

## Genetic dynamic analysis of the H5N1 Avian influenza virus NS1 gene isolated in Bali

Arief Mulyono,<sup>1</sup> Widya Asmara<sup>2</sup>

<sup>1</sup> Institute for Vector and Reservoir Control Research and Development, Salatiga. Indonesia

<sup>2</sup> Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada

### Abstrak

**Latar belakang:** Virus Avian Influenza H5N1 diperkirakan terus bermutasi, yang berpotensi meningkatkan kapasitas untuk melompati barrier spesies, dan dapat menular secara mudah antar manusia. Penelitian ini bertujuan untuk menganalisis dinamika genetik gen NS1 dan mengetahui adanya marka virulensi pada sekuen gen NS1 VAI H5N1 ayam asal Bali.

**Metode:** Metode yang digunakan dalam penelitian ini adalah isolasi RNA, amplifikasi gen NS1 dengan Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), elektroforesis dan sequencing. Data sekuen isolat virus Avian influenza H5N1 asal Bali tersebut selanjutnya dibandingkan dengan multiple alignment dengan isolat asal Indonesia lainnya dari berbagai hospes yang diakses melalui GenBank tahun 2005-2007, dan pembuatan pohon filogenetik.

**Hasil:** Keempat isolat uji mengalami substitusi P42S dan delesi 5 asam amino pada posisi 80-84 yang mengakibatkan potensi peningkatan virulensi virus, namun tidak dijumpai adanya substitusi D92E, F103L dan M106I. Analisis filogenetik menunjukkan keempat isolat uji mempunyai kekerabatan genetik lebih dekat dengan isolat asal kucing dan manusia. Dibandingkan dengan isolat Bali tahun 2005 isolat uji mengalami peningkatan substitusi nukleotida dan asam amino.

**Kesimpulan:** Isolat VAI H5N1 asal Bali mengalami dinamika genetik dan ditemukan marker virulensi pada sekuen gen NS1. (*Health Science Indones 2012;2:xx-xx*)

**Kata kunci:** avian influenza, H5N1, NS1

### Abstract

**Background:** H5N1 Avian Influenza virus is expected to continue to mutate, potentially increasing the capacity to jump the species barrier, and can be easily transmitted between humans. This study aimed to analyze the genetic dynamics of the NS1 gene and to recognize markers of virulence in VAI H5N1 NS1 gene sequences from Balinese poultry.

**Methods:** The method used was isolation of RNA, NS1 gene amplification by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), electrophoresis and sequencing. Data sequence Avian influenza H5N1 virus isolates from Bali is then compared with the multiple alignment with other Indonesian isolates from different hosts accessed through 2005-2007 GenBank, and constructing a phylogenetic tree.

**Results:** The four test isolates had substitutions and deletions P42S 5 amino acids at positions 80-84 resulting in the potential for increased virulence of the virus. But no D92E, F103L and M106I substitution were found. Phylogenetic analysis showed four test isolates have a closer genetic kinship with cats and human origin isolates. Compared to the 2005 Bali isolate, the test isolates had increased nucleotide and amino acid substitutions.

**Conclusions:** Avian influenza virus H5N1 isolates from Bali has dynamic genetic and virulence marker had found on NS1 gene sequence. VAI H5N1 isolates from Bali underwent genetic dynamics. Virulence markers were found in the NS1 gene sequences. (*Health Science Indones 2012;2:xx-xx*)

**Keywords:** Avian influenza, H5N1, NS1

Avian influenza H5N1 virus is expected to continue to mutate, potentially increasing the capacity to jump the species barrier, and can be easily transmitted between humans. Combination of all fragments of the genes (genetic makeup) VAI H5N1 appearing together determined whether a strain can infect humans or mamalia.<sup>1</sup> Generally the host range and pathogenesis of H5N1 VAI are polygenic, the determinant factor for the host range and pathogenesis can be found in all gene fragments. These genes include the gene for hemagglutinin (HA), the polymerase genes basic polymerase-1 (PB1), polymerase basic-2 (PB2) and polymerase acidic (PA), neuraminidase gene (NA), and the non-structural genes NS1 and NS2.<sup>2</sup>

