Makara Journal of Science

Volume 27
Issue 3 *September*

Article 10

9-25-2023

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Recommended Citation

Ramlah, Ramlah; Murtini, Sri; Soejoedono, Retno Damayanti; Natih, Ketut Karuni Nyanakumari; and Ningrum, Siti Gusti (2023) "Chicken Immune Responses to Vaccination by the Avian Influenza Subtype H5N1 against Avian Influenza," *Makara Journal of Science*: Vol. 27: Iss. 3, Article 10.
DOI: 10.7454/mss.v27i3.1367
Available at: https://scholarhub.ui.ac.id/science/vol27/iss3/10

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Cover Page Footnote

The author would like to thank the scholarships given by the Agricultural Human Resources Extension and Development Agency (BPPSDMP) and all the virology test unit staff at the National Veterinary Drugs and Assay Laboratory (BBPMSOH) of the Ministry of Agriculture

Makara Journal of Science, 27/3 (2023), 247–254
doi: 10.7454/mss.v27i3.1367

Chicken Immune Responses to Vaccination by the Avian Influenza Subtype H5N1 against Avian Influenza

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Received June 22, 2022 | Accepted July 12, 2023

Abstract

Avian Influenza (AI) is one of the strategic animal diseases still considered a priority for control by the Indonesian government. This study aimed to examine the immune response of chickens to various types of H5N1 subtype AI vaccines in Indonesia and to determine the correlation of factors influencing the post-vaccination AI antibody response. Serum samples were tested using the hemagglutination inhibition (HI) test with the standard AI antigen subtype H5N1 strain A/Chicken/Barru/BBVM/41-13/2013 (Clade 2.1.3) and strain A/Chicken/Semarang/04141225-07/2014 (Clade 2.3.2). Antibody titer was calculated using the Geometric Mean Titer (GMT). The correlation analyses were performed to assess the correlation of antibody titer against each of the following factors: age of chicken at the time of sampling, the interval between sampling time and the previous vaccination, and the number of vaccinations performed. The results showed that the average antibody titer value against the AI antigen subtype H5N1 strain A/Chicken/Barru/BBVM/41-13/2013 (Clade 2.1.3) was highest in samples from the South Sumatra Province, namely, Palembang City, which was $2^{6.42}$ HIU. The results showed a significant correlation (*p*-value <0,05) between antibody titer and the interval between sampling time and the previous vaccination, the number of vaccination performed and age of chicken at the time of sampling. Therefore, results showed that the immunity developed from vaccination using the AI vaccine seed subtype H5N1 could induce immunity with a protective value of ≥16.

Keywords: avian influenza, H5N1 clade 2.1.3, H5N1 clade 2.3.2, hemagglutination test

Introduction

Avian Influenza (AI) is a zoonotic disease; it can be transmitted from infected animals to humans. The percentage of AI cases in humans associated with AI outbreaks in poultry was 34% [1]. AI causes very high mortality in poultry, causing huge economic losses in the poultry industry in Indonesia. Komisi Nasional Flu Burung dan Pandemi Influenza estimates that Indonesia's economic losses due to the AI outbreak from 2004 to 2008 were about Rp. 4.3 trillion, excluding losses due to loss of job opportunities and the reduced consumption of animal protein in the community [2]. These conditions make AI a priority for the Indonesian government to control [3].

The AI disease outbreaks in Indonesia in 2003–2004 were caused by the AI virus subtype H5N1 Clade 2.1. Two years after the first outbreak, the AI virus subtype H5N1 Clade 2.1 developed into three sublineage viruses: Clades 2.1.1, 2.1.2, and 2.1.3 [4]. However, since 2008, AI Clades 2.1.1 and 2.1.2 viruses are no longer found in poultry and humans, whereas the AI Clade 2.1.3 viruses developed into three new AI viral groups, Clade 2.1.3.1, 2.1.3.2 and 2.1.3.3 [5]. At the end of 2012, the AI virus subtype H5N1 Clade 2.3.2 was the causative agent of outbreaks in domestic waterfowl, including ducks, in Central Java, East Java, and Jogjakarta [6]. The AI virus subtype H5N1 Clade 2.3.2 is a new virus that had never been reported in poultry in Indonesia. From 2008 to 2014, the dominant subtypes of H5N1 AI virus group found in poultry in Indonesia were Clade 2.1.3.2 and Clade 2.3.2.1 [7]. The AI virus subtype H5N1 Clades 2.1.3.2 and Clade 2.3.2.1 were isolated in the live bird

market (LBM) in several provinces in Indonesia from 2014 to 2019, and reassortments from the two clades have been reported [8].

