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

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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/

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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

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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

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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.

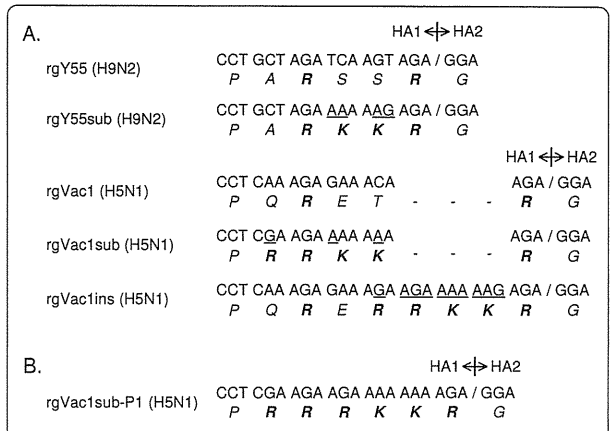

Figure 1 Nucleotide and amino acid sequences at the HA cleavage site of each virus. (A) Mutations were introduced into the HA genes of rgY55 (H9N2) and rgVac1 (H5N1) strains to give multibasic amino acids at their cleavage sites by site-directed mutagenesis. Mutation sites were underlined and deduced 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

depend on the presense of P3-like viruses in the inoculum.

The intranasal pathogenicity of the mutants of H9N2 virus was different from those of H5N1 mutants while these viruses replicated in MDCK cells in the absence of trypsin and killed chickens when inoculated via intravenous route (Tables 1 and 4). The viruses were recovered from the brain and the blood of some chickens infected with rgVac1 mutants (H5N1), and morbidity was closely associated with viral titers in the brain (Table 5). No viruses were recovered from the brain and the blood of chickens infected with rgY55 mutants (H9N2), indicating the reason why rgY55sub-P10 (H9N2) did not show intranasal pathogenicity. All the viruses passaged in the air sacs of chicks killed chicken embryos by 48 hours post allantoic inoculation (data not shown). rgVac1sub-P3 (H5N1) and rgVac1ins-P3 (H5N1) were more pathogenic to chicken embryos than rgY55sub-P10 (H9N2); the allantoic fluids obtained from the embryonated eggs inoculated with the H5N1 viruses passaged in the air sacs were turbid. It was reported that infection of a highly pathogenic virus was strictly confined to endothelial cells in chicken embryos or chickens [34,35]. Therefore, it is suggested that the difference of endotheliotropism between the H9N2 and H5N1 viruses passaged in the air sacs affected their intranasal pathogenicity. rgY55sub-P10 (H9N2) was not recovered from the brain and the blood of chickens although it caused systemic infection (Table 5), indicating that high levels of viremia followed by replication in the vascular endothelial cells was prerequisite for the virus to cross the blood-brain barrier and consequently replicated in the brain. This hypothesis is supported by the result that rgY55sub-P10 (H9N2) showed intravenous pathogenicity in chickens; direct injection of the virus to the blood vessels readily caused viremia, leading to invasion of the virus to the brain. (Table 1).

H9N2 viruses which have the PARSKR or PARSRR motifs at their HA cleavage site have been isolated from turkeys, ostriches, and chickens in Israel and quails in China [14] although PARSSR motif has been found in most H9N2 isolates, indicating that such substitutions with basic amino acid residues occur in nature. If serine at the c-terminus of the HA1 of the H9 virus was substituted with lysine, the amino acid motif would be consistent with that of rgY55sub (H9N2) which acquired intravenous pathogenicity on consecutive passages in the air sacs of chicks. LPAI caused by H9N2 strains in poultry is now causing serious economic losses [11-19], and its eradication is still difficult because of its low pathogenicity, frequently causing inapparent infections. The present study demonstrated that H9N2 viruses circulating in chicken flocks can acquire intravenous pathogenicity. It is predicted that co-infections of rgY55-sub-P10 (H9N2) with bacteria exacerbate not only intravenous pathogenicity but intranasal pathogenicity in chickens as shown in a previous study [21]. Therefore, continuous monitoring in poultry is important to prevent the emergence of pathogenic H9 viruses.

