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Avian Influenza H5N1 Isolate from Bali, its Safety and Potential as a New Vaccine Candidate

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ABSTRACT

Avian Influenza H5N1(AI-H5N1) is a highly contagious viral disease that causes high morbidity and mortality in chickens. As in many infected countries, the disease has been endemic in Indonesia since 2005, and vaccination to control it had massively been implemented but the outbreak has been frequently reported. One of the most critical of this condition was associated with the ability of the virus to mutate quickly leading to no protective antibodies, so it was crucial to find new locally viral candidates for a homolog vaccine. Fortunately, an AI-H5N1 isolate was successfully isolated in Bali the so-called A/Chicken/Bali9C/GAY/2019 and had been made for a new inactivated vaccine candidate and proven to be safely tested in a BSL-3 laboratory. However, this novel vaccine candidate has never been tested in the field, therefore, the aims of this research were to test the safety aspects of the vaccine in field. A total of 40 layers of Novogen Brow strain at 5 weeks old were used in this study and immunized once using the vaccine at the age of 5 weeks. Blood samples were collected one week before vaccination and repeated at one-week intervals for 5 weeks. The antibody response was tested using the HI test and hematological changes were examined using standard procedures. The results showed that antibody titers increased significantly from 1st to 4th weeks post-vaccination with a titer of ≥16 HI units, and after a further booster at week 4th, it reached a peak titer of about 256 HI units. Hematological data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's test using SPSS for windows. No significant difference (P>0.05) was observed in the hematological profile tested including total erythrocytes, hemoglobin, hematocrit, as well as the heterophils and lymphocytes ratio. The serological and hematological analyses suggested that this vaccine trial was valuable and safe coinciding with the clinical data that all the immunized chickens showed no clinical disease during the experimental studies. It was concluded that the A/Chicken/Bali9C/GAY/2019 had the potential to be used as novel and safe vaccine candidate, although other efficacy factors such as challenge studies and cellular immunity should be further evaluated.

Key words: Layer Chickens, Vaccination, AI-H5N1, Hematological Test, H/L Ratio.

INTRODUCTION

For many years, the avian influenza virus H5N1(AI-H5N1) has been considered as one of the most vital and fatal viral diseases in chicken, the so-called avian influenza (AI) (Kencana et al. 2021). The principal causative agent of the disease is the AI virus from the Orthomyxoviridae family which has potential zoonotic importance (OIE 2018). As in many infected countries, the disease is also endemic in Indonesia, and it is a very

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strategic infectious disease in chicken (Kencana et al. 2016; 2021). Based on their virulence, AI viruses can be classified into two, highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (OIE 2019). Avian influenza subtype H5N1 belongs to the group of a malignant type of AI, while the H9N2 subtype of type AI included in the group is not malignant. The introduction of Avian Influenza virus subtype H5N1 to Indonesia was estimated in 2003, which then spread widely to various parts of Indonesia. The dynamics of the development of the HPAI virus in Indonesia show significant changes including gene mutations, changes in pathogenicity, the phenomenon of escape mutants, and reassorting to the introduction of new types (OIE 2018). To control AI infection in Indonesia, many efforts and actions had been done, one of them was the application of combined ND-AI vaccines on chicken that resulted in high antibody titer of 5 logs 2 to 5 log 7 at two to four weeks post-vaccination (Kencana et al. 2014; Kencana et al. 2016). However, AI cases in the field had frequently been reported on those of vaccinated chickens which may be associated with mutation of AI subtype H5N1 (Ninyio et al. 2020).

