Identification of Pathogenecity of Avian Influenza Virus Subtype H5N1 from Waterfowls Base on Amino Acid Sequence of Cleavage Site Hemagglutinin Protein

R. Susanti1*, Retno D Soejoedono2, I Gusti Ngurah K Mahardika3, I Wayan T Wibawan2, and Maggy T Suhartono4

1.Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Semarang, Semarang  Indonesia
2.Department of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agriculture Institute, Bogor,  Indonesia
3.Biomedical and Animal Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali, Indonesia
4.Faculty of Agricultural Technology, Bogor Agriculture Institute, Bogor, Indonesia

Abstract
Identification of pathotype of Avian Influenza Virus (AIV) subtype H5N1 isolates is very important. This research aimed to identify the pathotype of AIV subtype H5N1 isolated from household waterfowls in West Java based on molecular markers of amino acid sequences of the Hemagglutinin (HA) cleavage site. Fragments of HA genes of 21 isolates were amplified using RT-PCR with a primer pair that flanking the cleavage site region, and sequenced with dideoxy-termination method with ABI automatic sequencer (Applied Biosystems). Multiple alignment of nucleotide and their deduced amino acid sequence were analyzed using ClustalW from MEGA 3.1 program. The result shows that all H5N1 isolates (21 isolates) possess polybasic cleavage sites with 2 patterns of amino acid sequence, i.e QRRERRRRKKR (20 isolates) and QRESRRKKR (1 isolate). This finding indicates that all of the viruses isolated in this research were of highly pathogenic avian influenza (HPAI) strains.

Keywords: cleavage site, waterfowls, HPAI

Introduction
Highly Pathogenic Avian Influenza (HPAI) Virus Subtype H5N1 is endemic in 31 of 33 provinces in Indonesia (Health Department, 2008). AI Virus subtype H5N1 is highly pathogenic on chicken and human, but clinical cases and death of waterfowls (ducks, muscovy ducks and geese) were not significant. Waterfowls are potential as vector of AI Virus subtype H5N1. Studies showed that as many as 21 isolates of AI Virus Subtype H5N1 from 460 samples of healthy unvaccinated waterfowls (ducks, geese, muscovy ducks) have been successfully isolated from household farms in West Java. The prevalence number of AI Virus H5N1 of each species are (in descending order) 6.67%, 4.85%, and 4.04% for geese, ducks, and muscovy ducks, respectively (Susanti et al., 2008; in press).

AI Virus subtype H5N1 isolated from waterfowls in household farms in West Java should be determined for its pathogenecity characteristics using molecular and biologi-
cal methods to understand the virulence of AI Virus H5N1 both on waterfowls and other hosts including mammals (human). AI Virus H5N1 isolated from a healthy duck in South China, have been found to be molecularly pathogenic, and biologically the virus was also highly pathogenic on chickens and mammals (mouse). The basic of molecular pathogenicity and the transmission ability across species (from fowls to mammals) clearly involved various virus genes, including hemagglutinin gene (HA) (Chen et al. 2004).

Cleavage site is an amino acid sequence acting as a splitting site of HA (HA0) precursor into HA1 and HA2 enzymatically by protease of host cells, and therefore fusion with endosome membrane can occur to facilitate infection of AI Virus into host cells. The existence of HA0 cleavage site relies on the presence of arginine (R) or lysine (K) base amino acid. A cleavage site is specific and certain specificity of protease limit the distribution of tissues infected by AI virus. Most of non-virulent or low pathogenic AI Virus have one base amino acid (monobasic) cleavage site, but highly pathogenic strains have more than one base amino acid (polybasic) on the site (Munch et al. 2001).

HA sequences with monobasic cleavage site (e.g. HA1-PSIQVR-GL-HA2) is cut by tryptase yielded from respiration and digestive tract epithelials (Whittaker 2001; Chen et al. 2004). HA sequences with polybasic cleavage site (e.g. HA1-KKREKR-GL-HA2), allow proteolitic process done by proteases such as furine and pro-proteine convertases 6 (PC6) found in Golgi apparatus of all cells (Horimoto et al. 1994). AI Virus with polybasic cleavage site have unlimited distribution network and may cause fatal sistemic infection (Whittaker 2001; Chen et al. 2004). Polybasic cleavage sites in AI Virus H5N1 are responsible for sistemic infection and therefore virus can be isolated from blood, cerebrospinal aqueous and feces (WHO et al. 2005).

