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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 35ºC.

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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REFERENCES

1. Capua, I., Marangon, S., Dalla Pozza, M., Terregino, C. and Cattoli, G. 2003. Avian influenza in Italy 1997–2001. *Avian Dis.* **47**: 839–843.
2. Cheng, M. C., Soda, K., Lee, M. S., Lee, S. H., Sakoda, Y., Kida, H. and Wang, C. H. 2010. Isolation and characterization of potentially pathogenic H5N2 influenza virus from a chicken in Taiwan in 2008. *Avian Dis.* **54**: 885–893.
3. Donatelli, I., Campitelli, L., Di Trani, L., Puzelli, S., Selli, L., Fioretti, A., Alexander, D. J., Tollis, M., Krauss, S. and Webster, R. G. 2001. Characterization of H5N2 influenza viruses from Italian poultry. *J. Gen. Virol.* **82**: 623–630.
4. Fouchier, R. A., Munster, V., Wallensten, A., Bestebroer, T. M., Herfst, S., Smith, D., Rimmelzwaan, G. F., Olsen, B. and Osterhaus, A. D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**: 2814–2822.
5. Garcia, M., Crawford, J. M., Latimer, J. W., Rivera-Cruz, E. and Perdue, M. L. 1996. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. *J. Gen. Virol.* **77 (Pt 7)**: 1493–1504.
6. Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. and Perez, D. R. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.* **146**: 2275–2289.
7. Horimoto, T., Rivera, E., Pearson, J., Senne, D., Krauss, S., Kawaoka, Y. and Webster, R. G. 1995. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. *Virology* **213**: 223–230.
8. Ito, T., Goto, H., Yamamoto, E., Tanaka, H., Takeuchi, M., Kuwayama, M., Kawaoka, Y. and Otsuki, K. 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. *J. Virol.* **75**: 4439–4443.
9. Ito, T. and Kawaoka, Y. 1998. Avian influenza. pp. 126–136. *In*: Textbook of Influenza (Nicholson, K. G., Webster, R. G., and Hay, A. J. eds.), Blackwell Science Ltd., Oxford.
10. Kishida, N., Sakoda, Y., Eto, M., Sunaga, Y. and Kida, H. 2004. Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. *Arch. Virol.* **149**: 2095–2104.
11. Lee, C. W., Senne, D. A. and Suarez, D. L. 2004. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J. Virol.* **78**: 8372–8381.
12. Lee, M. S., Chang, P. C., Shien, J. H., Cheng, M. C., Chen, C. L. and Shieh, H. K. 2006. Genetic and pathogenic characterization of H6N1 avian influenza viruses isolated in Taiwan between 1972 and 2005. *Avian Dis.* **50**: 561–571.
13. Nguyen, D. C., Uyeki, T. M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H., Xu, X., Balish, A., Klimov, A., Tumpey, T. M., Swayne, D. E., Huynh, L. P., Nghiem, H. K., Nguyen, H. H., Hoang, L. T., Cox, N. J. and Katz, J. M. 2005. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. *J. Virol.* **79**: 4201–4212.
14. OIE-Website. 2004. Disease Information, Avian influenza in Taipei China: follow-up report No.1 (final report) [cited 2010 November 1], Available from ftp://ftp.oie.int/infos_san_archives/eng/2004/en_040423v17n17.pdf. **17**: 118.
15. OIE. 2008. Avian influenza. pp. 465–481. *In*: Manual of Diagnostic Tests and Vaccines For Terrestrial Animals (Mammals, Birds and Bees), 6th Edition, Volume 1, Office Intl Des Epizooties, Paris.
16. Okamatsu, M., Saito, T., Mase, M., Tsukamoto, K. and Yamaguchi, S. 2007. Characterization of H5N2 influenza A viruses isolated from chickens in Japan. *Avian Dis.* **51**: 474–475.
17. Okamatsu, M., Saito, T., Yamamoto, Y., Mase, M., Tsuduku, S., Nakamura, K., Tsukamoto, K. and Yamaguchi, S. 2007. Low pathogenicity H5N2 avian influenza outbreak in Japan during the 2005–2006. *Vet. Microbiol.* **124**: 35–46.
18. Reed, M. and Muench, H. 1938. A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* **37**: 493–497.
19. Senne, D. A., Panigrahy, B., Kawaoka, Y., Pearson, J. E., Suss, J., Lipkind, M., Kida, H. and Webster, R. G. 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian Dis.* **40**: 425–437.
20. Soda, K., Ozaki, H., Sakoda, Y., Isoda, N., Haraguchi, Y., Sakabe, S., Kuboki, N., Kishida, N., Takada, A. and Kida, H. 2008. Antigenic and genetic analysis of H5 influenza viruses isolated from water birds for the purpose of vaccine use. *Arch. Virol.* **153**: 2041–2048.
21. Webster, R. G., Kawaoka, Y. and Bean, W. J. Jr. 1986. Molecular changes in A/Chicken/Pennsylvania/83 (H5N2) influenza virus associated with acquisition of virulence. *Virology* **149**: 165–173.
22. Webster, R. G. and Laver, W. G. 1967. Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. *J. Immunol.* **99**: 49–55.