



Research on the Selection of Vaccine Seeds with the HI Method Approach to Improve the Quality of Avian Influenza Vaccines

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Abstract

AI virus is a virus that is easily mutated because it has a single chain of RNA that is unstable, so many strains appear in the field. Currently in the field (in Indonesia), it is known that there are 2 vaccine strains that dominate, namely AI H5N1 Clade 2.1.3 and AI H5N1 Clade 2.3.2. The incompatibility of seed viruses used as vaccine seeds will hinder the eradication of AI disease in Indonesia. Therefore, it is necessary to select and develop seed viruses as new vaccine seed candidates to obtain vaccines with high quality, safe, homologous, and able to cover viruses in the field. Development activities carried out by researchers aim to obtain vaccine seeds that are able to protect against AI clade 2.1.3 virus and AI clade 2.3.2 virus. The vaccine candidates to be developed are several vaccine candidates derived from the isolation of chicken organs from AI outbreak areas in several areas on the island of Java which include West Java, Central Java and East Java which were found around 2010 to 2013. These isolates are processed and then propagated on the SPF TAB and passaged until a high viral titer is obtained. After that, identification, pathogenicity, immunogenicity and protective tests were carried out. From the results of this development, AI C-8 and C-11 virus seeds were obtained which are able to protect against both AI Clade 2.1.3 and AI Clade 2.3.2.

Introduction

Since 2003 until now, bird flu outbreaks (Avian Influenza) have caused very high poultry mortality and economic losses in various countries in Asia such as Vietnam, Thailand, China, Japan, South Korea, Laos and Indonesia (Ernawati, 2011; Yadav, 2022). In addition to causing high economic losses, the highly pathogenic Avian Influenza virus (HPAI/High Pathogen Avian Influenza) cause panic because it can pass through a Barrier between poultry and humans, which means that it can be contagious and deadly to humans and become a pandemic threat (Ernawati, 2011; Sims & Brown, 2016).

Many government efforts have been made to overcome the outbreak of bird flu, including the destruction of infected birds within a certain radius (stamping out/culling), biosecurity and vaccination. Vaccination is one of the government's programs to prevent bird flu outbreaks in Indonesia (Behrens et al., 2006; Indriani et al., 2011; Swayne, 2012).

Various AI vaccine preparations for poultry have been widely produced by several government agencies and private companies in Indonesia (Ernawati, 2011; Fournié et al., 2012). A common

preparation for AI vaccines in the form of emulsions with oil adjuvants. The vaccine can protect poultry from clinical symptoms and death, but does not suppress viral excretion. This raises doubts in preventing transmission of the virus between animals and increases the risk of new outbreaks and becoming a threat to public health (Bett et al., 2015; Ernawati, 2011).

Another problem in vaccination strategy is the determination of seed virus used as raw material in vaccine manufacturing (Venkataraman et al., 2021; Webster et al., 1992). AI vaccines are RNA viruses that are genetically easy to mutate, and quickly change their antigen structure so determining vaccine seeds that are guaranteed to be safe, effective, and genetically stable and antigenically homologous is a problem that is not easy (Ernawati, 2011; Smith et al., 2006; Charlesworth et al., 202).

Therefore, research was conducted on the selection of vaccine seeds as raw material seeds in making vaccines with the HI method to improve the quality of AI vaccines by making single vaccines which were then challenged with various kinds of local AI strain antigens owned by PT. Caprifarmindo Laboratories.

Methods

Virus isolation

Isolation of samples came from sick chickens in West Java, Central Java and East Java. AI virus sampling techniques were carried out with organ scouring techniques. The organ scouring technique is done by taking some pieces of organs such as respiratory organs (trachea and lungs), digestive organs (proventriculus, intestines), lymphoid organs (liver, spleen, bursa Fabricius), and brain. These organs are washed using PBS that has been added to antibiotics and crushed to make 10% suspension. Furthermore, the 10% suspension is carried out freeze-thawing As many as 3 times, then centrifuge at 6000 rpm for 10-15 minutes. The supernatant is taken and then inoculated on 3 grains of TAB (embryonated chicken eggs) which are SPF (Specific Pathogen Free) aged 9 - 11 days. Then incubated at a temperature of 37°C for 5 days. This TAB is observed to develop every 2 hours using candling. If there is a TAB whose embryo dies, it is put in the refrigerator temperature 4°C for distilling. Allantois fluid from TAB that has been in chilling was harvested for Rapid HA testing (Rapid Test) by reacting 1 part allantois liquid with 1 part RBC 10%. If there is an isolate sample that is positive for agglutination, a rapid-blocking agglutination test is carried out using AI serum to find out whether the isolate sample is an AI virus. If the blocking results show no agglutination, the isolate sample is positive for the AI virus. Positive samples are stored within -86°C. Furthermore, identification tests were carried out, including biomolecular tests, identification tests by method Cross micro technical HA reactions, virus content tests, pathogenicity tests on SPF embryonic chicken eggs, pathogenicity tests in chickens, and then evaluated immunogenicity and protectivity as the final product of the vaccine.

