤2.0, ≤2.5	–, ≤1.6, 3.3	–, ≤1.6, ≤2.3	–, ≤1.6, ≤2.3	–, –, –	–, –, –	ND
	3	14 (sacrificed)	ND	ND	ND	ND	ND	ND	ND	32, 32, 128
Taiwan08-P4	3	3 (sacrificed)	3.0, 4.3, 5.0	2.4, 2.8, 3.3	2.4, 2.7, 3.3	2.5, 3.3, 3.5	2.5, 4.3, 4.5	2.4, 2.7, 3.5	–, –, 2.4	ND
	1[d]	8 (dead)	7.7	2.5	3.5	≤2.0	3.7	2.5	–	ND
	2[d]	14 (sacrificed)	ND	ND	ND	ND	ND	ND	ND	64, 128
Taiwan08-P8	3	3 (sacrificed)	3.5, 3.7, 5.5	4.0, 4.5, 4.7	4.3, 4.7, 5.0	2.5, 3.7, 5.3	4.3, 4.5, 5.3	2.5, 3.7, 3.7	≤1.6, 3.7, 4.3	ND
	2[d]	6 (dead)	7.5, 8.5	2.4, 4.3	–, 3.0	–, 2.5	4.3, 6.5	2.5, 2.7	–, –	ND
	1[d]	14 (sacrificed)	ND	ND	ND	ND	ND	ND	ND	128

a) logEID_{50}/m*l*. b) ≤ 1.5 (≤ 0.5 for the blood samples). c) Not determined. d) Each chicken showed depression.

lated with Taiwan08-P4 or Taiwan08-P8 were higher than those of the chickens sacrificed at three d.p.i.

DISCUSSION

In recent years, outbreaks caused by low pathogenic H5N2 viruses have occurred in East Asia [2, 16, 17]. The causal viruses are classified into the American lineage. It has been unclear how such viruses invaded a chicken population in East Asia. Antigenic analysis revealed that the HAs of Taiwan03 and Taiwan08 were antigenically similar to those of nonpathogenic H5 viruses isolated from feral waterbirds (Table 1), indicating that the viruses were not completely adapted to the chicken populations in Taiwan. The antigenicities of the HAs of the Taiwan strains were different from that of Ibaraki05, a causal agent of LPAI in Japan in 2005; Ibaraki05 did not react with any of the MAbs recognizing H5 HA. These H5N2 virus strains also differed in the origins of their gene segments other than the HA and NA genes. These genes of Taiwan03 and Taiwan08 were derived from the Eurasian H6N1 viruses maintained in the chicken population in Taiwan for more than 38 years [2, 12], and those of Ibaraki05 were derived from the American H5N2 viruses [16]. These results suggest that the causal viruses independently evolved in the chicken flocks of Taiwan and Japan and that there were no relationships between the outbreaks in each country. Taiwan08 was similar to Taiwan03 antigenically and genetically, indicating that H5N2 viruses classified into the American lineage had been maintained in chicken flocks in East Asian countries for five years. Thus, continuous surveillance of avian influenza is important to prevent the emergence of pathogenic viruses like Taiwan08-P8 in the present study.

Taiwan08 had multiple basic amino acid residues at the HA cleavage site [2] and replicated in Madin-Darby Canine Kidney cells in the absence of trypsin (data not shown), indicating that the HA was cleavable by the ubiquitous proteases in the systemic organs of the chicken. These results indicate that the HA of Taiwan08 met the condition for the virus to exert pathogenicity in chickens. In the study of intranasal inoculation in 4 w chickens, Taiwan08 replicated in the systemic organs without showing any clinical signs (Table 5). It was reported that the acquisition of a polybasic HA cleavage site by an LPAIV was not sufficient for immediate transformation into an HPAIV [22]. Thus, it was concluded that Taiwan08 acquired high pathogenicity for chickens by the additional amino acid changes shown in Table 4.

