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Preparation of avian influenza H5N1 and Newcastle disease antigens for hemagglutination inhibition assay applications

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Abstract

Background Avian influenza (AI) and Newcastle disease (ND) are major poultry diseases in Indonesia, where monitoring of vaccination efficacy commonly relies on the hemagglutination inhibition (HI) assay. The HI assay requires viral antigens, which are generally obtained commercially from PUSVETMA. However, independent antigen preparation would be beneficial for private laboratories.

Objective This study aimed to prepare and validate in-house AI and ND antigens as HI assay reagents.

Methods Viruses were propagated in embryonated chicken eggs (ECE), inactivated using buffered neutral formalin (BNF), precipitated with polyethylene glycol (PEG-6000), and preserved with glycerol. Validation was conducted by parallel HI testing of 24 chicken sera for AI and 22 chicken sera for ND using both in-house and PUSVETMA's antigens. Antibody titers were analyzed using analysis of variance (ANOVA), with sensitivity (Se), specificity (Sp), and kappa (κ) tests performed for agreement**.**

Results ANOVA revealed no significant differences in geometric mean titers between in-house and PUSVETMA's antigens ($P<0.05$). Both the AI and ND in-house antigens demonstrated Se and Sp values of 100% and κ values of 1, indicating perfect agreement.

Conclusion These findings confirm that in-house AI and ND antigens are comparable to their commercial counterparts and can serve as reliable and cost-effective reagents for HI testing in private laboratories.

Keywords antigen preparation | avian influenza | hemagglutination inhibition assay | Newcastle disease | vaccine monitoring

Introduction

The two major poultry diseases in Indonesia are Avian influenza (AI) and Newcastle disease (ND). Avian influenza, or bird flu, was first reported in Indonesia in 2003 and infects both poultry and humans. By 2014, Indonesia had the highest number of human fatalities caused by AI worldwide (Pudjiatmoko *et al.*, 2014). The initial outbreak was associated with the AI virus subtype H5N1 clade 2.1.1, which later evolved into the clade 2.1.3. In 2012, clade 2.3.2, which showed high pathogenicity in waterfowl, as well as in humans (Kemenkes RI, 2017; Karo-Karo *et al.*, 2022). The AI H5N1 virus can infect broilers, layers, native chickens, quails, and ducks, with mortality rates of up to 90%. According to the World Organization for Animal Health (WOAH), since 2005 Indonesia has been categorized as an AI-endemic country alongside Egypt and Nigeria (Fournié *et al.* 2011, WOAH 2021a). AI viruses are classified as low-pathogenic avian influenza (LPAI) and highly

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pathogenic avian influenza (HPAIV) (WOAH 2021a). LPAI can mutate into HPAIV after adapting to new hosts, leading to severe systemic diseases with high mortality (Kamps *et al.*, 2006). Currently, the Indonesian poultry sector continues to face challenges of HPAIV H5N1 and LPAI H9N2.

In addition to AI, Newcastle disease (ND) remains an endemic threat that causes substantial economic loss in the Indonesian poultry industry. Caused by avian paramyxovirus serotype 1 (APMV-1), ND induces respiratory, digestive, and neurological disorders, with morbidity and mortality rates reaching up to 100% (Hewajuli & Dharmayanti, 2011; Susanti *et al.*, 2021). Serological assays, such as hemagglutination (HA) and HI, are widely used for ND diagnosis because of their simplicity and cost-effectiveness (Bello *et al.*, 2018).

Vaccination remains a primary control strategy, and its effectiveness is routinely monitored by measuring post-vaccination antibody titers using a hemagglutination inhibition (HI) assay (WOAH 2021a, WOAH 2021b). Commercially standardized AI and ND antigens in Indonesia are produced only by the Veterinary Farma Center PUSVETMA (Surabaya, Indonesia) or imported from Royal GD (Deventer, the Netherlands). This situation limits the accessibility of private laboratories. Therefore, independent preparation of AI antigens is essential to support broader surveillance and vaccine evaluation. Based on these considerations, this study aimed to prepare and validate AI H5N1 clade 2.1.3 and ND antigens for use as reagents in the HI assay and to compare their performance with commercially available antigens.

Methods

Virus, commercial antigens, and serum samples

Viruses and sera used in this study were obtained from the archives of the Immunology Laboratory, Division of Medical Microbiology, School of Veterinary Medicine and Biomedical Sciences, IPB University. The avian influenza (AI) virus used was H5N1 clade 2.1.3, whereas the Newcastle disease (ND) virus used was the LaSota strain (velogenic). Commercial antigens used for comparison included AI clade 2.1.3 antigen (PUSVETMA, Surabaya, Indonesia) and ND LaSota antigen (PUSVETMA, Surabaya, Indonesia).

