FULL PAPER *Avian Pathology*

Safety Test and Field Study of an Inactivated Oil-Adjuvanted H5N1 Avian Influenza Vaccine

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ABSTRACT. We previously reported the development of an inactivated oil-adjuvanted avian influenza vaccine using an apathogenic H5N1 strain of the same lineage as the Eurasian lineage viruses currently epidemic in Asia. In this study, we confirmed the safety and evaluated the efficacy of this vaccine in layer chicken farms by field trials. No problematic adverse reactions occurred in the safety test. In addition, no adverse effects were observed in the field trial, and the antibody titer exceeded a protective level (hemagglutination inhibition (HI) antibody titer of 16) at 3 weeks after a single injection. Based on the above findings, this vaccine was confirmed to be safe and induced a protective level of antibody titer with a single injection in the chickens at the farms.

KEY WORDS: avian influenza, field trial, H5N1, safety test, vaccine.

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Highly pathogenic avian influenza (HPAI) caused by H5 and H7 influenza viruses has caused serious economic damage to the poultry industry worldwide. In Japan, an outbreak of H5N1 HPAI infection occurred in Yamaguchi Prefecture in 2004, 79 years after the previous outbreak; this outbreak was followed by 4 outbreaks in Oita and Kyoto Prefectures at 4 poultry farms, and a total of about 275,000 chickens were culled [5, 7, 9, 10, 16]. Moreover, HPAI infection of chickens was confirmed in Miyazaki and Okayama Prefectures in 2007, and 170,000 chickens were culled [6].

In wild animals, H5N1 viruses were isolated from a dead mountain hawk-eagle in Kumamoto Prefecture in 2007 [6] and from dead Whooper Swans in Akita and Hokkaido Prefectures in 2008 [15].

H5N1 viruses isolated from chickens in Yamaguchi, Oita and Kyoto Prefectures were genetically highly homologous, and the Yamaguchi strain was confirmed to be related to a strain isolated from chickens in Korea in 2003 [9]. Viruses isolated from chickens in Miyazaki Prefecture and a mountain hawk-eagle in Kumamoto Prefecture in 2007 were related to those isolated from a wild bird in Qinghai Lake in western China, and this lineage was also isolated from wild birds and poultry in Mongolia, Russia, Europe, Africa and Korea after 2005 [6]. The viruses isolated from Whooper swans in Akita Prefecture belong to clade 2.3.2, and one of these viruses was reported to be genetically closely related to viruses isolated in Korea and Hokkaido during the same period [15].

These findings suggest that the viruses isolated in Japan were those that were epidemic in Asia and had invaded Japan. Thus, an avian influenza outbreak may occur at any time in Japan, and it is urgently necessary to prepare a suitable vaccine.

In Japan, HPAI outbreaks are dealt with by a test-and-cull program, but when infection persists within a region and rapid culling is difficult, ring vaccination is applied in which chickens in areas around the epidemic area (movement restriction or adjacent areas) are vaccinated. For this kind of emergency, about 2.7–5.4 million doses of vaccine using H5N2 or H5N9 North American lineage virus have been stockpiled yearly by the Japanese government since 2006. However, these vaccine strains were found to be distant from H5N1 (the epidemic type in East Asia in recent years) by phylogenetic analysis of hemagglutinin (HA) [11], and development of a vaccine using a strain close to the epidemic type is desired.

Using a Eurasian lineage apathogenic avian influenza virus, we developed an inactivated oil-adjuvanted vaccine induces sufficient immunity with a single injection [12–14].

The objective of this study was to confirm the safety and efficacy of the vaccine under field conditions.

MATERIALS AND METHODS

Viruses: The vaccine was prepared from A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/vac-1/04) [9], which is an apathogenic avian influenza virus (AIV) established by reassortment of A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) at Hokkaido University in 2004. The AI virus strain was grown in 11-day-old embryonated chicken eggs at 34–35°C for 72 hr.

Vaccine preparation: The allantoic fluid was concentrated 10 times by an ultrafiltration membrane module and was then inactivated by the addition of formalin for final concentration of 0.5% and incubated at 4°C for 3 days.