All of the chickens intranasally inoculated with Taiwan08-P8 developed viremia, and high titers of the viruses were detected in their respiratory organs at three d.p.i. (Table 5). In addition, the virus titers in the brain samples of the chickens at the time of death were substantially high. These results suggest that high-level replication in the brains followed by hematogenous dissemination is essential for the virus to exert intranasal pathogenicity in chickens. The chickens intranasally inoculated with Taiwan08-P2, which had amino acid mutations at the PA and NA, did not show any clinical signs (data not shown). The other mutations in PB2, HA, M1 and/or NS1 therefore appear to be responsible for Taiwan08 becoming more pathogenic for chickens. How and which amino acid changes observed in the passage study affected the function of viral proteins need to be clarified to understand the adaptation of influenza viruses to chickens.

In the present study, we demonstrated that Taiwan08 had the potential to become more pathogenic by short-term passages in chickens (Tables 2 and 5). Taiwan08 showed high pathogenicity in 6 w chickens by the intravenous route of infection, but it did not kill the 8 w chickens (Table 2). Therefore, the age of the chickens applied to the IVPI test should be taken into account, especially when causal virus show low pathogenicity. In actual fact, the outbreak caused by Taiwan08 was controlled by a stamping out procedure, suggesting that this procedure should be selected as a countermeasure even if a causal virus was identified as an LPAIV.

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

Kosuke Soda[1], Shingo Asakura[1], Masatoshi Okamatsu[1], Yoshihiro Sakoda[1], Hiroshi kida[1,2,3*]

Abstract

Background: Outbreaks of avian influenza (AI) caused by infection with low pathogenic H9N2 viruses have occurred in poultry, resulting in serious economic losses in Asia and the Middle East. It has been difficult to eradicate the H9N2 virus because of its low pathogenicity, frequently causing in apparent infection. It is important for the control of AI to assess whether the H9N2 virus acquires pathogenicity as H5 and H7 viruses. In the present study, we investigated whether a non-pathogenic H9N2 virus, A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2), acquires pathogenicity in chickens when a pair of di-basic amino acid residues is introduced at the cleavage site of its HA molecule.

Results: rgY55sub (H9N2), which had four basic amino acid residues at the HA cleavage site, replicated in MDCK cells in the absence of trypsin after six consecutive passages in the air sacs of chicks, and acquired intravenous pathogenicity to chicken after four additional passages. More than 75% of chickens inoculated intravenously with the passaged virus, rgY55sub-P10 (H9N2), died, indicating that it is pathogenic comparable to that of highly pathogenic avian influenza viruses (HPAIVs) defined by World Organization for Animal Health (OIE). The chickens inoculated with the virus via the intranasal route, however, survived without showing any clinical signs. On the other hand, an avirulent H5N1 strain, A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1), acquired intranasal pathogenicity after a pair of di-basic amino acid residues was introduced into the cleavage site of the HA, followed by two passages by air sac inoculation in chicks.

Conclusion: The present results demonstrate that an H9N2 virus has the potential to acquire intravenous pathogenicity in chickens although the morbidity via the nasal route of infection is lower than that of H5N1 HPAIV.

Background

Each of the known subtypes of the influenza A virus (H1 to H16 and N1 to N9) is circulating in water birds, especially in migratory ducks [1]. A highly pathogenic avian influenza virus (HPAIV) is generated when a non-pathogenic virus brought in by migratory birds from nesting lakes in the north is transmitted to chickens via domestic ducks, geese, quails, turkeys, etc. and acquires pathogenicity for chickens with repeated multiple infections in the chicken population [2-6]. The hemagglutinins (HAs) of HPAIVs differ from those of low pathogenic avian influenza viruses (LPAIVs) with a pair of di-basic amino acid residues at their cleavage site [7]. This structure permits ubiquitous proteases such as furin and PC6, which recognize multiple basic amino acids, to cleave the HA, leading to systemic infection in chickens. By contrast, HAs of LPAIVs are cleaved only by trypsin-like proteases which are expressed in the cells lining the respiratory or intestinal tracts, so that the

* Correspondence: kida@vetmed.hokudai.ac.jp
[1]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
Full list of author information is available at the end of the article

viruses cause only localized infections, resulting in mild or asymptomatic diseases. It is presently believed that the strains only with H5 or H7 HAs become HPAIVs during extensive infections in chicken populations [8]. The reason why the subtypes of HPAIVs are restricted to H5 and H7 is not known although a model demonstrating that H5 HA is cleaved by furin through molecular docking analyses have been proposed [9,10].

