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Research Article

Construction of Indonesian-Strain Avian Flu Virus Seed Vaccine Using Low Pathogenic Hemagglutinin Gene and Neuraminidase Pr8 Gene through Reverse Genetics

Abstract

Avian Influenza H5N1 has been spreading in Indonesia since 2003. The Avian influenza virus H5N1 actually infects various animals, including birds, mammalian and others. Since 2005, avian influenza virus has infected humans. According to data, there are 132 persons suspected of contracting this virus, and this was one of the highest cases of avian influenza H5N1 outbreak in the world. One of the means of protection against infection is vaccination. Until now there is no H5N1 influenza vaccine extant, because the virus has a high level of pathogenicity, which makes it difficult to obtain a high yield of seed vaccine.

In this research, we constructed H5N1 virus with low pathogenic Hemagglutinin gene and neuraminidase gene from PR8 (H1N1) using reverse genetic method. The new virus then inoculated in embryonated chicken eggs. We tested the H5N1 virus reverse genetic using molecular methods such as PCR and sequencing; and we used EID50 and Intravenous Pathogenicity Index for the pathogenicity test. The new H5N1 virus reverse genetic was also tested in mice to observe the antibody's titer and challenge.

The result, we found that the low pathogenic H5N1 virus reverse genetic was successfully created. Neuraminidase from PR8 played important role in reducing pathogens in chickens and mice. From molecular data we note that no amino acids were changed, the pathogenicity of H5N1 virus reverse genetic was low, with an Intravenous Pathogenicity Index of 0 and the highest titer was 256 GMT and the highest survival rate was 85.71%.

Abbreviations

AI: Avian Influenza; AFC: Antibody Forming Cell; APC: Antigen Presenting Cell; CD-8: Cluster of Differentiation 8; CTL: Cytotoxic T Cell; GFP: Green Fluorescence Protein; HA: Hemaglutinin; HA0: Hemaglutinin 0; HA1: Hemaglutinin 1; HA2: Hemaglutinin 2; HAU: Hemaglutinin Unit; HEK: Human Embryonic Kidney; HI: Hemaglutination Inhibition; HPAI: Highly Pathogenic Avian Influenza; IFN-γ: Interferon γ; IgA: Imunoglobulin A; IgE: Imunoglobulin E; IgG1: Imunoglobulin G1; IgG2a: Imunoglobulin G2a; IL-1β: Interleukin-1β; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-5: Interleukin-5; IL-12: Interleukin-12; IVPI: Intravenous Pathogenicity Index; LPAI: Low Pathogenic Avian Influenza; NA: Neuraminidase; PBS: Phosphate Buffer Saline; PCR: Polymerase Chain Reaction; PR-8: Puerto Rico 8; RG: Reverse Genetic; RNA: Ribonucleic Acid; SPF: Specific Pathogen Free; Th1: T helper 1; Th2: T helper 2; VLP: Virus Like Particle; WHO: World Health Organization

Introduction

The Avian Influenza (AI), or better known in Indonesia as *flu burung* ("Bird's Flu"), has spread in Indonesia since mid-2003. Initially, the AI virus infected animals (poultry, mammals, etc.). However, since 2005, AI has begun to infect humans. According to the latest data, the number of suspect human victims infected by Avian Influenza in Indonesia were 199 people [1], although in 2017 no human case reported but the number of death people in Indonesia caused by H5N1 virus still the largest number in the world.

Prevention and treatment of Avian Influenza in humans in Indonesia has been facing several obstacles ranging from disease sources such as poultry and other animals have not

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been fully controlled, until the understanding of the people recognize the early symptoms of Avian Influenza disease, which is difficult to distinguish from other influenza, as well as antiviral Avian Influenza not yet available in the community. Generally fatal victims were caused late in handling this AI disease [2].

The readiness of prevention and medication actions against this disease is necessary, and all efforts using Indonesia's potentials must be made. One of the most effective methods of prevention is through vaccination program. Therefore, if the control of Bird's Flu infection in humans uses the vaccination program, one of the requirements that must be met is the availability of seed vaccine that is already, and that can be adapted, to the human body, so that they can trigger the optimum specific antibodies in order to minimize or eliminate the infecting virus [3].

