Japanese Journal of Veterinary Research 56(4): 189-198, 2009

FULL PAPER

Evaluation of the potency, optimal antigen level and lasting immunity of inactivated avian influenza vaccine prepared from H5N1 virus

Takashi Sasaki[1,*], Norikazu Isoda[2], Kosuke Soda[2], Ryuichi Sakamoto[3], Kazue Saijo[4], Junko Hagiwara[5], Norihide Kokumai[1], Toshiaki Ohgitani[1], Takashi Imamura[3], Akira Sawata[4], Zhifeng Lin[5], Yoshihiro Sakoda[2] and Hiroshi Kida[2,6]

[1]Avian Biologics Department, Kyoto Biken Laboratories, Inc., Uji 611-0041, Japan
[2]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
[3]Division 2, Second Research Department, The Chemo-Sero-Therapeutic Research Institute, Kikuchi 869-1298, Japan
[4]Research Center for Biologicals, The Kitasato Institute, Kitamoto 364-0026, Japan
[5]Research Department, Nippon Institute for Biological Science, Ome 198-0024, Japan
[6]Research Center for Zoonosis Control, Hokkaido University, Sapporo 060-0818, Japan

Received for publication, November 18, 2008; accepted, February 4, 2009

Abstract

Test vaccines comprised of inactivated water-in-oil emulsions containing various antigen levels were prepared using a non-pathogenic H5N1 avian influenza (AI) virus, A/duck/Hokkaido/Vac-1/04 (H5N1). The potencies of these test vaccines were evaluated by two experiments. In the first experiment, the triangular relationship among the antigen levels of test vaccines, the hemagglutination inhibition (HI) antibody response, and the protective effect against challenge with a highly pathogenic avian influenza (HPAI) virus, A/chicken/Yamaguchi/7/04 (H5N1), was confirmed. Then lasting immunity of chickens after a single-shot vaccination was confirmed in the second experiment. As a result, complete protection after the challenge was observed in chickens immunized by test vaccines with an antigen level of 160 HA units/dose or higher. Thus, it was ascertained that the minimum antigen level in the AI vaccine was 160 HA units/dose, and the minimum HI antibody titer that could protect chickens from HPAI virus infection-related death was considered to be 1:16. Dose-dependent HI antibody responses were observed in chickens after the vaccination. Thus, 640 HA units/dose was thought to be similar to the optimal antigen level. Alternatively, the HI antibody titers of chickens, injected with the vaccine containing 640 HA units/dose, were maintained at 1:181 or higher for 100 weeks after the single-shot vaccination.

Key words: avian influenza vaccine, minimum antigen, minimum HI antibody, lasting immunity, optimal antigen level

*Corresponding author: Takashi Sasaki, Avian Biologics Department, Kyoto Biken Laboratories, Inc., 24-16 Makishima-cho, Uji 611-0041, Japan
Phone:+81-774-22-4518. Fax:+81-0774-24-1407. E-mail: keibyou_one@kyotobiken.co.jp

Introduction

In 2004, an outbreak of highly pathogenic avian influenza (HPAI) occurred in Yamaguchi Prefecture. It was the first outbreak for 79 years in Japan. Subsequent outbreaks then occurred in Oita and Kyoto prefectures at a total of four poultry farms, and about 275,000 fowls were eventually culled. The outbreak in Kyoto Prefecture caused the largest damage because the emergent condition at the poultry farm was intentionally hidden[3,8,15].

The H5N1 viruses isolated from birds in Yamaguchi, Oita, and Kyoto prefectures showed close genetic homology with each other[7]. A/chicken/Yamaguchi/7/04 (H5N1) also showed marked homology with a virus isolated from fowls in Korea in 2003[6]. Thus, these viruses isolated in Japan were strongly suspected to have originated from the Korean Peninsula, possibly being brought by migrating birds[5].

At the beginning of 2007, HPAI virus infection in chickens was confirmed on three poultry farms in Miyazaki prefecture and one in Okayama prefecture[4].

Under the present regulations in Japan, the practical application of avian influenza (AI) vaccine on actual poultry farms is permitted on limited occasions when the spread of the virus is impossible to curb by ordinary actions such as banning the movement of fowls or culling. However, it is very important to prepare a reliable vaccine against any AI outbreak in the future[1,2,10,13,14]. Thus, we tried to develop a vaccine that would induce a good immunological response to protect chickens from HPAI virus infection-related death by single-shot vaccination.

This study investigated the potency of our vaccine by two experiments. In the first experiment, the triangular relationship among the antigen levels of test vaccines, the hemagglutination inhibition (HI) antibody response, and the protective effect against challenge with a highly pathogenic avian influenza (HPAI) virus, A/chicken/Yamaguchi/7/04 (H5N1), was confirmed. Lasting immunity of the chickens after the single-shot vaccination was confirmed in the second experiment.

Materials and Methods

Viruses: A/duck/Hokkaido/Vac-1/04 (H5N1) (hereinafter referred to as Dk/Vac-1/04), a Eurasian lineage of a non-pathogenic AI virus produced as a reassortant virus of A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) in 2004 at Hokkaido University, was used for vaccine preparation[12].

A/chicken/Yamaguchi/7/04 (H5N1) (hereinafter referred to as Ck/Yamaguchi/04) was isolated from a dead chicken following an AI outbreak in 2004 in Yamaguchi Prefecture by the National Institute of Animal Health of Japan and was used as the challenge virus.

Each AI virus strain, Dk/Vac-1/04 and Ck/Yamaguchi/04, was inoculated into the allantoic cavity in 10- to 12-day-old embryonated chicken eggs, and incubated for 48 hr at 34℃ for the former, and 35℃ for the latter to prepare viral suspensions.

Vaccine preparation: A viral suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4℃. Inactivation of the virus was confirmed by the inoculation of embryonated chicken eggs.

The inactivated viral suspension was diluted with phosphate-buffered saline (PBS) to appropriate concentrations based on the hemagglutination (HA) titer. A 2.5 volume of each viral suspension with HA titers of 1:256, 1:64, and 1:32 was mixed with a 7.5 volume of oil adjuvant containing 3.9% anhydromannitol-octadecenoate-ether and light mineral oil to comprise the remaining volume. These mixtures were then homogenized using an ultra-homomixer to produce water-in-oil type Vaccines A, B, and C, respectively.

The antigen levels of Vaccines A, B, and C were calculated as 640, 160, and 80 HA units/dose, respectively, using the formula shown below:

$$\text{HA units/dose} = \frac{\text{Volume (μl) of viral suspension/dose}}{50\ \mu l} \times \text{HA titer of viral suspension}$$

Animals: Four-week-old specific pathogen-free (SPF) white leghorn chickens were used in this study. The chickens were hatched and fed in Kyoto Biken Laboratories, Inc. The chickens prepared for challenge test were transported to a biosafety level 3 facility at Hokkaido University 7 weeks after the vaccination. All procedures were performed according to the animal experiment guidelines of Hokkaido University.

HA antigen: The viral suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4℃, followed by dilution of the antigen with PBS to adjust the HA titer to 1:8.

HI test protocols: One volume of each serum was respectively mixed with 3 volumes of 10% chicken red blood cells (RBCs), and stored overnight at 4℃. The mixtures were centrifuged at 1,000 g for 5 min, and the supernatants were then collected as fourfold-diluted sera.

One-hundred μl of each of the supernatants described above was dispensed into several wells of the first lane of a plastic V-bottomed microtitration plate. Fifty μl of PBS was dispensed into all other wells, after which 50 μl of twofold serial dilutions of the supernatants were then added to the wells. Fifty μl of each HA antigen (1:8 HA titer) was then dispensed into all wells of the plates, and they were incubated for 30 min at room temperature. Finally, 100 μl of 0.5% chicken RBCs was dispensed into all wells, and they were incubated again for 60 min at room temperature.

HI antibody titers were expressed as the highest dilution of the serum sample that showed complete hemagglutination inhibition.

Experiment 1 (Confirmation of HI antibody responses of immunized chickens and the minimum vaccinal antigen level required for protection against HPAI virus challenge): Seven, ten, and eleven chickens were vaccinated intramuscularly in the lower thigh with 0.5 ml of Vaccines A, B, and C, respectively, and 13 other chickens were prepared as non-vaccinated controls. Sera of all chickens were collected every week after the vaccination, and the geometric mean of the HI antibody titer against Dk/Vac-1/04 was calculated by the method described above. Chicken groups injected with Vaccines A, B, and C were designated Groups A, B, and C, respectively. The chicken group without vaccination was designated the Control group.

All chickens were challenged intranasally with a 100-fold 50% chicken lethal dose ($10^{5.3}$ 50% egg infectious dose) of Ck/Yamaguchi/04 at 7 weeks after the vaccination. Clinical signs were monitored for 14 days post-challenge (p.c.).