Countries with AI endemics are advised to continue with AI virus surveillance and monitoring, implement biosecurity measures, and identify and reduce AI vaccination failures [9, 10]. Serological identification is a surveillance method to determine the pattern of AI disease spread in the field [12–14]. Naturally, if animals are exposed to viruses, their humoral immune response is stimulated and produces antibodies [15]. Antibody titers can be measured through a serological test, namely, the hemagglutination inhibition (HI) test. This study aimed to determine the diversity of immune responses when chickens are receive H5N1 subtype AI vaccines in Indonesia and to determine the correlation of factors that affect the resulting AI antibody response.

Methods

Sampling. Chicken serum samples were obtained from the National Veterinary Drug and Assay Laboratory Veterinary Drugs (BBPMSOH) AI study archive samples in 2020. A total of 600 chicken serum samples were obtained from six provinces: West Java, West Kalimantan, North Sumatra, West Sumatra, South Sumatra, and Bali. Sample data were obtained from questionnaires and included the sample's origin (province and district), type of chicken, chicken age, vaccination schedule, last vaccination, time of sampling, and type of vaccine. Data on circulating AI virus strains that had been identified from the place of origin of the sample were obtained from literature (secondary data). The serum samples were collected from laying hens aged from 11 to 81 weeks AI H5N1 vaccines clade 2.1.3 and or 2.3.2 consisted of vaccine A (*seed* virus *clade* 2.1 dan *clade* 2.3), vaccine B (*seed* virus clade 2.3.2), vaccine C (*seed* virus *clade* 2.1.3 dan *clade* 2.3.2), vaccine D (*seed* virus *clade* 2.3), vaccine E (*seed* virus *clade* 2.1.3), and vaccine F. Based on the questionnaire data that the implementation of AI vaccination was carried out twice until six times, and collecting chicken serum samples from one to 44 weeks post-vaccination, chickens were chosen randomly in each cage. Blood was taken through the brachial vein and stored at 4 ˚C until serum was extracted. This research was conducted from November 2020 to May 2022 in the Virology Laboratory of the National Veterinary and Drugs Laboratory (BBPMSOH).

Examination of antibody titer. Antibody titers of chicken serum samples were determined using the standard HI test [10] based on two types of standard AI antigens set by the government, namely, the AI antigen subtype H5N1 strain A/Chicken/Barru/BBVM/41-13/2013 (Clade 2.1.3) and strain A/Chicken/Semarang/ 04141225-07/2014 (Clade 2.3.2). The bottom of the microplate well "V" was filled with 25 µL of phosphate-buffered saline (Oxoid, England) pH 7.2. A volume of 25 µL of the serum sample was serially diluted in multiples of two, starting from the 1st microplate well to the 11th well. A total of 25 µL of AI 4 HAU antigen was added to each well of the microplate beginning from the 1st to the 11th well before shaking on amini shaker for 20 s and allowing the mixture to stand for 30 min at room temperature. A volume of 25 µL of 1% red blood cells (RBCs) suspension was added into the 1st to 12th wells before shaking for 20 s at medium speed. Hemagglutination test results were read after incubation for 40 min.

Table 1. Correlation Coefficient Intervals and Their Interpretations [21]

Coefficient Interval	Relationship Level
0,00–0,199	Very low
0,20–0,399	Low
0,40–0,599	Moderate
0,60–0,799	Strong
0,80–1,000	Very strong

The HI titre is the highest dilution of serum causing complete inhibition of 4HAU of Antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 25 µL RBCs and 50 µL PBS only) should be considered to show inhibition [11]. Data analysis involved calculating the antibody titer from each farm, which was differentiated based on the antigen used in the HI test. Antibody titers below 2^4 or 16 HI Units were interpreted as negative results, whereas titers $\geq 4\log_2$ were considered positive [11, 16]. Titers below 24 were considered less protective against challenges from the AI virus.