Materials and methods

Viruses

A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2) isolated from chicken meat imported from China upon quarantine was kindly provided by Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan) [36]. A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1) was generated by the standard genetic reassortment procedure from non-pathogenic viruses, A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) [37-39]. Viruses were propagated in ten-day-old embryonated chicken eggs for 48 hours at 35°C.

The complete nucleotide sequences of Y55 (H9N2) and Vac1 (H5N1) have been registered in GenBank/EMBL/DDBJ (Accession numbers: AB256671-AB256678 [36] and AB259709-AB259716 [37], respectively).

Reverse genetics

Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with the Y55 and Vac1 strains using a commercial kit (TRIzol LS Reagent, Sigma-Aldrich, St. Louis, MO, U.S.A) and reverse-transcribed with the Uni12 primer [40] 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 [41]. The PCR products were cloned into the vector pCR2.1-TOPO (Invitrogen) or pGEM-T Easy Vector (Promega, Mannheim, Germany). After confirmatory sequencing, T-vector clones were digested with *BsmBI* and inserted into the vector pHW2000 [42]. MDCK cells and 293T cells were maintained in Minimum Essential Medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% calf serum and D-MEM (Invitrogen) containing 10% FBS, respectively. Before transfection, confluent 293T and MDCK cells in 75 cm^2 flasks were trypsinized, and 10% of each cell line was mixed in 12 ml of Opti-MEM I (Invitrogen); 2 ml of the suspension was seeded into each well of six-well tissue culture plates (Nunc Inc., Naperville, IL). The cocultured 293T and MDCK cells were used for the transfection. TransIT-293 (Panvera, Madison, WI) was used to transfect cells according to the manufacturer's directions. Briefly, two microliters of TransIT-293 per microgram of DNA was mixed, incubated at room temperature for 45 minutes, and added to the cells. The transfection mixture was replaced with Opti-MEM I after six hours of incubation at 37°C. Thirty hours later, Opti-MEM I containing one microgram per microliter

of trypsin was added. At 48 to 72 hours post-transfection, the culture supernatant was collected and propagated in ten-day-old embryonated chicken eggs.

Site-directed-mutagenesis

To generate H9 and H5 mutant viruses with basic amino acid residue substitutions (sub) or insertions (ins) at the HA cleavage site, mutations were introduced into the HA genes of the Y55 and Vac1 strains using a QuikChange II site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The mutant viruses, rgY55sub (H9N2), rgVac1sub (H5N1), and rgVac1ins (H5N1), were rescued by reverse genetics as described above, and the entire genomes of the eight gene segments were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for one hour. Unbound viruses were removed by washing the cells with MEM. Cells were then overlaid with MEM containing 0.7% Bacto-agar (Difco, Sparks, MD) in the presence or absence of trypsin (5 μg/ml). After 48 hours of incubation at 35°C, cells were stained with 0.005% neutral red.

Consecutive passage in the air sacs of chicks

The caudal thoracic air sacs of three 3-day-old chicks were inoculated with 200 μl of each of the mutant Y55 and Vac1 viruses. The chicks were sacrificed, and their lungs and brains were collected at three days post-inoculation. Serial passages in the air sacs of three to six 3-day-old chicks were performed with 200 μl of a pooled 10% tissue suspension of infected organs. Brain samples were used as the inoculum when both samples (lungs and brains) tested positive for the virus. Isolates were identified by their parental strain's name, mutation (substitution or insertion), and number of passages. For example, the designation rgY55sub-P10 (H9N2) indicates that the amino acids at the HA cleavage site of the Y55 virus were substituted with basic amino acids as shown in Figure 1A, then passaged ten times in the air sacs. Passaged viruses were propagated in the allantoic cavities of ten-day-old embryonated chicken eggs for 48 hours at 35°C. The allantoic fluid was harvested and stored at -80°C.