Biomolecular Test

Biomolecular identification in this study was only carried out on isolate C-11, because isolate C-8 and other isolates had been carried out in previous research.

Polymerase Chain Reaction (PCR)

Virus testing using PCR is carried out by isolating DNA/RNA using Purelink RNA/DNA Kit (Invitrogen, 12280050). The procedure applied is adapted from the protocol issued by the manufacturer by the spin column method. The results of RNA/DNA isolation are identified and stored at -20°C for DNA and -86°C for RNA.

The RNA-isolated samples obtained were then converted to cDNA form using the Reverse Transcriptase enzyme in the Polymerase Chain Reaction (PCR) process. After that, samples were amplified using H5-155f: 5'-ACACATGCYCARGACATACT-3' and H5-699r: 5'-

CTYTGRTTYAGTGTTGATGT-3' primers with a target length of about 545 bp. PCR results are then detected using electrophoresis techniques.

Sequencing

PCR products are detected with amplification lengths according to the primary target, and then purification and nucleotide sequencing are carried out using Genetic Analyzer 3500 (Applied Biosystems). DNA sequences were analysed using Mega 5 Software.

Micro technical HA Test

The HA test is used to determine the antigen titer. The HA test procedure is microplate holes from A1 to A12 filled with 25µl PBS (Phosphate Buffer Saline) depending on the amount of shampoo. Samples are inserted as much as 25µl in holes A1, B1, and so on depending on the number of samples. Next, a serial dilution is carried out from holes A1 to A11, then remove 25µl from hole A11, because hole A12 is the control, and so on for the next sample. 1% RBC is inserted as much as 25µl from hole A1 to A12. Then incubated at room temperature for 30 – 45 minutes. The HA titer is read at the last hole that still causes agglutination.

Test Virus Content

A virus content test is a test to determine the dose of virus that can infect 50 percent of TAB expressed with EID₅₀ units (Effective Infectious dose). Virus content test procedure by providing 20 SPF TAB items. Then 8 threaded test tubes that have been filled with PBS of 9 ml each. Furthermore, the sample (harvested from allantois liquid) is inserted in the first tube as much as 1 ml, homogenize, then 1 ml is taken from the first tube and inserted into the second tube and so on 10x dilution is done. Furthermore, in 5 grains of SPF TAB with dilutions of -8, -7, -6, -5 respectively were injected as much as 0.1 ml in each dilution. Further incubated for 5 days on an incubator 37°C. And observations were made every day and his death was recorded. TABs that are dead in chilling. The assessment is carried out at the end of the observation, using the formula from Method calculation “Spearman – Karber” based on log₁₀ EID₅₀.

$$EID_{50} = X + \frac{d \sum r_i}{n} - \frac{1}{2} d$$

Description :

X = log of depletion that still gives positive results

∑ r_i = number of negatives

n = number of eggs used in each dilution

d = log of dilution factor

(log₁₀=1)

Pathogenicity in SPF eggs

The eggs used are embryonated SPF eggs with an incubation period of 10-11 days as many as 5 eggs. Each egg was inoculated with 0.1 ml virus containing 1000 virus particles, then incubated at 37°C and observed (candling) every 2 hours. Record the time of death and chilling first before the rapid agglutination test. A malignant virus is the fastest infecting embryo characterized by embryonic death and agglutination.

Pathogenicity in SPF Chickens

Inoculate the virus as much as 10⁷ in chickens aged 3-4 weeks, observe every day, and note the onset of clinical symptoms (start to illness) and death. In dead chickens, an autopsy is performed and changes in anatomical pathology (PA) are seen, the malignant virus is the virus

that most quickly infects chickens characterized by clinical symptoms (illness) which is usually followed by chicken death and changes in PA in organs.

Inactive bulk product manufacturing

Inactivated bulk products are raw materials in the manufacture of vaccines. The procedure is to first dilute the virus titer obtained from the virus content test to 10^3 per dose. Furthermore, 0.1 ml was injected in 30 grains of SPF TAB. Then incubated in an incubator at 37°C for 5 days. Chickens that die in chilling. After 5 days of harvesting allantois liquid from each TAB. The harvest of allantois liquid is centrifuge 2500 for 15 minutes. The supernatant is taken, then the antigen must first be inactivated with 37% Formaldehyde with a final concentration of 0.2%. Then incubate at room temperature overnight while turning.