Data analysis. Antibody titer values were log transformed to establish Geometric Mean Titer (GMT) values. Antibody titers from samples were determined using correlation analysis in the software R version 4.1.2 to determine the correlation of factors that influence antibody titers, namely, the chickens' age at the time of sampling, the last vaccination interval with sampling and the number of vaccinations. Guidelines for interpreting the correlation coefficient interval can be seen in Table 1.

Results and Discussion

Antibody titer response. Results of antibody titer determination against the AI antigen subtype H5N1 strain A/Chicken/Barru/BBVM/41-13/2013 (Clade 2.1.3) showed that 374 out of 600 serum samples (62%) were positive for AI antibodies and 226 serum samples (38%) were negative for AI antibodies. A total of 349 sera (58%) were positive and as many as 251 sera (42%) negative for AI antibodies using the AI antigen subtype H5N1 strain

A/Chicken/Semarang/04141225-07/2014 (Clade 2.3.2). The antibody titers produced by each sample from the six provinces varied against the government's two types of standard AI antigens.

The antibody titer results against the AI antigen subtype H5N1 strain A/Chicken/Barru/BBVM/41-13/2013 (Clade 2.1.3) for samples from North Sumatra Province, Simalungun District using vaccine C and South Sumatra, Banyuasin District using the E vaccine was 100%. The highest mean GMT value GMT ($2^{6.42}$HIU) of antibodies was recorded in samples from South Sumatra, Palembang City Province. Vaccine A was used in the City of Palembang. The lowest response was found in chickens from West Java, namely, those from the districts of Sukabumi and Cianjur, at 0%, and in the district of Sukabumi, the average value was 0 HIU. The chickens from Sukabumi and Cianjur regencies were vaccinated against AI using the F vaccine.

Samples from the six provinces in Indonesia showed the effectiveness of vaccination with antibody titer values (Table 2). Antibody titer results against the AI antigen subtype H5N1 strain A/Chicken/Semarang/04141225-07/2014 (Clade 2.3.2) were the highest in samples from North Sumatra Province, Deli Serdang District by 90%. The chickens were vaccinated using vaccine A. The highest average value in South Sumatra, Banyuasin District area was $2^{5.26}$ HIU. The type of vaccine used in the Banyuasin District was vaccine E. The lowest response was found in West Java Province, Cianjur District with 18% and an average value of $2^{1.64}$ HIU using the F vaccine.

AI subtype H5N1 vaccination of a flock is regarded as effective if it has induced immunity to the AI virus subtype H5N1 with an estimated antibody value >60% of the sample having an antibody titer $\geq 4\log_2$, which can reduce and prevent field AI virus challenges [17, 18]. The antibody response produced from vaccinated chickens in each province in Indonesia was different due to the various strains of the vaccine virus. The AI vaccines used had additional vaccine seed content from each manufacturer (Table 2). Based on the questionnaire data, it was found that the AI vaccine containing the AI virus seed subtype H5N1 Clade 2.1.3 was vaccine E, whereas vaccines A and C had the AI virus seed subtype H5N1 Clade 2.1.3 and clade 2.3.2. Vaccines B and D contained AI virus subtype H5N1 Clade 2.3.2 only. Based on questionnaire data, it is unknown which clade seed virus AI subtype H5N1 is contained.