Experimental infection of chickens with mutant virus strains

Four-week-old Boris Brown chickens were used to test the pathogenicity of the passaged viruses. Eight chickens were intravenously inoculated with 200 μl of each virus (1:10 diluted allantoic fluid), and examined for clinical signs at intervals of 24 hours over a period of ten days. Similarly, three chickens were infected intranasally with 100 μl of allantoic fluid containing each virus at a EID_{50} of $10^{6.5}$ and observed for 14 days. Specific antibodies against homologous viruses after 14 days of infection were detected in serum using the hemagglutination inhibition (HI) test and/or enzyme-linked immunosorbent assay (ELISA) as described previously [43]. To study viral replication, each virus was inoculated into three chickens at an EID_{50} of $10^{6.5}$. The birds were euthenized three days post-challenge, and tissue and blood were collected aseptically. To make a 10% suspension with MEM, the tissue samples were homogenized using a Muti-Beads Shocker (Yasui Kikai, Osaka, Japan). These suspensions were serially diluted ten-fold with PBS and inoculated into ten-day-old embryonated eggs and incubated at 35°C for 48 hours. Viral titers were calculated by the method of Reed and Muench [44] and expressed as EID_{50} per gram and milliliter of tissue and blood, respectively.

All experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 biosafety facility at 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.

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Authors' contributions

HK is the leader of the study group. KS carried out the experiments and wrote the manuscript. SA helped in passaging study. KS, MO, YS, and HK designed the experiments and analyzed the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Characterization of influenza A viruses isolated from wild waterfowl in Zambia

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Although the quest to clarify the role of wild birds in the spread of the highly pathogenic H5N1 avian influenza virus (AIV) has yielded considerable data on AIVs in wild birds worldwide, information regarding the ecology and epidemiology of AIVs in African wild birds is still very limited. During AIV surveillance in Zambia (2008–2009), 12 viruses of distinct subtypes (H3N8, H4N6, H6N2, H9N1 and H11N9) were isolated from wild waterfowl. Phylogenetic analyses demonstrated that all the isolates were of the Eurasian lineage. Whilst some genes were closely related to those of AIVs isolated from wild and domestic birds in South Africa, intimating possible AIV exchange between wild birds and poultry in southern Africa, some gene segments were closely related to those of AIVs isolated in Europe and Asia, thus confirming the inter-regional AIV gene flow among these continents. Analysis of the deduced amino acid sequences of internal proteins revealed that several isolates harboured particular residues predominantly observed in human influenza viruses. Interestingly, the isolates with human-associated residues exhibited higher levels of virus replication in the lungs of infected mice and caused more morbidity as measured by weight loss than an isolate lacking such residues. This study stresses the need for continued monitoring of AIVs in wild and domestic birds in southern Africa to gain a better understanding of the emergence of strains with the potential to infect mammals.

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INTRODUCTION

Avian influenza viruses (AIVs) are zoonotic pathogens maintained in nature mainly in wild aquatic birds (Olsen *et al.*, 2006; Webster *et al.*, 1992). Viruses of 16 different haemagglutinin (HA) (H1–H16) and nine neuraminidase (NA) (N1–N9) subtypes have been identified in waterfowl reservoirs. These viruses are usually non-pathogenic for their natural hosts. It is generally accepted that highly pathogenic AIVs (HPAIVs), particularly of the H5 and H7 subtypes, emerge from low-pathogenic AIV (LPAIV) precursors once introduced into poultry and that they may not be harboured by wild birds (Capua & Alexander, 2006; Röhm *et al.*, 1995). After emerging in China in 1996, H5N1 HPAIV spread rapidly throughout Asia, Europe, the

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are AB569476–AB569571.

Supplementary data on the phylogenetic relationships of AIVs from wild birds in Zambia are available with the online version of this paper.

Middle East and Africa, causing unprecedented outbreaks in wild birds, poultry and occasional human infections that have risen to pose a significant pandemic threat (Ducatez *et al.*, 2006; Li *et al.*, 2004; Smith *et al.*, 2006; Wang *et al.*, 2008). The rapid spread of the H5N1 HPAIV and the detection of H5N2 AIVs with an HP viral genotype in healthy wild waterfowl in Africa (Gaidet *et al.*, 2008) have heightened the possibility of the existence of a wild-bird reservoir for HPAIVs and underscore the need to improve our current understanding of the eco-epidemiological dynamics of AIVs in nature.