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Inactivation of viruses was confirmed by the absence of hemagglutination (HA) activity in allantoic cavity fluid after 2 passages in embryonated chicken eggs. Confirmation of inactivation was also performed at the National Veterinary Assay Laboratory (NVAL). The inactivated virus suspension was diluted with phosphate-buffered saline (PBS) to adjust the HA titer to 256, mixed with 8.2 volumes of oil adjuvant containing 2% polysorbate 80, 8% sorbitan monooleate and light liquid paraffin and emulsified using a pressure-type homogenizer to prepare a water-in-oil-type vaccine.

Safety test design (test 1): This test was performed at the Research Institute for Animal Science in Biochemistry & Toxicology (RIAS) (Sagamihara, Japan). Thirty-six (18 male and 18 female) specific pathogen-free (SPF) chickens (4-week-old White Leghorn, Line M, Nisseiken Co., Ltd.) were used. The chickens of each sex were randomly divided into 3 groups (6 male + 6 female chickens per group). One dose (0.5 m*l*) and three doses (1.5 m*l*) of the vaccine were injected into the lower thigh muscle at 4 weeks of age in Groups 1 and 2, respectively. No vaccine was injected in the control group (Group 3).

The chickens were checked daily for four weeks for reactions at the local injection site, clinical symptoms and the presence or absence of other abnormalities. Reactions at the local injection site were observed in comparison with the non-injected leg, and swelling and/or hardness were evaluated using the following 4 levels: normal (–), slight (+), moderate (++) and severe (+++), according to the standard scale used by the RIAS. All chickens were weighed weekly to observe their growth. Blood was collected just prior to and at 1, 2 and 4 weeks after vaccine injection and subjected to hematology (erythrocyte count, leukocyte count, hematocrit value, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and differential leukocyte count) and blood chemistry testing (lactate dehydrogenase, aspartate aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, total protein, albumin, globulin, A/G ratio, total cholesterol, triglycerides, glucose, total bilirubin, uric acid, creatinine, calcium, inorganic phosphorus, sodium, potassium and chloride). The hemagglutination inhibition (HI) antibody titer against DK/vac-1/04 was determined. The HI test was performed as described in our previous report [12]. Four weeks after vaccination, all chickens were euthanized by CO_2 inhalation, and pathological examination (necropsy, organ weight measurement [brain, liver, kidney, heart, spleen, lung, thymus, thyroid and bursa of fabricius] and histopathological examination [liver and the injection site]) was performed. Necropsy and histopathological examinations were evaluated using the following 4 levels: no findings (–), slight (+), moderate (++) and severe (+++).

In the statistical analysis, Bartlett's test for homogeneity was performed on the results of body weight measurement, hematology testing (except the differential leukocyte count), blood chemistry testing and organ weight measurement. When a variance was homogenous, a one-way analysis of variance was used. When the variance was not homogenous, the Kruskal-Wallis rank test was used. The significance level was set at 5%.

Field study design (test 2): This study was performed using 400 commercial conventional layer chickens (200 four-week-old Julia chickens at Farm A and 200 seventy six-week-old Bolice Brown chickens at Farm B). At each farm, chickens were randomly divided into 2 groups (100 chickens per group) and numbered. One dose of the vaccine was injected into the lower thigh muscle of chickens in Groups A-1 and B-1, but the vaccine was not injected into chickens in Groups A-2 and B-2. Vaccination was performed at 4 weeks of age in Group A-1 and 76 weeks of age in Group B-1. The vaccination schedules for vaccines other than the AI vaccine are shown in Tables 1 and 2.

Clinical conditions (activity, appetite, respiratory conditions and digestive conditions) were observed and recorded daily for 14 days after vaccination in all chickens of all groups. When an abnormality was noted thereafter during the study period, the abnormal findings were recorded.

Twenty-five chickens were randomly selected from each group and checked macroscopically and by palpation daily for 14 days for the presence or absence of reactions at the local injection site. Another 25 chickens were randomly selected and weighed 3 times, at the time of vaccination and 4 and 8 weeks after vaccination.

To calculate the rate of maturity (survival rate at Farm B), all chickens in the test and control groups were checked 3 times, at the time of vaccination and 4 and 8 weeks after vaccination.

At Farm B, the number of eggs was recorded 3 times, 1 week before vaccination (Day –6 to Day 0) and 3 and 7 weeks after vaccination (Day 22 to Day 28 and Day 50 to Day 56, respectively), to calculate the egg-laying rate.

