NOTE *Avian Pathology*

Protective efficacy of stockpiled vaccine against H5N8 highly pathogenic avian influenza virus isolated from a chicken in Kumamoto prefecture, Japan, in 2014

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ABSTRACT. H5 highly pathogenic avian influenza (HPAI) viruses have spread worldwide, and antigenic variants of different clades have been selected. In this study, the national stockpiled vaccine prepared from A/duck/Hokkaido/Vac-1/2004 (H5N1) strain was evaluated for the protective efficacy against H5N8 HPAI virus isolated in Kumamoto prefecture, Japan, in April 2014. In the challenge test, all of the vaccinated chickens survived without showing any clinical signs and reduced virus shedding. It was concluded that the present stockpiled vaccine was effective against the H5N8 HPAI virus.

KEY WORDS: disease control, efficacy, highly pathogenic avian influenza, low pathogenic avian influenza, vaccine

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H5 highly pathogenic avian influenza (HPAI) viruses have been isolated continuously, have spread worldwide to 64 countries/regions (http://www.fao.org/docrep/015/an333e/an333e00.pdf) and have been undergoing antigenic drift, evading vaccine protection [1–3]. There was an outbreak of HPAI caused by H5N8 HPAI virus in Kumamoto prefecture, located in the southern part of Japan in April 2014 [7]. By stamping out procedures, the HPAI was successfully eradicated with minimum damages. However, in the case of consecutive outbreaks of HPAI, avian influenza (AI) vaccines may be applied as an optional tool in addition to stamping out to contain the spread.

Ministry of Agriculture, Forestry and Fisheries (MAFF), therefore, stocks AI vaccines for such an emergency case. Because of the spread of antigenically different H5 HPAI viruses due to the use of vaccines in some countries, such as China, Indonesia, Vietnam and Egypt, it is concerned that AI vaccine prepared from a low pathogenic AI virus isolated from a feral water bird may not be effective against present antigenic variant HPAI viruses prevailing in poultry population in Asia [1–3].

National Veterinary Assay Laboratory (NVAL), MAFF established a vaccine strain selection committee for veterinary influenza vaccines (the selection committee) [4]. The main agenda involves determining whether AI and equine influenza vaccine strains that are currently in use need to be updated and selecting the most appropriate vaccine strains. In this study, the authors evaluated the protective efficacy of the stockpiled AI vaccine against H5N8 HPAI virus isolated in Kumamoto prefecture, Japan, in April 2014.

A commercial inactivated AI vaccine (oil adjuvant added) (Kaketsuken, Kumamoto, Japan) prepared from A/duck/Hokkaido/Vac-1/2004 (H5N1) strain (Vac-1 strain) [6, 8, 12–14, 16] which was purchased and stocked by MAFF was used in this study. A/chicken/Kumamoto/1-7/2014 (H5N8) strain (Kumamoto strain) [7] which was isolated from a dead chicken in Kumamoto prefecture in April 2014 and kindly provided by the National Institute of Animal Health (Tsukuba, Japan) was used in a challenge protection test. Furthermore, A/duck/Hokkaido/Vac-3/2007 (H5N1) strain [15, 16] which is one of the Japanese vaccine strains that shows similar antigenic characters to Vac-1 strain, A/chicken/Yamaguchi/7/2004 (H5N1) strain [8], A/whooper swan/Hokkaido/1/2008 (H5N1) strain [9], A/whooper swan/Hokkaido/4/2011 (H5N1) strain [11], A/peregrine falcon/Aomori/7/2011 (H5N1) strain [11] and Vac-1 strain were used as the hemagglutination (HA) antigens in hemagglutination inhibition (HI) test. These viruses were inoculated and propagated in the allantoic cavity of 10-day-old SPF embryonated chicken eggs, and the allantoic fluids were used as HA antigens or a challenge virus. In order to determine 50% chicken lethal dose (CLD_{50}) of Kumamoto strain, 4 groups of four 7-week-old chickens were challenged intranasally with serial dilution (10^4, 10^5, 10^6 and 10^7) of the 50% egg infectious dose (EID_{50}) of Kumamoto strain. Chickens were