For the hemagglutination inhibition (HI) test against the AI virus, 20 chicken serum samples (collected from AI-vaccinated chickens), one AI-positive serum sample (PUSVETMA, Surabaya, Indonesia), and three AI-negative serum samples (PUSVETMA, Surabaya, Indonesia) were used. For the HI test against the ND virus, 20 chicken serum samples (collected from ND-vaccinated chickens), one ND-positive serum sample (PUSVETMA, Surabaya, Indonesia), and one ND-negative serum sample (PUSVETMA, Surabaya, Indonesia) were used.

Virus propagation

Propagation of avian influenza (AI) and Newcastle disease (ND) viruses was carried out by inoculating five 9-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) with each virus via the allantoic cavity route. The inoculum was prepared by mixing the virus suspension with penicillin–streptomycin at a concentration of 10,000 IU/mL.

Embryos and air sacs were examined and marked. The eggshell surface was disinfected with 70% alcohol swabs and a small hole was drilled without damaging the underlying membranes. Subsequently, 0.2 mL of the virus inoculum was injected into the allantoic cavity through the prepared hole and passed through the air sac boundary. The inoculation site was sealed using glue or adhesive tape to ensure complete closure.

The inoculated eggs were incubated for 4 days at 37°C in an egg incubator, and candling was performed every 24 hours. Embryos that died within 24 hours post-inoculation were discarded as death was likely due to contamination. Allantoic fluid was harvested from embryos that died after 48 hours and examined for viral growth.

AI virus replication was indicated by the presence of hemorrhages in the embryo, reduced embryo size compared to normal, and the ability of allantoic fluid to agglutinate 5% chicken red blood cells (WOAH, 2021a). ND virus replication was characterized by similar findings, including embryo hemorrhage, reduced embryo size, and agglutination of 5% chicken red blood cells (WOAH, 2021b). Allantoic fluids showing hemagglutination activity were pooled and the virus titer was determined using a hemagglutination (HA) assay.

Preparation of avian influenza H5N1 clade 2.1.3 antigen

The AI antigen was prepared using a method adapted from Pawar *et al.* (2015). The AI antigen prepared in this study is hereafter referred to as "in-house AI antigen". Virus inactivation was performed by adding 1% neutral buffered formalin (NBF) in phosphate-buffered saline (PBS) to 10 mL of the AI virus suspension until a final concentration of 0.04% (v/v) was achieved. The mixture was homogenized by vortexing and incubated at 37°C for 16 hours. Virus inactivation was confirmed by inoculation of embryonated chicken eggs; the virus suspension was considered inactivated if no evidence of viral replication was observed.

The inactivated AI virus suspension was then precipitated using polyethylene glycol (PEG-6000) as follows: 10% PEG-6000 was added to the suspension and homogenized with a magnetic stirrer at 4°C for 120 minutes. The mixture was centrifuged at 225 × g at 4°C for 15 min, and the supernatant was subjected to a second centrifugation at 3180 × g at 4°C for 90 minutes. The resulting pellet was resuspended in PBS to a final volume of 1 mL and sonicated for 90 seconds. Subsequently, 1 mL glycerol was added, yielding a final antigen volume of 2 ml.

Preparation of ND Lasota antigen

Newcastle Disease antigens were prepared using the modified method described by Beard *et al.* (1975). The ND antigen prepared in this study is hereafter referred to as "in-house ND antigen". The virus suspension, consisting of harvested allantoic fluid, was centrifuged at 14,000 × g for 10 minutes at 4°C, and the lipid layer on the surface was carefully removed. A hemagglutination (HA) test was performed to determine the ND virus titer. The ND virus suspension was inactivated using 0.1% (v/v) neutral buffered formalin (NBF, 10%) and incubated at 4°C for 24 hours. Inactivation was con

firmed by inoculation into embryonated chicken eggs, with the suspension considered inactivated if no viral replication was observed.

The inactivated ND virus suspension was mixed with 10% (w/v) PEG-6000 and 2% (w/v) NaCl, homogenized with a magnetic stirrer at 4°C for 2 hours, and centrifuged at 8,000 g for 30 minutes at 4°C. After centrifugation, the supernatant was discarded and the pellet was resuspended in 1% PBS to a final volume equivalent to 1/20 of the original volume. The antigen was sonicated for 90 seconds at maximum intensity, followed by the addition of 50% (v/v) glycerol.