Based on its pathogenicity, avian influenza virus can be distinguished into two namely Low Pathogenic Avian Influenza (LPAI) with mild clinical symptoms, such as H7N9 subtype, and Highly Pathogenic Avian Influenza (HPAI), such as H5N1 subtype with more severe clinical symptoms than LPAI [4,5].

The constraints faced by the world today are virtually evolved nature of the virus, so its pathogenicity is unstable. The presence of antigenic drift in the H5N1 virus may affect the production of influenza vaccine, resulting in the production of caution in the selection of viral seed vaccines.

The considerations of producing good human Avian Influenza H5N1 vaccine are determined not only by the accuracy of selecting H5N1 virus as seed vaccine, but also by efficiency at production level. Avian Influenza virus H5N1 is highly pathogenic: if inoculated to embryonic chicken eggs as virus culture medium (egg based vaccine), it would soon kill the chicken egg's embryo. Consequently, high virus titration efficiency rate could not be obtained. One way to get a stable influenza vaccine seed is by reverse genetic method. This method is an attempt to reconstruct a virus from copying a full-length genome into a new virus clone as programmed. This method is commonly used in the field of modern virology with genetic control. The use of reverse genetic coupled with *in vitro* mutagenesis can be applied to accelerate progress in understanding the life cycle of influenza viruses, generate viruses for live-attenuated vaccines and use of influenza viruses as vaccines and gene delivery vectors.

The principle of the reverse genetic method is to eliminate the pathogenicity nature of the H5N1 virus used as a vaccine seed without losing its immunogenicity, so that seed vaccines are found to be capable of triggering specific antibodies against influenza viruses, especially H5N1 but not harmful to vaccinated individuals. How to eliminate the viral nature of the virus by reverse genetic method was by removing some amino acid arrangement in the multi-amino acid HA cleavage site of the seed of the virus, through the stages of mutagenesis virus seed vaccine, thus, pathogenicity will turn into non-pathogenicity. The result of the HA gene, which has been removed from the amino acid, was reconstituted into a new virus (rescue virus) ready to be propagated as a vaccine seed.

Airlangga University, through the Avian Influenza-zoonosis Research Center (AIRC), was able to develop and generate Avian Influenza H5N1 virus seed vaccine with original Indonesian strain in 2011 (unpublished). The preparation of Avian Influenza H5N1 virus seed vaccine construction has yielded a ratio of 1 (one) embryonic chicken's egg for 1 (one) dose. The construction was made of a genome order comprising of HA and NA genes of Avian Influenza H5N1 virus that infected humans in Indonesia in 2007. HA genes initially undergo *in vitro* mutagenesis, while the NA gene was not treated and match the original genome. Meanwhile, other genes were originated from PR-8 virus according to WHO recommendation. Even though the result was good, the yield of the propagation of the virus to be produced must be increased in order to satisfy mass-production requirements in the industry.

Therefore, efforts are needed to increase yields compared to previous construction. One of them was by replacing the position of Neuraminidase (NA) fragments that had been obtained from natural avian influenza virus with NA gene from non-pathogenic influenza virus, such as H1N1 PR8 and Seasonal H1N1 Flu viruses using reverse genetic method.

Based on research [6], it was mentioned that the difference in replication of reassortant virus in embryonated chicken eggs increased by replacing at least 2 HA residues (G186V and V226I) to improve HA receptor attachment capacity or by replacing at least 2 NA (E119Q and Q136K) residues to decrease NA enzymatic activity, so a balance between HA and NA activity were critical for the replication of influenza viruses in a different host. Until now, no preparation of avian flu virus seed virus had been done by using NA fragment from low pathogenic virus.

This study was expected to obtain the avian influenza H5N1 virus seed with higher viral yield compared with the construction of the avian flu H5N1 virus earlier in 2011, by altering the construction of the AI virus genome of avian influenza virus by replacing the neuraminidase genes from the HPAI Avian Influenza virus with NA gene derived from LPAI.

