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Molecular Study on The Pathogenicity of Avian Influenza Virus

M. Haryadi Wibowo[1]*, Heru Susetya[2], Tri Untari[1], Khrisdiana Putri[3], Charles Rangga Tabbu[4], and Widya Asmara[1]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Sekip Unit II Yogyakarta, Indonesia.
[2] Department of Veterinary Public Health, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.
[3] Graduate School of Veterinary Science, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.
[4] Department of Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

Abstract

Highly pathogenic avian influenza virus (HPAI) differ from Low pathogenic avian influenza virus (LPAI) based on multiple basic amino acid motif of the carboxylterminus of HA1, especially arginine and lysine. The propose of this work was toamplify and sequence the cleavage site region of HA gene of avian influenza virusisolated from both cases with characteristic or unspecific lesion, using reversetranscriptase polymerase chain reaction (RT-PCR). Primer desaigned for amplification and sequence was H5-F: 5' ggagactcagcaatcccatgaaaag 3' and H5-R:5'ccataccaaccgtctaccattcc 3', and expected product size was 246 bp. The result indicated that all avian influenza virus (AIV)-isolates originated from chicken with both specific and non specific lesion show a multiple basic amino acid motif -PQRERRRKKR/ /GLF- and classified as highly pathogenic avian influenza. Philogenetic study of HA genefragment indicated that each type of characteristic lesion created philo-groups.

Key words: avian influenza, lesion, hemagglutinin, cleavage site, phylogeny.

Introduction

Highly pathogenic Avian Influenza (HPAI) outbreak has been reported in Indonesia since 2003 and still continuously circulated, due to H5N1 sub-type. The field examination focused on clinical signs of the disease that indicated some changes in macroscopic lesions. Formerly the bird exhibited characteristic symptoms, e.g.: high mortality rate, hemorrhages on shank and hock joint, congestion and cyanosis of comb and wattle, which can be followed by edema gelatinous. Lately the specific symptom does not always evident anymore, although in our previous study all isolates were serologi cally identified as Avian Influenza Virus (AIV) H5N1 sub-type (Wibowo *et al.*, 2006).

Avian Influenza Virus is a member of Orthomyxoviridae family, consist of a segmented, single stranded, and negative sense RNA genome (Fenner *et al.*, 1993; Easterday *et al.*, 1997). The virus has a wide range of hosts. Poultry, mammalian, deep whale and human could be infected by AIV. So far the water fowls and wild birds considered as the potential reservoir of the virus, and exhibited without clinical signs (Zhou *et al.*, 1999; Lipatov *et al.*, 2004). Virions envelope have glycoprotein

*Corresponding author: M. haryadi Wibowo, Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Jl. Olah Raga Karangmalang, Yogyakarta 55281, Indonesia. E-mail: mhwibowo@ugm.ac.id

projection called Hemagglutinin (HA) and Neuraminidase (NA). Approximately 80% of the surface's projections are Hemagglutinin and the remaining Neuraminidase (McCaulay and Mahy, 1983). Antigenic properties of HA and NA glycoprotein determine the sub-type specifity of AIV. Currently, it has been recognized 16-H and 9-N (Suzuki and Nei, 2002; Fourchier *et al*., 2005). Hemagglutinin is viral glycoprotein which is res-ponsible for both receptor binding and membrane fusion that in turn will facilitate the infection (Matrosovich *et al.*, 1999; Mitnaul *et al.*, 2000; Suzuki *et al*., 2000). In addition to its binding and fusion functions it is the primary target for neutralizing antibodies.

Although the pathogenicity of AIV is polygenic trait, the HA's surface glycoprotein still play a central role (McCauley and Mahy, 1983; Ito *et al.*, 2000). The process of the infection, HA will be cleaved by certain protease enzyme into HA1 and HA2 sub-unit. The HA1 is receptor binding protein as the major target of immune responses, whereas HA2 is an anchor protein of the envelope and mediates fusion of the envelope and the cellular endosomal membrane (Suzuki and Nei, 2002). Another worker emphasize that the cleavage process on HA region will determine further infection.