Pathotype identification of AI Virus H5N1 is very important to determine whether the strain/isolate is low pathogenic (LPAI) or highly pathogenic (HPAI). Pathogenecity of AI Virus is determined based on molecular or biological analysis. Biologically, AI Virus is considered to highly pathogenic if the virus infects chicken aged 4-8 weeks intravenously it would cause 75% death within 8 weeks (WHO, 2002). Molecularly, virus pathogenecity can be quickly analyzed based on the melting temperature (Tm) curve using real-time reverse transcriptase polymerase chain reaction (RT-PCR). HPAI virus isolates has Tm as high as 77.43°C, whereas that of LPAI virus is 79.57°C (Payungporn et al. 2006). However, the weakness of that method was that we could not determine the pattern of amino acid sequence in the cleavage site.

This research aimed to determine AI Virus H5N1 pathotype isolates from household waterfowls in West Java, based on the amino acid sequence of cleavage site hemagglutinin protein by means of sequencing method.

**Materials and Methods**

As many as 21 AI viruses Subtype H5N1 isolates obtained from waterfowls (ducks, muscovy ducks, geese) in the household farms in West Java, were analyzed for its pathotype based on the amino acid sequence in the cleavage sites of hemagglutinin protein using sequencing method.

**RNA Virus Isolation**

RNAs from AI virus H5N1 were extracted using Trizol®LSReagent (Invitrogen) as guided in the manual.

**RT-PCR**

RT-PCR was done using Superscript™ III One-step RT-PCR system (Invitrogen). RT-PCR reaction was prepared at amount of
50 ml with composition of 25 ml 2x reaction mix, 2 ml forward primer (10 mM), 2 ml reverse primer (10 mM), 2 ml Superscript III RT/Platinum Taq Mix, 3 ml RNA sample and ultrapure H2O until reaching 50 ml. Primer used was the primer pair that flanking cleavage site region, they are H5-1 (5’GCCATTCCACAACATACACCC’3) and H5-3 (5’CCTCCCTGCTCAT TGCTA’3) (WHO 2005). RT-PCR program consists of process of transverse transcription 45°C for 60 minutes, pre-denaturation 95°C for 5 minutes, 35 cycles consist of denaturation 95°C for 30 seconds, annealing 55°C for 30 seconds, extension 72°C for 40 seconds, and post-extension 72°C for 10 minutes (WHO 2005). The specific DNA band resulted from PCR was identified by electrophoresis on 2% agarose gel.

DNA Sequencing

PCR products (219bp) from each isolate were sequenced in 1stBASE Malaysia in dideoxy method using ABI automatic sequencer (Applied Biosystems). Multiple alignment of nucleotide and their deduced amino acid sequence were analyzed using ClustalW from MEGA 3.1 program (Kumar et al. 2004). Pathotype of AI Virus was determined based on the cleavage site of amino acid sequence. Non-virulent or low pathogenic AI Virus has monobasic amino acid sequence of cleavage site (i.e: HA1-PSIQVR-GL-HA2), and highly pathogenic strain of AI virus has polybasic amino acid sequence on the cleavage site (i.e: HA1-KKRRKKR-GL-HA2) (Munch et al., 2001).

Results

Target of H5-1 and H5-3 primers (WHO, 2005) are nucleotides 915-1133. On this sequence there are amino acid marking genes in the cleavage site that determine the characteristics of virus, whether it is an HPAI or a LPAI. The result of RT-PCR avian influenza virus subtype H5N1 with H5-1 and H5-3 primer showed in Figure 1. The sequence of entirely hemagglutinin fragment gene and predicted amino acid sequences were presented in Figure 2. The sequencing result from 21 AI virus H5N1 isolates showed that all of the isolates were grouped into HPAI with polybasic amino acid sequence QRERRRRKKR (20 isolates) and QRESRRKKKR (1 isolate) on the cleavage site (Table 1).
Discussion

Analysis of amino acid sequence in the HA cleavage site of all AI viruses subtype H5N1 that cause death in human and poultry in Indonesia which was based on data from GenBank (http://www.ncbi.nlm.nih.gov/) showed that all of AI viruses Subtype H5N1 spread in Indonesia demonstrated the characteristics of HPAI molecular with varying cleavage site sequence (Table 2). Pattern of amino acid sequence in the cleavage site QRERRRKKR was typically the cause of death poultry in Hong Kong on 1997 and other Asian countries (2003-2007) (Guan et al. 2004; Smith et al. 2006; Stevens et al. 2006). Isolates of H5N1 AI virus that cause poultry death in Indonesia during 2003-2004 had amino acid sequence pattern in the cleavage site QRERRRKKR, except for A/Chicken/Kulonprogo/BBVet-XIII isolate which underwent deletion of one amino acid lysine (K) so that the cleavage site was QRERRK_R. Starting from 2005, H5N1 AI virus isolates emerged with QRESRRKKR, QIERRRRKR, QERRREKR, QGERRRRK, QERRRR_K and QRE_RRKKR cleavage site sequences.

