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Application of Reverse Genetics for Producing Attenuated Vaccine Strains against Highly Pathogenic Avian Influenza Viruses

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ABSTRACT. In this study, reverse genetics was applied to produce vaccine candidate strains against highly pathogenic avian influenza viruses (HPAIVs) of the H5N1 subtype. The H5 subtype vaccine strains were generated by a reverse genetics method in a biosafety level 2 facility. The strain contained the HA gene from the H5N1 subtype HPAIV attenuated by genetic modification at the cleavage site, the NA gene derived from the H5N1 subtype HPAI or the H5N3 subtype of avian influenza virus and internal genes from A/Puerto Rico/8/34. Vaccination with an inactivated recombinant virus with oil-emulsion completely protected chickens from a homologous viral challenge with a 640 HAU or 3,200 HAU/vaccination dose. Vaccination with a higher dose of antigen, 3,200 HAU, was effective at increasing survival and efficiently reduced viral shedding even when challenged by a virus of a different HA clade. The feasibility of differentiation of infected from vaccinated animals (DIVA) was demonstrated against a challenge with H5N1 HPAIVs when the recombinant H5N3 subtype viruses were used as the antigens of the vaccine. Our study demonstrated that the use of reverse genetics would be an option to promptly produce an inactivated vaccine with better matching of antigenicity to a circulating strain.
KEY WORDS: DIVA, H5N1 subtype HPAI, recombinant, reverse genetics, vaccine

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Dissemination of the H5N1 subtype of highly pathogenic avian influenza viruses (HPAIVs) among poultry and wild birds in Asia, the Middle East, Europe and Africa has caused huge economic losses by damaging commercial poultry production [1, 15]. Eradicating affected poultry flocks is the first option to control outbreaks in poultry when the outbreaks occur sporadically [2]. Detecting and destroying affected flocks as early as possible might be an effective way to contain an HPAIV at a site. An eradication approach was successful in Japan in 2004, 2007 and 2010–2011 to control outbreaks of H5N1 HPAIVs in commercially raised chickens.

Vaccination of poultry has been adopted to control outbreaks and to reduce human infection in countries where HPAIV establishes endemicity in poultry, such as China, Vietnam, Egypt and Indonesia [2]. A successful vaccination program might reduce morbidity and mortality in poultry and losses in egg production by decreasing virus excretion through the respiratory and alimentary tracts; these effects consequently reduce opportunities for transmission in poultry as well as to humans [11]. Several disadvantages of vaccination against HPAIV exist and are described as follows. First, vaccinated poultry are considered silent spreaders of the viruses when suppression of virus excretion is not completely achieved [7]. Second, the differentiation of poultry that are infected or vaccinated is difficult when they are vaccinated with commercially available oil-adjuvant inactivated whole virion vaccines [14]. In addition, immune pressure obtained by vaccination might lead to antigenic drift of the hemagglutinin (HA) protein of the virus, thereby reducing the efficacy of the vaccine when an antibody raised by vaccination fails to prevent virus replication in the body [3, 5].

Although the addition of an oil adjuvant potentiates the immunogenicity of an antigen, the antigenicity of currently available inactivated vaccines against H5 HPAIV for poultry use is not well matched with that of epidemic strains. In Japan, the first priority for preventive measures for avian influenza viral infection was swift eradication of poultry; vaccination of poultry was carried out only in cases where it would be considered difficult to inhibit spreading the infection by the eradication and movement restriction of poultry and poultry products. Several types of inactivated vaccines against H5 avian influenza viruses produced from low pathogenicity avian influenza viruses of the H5 subtype are approved and commercially available in some countries. Previous reports demonstrated that in vaccines produced from A/chicken/Hidalgo/232/1994 (H5N2) and A/turkey/Wisconsin/1968, the HA gene of both strains belongs to a genetically distinct lineage from the H5N1 subtype HPAIVs circulating in Asian countries and could not reduce viral shedding from vaccinated chickens challenged by the Asian H5N1 subtype HPAIVs [4]. The vaccine produced with a reassortant virus, obtained from a mixed inoculation of A/duck/Hokkaido/101/2004 (H5N3) and A/duck/Hokkaido/262/2004 (H6N1) in embryonated eggs, has currently been stockpiled for emergency use in Japan [9]. Because the H5N1 subtype HPAIV has been