Blood was collected at the time of vaccination and at 3 and 8 weeks after vaccination from the 25 chickens that had their local injection sites checked. The blood was examined to confirm whether the HI antibody titer against Dk/vac-1/04 rose to an HI antibody titer of 16 or higher, which is the level established as the protection level against the epidemic strain (A/chicken/Yamaguchi/7/04 (H5N1))[12].

In the statistical analysis, the *t*-test was performed for body weight, the Welch's test was performed for the rate of maturity and the chi-square test was performed for the survival and egg-laying rates. The significance level was set at 5% in all tests.

RESULTS

Safety test (test 1): No changes were noted in general condition in either Group 1 vaccinated with one dose or Group 2 vaccinated with three doses.

Slight swelling was noted at the injection site. In Group 1, swelling was noted in 2–10 chickens 1–6 days after vaccination, but the swelling disappeared by day 7. In Group 2, which was treated with 3 doses, swelling was noted in 3–12

Table 1. Schedule of other vaccines at Farm A

Age in days	Vaccine
Day 0	Marek's disease vaccines (HVT, MDV1; live). Fowlpox vaccine (live).
Day 2	Avian infectious bronchitis (IB) vaccine (live).
Day 9	Newcastle disease and avian infectious bronchitis vaccine (live).
Day 15	Newcastle disease and avian infectious bronchitis vaccine (live).
Day 24	Infectious bursal disease vaccine (live).
Day 31	Newcastle disease and avian infectious bronchitis vaccine (live).
Day 44	Newcastle disease and avian infectious bronchitis vaccine (live).
Day 51	Avian infectious bronchitis vaccine (live).
Day 66	Newcastle disease and avian infectious bronchitis vaccine (live).

Table 2. Schedule of other vaccines at Farm B

Age in days	Vaccine
Day 0	Marek's disease vaccines (HVT, MDV1; live). Fowlpox vaccine (live).
Day 14	Newcastle disease and avian infectious bronchitis vaccine (live).
Day 21	Infectious bursal disease vaccine (live).
Day 28	Infectious bursal disease vaccine (live). Newcastle disease and avian infectious bronchitis vaccine (live).
Day 35	Infectious bursal disease vaccine (live).
Day 50	Fowlpox vaccine (live).
Day 70	Mycoplasma gallisepticum vaccine (inactivated). Newcastle disease and avian infectious bronchitis vaccine (live).
Day 110	Mycoplasma gallisepticum vaccine (inactivated). Newcastle disease and avian infectious bronchitis vaccine (live).
Day 200	Newcastle disease vaccine (live).
Day 290	Newcastle disease vaccine (live).
Day 380	Newcastle disease vaccine (live).

Table 3. Number of chickens with reactions at the local injection site in the safety test

Group	Inoculum	Dosage	Days after vaccination										
			0	1	2	3	4	5	6	7	8	9	10	11–28
1	Vaccine	1	0/12[a)]	10/12	6/12	6/12	4/12	4/12	2/12	0/12	0/12	0/12	0/12	0/12
2	Vaccine	3	0/12	12/12	12/12	12/12	12/12	12/12	11/12	9/12	6/12	3/12	3/12	0/12

a) Number of chickens with slight swelling (+) / total.

chickens 1–10 days after vaccination, but all swelling disappeared by day 11 (Table 3).

The mean body weight was slightly lower in males and slightly higher in females in Groups 1 and 2 compared with those in the control group (Group 3), but no dose-dependency or significant difference was observed (Figs. 1 and 2).

No changes were observed that were thought to be caused by vaccination in the results of hematology and blood chemistry testing of the one-dose group. In the three-dose group, the ratio of the eosinophil count to the total white blood cell count was significantly increased (7 and 14 days after vaccination, Table 4), and the white blood cell count was significantly decreased (7 days after vaccination, Table 5). In the results of blood chemical testing of the three-dose group, the aspartate aminotransferase level was significantly increased or tended to increase (7–28 days after vaccination, Table 6), the lactate dehydrogenase level was significantly elevated (Table 7), the potassium level was significantly decreased (7 days after vaccination, Table 8) and the chloride levels were significantly decreased (28 days after vaccination, Table 9).