Cloacal swabs on day 4 p.c. were individually collected from all surviving chickens to detect viral shedding. Both tracheal and cloacal swabs were also collected individually at the time of death and euthanasia on day 14 p.c. As primary screening for viral shedding, swabs were individually suspended in 1.0 ml of Eagle's minimum essential medium (MEM) containing a moderate amount of antibiotics. A 0.1-ml aliquot of each suspension was then inoculated into the allantoic cavity in 10-day-old embryonated chicken eggs and incubated at 35℃ for 48 hr, followed by refrigeration at 4℃. The allantoic fluids showing typical HA activity were judged as an indication of positive virus growth.

For the quantification of viral shedding, suspensions that were positive in the primary screening test were serially diluted tenfold with MEM, and inoculated into the allantoic cavity in 10-day-old embryonated chicken eggs to calculate the recovered viral titers by the same method and conditions described above. The recovered viral titers from swabs were judged by typical HA activity of the allantoic fluids. These were calculated by the method of Reed and Muench and expressed as the 50% egg infectious dose per ml (EID_{50}/ml)[11].

Experiment 2 (Immunization of chickens and se-

rum sampling until 100 weeks after the vaccination to confirm lasting immunity): Vaccine A was considered similar to the optimal antigen level based on the results of Experiment 1. We designed another experiment to determine if there was lasting immunity. Eight chickens were vaccinated intramuscularly with 0.5ml of Vaccine A in the lower thigh, and the other 3 chickens were used as non-vaccinated controls. Sera from all chickens were collected every week after the vaccination until 7 weeks, and then collected regularly at 9- to 12-week intervals until 100 weeks. The HI antibody titer against Dk/Vac-1/04 was calculated by the method described above. The chicken group injected with Vaccine A was designated the Test group, and the chicken group without vaccination was designated the Control group.

Results

Experiment 1 (Confirmation of HI antibody responses of immunized chickens and the minimum vaccinal antigen level required for protection against HPAI virus challenge)

HI antibody titers of chickens after vaccination with test vaccines are presented in Table 1. The geometric means of HI antibody titers every week after vaccination were <1:4, 1:58, 1:1,131, 1:2,497, 1:1,522, 1:1,248, and 1:1,248 in Group A, <1:4, 1:9, 1:294, 1:832, 1:724, 1:724, and 1:832 in Group B, and <1:4, <1:4, 1:9, 1:41, 1:56, 1:106, and 1:73 in Group C. Dose-dependent HI antibody responses were observed in chickens after the vaccination.

Clinical signs observed for 14 days after the challenge in all chickens are presented in Table 2. There were no clinical signs in any chickens in Groups A and B with HI antibody titers of 1:1,024 to 1:4,096 and 1:512 to 1:2,048, respectively, at the time of challenge. However, the HI antibody titers of Group C showed variable results ranging from 1:4 to 1:512. Chicken No. 148, which had an HI antibody titer of 1:4 at the time of challenge showed typical clinical signs (gloom, anorexia and nervous symptoms) from day 3 p.c. and died on day 8 p.c. Moreover, chicken No. 143, which had an HI antibody titer of 1:8 at the time of challenge also showed typical clinical signs from day 3 p.c. and died on day 4 p.c. Chickens with HI antibody titers of 1:16 or higher did not show any clinical signs for 14 days after the challenge. All chickens in the Control group showed HI antibody titers of <1:4 at the time of challenge and died on day 2 or 3 p.c.

Viral shedding in each group is presented in Table 3. None of the chickens in Groups A and B showed any virus shedding at any time investigated after the challenge. The HI antibody titers of vaccinated chickens in these groups were 1:512 or higher at the time of challenge. In Group C, $10^{1.0}$ EID_{50}/ml of the virus was recovered on day 4 p.c., and subsequently, $10^{4.3}$ and $10^{3.7}$ EID_{50}/ml of the virus were recovered from cloacal and tracheal swabs, respectively, at the time of death on day 8 p.c. in chicken No. 148, which showed an HI antibody titer of 1:4 at the time of challenge. Moreover, $10^{2.3}$ and $10^{3.5}$ EID_{50}/ml of the virus were recovered from cloacal and tracheal swabs, respectively, at the time of death on day 4 p.c. in chicken No. 143, which had an HI antibody titer of 1:8 at the time of challenge. None of the chickens with an HI antibody titer of 1:16 or higher showed any viral shedding at any time point examined. All chickens in the Control group demonstrated viral shedding from the cloaca and trachea at the time of death, and the maximum titer of recovered virus from the swabs was $10^{7.3}$ EID_{50}/ml.

Experiment 2 (Immunization of chickens and serum sampling until 100 weeks after the vaccination to confirm lasting immunity)

HI antibody titers of chickens until 100 weeks after vaccination are presented in Table 4. All chickens exceeded an HI antibody titer of 1:64 within 2 weeks after vaccination. The geometric mean of HI antibody titers reached a maximal value of 1:1,722 at 4 weeks after vaccination, and then slowly decreased. However, the geometric mean of HI antibody titers in the Test group remained 1:181 or higher for 100 weeks after vacci-

Table 1. HI antibody titers of chickens after the vaccination with test vaccines

Group	HA units/dose	Chicken No.	HI antibody titers at the following weeks after vaccination						
			1	2	3	4	5	6	7
A	640	105	<4	64	1,024	2,048	1,024	1,024	1,024
		109	<4	8	1,024	2,048	1,024	1,024	1,024
		115	<4	128	1,024	4,096	2,048	1,024	1,024
		116	<4	128	1,024	2,048	2,048	1,024	1,024
		117	<4	128	1,024	2,048	1,024	1,024	1,024
		120	<4	64	2,048	2,048	1,024	1,024	1,024
		114	<4	32	1,024	4,096	4,096	4,096	4,096
	GM[a)]		<4	58	1,131	2,497	1,522	1,248	1,248
B	160	123	<4	8	256	512	512	256	512
		128	<4	128	2,048	2,048	1,024	512	512
		131	<4	32	1,024	1,024	1,024	512	512
		133	<4	<4	128	256	256	512	512
		138	<4	<4	16	128	128	512	512
		122	<4	<4	256	1,024	1,024	1,024	1,024
		125	<4	16	128	1,024	1,024	1,024	1,024
		127	<4	—[b)]	128	2,048	1,024	1,024	1,024
		134	<4	4	256	1,024	1,024	1,024	2,048
		135	<4	32	4,096	2,048	2,048	2,048	2,048
	GM		<4	9	294	832	724	724	832
C	80	148	<4	<4	<4	<4	4	4	4
		143	<4	<4	<4	4	8	8	8
		154	<4	<4	<4	4	8	32	16
		141	<4	<4	<4	16	32	32	32
		151	<4	<4	<4	32	32	128	128
		153	<4	<4	128	512	256	256	128
		158	<4	<4	16	64	128	256	128
		147	<4	<4	16	128	128	256	256
		155	<4	<4	32	128	128	512	256
		159	<4	<4	64	512	512	1,024	256
		146	<4	<4	16	256	256	512	512
	GM		<4	<4	9	41	56	106	73
Control		161	<4	<4	<4	<4	<4	<4	<4
		164	<4	<4	<4	<4	<4	<4	<4
		165	<4	<4	<4	<4	<4	<4	<4
		166	<4	<4	<4	<4	<4	<4	<4
		167	<4	<4	<4	<4	<4	<4	<4
		170	<4	<4	<4	<4	<4	<4	<4
		172	<4	<4	<4	<4	<4	<4	<4
		173	<4	<4	<4	<4	<4	<4	<4
		162	<4	<4	<4	<4	<4	<4	<4
		163	<4	<4	<4	<4	<4	<4	<4
		168	<4	<4	<4	<4	<4	<4	<4
		169	<4	<4	<4	<4	<4	<4	<4
		175	<4	<4	<4	<4	<4	<4	<4
	GM		<4	<4	<4	<4	<4	<4	<4

a) GM: geometric mean, b) not tested

Table 2. Clinical signs of chickens after challenge with a highly pathogenic avian influenza virus

Group	HA units/dose	Chicken No.	HI antibody titer[a)]	Clinical signs on days after challenge													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	640	105	1,024	-[b)]	-	-	-	-	-	-	-	-	-	-	-	-	-
		109	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		115	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		116	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		117	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		120	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		114	4,096	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	160	123	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		128	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		131	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		133	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		138	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		122	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		125	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		127	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		134	2,048	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		135	2,048	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	80	148	4	-	-	+	+	+	+	+	D						
		143	8	-	-	+	D										
		154	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		141	32	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		151	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		153	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		158	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		147	256	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		155	256	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		159	256	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		146	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control		161	<4	-	D												
		164	<4	-	D												
		165	<4	-	D												
		166	<4	-	D												
		167	<4	-	D												
		170	<4	-	D												
		172	<4	-	D												
		173	<4	-	D												
		162	<4	-	+	D											
		163	<4	-	+	D											
		168	<4	-	-	D											
		169	<4	-	-	D											
		175	<4	-	-	D											

a) HI antibody titer at the time of challenge, b) -: No abnormal signs, +: typical clinical signs (gloom, anorexia and nervous symptoms), D: Death

Table 3. Virus isolation from cloacal and tracheal samples of chickens after challenge with a highly pathogenic avian influenza virus