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continuously evolving, the antigenicity of the stockpiled vaccine might not adequately match a virus entering Japan in the future. To achieve adequate efficacy of the inactivated vaccine, antigenic matching between a vaccine production strain and a circulating virus is required. For pandemic preparedness against the H5N1 subtype HPAIV in humans, viral strains for vaccine production are selected by the WHO Collaborating Centers of the Global Influenza Surveillance and Response System (GISRS) through antigenic and genetic analysis of HPAIVs. Based on the information from this monitoring system, pandemic vaccine candidates have been generated by a reverse genetics strategy to accommodate antigenicity with a circulating strain and to attenuate virulence against the embryonated eggs in which vaccine strains are propagated [1, 12]. Attenuation is achieved by modification of the HA cleavage site from the HPAIV to that of the LPAIVs. The internal genes of A/Puerto Rico/8/34 (PR8), used for reassortment to produce a vaccine strain against seasonal type A influenza virus, are utilized to ensure high viral multiplication in the embryonated eggs [12] for vaccine production. Adopting this strategy for the AI vaccine for poultry use would ensure better antigenic matching between a vaccine strain and a circulating strain, as well as a higher yield of antigens in embryonated eggs. Several experiments demonstrated that the reverse genetics-based strategy to produce human pandemic vaccine strains has been utilized to generate vaccine candidate strains for poultry use [12]. In this study, we produced vaccine strains by such a strategy to achieve an antigenic match with the circulating strains and showed that antigenic matching provided better protection against challenging with homo- and hetero-clade viruses of the Asian H5N1 HPAIVs. The vaccine strains produced in this study included the strains that possess a neuraminidase protein of the N3 subtype to practice the differentiation of infected from vaccinated animals (DIVA) system and to distinguish the anti-NA antibody raised by the challenging strains and a vaccine strain. This study demonstrates that the strategy of vaccine strain production by the reverse genetic method is applicable for use in poultry.

MATERIALS AND METHODS

Vaccines: Four recombinant viruses were generated by the reverse genetics method. Each virus was designated as MY, MM, MS or AA, based on their combination of surface antigen genes. The HA and NA segments of MM and the HA segments of MY and MS were derived from an HPAIV, A/chicken/Miyazaki/K11/2007 (Miyazaki, H5N1); the NA segment of MY was from an HPAIV, A/chicken/Yamaguchi/7/2004 (H5N1), and that of MS was from an LPAIV, A/whistling swan/Shimane/580/2002 (H5N3). The HA and NA segments of AA were from an HPAIV, A/whooper swan/Akita/1/2008 (Akita, H5N1). Multiple basic amino acids at the cleavage site of the HA gene segment were replaced with the motif of a low pathogenic H5 AIV, RETR, based on the published sequence (Accession No. DQ999887) for attenuation. The internal gene segments of those viruses were from PR8. All of the genes of those segments were inserted into the pHW2000 plasmid provided by Dr. Erich Hoffmann of St. Jude Children's Research Hospital. Eight plasmids, with the insertion of PB2, PB1, PA, HA, NP, NA, M and NS genes into pHW2000, were simultaneously transfected to 293T cells in opti-MEM (Life Technologies Corporation, Van Allen Way Carlsbad, CA, U.S.A.) by TransIT-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI, U.S.A.). Forty-eight hours post transfection, the supernatant of the 293T cells was transferred and cultured in Madin-Darby canine kidney (MDCK) cells with 5 μg/m*l* of acetylated trypsin to propagate the viruses. The viruses were cultured in 10-day-old embryonated eggs to produce the antigen for inactivation. The infectious allantoic fluid was inactivated at 4°C adding 0.01% (vol/vol) formaldehyde for 2 weeks, and the inactivation of the virus was confirmed by inoculation to 10-day-old embryonated eggs twice. The inactivated virus was purified by ultra-centrifuging at 27,000 rpm for 2 hr at 4°C through 25 and 70% of sucrose discontinuous gradient. A hemagglutinating assay with 0.5% chicken red blood cells was performed to adjust the virus content in the vaccine. The inactivated viral antigen was emulsified by incomplete Freund's adjuvant to generate the experimental vaccines.