At necropsy, yellow granular substances assumed to be vaccine residue were noted in and between muscles at the injection site. In Group 1, 100–180 mm^3 of the substances was found in 2 males and 1–90 mm^3 of the substances was found in 2 females. In Group 2, 36–150 mm^3 of the substances was found in 4 males and 26–600 mm^3 of the sub-

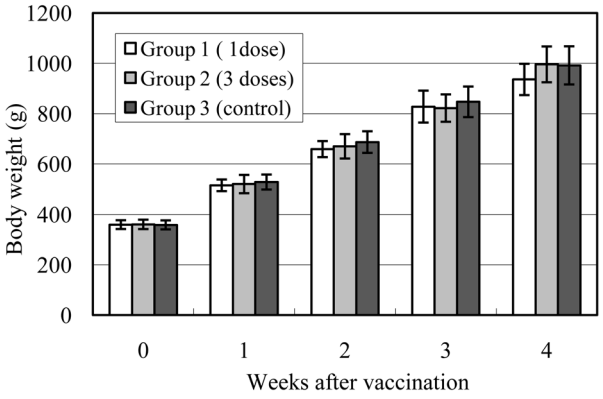

Fig. 1. Mean body weight in males in the safety test. Six male chickens in each group were weighed weekly for 4 weeks. Group 1 was injected with one dose, Group 2 was injected with three doses and Group 3 was not injected (control). Standard deviation error bars are shown.

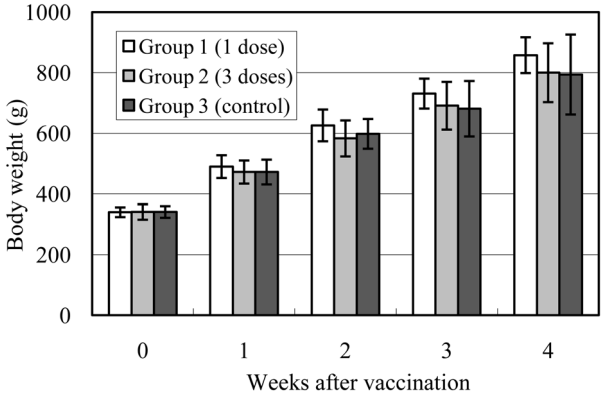

Fig. 2. Mean body weight in females in the safety test. Six female chickens in each group were weighed weekly for 4 weeks. Groups 1 was injected with one dose, Groups 2 was injected with three doses and Group 3 was not injected (control).

Table 4. Results of hematology in the safety test (eosinophil count to the total white blood cell count)

Group	Inoculum	Dosage	Sex	Number of chickens	Eosinophil count to the total white blood cell count (%)			
					Days after vaccination			
					–1	7	14	28
1	Vaccine	1	Male	6	2 ± 1	6 ± 3	5 ± 3	4 ± 4
			Female	6	3 ± 2	6 ± 5	7 ± 7	4 ± 5
2	Vaccine	3	Male	6	3 ± 3	15 ± 3**	16 ± 7**	7 ± 5
			Female	6	2 ± 2	7 ± 4	12 ± 7**	5 ± 3
3	–	–	Male	6	3 ± 2	3 ± 2	3 ± 1	3 ± 1
			Female	6	4 ± 2	3 ± 2	2 ± 1	2 ± 2

a) Geometric mean ± standard deviation.
Values followed by asterisks (**) are significantly different from those of group 3 ($P<0.01$).

Table 5. Results of hematology in the safety test (leukocyte count)

Group	inoculum	Dosage	Sex	Number of chickens	Leukocyte count ($10^2/\mu l$)			
					Days after vaccination			
					–1	7	14	28
1	Vaccine	1	Male	6	238 ± 29[a)]	248 ± 63	233 ± 31	240 ± 31
			Female	6	256 ± 15	256 ± 15	256 ± 15	256 ± 15
2	Vaccine	3	Male	6	249 ± 30	155 ± 37**	288 ± 42	263 ± 24
			Female	6	256 ± 30	256 ± 15	288 ± 42	256 ± 15
3	–	–	Male	6	256 ± 15	251 ± 22	247 ± 28	248 ± 31
			Female	6	256 ± 15	251 ± 22	247 ± 28	248 ± 31

a) Geometric mean ± standard deviation.
The value followed by asterisks (**) is significantly different from that of group 3 ($P<0.01$).

stances was found in 5 females (Table 10).