Table 2. H5N1 Subtype AI Antibody Titer Results

No	Provinces	Vaccine Type	Prevalence of Sero Positive		GMT[a]	
			Ag. AI H5N1 *Clade* 2.1.3	Ag. AI H5N1 *Clade* 2.3.2	Ag. AI H5N1 *Clade* 2.1.3	Ag. AI H5N1 *Clade* 2.3.2
1	West Java_Cianjur	Vaccine F	0%	18%	$2^{0.04}$	$2^{1.64}$
	West Java_Sukabumi	Vaccine F	0%	48%	0	$2^{2.94}$
2	West Kalimantan_Singkawang	Vaccine B *clade* 2.3.2	64%	50%	$2^{4.36}$	$2^{3.48}$
	West Kalimantan_Ambawang	Vaccine B *clade* 2.3.2	52%	58%	$2^{3.2}$	$2^{3.88}$
3	North Sumatra_Deli Serdang	Vaccine A *clade* 2.1 dan 2.3	70%	90%	$2^{3.90}$	$2^{4.58}$
	North Sumatra_Simalungun	Vaccine C *clade* 2.1.3 dan 2.3.2	100%	70%	$2^{6.08}$	$2^{4.54}$
4	West Sumatra_Tanah Datar	Vaccine F	90%	66%	$2^{5.68}$	$2^{4.16}$
	West Sumatra_Lima Puluh Kota	Vaccine F	58%	40%	$2^{4.28}$	$2^{2.36}$
5	South Sumatra_Palembang	Vaccine A *clade* 2.1 dan 2.3	94%	76%	$2^{6.42}$	$2^{4.32}$
	South Sumatra_Banyuasin	Vaccine E *clade* 2.1.3	100%	84%	$2^{6.14}$	$2^{5.26}$
6	Bali_Bangli	Vaccine D *clade* 2.3	66%	64%	$2^{3.78}$	$2^{4.14}$
	Bali_Karangasem	Vaccine D *clade* 2.3	54%	34%	$2^{3.4}$	$2^{2.76}$

Each district has 50 laying hens
a Geometric Mean Titer

Vaccines A and C induced immunity above 60% when tested with the common viruses, indicating that the vaccine's effectiveness in inducing immunity was quite good. Although the E vaccine contained only AI virus seed subtype H5N1 Clade 2.1.3, it induced protective immunity more than 60% both with homologous standard viruses (AI antigen subtype H5N1 strain A/Chicken/Barru/BBVM/ 41-13/2013) and heterologous standard antigens. The average antibody titer induced also reached a protective titer ($\geq 4\log_2$) both with homologous and heterologous test antigens. It could be due to a cross-reaction between antigen clade 2.1.3 and AI virus clade 2.3.2 or during field infection exposure to the AI virus subtype H5N1 Clade 2.3.2.

Chickens vaccinated with an AI virus strain (Sukabumi strain), which was different from the test standard virus strain (Karanganyar strain), still showed antibodies that had a protective titer of $2^{4,6}$ [19]. The homology value between AI Clade 2.1.3 and clade 2.3.2 was 93–94% [6]. This high homology level allows cross-reactions [12]. Serum samples from 15 laying hens were 92.31% to 92.86% positive for antibodies to the AI virus subtype H5N1 Clade 2.3.2 with a titer of $\geq 4\log_2$ even though the chickens in these farms had never received AI vaccination subtype H5N1 Clade 2.3.2 [12]. Antibodies to the AI virus subtype H5N1 Clade 2.3.2 were formed due to a cross-reaction of AI vaccine subtype H5N1 Clade 2.1.3 or field infection with AI virus subtype H5N1 Clade 2.3.2. Chickens exposed to the virus can occur naturally in field infections or artificially by vaccination. The immune system of chickens recognizes the presence of foreign agents that enter the body and forms antibodies to eliminate infectious agents. A booster vaccination will increase the immune response in the chicken body. Booster vaccines activate memory cells, proliferate rapidly, in large numbers, and produce specific antibodies with a faster and higher increase in antibody titers [15].

Indonesia is one of the countries in which the AI virus is endemic since 2003, and uses the AI H5 vaccine as it has been infected with the AI virus [20]. Since 2013, viruses from clades 2.1.3.2 and clade 2.3.2.1 are the dominant group of viruses found in poultry in Sumatra, Java, Bali, Kalimantan, Sulawesi, and Papua [7]. The latest data from iSIKHNAS showed that the spread of AI virus clade 2.1.3 occurred in most North Sumatra and West Kalimantan Provinces, whereas the spread of the AI virus clade 2.3.2, isolates occurred in almost all areas of Indonesia [21]. The strains and clades of AI H5N1 viruses had similarities in several regions in Indonesia. The AI virus subtype H5N1 was isolated in the LBM in the provinces of Banten, West Java, Central Java, East Java, and DKI Jakarta in Indonesia was in the clade 2.1.3.2 and clade 2.3.2.1 groups [8].

Vaccines B and D had a lower ability to induce immunity in chickens than vaccines A, C, and E. Vaccination with Vaccine B was carried out twice, sampling was carried out at 36 to 68 weeks of age of chickens, and the sampling interval was 8 to 12 weeks after the previous vaccination. Likewise, vaccination with vaccine D was performed twice, sampling was carried out at 7 to 26 weeks of age of chickens, and the sampling interval was 6 to 29 weeks after the previous vaccination.

