

Subtype Identification Of Avian Influenza Virus Isolated from Laying Duck In Sidenreng Rappang, South Sulawesi With Hemagglutination Inhibition Assay

Bagus Nanang Luwito^{1,2,a)*}, Ahmad Nadif^{1,3,b)}, Retno D Soejoedono^{4,c)}, I Wayan T Wibawan^{4,d)}

¹ Graduate School of IPB, Bogor Agricultural University

Jl. Agatis, Kampus IPB Dramaga, Bogor

² Agricultural Quarantine Agency, Ministry Agriculture Republic of Indonesia

Jl. Harsono RM Nomor 3 Ragunan, Pasar Minggu Jakarta 12550 Grdung E Lt.1, 5, 7

³ Pare – pare Agricultural Quarantine Unit of South Sulawesi

JL Tarakan, No. 1, Cappa Ujung, Parepare, Sulawesi Selatan 91114, Indonesia

⁴Departemen of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine,

Jl. Agatis, Kampus IPB Dramaga, Bogor

^{a)}Corresponding author: bagus240456@gmail.com

^{b)}ahmadnadif1981@gmail.com

^{c)}retnodmail@yahoo.com

^{d)}wibawan@yahoo.com

Abstract

Avian Influenza virus (AIV) have caused significant economic losses in poultry sector worldwide. Results of genetic mapping showed that circulating AIV in Indonesia were belong to clade 2.1.1, 2.1.2, 2.1.3 and 2.3.2, however the AIV subtype were merely H5N1. Our study aim to identify the hemagglutinin protein of AIV isolated from native laying duck in Sidenreng Rappang, South Sulawesi, Indonesia using HI assay. Three cloacal swab samples: Sidrap3 (S3), Sidrap5 (S5), and Sidrap10 (S10) were isolated from native laying duck at three duck farms at Sidenreng Rappang during AI outbreaks in 2016. These samples were propagated on spesific antibody negative (SAN) embryonated chicken eggs, followed by subtype identification using hemagglutination inhibition (HI) test using spesific antibody of AIV subtype H5N1, AIV subtype H5N2, AIV subtype H5N3 and ND Lasota virus. Our study showed that H5N1 spesific antibody has titer of 2⁴ against virus isolates Sidrap3 and Sidrap10, titer of 2⁶ against virus isolates Sidrap5. H5N2 spesific antibody has titer 2⁴ against virus isolates Sidrap3 and Sidrap5, titers of 2⁶ against virus isolates Sidrap10. H5N3 spesific antibody has titers of 2⁶ against virus isolates Sidrap3, Sidrap5 and Sidrap10. Whilst, spesific antibody of ND Lasota did not react with all of virus isolates. Our results showed that all AIV isolates gives highest reaction with spesific antibody of AIV H5N3. According to the result, it is likely that AIV H5N3 might circulating among laying duck in Sidenreng Rappang.

Keywords: avian influenza, subtype, hemagglutination inhibition, Sidenreng Rappang

1. INTRODUCTION

Avian Influenza Virus (AIV) outbreaks have caused significant economic losses in poultry sector worldwide. Since 1997, Asian lineage highly pathogenic AIV (H5N1) has spread from Asia to Europe, the Middle East and Africa (Bragstad *et al.* 2007). The virus has been associated with significant mortality in wild bird (Uchida *et al.* 2008). Furthermore, humans and some other species of mammals have contracted the disease by close contact with infected bird (Hayden and Croisier 2005; Songserm *et al.* 2006).

AIV is a lipid-envelope virus of the family *Orthomyxoviridae* and genus *Influenzavirus A*. Type A influenza viruses can be divided into 16 hemagglutinin (HA; H1-H16) and nine neuraminidase (NA; N1-N9) subtypes based on the genetic sequence for the envelope protein (Spackman 2008), although in 2012 it has been found H17N10 type A influenza virus isolated from bats (Tong *et al.* 2012). The hemagglutinin protein on the surface of type A influenza virus particles is capable of binding to N-acetylneuraminic acid-containing protein on avian and mammalian erythrocytes (Killian 2008). Agglutination of erythrocytes is the basis of the hemagglutination (HA) assay, and inhibition of the agglutination reaction by subtype-specific antisera is the basis of the hemagglutination inhibition (HI) assay (Pedersen 2008).

Results of genetic mapping showed that circulating AIV in Indonesia were belong to clade 2.1.1, 2.1.2, 2.1.3 and 2.3.2 (Dharmayanti *et al.* 2013), however the AIV subtype were merely H5N1. Since its first outbreak in Indonesia in late 2003 until mid 2012, AIV caused morbidity and mortality in chickens, whilst duck were considered as reservoir. However, in late 2012, there is AIV outbreak among commercial duck in Central Java, this incident proves that duck were also susceptible of AIV. AIV which infected duck were belong to subtype H5N1 clade 2.3.2 (Wibawa *et al.* 2012). After its introduction of new AIV clade 2.3.2, this clade has been spread to other regions in Indonesia. Some epidemiological studies revealed that free-range domestic ducks played a prominent role in regional spread of AIV H5N1 in Southeast Asia. Asymptomatically infected domestic ducks can shed the virus continuously from their oral cavity and cloaca, contributing significantly to silent spread of AIV H5N1 (Songserm *et al.* 2006).