Materials and Methods

Cells and viruses

Human embryonic kidney 293T (HEK-293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 5% FBS.

Generation of vaccine seed viruses

To generate reassortant viruses, we used plasmid-based reverse genetics (Neumann et al., 1999). Viral RNA from D42007 was extracted from allantoic fluid by using a commercial kit (Qiagen) and was converted to cDNA using reverse transcriptase (SuperScript III; GIBCO-BRL) and primers based on the consensus sequences of the 3-prime ends of the RNA segments for the H5 viruses.

By using an inverse PCR method, we altered the HA cleavage site sequence (RERRRKKR) of the wild-type virus to create

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the avirulent type sequence (RETR), as described previously [7]. Seven pPolI plasmids, each containing an internal gene of PR8(Cambridge) that were constructed from NIBRG-14. Using these PolI plasmids, we conducted reverse genetics (12-plasmid) systems [8]. Briefly, a total of 12 plasmids containing 8 PolI and 4 plasmids expressing PR8(UW) NP, PA, PB1, or PB2 were transfected to 293T cells [9]. Supernatants containing infectious viruses were harvested and used in further experiments.

In Vitro virus replication

The Avian influenza virus reverse genetic (RG) that had been harvested from the 293T cells, then adapted to embryonated chicken eggs, was purified. Avian influenza virus (RG) was dissolved in PBS isotonic buffer fluid with pH 7.0-7.4 containing antibiotics. Antibiotics were used: penicillin 2000 units per ml, streptomycin 2 mg per ml, gentamicin 50 ug per ml and mycostatin 1,000 units per ml. This suspension was incubated for one hour and inoculated virus solution in embryonated chicken eggs and incubated at 37° C.

The death of the embryo was observed every day, up to the third day after inoculation. The fourth day, all embryonated chicken eggs were stored at 4° C for 24 hours. All eggs were harvested by allantoic, then Hemagglutinin test (HA test) was performed.

Pathogenicity studies in chickens

The procedures of IVPI was the allantoic fluid of the influenza virus with a HA titre greater than 1/16 diluted 1:10 in sterile isotonic fluid. From the dilution of the virus each of chickens SAN (Specific Antibody Negative) then injected 0.1 ml. The chickens were observed at intervals of 24 hours for 10 days. At each observation, the chickens were scored '0' if normal, '1' if they looked sick, '2' when they felt severe pain, and '3' if the chickens were dead.

Calculation of IVPI was an average per chickens per observation over a period for 10 days. An index value of 3.00 means that the chickens died within 24 hours; whereas an index value of 0.00 means that no chickens showed any clinical symptoms during the 10 days of observation [10].

Pathogenicity studies in mice

The mice used in this study were specific pathogen free (SPF) and strain BALB/C (*Mus musculus*) mice. The study was done in the cages insulators in Animal BSL - 3 facilities. To conduct immunology studied, we performed vaccination using mice that were divided into five groups, wherein each group consists of 7 mice. For challenge studied we performed anaesthetic from 0.03 to 0.04 ml of a mixture of 5 ml ketamine HCl (100 mg//ml) and 1 ml xylazine (20 mg//ml). Furthermore, the virus was infected intranasal as much as 0.5 ml with a concentration of 10^7 TCID - 50 [11].