Antibody titer response based on statistical analysis. There were differences in antibody titer values obtained for chicken serum samples from each province. Apart from differences in vaccine seed content, there were also several factors, including the age of chicken at the time of sampling, the interval since last vaccination, and the number of vaccinations received by the sampled chickens. These factors were analyzed for correlation using the software R version 4.1.2. The results of the influence of age of laying hens at the time of sampling are presented in Figure 1.

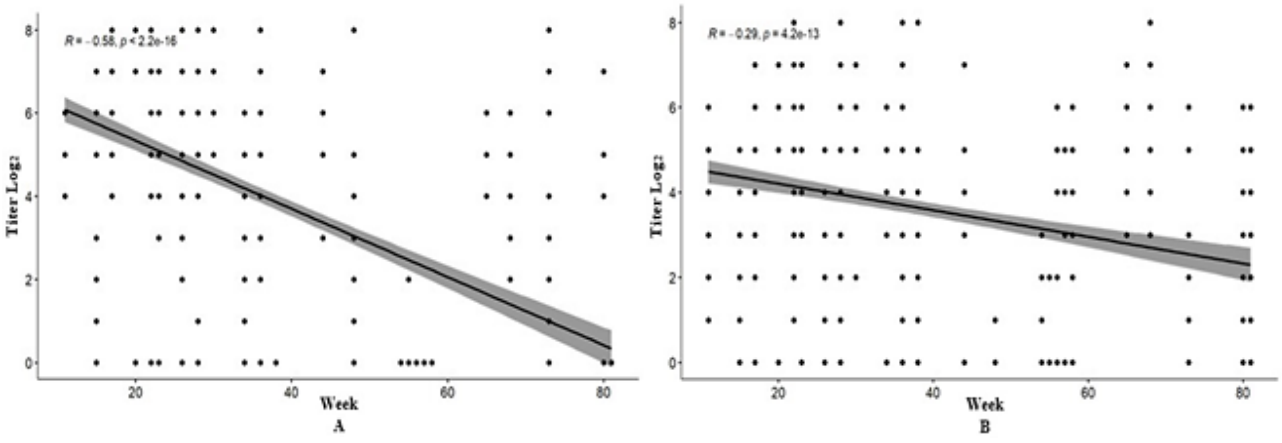

Figure 1. Correlation Between Antibody Titer and the Age of Chicken at the Time of Sampling for AI Subtype H5N1 (A) Antigen Clade 2.1.3 and (B) Clade 2.3.2

The test results (Figure 1) showed a significant correlation between the age of the chickens at the time of sampling and the AI Clade 2.1.3 antigen titer ($p < 0.05$). The value of the correlation coefficient R was −0.58, indicating a moderate negative correlation between the two factors [22]. The antibody titer against the AI Clade 2.3.2 antigen exhibited significant correlation with the age of chicken at the time of sampling ($p < 0.05$). The value of the correlation coefficient was −0.29, indicating a weak correlation between the two factors [21]. The older the age of the chickens at the time of sampling, the lower the antibody titer formed. In Figure 1, the age at time of sampling showing an antibody response 4log2 to AI antigens clades 2.1.3 and clade 2.3.2 was between 20 and 40 weeks. Based on the questionnaire data, the chickens were vaccinated at more than 25 weeks to 30 weeks of age. However, data on regular vaccination programs for each farm in the province were not well documented.

Figure 2 shows the data spread over the block spot area. There was a significant correlation between the interval since the previous vaccination and antibody titer against the AI antigen subtype H5N1 Clade 2.1.3 and AI antigen subtype H5N1 Clade 2.3.2 (each *p*-value < 0.05). The values of the correlation coefficients were −0.61 and −0.43, respectively, indicating a strong correlation between the factors [22]. The closer the interval between the previous vaccinations and sampling, the higher the antibody titer response to clades 2.1.3 and 2.3.2 AI antigens.

