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FULL PAPER

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

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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

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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 (H5N 2) 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:

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

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.

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