As early as 1961, Africa recorded the first outbreak of HPAIV in wild birds, which caused the death of approximately 1300 common terns (Capua & Alexander, 2006). Until 2004 when H5N2 HPAIV caused an outbreak in South African ostriches, there had been no reported cases of HP avian influenza in Africa. The continent's first experience with the Asian-origin H5N1 HPAIV was in 2006 in Nigeria (Ducatez *et al.*, 2006). The virus has since spread to several African countries, affecting a range of avian species with sporadic spillover into humans. Egypt is the African country that has recorded the highest number of human infections with the H5N1 HPAIV to date, with 115 confirmed cases, of which 38 were fatal (World Health Organization, 2010). Despite the significance of these events, which pose a serious threat to animal and public health, as well as to food security in Africa, very little is known about AIVs circulating in wild birds in Africa. Presently, there is very limited GenBank coverage (no more than three complete genomes) of non-pathogenic/LP viral genes of AIVs isolated from African wild birds.

Repeated direct transmissions of AIVs from poultry to humans and other mammals have stimulated investigations into the pathogenicity and transmission mechanisms of AIVs in mammals. Prior to the H5N1 'bird flu' incident in Hong Kong in 1997, which marked the first recorded instance of a purely AIV infecting and causing death in humans (Peiris *et al.*, 2007), investigations on the potential of AIVs from waterfowl to infect mammals, including humans, monkeys, pigs, ferrets and cats, have revealed a spectrum of replication, mostly with no significant disease signs (Beare & Webster, 1991; Hinshaw *et al.*, 1981; Kida *et al.*, 1994; Murphy *et al.*, 1982). In recent years, considerable advances have been made in elucidating the determinants of pathogenicity and adaptation of AIVs in mammals, especially for HP isolates involved in human infections (de Wit *et al.*, 2008). However, the mechanisms of pathogenicity and replicative capacity of LPAIVs isolated from wild birds in mammals are still poorly understood.

Sub-Saharan Africa where Zambia is located supports large populations of indigenous waterfowl and is an overwintering area for some Eurasian birds (Olsen *et al.*, 2006). Hitherto, no cases of H5N1 HPAIV have been recorded in southern Africa. Thus, AIV surveillance in wild birds and poultry in this region could provide timely information on the possible introduction of H5N1 HPAIV for mitigation purposes. Additionally, data obtained on LPAIVs in wild birds would expand our current understanding of the ecology and epidemiology of AIVs in this region.

During AIV surveillance conducted between 2008 and 2009 in Zambia, 12 viruses were isolated from wild waterfowl in Lochinvar National Park. Whole-genome sequencing was performed on each isolate, and bioinformatics approaches were employed to characterize the viruses genetically. Furthermore, based on genetic characterization results, we evaluated the replication and pathogenicity of some of the isolates in a mouse model.

RESULTS

Surveillance and virus isolation

AIV surveillance has been ongoing in Zambia since 2006 (Simulundu *et al.*, 2009). Between April 2008 and November 2009, a total of 3094 wild waterfowl faecal specimens were collected in Lochinvar National Park. On average, about 200 faecal specimens were collected every month except during the rainy season (December to March) when the wetland becomes inaccessible due to extreme flooding. Twelve AIVs were isolated (Table 1). Of the 12 isolates, seven were from ducks, four from geese and one from a great white pelican (*Pelecanus onocrotalus*). We identified five different HA (H3, H4, H6, H9 and H11) and NA (N1, N2, N6, N8 and N9) subtypes (Table 1). Among these subtypes, the H11N9 subtype is relatively uncommon, whilst H9N1 is a rare HA/NA combination. Currently, only ten H9N1 isolates are available in GenBank and none has been reported from Africa or Europe.

Phylogenetic analysis of the HA and NA genes

To understand the evolutionary relationships of AIVs isolated from wild birds in Zambia in detail, we sequenced the entire genome of each isolate and conducted phylogenetic analyses. To include, as much as possible, some AIV sequences of isolates from African birds in our analyses, some partial sequences were used. The HA and NA genes of all the viruses characterized in this study belonged to the Eurasian avian lineage (Figs 1 and 2 and Supplementary Figs S1 and S2, available in JGV Online). They clustered mostly with those of AIVs isolated in southern Africa. It was noted that the HA and NA genes of H11N9 viruses reported here formed a distinct sublineage within the Eurasian lineage (see Supplementary Figs S1c and S2c). In this report, only the H6 and H9 HA and the N2 and N8 NA gene trees are described in more detail, because these subtypes have been involved in avian influenza outbreaks in southern Africa.