On histopathological examination, oil cysts of various sizes surrounded mainly by macrophages and inflammatory cell infiltration, mainly lymphocytes, were observed at the injection site in most chickens, and follicle-like accumulations of lymphocytes were frequently observed. In addition, proliferation of fibroblasts was noted (Table 10). No changes were noted in organs or tissues beyond the injection site.

No statistical differences in organ weights were observed

Table 6. Results of blood chemical testing in the safety test (aspartate aminotransferase)

Group	Inoculum	Dosage	Sex	Number of chickens	Aspartate aminotransferase (IU/*l*)			
					Days after vaccination			
					–1	7	14	28
1	Vaccine	1	Male	6	178 ± 16[a)]	165 ± 11	165 ± 9	188 ± 8
			Female	6	176 ± 14	165 ± 10	157 ± 9	181 ± 23
2	Vaccine	3	Male	6	196 ± 21	203 ± 17**	198 ± 12**	209 ± 13**
			Female	6	179 ± 23	189 ± 13*	188 ± 26*	193 ± 17
3	–	–	Male	6	189 ± 19	162 ± 13	164 ± 11	178 ± 11
			Female	6	180 ± 20	163 ± 11	163 ± 12	188 ± 21

a) Geometric mean ± standard deviation.
Values followed by an asterisk (*) are significantly different from those of group 3 ($P<0.05$).
Values followed by asterisks (**) are significantly different from those of group 3 ($P<0.01$).

Table 7. Results of blood chemical testing in the safety test (lactate dehydrogenase)

Group	Inoculum	Dosage	Sex	Number of chickens	Lactate dehydrogenase (IU/*l*)			
					Days after vaccination			
					–1	7	14	28
1	Vaccine	1	Male	6	1263 ± 223[a)]	1035 ± 131	961 ± 66	974 ± 123
			Female	6	1053 ± 90	855 ± 93	816 ± 68	888 ± 132
2	Vaccine	3	Male	6	1349 ± 204	1173 ± 100	1108 ± 149	1073 ± 145
			Female	6	1149 ± 128	1150 ± 121*	1040 ± 132	982 ± 120
3	–	–	Male	6	1181 ± 158	945 ± 196	940 ± 87	906 ± 99
			Female	6	1177 ± 124	924 ± 71	892 ± 54	907 ± 111

a) Geometric mean ± standard deviation.
The value followed by an asterisk (*) is significantly different from that of group 3 ($P<0.05$).

Table 8. Results of blood chemical testing in the safety test (potassium)

Group	Inoculum	Dosage	Sex	Number of chickens	Potassium (mEq/*l*)			
					Days after vaccination			
					–1	7	14	28
1	Vaccine	1	Male	6	4.94 ± 0.29[a)]	5.34 ± 0.43	5.29 ± 0.22	5.38 ± 0.38
			Female	6	4.96 ± 0.20	5.36 ± 0.47	5.31 ± 0.40	5.57 ± 0.36
2	Vaccine	3	Male	6	4.91 ± 0.30	5.11 ± 0.36	5.34 ± 0.28	5.73 ± 0.66
			Female	6	4.84 ± 0.11	4.47 ± 0.26*	5.55 ± 0.32	5.88 ± 0.44
3	–	–	Male	6	4.78 ± 0.26	5.22 ± 0.51	5.36 ± 0.46	5.62 ± 0.61
			Female	6	4.86 ± 0.21	5.17 ± 0.46	5.41 ± 0.20	6.02 ± 0.47

a) Geometric mean ± standard deviation.
The value followed by an asterisk (*) is significantly different from that of group 3 ($P<0.05$).

between the vaccinated groups (Groups 1 and 2) and the control group (Group 3; data not shown).

The antibody titer increased in a dose-dependent manner from 2 weeks after vaccination in both Groups 1 and 2 and further increased at 3 weeks after vaccination (Table 11). The antibody response in chickens vaccinated with one dose was comparable to that from an in-house test (data not shown).