Recently, AIV identification were determined using molecular technique such as RT-PCR and nucleotide sequence. These technique enabled us to identify pathogen to the species, strain and clade. However, conventional technique as HI assay were still considered as standard test for AIV identification (OIE 2015). Our study aim to identify the hemagglutinin protein of AIV isolated from native laying duck in SidenrengRappang, South Sulawesi, Indonesia using HI assay. Result of this research might be useful as first information AIV subtype for genetic analysis.

2. MATERIAL AND METHOD

Samples

Three cloacal swab samples: Sidrap3 (S3), Sidrap5 (S5), and Sidrap10 (S10) were isolated from native laying duck at three duck farms at SidenrengRappang during AI outbreaks in 2016.

Isolation and Propagation Virus

Each swab samples were propagated in three embryonated Specific Antibody Negative (SAN) chicken eggs on 9 day old, each egg were inoculated with 0.2 ml swab medium , after 60 hours (h), or when the embryo died before that time, the allantois fluid was harvested. Allantois fluid were tested using HA assay following standard procedure (OIE 2015).

Hemagglutination Assay

HA tests were performed by combining 50 µl chorioallantoic fluid (CAF) from one egg and 50 µl chicken red blood cells (CRBCs) 5%. The mixture of CAF and CRBCs was gently agitate for 15 to 30 second. A positif result with aglutination indicate the presence of the AIV in the CAF sample tested. The HA test was performed on each egg individually.

Hemagglutination Inhibition Assay

AIV subtype identification were determined using HI assay. Four specific antibody of AIV H5N1, AIV H5N2, AIV H5N3 and Newcastle diseases virus (NDV) Lasota were used for HI assay. The HI tests were carried out using 4 HAU of the AIV isolates. The serum samples were made into two fold dilution, and the titer were expressed as the reciprocal of the last serum dilution that caused complete inhibition of agglutination (OIE 2015)

3. RESULTS

Virus Titer

All AIV isolates showed virus positive. Results of virus HA titre were presented in Table 1.

TABLE 1. Virus titer

Isolat	HA titers (HAU)*
Dk/Sidrap/(S3)/2016	2 ¹⁰
Dk/Sidrap/(S5)/2016	2 ⁹
Dk/Sidrap/(S10)/2016	2 ¹¹

*HA titer were presented in log2

Virus-specific Antibody Reaction

AIV subtype identification were determined using HI assay using specific antibody of AIV H5N1, AIV H5N2, AIV H5N3 and NDV Lasota, the result were presented in Table 2.

TABLE 2. Virus-specific antibody reaction

Sample	HI titre (Log ₂)			
	H5N1	H5N2	H5N3	ND Lasota
Dk/Sidrap/(S3)/2016	2 ⁴	2 ⁴	2 ⁶	< 2 ¹
Dk/Sidrap/(S5)/2016	2 ⁶	2 ⁴	2 ⁶	< 2 ¹
Dk/Sidrap/(S10)/2016	2 ⁴	2 ⁶	2 ⁶	< 2 ¹

DISCUSSION

The AIV is type A influenza isolated from and adapted to avian host. Type A influenza belongs to the *orthomyxoviridae* virus family, is enveloped, and pleiomorphic with a size ranging from 80–120 nm. Type A influenza strains are classified by the serological subtypes of the primary viral surface proteins, the HA and NA. With a new serotype (H17) of hemagglutinin recently being discovered, there are now 17 serotypes (H1 to H17) of influenza A viruses in total (Tong *et al.* 2012). There are currently 17 HA subtypes (H1 to H17) and 10 NA subtypes (N1 to N10) known. 16 subtypes have been found in ducks, gulls, or shore birds, the natural reservoir host species of the virus. However, in these species certain subtypes are more common than others; for example, H3, H4, and H6 are most common in ducks in North America and although there is no clear association between host range or host restriction based on HA subtype, some subtypes are more common in some species than others, for example H1 and H3 in swine, H3 in horses, and H5 and H7 in chickens (Spackman 2008), and new virus genome subtype H17 identified from bat (Tong *et al.* 2012).

The rapid detection and diagnosis of AI virus in poultry, wild birds, and other species are critical to control the virus. Subsequent characterization provides vital information about the ecology and biology of the virus. Because of the clinical importance of the presence of a viable virus isolate, and the necessity of having a virus isolate to be characterized, virus isolation is probably the most critical of these methods. Classical methods such as HI assay, neuraminidase inhibition assay, and *in vivo* pathotyping are still the reference standards required.

Inhibition of RBCs agglutination in HI assay were caused by antigen-antibody bond. Influenza virus membranes contain two subtype specific glycoprotein which is hemagglutinin (HA) and neuraminidase (NA) on their surface envelope. Hemagglutinin mediates cell surface sialic acid receptor binding to initiate virus infection, while neuraminidase play role on biological activity in removing N-acetyl neuraminic acid or sialic acid from sugar chain of glycoprotein and as major antigenic determinant (Pedersen 2008).