The topology of the H6 HA phylogenetic tree conformed to that described previously by Bahl *et al.* (2009), particularly in the classification of isolates into the American and

Table 1. AIVs isolated from wild waterfowl in Zambia (2006–2009)

Host	Strain name	Abbreviation	Sampling date
Wild duck	A/duck/Zambia/02/08 (H6N2)	Zb02 (H6N2)	June 2008
	A/duck/Zambia/03/08 (H6N2)	Zb03 (H6N2)	June 2008
	A/duck/Zambia/04/08 (H3N8)	Zb04 (H3N8)	June 2008
	A/duck/Zambia/08/09 (H6N2)	Zb08 (H6N2)	August 2009
	A/duck/Zambia/10/09 (H6N2)	Zb10 (H6N2)	September 2009
	A/duck/Zambia/11/09 (H11N9)	Zb11 (H11N9)	September 2009
	A/duck/Zambia/12/09 (H11N9)	Zb12 (H11N9)	September 2009
Wild goose	A/goose/Zambia/05/08 (H3N8)	Zb05 (H3N8)	July 2008
	A/goose/Zambia/06/08 (H3N8)	Zb06 (H3N8)	July 2008
	A/goose/Zambia/07/08 (H4N6)	Zb07 (H4N6)	September 2008
	A/goose/Zambia/09/09 (H11N9)	Zb09 (H11N9)	September 2009
Wild pelican	A/pelican/Zambia/01/06 (H3N6)*	Zb01 (H3N6)	August 2006
	A/pelican/Zambia/13/09 (H9N1)	Zb13 (H9N1)	November 2009

*The first influenza virus isolate from an avian host in Zambia (Simulundu *et al.*, 2009).

Eurasian/American lineages (Fig. 1a). The H6 HA genes reported in this study belonged to a group of viruses of the Eurasian/American lineage that consisted of contemporary H6 strains isolated from wild aquatic birds in Africa, Asia and America, including those viruses that were introduced into terrestrial poultry in Taiwan and South Africa (Fig. 1a). They shared a common ancestor with an H6N8 virus that caused avian influenza in South African ostriches in 2007 (Abolnik *et al.*, 2010) (Fig. 1a). The H6 HA genes of the isolates obtained in Zambia were distinct from those that caused an avian influenza outbreak in chickens in South Africa in 2002.

Genetic and antigenic analyses of the HA genes of H9N2 AIVs have shown that these viruses separate into three main Eurasian lineages (Xu *et al.*, 2007). These lineages are represented by chicken/Beijing/1/94, quail/Hong Kong/G1/97 and duck/Hong Kong/Y439/97 (Fig. 1b). The HA gene of Zb13 (H9N1) belonged to the duck/Hong Kong/Y439/97-like lineage (also called the Korean lineage) and was most closely related to that of ostrich/South Africa/AI1586/08 (H9N2).

In the N2 NA gene tree, AIVs isolated in this study fell in a Eurasian sublineage composed of viruses isolated mainly from wild aquatic birds in Asia, Europe and Africa (Fig. 2a). They clustered together with strains isolated from an ostrich and from a wild goose in South Africa in 2008, as well as two other strains isolated in China and Japan. The H5N2 AIVs with a genotype characteristic of HPAIVs detected from wild ducks in Nigeria also belonged to this sublineage. The N2 NA phylogeny further revealed that the N2 genes of viruses that caused outbreaks of avian influenza in South Africa in 2002 were distinct from those characterized in this study, a finding that is in concordance with their HA phylogenetic comparisons (Figs 1a and 2a).