H9N2 avian influenza virus strains have caused outbreaks in poultry, resulting in serious economic losses in Asia and the Middle East [11-19]. The causal strains, however, are avirulent and none of them have multiple basic amino acid residues at the cleavage site of the HA [12,15]. No specific-pathogen-free chickens experimentally infected with H9N2 isolates from diseased chickens showed any clinical symptoms [20]. Co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus* and *Haemophilus paragallinarum* or with attenuated coronavirus vaccine exacerbated the disease [19,21-23].

Since H9N2 viruses have been isolated not only from domestic birds but also from pigs and humans, the H9 virus has the potential to cause a next pandemic in humans [17,24-27]. It is important for controlling avian influenza and for preparing for pandemic influenza to assess whether the H9N2 virus aquires pathogenicity as H5 and H7 viruses. In the present study, we introduced a pair of di-basic amino acid residues into the cleavage site of the H9 and H5 HAs of non-pathogenic strains. These mutant H9 and H5 viruses were then serially passaged in the air sacs of chicks and their pathogenicity was assessed by inoculation to four-week-old chickens via intravenous and intranasal routes.

Results

Generation and characterization of mutant viruses

To investigate whether a non-pathogenic H9 influenza virus, A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2) acquires pathogenicity on the introduction of a pair of di-basic amino acid residues at their HA cleavage site, rgY55sub (H9N2) was generated by site-directed-mutagenesis and reverse genetics. Amino acid sequences at the HA cleavage site of the mutant strain are shown in Figure 1A. The RKKR motif was introduced into the H9 HA cleavage site to give a pair of di-basic amino acid residues that is known to be a *sine qua non* for H5 and H7 viruses to become highly pathogenic to chickens. The virus with the insertion of basic amino acid residues at the H9 HA cleavage site was not rescued from plasmid-transfected cells (data not shown). As a positive control, rgVac1ins (H5N1) was generated by inserting the RRKKR motif, rather than RKKR, into the HA of the non-pathogenic virus A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1) since recent H5 HPAIV isolates have the motif as insertion mutation. rgVac1sub (H5N1) was also generated to examine whether subtitution mutation with basic amino acid residues at the HA cleavage site contributed to acquisition of pathogenicity for chickens.

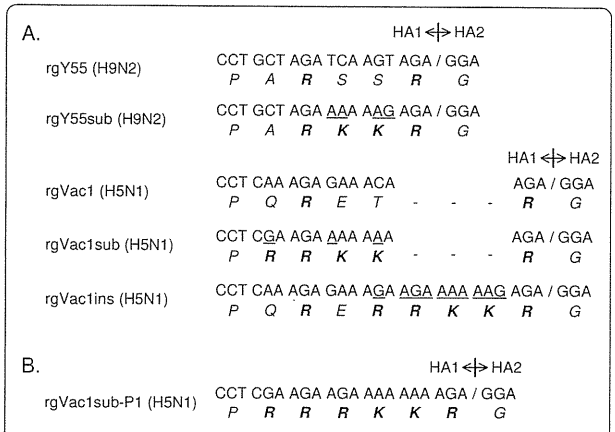

Figure 1 Nucleotide and amino acid sequences at the HA cleavage site of each virus. (A) Mutations were introduced into the HA genes of rgY55 (H9N2) and rgVac1 (H5N1) strains to give multibasic amino acids at their cleavage sites by site-directed mutagenesis. Mutation sites were underlined and deduced amino acid sequences were depicted in italics. Basic amino acids were shown in bold. (B) Nucleotide and amino acid sequences at the HA cleavage site of rgVac1sub-P1 (H5N1). rgVac1sub (H5N1) acquired an additional argenine residue represented by the codon AGA after one passage in chickens.