Results and Discussions

Construction of low pathogenic avian influenza virus

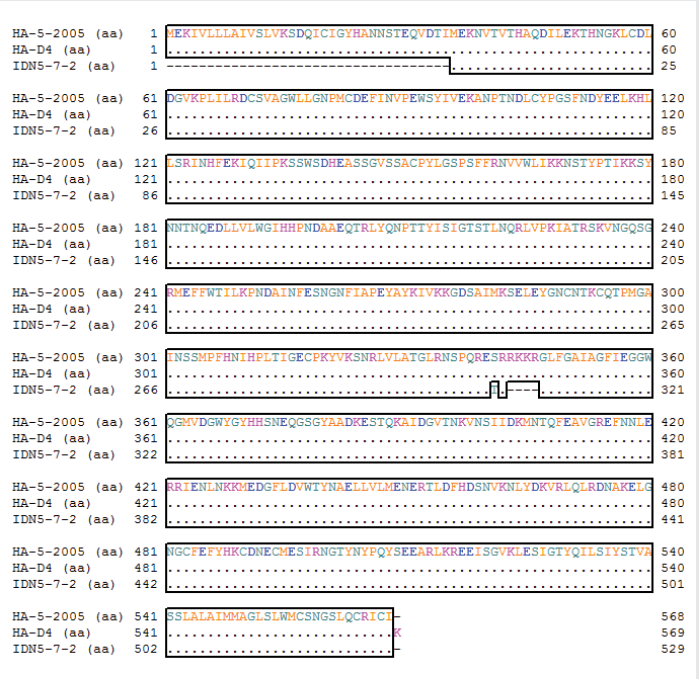
The construction of avian influenza virus (RG) in this study used the reverse genetic method, which was one of the latest molecular biology techniques, especially in studying Influenza virus. Using this method, we were able to develop and improve prophylactic and therapeutic intervention against influenza virus [12].

The hemagglutinin gene used to construct low pathogenic avian influenza virus was HA gene of highly pathogenic H5N1 Indonesia strain (A//Indonesia//D4//2007 (H5N1)). It was then transformed into low pathogenic strain using reverse genetic. Mutagenesis was done by eliminating (deleting) a nucleotide sequence that encodes the amino acid lysine (K) and arginine (R), and by changing (substituting) amino acid sequence that encodes the amino acid serine (S) to threonine (T) at the cleavage site, so the sequence of amino acid, originally RRKKR (poly lysine and arginine), changed into RETR.

The result of nucleotide sequencing from this mutation process as shown in Figure 1, which showed the amino acid in the region 360 of the Hemagglutinin (HA) protein of the wild type H5N1 which was original virus had deletion of amino acid RRKK and substitution of amino acid S (serine) to T (threonine).

Based on the analysis of amino acids, we did not find the mutation in the amino acid sequence of the HA gene cleavage site, except in the regions that were expected to change as desired.

Figure 1: Alignment of HA-H5 gene. Amino acid alignment of HA-H5 –RG and original virus H5-D4. HA-D4 was original strain and IDN5-7-2 was low pathogenic mutated HA. Amino acid reference HA-5-2005 was taken from GenBank accession no. CY116646, while HA-D4 from AIRC collection data.



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The results of this study, which was performed in accordance with the methods recommended by WHO guidelines, show that reverse genetic methods and recombination of mutagenesis cleavage site of HA genes to eliminate the virulence of H5N1 virus are further tested for their pathogenicity in embryonated chicken eggs and using the Intravenous Pathogenicity Index [13].

Construction of low pathogenic neuraminidase (NA) avian influenza virus

This was studied using neuraminidase (NA) gene derived from the H1N1 virus Puerto Rico, (A//Puerto Rico//08//1934) H1N1, which was obtained from the Institute of Medical Science of the University of Tokyo, Japan. NA gene was then inserted in pHH21 plasmid for vRNA together with seven other influenza virus genes (HA, NP, NS1, PB1, PB2, PA, M).

The plasmid combination of pHH21 - NA was reproduced through cloning by using *E.coli* DH5α, in order to discover the cloning process and the combination of electrophoresis. The best colony was then taken to be used as transfection agent. Plasmid - NA pHH21 of 4500 bp. The clone was then sequenced to find out the sequence of nucleotides and amino acids, in order to ensure that these clones and combinations did not undergo either undesirable nucleotide or amino acid mutations.

Figure 2: Alignment of neuraminidase gene. Neuraminidase gene sources with accession no. EF190984 (NA-PR8-LV), EF190976 (NA_PR8-HV) and AB671290 (NA-PR8-Tokyo) were obtained from GenBank.