Immunogenicity and Protectivity

This test was carried out using 3 batches of vaccines containing AI Capri-8 and AI Capri-11 antigens injected with 1 dose/head in SPF chickens aged 4 weeks. Each batch required 30 vaccination groups and 30 control groups (unvaccinated) Three weeks after vaccination, blood was taken and serum, for titer (HI) testing, and then all chickens were inoculated with AI challenge virus with a content of 10^6 ELD₅₀. Observation 14 days after virus inoculation challenge.

Vaccine Manufacturing

The vaccine is made in an emulsion preparation using an oil adjuvant in the ratio of 30% antigen and 70% adjuvant.

Antisera Manufacturing

The vaccine is injected into 10 4-week-old SPF chickens as much as 0.3 ml per dose. Three weeks later the chicken had its blood drawn to separate its serum. The obtained serum is inactivated on a water bath at 56°C for 30 minutes, for further HI testing (Haemagglutination Inhibition) to determine the titer of antibodies elicited by the vaccine.

Result and Discussion

Biomolecular Test (of AI H5N1 Isolate C-11)

Polymerase Chain Reaction (PCR)

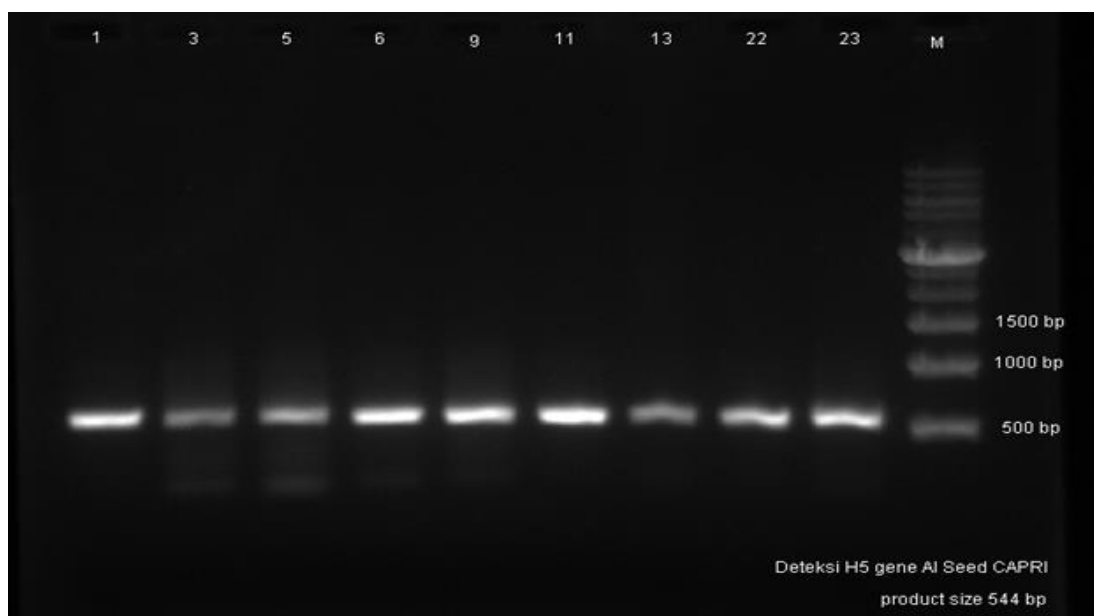


Figure 1. The electrophoresis yield of agarose gel 2%. M: marker 500 bp – 1 kb; 11: AI H5N1 C 11

The PCR product in the sample has been successfully amplified with a length of approximately 544 bp, according to the primary target used. This is shown in the results of electrophoresis on 2% agarose gel (Figure 1).