rgY55sub (H9N2) and rgVac1ins (H5N1) required trypsin to replicate in MDCK cells, and showed similar levels of growth to their parental viruses (Table 1). Chickens intravenously inoculated with rgY55sub (H9N2) or rgVac1ins (H5N1) did not show any signs of disease. rgVac1sub (H5N1) replicated in MDCK cells without exogenous trypsin, and one of the eight chickens inoculated with the virus showed slight depression at one day post-infection.

Consecutive passages of the viruses in the air sacs of chicks

The H9 mutant virus was serially passaged in the air sacs of chicks to assess whether it acquires pathogenicity as did H5 viruses. The passaged viruses were tested for their growth potential in MDCK cells and pathogenicity for chickens (Table 1). rgY55sub (H9N2) replicated in MDCK cells in the absence of trypsin and killed all of the chicks after six consecutive passages. Two of the eight four-week-old chickens inoculated intravenously with rgY55sub-P8 (H9N2) died within five days. Consequently, over 75% of the chickens intravenously infected with rgY55sub-P10 (H9N2) died by two days post inoculation, and its pathogenicity was comparable to that of the known HPAIVs [28].

H5N1 mutant viruses acquired intravenous pathogenicity by passaging twice in chicks; all of the chickens

Table 1 Growth potential in MDCK cells and pathogenicity for chicken of each virus

Viruses	Plaque formation (log PFU/ml)		Pathogenicity (number of dead/sick/total)	
	With trypsin	Without trypsin	3-day-old chicks (air sac inoculation)	4-week-old chickens (intravenous inoculation)
rgY55 (H9N2)	8.1	-[a]	NT[b]	0/0/8
rgY55sub (H9N2)	8.0	-	0/0/3	0/0/8
rgY55sub-P5 (H9N2)	7.6	-	0/0/4	0/0/8
rgY55sub-P6 (H9N2)	7.2	6.7	3/3/3	0/1/8
rgY55sub-P7 (H9N2)	7.8	7.5	3/3/3	0/5/8
rgY55sub-P8 (H9N2)	7.6	7.6	3/3/3	2/7/8 (4.0)[c]
rgY55sub-P9 (H9N2)	7.2	6.9	3/3/3	1/8/8 (10.0)
rgY55sub-P10 (H9N2)	6.5	6.1	3/3/3	6/8/8 (1.8)
rgVac1 (H5N1)	7.6	-	NT	0/0/8
rgVac1sub (H5N1)	7.6	7.8	0/0/3	0/1/8
rgVac1sub-P1 (H5N1)	6.8	6.8	3/3/3	3/5/8 (5.3)
rgVac1sub-P2 (H5N1)	6.4	6.5	6/6/6	8/8/8 (2.6)
rgVac1ins (H5N1)	7.3	-	0/0/3	0/0/8
rgVac1ins-P1 (H5N1)	7.8	7.1	3/3/3	6/7/8 (6.8)
rgVac1ins-P2 (H5N1)	7.1	7.1	4/4/4	6/8/8 (4.4)

[a] A plaque was not observed.

[b] Not tested.

[c] Mean death days are shown in parentheses.

died after intravenous inoculation with rgVac1sub-P2 (H5N1) or rgVac1ins-P2 (H5N1).

Amino acid changes of the viruses during consecutive passages in the air sacs of chicks

Nucleotide sequences of the eight segmented genomes of the viruses passaged in the air sacs of chicks were analyzed and compared with those of each parental virus. Leu234 (equivalent to position 226 of H3 HA) in the HA of rgY55sub (H9N2) was substituted with glutamine at the initial passage (Table 2). No other amino acid change was observed up to the fifth passage. Four amino acids in the HA, NA and M2 changed at the sixth passage. One of the asparagine-linked glycosylation sites on the HA was lost by Asn29His mutation. In total, eight amino acid differences were found between rgY55sub (H9N2) and rgY55sub-P10 (H9N2). Five and one amino acid changes were found in the PA, HA, M1 and M2 of rgVac1sub-P2 (H5N1), and the HA of rgVac1ins-P2 (H5N1), respectively (Table 3). It is worth noting that one argenine was inserted at the HA cleavage site of rgVac1sub (H5N1) after one passage in chickens (Figure 1B).