NS1 protein is a multifunctional protein that plays a key role in the pathogenesis and virulence VAI.<sup>3</sup> Amino acid substitutions P42S, D92E, F103I, M106I, and deletion of five amino acids at positions 80-84 reportedly NS1 gene increased virulence of VAI H5N1.<sup>4-6</sup>

Cases of Avian influenza outbreak in the province of Bali began on poultry farm in 2003. It was first reported in the Karangasem regency, allegedly due to the inclusion of sick birds from the outbreak on Java. In Bali, the location poultry and pig farms were close to human settlements. This makes it possible for transmission of the virus from animals to humans, which can potentially reabsorbs the genetic material that could create a new virus subtype. Cases of Avian Influenza in humans in Indonesia was 79%, which showed a history of direct or indirect contact with sick poultry.<sup>7</sup>

Several studies have shown VAI H5N1 circulating in Indonesia is experiencing dynamic changes. Genetic changes VAI H5N1 AI virus has the potential to bring a new, more virulent and will cause a pandemic. Therefore, the NS1 gene mutations needs to be anticipated and an early warning system needs to be created for the possible occurrence of a pandemic.

This study aimed to analyze the genetic dynamics of the NS1 gene and to recognize markers of virulence in VAI H5N1 NS1 gene sequences from Balinese poultry.

## METHODS

The research was conducted from August 2010 to February 2011 in the Laboratory for Avian Influenza, Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta.

The sample was isolated virus from cloacal swab of local poultry in the Klungkung regency of Bali in 2009. Isolates were then propagated in sterile embryonic eggs. Allantois fluid resulted from the virus propagation was first tested by hemagglutination activity assay (Hemagglutination Assay) and then used as sample in this study. There were 4 isolates.

This study included the preparation of samples of H5N1 virus isolates, RNA isolation of the study sample, NS1 gene amplification using the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), electrophoresis, and sequencing.

Isolation of RNA virus used procedures by PureLink™ Micro-to-Midi Total Purification System (Invitrogen). Ribonucleic acid (RNA) obtained was used as a template for RT-PCR reactions to amplify the NS1 gene. Analysis of RT-PCR amplification products was done by electrophoresis. The sequencing process was done using the method of dye terminator sequencing cycle AB PRISM 377A sequencer in the PT Charoen Pokphan biotechnology laboratory.

Analysis of the results was done descriptively. Nucleotide sequencing of the NS1 gene of chicken isolates of Bali were then compared with isolates that have been selected from the Genbank sequences of H5N1 viruses of 2005-2007. The results of the nucleotide sequence of the NS1 gene then positioned (aligned) using Clustal X 1.81 and BioEdit 5.0.6 software using software 2.6.002 Genedoc nucleotide sequence and was visualized in the form of protein and amino acid sequence. Phylogenetic analysis was done using the Clustal X 2 software with neighborjoining bootstrap analysis methods (replication 1000) and then visualized with the TreeView program. Outgroup using A/Goose/Guangdong/1/96.

## RESULTS

The results of electrophoresis of four RT-PCR amplification products isolates were studied, namely A/Ck/Klungkung/A/09, A/Ck/Klungkung/K/09, A/Ck/Klungkung/T/09, and A / Ck / Klungkung / Y/09 showed specific bands with a length of 683 bp. The sequencing results indicated the nucleotides read ranged from no. 32 to no. 672.

The results of the alignment at the nucleotide level for the four test isolates indicated the degree of similarity

or homology between 98.2-99.1%. While at the amino acid level of the four test isolates showed homology levels were between 97.5-99%.