Field study (test 2): No vaccination-induced clinical symptoms were noted throughout the observation period in either vaccinated group at Farm A or B (A-1 or B-1, respectively). No swelling or induration developed in either Group A-1 or B-1 during the observation period.

No vaccination-induced abnormality regarding body weight occurred in any group during the study period. On analysis of significant differences, the body weight at 8

Table 9 . Results of blood chemical testing in the safety test (chloride)

Group	Inoculum	Dosage	Sex	Number of chickens	Chloride (mEq/*l*)			
					Days after vaccination			
					–1	7	14	28
1	Vaccine	1	Male	6	105 ± 2[a)]	106 ± 2	106 ± 1	108 ± 5
			Female	6	108 ± 1	107 ± 1	108 ± 1	108 ± 3
2	Vaccine	3	Male	6	105 ± 1	106 ± 1	107 ± 2	104 ± 2*
			Female	6	107 ± 1	108 ± 0	108 ± 1	109 ± 1
3	–	–	Male	6	105 ± 3	105 ± 1	105 ± 1	108 ± 2
			Female	6	107 ± 1	107 ± 1	109 ± 2	107 ± 3

a) Geometric mean ± standard deviation.
The value followed by an asterisk (*) is significantly different from that of group 3 (P<0.05).

Table 10. Results of necropsy and histopathological examination at the vaccine injection site in the safety test

Group	Inoculum	Dosage	Sex	Number of chickens	Necropsy finding	Histopathological findings[b)]											
					Yellow granular substances	Inflammatory cell infiltrations				Proliferation of fibroblasts				Oil-cysts			
						–	+	++	+++	–	+	++	+++	–	+	++	+++
1	Vaccine	1	Male	6	2[a)]	4[a)]	2	0	0	0	3	3	0	0	6	0	0
			Female	6	2	5	1	0	0	0	1	4	0	0	4	2	0
2	Vaccine	3	Male	6	4	0	2	4	0	0	2	5	0	0	3	3	0
			Female	6	5	0	1	5	0	0	1	4	0	0	0	6	0

a) Number of chickens with observed changes.
b) None = –; slight = +; moderate = ++; severe = +++.

Table 11. Antibody responses of chickens in the safety test

Group	Inoculum	Dosage	Weeks after vaccinations		
			0	2	4
1	Vaccine	1	0[a)] (<4)[b)]	92 (38)	100 (861)
2	Vaccine	3	0 (<4)	100 (90)	100 (1,625)
3	–	0	0 (<4)	0 (<4)	0 (<4)

a) Percentage of chickens having an HI titer higher than 4.
b) Numbers in parentheses show the mean antibody titers of 12 chickens (GM).

Table 12. Antibody responses of chickens in the field study

Farm	Group	Inoculum	Weeks after vaccinations		
			0	3	8
A	A–1	Vaccine	0[a)] (<4)[b)]	100 (388)	100 (357)
	A-2	-	0 (<4)	0 (<4)	0 (<4)
B	B-1	Vaccine	0 (<4)	100 (377)	100 (223)
	B-2	-	0 (<4)	0 (<4)	0 (<4)

a) Percentage of chickens having an HI titer higher than 4.
b) Numbers in parentheses show the mean antibody titers of 25 chickens (GM).

weeks after vaccination was significantly higher in Group A-1 (vaccinated) than in A-2 (control) at Farm A (p=0.0442; Fig. 3). At Farm B, no significant differences were noted between the vaccinated and control groups (Fig. 4).

The rate of maturity (survival rate) in the study period was 100% in all vaccinated (A-1 and B-1) and control (A-2 and B-2) groups, showing no vaccination-induced abnormality.

There was no significant difference in the egg-laying rate between Groups B-1 (vaccinated) and B-2 (control) during the study period, indicating no vaccination-induced abnormality (Fig. 5).

An HI antibody titer of 16 or higher was detected in 0/25 chickens (0%, <4) at the time of vaccination, 25/25 chickens (100%, geometric mean [GM] HI antibody titer: A-1, 388; B-1, 377) at 3 weeks after vaccination and 25/25 chickens (100%, GM antibody titer: A-1, 357; B-1, 223) at 8 weeks after vaccination in the vaccinated groups, whereas the titer was less than 4 in the control groups (A-2 and B-2; Table 12). All chickens in the vaccinated groups that had their HI antibody titers measured showed an HI antibody titer of 16 or higher at 3 and 8 weeks after vaccination.

