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

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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 Q**R**E**RRRKKR** (20 isolates) and Q**R**ES**RRKKR** (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-

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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 pathogenecity 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 (HA_0) precursor into HA_1 and HA_2 enzimatically 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 HA_0 cleavage site relies on the presence of arginine (R) or lysine (K) base amino acid. A cleavage site is specific and certain specifity 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. HA_1-PSIQVR-GL-HA_2) 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. HA_1-KKREKR-GL-HA_2), 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). P*olybasic* 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 H_2O 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'CTCCCCTGCTCAT 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 1st BASE 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: HA_1-PSIQV**R**-GL-HA_2), and highly pathogenic strain of AI virus has polybasic amino acid sequence on the cleavage site (i.e: HA_1-KK**R**EK**R**-GL-HA_2) (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 characeristics 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 QRE**R**RRKKR (20 isolates) and QRE**S**RRKKR (1 isolate) on the cleavage site (Table 1).

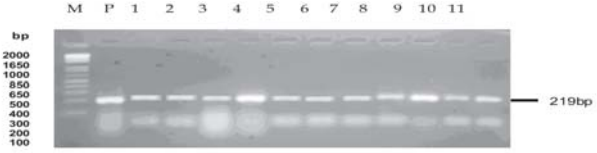

Figure 1. Electrophoregram of H5 gene RT-PCR VAI subtype H5N1 using H5-1 and H5-3 primer (product 219bp). Well M: DNA *ladder* 100bp. Well P: positive control of AIV subtype H5N1. Well 1-11: positive sample of AIV subtype H5N1

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                                ♣ ♣ ♣ TAT GTG AAA TCA AAC AGA TTA GTC CTT GCG ACT GGA
CTC AGA AAT ACC CCT CAA AGA GAG  972
 Y  V  K  S  N  R  L  V  L  A  T  G  L  R  N  T  P  Q  R  E  324
♣  ♣  ♣  ♣  ♣  ♣
AGA AGA AGA AAA AAG AGA GGA CTA TTT GGA GCT ATA GCA GGT TTT ATA GAG GGA GGA
TGG 1032
 R  R  R  K  K  R  G  L  F  G  A  I  A  G  F  I  E  G  G  W  344

CAG GGA ATG GTA GAT GGT TGG TAT GGG TAC CAC CAT AGC AAT GAG CAG GGG AGT GGG
TAC 1092
 Q  G  M  V  D  G  W  Y  G  Y  H  H  S  N  E  Q  G  S  G  Y  364

GCT GCA GAC AAA GAA TCC ACT CAA AAG GCA ATA GAT GGA GT                 1133
 A  A  D  K  E  S  T  Q  K  A  I  D  G                  377
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Figure 2. The hemagglutinin fragment gene sequences and their deduced amino acid sequences of virus A/ muscovy duck/Klapanunggal/IPB1-RS (H5N1). ♣: cleavage site

Table 1. Amino acid sequens of *cleavage site* hemagglutinin protein of avian influenza virus subtype H5N1 isolated from household waterfowls