Vaccination has become one of the main strategies for dealing with AI (OIE 2018), and therefore a good vaccine ideally possesses the ability to continuously stimulate relevant protective antibody formation (Balqis et al. 2011). Furthermore, a good vaccine should ideally possess genetic and antigenic homology that are homologous to the virus circulating in the area concerned (Mahardika et al. 2009). The success rate of AI vaccination is highly dependent on the degree of compatibility of the AI virus strain in the field, the vaccine used, the quality of the vaccine, the vaccination program, and its application (Kencana et al. 2014). AI vaccination often failed to give full protection which is associated to many factors (Suardana et al. 2023), mainly cold chain and antigenic drift of the virus. For this reason, monitoring and continuous research in the framework of the AI virus vaccine efficacy are crucial. In Indonesia, Kencana et al. (2020) have succeeded in isolating and producing an AI-H5N1 vaccine seed isolate from Bali the so-called A/Chicken/Bali9C/GAY/2019 as a vaccine candidate. Further research was done in collaboration with PT Sanbio Laboratories, Bogor, to evaluate the vaccine candidate's efficacy (Kencana et al. 2020). As the dynamics of the development of the HPAI virus has been very quick, the potential of AI vaccines needs to be monitored at least by checking the chicken antibody titer after the vaccination (Kencana et al. 2015). Vaccine safety also needs to be investigated because, in addition to being beneficial, vaccination can also cause stress in chickens (Khan et al. 2003). Natural stress on layer chickens could potentially cause a decrease in egg production by up to 25% and also affect the quality of the eggs produced (Prayitno and Sugiharto 2015). Stress due to vaccination is usually of short duration but may increase serum adrenocorticotropic (ACTH) and immunological effects that affect the growth of chickens (Wang et al. 2023). The adrenocorticotropic hormone affects lymphoid tissue that causes a decrease in lymphocytes and increasing the heterophile which also stimulates the secretion of corticosteroid hormones such as cortisol and corticosterone which is closely associated with poultry response to stress (Khan et al. 2003). Stress due to vaccination also increases cortisol and triggers the apoptosis of lymphocytes in the spleen (Li et al. 2020). The level of the hormone corticosterone is increased when poultry is under stress, which reduces weight gain and feeds conversion ratio (Prayitno and Sugiharto 2015). In broiler, the effect of stress was a decrease in muscle gain and an increase in belly fat accumulation (Wang et al. 2015). Ideally, any stressors should be minimal, especially during parenteral vaccination (Suartha et al. 2018) although they could not be avoided unless vaccination is provided orally, and the effect of the parenteral vaccination will also be evaluated in this experimental trial.

Physiologically, indicators of stress in chickens can be evaluated by analyzing the value of the percentage ratio of heterophils/lymphocytes (H/L) (Sugito and Delima 2009; Cotte 2015). The H/L ratio determined body resistance in poultry, the H/L ratio of 0.2, 0,5 and 0,8 were considered as low, normal and high categories respectively (Apriliyani et al. 2013), although Prayitno and Sugiharto (2015) stated that the normal ratio of H/L was 0.4. In stress condition, changes in H/L were associated with the elevated secretion of adrenal gland hormones such as corticosterone, mainly glucocorticoid hormone that stimulates lymphocytes which enter blood circulation to the skin, spleen, and lymph nodes (Chourpiliadis and Aeddula 2022). At the same time, heterophils from the bone marrow also enter the blood circulation (Cotter 2021). Furthermore, H/L ratio has mostly been used in physiological research as a simple but convincing measure of physiological stress (Minias 2019). In this study, the potential and safety aspects of a vaccine candidate contained an A/Chicken/Bali9C/GAY/2019 isolate in layer chickens were investigated based on the evaluation of antibody response and hematological changes (erythrocytes, hemoglobin, and hematocrit) and stress indicators of the heterophile to lymphocytes ratio (H/L) post-vaccination.

MATERIALS AND METHODS

Ethical statement

This research has been approved by the Ethical Commission at Udayana University, Denpasar Bali of Indonesia, with letter No. B125/UN14.2.9/PT.01.04/2021.

Research design and sample

A total of 40 layers of chicken of the Novogen Brown strain were used in this study and kept since day-old chicken (DOC) in a closed house cage. After 5 weeks of age, the 40 layers of chickens were injected intramuscularly with 0.5 ml/head inactivated vaccine containing HPAI isolate of A/Chicken/Bali9C/GAY/2019 and kept in grower cages. Blood samples were taken aseptically once before vaccination and five times after vaccination at weekly interval using a tube containing ethylene diamine tetra acetic acid (EDTA).

Blood examination

The serum samples were tested using the HI test (Kencana et al. 2018a), meanwhile, the blood samples

were tested for total erythrocytes, hemoglobin levels, hematocrit, and leukocyte differential, using the published methods (Feldman et al. 2000). All tests were done at disease investigation center (DIC) Denpasar with ISO: 17025:2008 protocols. Antibody tires were assessed with Hemagglutination Inhibition (HI) test.