The results of the alignment at the nucleotide level between the isolates tested with isolates of Avian origin H5N1 VAI Java and Bali in 2005-2007 showed homology ranged from 94.3-97.2%. While the level of amino acid homology ranged from 91.7 to 97.1%. Homology at the nucleotide level between the isolates tested with isolates of human origin H5N1 VAI in 2005-2007 ranged from 95.1-97.2%. While at the amino acid level ranged from 93.2-97.1%.

Homology at the nucleotide level between the isolates tested with swine-origin H5N1 isolates VAI and cats in 2005-2007 ranged from 94.5-97.1%. While at the

level of amino acid homology ranged from 92.2-97.1%. Analysis of the amino acid sequence of molecular markers for pathogenicity showed all test isolates had amino acid substitution at position 42 from proline (P) to serine (S) as well as the deletion of five amino acids at positions 80-84. No amino acid positions 92, 103, and 106. All isolates test did not indicate a substitution. At position 92 the amino acid aspartic test isolates (D), position 103 phenyl alanine (F) and position 106 methionine (M).

In phylogenetic analysis, the test isolates were compared with 47 isolates from various regions in Indonesia and countries in Asia that were isolated from different host from 1996 to 2007. Selection of isolates from different hosts between 1996 and 2007 was to look at the possibility the occurrence of genetic dynamics (Figure 1).

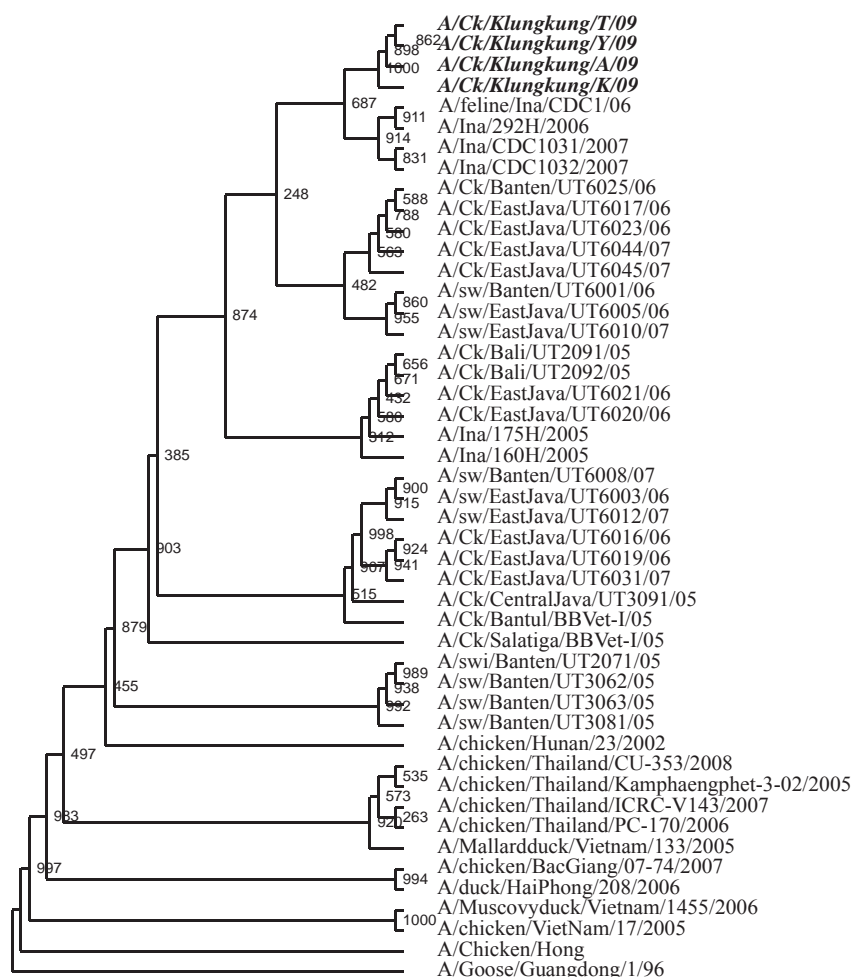


Figure 1. Phylogenetic relationships between H5N1 virus NS1 gene fragment which comprised of 47 isolates from Indonesia and other countries in Asia. Isolates in bold were the test isolates. Analysis was performed with 1000 bootstrap replication using Clustal-X and visualized using TreeView. Outgroup used Goose/Guangdong/1/1996 isolates.