Alignment result from the NA-PR8 gene plasmid showed no amino acid difference compared to the original NA-PR8 gene (NA-PR8-Tokyo), so the NA-PR8 gene plasmid could be used for transfection in subsequent studies.

Previous research had shown that virus neuraminidase largely had the role of releasing and spreading replicated virus in cells [14]. Sources of neuraminidase originally derived from the H5N1 virus was converted to a neuraminidase derived from H1N1, thereby increasing the low pathogenic avian influenza virus (RG). The neuraminidase gene selected in this study was neuraminidase PR8 gene because it was low pathogenic. The neuraminidase gene had a sequence as well as a length on the stalk region of the region that varies depending on each influenza virus. Based on [15], the balance of HA and NA gene function in an influenza virus greatly influences the growth of the virus. Therefore, in this studied we picked neuraminidase from PR8 that could increase viral yield and had high immunogenicity level so that it could be used as seed vaccine influenza H5N1.

In Vitro virus replication

In the transfection process, we used 293T cell as propagation media. 293T cells were eukaryotic (human embryonic kidney) cells, which leads to the transcription of the reporter gene by cellular RNA polymerase I, thereby generating influenza virus-like RNA. Based on the research results of [8], which showed that cells 293T transfected with plasmids encoding a structural protein of influenza A viruses; and with plasmids encoding influenza virus-like vRNA (which contains a copy of antisense of cDNA GFP (Green Fluorescence Protein)); were flanked by the promoter of RNA polymerase I and terminator, generated more than 104 VLP (Virus Like Particle) influenza containing GFP vRNA. This means that 293T cells could be used as a host in the process of influenza a virus transfection.

The result of EID_{50} titer for viral propagation, originating from inoculation of embryonated chicken eggs, generated titer for Avian Influenza virus (RG) low pathogenic (HA-H5 (RG)/NA-PR8) was 1.9304 x 10^5/ml. The resulting titer was lower than the titer of avian influenza virus (RG) low -high (HA-H5 (RG)/NA-H5), which was 3.1623 x 10^7/ml. Complete data of each measurement titer can be seen in table 1.

Hemagglutinin and neuraminidase had an important role in penetration and replication in vaccine development media and bullying the emergence of an expected immune response. As previous research had shown, the role of Hemagglutinin and neuraminidase was always synergistic [6]. Hemagglutinin proteins will bind to surface receptors and envelop viruses that hydrolyze hemagglutinin proteins into HA1 and HA2 subunit proteins, then interact with the host membrane and cause membrane fusion.

Differences of hemagglutinin proteins derived from highly pathogenic and low pathogenic viruses were determined by the presence of amino acid polybasic on cleavage sites. The role of protease enzymes in the host cell was to hydrolyze the cleavage

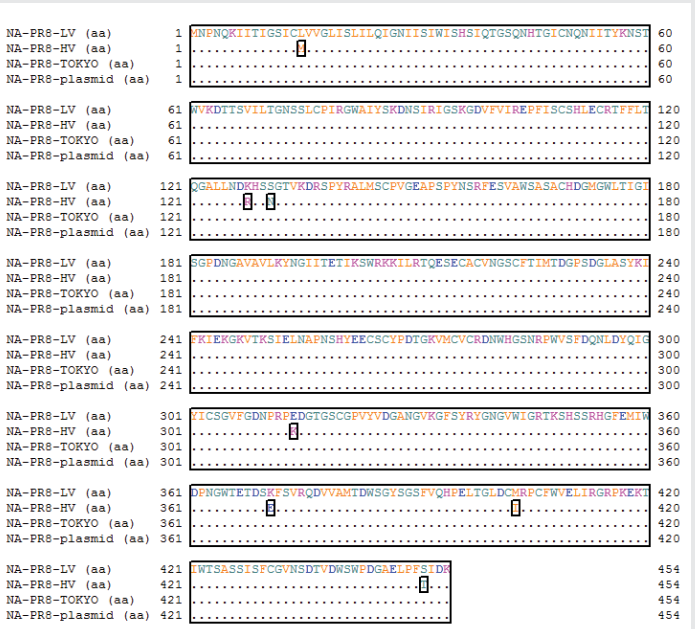

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Table 1: EID_{50} of Influenza H5N1 virus (RG).