Sequencing

Data sequencing virus AI H5N1 C-11 (A/CK/C-11/Jatim/2010)

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CTGGAAAGGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTA
ATTTTAAAAGATTGCAGTGTAGCTGGATGGCTCCTCGGGAACCCAATGTGTGACG
AATTCATCAAAGTACCAGAATGGTCTTACATAGTGGAGAAGGCCAATCCGACCA
ACGACCTCTGTTACCCAGGGAGTTTCAACGACTATGAAGAAGTAAACACCTATT
AAGCAGAATAAACCATTTTCGAGAAAATTCAGATCATCCCTAAAAGTTCTTGGTCC
GATCATGAAGCATCATCAGGAGTGAGCTCAGCATGCCCATACCTGGGAAGTCCC
TCCTTTTTTAGAAATGTGGTATGGCTTATCAAAAAGAACAGTACGTACCCAACAA
TAAAGAAAACCTACAATAATACCAACCAAGAAGATCTTTTGATACTGTGGGGAA
TTCATCATCCTAATAATGAGGCAGAGCAGACAATGCTATATCAAAACCCAACCA
CCTATATTTCCATTGGGACAT
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The results of nucleotide sequence alignment of AI seed C-11 gene H5 using BLAST NCBI (table 1.2) showed a 99% level of proximity (identity) with Influenza A virus (A/chicken/Indonesia/SmiWN18/2009(H5N1)) accession no. JF302895.1 and Influenza A virus (A/chicken/West Java/M35/2010(H5N1)) accession no. KR078232.1 originating from West Java.

Based on the phylogenetic tree by including several other vaccine seeds, AI Capri -11 has proximity to AI C-8 seeds and several West Java AI seeds (Table 1.1).

Table 1. Alignment Results Using NCBI BLAST

Sample Code	Alignment Result			
	Description	Country	Identity	Accession
AI-C-11	Influenza A virus (A/chicken/Indonesia/SmiWN18/2009(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	Indonesia	99%	JF302895.1
	Influenza A virus (A/chicken/West Java/M35/2010(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	West Java, Indonesia	99%	KR078232.1
	Influenza A virus (A/chicken/Indonesia/D10014/2010(H5N1)) HA gene for hemagglutinin, complete cds	Indonesia	99%	AB621353.1

Phylogenetic Tree Using Mega 5 Software

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.93985840 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 216 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.



Figure 1. evolutionary relationships of taxa.

Micro technical HA Test

Table 2. Result of Rapid Blocking Agglutination Test

Sample	Hyperimmun Antisera		
	ND	EDS	AI PWT
AI C-8 Antigen	Agglutination	Agglutination	Negative
AI C-11 Antigen	Agglutination	Agglutination	Negative

Source: RnD Report, 2014

Pathogenesis

Pathogenicity in SPF eggs

Table 3. The Mean Death Time and Anatomical Pathology Appearance of The Embryos

Sample	Number of Particles	Mean Death Time	PA
AI C-8	10 ³	24-26 hours	Haemorrhage throughout the embryo's body
AI C-11	10 ³	22-24 hours	Haemorrhage throughout the embryo's body

Pathogenicity in SPF Chickens

Table 4. The Mean Death Time and Anatomical Pathology Appearance of The Chickens

Sample	Number of Particles	Mean Death Time	PA
AI C-8	10 ⁷	19-22 hours	Haemorrhages in all organs, Cyanosis in the comb, wattles and feet
AI C-11	10 ⁷	16-17 hours	Haemorrhages in all organs

Assurance of Immunogenicity of Seed Vaccine Cross Reaction Test HI

The selected isolates included the previous isolates, each of which was made into a single vaccine, namely C 8, C 10, C 11, and C 14. In addition, a single vaccine containing the AI

clade 2.1.3 virus from the government was also made as a comparison, namely AI PWT, AI SMI, AI GARUT, and AI Clade 2.3.2 viruses, namely AI P4 (Sukoharjo), AI P6 (Sleman), while for the latest AI H5N1 Clade 2.1.3, strain A/Chicken/Barru/BBVM 41-13/2013 and the latest AI H5N1 Clade 2.3.2, strain A/Chicken/Semarang/04141225-07/2014, were tested using hyperimmune that had been made previously. All serum obtained from the results of single vaccinations and internal hyperimmune reference, then tested for HI against antigens contained in vaccines and antigens recommended by the government, including: AI PWT, AI SMI, AI P6, AI GARUT, C 5, C 6, C 8, C 10, C 14, Barru and Semarang.

Table 5. Results of Cross Reaction of AI Vaccine Challenged with Several Antigens