## DISCUSSION

The high homology between the isolates tested with isolates of avian origin H5N1 VAI Java and Bali in 2005 to 2007 showed there was an association between the isolates tested with poultry isolates from Java and Bali. Java is known as a center for the development and spread of H5N1 VAI to the surrounding islands through trade unggas.<sup>10</sup> In the Bali Province, AI outbreak was first reported in the Karangasem regency in October 2003, allegedly due to the inclusion of sick poultry from outbreak areas in Jawa.<sup>8</sup>

The position of amino acids in the NS1 gene designated as virulence markers of VAI H5N1 were amino acid no. 42, 92, 103, and 106. At amino acid position no. 42 all test isolates read serine (S). The substitution of proline for serine (P42S) will cause an increase in the virulence of the virus through the mechanism of inhibition of the host IFN response, including the NF- $\kappa$ B pathway and the IRB-3.<sup>5,7</sup> Deletions 5 amino acids at positions 80-84 are found in all isolates tested.

Deletion of 5 amino acids increased the degree of virulence and virus replication both in poultry and mamalia.<sup>9</sup> When viewed from the 3-dimensional structure of the five amino acid deletions at positions 80-84 caused the linker connecting the RNA binding domain to the effector domain to become shorter. This change shortened the orientation and stability of the RNA binding domain, or both. Changing the orientation and stability of the RNA binding domain contributes to increased virulence of the virus either by modulating increased dsRNA binding by the RNA binding domain and interaction with host proteins.<sup>10</sup>

The results of phylogenetic analysis showed that the reference virus from Indonesia on the phylogenetic tree was grouped into one but one deviated away from the group of viruses from Thailand, Vietnam and Hong Kong. Isolates from Indonesia on the phylogenetic tree is also divided into several groups. It showed the H5N1 virus has developed, evolved, and diversified in Indonesia.

The existence of genetic variations in a region as shown by isolates from East Java are grouped into two even though isolated in the same year. This is consistent with the results of research conducted by Takano et al. in 2009. Avian influenza viruses circulating on the island of Java in 2004 to 2007 have high genetic diversity.<sup>11</sup>

Although isolated in the same area, four isolates were not clustered into a single test with two isolates from Bali which was isolated in 2005 (A/CK/Bali/UT2091/05, A/CK/Bali/UT2092/05). It shows the genetic dynamics. But the genetic dynamics of H5N1 in Bali VAI cannot be inspected every year because of the limitations of isolates in GeneBank. VAI H5N1 isolates from Bali available at GeneBank were only isolates of 2005.

Multiple sequence alignment of the four NS1 gene test isolates showed an increase in nucleotide and amino acid substitutions when compared with 2 isolates from Bali of 2005. According to (FAO-OIE-WHO, 2010), the population of all influenza virus has highly variable and evolved rapidly including H5N1 AI virus through mutation and genetic reabsorbs. RNA polymerase mutations occur because the virus does not have the ability to proofreading progeny genomic RNA so that the frequency of nucleotide substitutions tinggi.<sup>12</sup>

In terms of the four host isolates, the Klungkung isolate has a closer kinship to human and cat isolates compared with isolates from other hosts. This closeness meant the test that isolates had close genetic to isolates of human and cats.

It can be concluded that the VAI H5N1 isolates from Bali underwent genetic dynamics. The VAI H5N1 NS1 gene from Bali had an amino acid substitution from proline to serine at position 5 P42S and underwent deletions of amino acids at positions 80-84 which is a virus virulence markers.

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