Cleavage site region of HPAI viruses differ from those of avirulent influenza a viruses by the virtue of possessing multiple basic amino acid of the carboxyl terminus of HA1, especially arginine and lysine. This feature permits cellular protease such as furine will recognize multiple basic amino acid, to cleave the HA and render the virus infection and able to spread to a variety organs, leading to systemic infection. Whereas most avirulent strain have a single arginine or monobasic cleavage site that will cleaved only by tripsin which secreted from cells in the respiratory and intestinal tract, so the virus only produced localized infection (Harimoto and Kawaoka, 2001; Ito *et al*., 2001). Specific motif consisting of a basic amino acid series at the cleavage site is RRRKKR/ /GL- (Dharmayanti *et al*., 2005) or –RRRKKR/ /G- (Harimoto and Kawaoka, 1994). Most of the motif has been identified for AIV H5N1 sub-type in Indonesia. The presence of the polybasic amino acid region is important as pathotypic marker of AIV (Bank and Polywright, 2003).

Based on fascinating fact, the aim of this study is to determine the molecular pathogenicity marker of both AIV isolates by amplifying and sequencing the cleavage site region of the hemagglutinin.

Materials and Methods

Ten isolates have been identified using RT-PCR. Four isolates were obtained from birds without specific symptoms. Six isolates were obtained from birds that indicated characteristic lesion of avian influenza virus infection.

Viral RNA extraction

Viral RNA was extracted from allantoic fluid using RNA extraction kit Invitrogen Pure Link™ Micro to Midi 50xRxn Total RNA Purification System (Cat. no: 12183-018) according to the manufacturer's protocol. To purify total RNA from 0.2 ml allantoic fluid, in a 1.5 ml RNAse-free micro centrifuge tube was added 0.2 ml of RNA lysis solution contain 1% (v/v) 2-mercaptoethanol. The mixture was vortexed throughly to disrupt lyse blood cells, and centrifuged at 12,000xg for 2 min at room temperature. Supernatant was transferred to clean 1.5 ml RNAse-free microcentrifuge tube and 200 ml of 100% ethanol was added. Any precipitate was dispersed by vortexing or pipetting up and down several times. The sample was transferred to the RNA spin cartridge, centrifuged at 12,000xg for 15 s at room temperature. The flow-through was

discarded. This step was repeated once. The spin cartridge was centrifuged at 12,000xg for 1 min at room temperature to dry the membrane, and the cartridge was moved into an RNA recovery tube. Fifty microliters of RNase-free water was added to elute the RNA and incubated for 1 min at room temperature then centrifuged for 2 min at 12,000xg at room temperature. This step was repeated once, after that the cartridge was discarded and elutes was stored at -4°C.

Thermocycling condition

One step Reverse transcriptase-PCR (RT-PCR) was carried out in GeneAmp® PCR System 2400 machine and performed using Invitrogen SuperScript™ III One-Step RT-PCR System. Cycling conditions included a reverse transcription step at 50°C for 30 min. After an initial denaturation step at 94°C for 5 min, amplification was performed during 40 cycles including denaturation (94°C for 30 s), annealing (50°C for 1 min) and extension (68°C for 45 s), followed by final extension at 68°C for 5 min. Primer designed for this amplification and sequence was H5-F: 5' ggagactcagcaatcccatgaaaag 3' and H5-R: 5'ccataccaaccgtctaccattcc 3', which has expected product size was 246 bp (Ito *et al*., 2001).

Electrophoresis of PCR product

PCR product were electrophoresis in 1.5% agarose gel in 1x TBE, using Electrophoresis tank (MSMIDIDUO, Cleaver Scientific Ltd), at 100V for 40 min, and read under UV transillumination at 302 nm wave length to determine the size of PCR product fragment.

Sequencing of PCR product

Fragment HA sequencing was performed with Dye Terminator Cycle Sequencing method using ABI PRISM 377A model version 3.4.1. All process was done at BPPT, Gene Technology Laboratory, Tangerang, Jawa Barat.

Sequence Analyses

Nucleotide sequences were analyzed by using Clustal W version 1.83 and Phyllip version 3.65 to determine their genetic relationship.