Viruses: Four H5N1 subtype HPAIVs were used for the challenge in the vaccinated chickens, namely A/chicken/Miyazaki/K11/200 (clade 2.2), A/chicken/Shimane/1/2010 (Shimane, clade 2.3.2.1), A chicken/Pyigyitagon/204/2006 (Pyigyitagon, clade 7) and A/whooper swan/Akita/1/2008 (clade 2.3.2.1). The HPAIVs were cultured in 10-day-old embryonated eggs and adjusted to 6 $\log_{10}EID_{50}$/chicken with PBS for the challenge. The cross reactivity among the 4 viruses used for the challenge was examined with the antiserum to each virus by the hemagglutinin inhibition (HI) test according to the WHO manual on animal influenza diagnosis and surveillance [13].

Animal experiment: Four-week-old specific pathogen-free (SPF) white leghorn chickens, L-M-6 strain (Nisseiken Co., Ltd., Tokyo, Japan), were inoculated with MM, MS or AA vaccines in the thigh muscles. Two weeks after inoculation with the vaccines, the chickens in each vaccinated group and the non-vaccinated control group were challenged intranasally with 6 $\log_{10}EID_{50}$/chicken of each H5N1 HPAIV subtype. The viral challenge was carried out in biosafety level 3 facilities at the National Institute of Animal Health, Japan, and was approved by the ethics committee of the institute.

Virus titration: The tracheal and cloacal swabs taken at 3, 5, 7 and 10 days after the viral challenge or at death were collected and dipped into 2.0 m*l* of minimum essential medium (MEM) containing 0.5% bovine serum albumin (BSA), 25 g/m*l* of Fungizone, 1,000 units/m*l* of penicillin, 1,000 g/m*l* of streptomycin, 0.01 M HEPES and 8.8 mg/m*l* NaHCO3. The swabs were removed from the MEM and stored at −80°C until titration. The frozen samples were thawed and centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was subjected to viral titration by EID_{50} using the Reed and Muench method [6].

Detection of antibodies: The chicken serum was collected at pre-vaccination, 14 days post-vaccination and at the end of the observation period 10 days from the viral challenge

Table 1. Prechallenge HI titers induced by vaccinations against three antigens

Vaccine	Antigen				
	MM*		MS		AA
	640 HAU	3,200 HAU	640 HAU	3,200 HAU	3,200 HAU
MM*	7/10** (12.31)***	7/8 (23.78)	3/10 (7.58)	7/8 (23.78)	1/8 (5.45)
MS	12/12 (14.98)	6/8 (14.14)	5/12 (7.07)	6/8 (16.82)	0/8 (<10)
AA	-	0/8 (<10)	-	0/8 (<10)	5/8 (10)

*MM: Both HA and NA segments are derived from strain Miyazaki, H5N1 (clade 2.2). MS: HA segment is derived from strain Miyazaki, H5N1, and NA is from strain Shimane, H5N3 (clade 2.3.2.1). AA: Both HA and NA segments are derived from strain Akita, H5N1 (clade 2.3.2.1). **Number of positive sera/total. ***Geometric mean HI titer in $\log_2$.