Phylogenetic analysis of the N8 NA genes showed several sublineages within the Eurasian avian lineage, namely, early 1 and 2, contemporary 1 and 2 and European gull isolates (Fig. 2b). The NA genes of Zb04 (H3N8), Zb05 (H3N8) and Zb06 (H3N8) belonged to the contemporary 1 sublineage, which consisted of AIVs isolated mostly from wild birds in Europe and southern Africa.

Phylogenetic analysis of the internal protein genes

Broadly, the topologies of the internal protein gene trees showed assortment of the AIVs into the American and Eurasian avian lineages, with early and contemporary sublineages being identifiable in the latter lineage, as described previously by Duan *et al.* (2007). The Eurasian contemporary sublineage was further divided into two to three groups.

Phylogenetic analysis of the PB2 polymerase subunit gene showed that, except for Zb04 (H3N8) which fell in group 2 of the Eurasian contemporary sublineage, all the viruses isolated in Zambia from 2006 to 2009 clustered together in group 1 (Fig. 3a). H5N1 HPAIVs also joined this group, but they were not closely related to the viruses reported here. The PB2 gene of Zb04 (H3N8) showed a close relationship to those of two H5N2 viruses isolated from domestic ducks in China.

In the PB1 polymerase subunit gene tree, three groups were observed in the Eurasian contemporary sublineage (Fig. 3b). All isolates from waterfowl in Zambia belonged to group 3. Whilst the majority of the strains isolated between 2006 and 2009 in Zambia grouped together as an independent branch, the PB1 genes of Zb04 (H3N8), Zb08 (H6N2), Zb10 (H6N2) and Zb13 (H9N1) belonged to a cluster of viruses that included two recent wild-bird isolates from South Africa, two H5N2 influenza viruses isolated from pigs in South Korea and two H5N1 HPAIVs isolated in Laos (Fig. 3b).

In the PA polymerase subunit phylogeny, all the viruses reported here belonged to group 1 and the majority of the