Discussion

In order to AIV molecular identification, the important and basic thing in this study was to understand virus isolates pathogenicity HPAI similarity which taken from different clinical character cases, by identified the amino acid motif on cleavage site region. We found polybasic amino acid region on HPAI that especially being filled by arginine and lysine. In LPAI virus, in that restricted site only have single arginine, which is why this motif could be used as AIV pathotypic marker (Kawaoka *et al.*,1984; Swayne and Suarez, 2000; Banks and Polywright, 2003). The amplification of cleavage site of HA gene fragment at position 775 to 1021 was based on Ito *et al.* (2001), and the result can be seen in the Figure 1.

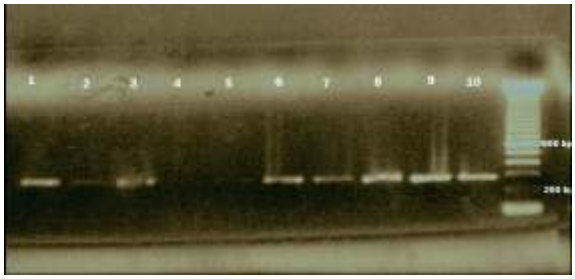


Figure 1. Gel electrophoresis indicated DNA band of expected product size (246 bp), on cleavage site region.Line no 1,2,3,& 6 are samples without specific symptoms; Line no 4,5,7,8,9 & 10 are samples with characteristic lesion of AIV

All 10 RT-PCR products were sent to BPPT to have them sequenced. Only 7 out of 10 samples give good results amino acids sequences at cleavage site deducted from

nucleotide sequences (Table 1).

Table 1. Translation of amino acid sequence at cleavage site region of seven AIV isolates.

No	Sample No & Code	Amino Acid sequence
1	Sample 1 A/layer/K. Progo/01/2004 (H5N1) Sample 2	PQRERRRKKR/GL
2	A/puyuh/Bantul/02/2005 (H5N1) Sample 3	PQRERRRKKR/GL
3	A/layer/Magelang/04/2005 (H5N1) Sample 6	PQRERRRKKR/GL
4	A/broiler/Klaten/09/2005 (H5N1) Sample 8	PQRERRRKKR/GL
5	A/layer/Purwokerto/12/2003 (H5N1) Sample 9	PQRERRRKKR/GL
6	A/layer/Klaten/13/2003 (H5N1) Sample 10	PQRERRRKKR/GL
7	A/layer/Lampung/14/2004 (H5N1)	PQRERRRKKR/GL

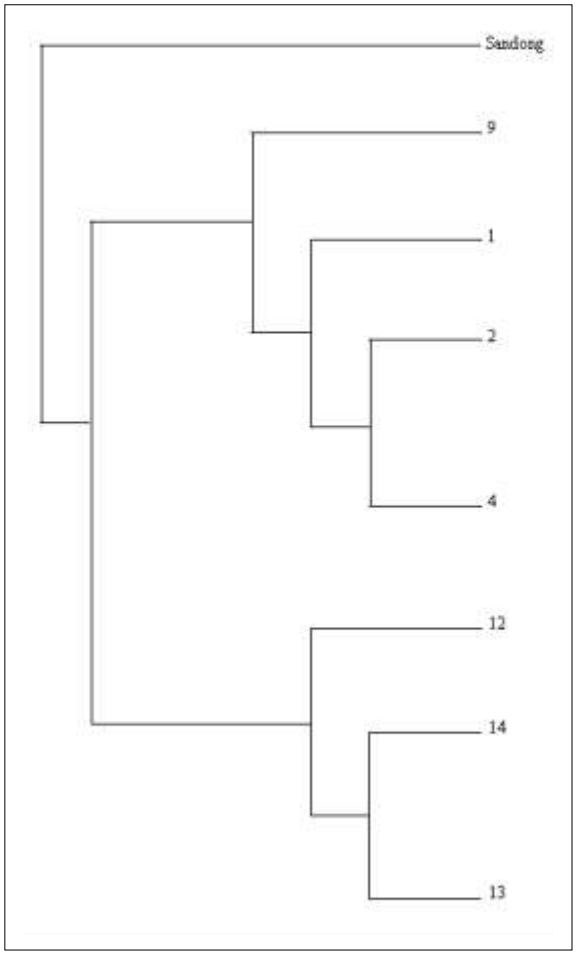
According to Senne *et al.*, (1996), minimal motif amino acid concept is BXBR//, which B is basic amino acid, X is non basic amino acid and R is arginine that related to restriction area (sign //). Follow in amino acid level conversion, samples number 1, 2, 3 and 9 that are virus isolates from unspecific lesion case have same motif with samples number 4, 5, and 7 from characteristic case of AI disease. Amino acid motif in the restriction area above (Tab-2) fulfill the minimal concept of HPAI virus according to Senne *et al.*, (1996) and it still consistent with few kind of studies that reported by another researcher.