Table 2 Amino acid changes during consecutive passages of rgY55sub (H9N2)

Passage number	PB2	HA				NP	NA	M2
	271[a]	29	234	357	391	54	195	51
P0	T	N	L	A	N	W	T	I
P1-P5	.[b]	.	Q	.	.	.	.	.
P6	.	H	Q	D	.	.	A	T
P7	.	H	Q	D	N/D[c]	.	A	T
P8	T/A	H	Q	D	N/D	.	A	T
P9	T/A	H	Q	D	N/D	G	A	T
P10	A	H	Q	D	D	G	A	T

[a] Methionine encoded by the AUG start codon is defined as position 1.

[b] Periods indicate same amino acids as the parental virus.

[c] Amino acid quasispecies are observed.

Pathogenicity of the viruses on intranasal infection in chickens

To examine whether the pathogenicity of each virus via the intranasal route of infection correlates with that via intravenous route, three 4-week-old chickens were intranasally inoculated with the viruses of $10^{6.5}$ 50% egg infectious dose (EID_{50}) and observed for clinical signs until day 14 post-infection (Table 4). All chickens inoculated with rgY55sub-P10 (H9N2) or its parental rgY55sub (H9N2) survived without showing any clinical signs, and serum antibodies were detected (1:128-2,048 HI titers), indicating that virus replication occurred.

One of the three chickens inoculated with rgVac1sub (H5N1) or rgVac1ins (H5N1) showed seroconversion after 14 days while no chickens were susceptible to infection with rgVac1 (H5N1). Both of rgVac1sub-P2 (H5N1) and rgVac1ins-P2 (H5N1) were pathogenic, killing two of the three chickens by day 11 post-inoculation.

Additional passages of the Vac1-based viruses in chickens

One of the three chickens intranasally inoculated with rgVac1sub-P2 (H5N1) or rgVac1ins-P2 (H5N1) did not

Table 3 Amino acid changes during consecutive passages of rgVac1 mutants

Passage number	rgVac1sub (H5N1)									rgVac1ins (H5N1)				
	PA		HA		NP		M1		M2	HA			NA	M1
	65[a]	672	308	338	213	374	89	101	45	157	298	465	171	130
P0	S	L	H	-	R	M	D	R	R	S	M	D	N	L
P1	.[b]	.	Q	R[c]	.	.	.	.	H	P	.	E	.	.
P2	T	.	Q	R	.	.	.	K	H	P	.	.	.	.
P3	.	F	Q	R	Q	V	N	K	H	P	I	.	N/H[d]	I

[a] Methionine encoded by the AUG start codon is defined as position 1.

[b] Periods indicate same amino acids as the parental virus.

[c] Arginine was inserted at the HA cleavage site.

[d] Amino acid quasispecies were observed.

show any clinical signs (Table 4), indicating that the viruses did not extensively replicate in chickens. rgVac1-sub-P3 (H5N1) and rgVac1ins-P3 (H5N1) were prepared from the brain homogenates of the chickens that died on day 11 post-intranasal inoculation with the P2 viruses. Additional amino acid changes were found in P3 viruses (Table 3). To investigate whether the P3 viruses show higher pathogenicity in chicken, the viruses were inoculated via intranasal route. Mortality rate of chickens inoculated with the P3 viruses was equal to that with P2 viruses (Table 4).