Table 2. Survival rate and antibody positive rate before and after viral challenge of vaccinated chickens

Vaccine	Challenged Virus	Survival rate		Antibody positive rate to vaccinated antigen before challenge		Antibody positive rate to vaccinated antigen after challenge		Antibody positive rate to challenged virus after challenge	
		640 HAU	3,200 HAU	640 HAU	3,200 HAU	640 HAU	3,200 HAU	640 HAU	3,200 HAU
MM*	Ck/Miyazaki/K11/2007	4/4**	4/4	2/4***	3/4	3/4	4/4	3/4	4/4
	Ck/Shimane/1/2010	2/3	4/4	3/3	4/4	2/2	4/4	0/2	1/4
	Ck/Pyigyitagon/204/2006	2/3	-	2/3	-	2/2	-	2/2	-
MS	Ck/Miyazaki/K11/2007	4/4	4/4	2/4	2/4	4/4	4/4	4/4	4/4
	Ck/Shimane/1/2010	3/4	4/4	2/4	4/4	2/3	4/4	1/3	3/4
	Ck/Pyigyitagon/204/2006	3/4	-	1/4	-	3/3	-	2/3	-
AA	Whooper swan/Akita/1/2008	-	4/4	-	2/4	-	4/4	-	4/4
	Ck/Shimane/1/2010	-	4/4	-	3/4	-	4/4	-	3/4

*MM, MS and AA abbreviated strains used for HI tests and vaccination as described in Table 1. **Numbers of chickens surviving/total. ***Number of positive sera/total.

to observe the increase in antibodies against the vaccine antigen and challenged viruses. The detection of antibodies against HA and NA was examined by the HI and neuraminidase inhibition (NI) tests according to the WHO manual on animal influenza diagnosis and surveillance [13]. HI and NI titers equal to or more than 10 were judged as positive.

RESULTS

As a preliminary study, we examined the replication ability and the genetic stability of the MY generated by reverse genetics, after passages in embryonated eggs 5 times. The MY was able to efficiently multiply in the embryonated eggs throughout the passages; the yield of the strain reached $10^{8.2}$ EID_{50}/ml at the fifth passage. No substitution in the HA gene was found in the recombinant after the passages; thus, the possibility of reversion to highly pathogenic characteristics and antigenic drift from the original strain was negligible (data not shown).

The pathogenicity of a vaccine candidate strain generated by the same method, MM, for chickens was examined by the intravenous pathogenicity index (IVPI) test described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by the World Organization for Animal Health (OIE). This test was performed to ensure that the removal of multiple basic amino acids at the HA cleavage site abolishes the pathogenicity of the H5 strain. All of the chickens survived without showing any clinical signs of intravenous inoculation of the MM virus, confirming that recombinant viruses generated by this method were well attenuated (data not shown).

The immunogenicity of the vaccine candidates produced by reverse genetics was examined by hemagglutination inhibition (HI) tests of the chicken serum immunized with the experimental formalin-inactivated adjuvant vaccines with different antigen contents. It was demonstrated that the quantity of HAU affected the immunogenicity of the vaccines. The sera obtained from the chickens vaccinated with 3,200 HAU of MM and MS showed HI titers approximately twice as high as those of the lower dose vaccinations, 640 HAU, when the HI titers against the homologous antigens were compared (Table 1). In the 3,200 HAU of MM vaccination group, more than a three-fold increase in the HI titer against the heterologous, MS, antigen was seen, whereas those of the MS vaccination groups of 640 HAU and 3,200 HAU doses were similar.

The efficacy of the vaccines was demonstrated, because all of the chickens vaccinated with the vaccines survived challenges by the viruses possessing homologous HA proteins to the vaccine antigens (homologous challenge) (Table 2). The antibody positive rates as well as the HI titers against the vaccinated antigen were increased after the homologous challenges. The increase in the HI titers against the homologous antigens was much higher in the chickens immunized