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Table 1. Antigenic analysis of H5 avian influenza viruses using antisera against Vac-1 strain

Virus	HI titers with antiserum to Vac-1
A/duck/Hokkaido/Vac-1/2004	2,048
A/duck/Hokkaido/Vac-3/2007	2,048
A/chicken/Kumamoto/1-7/2014	8
A/whooper swan/Hokkaido/1/2008	256
A/whooper swan/Hokkaido/4/2011	128
A/chicken/Yamaguchi/7/2004	2,048
A/peregrine falcon/Aomori/7/2011	128

observed for disease manifestation and mortality for a period of two weeks. The titer of CLD_{50} was calculated by the method of Reed and Muench [10].

The challenge protection test was performed as follows; chickens were divided into three groups (a test group composed of 10 chickens, a positive control group composed of 2 chickens and a negative control group composed of 3 chickens). The test group and the positive control group were vaccinated intramuscularly in the lower thigh with 0.5 m*l*/chicken of the AI vaccine, according to the manufacturer's recommendation. The test group and the negative control group were challenged with 100 CLD_{50} ($10^{7.8}$ EID_{50}) of Kumamoto strain in a volume of 0.5 m*l* by intranasal route at 5 weeks after the vaccination. Disease manifestations were observed for 14 days after the challenge. Swabs were individually collected from both cloaca and laryngopharynx on the 2 and 6 days after the challenge for the detection of virus shedding. The swabs were individually mixed in 1.0 m*l* of PBS with antibiotics. As primary screening, a 0.1 m*l* of each swab was inoculated into an allantoic cavity of 10-day-old SPF embryonated chicken egg and incubated at 34°C for 48 hr. The allantoic fluids showing typical HA activity were regarded as positive virus growth. Then, the positive swabs were serially diluted tenfold, and serial dilutions (10^1–10^6) were inoculated into allantoic cavities of 10-day-old SPF embryonated chicken eggs and incubated at 34°C for 48 hr to calculate virus titers by HA. The virus titers were calculated by the method of Reed and Muench [10]. The experiment was carried out in a BSL3 facility and was approved by NVAL animal ethics committee (approval number: 25-014). The HI test was performed according to the potency test of AI vaccine described in the Minimum Requirements for Veterinary Biological Products [6, 14].

The HI antibody titers of hyper-immune antisera against Vac-1 strain were 1:2,048 and 1:8 with Vac-1 strain and Kumamoto strain, respectively (Table 1). Kumamoto strain showed lower HI titers compared with the previously isolated field strains, such as A/whooper swan/Hokkaido/4/2011 (H5N1) and A/peregrine falcon/Aomori/7/2011 (H5N1).

Disease manifestations after challenge are shown in Table 2. All the vaccinated chickens were completely protected from disease manifestations and death. On the other hand, all the chickens in the negative control showed gloom on the 4th or 5th day and died on the next day. As shown in Table 3, the challenge virus was not recovered from any of the swabs from vaccinated chickens, while $10^{5.8}$–$10^{6.8}$ EID_{50}/m*l* and $10^{2.5}$–$10^{4.5}$ EID_{50}/m*l* of the challenge virus were recovered from swabs of cloaca and laryngopharynx, respectively, of all chickens in the negative control group at the time of death, the second day after challenge. All the vaccinated chickens developed high HI titers (1:256–512) against the vaccine strain prior to the challenge, but showed substantially lower HI titers against Kumamoto strain (1:<4–8). Two weeks after challenge, a 2–8 fold increase in HI titers against Kumamoto strain was observed in all the vaccinated chickens, while no increase in HI titers against the vaccine strain was observed in most of the vaccinated chickens (Table 4). These data indicated the infection of the virus occurred in the vaccinated chickens.