Based on Figure 2, the highest antibody titer responses came from samples from South Sumatra Province, Banyuasin District and Palembang City, with a sampling interval of 1 week after the previous vaccination. Similar responses were recorded for representatives from the Province of North Sumatra, Deli Serdang District with an interval of 6 to 7 weeks after the previous vaccination. The other four regions, West Kalimantan, Bali, West Sumatra, Lima Puluh Kota Regency, and West Java, had lower average antibody titers due to long intervals since the previous vaccination interval of 6 o 41 weeks.

IgM levels peaked about seven days after antigen exposure. The presence of IgY could be detected in the serum 6 to 7 days after exposure. The IgM concentration began to decrease before IgY levels reached their peak, 10 to 14 days after antigen exposure. Although antibody levels then fell, they were usually still detectable 4 to 5 weeks after exposure [15]. In the first month post-vaccination, the protective titer was 80%, in the second month, it increased to 95%, but in the third month, the AI protective titer decreased to 75% [23].

There was a significant correlationship between the number of vaccinations and antibody titers against AI antigen subtype H5N1 Clade 2.1.3 and AI antigen subtype H5N1 Clade 2.3.2 (each *p*-value < 0.05). The correlation coefficient values were 0.45 and 0.31, respectively, which indicated a moderate positive correlation between the factors [22]. These results suggest that the higher the frequency of revaccination, the higher the antibody titer value for both clade 2.1.3 and clade 2.3.2 AI antigens.

Revaccination can form a secondary immune response showing a shorter lag phase and activate memory cells of the immune system so that there will be a faster and higher increase in antibody titers resulting in the production of an elevated antibody level [15]. Based on Figure 3, the high antibody titer response in serum samples from the Province of South Sumatra from Banyuasin District and North Sumatra Province from Deli Serdang District and Simalungun District was thought

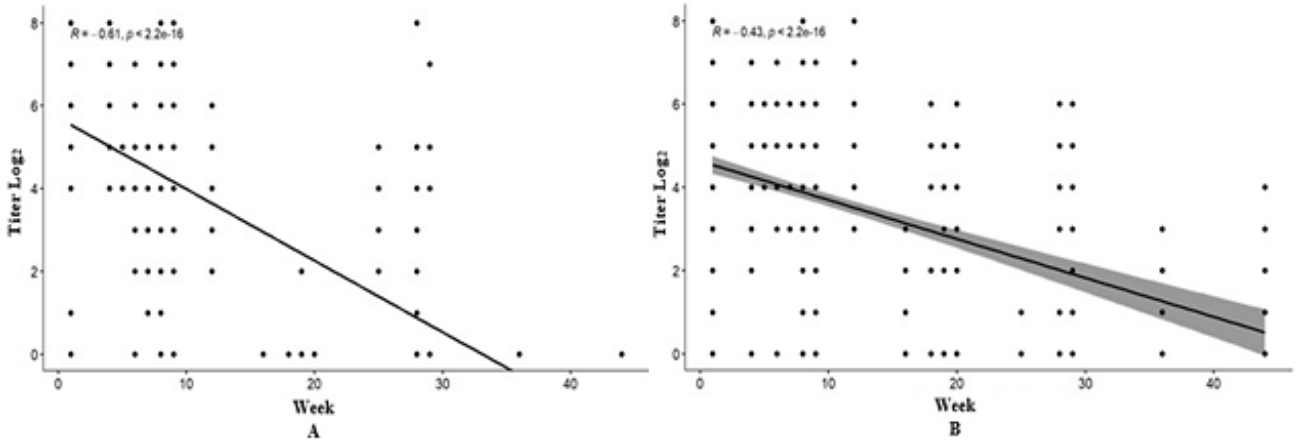

Figure 2. Correlation Between Antibody Titer and the Interval Between Sampling and the Previous Vaccination for AI Subtype H5N1 (A) Antigen Clade 2.1.3 9 and (B) Clade 2.3.2