Group	HA units/dose	Chicken No.	HI antibody titer[a]	Virus titers on days after challenge									
				2		3		4		8		14	
				C[b]	T	C	T	C	T	C	T	C	T
A	640	105	1,024					-[c]				-	-
		109	1,024					-				-	-
		115	1,024					-				-	-
		116	1,024					-				-	-
		117	1,024					-				-	-
		120	1,024					-				-	-
		114	4,096					-				-	-
B	160	123	512					-				-	-
		128	512					-				-	-
		131	512					-				-	-
		133	512					-				-	-
		138	512					-				-	-
		122	1,024					-				-	-
		125	1,024					-				-	-
		127	1,024					-				-	-
		134	2,048					-				-	-
		135	2,048					-				-	-
C	80	148	4					1.0[d]		4.3	3.7		
		143	8					2.3	3.5				
		154	16					-				-	-
		141	32					-				-	-
		151	128					-				-	-
		153	128					-				-	-
		158	128					-				-	-
		147	256					-				-	-
		155	256					-				-	-
		159	256					-				-	-
		146	512					-				-	-
Control		161	<4	5.0	6.8								
		164	<4	7.0	6.0								
		165	<4	6.3	5.5								
		166	<4	4.3	5.5								
		167	<4	5.3	7.3								
		170	<4	5.3	5.5								
		172	<4	5.5	6.6								
		173	<4	7.3	6.3								
		162	<4			1.5	4.8						
		163	<4			3.8	5.8						
		168	<4			4.5	3.3						
		169	<4			4.7	3.5						
		175	<4			5.8	3.5						

a) HI antibody titer at the time of challenge, b) C: Cloaca, T: Trachea, c) -: Not detected,
d) virus titer ($\log_{10}$ EID_{50}/ml)

Table 4. HI antibody titers of chickens until 100 weeks after the vaccination

Group	HA units/dose	Chicken No.	HI antibody titers at the following weeks after vaccination														
			1	2	3	4	5	6	7	16	28	40	52	64	76	88	100
Test	640	1	<4	512	1,024	1,024	2,048	2,048	1,024	1,024	256	256	128	128	128	128	128
		2	<4	128	2,048	4,096	2,048	2,048	2,048	1,024	512	512	512	256	256	256	256
		3	<4	256	1,024	2,048	2,048	2,048	2,048	2,048	1,024	1,024	1,024	512	512	512	512
		4	<4	64	512	1,024	1,024	1,024	1,024	1,024	512	256	256	256	256	256	128
		5	<4	128	2,048	2,048	2,048	1,024	1,024	1,024	256	256	128	128	128	128	128
		6	<4	128	512	1,024	1,024	1,024	1,024	1,024	256	256	256	128	128	128	128
		7	<4	128	512	2,048	2,048	1,024	1,024	256	128	128	128	128	128	128	128
		8	<4	512	1,024	2,048	2,048	2,048	1,024	1,024	512	512	512	512	512	512	256
	GM[a)]		<4	181	939	1,722	1,722	1,448	1,218	939	362	332	279	215	215	215	181
Control		9	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
		10	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
		11	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	GM		<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4

a) GM: geometric mean

nation.

Discussion

Based on the results of Experiment 1, it was demonstrated that the HI antibody responses of immunized chickens were correlated with the antigen levels of test vaccines. The minimum HI antibody titer that could protect chickens from HPAI virus infection-related symptoms and death was considered to be 1:16, because chickens with HI antibody titers of 1:4 and 1:8 at the time of challenge died on days 8 and 4 p.c., respectively, and distinct viral shedding was observed. In contrast, chickens with an HI antibody titer of 1:16 or higher survived without symptoms of AI and there was no viral shedding after the challenge.

Vaccine A, containing 640 HA units/dose, provided excellent protection for chickens by single-shot vaccination without viral shedding. Transit of the HI antibodies and the presence of anti-NS-1 antibodies (considered an indicator of infection by AI virus[9)]) after the challenge were not monitored in this study. We could not clearly assess the presence or absence of AI virus infection in the surviving chickens. However, Vaccine A might be able to protect chickens from HPAI virus infection. Vaccine B, containing 160 HA units/dose, induced an antibody response at 1:16 or higher in all chickens at 3 weeks after the vaccination. This was sufficient to protect the chickens from HPAI virus infection-related symptoms and death. Vaccine C, containing 80 HA units/dose, was inadequate to protect the chickens from HPAI virus infection-related death by single-shot vaccination, because it did not induce an adequate HI antibody response in any of them 2 weeks after vaccination. Moreover, the geometric mean 3 weeks after the vaccination was only 1:9. Two chickens died on days 4 and 8 after challenge. It would be possible to employ an emergency vaccination against severe outbreaks of HPAI if the vaccine contains an amount of antigen similar to that in Vaccine A. In this experiment, challenge was performed 7 weeks after the vaccination. Though it seems desirable to confirm the potency of an AI vaccine for emergency use via a challenge test as early as possible after vaccination, observation of the antibody response several weeks after the vaccination was also an important objective of the experiment. Thus, the challenge was performed at a relatively late time after vaccination, when the antibody titer had reached a plateau.

Based on the results of Experiment 2, Vaccine A, containing 640 HA units/dose, showed a good

immunological response. It showed rapid as well as long-lasting high-level immunity for 100 weeks after a single-shot vaccination. We are currently planning to continue the long-term observation further to determine the duration of the HI antibody titer, and to conduct an eventual challenge with HPAI at an appropriate time around 200 weeks after the vaccination. However, we speculate that the chickens of the vaccinated group could be protected from death even at that time because the HI antibody titers of all the chickens in the Test group remained 1:128 or higher for 100 weeks after single-shot vaccination, and the decrease of the HI antibody level to date has been extremely slow. Based on the results of Experiment 1, those HI antibody titers were considered sufficient to protect chickens from challenge with HPAI virus.

References

1) Ellis, T.M., Leung, C.Y., Chow, M.K., Bissett, L. A., Wong, W., Guan, Y. and Malik, Peiris, J.S. 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. *Avian Pathol.,* **33**: 405-412.
2) Garcia, A., Johnson, H., Srivastava, D.K., Jayawardene, D.A., Wehr, D.R. and Webster, R. G. 1998. Efficacy of inactivated H5N2 influenza vaccines against lethal A/Chicken/Queretaro/19/95 infection. *Avian Dis.,* **42**: 248-256.
3) Highly Pathogenic Avian Influenza Infection Route Elucidation Team. 2004. Routes of infection of highly pathogenic avian influenza in Japan. www.maff.go.jp/tori/20040630report.pdf.
4) Highly Pathogenic Avian Influenza Infection Route Elucidation Team. 2007. Routes of infection of highly pathogenic avian influenza in Japan. www.maff.go.jp/j/syouan/douei/tori/pdf/report2007.pdf.
5) Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X.W., Zhang, X.L., Zhao, D., Wang, G., Feng, Y., Ma, J., Liu, W., Wang, J. and Gao, G.F. 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science,* **309**: 1206.
6) Mase, M., Kim, J.H., Lee, Y.J., Tsukamoto, K., Imada, T., Imai, K. and Yamaguchi, S. 2005. Genetic comparison of H5N1 influenza A viruses isolated from chickens in Japan and Korea. *Microbiol. Immunol.,* **49**: 871-874.
7) Mase, M., Tsukamoto, K., Imada, T., Imai, K., Tanimura, N., Nakamura, K., Yamamoto, Y., Hitomi, T., Kira, T., Nakai, T., Kiso, M., Horimoto, T., Kawaoka, Y. and Yamaguchi, S. 2005. Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. *Virology,* **332**: 167-176.
8) Nishiguchi, A., Yamamoto, T., Tsutsui, T., Sugizaki, T., Mase, M., Tsukamoto, K., Ito, T. and Terakado, N. 2005. Control of an outbreak of highly pathogenic avian influenza, caused by the virus sub-type H5N1, in Japan in 2004. *Rev. Sci. Tech.,* **24**: 933-944.
9) Ozaki, H., Sugiura, T., Sugita, S., Imagawa, H. and Kida, H. 2001. Detection of antibodies to the nonstructural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. *Vet. Microbiol.,* **82**: 111-119.
10) Qiao, C., Yu, K., Jiang, Y., Li, C., Tian, G., Wang, X. and Chen, H. 2006. Development of a recombinant fowlpox virus vector-based vaccine of H5N1 subtype avian influenza. *Dev. Biol. (Basel),* **124**: 127-132.
11) Reed, L. J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.,* **27**: 493-497.
12) Soda, K., Sakoda, Y., Isoda, N., Kajihara, M., Haraguchi, Y., Shibuya, H., Yoshida, H., Sasaki, T., Sakamoto, R., Saijo, K., Hagiwara, J. and Kida, H. 2008. Development of vaccine strains of H5 and H7 influenza viruses. *Jpn. J. Vet. Res.,* **55**: 93-98.
13) Swayne, D.E., Beck, J.R. and Mickle, T.R. 1997. Efficacy of recombinant fowl poxvirus vaccine

in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. *Avian Dis.,* **41**: 910-922.