Hemagglutination assay

A hemagglutination (HA) assay was performed to determine the antigen titers of avian influenza (AI) and Newcastle disease (ND) viruses, which were subsequently used as reagents in the hemagglutination inhibition (HI) assay. Antigen titers were standardized to 4 HAU and verified by back-titration. The HA assay was performed according to the following procedure (WOAH, 2021a; WOAH, 2021b):

A total of 25 µL of 1% PBS was dispensed into each well of the microplate from rows A to F. Then, 25 µL of the prepared viral suspension (allantoic fluid) was added to the first well. To obtain more accurate HA results, the ratios of the virus suspension to 1% PBS in the first wells of rows A to E were adjusted to 1/2, 1/3, 1/5, 1/7, and 1/9, respectively. Subsequently, two-fold serial dilutions were made across rows A to E by transferring 25 µL of the virus suspension from the first well to the second, mixing thoroughly, and then transferring 25 µL to the next well, continuing until the 12th well. From the 12th well, 25 µL of the viral suspension was discarded. Row F served as a negative control and contained no viral suspension.

Next, 25 µL of 1% PBS was added to each well, followed by 25 µL of 1% (v/v) chicken red blood cells (RBCs), starting from Column 12. The RBCs were gently mixed by scratching the bottom of the plate. The microplate was incubated at room temperature for 40 minutes. The results were recorded once the RBCs in the negative control wells completely settled at the bottom. The test was interpreted as negative when RBCs sedimented and formed a tear-drop shape upon tilting the plate, while a positive result was indicated by agglutinated RBCs.

HA was assessed by gently tilting the microplate and examining whether red blood cells (RBCs) displayed tear-shaped streaming. The endpoint of titration is defined as the highest dilution that produces complete hemagglutination (absence of streaming), which corresponds to one hemagglutination unit (HAU) and can be precisely calculated from the initial dilution series.

Data analysis

Antibody titers were statistically analyzed using one-way analysis of variance (ANOVA) to compare the means of two or more groups. When significant differences were observed among groups, post-hoc testing was performed. The sensitivity, specificity, and kappa values were determined by comparing the in-house antigen with the PUSVETMA's antigen, following the method described by Dohoo *et al.* (2010). Data analysis, including sensitivity, specificity, and kappa statistics, are summarized in **Table 1**.

Sensitivity was defined as the proportion of true-positive samples correctly identified by the test among all samples that were truly positive, thereby reflecting the ability of the assay to detect the presence of the disease. Specificity was defined as the proportion of true-negative samples correctly identified among all truly negative samples, indicating the ability of the assay to exclude individuals without the disease. Cohen's kappa (κ) is a statistical measure commonly used to assess inter-rater agreement for categorical data, while accounting for agreements that may occur by chance. The κ coefficient categorized as follows: <0 as poor, 0.00–0.20 as slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement (Dohoo *et al.*, 2010).

Table 1 Diagnostic evaluation of in-house antigen compared with PUSVETMA's antigen

	PUSVETMA's antigen		Total
	(+)	(-)	
In-house antigen			
(+)	a	b	m_1
(-)	c	d	m_0
Total	n_1	n_0	n

Results

HI assay using in-house antigen versus PUSVETMA's antigen

The HI assay was performed using both an in-house antigen and the PUSVETMA's antigen on all serum samples in parallel and in duplicate. Avian influenza antibody titer data in $\log_2$ scale are presented in Table 2, the result showed replicates 1 and 2 of the in-house AI antigen produces geometric mean titer (GMT) of 6.8 $\log_2$ and 6.75 $\log_2$ whereas the PUSBETMA's AI antigen produced GMT of 6.35$\log_2$ in replicate 1 and 6.5 $\log_2$ in replicate 2. ANOVA analysis of AI antibody titer data (**Table 2**) showed no significant difference ($P>0.05$) between the GMT obtained from the HI assay using in-house AI antigen and PUSVETMA's AI antigen. Similarly, the GMT values from replicates 1 and 2 did not differ significantly ($P>0.05$), regardless of whether the assay was performed using in-house or the PUSVETMA's AI antigen.

The Newcastle disease antibody titer data on the $\log_2$ scale are presented in **Table 3**. Replicates 1 and 2 of the in-house ND antigen showed an identical GMT of 8 $\log_2$, whereas the PUSVETMA's ND antigen produced a GMT of 7.45 $\log_2$ in replicate 1 and 7.40 $\log_2$ in replicate 2. ANOVA analysis indicated no significant difference ($P>0.05$) between the GMT obtained from the HI assay using the in-house ND antigen and the PUSVETMA's ND antigen. Similarly, the GMT values from HI replicates 1 and 2 did not show significant differences ($P>0.05$) for either the in-house ND antigen or the PUSVETMA's ND antigen.