In this study, the challenge protection test using Kumamoto strain indicated that the present stockpiled AI vaccine (Vac-1 strain) induced sufficient immunity for preventing disease manifestations and reducing virus shedding; however, im-

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

Group	Chicken No.	Clinical signs on days after challenge													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vaccine	605	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	611	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	613	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	615	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	617	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	619	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	624	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	629	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	635	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
	638	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-
Negative control	610	-	-	+	D										
	618	-	-	-	+	D									
	619	-	-	-	+	D									

-: No abnormal clinical signs, +: Abnormal clinical signs, D: Death, NT; Not tested.

Table 3. Virus isolation from cloaca and laryngopharynx swabs of chickens after challenge with a highly pathogenic avian influenza virus, Kumamoto strain

Group	Chicken No.	Virus titers on the days after challenge ($log_{10}EID_{50}/ml$)			
		2[a]		6[a]	
		C	L	C	L
Vaccine	605	-	-	-	-
	611	-	-	-	-
	613	-	-	-	-
	615	-	-	-	-
	617	-	-	-	-
	619	-	-	-	-
	624	-	-	-	-
	629	-	-	-	-
	635	-	-	-	-
	638	-	-	-	-
Negative control	610[b]	3.0	-	NT	4.5
	618[c]	3.5	-	6.8	3.0
	619[c]	2.8	-	5.8	2.5

C; Cloaca swab, L; Laryngopharynx swab, NT; Not tested, -; The titer is below the limit of detection (0.5), a) Days after challenge, b) Dead on 4th day, c) Dead on 5th day.

Table 4. HI titers of chicken sera at 0 and 14 days after the challenge with Kumamoto strain

Group	Chicken No.	HI titers on the days after challenge			
		0[a]		14[a]	
		Vac-1[b]	Kum/14[c]	Vac-1	Kum/14
Vaccine	605	256	<4	256	8
	611	256	<4	256	16
	613	256	4	256	16
	615	256	8	256	16
	617	256	<4	256	8
	619	512	<4	512	4
	624	256	8	256	8
	629	256	<4	512	8
	635	512	4	512	8
	638	256	<4	256	4
Negative control[d]	610	<4	<4	NT	NT
	618	<4	<4	NT	NT
	619	<4	<4	NT	NT
Positive control[e]	600	256	<4	256	<4
	606	256	4	256	4

NT; Not tested, a) Days of bleeding after challenge, b) A/duck/Hokkaido/Vac-1/2004 (H5N1), c) A/chicken/Kumamoto/1-7/2014 (H5N8), d) Not vaccinated and challenged., e) Vaccinated and not challenged.

munity sufficient to defend infection was not induced. The selection committee has determined that if the survival rate of vaccinated chickens is equal to or more than 80% following challenge with field strains [5], the AI vaccine should be regarded as effective against the field strain, and the AI vaccine strain needs not to be changed. Therefore, we concluded that the present stockpiled vaccine (Vac-1 strain) is effective against Kumamoto strain. Although the challenge test was not conducted, A/duck/Hokkaido/Vac-3/2007 (H5N1) strain (Vac-3 strain) which shows very similar antigenic characters to Vac-1 strain [15, 16] is considered to be effective against Kumamoto strain. Therefore, the present AI vaccine strains, both Vac-1 strain and Vac-3 strain, need not to be changed.

The misuse of AI vaccines appears to have given rise to antigenically drifted AI viruses [1–3]. Okamatsu *et al.* demonstrated that the antigenic character of H5N1 HPAIs has drastically changed since 2007 [9]. The results shown in Table 1 might indicate that the Kumamoto strain exhibits more antigenic drift compared with the strains isolated in 2010 and 2011. Since China, Indonesia, Vietnam and Egypt have used AI vaccines for more than 10 years, AI viruses have been undergoing antigenic drift due to the presence of

immune pressure and could therefore escape from vaccine protection. Although Japan has successfully eradicated each of HPAI and LPAI outbreaks without the use of vaccine, the antigenic characters of field strains isolated from Japan and neighboring countries should continuously be monitored in preparation for emergency vaccination programs.

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