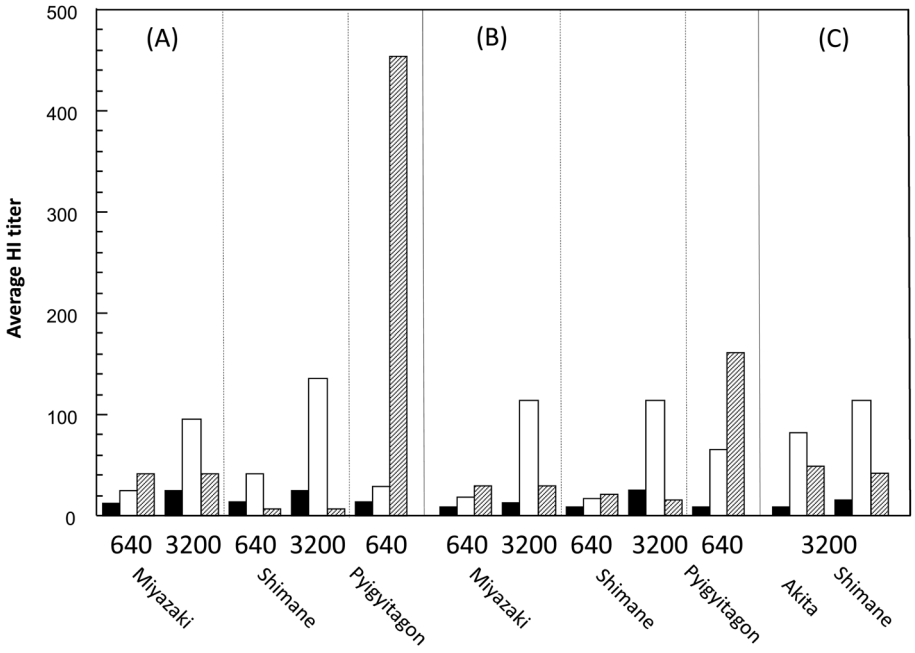

Fig. 1. The HI titers against the challenged viruses in the serum of chickens vaccinated with 640 or 3,200 HAU of MM (A), MS (B) and AA (C). MM, MS and AA were abbreviated as described in Materials and Methods. The black, white and slashed box represented the geometric means of the HI titers against the vaccine antigen before the challenge, against the vaccine antigen after the challenge and against the challenging virus after the challenge, respectively. The upper line of footnotes at the X-axis represents the amount of vaccine antigen (HAU/dose), and the lower line represents the abbreviated names of the challenging viruses as described in Materials and Methods.

with the 3,200 HAU dosage of the vaccines.

When the immunized chickens were challenged by the viruses possessing heterologous HA proteins to the vaccine antigens, which demonstrated cross-reactivity as a result in the HI test shown in Table 3, protection from the challenge was not complete in the 640 HAU immunized groups, whereas the survival rate of the heterologous challenges of the 3,200 HAU immunized groups was 100%. The HI titers against the vaccinated antigens increased after heterologous challenges in the 3,200 and 640 HAU vaccinated chickens (Fig. 1), such as an increase of the HI titers was more apparent in the 3,200 HAU vaccinated groups than in the 640 HAU vaccinated groups. These results indicated that an inadequate quantity of an antigen did not induce immunity to completely protect against a heterologous viral challenge. In addition, reduction of the HI titers against the challenged viral antigens was observed in the chickens vaccinated with 3,200 HAU antigens; this effect was the opposite of that in chickens with 640 HAU antigens, except for a group of chickens immunized by 640 HAU MM and then challenged by Shimane.

All of the chickens died after the challenge in the 640 HAU vaccinated group and, in the trachea swab, excreted virus at levels similar to the average titers of the control group; virus excretion was also observed in the cloaca swabs of the dead chickens (Table 4). A certain amount of virus excretion was detected in the surviving chickens in the 640 HAU vaccinated group after the homologous and heterologous viral challenges. The range of viral titers in the trachea and cloaca swabs was 0.33–2.33 $\log EID_{50}$/m*l*, which was lower than that of the controls. Although all of the chickens vaccinated with 3,200 HAU survived the homologous and heterologous challenges, replication of the virus was detected in some chickens in each challenge group. The viral titers detected from the surviving chickens were in a range of 0.3 to 0.6 $\log EID_{50}$/m*l*. This result showed that the 3,200 HAU vaccine could inhibit viral replication more efficiently than could the 640 HAU vaccine in chickens.