Virus	Concentration (per mL)
	EID_{50}
HA-H5 (RG)/NA-PR8	1.9304 x 10^5
HA-H5 (RG)/NA-H5	3.1623 x 10^7

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site to HA1 and HA2, leading to highly pathogenic infection and systemic conditions (the virus spreads throughout the body through the bloodstream). In contrast to low pathogenic viruses did not cause systemic conditions [16,17].

Neuraminidase was a type II membrane protein that acted as a plasminogen receptor on the cell surface and participates in the conversion of plasminogen to plasmin which can lead to hydrolysis of the cleavage site by plasmin, so that the role of NA protein can determine highly pathogenic or low pathogenic influenza a virus [18]. Although no direct tests were conducted on the role of plasminogen and plasmin enzymes after the replacement of neuraminidase sources from highly pathogenic, low pathogenic influenza A viruses, the pathogenicity test showed a balance between hemagglutinin and neuraminidase proteins in the construction of avian influenza virus (RG) low pathogenic.

Pathogenicity study in chickens

The results showed that the index score Intravenous Pathogenicity Index of the low pathogenic bird flu virus (HA-H5-RG/NA-PR8) was 0 (zero), which is equal to the score of the low-high bird flu virus (HA-H5-RG/NA-H5). Both viruses have 0 (zero) score, meaning that both of the viruses had low pathogenicity. The result could be seen in table 2.

Intravenous Pathogenicity Index testing performed for 10 days with each virus as many as 10 chickens. The value obtained from the results of this test was index score. Calculation of Intravenous Pathogenicity Index score was average of each chicken in each observation divided by 10 days of observation. The result of this study showed that the index of IVPI score of (HA-H5-RG/NA-PR8) virus was 0 (zero) equal to the score of (HA-H5-RG/NA-H5) virus. It can be said that both viruses were low pathogenic, so it did not show any symptoms on the chickens tested.

Table 2: Intravenous Pathogenicity Index of virus.

Clinical Signs	Observation Day											
	1	2	3	4	5	6	7	8	9	10	Total	Score
PBS (Negative Control)												
Normal	10	10	10	10	10	10	10	10	10	10	100 x 0	0
Sick	0	0	0	0	0	0	0	0	0	0	0 x 1	0
Paralyzed	0	0	0	0	0	0	0	0	0	0	0 x 2	0
Dead	0	0	0	0	0	0	0	0	0	0	0 x 3	0
Total												0
Index												0.00
Low Pathogenic Virus (HA-H5-RG/NA-PR8)												
Normal	10	10	10	10	10	10	10	10	10	10	100 x 0	0
Sick	0	0	0	0	0	0	0	0	0	0	0 x 1	0
Paralyzed	0	0	0	0	0	0	0	0	0	0	0 x 2	0
dead	0	0	0	0	0	0	0	0	0	0	0 x 3	0
Total												0
Index												0
Low-High Virus (HA-H5-RG/NA-H5)												
Normal	10	10	10	10	10	10	10	10	10	10	100 x 0	0
Sick	0	0	0	0	0	0	0	0	0	0	0 x 1	0
Paralyzed	0	0	0	0	0	0	0	0	0	0	0 x 2	0
Dead	0	0	0	0	0	0	0	0	0	0	0 x 3	0
Total												0
Index												0.00

Pathogenicity study in mice

The result of immunology study in mice of low pathogenic (HA-H5-RG/NA-PR8) vaccine, it was known that the value of the highest HI titer in all treatment groups demonstrated on day 21 post-vaccination. HI titers were obtained at 2^8 or GMT (Geometric Mean Titer) was 256. Meanwhile HI titer value of low high (HA-H5-RG/NA-H5) vaccine was 26.14 or 70.52 GMT. Statistical tests against HI titer results of day 14 to day 21 post-vaccination showed that there was a significant difference in value: HI titers 21 days post-vaccination were all higher than the 14 days post-vaccination titers. Tests were performed with the Wilcoxon test with $p = 0.000$ ($p < 0.05$).