14) Swayne, D.E., Lee, C.W. and Spackman, E. 2006. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. *Avian Pathol.,* **35**: 141-146.

15) Webster, R.G., Guan, Y., Poon, L., Krauss, S., Webby, R., Govorkovai, E. and Peiris, M. 2005. The spread of the H5N1 bird flu epidemic in Asia in 2004. *Arch. Virol. Suppl.,* **19**: 117-129.

Vaccine 27 (2009) 5174–5177

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short communication

Long lasting immunity in chickens induced by a single shot of influenza vaccine prepared from inactivated non-pathogenic H5N1 virus particles against challenge with a highly pathogenic avian influenza virus

Takashi Sasaki[a,*], Norihide Kokumai[a], Toshiaki Ohgitani[a], Ryuichi Sakamoto[b], Noriyasu Takikawa[c], Zhifeng Lin[d], Masatoshi Okamatsu[e], Yoshihiro Sakoda[e], Hiroshi Kida[e,f]

[a] Avian Biologics Department, Kyoto Biken Laboratories, Inc., 24-16 Makishima-cho, Uji, Kyoto 611-0041, Japan
[b] Division 2, Second Research Department, The Chemo-Sero-Therapeutic Research Institute, Kikuchi, Kumamoto 869-1298, Japan
[c] Research Center for Biologicals, The Kitasato Institute, Kitamoto, Saitama 364-0026, Japan
[d] Research Department, Nippon Institute for Biological Science, Ome, Tokyo 198-0024, Japan
[e] Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
[f] Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

ARTICLE INFO

Article history:
Received 12 May 2009
Received in revised form 12 June 2009
Accepted 22 June 2009
Available online 9 July 2009

Keywords:
Avian influenza vaccine
Lasting protective immunity
H5 virus

ABSTRACT

An influenza vaccine was prepared from inactivated whole particles of the non-pathogenic strain A/duck/Hokkaido/Vac-1/04 (H5N1) virus using an oil adjuvant containing anhydromannitol-octadecenoate-ether (AMOE). The vaccine was injected intramuscularly into five 4-week-old chickens, and 138 weeks after vaccination, they were challenged intranasally with 100 times 50% chicken lethal dose of the highly pathogenic avian influenza (HPAI) virus A/chicken/Yamaguchi/7/04 (H5N1). All 5 chickens survived without exhibiting clinical signs of influenza, although 2 days post-challenge, 3 vaccinated chickens shed limited titres of viruses in laryngopharyngeal swabs.

1. Introduction

Influenza A viruses are divided into H1–H16 and N1–N9 subtypes on the basis of antigenic specificity of two glycoproteins [1,2]. Each of the subtypes has been isolated from migrating waterfowls, which play a role in the spread of the influenza A viruses [3,4]. During repeated passage through a chicken population, the viruses acquire transmissibility and pathogenicity against chickens, resulting in highly pathogenic avian influenza (HPAI) outbreaks in domestic poultry [5–9].

HPAI caused by H5 and H7 subtype viruses have occurred in many parts of the world, and such outbreaks have resulted in huge economic losses in poultry industries. A recent outbreak of H5N1 virus infection emerged in South Asia and spread through Eurasia and Africa. In addition, direct transmission of H5N1 viruses from birds to humans with high mortality occurred. Since 2003, more than 400 human cases with 60% mortality have been reported as of 11 May 2009 [10].

The standard measure undertaken for the control of HPAI in poultry is stamping out. Vaccination is allowed as an optional-tool to decrease the amount of viruses shed from infected chickens when standard measure cannot enough to control the outbreak in the field [11]. Although commercial vaccines prepared from viruses of the North American lineage are available, they may be less effective for the control of HPAI outbreaks caused by infection by viruses of the Eurasian lineage [12]. Thus, a vaccine prepared from a Eurasian lineage virus may provide better protection against Asian HPAI virus infections [13–16].

We have developed an H5N1 reassortant virus of the Eurasian lineage that is non-pathogenic for chickens and chicken embryos, and exhibits good growth in embryonated chicken eggs [12]. Subsequently, we prepared test vaccines using this reassortant virus, and confirmed the optimal antigen concentration and its protective potency against a currently prevalent Eurasian lineage HPAI virus [17]. We demonstrated that the test vaccine is able to induce protective immunity against HPAI virus starting 8 days post-vaccination and that chickens challenged with a HPAI virus, A/whooperswan/Mongolia/3/2005 (H5N1) strain did not show clinical signs of HPAI [14]. In the present study, we examined whether the vaccine induces long lasting immunity (138 weeks) by challenging vaccinated chickens with HPAI virus.

* Corresponding author. Tel.: +81 774 22 4518; fax: +81 774 24 1407.
E-mail address: keibyou_one@kyotobiken.co.jp (T. Sasaki).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.06.070

2. Materials and methods

2.1. Viruses

The A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/Vac-1/04) virus belonging to the Eurasian lineage of a non-pathogenic avian influenza (AI) virus, generated as a reassortant virus between A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1), was used for vaccine preparation [12].

A/chicken/Yamaguchi/7/04 (H5N1) (Ck/Yamaguchi/04) virus, isolated by the National Institute of Animal Health (Ibaraki, Japan) from a dead chicken during the HPAI outbreak in 2004 in Japan, was used as the challenge virus [18,19].

To prepare virus suspensions, the Dk/Vac-1/04 and Ck/Yamaguchi/04 viruses were inoculated into the allantoic cavity of embryonated chicken eggs and incubated at 34 C for 48 h and 35 °C for 24 h, respectively.

2.2. Vaccine preparation

A virus suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4 °C. Virus inactivation was confirmed by inoculation of the formalin-treated samples into embryonated chicken eggs.

The inactivated Dk/Vac-1/04 virus suspension was diluted with phosphate-buffered saline (PBS) to appropriate concentrations based on hemagglutination (HA) titres. A 2.5 volume of viral suspension with HA titre of 1:256 was mixed with a 7.5 volume of oil adjuvant containing 3.9% anhydromannitol-octadecenoate-ether (AMOE) and sufficient light mineral oil to comprise the remaining volume. This mixture was homogenized using an ultra-homomixer (PRIMIX Co. Ltd.) to produce a water-in-oil type of adjuvant test vaccine. The virus concentration in the test vaccine was 640 HA units per dose [17].

2.3. Animals and serum sampling

Specific pathogen-free white leghorn chickens were obtained from Kyoto Biken Laboratories, Inc., Kyoto, Japan. Ten 4-week-old chickens were vaccinated intramuscularly in the lower thigh with 0.5 mL of the test vaccine, and 4 other 4-week-old chickens were used as non-vaccinated controls. The two groups of chickens were reared separately for 138 weeks after the vaccination. Five vaccinated chickens, however, kept separately, died due to air conditioning accident of rearing facility 44 weeks after vaccination. Thus we used the remaining 5 vaccinated chickens for the experiment. For the Ck/Yamaguchi/04 challenge test, the chickens were transported to a bio-safety level 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All procedures were performed according to the animal experiment guidelines of Hokkaido University. A blood sample was obtained from each chicken every week after vaccination for 5 weeks, and then at 11–14-week intervals until 138 weeks. In addition, blood samples were obtained from all surviving chickens 2 weeks after the Ck/Yamaguchi/04 challenge.

2.4. Hemagglutination-inhibition (HI) test

The HI test was performed according to the Japanese Standards for Veterinary Biological Products. Briefly, 1 volume of each serum sample was mixed with 3 volumes of 10% chicken red blood cells (RBCs) and incubated overnight at 4 °C. The mixtures were centrifuged at 1000 × *g* for 5 min and the supernatants collected as four-fold diluted sera. One-hundred microliters of each supernatant were dispensed into wells in the first lane of a plastic V-bottomed microtitration plate. Fifty microliters of PBS was dispensed into all other wells, after which 50 μL of two-fold serial dilutions of the supernatants were added to the PBS-containing wells. The Dk/Vac-1/04 and Ck/Yamaguchi/04 virus suspensions were inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4 °C and 0.1% for 7 days at 4 °C, respectively, followed by dilution of the antigen with PBS to adjust the HA titre to 1:8. Fifty microliters of each HA antigen was then dispensed into all wells of the plates and they were incubated for 30 min at room temperature. Finally, 100 μL of 0.5% chicken RBCs was dispensed into all previously prepared wells and the plates were incubated again for 60 min at room temperature. The HI antibody titres were expressed as the highest dilution of the serum sample that showed complete inhibition of hemagglutination.

2.5. Protection test of vaccinated chickens against HPAI virus challenge

All chickens were challenged intranasally with 100 times 50% chicken lethal dose (i.e. $10^{5.5}$ times 50% egg infectious dose) of Ck/Yamaguchi/04 at 138 weeks after the Dk/Vac-1/04 vaccination. Clinical signs, such as lethargy, loss of appetite and nervous symptoms, were monitored for 14 days post-challenge (p.c.). To detect virus shedding, laryngopharyngeal and cloacal swabs were individually collected from all surviving chickens on days 2 and 4 p.c. Laryngopharyngeal and cloacal swabs were also collected individually at the time of death or at euthanasia on day 14 p.c. Swabs were individually suspended in 1.0 mL of minimal essential medium. A 0.1-mL aliquot of each suspension was then inoculated into the allantoic cavity of embryonated chicken eggs. The infectivity titres of the swabs were calculated using the method of Reed and Muench [20], and expressed as 50% egg infectious dose per millilitre (EID_{50}/mL).