Since July 2005 to 2007, there happened cases of human death in Indonesia due to AI virus H5N1 with cleavage site sequence QRESRRKKR. In this research it was found that 1 isolate of AI virus H5N1 from a duck (IPB10-RS) has cleavage site pattern of QRESRRKKR. This QRESRRKKR pattern is specific in AI virus H5N1 causing human death in Indonesia during 2005-2007 (CDC 2007). The findings that the pattern (QRESRRKKR) was found clinically on healthy waterfowls, supported the hypothesis that ducks seem to play an important role as source of AI virus subtype H5N1 and transmission of these virus to terrestrial poultry and humans. On the other hand, QRESRRKKR pattern found on ducks indicate that ducks may act as evolution site of AI virus Subtype H5N1. This result corresponds with the previous findings that AI virus H5N1 evolve in the body of clinically healthy ducks in South China on 1999-2002, and from year to year it becomes more pathogenic to mammals (Chen et al. 2004).

Although the amino acid sequence of the cleavage site of the 21 AI viruses Subtype H5N1 isolates obtained from waterfowls in this research are highly pathogenic clinical symptoms (Lipatov et al. 2004; Hulse-Post et al. 2005; Sturm-Ramirez et al. 2005; Webster et al. 2007). Virus adaptation on this host occurred for years, because
waterfowls acting as reservoir might also cause avirulence of HPAI virus H5N1 infection on waterfowls (Webster et al., 1992). The low level of HPAI virus H5N1 pathogenicity on waterfowls was said to be related to the limited amount and capability of waterfowls proteases to cut HA₀ on the cleavage sites (Siegel, 2006).

As natural host, waterfowls also act as a host adaptation for influenza virus (Hulse-Post et al., 2005). The non-pathogenic characteristics of HPAI virus H5N1 on waterfowls showed that the biological evolution of virus have reached equilibrium point on this natural hosts (Horimoto and Kawaoka, 2001; Hulse-Post et al., 2005; Sturm-Ramirez et al., 2005). Most of the virus may be eliminated by immune responses of the waterfowls, but a part of virus population would remain replicate and excreted with feces (Hulse-Post et al., 2005; Liu, 2007).

Outbreak of AI virus H5N1 in Hongkong late 2002 that caused death on migratory birds and domestic waterfowls including ducks, was the first report since 1961. On 1961, H5N3 AI virus infection was lethal to about 13,000 of Sterna hirundo in South Africa (Sturm-Ramirez et al., 2004; Beato et al. 2007; Stallknecht & Brown, 2007). HPAI Virus H5N1 has caused outbreak that killed thousands of wild waterfowls (60 species) on Qinghai Lake, China, on 2005 (Zhou et al. 2006; Stallknecht and Brown, 2007). The pathogenicity of AI virus H5N1 on waterfowls was an adaptation process of the virus on waterfowls, and kept mutating and/or reassorting until the virus really adapted to natural hosts (Hulse-Post et al., 2005).

The fact that waterfowls are source of HPAI virus H5N1 infection has made the implementation of prevention and control programs against virus became more complicated. Water as waterfowl habitat, is a persistence media and a source of HPAI virus H5N1 infection. Although the virus shedding from ducks was not persistently occur (only 2-4 weeks post-infection), the virus may still infective in the water for up to 30 days at temperature of 0°C and 4 days at 22°C. AI viruses on waterfowl feces may remain infective for up to 30 days at 4°C, and up to 7 days at 20°C and up to 4 days at 25°C (Spencer et al., 2007). Asian strain of HPAI Virus H5N1 was also persistent on water at 17°C and 18°C (Brown et al., 2007).

Since waterfowls live on waters, water as a place for swimming, eating and drinking activities, is too risk as the source of HPAI virus H5N1 spread to other waterfowls, terrestrial poultry and humans (Hulse-Post et al. 2005; Liu 2007). Waterborne transmission is the mechanism for influenza virus to keep survive on waterfowls as its natural habitat (Ito et al. 1995; Liu, 2007).