Data analysis

On the other hand, hematological data of erythrocytes, hemoglobin, and hematocrit were analyzed for one-way analysis of variance (ANOVA) followed by Duncan's test. Leukogram data (the result of leukocyte calculation) were analyzed for one-way analysis of variance (ANOVA) (Khan et al. 2003; Cotter 2015), and the IBM statistical product and service solutions (SPSS) version 25 was used.

RESULTS

The HI test revealed the gradual increase of antibody titer from 1st to 4th weeks post-vaccination with titer of 2 log 4.5 (≥16 HI units). Moreover, after a further booster was administered at week 4th, antibody titers increased sharply and reached a peak titer of 2 log 7.9 (256 HI units) at week 7 (Fig. 1).

Analysis of the mean-variance of the hematological profile of layer chickens' post-vaccination with A/Chicken/Bali9C/GAY/2019 isolate is presented in Table 1. Analysis of variance of total erythrocytes (Table 1) showed that the vaccine contained the A/Chicken/Bali9C/GAY/2019 isolate had no significant effect (P≥0.05) on total erythrocytes in layer chickens with a value of significant 0.69 and slightly fluctuated during vaccine experiment, but they were in the normal range. Analysis of the variance of hemoglobin levels showed a significant difference (P≤0.05), but also in the normal range, so Duncan's test could be continued (Table 2). Similarly, analysis of hematocrit variance showed a significant effect (P≤0.05) on the hematocrit value and was in the normal range so that Duncan's test can be continued as shown in Table 2.

The absolute mean of heterophils, lymphocytes, H/L ratio, and sampling time are presented in Table 3. The average results showed that the stress effect of using the A/Chicken/Bali9C/GAY/2019 isolate was not significantly different (P>0.05).

As can be seen from Table 3, heterophil profile at the 3rd and 4th weeks post-vaccination showed significant difference (P≤0.05) compared to week 0 (pre-vaccination), 1st, 2nd, and 5th weeks post-vaccination. Heterophils and lymphocytes profiles post-vaccination were statistically higher with significant difference (P≤0.05) at 1st, 3rd, 4th, and 5th weeks post-vaccination but no significant difference (P≥0.05) in 2nd week. The average H/L ratio at 0 week (pre-vaccination) was 0.44±0.06. The highest H/L ratio occurred in the 4th week with a value of 0.59±0.06, and the lowest mean H/L ratio occurred in the 5th week with a value of 0.36±0.06. The graph of the pre-vaccination and post-vaccination H/L ratio values from 1st to 5th week is shown in Fig. 2.

DISCUSSION

The AI vaccination program for layer chickens in the field is frequently carried out by giving more than one vaccination, to get the secondary immune response, using the same immunogen (Suardana et al. 2009). The emergence of post-vaccination antibodies will result in changes to blood components (Wei et al. 2022), particularly a clear change in the white blood cells that function as the forerunner of forming antibodies but did not significantly affect the red blood cells (Perumal et al. 2021). Roit (2011) and Tizard (2000) stated that vaccination resulted in an increase in blood protein and returned to normal in three to five weeks after vaccination so that the blood picture also changed. Potential and safe vaccines are demonstrated by the production of high titer of protective antibodies without any side effects on vaccinated animals. In this study, a vaccine candidate containing the A/Chicken/Bali9C/GAY/2019 isolate released a high titer of antibodies, higher than a recommended titer of ≥16 HI units at a single vaccination for at least 4 weeks and a higher titer of 256 HI units after the second vaccination, suggesting the vaccine possessed immunogenic proteins as the potential vaccine component (Chaudhary et al. 2021). Moreover, the vaccine had no significant effect (P≥0.05) on the total erythrocyte post-vaccination. In other words, the antigen