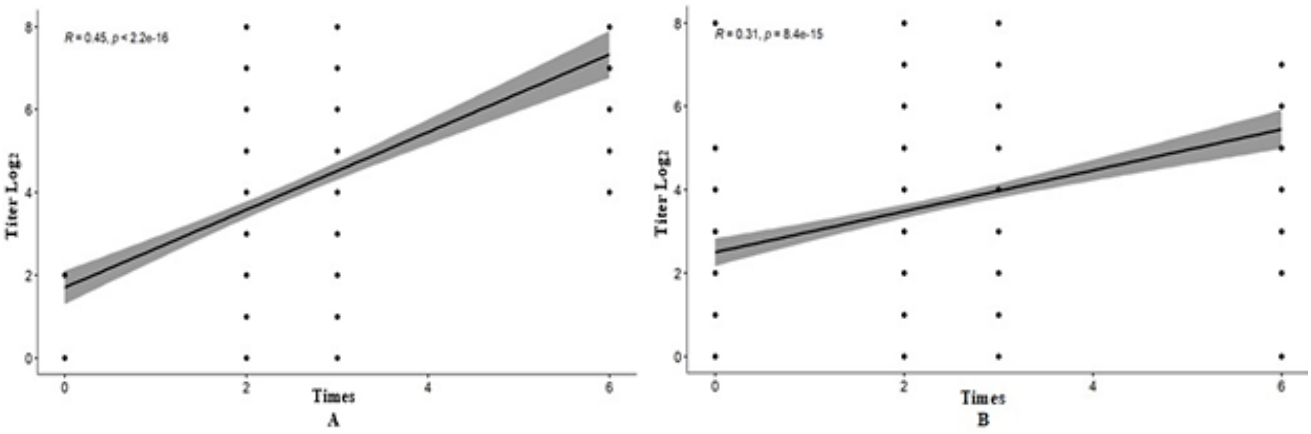

Figure 3. Correlation Between Antibody Titer and Vaccination Amount for AI Subtype H5N1 (A) Antigen Clade 2.1.3 and (B) Clade 2.3.2

to be due to higher number of repeated vaccinations of more than twice (i.e., three and five times) resulting in titers reaching a level of protection. Samples from the Provinces of South Sumatra (Palembang City), West Kalimantan, West Sumatra, Bali, and West Java were only vaccinated twice. With repeated vaccinations of three times before 18 weeks of age and two to three AI vaccinations after 18 weeks of AI vaccination, the antibody titers of chickens could reach >4log$_2$ [18]. AI vaccinations of five to six times in chickens are less economical than that of a total of three vaccinations, at six weeks, 16 weeks, and 45 weeks [24].

Based on observations of the conditions of sampled laying hen chicken farms, the farms had generally implemented a biosecurity program. The biosecurity program mainly involved cleaning the manure cage once a week to once a month, cleaning the cage once after 1 to 2 weeks, using disinfectants, and washing the cage equipment with water or using soap. However, restrictions related to the traffic of people on chicken farms were rarely implemented. The application of biosecurity and biosafety in livestock must be implemented to reduce HPAI infections [25–27].

AI serological monitoring of vaccination results is important for evaluation of AI vaccine efficacy. Immunity to AI is not absolute in the field and vaccinated chickens can potentially still be exposed to field viruses and become a source of virus contamination to the environment. Poultry farms usually rely only on vaccine manufacturers' recommendations to achieve protective herd immunity against infection with the H5N1 subtype AI virus [18]. However, AI serological monitoring of vaccination results is necessary to evaluate the efficacy of AI vaccines. Serological monitoring identifies the level of immunity of chickens on vaccinated farms and helps to identify factors that may cause vaccination failures. Based on the evaluation of the vaccination, the right time for revaccination can be determined to obtain optimal immunity in farmed chickens.

Conclusions

The results showed that the immunity induced by vaccination using the AI vaccine seed subtype H5N1 was above the protective value and the population was 60% immune if the booster vaccination was carried out more than twice, with the intervals of 4 to 5 weeks between sampling and the previous vaccination. This study showed a significant correlation (p-value < 0.05) between antibody titers and the length of interval between sampling and previous vaccination. In the AI post-vaccination surveillance program, it was necessary to take post-vaccination AI H5N1 vaccination samples, with the interval since previous vaccination of 4 to 5 weeks, so that the post-vaccination AI H5N1 antibody titer can induce immunity protective values >60%.

Acknowledgement

The author would like to thank the scholarships given by the Agricultural Human Resources Extension and Development Agency (BPPSDMP) and all the virology test unit staff at the National Veterinary Drugs and Assay Laboratory (BBPMSOH) of the Ministry of Agriculture.

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