The feasibility of DIVA by detecting the NI antibody was examined in the antiserum collected from the chickens immunized with the 3,200 HAU vaccine. The anti-neuraminidase antibodies were measured in the serum collected from the chickens immunized with MM and MS and challenged with Miyazaki or Shimane. The MM possessing N1 NA and the MS possessing N3 NA as well as the challenging strains were used for the antigens of the conventional NI test. The result of the NI test indicated that specific antibodies against the N3 subtype were detected from all of the chickens immunized with the MS vaccine before and after the viral challenge (Table 5). In the MS-vaccinated groups, the N3 antibody and the N1 antibody were detected after the challenge by the H5N1 subtype virus. This finding indicated that the N3 antibody was induced by the MS vaccine and that the N1 antibody caused a viral infection. This strategy could

Table 3. The cross reactivity of challenged viral antigen and antibodies against challanted virus by HI test

Strain	Clade	Hyper immuned antiserum			
		Miyazaki*	Shimane	Pyigyitagon	Akita RG**
Ck/Miyazaki/K11/07	2.2	1,280	40	160	80
Ck/Shimane/1/10	2.3.2.1	320	2,560	80	1,280
Ck/Pyigyitagon/204/06	7	640	<10	640	640
Whooper swan/Akita/1/08	2.3.2.1	320	640	80	1,280
Whooper swan/Akita/1/08 RG**		1,280	2,560	160	2,560

*Abbreviation of strain names Miyazaki: Ck/Miyazaki/K11/2007, Shimane: Ck/Shimane/1/2010, Pyigyitagon: Ck/Pyigyitagon/204/2006 and Akita: Whooper swan/Akita/1/2008. ** This virus was generated by reverse genetics methods, and antibodies against Akita RG were produced.

Table 4. Viral replication in the respiratory or intestinal tract after viral challenge

(a) 640 HA

Vaccine	Challenge strains	Total chicken number	Viral replication							
			Trachea				Cloaca			
			Surviving	Average of $logEID_{50}/ml$	Dead	Average of $logEID_{50}/ml$	Surviving	Average of $logEID_{50}/ml$	Dead	Average of $logEID_{50}/ml$
Control	Ck/Miyazaki/K11/2007	3	0/0**	-***	3/3**	2.73****	0/0	-	2/3	1.99
	Ck/Shimane/1/2010	4	0/0	-	4/4	3.24	0/0	-	1/4	3.33
	Ck/Pyigyitagon/204/2006	4	0/0	-	4/4	4.71	0/0	-	4/4	4.31
MM*	Ck/Miyazaki/K11/2007	4	1/4	0.53	0/0	-	0/4	-	0/0	-
	Ck/Shimane/1/2010	3	0/2	-	1/1	3.38	0/2	-	1/1	1.08
	Ck/Pyigyitagon/204/2006	3	0/2	-	1/1	4.53	1/2	2.33	1/1	3.87
MS	Ck/Miyazaki/K11/2007	4	0/4	-	0/0	-	1/4	2.08	0/0	-
	Ck/Shimane/1/2010	4	1/3	1.53	1/1	5.2	1/3	1.87	1/1	2.08
	Ck/Pyigyitagon/204/2006	4	1/3	0.33	1/1	4.2	1/3	3.7	1/1	1.2