	PWT	SMI	GRT	Slm	C 5	C6	C8	C 10	C11	C13	C 14	Barru	Smrg
PWT	9,5	3.90	4.70	4.60	3.30	3.70	4.60	1.00	5.65	1.78	1.40	1.22	1.03
SMI	7.56	4.30	5.00	4.10	3.80	4.00	6.56	1.90	4.44	1.80	2.11	1.65	2.05
GARUT	3.80	2.89	7.20	3.80	2.33	5.90	5.70	1.90	6.45	1.63	2.40	1.35	2.10
Sleman	4.00	2.50	4.00	7.50	1.75	3.50	7.00	1.38	7.22	3.63	3.90	2.25	5.50
Sukoharjo	3.70	3.00	5.20	8.10	2.50	3.30	6.00	2.00	6.05	3.60	3.80	1.50	5.25
C8	4.00	2.90	5.10	5.20	5.60	4.30	9.10	3.80	7.65	2.90	3.70	2.90	4.86
C 10	2.60	2.50	3.90	5.11	2.00	2.90	4.30	0.40	6.56	3.33	3.00	1.25	2.35
C 11	5.30	4.70	6.60	7.10	4.40	5.40	8.30	6.44	8.55	4.22	4.20	5.20	4.50
C14	3.50	2.90	4.33	3.00	2.50	2.90	5.70	2.22	4.20	1.10	2.00	2.15	3.35
Barru	1,50	2.05	2.53	3.05	1.30	2.55	2.50	1.05	4.35	2.05	2.01	7.95	4.20
Semarang	1.02	2.55	2.15	5.40	2.02	3.22	4.25	2.22	4.25	2.55	1.85	3.45	7.55

Note: The antibody titer in the table is the average titer log 2.

Serological results that can be said to be homologous between serum and its antigens are those with titers $\geq 2^5$. Based on the table above, serum C 8 and C 11 derived from field isolates have a wide range of antigen binding homology compared to other serums. According to Darmawan and Wahyuningsih, 2010 in Ernawati et al. (2011), that vaccine candidates must have antigen binding with a wide scope because it is one of the requirements for vaccine candidates. The selection of the IR test in determining vaccine seeds is one of the government regulations in accordance with the results of *antisera cartography* (OFFLU Workshop and AAHL 2009) where AI vaccine seeds must go through *Haemagglutination Inhibition* challenge tests with predetermined antisera panels (with titers $\geq 2^4$) (Ernawati et al., 2011).

Ensuring the Efficacy of Vaccine Finished Products

Each vaccine candidate that has been inoculated with the AI H5N1 Clade 2.1.3 strain C-8 and C-11 based vaccine is injected into 30 chickens 4 weeks old at 1 dose/bird, after three weeks then the blood and serum are taken for serological testing using the HI method and respectively. -each batch was challenged 3 weeks after vaccination with AI H5N1 Clade 2.1.3 and AI H5N1 Clade 2.3.2 viruses with a content of 10^6 ELD₅₀/dose.

Table 6. Efficacy of Single AI H5N1 Clade 2.1.3 strain C-8 and C-11 Vaccine Based Products

Groups	Batch No.	Serology titer average (3 wpv)			Challenge Test (% protectifitas)		
		Clade 2.1 (C-8)	Clade 2.1 (C-11)	Clade 2.3 (Slm)	Clade 2.1 (C11)	Clade 2.1 (PWT)	Clade 2.3 (Slm)
Group 1	RC09B12 (Single C11)	7.40	6.80	4.20	100	100	100
Group 2	RE09B15 (Single C-8)	7.10	5.00	2.70	100	100	90

Group 3	RE09B16 (C11+C8)	7.80	6.40	3.40	100	100	100
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AI-based vaccines H5N1 Clade 2.1.3 strains C-8 and C-11 can stimulate the formation of antibodies at 3 weeks post-vaccination with titers greater than 2 to the power of 5 (Minimum standard titer antibody tested positive), in accordance with those stipulated in the pharmacopoeia of Indonesian Veterinary Medicine (Abdullah et al., 2023; Hartanti et al., 2023) against AI H5N1 Clade 2.1.3 or homologous to contained vaccines, however, the titer produced less than the standard against the AI H5N1 clade 2.3.2.

The protection of AI H5N1 Clade 2.1.3 strains C-8 and C-11 based vaccines products, can be seen from the results of challenge tests, where both using AI H5N1 Clade 2.1.3 challenge seeds both homologous, and heterology by using AI H5N1 Clade 2.3.2 still produces 90-100% protection, where after being challenged at 3 weeks after vaccination chickens in each treatment still survive or live without specific clinical symptoms against AI up to 14 days of observation.

Conclusion

Antigens derived from C 8 and C 11 vaccines can be used as vaccine seed candidates because serologically with HI assays have a wide coverage of homology against various challenging viruses, both homologous and heterology. However, this needs to be continued by conducting challenge tests followed by virus shedding tests, so that the efficacy and protectivity of AI-based vaccines H5N1 Clade 2.1.3 strains C-8 and C-11 can be analysed by anticipating shedding or virus excretion, especially heterology which can be a risk factor for outbreaks in the field.

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