Statistical measurement by Kruskal Wallis test on day 14 post-vaccination revealed, from the three groups did not show significant difference from HI test result with $p = 0.149$ ($p > 0.05$). Meanwhile, on day 21 post vaccination showed there was a significant difference ($p<0.05$) between virus HA-H5-RG/NA-PR8 and HA-H5-RG/NA-H5 where virus HA-H5-RG/NA-PR8 was higher than HA-H5-RG/NA-H5 (Table 3).

The body weight and temperature of mice were also observed, to see if there are any changes in mice due to vaccination. According to [19], body surface temperature was said to be normal if it did not increase more than 4 to 5 degrees from the initial temperature, while body weight was called normal if it did not decrease 20% or more than the initial body weight. In addition, sneezing and diarrhea were also observed post-vaccination. From the observation, it was found that there was no nasal fluid coming out or diarrhea during observation. This suggested that the vaccine given to the mice has indeed been completely inactivated and did not cause pathogens.

The observation of body weight and temperature were on day 0, 7, 14, 21 post-vaccinations. The overall body weight data of the mice during the test can be seen on figure 3a, it indicated that weight of the mice was stable during the vaccination process.

The measurements of body temperature of mice figure 3b) showed no significant temperature changes in each treatment, the temperature was relatively stable, with the exception of the 21st day higher. It was possible that on the 21st day a challenge test was performed on all groups of mice.

Figure 3: Body weight and temperature performance of mice. (a) Body weight of mice until day 21 post-vaccination in grams; (b) temperature of mice until day 21 post-vaccination in Celsius degree.

21 days after the vaccination, the mice were challenged with H5N1 Indonesia influenza virus. The result was survival rate of mice as expressed in percentage. The challenge test result showed that the highest survival rate was obtained by the group of low pathogenic (HA-H5-RG/NA-PR8) vaccine is

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85.71%. Testing was performed using 7 mice in each group were observed daily and includes the clinical symptoms of sneezing and weight changes (Table 4).

In this study, the vaccination process was done twice (booster vaccination), where after day 14 post-vaccination, mice were given the vaccine again with the same dose as before and observed until day 21. This was done to increase antibody titer in mice, according to the process described in [20]. Exposure to early antigen elicits the response of additional follicular production, which causes rapid emergence of low IgG antibody titer. As the B-cells proliferate in the germinal Center and differentiate into plasma cells, IgG antibody titer increased to a peak value within usually up to 4 weeks after immunization. The short life span of plasma cells produces a rapid decline of antibody titers, with the final result of antibody titer returning to the base level [20].