Growth potential of the H9N2 and H5N1 viruses in chickens

To investigate whether tissue tropism of the viruses was involved in their pathogenicity, we determined viral titers in the tissue and blood samples from four-week-old chickens intranasally inoculated with each virus on three days post infection (Table 5). rgY55 (H9N2) and rgVac1 (H5N1) were scarcely recovered from the samples, and the mutant strains before passage in chicks showed broader tissue tropism than the parental viruses. None of the chickens inoculated with rgY55sub-P10 (H9N2) showed any signs of disease, and viruses were recovered from each of the samples except the brain and the blood. One of the three chickens inoculated with rgVac1sub-P2 (H5N1) showed clinical signs such as depression, and the viruses were recovered from virtually all of the organs and blood samples. The remaining two did not show disease signs nor the virus was recovered from any of the tissues tested. Two of the three chickens inoculated with rgVac1ins-P2 (H5N1) showed disease signs, one of them died two days post inoculation, and the virus was recovered from almost all samples. P3 viruses efficiently replicated in each of the tested tissues in chickens as compared with P2 viruses. Throughout the study, the viruses were recovered from the brains of all of the chickens showing clinical signs.

Table 4 Pathogenicity of each virus for chicken via intranasal route

Inoculated viruses	Seroconversion at 14 d.p.i.[a]	Clinical signs	Mortality (dead days)
rgY55 (H9N2)	3/3[b]	0/3	0/3
rgY55sub (H9N2)	3/3	0/3	0/3
rgY55sub-P10 (H9N2)	3/3	0/3	0/3
rgVac1 (H5N1)	0/3	0/3	0/3
rgVac1sub (H5N1)	1/3	0/3	0/3
rgVac1sub-P2 (H5N1)	0/1	2/3	2/3 (4, 11)
rgVac1sub-P3 (H5N1)	1/1	3/3	2/3 (7, 8)
rgVac1ins (H5N1)	1/3	0/3	0/3
rgVac1ins-P2 (H5N1)	1/1	2/3	2/3 (8, 11)
rgVac1ins-P3 (H5N1)	0/1	2/3	2/3 (4, 6)

[a] Examined for the survived chickens by HI test and ELISA.

[b] The number of positive animals/total.

Discussion

Here, we demonstrated that the H9N2 influenza virus acquired intravenous pathogenicity after a pair of dibasic amino acid residues was introduced into the cleavage site of the HA and serially passaged in chicks. Since rgY55sub-P10 (H9N2) killed 75% of chickens inoculated via intravenous route, the pathogenicity was comparable to that of HPAIVs (Table 1). On the other hand, chickens intranasally inoculated with rgY55sub-P10 (H9N2) did not show any clinical signs of disease (Table 4). These results are consistent with those of previous study showing that some H10 influenza viruses did not show intranasal pathogenicity for chicken while their intravenous pathogenicity index was over 1.2 and classified as HPAIV according to the definition by European Union [29]. Amino acid changes during consecutive passages in the air sacs of chicks (Table 2) are considered to be responsible for the acquisition of