Sensitivity, specificity, and kappa values of the in-house antigen versus PUSVETMA's antigen

The HI assay results were presented as binary/qualitative data (positive or negative) to determine the sensitivity (Se),

Table 2 Avian influenza antibody titers using in-house AI antigen and PUSVETMA's AI antigen

Serum number	AI antibody titer (log_2)			
	AI in-house antigen		PUSVETMA's antigen	
	HI 1	HI 2	HI 1	HI 2
1	8	8	8	8
2	7	5	5	6
3	6	7	5	6
4	8	7	5	7
5	8	7	4	6
6	7	6	8	6
7	7	7	8	8
8	7	6	8	6
9	4	4	6	6
10	5	7	6	6
11	8	8	8	6
12	6	7	5	6
13	4	5	4	5
14	4	6	4	6
15	8	7	5	8
16	7	7	8	6
17	8	7	6	7
18	8	8	8	6
19	8	8	8	8
20	8	8	8	7
GMT ± SD	6.8 ± 1.47	6.75 ± 1.12	6.35 ± 1.63	6.5 ± 0.89
SP	8	8	8	8
SN1	0	0	0	0
SN2	0	0	0	0

HI 1: replicate 1, HI 2: replicate 2, GMT±SD: geometric mean titer ± standard deviation, SP: AI-positive serum, SN: AI-negative serum

Table 3 Newcastle disease antibody titers using in-house ND antigen and PUSVETMA's ND antigen

Serum number	ND antibody titer (log_2)			
	ND in-house antigen		PUSVETMA's antigen	
	HI 1	HI 2	HI 1	HI 2
1	8	8	8	8
2	8	8	8	8
3	8	8	8	8
4	8	8	8	8
5	8	8	8	8
6	8	8	8	8
7	8	8	8	8
8	8	8	8	8
9	8	8	8	8
10	8	8	8	8
11	8	8	8	8
12	8	8	8	8
13	8	8	6	6
14	8	8	8	8
15	8	8	8	8
16	8	8	8	8
17	8	8	8	8
18	8	8	6	5
19	8	8	8	8
20	8	8	8	8
GMT ± SD	8 ± 0	8 ± 0	7.8 ± 0.62	7.75 ± 0.79
SP	8	8	8	8
SN	0	0	0	0

HI 1: replicate 1, HI 2: replicate 2, GMT±SD: geometric mean titer ± standard deviation, SP: AI-positive serum, SN: AI-negative serum

specificity (Sp), and kappa values. According to WOAH (2025), the minimum protective antibody titer against the AI virus in poultry is ≥ 3 log_2; therefore, antibody titers of 3 log_2 or higher were categorized as positive. However, for ND, WOAH (2021b) states that serum samples are considered antibody-positive when the HI titer is ≥ 4 log_2. The sensitivity and specificity values of the in-house AI antigen are presented in **Table 4**, which shows that both Se and Sp

reached 100%. The κ value was calculated using the formula described by Dohoo *et al.* (2010) to be 1. In contrast, in-house ND also showed a Se of 100%, a Sp of 100%, and a κ value of 1 when compared with the PUSVETMA's ND antigen (**Table 5**). These results indicate that the in-house antigen is highly suitable for use in the HI assay, with a κ value of 1, demonstrating almost perfect agreement with the PUSVETMA's antigen.

Table 4 The sensitivity, specificity and kappa values of in-house AI antigen

HI result	PUSVETMA's antigen		Total
	(+)	(-)	
in-house AI antigen			
(+)	21	0	21
(-)	0	3	3
Total	21	3	24
Sensitivity	100%		
Specificity	100%		
Kappa	1		

Table 5 The sensitivity, specificity and kappa values of in-house ND antigen

HI result	PUSVETMA's antigen		Total
	(+)	(-)	
in-house ND antigen			
(+)	21	0	21
(-)	0	1	1
Total	21	1	22
Sensitivity	100%		
Specificity	100%		
Kappa	1		

Discussion

The hemagglutination inhibition (HI) assay is the gold standard serological test for determining antibody titers against avian influenza (AI) and Newcastle disease (ND). This assay requires a standardized antigen as one of the essential reagents for its implementation (WOAH, 2021a; WOAH, 2021b). In Indonesia, standardized AI and ND antigens are manufactured only by PUSVETMA, which limits their availability. Therefore, it is necessary for private laboratories conducting HI tests to develop the capacity to produce their own standardized antigens. This study aimed to prepare AI and ND antigens and validate in-house antigens against those provided by PUSVETMA.