In general amino acid motif at cleavage site of HPAIV-H5 is: -RRRKKR//GL- (Dharmayanti *et al.*, 2005) or –RRKKR//GL- (Ito *et al.*, 2001). Basic amino acid series in this study have same motif with isolates that reported by Zhou *et al.*, (1999) to upstream-10: -PQRERRRKKR//. The origin of this basic amino acid series was from human isolate (A/Hong Kong/156/97), 11 isolates from chicken, i.e.: A/chicken/Hong Kong/1997 and 2 duck isolates (A/Duck/Hong Kong/1997) that isolated in Hong Kong (1997). All isolates have been confirmed as AIV pathogenic for chicken based on the pathogenic marker of the cleavage site position. This amino acid motif in this study show a bit different with motif that reported by Senne *et al.* (1996). In the cleavage site, we found 3 different pattern that showed until upstream-10 and downstream+3: -PQ—RKRKKR//GLF or –PQRETRRQKR//GLF- or –PQ—RKRKTR//GLF- (Senne *et al.*, 1996). The first motif is very clear because of basic amino acid repetition to upstream–6, whereas the second motif still consistent in minimal concept: BXBR//. In the third motif, amino acid position on upstream-2 filled by Threonine (T) that belonging to alcohol group, replacing Arginine or Lycine. This unusual phenomenon still considered as HPAI because of the basic amino acid repetition in upstream-3 to -6. In his view the second position became unimportant, as far as this motif still has a repetition of basic amino acid in upstream -3 to -6.

Most AIV have Arginine (R) on terminal carboxy HA-1 at upstream-1 position,

whereas upstream-9 and -10 is the proximal end that according to another similar study this position always placed by amino acid Glutamine at position-9 and Proline at position-10 and these position so far considered as conserved region (Senne *et al.*, 1996; Zhou *et al.*, 1999). Isolate A/Gull/PA/4175/83 have been reported that on upstream-1 position Arginine (R) was substituted with Lycine (K), and the motif is – RETK/ /GL- (Senne *et al.*, 1996). Amino acid position at downstream +1, +2 so far still Glycine (G) and Leucine (L), both are aliphatic amino acid. This result supported by Dharmayanti *et al.* (2005), Ito *et al.* (2001), S*enne et al.* (1996), and Zhou *et al.* (199*9).* In this study, we still *c*an found consistency of amino acid motif at upstream-1 which is Arginine (R), Glutamine (Q) at upstream-9 and Proline (P) at upstream-10 as well as Glycine (G) and Leusin (L) at downstream+1 and +2.

The philogenetic tree analysis of the 230 bp of HA gene fragment around the cleavage site can be seen in the Figure 2. The result is very interesting since all isolates analyzed here, have the same multiple basic amino acid motif in the cleavage site region but created two kind of philogroups, based on Sandong isolate, gene bank accesion number DQ767725. From the diagram shown that our isolates were have different genetic relationship from Sandong AIV isolate. Avian influenza virus isolates codes number 1, 2, 4 and 9 have closely genetic relationship among other and created as one cluster meanwhile isolates codes number 12, 13 and 14, create another cluster that may different from that one. According to the characteristic type of lesion isolates codes number: 1, 2, 4 and 9 taken from unspecific lesion to AIV whereas reset of three isolates obtained from specific lesion for AIV.



Conclusion

1. All AIV-H5 isolates originated from chicken with specific or non specific lesion have multiple basic amino acid motif at cleavage site, that is -PQRERRRKKR/ /GLF- and classified as highly pathogenic avian influenza.

2. Philogenetic analysis of 230 bp of HA gene fragment around the cleavage site region, created two kinds of philo-groups for each type of characteristic lesion.

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