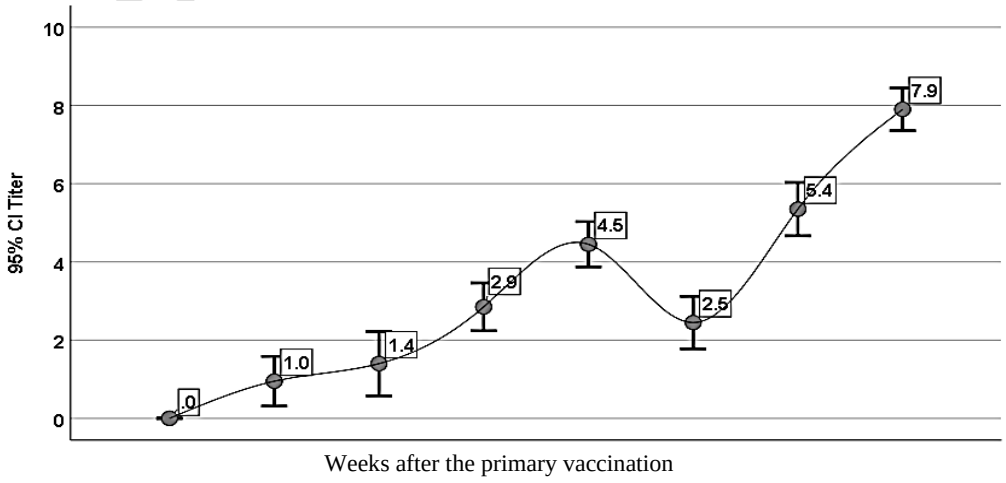

Fig. 1: Titers antibody following vaccination showed a gradual increase to a pick of 7.9 at week 7.

Table 1: Results of the Analysis of the Mean Variety of Hematological Profiles of Layer Chickens and Significance Values Post Vaccination with A/Chicken/Bali9C/GAY/2019 isolate.

Weeks	Parameters		
	Erythrocytes ($\times10^6$/µL)	Hemoglobin (g/dL)	Hematocrit (%)
0	2.26±0.11	9.04±0.39^{b}	24.90±1.77^{a}
1	2.23±0.11	9.08±0.47^{b}	25.95±1.43^{b,c}
2	2.21±0.13	8.75±0.48^{a,b}	26.10±1.07^{b,c}
3	2.30±0.11	9.12±0.55^{b}	26.75±1.83^{c}
4	2.23±0.45	8.36±1.53^{a}	25.60±1.31^{a,b}
5	2.30±0.10	8.59±0.41^{a,b}	24.85±1.78$^{a.}$
Normal value	2.0-3.5[1)]	7.0-13.0[1)]	22-35[1)]
Significance	0.691	0.008	0.001

Description: 1) Schalm (2010) Note: Week 0 was pre-vaccination, weeks 1, 2, 3, 4, and 5 were post-vaccination. Different letters (superscripts) show significant difference (P≤0.05) Meanwhile, the same letters (superscript) show no significant difference (P>0.05).

Table 2: Results of Duncan's Test of Hemoglobin Levels and Hematocrit Values of Layer Chickens Post Vaccination with A/Chicken/Bali9C/GAY/2019 isolate.

Weeks	Hemoglobin (g/dL)	Hematocrit (%)
0	9.04±0.39^{b}	24.90±1.77^{a}
1	9.08±0.47^{b}	25.95±1.43^{b,c}
2	8.75±0.48^{a,b}	26.10±1.07^{b,c}
3	9.12±0.55^{b}	26.75±1.83^{c}
4	8.36±1.53^{a}	25.60±1.31^{a,b}
5	8.59±0.41^{a,b}	24.85±1.78^{a}

Note: Different letters (superscripts) show significant difference (P≤0.05). Meanwhile, the same letters (superscript) show no significant difference (P>0.05). Week 0 = pre-vaccination 1st, 2nd, 3rd, 4th, and 5th week = post-vaccination.

Table 3: Average absolute number of heterophile cells, lymphocyte cells and H/L ratio.