DISCUSSION

Generally, the antibody responses in chickens inoculated with oil-adjuvanted vaccines are high and are expected to last for a long period, but they are often accompanied by

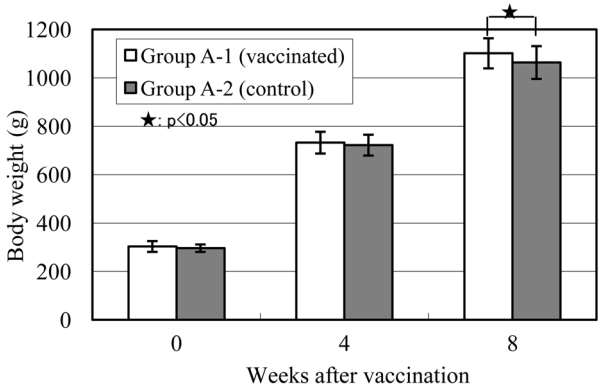

Fig. 3. Mean body weight at Farm A in the field study. Twenty-five chickens in each group were weighed 3 times, at the time of vaccination and 4 and 8 weeks after vaccination. Group A-1 was injected with one dose, and Group A-2 was not injected (control). Standard deviation error bars are shown.

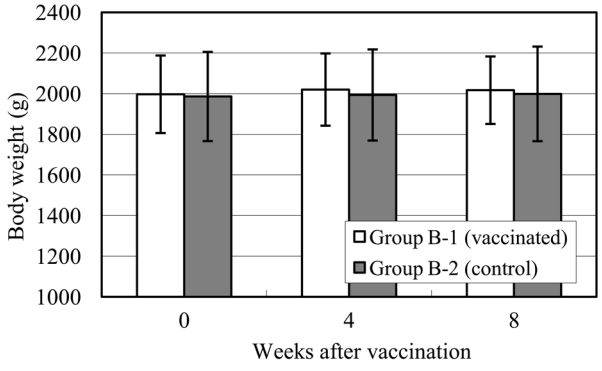

Fig. 4. Mean body weight at Farm B in the field study. Twenty-five chickens in each group were weighed 3 times, at the time of vaccination and 4 and 8 weeks after vaccination. Group B-1 was injected with one dose, and Group B-2 was not injected (control).

adverse reactions such as residual oil (oil cyst formation) and inflammatory cell infiltration [2–4].

In the safety test, no abnormalities were induced by intramuscular vaccine injection with one dose (0.5 m*l*, Group 1) or three doses (1.5 m*l*, Group 2) in the following areas: general condition, food intake, necropsy findings and organ weight. The changes detected in the data from hematology testing of the three-dose group were transient, and the differences in the findings in the blood chemistry testing compared with those from the control group were mostly within the baseline ranges of background data at the test facility. In addition, no marked changes were detected in the results of the hematology and blood chemistry testing in the one-dose group, suggesting that there are no problems with one-dose administration. Mild swelling and yellow granular substances in and between muscles were noted at the injection site in both the one-dose and three-dose groups at necropsy,

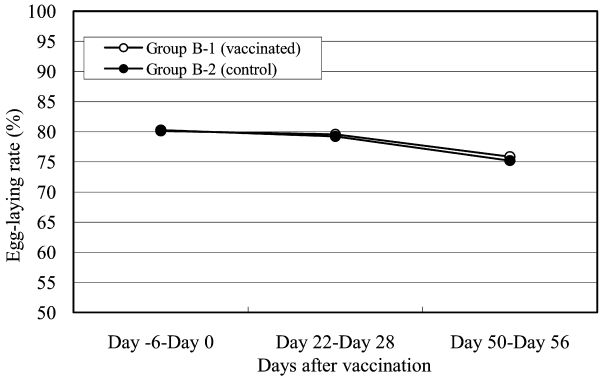

Fig. 5. Egg-laying rate at Farm B in the field study. The number of eggs was recorded 3 times, 1 week before vaccination and 3 and 7 weeks after vaccination. Group B-1 was injected with one dose, and Group B-2 was not injected (control).

and oil cysts with various sizes, slight inflammatory cell infiltration and proliferation of fibroblasts were observed on histopathological examination; however, the severity was relatively mild for all findings. These changes are generally induced by oil vaccines and are not problematic. In another test, oil cysts with various sizes surrounded mainly by macrophages were present at the injection site at 16 weeks after vaccination, but not at 20 weeks (data not shown). The severity of oil retention was also equivalent to or milder than that caused by general oil vaccines.

