academicJournals

Vol. 8(3), pp. 244-251, 15 January, 2014
DOI: 10.5897/AJMR2013.5609
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

*Full Length Research Paper*

# Genetic characterization of H5N1 avian influenza viruses isolated from pet bird and chickens from live bird market in Bali and Bekasi (Indonesia), 2011

**Ni Luh Putu Indi Dharmayanti*, Atik Ratnawati[1], Dyah Ayu Hewajuli and Risa Indriani**

Indonesian Research Center for Veterinary Science, JL RE Martadinata 30, Bogor 16114, West Java, Indonesia.

Accepted 16 September, 2013

**Indonesia has endemic status of H5N1 and the largest number of human cases of H5N1. Sources of human infections include mostly intensive contact with H5N1 virus or the environment contaminated by this virus. This study shows that feaces from pet bird victims and chickens were sold in live bird market (LBM) suspected to be the source of infection for human H5N1 in Bali and Bekasi in 2011. This study focused on the virus' genetic character that originated from these areas, especially HA1, M, NS1 and PB2 genes. The results show that Bali and Bekasi viruses have similar genetic characters with human H5N1 viruses in Indonesia. The viruses have multiple basic amino acids in hemagglutinin that showed highly pathogenic, specific motif amino acid in the Matrix protein and ESEV motif on NS1. The viruses have no mutations in PB2 gene. The findings support that most cases of human H5N1 infection are as a result of exposure of birds to H5N1 virus.**

**Key words:** Genetic character, H5N1, birds, LBM, source.

## INTRODUCTION

Since identified in 2003, the H5N1 avian influenza virus in Indonesia has been circulating for almost 9 years (Dharmayanti et al., 2004; Wiyono et al., 2004). Various strategic measures to control and eradicate the disease have been done, but until now the virus is still circulating and even has become endemic throughout the year 2012. As at March 2012, there were 187 people confirmed infected due to AI/H5N1 virus and 155 are fatal cases (WHO, 2012).

Vaccination, bio-security and depopulation were some strategics measurenments taken by the Indonesian Government to control avian influenza disease. The strategies resulted in the reduction of the number of H5N1 cases in the field. The decreasing number of H5N1 cases in poultry sector was not followed by H5N1cases in human.

Cases of H5N1 still occur sporadically in some areas. Most of the source of human H5N1 infection in Indonesia is not yet known with certainty, even the highest human cases of H5N1 reported no history of contact with poultry or birds. But Aditama et al. (2012), in their findings, showed that most cases of AI/H5N1 infection were as a result of exposure to zoonotic sources of virus.

Data on the characters and genetic information related to dead victims caused by H5N1 virus infections of birds' origin in Indonesia are still limited. Continuous exposure to H5N1 virus by humans will increase the possibility of influenza pandemic in humans. Avian virus can adapt more efficiently in human through reassortment with other influenza strains in humans (Webster et al., 1992; Taubenbarger et al., 2005).

Several H5N1 infection cases were family clusters; however, it can be stated that virus transmission from human to human is still very limited (Ungehusak et al., 2005).

Viral transmission inter human is not yet proven, thus

*Corresponding author. Email: nlpdharmayanti@yahoo.com.

**Table 1.** The specimens origin during our surveillance on human animal interface in 2011.

| H5N1 human case | Date of collected samples | District | Location | Type of sample | Number of sample | RT-PCR of positive for H5N1 | Positive for viral isolation | Remark |
|---|---|---|---|---|---|---|---|---|
| Female, 31 y.o | 3 March 2011 | Bekasi, West Java | Live Bird Market | Chicken | 21 | 11 | 2 | 100 m from victim house |
| | | | | Environment | 1 | 1 | 0 | |
| | | | Near victim house | Pet Bird | 61 | 14 | 0 | |
| | | | | Goose | 3 | 0 | 0 | |
| Female, 5 y.o; Male 10 y.o | 13-15 October 2011 | Bangli, Bali | Near victim house | Pet Bird (cendet bird like) | 2 | 1 | 1 | Victims kept the bird in their house |
| | | | Chicken collector | Chicken | 24 | 0 | 0 | |
| | | | | Muscovy Duck | 1 | 0 | 0 | |
| | | | | Duck | 5 | 1 | 1 | |

most cases in humans occur due to virus spread from infected birds (World Health Organization 2005a). In this study, we reported the molecular character of human H5N1 viruses isolated from birds in Indonesia.