Table 5 Virus recovery from the chickens intranasally inoculated with each virus

Inoculated viruses	No. of chickens	Days p.i. (Health status)	Virus recovery (log EID_{50}/g)						
			Brain	Trachea	Lung	Liver	Kidney	Colon	Blood[c]
rgY55 (H9N2)	3	3 (sacrificed)	-, -, -[b]	-, -, ≦ 1.7	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -
rgY55sub (H9N2)	3	3 (sacrificed)	-, -, -	5.5, 5.7, 6.5	-, 6.7, 2.7	-, 2.7, -	-, -, 2.5	-, -, -	-, -, -
rgY55sub-P10 (H9N2)	3	3 (sacrificed)	-, -, -	-, -, 3.3	-, 3.7, 6.0	-, -, 2.5	-, 4.3, 4.5	-, 3.3, 4.5	-, -, -
rgVac1 (H5N1)	3	3 (sacrificed)	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -
rgVac1sub (H5N1)	3	3 (sacrificed)	-, -, 2.7	-, -, -	-, -, 2.5	-, -, -	-, -, -	-, -, ≦ 2.0	-, -, -
rgVac1sub-P2 (H5N1)	2	3 (sacrificed)	-, -	-, -	-, -	-, -	-, -	-, -	-, -
	1[a]	3 (sacrificed)	5.0	3.8	5.0	5.2	7.5	5.2	4.3
rgVac1sub-P3 (H5N1)	2[a]	3 (dead)	5.7, 6.7	5.3, 6.5	4.7, 8.2	3.5, 6.0	7.3, 9.0	5.5, 6.5	NA[d]
	1[a]	3 (sacrificed)	6.3	6.5	6.7	6.5	6.5	6.5	5.8
rgVac1ins (H5N1)	3	3 (sacrificed)	-, ≦ 2.6, -	-, 3.5, -	-,3.3, 3.7	-, -, 3.5	-, 2.7, 3.0	-, 3.0, 2.5	-, ≦ 1.6, 2.8
rgVac1ins-P2 (H5N1)	1[a]	2 (dead)	3.5	3.4	4.7	3.7	4.8	4.7	NA
	1[a]	3 (sacrificed)	3.8	3.7	3.0	≦ 2.0	4.7	≦ 2.0	-
	1	3 (sacrificed)	-	-	-	-	-	-	-
rgVac1ins-P3 (H5N1)	3[a]	3 (sacrificed)	3.4, 4.7, 5.5	4.7, 3.5, 4.2	5.5, 5.2, 6.7	4.3, 4.5, 5.7	4.5, 4.7, 5.2	5.3, 4.5, 5.3	3.0, 2.5, 3.5

[a] Each chicken showed depression.

[b] 1.5 ≧ (0.5 ≧ for blood samples).

[c] log EID50/ml.

[d] Not applicable.

intravenous pathogenicity, and their effects on the functions of viral proteins should be clarified further. Here we focused on two substitutions at positions 29 and 234 of the H9 HA molecule. It has been reported that residue 226, based on the H3 HA numbering (234 in the present study), relates to receptor specificity and cell tropism [30]. Strain Y55 (H9N2) originally had a leucine at this position, and the change to glutamine after serial passages in the air sacs of chicks indicates that the passaged rgY55sub (H9N2) was further adapted to chicken. One of the asparagine-linked glycosylation sites on the HA of rgY55 (H9N2) lost a carbohydrate attachment with the substitution of Asn29His. The site locates sterically in the vicinity of the HA cleavage site, suggesting that the deletion of the carbohydrate chain affected the susceptibility of the HA to the host protease [31]. This notion is also supported by the present finding that the rgY55sub viruses (H9N2) after six passages in the air sacs of chicks replicated in MDCK cells in the absence of trypsin (Table 1). Ohuchi et al. (1991) reported that the insertion of additional basic amino acids into the H3 HA cleavage site resulted in intracellular proteolytic cleavage. Other groups reported that H3 and H6 HAs tolerated amino acid mutations into their cleavage sites and the viruses with the mutated HAs replicated in MDCK and/or QT6 cells in the absence of trypsin [32,33]. The results in the present study are in agreement with these, namely, cleavage-based activation by an ubiquitous protease is not restricted to the H5 and H7 HAs.

rgVac1sub (H5N1) and rgVac1ins (H5N1) acquired marked intravenous and intranasal pathogenicity after a few passages in chicks (Table 1). It was reported that an avirulent H5 virus isolated from a swan became highly pathogenic in chickens after 24 consecutive passages in the air sacs, followed by five passages in the brains of chickens [3]. The differences in time required for the viruses to become highly pathogenic between these studies depended on the amino acid motif at the HA cleavage site prior to passaging. rgVac1sub (H5N1) acquired an arginine at the HA cleavage site after only one passage in chickens (Figure 1B and Table 3), suggesting that an additional insertion of basic amino acid residues efficiently occurred in the serial basic amino acid residues at the cleavage site. One third of the chickens inoculated intranasally with rgVac1sub-P2 (H5N1) or rgVac1ins-P2 (H5N1) survived 14 days (Table 4). In addition, one of the birds was not susceptible to infection with rgVac1sub-P2 (H5N1), indicating that viral replication may