(b) 3,200 HA

Vaccine	Challenge strains	Total chicken number	Viral replication							
			Trachea				Cloaca			
			Survived	Average of $logEID_{50}/ml$	Dead	Average of $logEID_{50}/ml$	Survived	Average of $logEID_{50}/ml$	Dead	Average of $logEID_{50}/ml$
Control	Ck/Miyazaki/K11/2007	4	0/0	-	4/4	5.4	0/0	-	4/4	2.9
	Ck/Shimane/1/2010	4	0/0	-	4/4	4.3	0/0	-	4/4	1.6
	Whooper swan/Akita/1/2008	4	0/0	-	4/4	4.9	0/0	-	4/4	2.3
MM	Ck/Miyazaki/K11/2007	4	0/4	-	0/0	-	2/4	0.3	0/0	-
	Ck/Shimane/1/2010	4	0/4	-	0/0	-	2/4	0.5	0/0	-
MS	Ck/Miyazaki/K11/2007	4	2/4	0.3	0/0	-	0/4	-	0/0	-
	Ck/Shimane/1/2010	4	1/4	0.6	0/0	-	0/4	-	0/0	-
AA	Whooper swan/Akita/1/2008	4	1/4	0.3	0/0	-	0/4	-	0/0	-
	Ck/Shimane/1/2010	4	1/4	0.3	0/0	-	0/4	-	0/0	-

*MM, MS and AA abbreviated strains used for HI tests and vaccination as described in Table 1. **Numbers of chickens shedding virus/numbers of chickens surviving or dead. ***Viral titer was below detection limit. ****The highest viral titer in trachea or cloaca during observation period was used for calculation of geometric means of the titers in this Table.

distinguish between vaccination and infection and would be useful for DIVA.

DISCUSSION

Antigenic differences between epidemic strains and a vaccine strain could result in vaccine failure, leading to lethal consequences for vaccinated poultry or survival with a shedding virus. Consequently, this situation would lead to an endemic and economic loss in the poultry industry and could cause human infection by the virus. As the result of constant evolution, the HPAIVs of the H5N1 subtype are classified into 12 clades based on the phylogenetic analysis of the HA gene by the WHO/OIE/FAO in 2011. The antigenicity of the viruses has changed with genetic evolution. Antigenic profiling of H5N1 HPAIV in Asia from 2002 to 2007 with monoclonal antibodies showed that seven clades of the H5N1 subtype HPAIVs were distinguished into four

Table 5. NI test of vaccinated chicken serum before and after viral challenge

Vaccine	Challenge strains	Positive rate of anti-viral antibodies before challenge				Positive rate of anti-viral antibodies after challenge			
		vs. MM (N1) # of chickens	vs. MS (N3) # of chickens	vs. Miyazaki (N1) # of chickens	vs. Shimane (N1) # of chickens	vs. MM (N1) # of chickens	vs. MS (N3) # of chickens	vs. Miyazaki (N1) # of chickens	vs. Shimane (N1) # of chickens
MM*	Ck/Miyazaki/K11/2007	1/4**	0/4	1/4	0/4	3/4	0/4	3/4	0/4
MM	Ck/Shimane/1/2010	0/4	0/4	0/4	0/4	4/4	0/4	0/4	4/4
MS	Ck/Miyazaki/K11/2007	0/4	4/4	0/4	0/4	0/4	4/4	0/4	0/4
MS	Ck/Shimane/1/2010	0/4	4/4	0/4	0/4	0/4	4/4	0/4	3/4

*MM, MS, Miyazaki and Shimane abbreviated strains used for NI tests and vaccination as described in Tables 1 and 3. **Number of positive sera/total.

antigenic groups [16]. Thus, it is necessary to update a vaccine strain to accommodate an endemic strain to avoid vaccine failure. Embryonated eggs are conventionally used as a substrate to yield vaccine antigens for avian influenza. It would be difficult to obtain a good quantity of an antigen, if a vaccine strain was an HPAIV because of its lethality to embryonated eggs. The reverse genetics method described in this study enables us to immediately produce a vaccine strain that adequately fits the antigenicity of an endemic strain in BSL2 facilities when a strain with novel antigenicity emerges.