Weeks	Variable		H/L
	Heterophile ($\times10^3$/µL)	Lymphocytes ($\times10^3$/µL)	
0	22.31±2.36 ^{a}	51.69±2.44 ^{a}	0.44±0.06
1	23.39±2.36 ^{a}	56.48±2.44 ^{a,b}	0.45±0.06
2	23.25±2.36 ^{a}	49.06±2.44 ^{a}	0.48±0.06
3	28.96±2.36 ^{a,b}	54.00±2.44 ^{a,b}	0.57±0.06
4	31.42±2.36 ^{b}	59.91±2.44 ^{b,c}	0.59±0.06
5	23.10±2.36 ^{a}	65.98±2.44 ^{c}	0.36±0.06

Note: Different letters (superscripts) in the same column indicate significant difference (P≤0.05). Conversely, the same letters (superscript) show no significant difference (P≥0.05).

did not adversely affect red blood cell count that was in the normal range with an average of 2.21-2.30×10^6/µL coinciding with the normal value of erythrocytes in chickens of 2.0-3.2x10^6/µL, with an average of 3.0x10^6/µL (Schalm 2010). Similarly, hemoglobin and hematocrit values were also within the normal threshold, indicating no failure associated with oxygen transported to the tissue (Guyton and Hall 2010). Taken together, the mean of total erythrocytes, hemoglobin, and hematocrit levels detected in this study were within the normal threshold, confirming that the antigen used was safe. Furthermore, normal values of hematological changes observed in this study, implied the continuity of oxygen and nutrient transport in the body in accordance with the health performance of immunized chickens (Etim et al. 2013). Abasi et al. (2014), stated that a low hematocrit value indicates that the chicken is anemic, while an increase in the hematocrit value indicates that the chicken is dehydrated. An increase or decrease in the value of the hematocrit in the blood will have an impact on the viscosity (thickness) of the blood (Kishimoto et al. 2020) which may cause circulation disorder associated with oxygen deficiency (Cunningham 2002).

Measuring the H/L ratio is generally a well-recognized indicator of stress in poultry. In the current study, we did not find any significant effect of the antigen used for the vaccine trial. In the 1st week post-vaccination, there was an increase in the H/L ratio from 0.44±0.06 to 0.45±0.06 but statistically within the normal range. Furthermore, the application of the inactivated vaccine of A/Chicken/Bali9C/GAY/2019 isolate was administered intramuscularly so it did not cause stress and vaccination was able to increase the immune response. Inactivated vaccines have a longer duration immunity compared to active vaccines (Speiser and Bachmann 2020), associated with the valuable effect of an oil adjuvant content in the vaccine that functions as an antigen depot so that the vaccine antigen is released slowly (Aiyer-Harini et al. 2013; Kencana et al. 2016; Bhakty et al. 2018). This condition was confirmed by the significant increase of total lymphocytes post-vaccination, suggesting activation of both humoral and cellular immune responses, although in future challenge studies are crucially required to confirm the efficacy of the current vaccine candidate (Sadarangani et al. 2021).

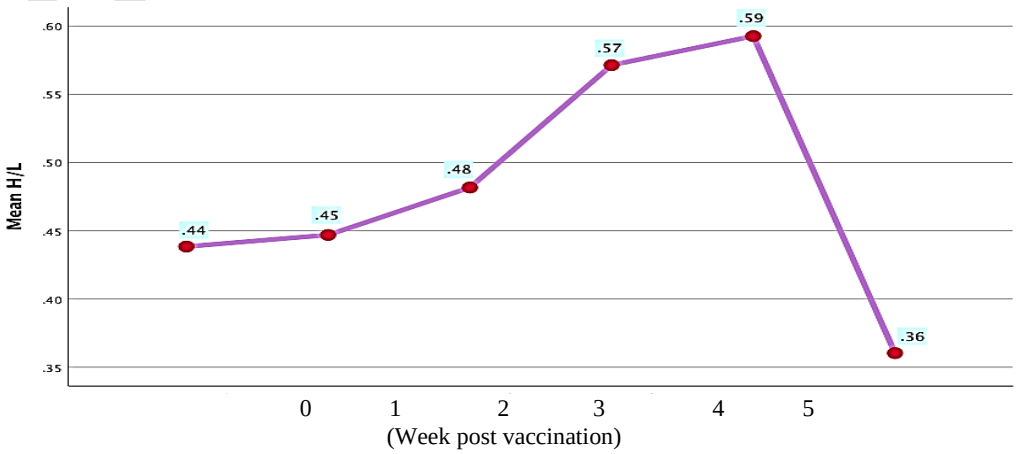

Fig. 2: H/L Ratio Post-Vaccinated with A/Chicken/Bali9C/GAY/2019 isolate.