3. Results

The serum HI antibodies against Dk/Vac-1/04 reached a maximum geometric mean (GM) titre of 1:2048 at 4 weeks after vaccination, and then gradually decreased until reaching a GM titre level of 1:111 at 138 weeks after vaccination (Table 1). The serum HI titres of 5 vaccinated chickens that died due to an accident were basically the same as those of the 5 chickens in the present results at every points of time until the time of the accident (44 weeks after vaccination, data not shown).

During the challenge test, all chickens in the vaccinated group survived for 14 days p.c. without showing any clinical signs of HPAI (Table 2). In contrast, all chickens in the non-vaccinated group showed typical HPAI symptoms 1–2 days p.c. and died within 3 days p.c.

A marked secondary antibody response was observed 14 days p.c. in serum HI titres of chickens vaccinated with Dk/Vac-1/04 and challenged with Ck/Yamaguchi/04 viruses (Table 3). In addition, $10^{1.3}$–$10^{1.7}$ EID_{50}/mL of challenge virus were recovered from the laryngopharyngeal swabs from 3 of the 5 vaccinated chicken 2 days p.c. but there was no virus recovery at 4 and 14 days p.c. However, $10^{4.5}$–$10^{7.5}$ EID_{50}/mL of the challenge virus were recovered from laryngopharyngeal and cloacal swabs of the non-vaccinated chickens.

These findings demonstrated that all of the chickens vaccinated with Dk/Vac-1/04 survived the Ck/Yamaguchi/04 challenge without exhibiting any clinical signs. Three of those chickens shed small amounts of Ck/Yamaguchi/04 virus in their laryngopharyngeal swabs, only on day 2 p.c.

Table 1
HI antibody titres against Dk/Vac-1/04 in chickens during the 138 weeks following Dk/Vac-1/04 vaccination.

Group	Chicken No.	HI antibody titres and weeks after vaccination															
		1	2	3	4	5	16	28	40	52	64	76	88	100	112	124	138
Vaccinated	2	<4	128	2048	4096	2048	1024	512	512	512	256	256	256	256	256	256	256
	3	<4	256	1024	2048	2048	2048	1024	1024	1024	512	512	512	512	256	256	128
	6	<4	128	512	1024	1024	1024	256	256	256	128	128	128	128	64	64	64
	7	<4	128	512	2048	2048	256	128	128	128	128	128	128	128	64	64	64
	8	<4	512	1024	2048	2048	1024	512	512	512	512	512	512	256	128	128	128
GM[a]		<4	194	891	2048	1783	891	388	388	388	256	256	256	223	128	128	111
Non-vaccinated	9	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	10	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	11	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	12	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
GM[a]		<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4

[a] GM: geometric mean.

Table 2
Clinical signs of influenza in vaccinated chickens after challenge with the HPAI virus Ck/Yamaguchi/04.

Group	Chicken No.	Serum HI antibody titre[a] and virus strain		Clinical signs on days following HPAI challenge													
		Dk/Vac-1/04	Ck/Yamaguchi/04	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vaccinated	2	256	256	–[b]	–	–	–	–	–	–	–	–	–	–	–	–	–
	3	128	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	6	64	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	7	64	64	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	8	128	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Non-vaccinated	9	<4	<4	–	+[b]	D[b]											
	10	<4	<4	+	D												
	11	<4	<4	–	D												
	12	<4	<4	–	+	D											

[a] HI antibody titre at the time of challenge.
[b] '–' no abnormal signs; '+': typical clinical signs (lethargy, loss of appetite and nervous symptoms); D: death.

Table 3
Antibody response and virus isolation from laryngopharyngeal and cloacal swabs of chickens after challenge with the Ck/Yamaguchi/04 virus.

Group	Chicken no.	Serum HI antibody titre and virus strain				Virus titres[a] on the following days after challenge							
		Dk/Vac-1/04		Ck/Yamaguchi/04		2		3		4		14	
		Pre[b]	Post[b]	Pre	Post	L[c]	C[c]	L	C	L	C	L	C
Vaccinated	2	256	2048	256	2048	–[d]	–	NT[d]	NT	–	–	–	–
	3	128	8192	128	8192	1.7	–	NT	NT	–	–	–	–
	6	64	512	128	512	–	–	NT	NT	–	–	–	–
	7	64	2048	64	2048	1.5	–	NT	NT	–	–	–	–
	8	128	512	128	256	1.3	–	NT	NT	–	–	–	–
Non-vaccinated	9	<4	NT	<4	NT	6.8	5.5	6.0	4.5	NT	NT	NT	NT
	10	<4	NT	<4	NT	4.5	5.5	NT	NT	NT	NT	NT	NT
	11	<4	NT	<4	NT	6.5	4.8	NT	NT	NT	NT	NT	NT
	12	<4	NT	<4	NT	7.5	5.5	4.5	4.8	NT	NT	NT	NT

[a] Virus titre expressed as $\log_{10}$ EID_{50}/mL.
[b] Pre: at the time of challenge; Post: 14 days post-challenge.
[c] L: laryngopharynx; C: cloacal.
[d] '–': Indicates a virus recovery titre lower than 0.5 $\log_{10}$ EID_{50}/mL; NT: not tested.

4. Discussion

In the present study, we demonstrated that an avian influenza test vaccine produced using an oil adjuvant containing AMOE as a surfactant induced a high level of HI antibody response in chickens, lasting as long as 138 weeks after vaccination. Oda *et al.* reported that the surfactant contained in our oil adjuvant plays a key role in stimulation of an antibody response [21]. They also described that oligosaccharide oleate ester is the most important element in the adjuvant activity of AMOE. It is, thus, considered that AMOE is a potent adjuvant equivalent to QS-21 saponin or alum adjuvants in mice.

Other elements of AMOE may play an important role in the creation of stable water-in-oil emulsions of light mineral oil. Such emulsion stability may improve and prolong the adjuvant activity of AMOE by enhancing the potency of the vaccine as a foreign substance and by capturing the antigen at the injection site. Recently, Hikida *et al.* reported that phospholipase C-gamma 2 is essential for formation and maintenance of memory B cells [22]. It is possible that the long-lasting immunological memory and the marked

antibody production following the HPAI virus challenge in the present vaccinated chickens may have been regulated through similar immunological mechanisms.

Both Dk/Vac-1/04 and Ck/Yamaguchi/04 were viruses of the Eurasian lineage, and both showed similar antigenic cross reactivity in the HI test. This suggests that the potency of a vaccine prepared from a virus strain belonging to the same lineage as the outbreak virus may be higher than that of a vaccine prepared from a virus strain belonging to a heterologous lineage strain [12,13].

To assure the safety of vaccines in humans, ether-split influenza vaccines are widely adopted for human use. We chose formalin-inactivated whole virus particles as an antigen in order to maintain efficacy while reducing the cost of the vaccine. Recently, Hagenaars *et al.* reported that whole inactivated virus particles, containing all viral components, produced the best results in potency tests as compared to split, subunit and virosomes presented through intramuscular and intranasal routes [23]. Their report supports our approach of using whole virus particles for vaccine development. Furthermore, it is anticipated that a whole virus particle vaccine should be effective against H5 viruses, even on an antigenically drifted virus [14]. Thus, whole virus particles appear to be one of the best candidates for chicken vaccine development.

The present results show that the test vaccine constructed using an apparently optimum adjuvant composition and an appropriate lineage strain of whole inactivated virus particles induces long-lasting protective immunity in chickens.

It is possible that potency of the test vaccine may be different for SPF from conventional lines of chicken. Accordingly, studies to compare the immunological response to vaccination in SPF and conventional lines of chicken are under way.

References

[1] Alexander DJ. A review of avian influenza in different bird species. Vet Microbiol 2000;74(1–2):3–13.

[2] Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79(5):2814–22.

[3] Kida H, Yanagawa R, Matsuoka Y. Duck influenza lacking evidence of disease signs and immune response. Infect Immun 1980;30(2):547–53.

[4] Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56(1):152–79.

[5] Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, et al. The evolution of H5N1 influenza viruses in ducks in southern China. Proc Natl Acad Sci USA 2004;101(28):10452–7.

[6] Ito T, Goto H, Yamamoto E, Tanaka H, Takeuchi M, Kuwayama M, et al. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol 2001;75(9):4439–43.

[7] Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR. Replication and transmission of influenza viruses in Japanese quail. Virology 2003;310(1):8–15.

[8] Perez DR, Lim W, Seiler JP, Yi G, Peiris M, Shortridge KF, et al. Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. J Virol 2003;77(5):3148–56.