The AI and ND antigens were successfully prepared from the AI H5N1 clade 2.1.3 virus and the ND Lasota strain, following the methods described by Pawar *et al.* (2015) and Beard *et al.* (1975). The preparation process included virus propagation in embryonated chicken eggs (ECE), inactivation with 0.04% buffered normal formalin (BNF), precipitation with PEG, and preservation with glycerin. Validation of the in-house antigens against the PUSVETMA's antigens was assessed using ANOVA analysis, sensitivity (Se), specificity (Sp), and the kappa statistic.

The ANOVA results for AI antigen indicated no significant differences in the HI test outcomes (HI 1 and HI 2), whether using the in-house AI antigen or the PUSVETMA's AI antigen. This finding suggests that the in-house AI antigen was stable and yielded consistent results in duplicate HI assays. Furthermore, ANOVA analysis of antibody titers obtained with the in-house antigen revealed no significant differences compared to those obtained with the PUSVETMA's AI antigen. These results indicate that the in-house AI antigen can substitute the PUSVETMA antigen in HI assays. According to Bibby et al. (2022), the application of ANOVA in HI testing is used to detect significant differences in GMT between test results, ensuring both the consistency and validity of HI outcomes.

HI assay in this study demonstrated 100% sensitivity and specificity. Both antigen (in-house AI antigen & PUSVETMA's AI antigens) used in the HI assay originated from the AI H5N1 clade 2.1.3, which likely contributed to the high sensitivity and specificity in chicken serum. This aligns with the findings of Comin *et al.* (2013), who reported that HI assays using antigens derived from H5N1 viruses could specifically and sensitively detect antibodies against H5N1 clade 2.1.3. However, the sensitivity of H5N1 antigens decreases when H5N1 antibodies are identified in human serum owing to cross-reactivity (Rowe *et al.*, 1999). The κ value between the in-house AI antigen and PUSVETMA's AI antigen was 1, indicating an almost perfect agreement. In this study, the κ value was used to measure inter-test reliability, confirming that the H5N1 antigen preparation method developed can serve as a reference for preparing AI antigens as reagents in HI assays (McHugh, 2012).

For the ND antigen, the ANOVA results also showed no significant differences between HI 1 and HI 2 outcomes, regardless of whether the in-house or commercial PUSVETMA ND antigen was used. This suggested that the in-house ND antigen was stable and produced consistent results in duplicate HI assays. Similarly, ANOVA analysis of antibody titers revealed no significant differences between the in-house and commercial PUSVETMA's ND antigens, indicating that the self-prepared ND antigen can be substituted for the commercial antigen.

The in-house ND antigen demonstrated a 100% sensitivity and specificity. Specificity (Sp) represents the proportion of non-immune sera correctly identified as negative, whereas Se indicates the proportion of serum correctly identified as positive (Moore *et al.*, 2013). The antibody titers obtained using the commercial PUSVETMA's ND antigen served as valid reference standards. According to the HI test with the commercial antigen, 21 sera were immune and one was non-immune. Testing with the in-house ND antigen yielded similar results. These findings indicate that the in-house ND antigen can measure ND antibody titers with high specificity and sensitivity. Achieving 100% Se and Sp demonstrated that the in-house ND antigen has a high level of accuracy for qualitatively diagnosing ND antibody immunity (West & Kobokovich, 2020). Based on Kappa analysis, the HI assay using the in-house and PUSVETMA's ND antigens achieved almost perfect agreement. This high level of concordance reflects a complete consensus between in-house and commercial ND antigens, without misclassification or misinterpretation (McHugh, 2012).

Conclusion

This study successfully prepared and validated in-house AI and ND antigens for use in hemagglutination inhibition (HI) assays. Both antigens demonstrated stability and consistency, as confirmed by ANOVA, and achieved 100% sensitivity and specificity when compared with PUSVETMA's standardized antigens. The κ values indicated almost perfect agreement, underscoring the reliability of in-house antigens. These findings suggest that AI and ND antigens prepared in-house can serve as reliable substitutes for commercial PUSVETMA antigens in HI testing. Establishing such independent antigen preparation methods provides a valuable alternative for private laboratories, supporting greater self-sufficiency in serological diagnostics of avian influenza and Newcastle disease in Indonesia.

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Author contribution ONP: Conceptualization, methodology, analysis, resource, supervision, and writing – original draft, review & editing; SS: investigation, data curation, formal analysis; NAP: investigation, data curation, formal analysis.

Availability of data and materials All data are available in the manuscript.

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