In the 2nd week post-vaccination, there was an increase in the value of the H/L ratio from 0.45±0.06 to 0.48±0.06 which was still at zero standards. Whalan (2015) stated that stress can occur because animals experience leukopenia, lymphopenia, and heterophils. However, the results reported here indicated that chickens did not have heterophile. Stress in chickens can increase corticosteroid hormones (Abo-Al-Ela et al. 2021), these hormones can cause leukopenia in the first hour and leukocytosis at 4-12 hours of vaccination (Kencana et al. 2018b) which may cause the H/L ratio to be in a normal state (Dhama et al. 2020). The layer chickens in this study did not experience stress even though there was a decrease in lymphocytes and heterophil cells which were still at normal standards. In contrast, significant changes in the ratio of H/L from 0.11 to 0.54 in vaccinated poultry were considered mild stress markers (Ka'b 2017). The researcher used a vaccine without adjuvant and applied an intra-ocular route that may cause a moderate stimulation of the acute phase and stress response. In this study, mild stress occurred in the 3rd and 4th weeks after vaccination but statistically, the H/L ratio was not significantly different. In general, stress may be due to several factors including feed, temperature, cage density, transportation, vaccination, pathological conditions such as ascites, coccidiosis, and arthritis (Panda and Cherian 2014; Akiyemi and Adewole 2021), noise (Elitok and Bingular 2018), handling and presence of new people (Kencana et al. 2018b). It was unclear what caused the mild stress that occurred in the 3rd and 4th weeks post-vaccination observed in this study. It was probably due to the frequent blood sample collection that was done at weekly intervals as well as the minor unwanted side effect (Oberländer et al. 2020) of the A/Chicken/Bali9C/GAY/2019 isolate. Vaccination generally causes mild stress in chickens and it is temporary in nature but has the effect of increasing immunity (Gottstein et al. 2015; Li et al. 2020).

Increased heterophile also occurred in the 3rd and 4th week post-vaccination which may be due to the induction of the hypothalamic-pituitary-adrenal axis (Hassan and El-Diem 2016) which further induces glucocorticoids and also results in the release of spare heterophils in the bone marrow (Sugito and Delima 2009). Heterophilic due to acute stress can enhance immunity by mobilizing heterophile into the bloodstream and making them available in larger quantities for recruitment and activation of inflammation (Tang et al. 2022). Heterophiles play an important role in the inflammatory reaction, increasing the recruitment of heterophils that can increase the inflammatory phase of the innate immune response (Dhabhar 2002). Clearly, the used of A/Chicken/Bali9C/GAY/2019 isolate as a new vaccine candidate in this study was characterized by a decrease in the H/L ratio from 0.59±0.06 to 0.36±0.06, and a decrease in heterophile cells from 31.42±2.36x10^3/µL to 23.10±2.36×10^3/mL, and an increase of lymphocytes total from 59.91±2.44x10^3/µL to 65.98±2.44×10^3/µL, suggesting the antigen under study was safe, did not cause significant stress on the immunized chicken, although blood samples were collected at weekly intervals and it potentially triggered the appearance of immune responses.

Conclusion

The Avian Influenza H5N1 isolate from Bali, the A/Chicken/Bali9C/GAY/2019 isolate investigated in this study based on the production of high titer of desired antibodies and hematological analysis, was considered safe and could potentially be used as a new vaccine candidate. This finding could essentially overcome vaccination problems associated with the ability of the AI virus to mutate quickly in nature. Further work is required to confirm the efficacy factors of the vaccine including challenge studies, cellular immunity, and duration of immunity.

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Author's Contribution

All authors were actively involved with different responsibilities. Gusti Ayu Yuniati Kencana and Nyoman Suartha: preparing research proposal and completion of the manuscript; Putu Teza Juliantari and Kevin Tri Tama: collecting sample; Anak Agung Sagung Kendran and Tegar Aprilian: statistical analyses, Wayan Masa Tenaya and Kadek Karang Agustina: laboratory testing and analyses.

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