[9] Webster RG, Guan Y, Peiris M, Walker D, Krauss S, Zhou NN, et al. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. J Virol 2002;76(1):118–26.

[10] WHO. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO; 2009 http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_03_11/en/index.html.

[11] Capua I, Marangon S. The use of vaccination as an option for the control of avian influenza. Avian Pathol 2003;32(4):335–43.

[12] Soda K, Sakoda Y, Isoda N, Kajihara M, Haraguchi Y, Shibuya H, et al. Development of vaccine strains of H5 and H7 influenza viruses. Jpn J Vet Res 2008;55(2–3):93–8.

[13] Bublot M, Le Gros FX, Nieddu D, Pritchard N, Mickle TR, Swayne DE. Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian influenza isolate from a chicken in Thailand. Avian Dis 2007;51(1 (Suppl.)):332–7.

[14] Isoda N, Sakoda Y, Kishida N, Soda K, Sakabe S, Sakamoto R, et al. Potency of an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 reassortant virus generated between isolates from migratory ducks in Asia. Arch Virol 2008;153(9):1685–92.

[15] Sakabe S, Sakoda Y, Haraguchi Y, Isoda N, Soda K, Takakuwa H, et al. A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus in chickens. Vaccine 2008;26(17):2127–34.

[16] Tumpey TM, Kapczynski DR, Swayne DE. Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine. Avian Dis 2004;48(1):167–76.

[17] Sasaki T, Isoda N, Soda K, Sakamoto R, Saijo K, Hagiwara J, et al. Evaluation of the potency, optimal antigen level and lasting immunity of inactivated avian influenza vaccine prepared from H5N1 virus. Jpn J Vet Res 2009;56(4):189–98.

[18] Isoda N, Sakoda Y, Kishida N, Bai GR, Matsuda K, Umemura T, et al. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch Virol 2006;151(7):1267–79.

[19] Mase M, Tsukamoto K, Imada T, Imai K, Tanimura N, Nakamura K, et al. Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. Virology 2005;332(1):167–76.

[20] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938;27:493–7.

[21] Oda K, Sato Y, Katayama S, Ito A, Ohgitani T. Separation and characterization of adjuvant oligosaccharide oleate ester derived from product mixture of mannitol-oleic acid esterification. Vaccine 2004;22(21–22):2812–21.

[22] Hikida M. PLC-gamma 2 is essential for formation and maintenance of memory B cells. J Exp Med 2009;206(3):681–9.

[23] Hagenaars N. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine 2008;26:6555–63.

Arch Virol (2009) 154:1517–1522
DOI 10.1007/s00705-009-0467-9

BRIEF REPORT

Characterization of H3N6 avian influenza virus isolated from a wild white pelican in Zambia

Edgar Simulundu · Aaron S. Mweene · Daisuke Tomabechi · Bernard M. Hang'ombe · Akihiro Ishii · Yuka Suzuki · Ichiro Nakamura · Hirofumi Sawa · Chihiro Sugimoto · Kimihito Ito · Hiroshi Kida · Lewis Saiwana · Ayato Takada

Received: 31 March 2009 / Accepted: 8 July 2009 / Published online: 5 August 2009

Abstract We characterized an influenza virus isolated from a great white pelican in Zambia. Phylogenetic analysis showed that all of its gene segments belonged to the Eurasian lineage and that they appear to have evolved in distinct geographical regions in Europe, Asia, and Africa, suggesting reassortment of virus genes maintained in wild aquatic birds whose flyways overlap across these continents. It is notable that this virus might possess some genes of the same origin as those of highly pathogenic H7 and H5 viruses isolated in Eurasia. The present study underscores the need for continued monitoring of avian influenza viruses in Eurasia and Africa.

Aquatic birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds) are thought to constitute the major natural reservoir for avian influenza (AI) A virus [20, 24]. All known influenza A virus subtypes with respect to two surface glycoproteins, hemagglutinin (HA) (H1–H16) and neuraminidase (NA) (N1–N9) and most HA/NA combinations have been identified in wild birds and poultry [11, 24]. Influenza A viruses of avian origin have been implicated in outbreaks of influenza in other hosts [13, 20, 24], indicating that a vast influenza virus gene pool for future epidemics in other animal species, including human pandemics, exists in avian sources.

Highly pathogenic (HP) H5N1 AI virus has spread from Asia to other regions, including Europe, the Middle East, and Africa, causing outbreaks in domestic poultry and wild birds [5, 10, 15]. As of 17 June 2009, Egypt had recorded the highest number of H5N1 human infections in Africa: 78 confirmed cases with 27 fatalities [25]. The origins and transmission routes of HP H5N1 virus from Asia to Africa remain unclear. The potential spread of HP H5N1 virus by wild birds over large geographical regions and the direct

Electronic supplementary material The online version of this article (doi:10.1007/s00705-009-0467-9) contains supplementary material, which is available to authorized users.

E. Simulundu · D. Tomabechi · A. Ishii · Y. Suzuki · I. Nakamura · H. Sawa · C. Sugimoto · K. Ito · H. Kida · A. Takada (✉)
Department of Global Epidemiology,
Hokkaido University Research Center for Zoonosis Control,
Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan
e-mail: atakada@czc.hokudai.ac.jp

A. S. Mweene · I. Nakamura · H. Sawa · C. Sugimoto · A. Takada
Department of Disease Control, School of Veterinary Medicine,
The University of Zambia, 32379 Lusaka, Zambia

B. M. Hang'ombe
Department of Paraclinical Studies,
School of Veterinary Medicine,
The University of Zambia, P. O. Box 32379,
Lusaka, Zambia

A. Ishii · Y. Suzuki
Hokudai Center for Zoonosis Control in Zambia,
School of Veterinary Medicine, The University of Zambia,
P. O. Box 32379, Lusaka, Zambia

H. Kida
Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

H. Kida
OIE Reference Laboratory for Highly Pathogenic Avian Influenza, Sapporo, Japan

L. Saiwana
Zambia Wildlife Authority, Kafue Road, Private Bag 1,
Chilanga, Zambia

Table 1 Antigenic characterization of Zb06 by HI and NI assays

Virus	Subtype	HI titer of chicken antiserum				Virus	Subtype	NI titer of chicken antiserum				
		Zb06	Aichi	Mem96	PR8			Zb06	Eng56	MD77	Czec56	PR8
Zb06	H3N6	**5,120**	320	<40	<40	Zb06	H3N6	**2,560**	2,560	640	320	<10
Aichi	H3N2	5,120	**10,240**	160	<40	Eng56	H11N6	1,280	**2,560**	320	320	<10
Mem96	H3N2	2,560	<40	**10,240**	<40	MD77	H13N6	5,120	2,560	**1,280**	640	<10
DHK836	H3N1	2,560	640	<40	<40	Czec56	H4N6	2,560	2,560	1,280	**1,280**	<10
PR8	H1N1	<40	<40	<40	**5,120**	PR8	H1N1	<10	<10	<10	<10	**1,280**

Homologous HI and NI titers are in boldface type and are underlined

zoonotic threat posed by several AI viruses of the Eurasian lineage underscore the need for more information on the ecology and evolution of AI A viruses circulating in the wild bird reservoir globally.

In attempting to narrow the knowledge gap that exists in the ecology of AI viruses circulating in wild birds in Africa, virologic surveillance studies were initiated in Zambian wetlands frequented by migratory birds. We report the characterization of the first influenza virus isolate from an avian host in Zambia.

In August 2006, 51 fresh fecal samples were collected from apparently healthy pelicans in Lochinvar National Park (15°40′S; 27°15′E), in Southern province of Zambia. Virus isolation was attempted in 10- to 11-day-old embryonated chicken eggs. One influenza virus isolate was obtained and designated A/pelican/Zambia/01/06 (H3N6) (Zb06) following subtyping by standard hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using specific antisera to the reference strains of influenza viruses. We then prepared chicken antisera against Zb06. Briefly, purified virus was inactivated with 0.1% formalin at 4°C for 1 week. Three-month-old specific-pathogen-free chickens were immunized intramuscularly and subcutaneously with a 100-µl suspension containing 300 µg inactivated virus with complete Freund's adjuvant (DIFCO). The chickens were re-immunized 2 weeks later in a similar way, but with incomplete Freund's adjuvant. The chickens were given a third intravenous booster injection without adjuvant 3 weeks after the second immunization. One week after the final immunization, the chickens were killed to obtain serum. We used A/Puerto Rico/8/34 (H1N1) (PR8), A/duck/Hong Kong/836/80 (H3N1) (DHK836), A/Aichi/2/68 (H3N2) (Aichi), A/Memphis/1/96 (H3N2) (Mem96), A/Czeckoslovakia/56 (H4N6) (Czec56), A/duck/England/1/56 (H11N6) (Eng56), A/gull/Maryland/704/77 (H13N6) (MD77), and Zb06 for antigenic characterization by HI and NI assays. Chicken antiserum was raised against all of these viruses except DHK836. Chicken erythrocytes (0.5%) and fetuin (CALBIOCHEM) were used in the HI and NI assays, respectively.