In the current study, we aim to identify hemagglutinin protein of AIV isolates. Our result showed that all isolates were able to react with specific antibody of AIV H5N1, AIV H5N2 and AIV H5N3, but did not react with specific antibody of NDV Lasota. This result indicated that all AIV isolates were belong to H5 subtype. In diagnostic assay, an AIV with novel hemagglutinin subtype would not be produce a false-negative result in HI assay using a known HA subtypes specific antibody. However, usage of NA subtype specific antibody will

help to avoid false-positive reaction on identification using HI assay. When the NA subtype is unknown, it is necessary to perform HI assay with several NA subtypes specific antibody since it will help to eliminate false-positive result due to steric inhibition from the NA antibody (Pedersen 2008).

Our results also showed that all AIV isolates gives highest reaction with specific antibody of AIV H5N3. According to the result, it is likely that AIV H5N3 might circulating among laying duck in Sidenreng Rappang. However, in the current study, we only performed HI assay for subtype determination, we realize that more general statement need another assay such as reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleotide sequence to be done. However, we did not performed other assay since it is beyond our purpose of study. Therefore we cannot give a strong conclusion based only HI assay result. Nevertheless, our result were in accordance with previous study of Susanti (2008) which is able to isolated AIV subtype H5Nx from native duck in West Java indicating that there might be other than N1 exist. A continuous surveillance of AIV in Indonesia are needed to understand the evolution, genetic and antigenic character of circulating virus. For better surveillance result, characterization of AIV should be done up to identifying HA and NA subtype.

REFERENCES

1. E. Spackman, A Brief Introduction to the Avian Influenza Virus, In: *Methods in Molecular Biology :Avian Influenza Virus*, edited by E. Spackman (HumanaPress, 999 Riverview Drive, Suite 208, Totowa, NJ 07512 USA 2008), pp. 1-6
2. F. Hayden, and A. Croisier. Transmission of avian influenza viruses to and between humans. *J. Infect. Dis.* 192:1311–1314. (2005).
3. H. Wibawa, W. B. Prijono, N. L. P. I. Dharmayanti, S. H. Irianingsih, Y. Miswati, A.Rohmah, EAndesyha, Romlah, R. S. D.Daulay, K. Safitria, Investigasi wabah penyakit pada itik di Jawa Tengah, Yogyakarta, dan JawaTimur: Identifikasi sebuah clade baru virus avian influenza sub tipe H5N1 di Indonesia. *Bul Lab Vet.* 12:2-9. (2012)
4. J. C. Pedersen, Hemagglutination-Inhibition Test for Avian Influenza Virus Subtype Identification and the Detection and Quantitation of Serum Antibodies to the Avian Influenza Virus, In: *Methods in Molecular Biology :Avian Influenza Virus*, edited by E. Spackman (HumanaPress, 999 Riverview Drive, Suite 208, Totowa, NJ 07512 USA 2008), pp. 53-66
5. K. Bragstad, P. H. Jørgensen, K. Handberg, A. S. Hammer, S. Kabell, and A. Fomsgaard. First introduction of highly pathogenic H5N1 avian influenza A viruses in wild and domestic birds in Denmark, Northern Europe. *Virol. J.* 4:43. (2007)
6. M. L. Killian, Hemagglutination Assay for the Avian Influenza Virus, In: *Methods in Molecular Biology :Avian Influenza Virus*, edited by E. Spackman (HumanaPress, 999 Riverview Drive, Suite 208, Totowa, NJ 07512 USA 2008), pp. 47-52
7. N.L.P.I.Dharmayanti, R.Hartawan, D. A.Hewajui, Hardiman, H.Wibawa, Pudjiatmoko..KarakteristikMolekulerdanPatogenesitas Virus H5N1 Clade 2.3.2 asal Indonesia. *JurnalIlmuTernakdanVeteriner* 18(2):99-113 (2013)
8. [OIE] Office International des Epizooties, Avian Influenza, In: *Manual of Diagnostic Test and Vaccines for Terrestrial Animals*, 7th ed., chapter 2.3.4., 2, <http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/pdf> (2015)
9. R.Susanti, R. D Soejoedono, I. G. N. K. Mahardika, I. W. T Wibawandan M. T.Suhartono,Potensi Unggas Air sebagai Reservoir Virus High Pathogenic Avian Influenza Sub tipe H5N1, *JITV* 12 (2), pp. 160-166(2007)
10. S.Tong , *et al.* . A distinct lineage of influenza A virus from bats. *Proc. Natl. Acad. Sci. U. S. A.* 109:4269–4274. (2012)

11. T. Songserm, , R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster, Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerg. Infect. Dis.* pp:575–581. (2006)
12. Y.Uchida, M. Mase, K. Yoneda, A. Kimura, T. Obara, S. Kumagai, T. Saito, Y. Yamamoto, K. Nakamura, K. Tsukamoto, and S. Yamaguchi. Highly pathogenic avian influenza virus (H5N1) isolated from whooper swans, Japan. *Emerg. Infect. Dis.* 14:1427–1429. (2008)