## MATERIALS AND METHODS

### Samples collection and virus isolation

We isolated viruses from cloacal swab samples collected from birds or chickens near H5N1 victim houses in Bekasi (West Java) and Bangli (Bali) in 2011 (WHO, 2011). Table 1 showed that the specimens collected from animal/environment side related with H5N1 human cases infection. H5N1 viruses from Bali were A/Bird/Bali1/2011 collected from feaces of the pet bird which has been kept by victims and A/Duck/Bali/2011 virus isolated from ducks and chicken located near houses of victims. The ducks look healthy and have no clinical sign. The other isolates (A/Chicken/West Java/Bks9/2011 and A/Chicken/West Java/Bks12/2011) were collected from LBM near the houses of victims in Bekasi (Tabel 1).

Sterile cotton-tipped swabs were used for sampling and were subsequently stored in viral transport medium. The transport medium consisted of Dulbecco's Modified Eagle Medium (DMEM) with 1000 IU penicillin and streptomycin. The samples were immediately transported to the laboratory after collection and were stored at -70°C. A 1000 µl sample in transport medium was homogenized by vortex and centrifuged with the speed of 2500-3000 rpm. The supernatants were then inoculated in embryonated specific pathogen free (SPF) eggs of 9-11 old days. The allantoic fluid was extracted using QIAmp RNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The extracted RNA was tested for H5 subtype by RT-PCR using H5-155F and H5-690R primers (Lee et al., 2001). Thermo cycling was performed in ABI 9700 and 2700 PCR machines.

### DNA sequencing and visualization of 3D-protein prediction

To amplify full length HA, the primer of Senne et al. (1996) was used to amplify HA1 region, and HA2 was modified using H5-155F (Lee et al., 2001); NS890 primers were published by Hoffman et al. (2001). For NA gene amplification, primer of Komadina (2006, personal communication) was used, while for the amplification of M and NS genes, primer of Hoffman et al. (2001) was used. The primers for amplifying PB2 gene were desinged by the authors. PCR products were separated in 1% agarose by electrophoresis and the amplicon was excised and purified using QIAquick gel purification kit (Qiagen). The sequencing method used was direct sequencing using Cycle sequencing kit (BigDye Terminator version 3.1; Applied Biosystem) on Genetyx Analyzer 3130 (Applied Biosystems, USA). The nucleotide sequencing data obtained in this study were analyzed together with the genetic data available in the avian influenza database (NCBI) based on each gene. The production of multiple alignments of each gene and residue analysis were carried out by using BioEdit version 7 (http://www.mbio.ncsu.edu/BioEdit). Phylogenetic trees were generated by neighbor-joining bootstrap analysis (1,000 replicates), using the Tamura-Nei algorithm in MEGA version 4 (Http: //www.megasoftware.net). All of the viruses used in this study have been submitted to GenBank (www.ncbi.nlm.nih.gov) with accession number (yet to be submitted to NCBI).

## RESULTS

The phylogenetic of HA gene (Figure 1) showed that Bekasi 2011 (Bks9/2011; Bks12/2012) and Bali 2011 (Bali1/2011; Bali9/2011) viruses (Bali1/2011; Bali9/2012) were in the same group with other Indonesian H5N1 viruses isolated in