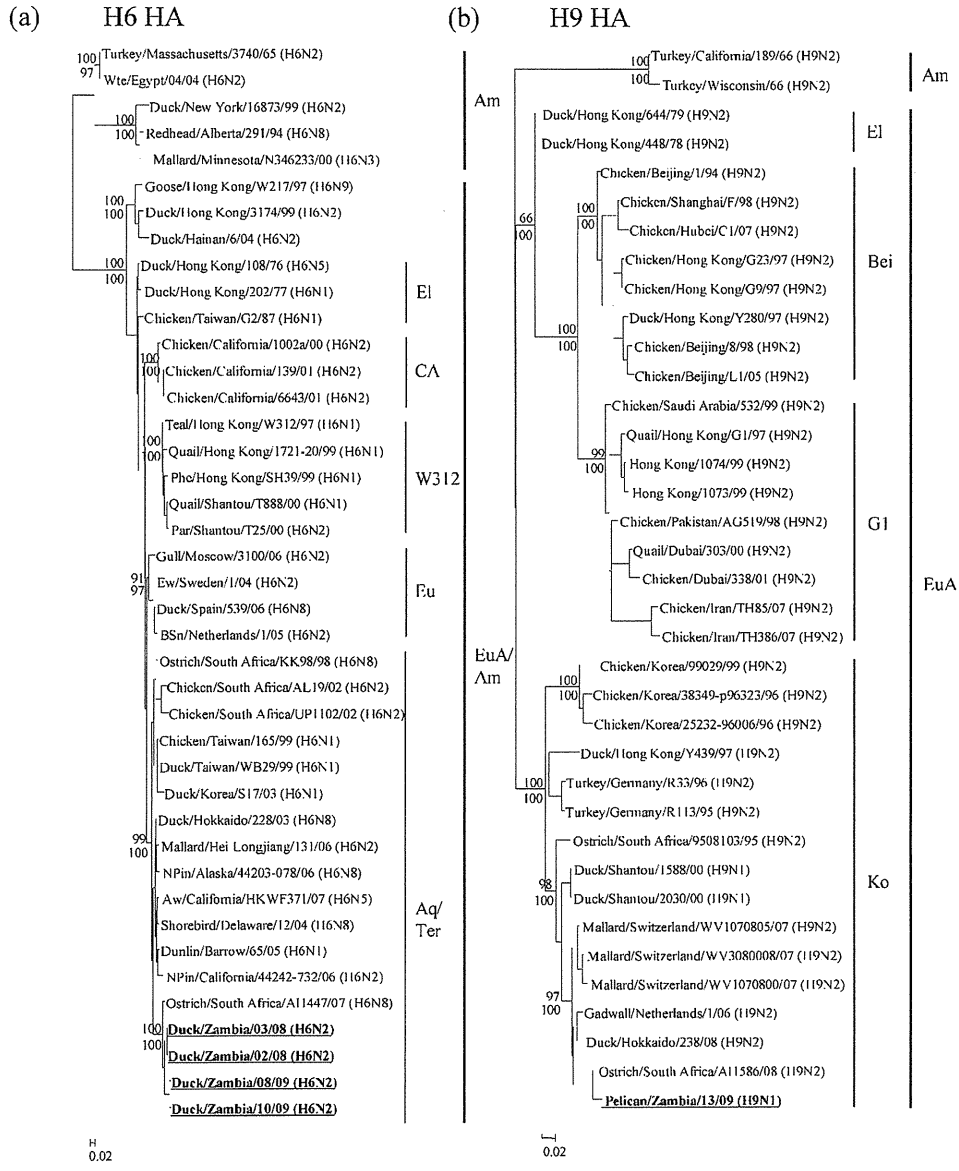

Fig. 1. Phylogenetic relationships of the H6 HA (a) and H9 HA (b) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 44–1066 (1023 bp) of H6 HA and 97–1228 (1132 bp) of H9 HA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are bold and underlined. Bars, number of substitutions per site. Lineages: Am, American; Aq/Ter, aquatic/terrestrial; Bei, chicken/Beijing/1/94-like; CA, California; El, early; Eu, Europe; EuA, Eurasian; G1, quail/Hong Kong/G1/97-like; Ko, Korean-like; W312, teal/Hong Kong/W312-like. Strain names: Aw, American wigeon; BSn, Bewick's swan; Ew, Eurasian wigeon; NPin, northern pintail; Par, partridge; Phe, pheasant; Wte, whiskered tern.

viruses grouped with those of AIVs isolated from wild birds and ostriches in South Africa (Fig. 3c). The PA gene of Zb04 (H3N8) showed a close relationship to viruses isolated from wild birds in the Netherlands. The PA gene of Zb01 (H3N6) belonged to group 2 and clustered with those of the Asian H5N1 HPAIVs, as we reported previously (Simulundu *et al.*, 2009).

The nucleoprotein (NP) gene tree showed the division of the Eurasian contemporary sublineage into three groups (Fig. 4a). All the viruses characterized in this study assorted to group 3. Eight of these strains, along with those isolated recently from wild and domestic birds in South Africa, formed a distinct cluster within this sublineage. The other four isolates clustered with viruses isolated mostly from