In the field study, one chicken in the vaccinated group (A-1) developed leg paralysis in the non-injected leg and one chicken in the control group (A-2) developed hemophthalmia at Farm A, but no abnormality with a causal relationship to the vaccine occurred. At Farm B, 2 chickens suddenly died in Group B-2 (control), but no abnormality was noted in Group B-1 (vaccinated). In regard to body weight measurement, body weight at 8 weeks after vaccination was significantly higher in Group A-1 (vaccinated) than in A-2 (control); the reason for such a significant weight difference is not clear. However, both the A-1 and A-2 weights were within the standard that the farm had set, so it was judged not to be a problem. There were no other differences between the vaccinated and control groups. The rates of maturity (survival rate) and egg-laying of Group B-1 (vaccinated) were equivalent to those of Group B-2 (control). Based on the above findings, it was concluded that there is no safety issue with this vaccine with regard to administration to commercial layer chickens at one dose.

Because this clinical trial was the first one conducted for the avian influenza vaccine in Japan, the processes from the beginning to the end of the trial were done in consultation with the Committee of Emergency Development Project for Avian Influenza Vaccine, which resulted in stricter controls compared with usual clinical trials. For example, inactivation of the virus, which was confirmed by an in-house test, was also confirmed by an official assay of the NVAL in Japan. The number of chickens per group had to be 100

birds. It is the minimum number needed for the field trial which is described in the official notification by the Director General, Livestock Industry Bureau, the Ministry of Agriculture, Forestry and Fisheries of Japan (Ref.No.12-Chiku-A-725). In addition, the birds were tagged and numbered. Monitoring, such as virus isolation from tracheal and cloacal swabs and serological testing using an agar gel diffusion precipitin test for antibody to avian influenza virus, was performed one week before vaccination and 3, 6 and 9 weeks after vaccination according to the special guidelines for prevention of domestic animal infectious disease concerning highly pathogenic avian influenza (released by the Minister of Agriculture, Forestry and Fisheries on November 18, 2004). These specimens were collected in the presence of the prefectural officials. Carcasses, excrement and eggs were disinfected, put into double plastic bags and transported to our facility or a livestock hygiene service center where they were incinerated.

Regarding the efficacy of this vaccine, we previously reported that chickens with an HI antibody titer of 16 or higher were protected from challenge with a virulent Yamaguchi strain (A/chicken/Yamaguchi/7/04 (H5N1); clade 2.5) having 92.2% amino acid homology of HA to the vaccine strain [12]. In addition, chickens injected with this vaccine developed no clinical symptoms when they were challenged with the current epidemic strain in Asia belonging to the clade 2.3.2 lineage, A/whooper swan/Hokkaido/1/2008 (amino acid homologies of HA protein with the vaccine and Yamaguchi strains were 90.3 and 94.5%, respectively), and virus isolation from tracheal and cloacal swabs was negative (personal communication with Dr. Yamamoto at Hokkaido University).

The viruses used for preparation of stockpiling vaccine in Japan are H5N2 and H5N9 of North American lineage isolated before 1994. The HA1 amino acid homologies of a North American lineage H5N2 virus, A/chicken/Mexico/232/94 (H5N2), with the Yamaguchi and Hokkaido strains are comparatively lower at 85.5 and 83.0%, respectively, suggesting that a vaccine prepared from an antigenically closer Eurasian lineage virus strain would be more effective against Eurasian lineage viruses currently epidemic in Asia than vaccines prepared from the North American lineage virus strains [1, 8]. In addition, the currently stockpiled vaccine requires 2 injections, but our vaccine was confirmed to induce antibody production to a defensive titer level with a single injection at chicken farms, which is more suitable for practical use.

This vaccine exhibited no adverse effects not only in laboratories but also at chicken farms and induced antibody production to a titer sufficient to protect chickens against avian influenza with a single injection, confirming its usefulness.

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