Problems with the antigenic differences between the vaccine and epidemic strains could be resolved by producing recombinant viruses via the reverse genetics method each time; however, frequent changes in the vaccine strain would require a significant amount of time and expense. In this study, we demonstrated that the efficacy of the vaccine, determined by the survivability of the chickens and the decrease in the viral load after the viral challenge, could be improved by the quantity of the antigen incorporated in a vaccine. Vaccination at the dose of 640 HA did not completely protect the chickens from viral infection by a heterologous viral challenge, whereas a dose of 3,200 HA protected all of the chickens from death by the viral challenge, irrespective of the different viral clades. Although the vaccinations at the dose of 3,200 HA did not completely prevent virus replication, these vaccinations reduced viral replication significantly compared to the 640 HA vaccinations. The homology of the HA amino acids among Miyazaki, Pyigyitagon and Shimane that were classified into different clades in the phylogenetic tree was more than 93.2%. Several amino acid substitutions were observed between Phigyitagon and Shimane against Miyazaki in the antigenic HA sites. Compared to the antigenic sites of Miyazaki HA, there are 7 aa substitutions, as follows: 4 in site A, 2 in site B and 1 in site E of the HA of Pyigyitagon as well as 4 substitutions in the HA of Shimane, including 2 in site A and 1 each in sites B and E. The chickens vaccinated with 640 HAU of MM and MS that were challenged by the homologous Miyazaki virus shed fewer viruses than did the chickens challenged by the heterologous viruses. Although the number of HA amino acid substitutions of Pyigyitagon against Miyazaki was more than those of Shimane, the difference in the viral load was not seen between the chickens challenged with viruses. In this study, the antibodies raised to common amino acids at the antigenic sites among the challenged viruses appeared to serve as protective antibodies for the viruses of different clades.

Several DIVA strategies have been proposed and utilized for the usage of AI vaccines [10]. Unvaccinated sentinel birds are cohabited in a vaccinated flock, and whether they acquire anti-influenza virus antibodies could be monitored. Subunit vaccines, vectored vaccines and vaccines using protein expressed in a protein expression system are useful for DIVA. Birds inoculated with a fowl pox-vectored recombinant vaccine that expresses influenza viral HA protein would not develop antibodies to the influenza viral internal protein, MA or NP, whereas birds infected with the influenza virus would produce antibodies to those proteins even if they survive the infection by the vaccination. Detecting antibodies to influenza nonstructural protein 1 (NS1) is described as an alternative DIVA strategy. An antibody against the NS1 protein is only detected in animals infected with an influenza virus, because inactivated virus in a vaccine does not contain the NS1 protein. A heteroserological neuraminidase strategy for DIVA has been used for outbreak cases in Italy. Vaccines containing the H7N3 subtype avian influenza virus were used against outbreaks caused by the H7N1 subtype virus in 2000. For the outbreak caused by the H7N3 subtype avian influenza virus in 2002 to 2003, the H7N1 subtype avian influenza viral vaccine was used [1]. Currently, fluorescent 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt is available as a substrate for the micro-NI assay to allow the examination of more samples at a time than the authentic NI assay [4]. As such, this strategy is useful as a countermeasure when the subtype of HA and NA of the epidemic virus is known. Our study confirmed the feasibility of a heteroserological neuraminidase strategy as a DIVA in the event of HPAI and showed that the application of reverse genetics had an advantage in combining the HA protein that matches the antigenicity and the NA protein that differs in the subtype of an epidemic strain.

Our study demonstrated that the obstacles regarding the usage of inactivated whole virion vaccines against HPAIV could be overcome by the application of reverse genetics. Recently, Shichinohe *et al.* reported that compared to a vaccine prepared from low pathogenicity H5 avian influenza virus, a vaccine made by a reverse genetics-based strategy similar to ours provided better protection in terms of virus secretion [8]. This vaccine strategy provides a good quantity of a vaccine antigen and proper matching between a vaccine antigen and a circulating strain, resulting in better vaccine

efficacy and allowing a DIVA strategy when a particular circulating strain is targeted. Reverse genetics allows us to promptly produce a vaccine production strain against a circulating HPAIV that constantly evolves until an innovative vaccine-producing strategy is established.

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