No	Isolates	Amino acid Cleavage site	Pathotype
1	A/muscovy duck/Klapanunggal/IPB1-RS (H5N1)	QRERRRKKR/G	HPAI
2	A/goose/Bojonggenteng/IPB2-RS (H5N1)	QRERRRKKR/G	HPAI
3	A/duck/Leuwiliang/IPB3-RS (H5N1)	QRERRRKKR/G	HPAI
4	A/goose/Leuwiliang/IPB4-RS (H5N1)	QRERRRKKR/G	HPAI
5	A/muscovy duck/Cileungsi/IPB5-RS (H5N1)	QRERRRKKR/G	HPAI
6	A/duck/Nagrak/IPB6-RS (H5N1)	QRERRRKKR/G	HPAI
7	A/goose/Klapanunggal/IPB7-RS (H5N1)	QRERRRKKR/G	HPAI
8	A/duck/Parung/IPB8-RS (H5N1)	QRERRRKKR/G	HPAI
9	A/duck/Parung/IPB9-RS (H5N1)	QRERRRKKR/G	HPAI
10	A/duck/Bojonggenteng/IPB10-RS (H5N1)	QRE**S**RRKKR/G	HPAI
11	A/muscovy duck/Cidahu/IPB11-RS (H5N1)	QRERRRKKR/G	HPAI
12	A/duck/Ciseeng/IPB12-RS (H5N1)	QRERRRKKR/G	HPAI
13	A/duck/Ciseeng/IPB13-RS (H5N1)	QRERRRKKR/G	HPAI
14	A/duck/Ciseeng/IPB14-RS (H5N1)	QRERRRKKR/G	HPAI
15	A/duck/Cileungsi/IPB15-RS (H5N1)	QRERRRKKR/G	HPAI
16	A/duck/Klapanunggal/IPB16-RS (H5N1)	QRERRRKKR/G	HPAI
17	A/duck/Leuwiliang/IPB17-RS (H5N1)	QRERRRKKR/G	HPAI
18	A/duck/Leuwiliang/IPB18-RS (H5N1)	QRERRRKKR/G	HPAI
19	A/muscovy duck/Cibinong/IPB19-RS (H5N1)	QRERRRKKR/G	HPAI
20	A/muscovy duck/Parung/IPB20-RS (H5N1)	QRERRRKKR/G	HPAI
21	A/goose/Parung/IPB21-RS (H5N1)	QRERRRKKR/G	HPAI

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 QRE**S**RRKKR, Q**I**ERRRKKR, QRERRR**E**KR,Q**G**ERRRKKR, QRERRRK_R and QRE_RRKKR cleavage site sequences.

Table 2. Variation of cleavage site amino acid sequence of avian influenza virus subtype H5N1 in Indonesia from 2003 to 2007 (Data from GenBank http://www.ncbi.nlm.nih.gov/)

No	Cleavage site	Year Isolation	Species/Isolates
1	QRERRRKKR	2003-2007	Humans, chickens, ducks, quails, turkeys
2	QRESRRKKR	2005-2007	Humans, chickens, ducks, muscovy ducks, quails
3	QRERRRK_R	2004	A/Chicken/Kulonprogo/BBVet-XIII-1 A/Chicken/Kulonprogo/BBVet-XIII-2
4	QIERRRKKR	2005	A/Duck/Pali/BVW1358
5	QRERRREKR	2005	A/Duck/Bufeleng BPPVI
6	QRE_RRKKR	2005	A/Chicken/Wates83
7	QGERRRKKR	2005	A/Duck/Badung Bali/05

Since July 2005 to 2007, there happened cases of human death in Indonesia due to AI virus H5N1 with cleavage site sequence of QRE**S**RRKKR. But in 2006 there was also incident of AI virus H5N1 with cleavage site sequence QRERRRKKR in human isolates. AI virus H5N1 isolate data on Indonesian poultry in GenBank (http://www.ncbi.nlm.nih.gov/) showed that cleavage site sequence QRE**S**RRKKR were the highes (86.67%) among all other isolates obtained from Western part of Java Island (West Java and Jakarta). Substitution of HA cleavage site of AI virus Subtype H5N1 (from QRERRRKKR to QRE**S**RRKKR) may correlate with virus adaptation to mammalian host especially human. This was also supported by data on human death cases caused by AI virus H5N1 which were the highest in West Java (Health Department . 2008).

In this research it was found that 1 isolate of AI virus H5N1 from a duck (IPB10-RS) has cleavage site pattern of QRE**S**RRKKR. 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, QRE**S**RRKKR 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 evolute 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 pathogenecity on waterfowls was said to be related to the limited amount and capability of waterfowls proteases to cut HA_0 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 pathogenecity 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 Southeast 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 virus 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 QRE**R**RRKKR (20 isolates) and QRE**S**RRKKR (1 isolate).

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