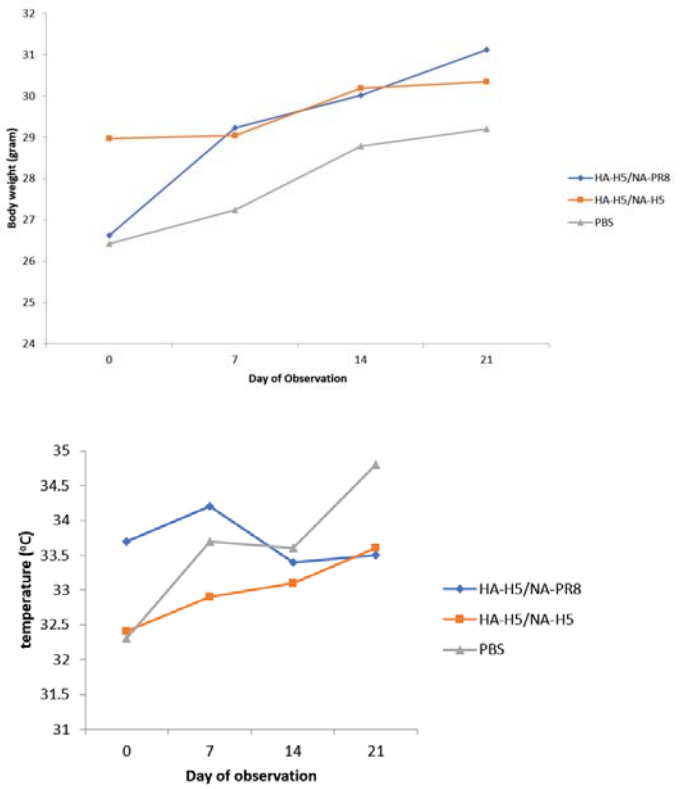

Figure 3: Body weight and temperature performance of mice. A. Body weight performance of mice until day 21 post-vaccination in gram,
B. Temperature performance of mice until day 21 post-vaccination in degree of celcius.

Table 3: HI titer post vaccination in mice.

Virus	Day-14 post vaccination			Day-21 post vaccination		
	Log2	GMT	p	Log2	GMT	p
HA-H5-RG/NA-PR8	5.14 ± 0.690	35.26	0.007	8 ± 0.577	256	0.002
HA-H5-RG/NA-H5	5.29± 0.756	39.12		6.14±0.900	70.52	
PBS	0	1		0	1	

Table 4: Challenge test result.

No.	Group	Total Number of Mice	Total Number of Mice Death	Survival Rate (%)
1	HA-H5-RG/NA-PR8	7	1	85.71
2	HA-H5-RG/NA-H5	7	4	42.86
3	PBS	7	7	0

The results showed that mice vaccinated with Ha-H5-RG/NA-PR8 virus had higher immunity than mice vaccinated with HA-H5-RG/NA-H5 and PBS as placebo, but there was still mortality in mice.

Several approaches to the study indicated that there were factors that influence the results of vaccination challenge, such as the accuracy of vaccine dose, the follow-up effect that arises from the injection of the vaccine; character or specification of mice as model animals [21,22].

In this study, the doses used refer to the general dose for humans, not specifically for mice, while the conversion of human doses with mice to avian influenza vaccine was not yet available. In addition, in this study no adjuvant was used on the given vaccine formulations.

The addition of an effective adjuvant will increase the protective immune response by using the vaccine antigen to a minimum, so that the adjuvant vaccine will increase the coverage of the vaccine.

When the antigen contained in the vaccine enters an organism, the amount was reduced to stimulate the macrophages, which in turn stimulates the activation of helper T cells and B cells, so that B cells formed as antibody-producing cells will also decrease. This will also cause a small amount of the resulting antibody. The use of adjuvants that affect Toll-like Receptors (TLRs) in the Presenting Cell Antigen (APC) and induce Th1 cells may enhance protective immune responses [23].

The use of mice as model animals in the vaccine virus seed testing at the level of preclinical testing, had the advantage that it was small in size, relatively cheap, and there was large availability of reagents for testing the immune response. The weakness was the virus challenge should be able to adapt with mice, anatomy and distribution of respiratory tract was different from the human respiratory tract. This situation was very different from the use of ferret as an animal flu vaccine model.

The vaccinated mice will produce cytokines from Th1 cells (IL2, IFN-γ) and Th2 (IL-4, IL-5 and IL-10). The production of these two responses differed in each strain of the experimental

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animal including the mice strain used. In addition, strains of mice also affect immune response due to vaccine and challenge test. The use of BALB / c strain mice will improve Th2 response, while C57BL / 6 strain mice will improve Th1 response [22]. The use of BALB / c strain mice in this study was because easy to obtain and the cost was relatively cheap. The relationship of mice mortality in this study also needs to be studied further.

Conclusion

The results of the study show that the low pathogenic avian influenza H5N1 virus (RG) was successfully constructed. It showed no molecular changes, whether at nucleotide or amino acid levels. Neuraminidase from PR8 plays an important role in reducing pathogens in chickens and mice. Pathogenicity test result showed that HA-H5-RG/NA-PR8 virus had lower pathogenicity than the HA-H5-RG/NA-H5 virus developed in the previous study, yet it has higher virus yield (replication) than its predecessor virus.

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