In antigenic analysis, chicken antiserum raised against Zb06 showed high HI and NI titers, roughly equal to those of all the H3 and N6 influenza viruses tested, including the relatively recent human strain, Mem96 (Table 1), indicating that chicken antiserum raised against Zb06 has high cross-reactivity. The reason for the high cross-reactivity is unclear, but one possibility is that antibodies raised against Zb06 predominantly recognize the conserved epitopes of the surface glycoproteins of the viruses tested. Chicken antiserum raised against Zb06 could therefore be useful in diagnosis of H3 and N6 influenza viruses. On the other hand, Zb06 did not react with chicken antisera raised against Mem96 but reacted with antiserum against Aichi (HI titers <40 and 320, respectively), confirming antigenic drift, which has been observed since this virus was first introduced into the human population [2].

For genetic analysis, viral RNA was extracted and amplified by RT-PCR as described previously [12]. PCR products were purified from agarose gels and then sequenced directly using a BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). The nucleotide sequences obtained in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB470293 to AB470300. Phylogenetic trees were constructed using the neighbor-joining bootstrap method (1,000 replicates) in MEGA4.

The entire genome of Zb06 was completely sequenced and analyzed with the basic local alignment search tool (BLAST) available from GenBank (Table 2). We found that the HA, PB2, and NS genes were highly similar (97–99%) to duck/South Africa/1108/04 (H3N8) (SAH3). The NP and PB1 genes showed 97% nucleotide similarity with H7N1 and H7N3 influenza viruses isolated from Italian poultry. The closest relative of the Zb06 M gene was duck/Mongolia/54/2001 (H5N2) (98% nucleotide similarity). The NA segment was closely related to mallard/Germany/Wv1806-09k/03 (H4N6), with 96% nucleotide identity. The PA gene showed close sequence identity (98%) to H5N3 virus, teal/Italy/3812/05.

Table 2 Influenza viruses with the highest nucleotide sequence similarity to Zb06

Gene (nucleotide positions of Zb06 compared)	Virus with highest degree of sequence identity	Subtype	Identity (%)	GenBank accession no.
HA (77–1,063)	A/duck/South Africa/1108/2004	H3N8	97	EF041487
NS (57–711)	A/duck/South Africa/1108/2004	H3N8	98	EF041491
PB2 (1,468–2,193)	A/duck/South Africa/1108/2004	H3N8	99	EF041493
NP (751–1,483)	A/turkey/Italy/3560/1999	H7N1	97	CY025168
PB1 (1,429–2,178)	A/turkey/Italy/9739/2002	H7N3	97	CY031617
M (197–868)	A/duck/Mongolia/54/2001	H5N2	98	AB301916
NA (38–1,264)	A/mallard/Germany/Wv1806-09k/2003	H4N6	96	AM933235
PA (1,456–2,149)	A/teal/Italy/3812/2005	H5N3	98	CY022650

The HA, NA, NP, and PA gene phylograms are shown in Fig. 1. Phylogenetic analysis of the HA gene of Zb06 showed the separation of the viruses into the Eurasian, American, and human-swine lineages (Fig. 1a). Sublineages 1–3 are distinguishable within the Eurasian lineage. The HA gene of Zb06 was closely related to that of SAH3, and belonged to the first sublineage, comprising viruses isolated mainly from the Far East and Europe. The much older virus, duck/Ukraine/1/63 (H3N8) and a swine isolate from Mongolia constituted the second Eurasian sublineage. The third sublineage is composed of H3N2 viruses isolated from fecal specimens collected from live poultry markets in Korea [21]. The NA gene tree of Zb06 revealed the assortment of viruses into the Eurasian, Eurasian–American, Oceania, and American lineages (Fig. 1b). Two sublineages were apparent within the Eurasian lineage: "contemporary" and 1970s and 1980s viruses (designated 1 and 2, respectively). The NA gene of Zb06 fell in the "contemporary" sublineage and was closely related to H4N6 viruses isolated from Germany and Norway. Aside from Zb06 and the two H4N6 European strains, all viruses of the "contemporary" sublineage were of Asian origin. Except for three viruses, Eng56, Czec56, and duck/Siberia/272/98 (H13N6), the Eurasian–American lineage was composed exclusively of shorebird and gull viruses isolated in America and Eurasia.

Phylograms of the internal protein genes (NP and PA) (Fig. 1c, d) of Zb06 showed the clustering of strains of the Eurasian lineage into sublineages as described previously [9]. In the NP phylogeny, four groups or sublineages are recognized. The first group consists of recent isolates from Europe, Asia and South Africa, including HP H5N1 viruses isolated from ducks and chickens in China, and from whooper swans in Japan. The NP gene of Zb06 belonged to the second group, consisting of "early" European strains represented by Dk/Potsdam/2216-4/84 (H5N6) and some recent isolates, including H7N1 Italian poultry viruses [1]. The third sublineage was composed of a single isolate, Dk/Hokkaido/120/01 (H6N2). The fourth group of the Eurasian lineage comprises 3 strains isolated from 1956 to 1961. The PA phylogram was topologically similar to that of the NP gene tree. In contrast to the NP gene, which clustered with H7N1 Italian poultry viruses, the PA gene of Zb06 was closely related to that of teal/Italy/3812/05 (H5N3) and grouped together with those of the Asian HP H5N1 viruses, suggesting a common source of the PA gene of these viruses.

The close relationship of the HA and internal (NS and PB2) protein genes (Supplementary Fig. S1) of Zb06 to those of wild bird isolates from South Africa suggests that some reassortment may have occurred within sub-Saharan Africa due to the interaction of wild birds through the intra-African flyways. Ring recoveries of water birds in southern Africa have shown that some waterbirds are migratory within southern Africa, while others show dispersal as far as central Africa [23]. Phylogenies of the NP and other internal (NS, PB1, and M) protein genes (Supplementary Fig. S1) of Zb06 showed that they were closely related to H7 influenza viruses isolated from Italian poultry in 1999, suggesting that viruses of the same origin as Zb06 may have contributed some internal protein genes to viruses that caused epidemics of AI of H7 viruses that have been observed in Europe since 1997 [1, 3, 4, 8]. Phylogenetic analysis of AI viruses isolated from wild ducks and domestic poultry in Italy revealed that the precursor H7 virus for AI in domestic poultry was introduced directly from migratory birds [4]. The close similarity of these genes of Zb06 to those of the viruses isolated in Italy leads us to speculate that these viruses may have infected their avian hosts on the Black Sea/Mediterranean flyway, which, together with the East Africa/West Asia flyway passes through Zambia. We acknowledge the need for caution in interpreting our data because only very limited sequence data from African wild birds are available in GenBank.

Until now, there has not been a report of influenza virus isolation from a great white pelican (*Pelecanus onocrotalus*). Influenza virus (H6N1) isolation from a great cormorant, a member of the order Pelecaniformes, has been