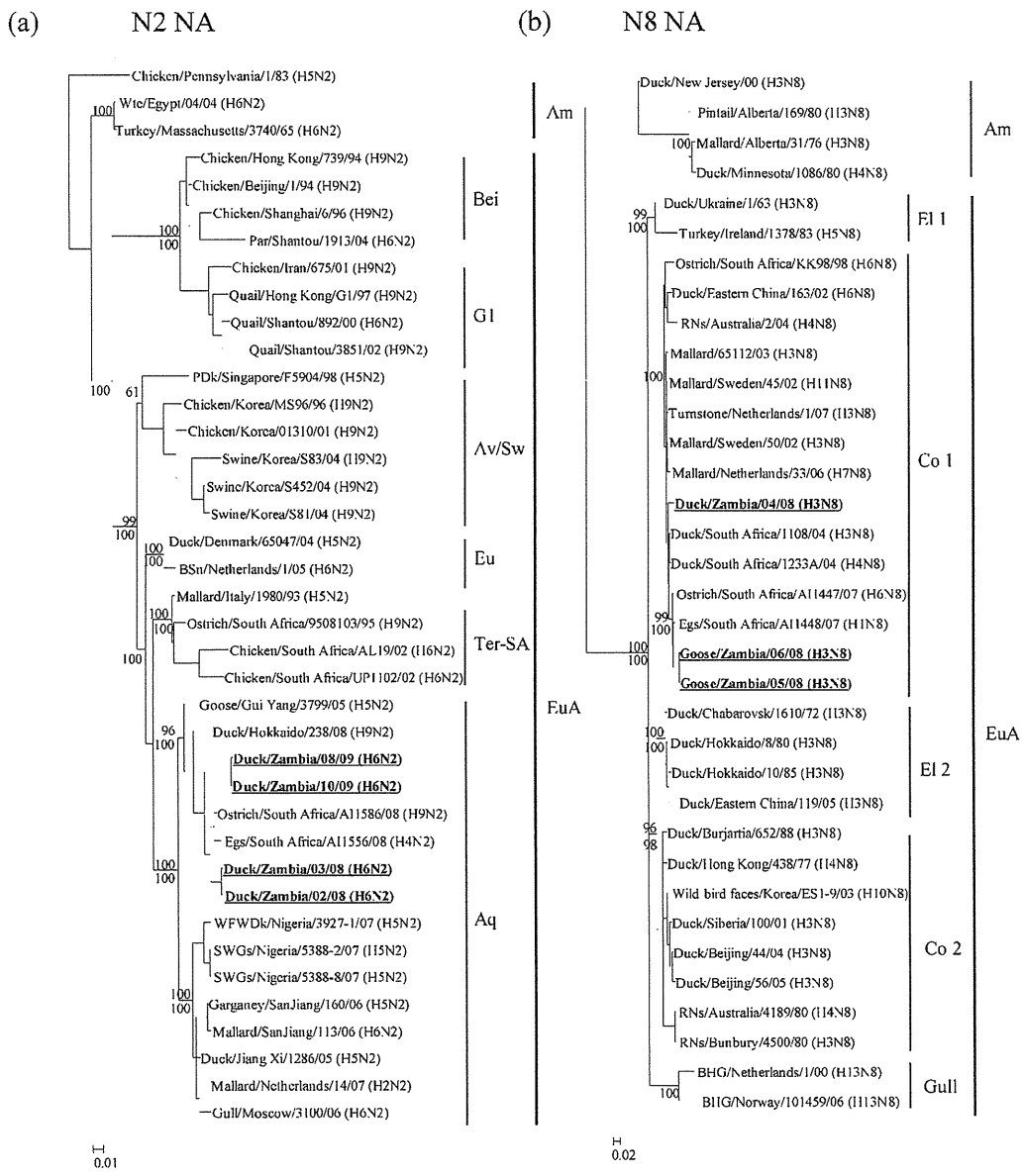

Fig. 2. Phylogenetic relationships of the N2 NA (a) and N8 NA (b) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 78–1396 (1319 bp) of N2 NA and 54–1343 (1290 bp) of N8 NA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined. Bars, number of substitutions per site. Lineages: Aq, aquatic; Av/Sw, avian/swine; Co, contemporary; Ter-SA, terrestrial, South Africa. Strain names: BHG, black-headed gull; Egs, Egyptian goose; PDk, pekin duck; RNs, red-necked stint; SWGs, spur-winged goose; WFWDk, white-faced whistling duck. Other abbreviations are listed in the legend of Fig. 1.

Europe. The NP gene of Zb01 (H3N6) was closely related to that of ostrich/South Africa/AI1447/07 (H6N8) and both these strains belonged to a group of viruses comprising early and contemporary strains.

The matrix (M) gene tree showed that all the viruses isolated from wild birds in Zambia were in group 3, but they did not all cluster together (Fig. 4b). The majority of the viruses reported in this study grouped with isolates obtained from wild and domestic birds in South Africa and appear to have been derived from A/mallard/Netherlands/1/06 (H8N4)-like viruses. The M genes of Zb01 (H3N6) and Zb04 (H3N8) were closely related to that of turkey/Italy/3620/99 (H7N1), whilst those of Zb02 (H6N2), Zb03 (H6N2), Zb05 (H3N8) and Zb06 (H3N8) grouped with that of an H9N2 virus isolated from an ostrich in South Africa.