Farming and agriculture systems involving various components of plant and animal species might increase the opportunity cross-infection among species (Cristalli and Capua, 2007). Farming of many terrestrial poultry species (even mixed with mammals) in one area may increase the risk of virus spread among species and may also increase the chance to create new virus strains due to reassortment process (Liu, 2007). Free-grazing ducks, especially during rice harvest time was also known as a critical factor in HPAI virus H5N1 persistence and spread (Gilbert et al. 2006; Liu 2007). The prevalence of AI virus H5N1 infection on domestic chicken/poultry correlates with duck distribution grazing in free range area (Songserm et al., 2006).

In East and Southeast Asia, billions of domestic waterfowl are raised in free range which facilitate to form ecological interfaces between wild aquatic birds and domestic waterfowls and between domestic waterfowls and other animals and humans. Therefore, AIV H5N1 can be transmitted...
from wild aquatic birds via domestic waterfowls to other animals, especially terrestrial poultry. Consequently, domestic waterfowls is not only a reservoir for AIV H5N1 but also play important role in the maintenance, evolution and perpetuation of the viruses and in interspecies transmission and epidemics (Liu, 2007).

Waterfowl elimination could not be done for the sake of logistics, environment and biodiversity reasons (FAO, 2007). Waterfowls may play an important role in the maintenance of aquatic ecosystem biodiversity, by passive dispersal of invertebrates and aquatic plants. The capability of waterfowls as a important vectors for the passive dispersal of those aquatic invertebrate and plant relate to the digestive system anatomy that provide an appropriate environment for aquatic organisms (Figuerola et al., 2003; Figuerola et al., 2004). In certain countries of East Asia and South-East Asia, domestic waterfowls (ducks, geese, muscovy ducks) are one of the main sources of protein for human consumption (Liu, 2007). In addition to part of the ecosystem, domestic waterfowls are also the main source of protein for human consumption, and the elimination of waterfowls may impact on the environment, the farmer’s economy and also the accompanying social life.

Prevention and control of HPAI H5N1 on waterfowls may be carried out by such activities as intensive monitoring of AI virus H5N1 on waterfowls, vaccination, farm restructuring and strict biosecurity application to the farms. Farm restructuring include the change of the farming system from open system to closed system. This way the contact between domestic waterfowls and wild waterfowls may be minimized. The system would also prevent AI virus transmission from waterfowls to terrestrial poultry. The mixed farm to breed waterfowls and terrestrial poultry in one area may no longer be recommended (Liu, 2007).

Waterfowls vaccination is one of ways to prevent contamination to humans and terrestrial poultry (Veits et al., 2006). It was reported that conventional vaccination using AI virus H5N1 isolated from ducks may prevent the occurrence of clinical symptoms, virus shedding and virus colonization in meat and internal organs. Vaccination on day 0 and day 30 would be very suitable for implementation in duck farms in Asia. On age 0-30 days, ducks may be still kept in cages and they will be released to open farming areas only after 30 days (Beato et al., 2007).

Measures to prevent the spread of HPAI H5N1 from waterfowls can also be done by regulating the live poultry markets to avoid the mixture of all kinds of poultry in one area (Capua and Marangon, 2006; Cristalli and Capua, 2007). AI virus transmission from waterfowls to other kinds of poultry have been found in the markets, where animal contact between waterfowls and other kinds of bird such as chickens, quails, and other birds could not be avoided (Capua and Marangon, 2006; Gilbert et al., 2006; Xue et al., 2007).

Prevention and control programs of AI virus H5N1 related to the role of waterfowls need to be immediately carried out and should involve many sectors as well participation of the policy makers. President Decree No. 1 year 2007 on the Handling and Control of Avian Influenza Virus does not yet specifically regulate the waterfowl farmings as well as the handling and the prevention.

As the conclusion of this research, all avian influenza virus subtype H5N1 (21 isolates) obtained from household waterfowls in West Java were highly pathogenic with 2 patterns of cleavage site amino acid sequence, they are QRERRRRKKKR (20 isolates) and QRESRRRKKR (1 isolate).
Acknowledgement

This research was partly supported by Indonesian state ministry of research and technology through Applications Research Incentive project

References


Horimoto, T., Nakayama, K., Smeekens, S.P., and Kawaoka, Y., 1994. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of