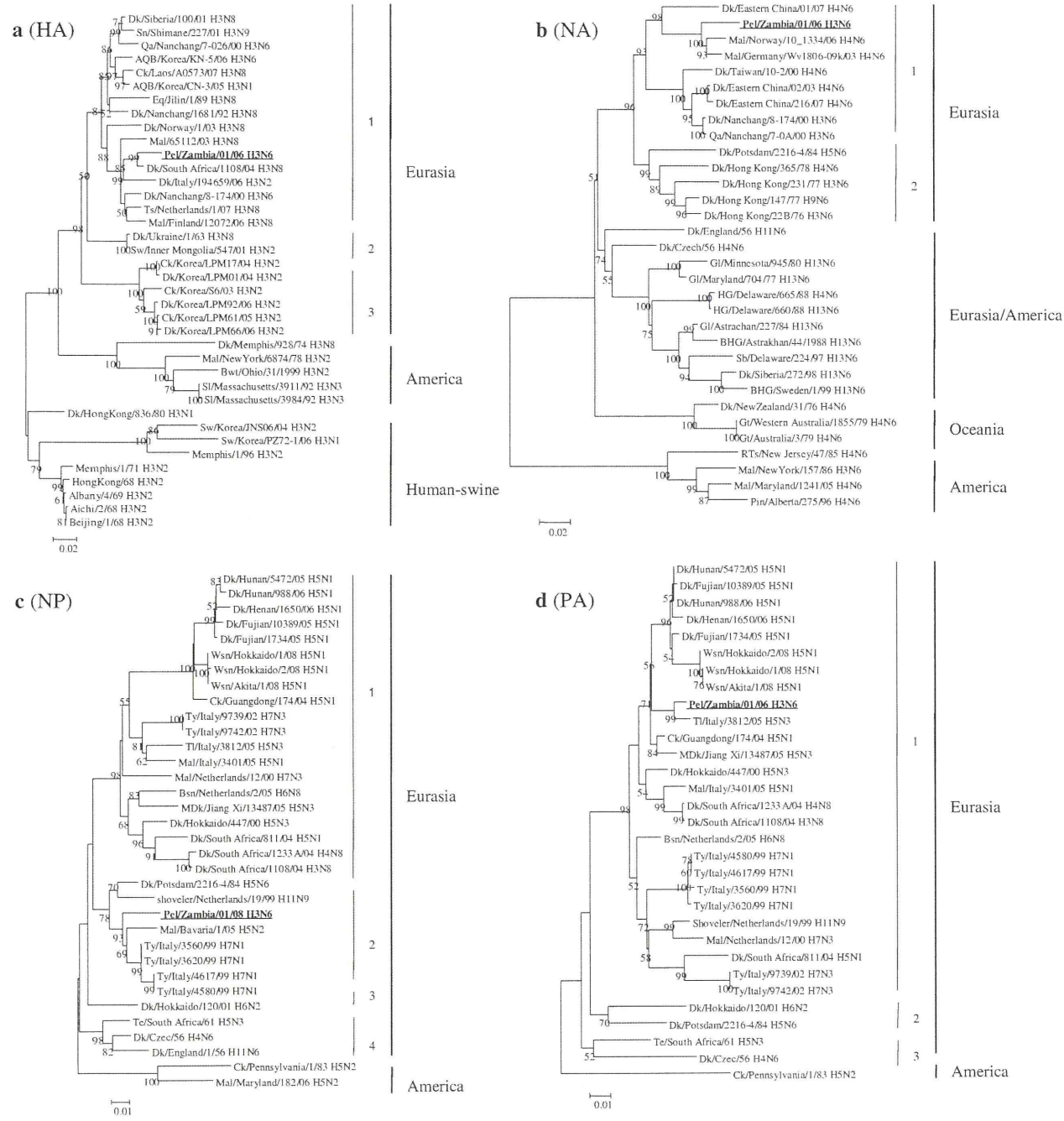

Fig. 1 Phylogenetic relationships of the HA (**a**), NA (**b**), NP (**c**), and PA (**d**) genes of Zb06. *Numbers* next to the branches indicate neighbor-joining bootstrap values of ≥50%. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The virus strain sequenced in this study is in *bold* and *underlined*. Analysis was based on the following nucleotides: HA (77–1,063), NA (38–1,264), NP (751–1,483), and PA (1,456–2,149). *AQB* aquatic bird, *BHG* black-headed gull, *Bsn* Bewick's swan, *Ck* chicken, *Dk* duck, *Eq* equine, *Gl* gull, *Gt* gray teal, *HG* herring gull, *MDk* migratory duck, *Mal* mallard, *Pel* pelican, *Pin* pintail, *Qa* quail, *RTs* ruddy turnstone, *Sb* shorebird, *Sl* seal, *Sn* swan, *Sw* swine, *Te* tern, *Ts* turnstone, *Ty* turkey, *Wsn* whooper swan

reported [22]. Other studies did not yield positive results of influenza virus isolation from this order [17, 19, 20]. The great white pelican is native to southern Africa. Limited breeding sites exist in the region, including two in South Africa and one in Namibia [6, 7]. Large colonies of white pelicans congregate in Lochinvar National Park, sharing the same habitat with other bird species in which AI viruses have been frequently isolated worldwide. The role of

"minor" bird reservoirs in influenza virus ecology is unclear. It remains to be determined in which of these species influenza viruses are endemic and in which ones the virus is a temporary pathogen [19, 20].

The available evidence suggests that the rapid spread of HP H5N1 virus from Qinghai Lake, China, to Europe and Africa may have involved migratory birds and possibly the poultry trade [14]. The close relationship of the PA gene of Zb06 to those of the Asian HP H5N1 viruses implies that wild birds could carry and spread, at least in part, genes of the same origin as those of HP AI viruses over large geographical regions. The overlap of multiple migratory flyways within Eurasia and Africa permits virus-infected birds of different bird populations to transmit pathogens to new hosts that may carry them to new areas [20].

While AI A viruses have evolved into two genetically distinct lineages, Eurasian and American, possibly due to long-term confinement of birds to distinct flyways [20, 24], transcontinental introduction of AI virus genes has been described between the two lineages [16, 18]. For instance, PB2 and PA genes of the American lineage were detected in H2 viruses isolated in Japan, and the H2 HA genes of Eurasian lineage was present in American birds. Our findings highlight that the gene segments of Zb06 appear to have been derived from multiple virus sources in Eurasia and Africa. Furthermore, our results indicate that wild waterfowl could play a role in the dissemination of genes of common origin as those of HP AI viruses over large geographical regions, thus underscoring the need for continued AI virus surveillance in Zambian wetlands as part of a global program.

Acknowledgments We thank the Zambia Wildlife Authority for supporting the wild bird influenza A surveillance program in Zambia. We also thank K. Matsuno, H. Miyamoto, A. Ohnuma, and A. Yokoyama for excellent technical assistance. This work was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References

1. Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, Cordioli P, Fioretti A, Alexander DJ (2001) Changes in the hemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 146:963–973
2. Bean WJ, Schell M, Katz J, Kawaoka Y, Neave C, Gorman O, Webster RG (1992) Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J Virol 66:1129–1138
3. Campitelli L, Di Martino A, Spagnolo D, Smith GJD, Di Trani L, Facchini M, De Marco MA, Foni E, Chiapponi C, Martin AM, Chen H, Guan Y, Delogu M, Donatelli I (2008) Molecular analysis of avian H7 influenza viruses circulating in Eurasia in 1999–2005: detection of multiple reassortant virus genotypes. J Gen Virol 89:48–59
4. Campitelli L, Mogavero E, De Marco MA, Delogu M, Puzelli S, Frezza F, Facchini M, Chiapponi C, Foni E, Cordioli P, Webby R, Barigazzi G, Webster RG, Donatelli I (2004) Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. Virology 323:24–36
5. Chen H, Smith GJD, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JSM, Guan Y (2005) H5N1 virus outbreak in migratory waterfowl. Nature 436:191–192
6. Crawford RJM, Cooper J, Dyer BM (1995) Conservation of an increasing population of great white pelicans *Pelecanus onocrotalus* in South Africa's Western Cape. S Afr J Mar Sci 15:33–42
7. Crawford RJM, Cooper J, Shelton PA (1981) The breeding population of white pelicans *Pelecanus onocrotalus* at Bird Rock Platform in Walvis Bay, 1949–1978. Fish Bull S Afr 15:67–70
8. de Marco MA, Foni E, Campitelli L, Delogu M, Raffini E, Chiapponi C, Barigazzi G, Cordioli P, di Trani L, Donatelli I (2005) Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 poultry epidemic periods (1998–2000). Avian Pathol 34:480–485
9. Duan L, Campitelli L, Fan XH, Leung YH, Vijaykrishna D, Zhang JX, Donatelli I, Delogu M, Li KS, Foni E, Chiapponi C, Wu WL, Kai H, Webster RG, Shortridge KF, Peiris JS, Smith GJ, Chen H, Guan Y (2007) Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. J Virol 81:7529–7539
10. Ducatez MF, Olinger CM, Owoade AA, De Landtsheer S, Ammerlaan W et al (2006) Avian flu: multiple introductions of H5N1 in Nigeria. Nature 442:37
11. Fouchier RAM, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus ADME (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79:2814–2822
12. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146:2275–2289
13. Horimoto T, Kawaoka Y (2001) Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev 14:129–149
14. Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP et al (2006) Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci USA 103:19368–19373
15. Li KS, Guan Y, Wang J, Smith GJD, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepangestie ATS, Chaisingh A, Auewarakul P, Long HT, Hanh NTH, Webby RJ, Poon LLM, Chen H, Shortridge KF, Yuen KY, Webster RG, Pieris JSM (2004) Genesis of highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430:209–213
16. Liu J, Okazaki K, Bai G, Shi W, Mweene A, Kida H (2004) Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. Virus Genes 29:81–86
17. Mackenzie JS, Edwards EC et al (1984) Isolation of ortho- and paramyxoviruses from wild birds in Western Australia, and the characterization of novel influenza A viruses. Aust J Exp Biol Med Sci 62(Pt 1):89–99
18. Makarova NV, Kaverin NV, Krauss S, Senne D, Webster RG (1999) Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. J Gen Virol 80:3167–3171
19. Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A et al (2007) Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 3(5):e61. doi:10.1371/journal.ppat.0030061

20. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA (2006) Global patterns of influenza A virus in wild birds. Science 312:384–388
21. Song MS, Oh TK, Moon HJ, Yoo DW, Lee EH, Lee JS, Kim CJ, Yoo GJ, Kim H, Choi YK (2008) Ecology of H3 avian influenza viruses in Korea and assessment of their pathogenic potentials. J Gen Virol 89:949–957
22. Suss J, Schafer J et al (1994) Influenza virus subtypes in aquatic birds of eastern Germany. Arch Virol 135(1–2):101–114
23. Underhill LG, Tree AJ, Oschadleus HD, Parker V (1999) Review of ring recoveries of waterbirds in Southern Africa. Avian Demography Unit, University of Cape Town, Cape Town
24. Webster RG, Bean WJ, Gorman TO, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza viruses. Microbiol Rev 56:152–179
25. WHO (2009) Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) reported to WHO. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_06_02/en/index.html. Accessed 17 June 2009