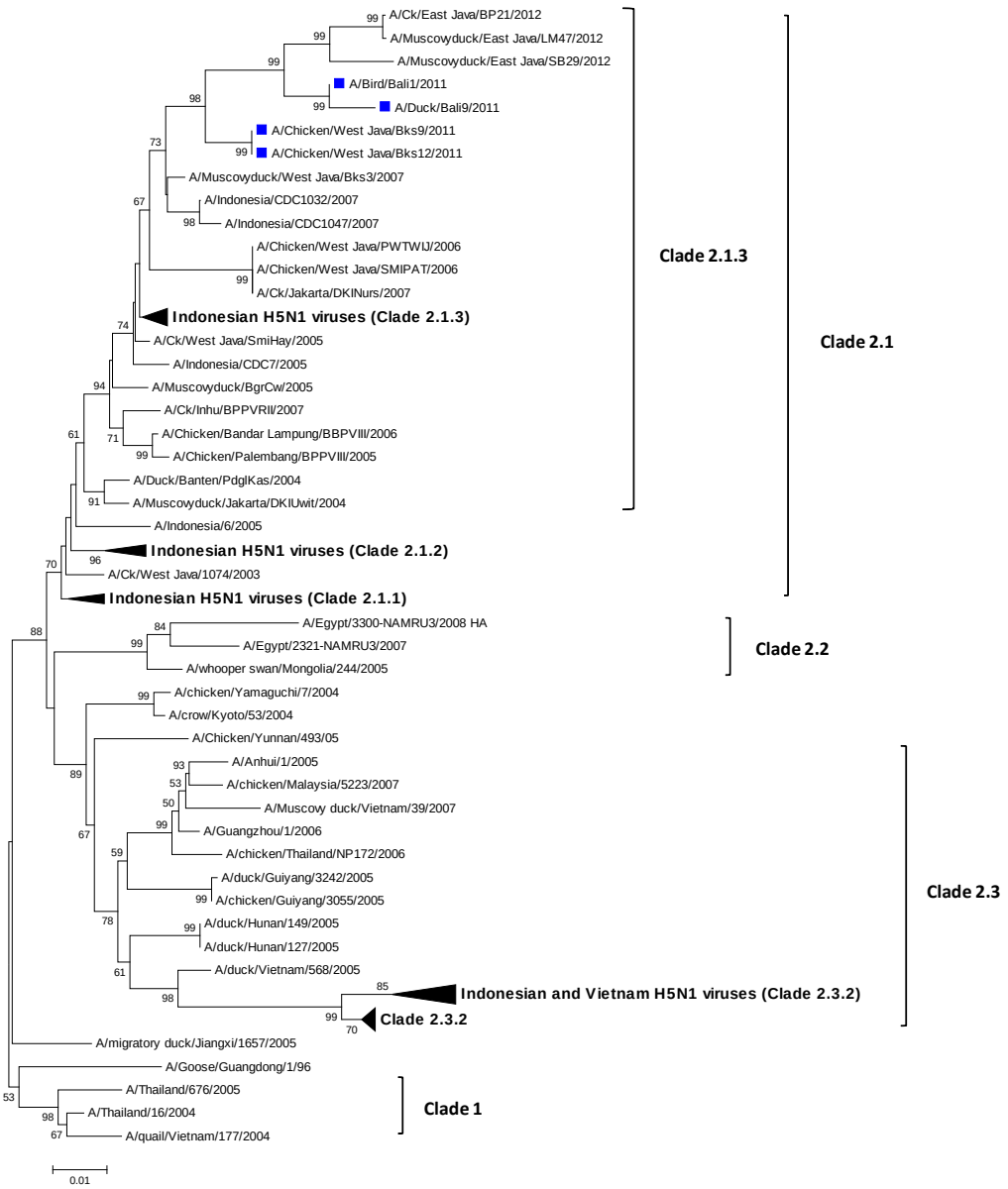


**Figure 1.** Phylogenetic of HA gen of H5N1 Viruses. Figure 3) Phylogenetic of M1, Figure 4) Phylogenetic of PB2; The viruses used in this study showed by blue color and in one group with Indonesian Human H5N1 viruses.

2011. Bekasi 2011 and Bali 2011 viruses belong to clades 2.1.3. which are the predominant clades in Indonesia. The genetic characteristic of Bekasi 2011 and Bali 2011 in terms of HA protein had amino acid QSG at positions 222, 223, 224, respectively (Figure 2). The condition showed that the viruses still recognize the 2,3 *avian receptor*. The cleavage site of HA protein sequences has PQRESRRKKR//G. Bekasi and Bali isolates have no significant differences with the majority of H5N1 avian influenza virus from Indonesia. Another substitution such as amino acid at position 53 is replaced with amino acid R and K amino acid (R→K). Substitutions are similar to the human H5N1 viruses A/Indonesia/CDC1047/2007, A/Indonesia/CDC370/2006, A/Indonesia/CDC390/2006). If Bali 2011 viruses are compared with previous viruses from Bali and other Indonesian AI viruses, there are differences at positions 183, 184 and 189. But the substitution of amino acids at positions 183 (D→N), 184 (A→G) and 189 (R→M) is similar to the Bekasi 2011 viruses.

Dharmayanti et al. (2011a) showed that human AI/H5N1 virus isolated from birds has a genetic similarity with Indonesian human AI/H5N1viruses and has specific character with the Matrix protein from birds collected from AI/H5N1 cases in humans. In this study (Table 2) showed that most of Indonesian H5N1 human viruses, Bekasi/2011

**Table 2.** Amino acid specific on Matrix protein belong to H5N1 Bali and Bekasi viruses.

| Virus | M1 amino acid position | | | | M2 amino acid position | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 37 | 95 | 137 | 249 | 8 | 18 | 20 | 27 | 50 |
| West Java/Bks9/2011 | A | K | A | H | Y | K | I | A | F |
| West Java/Bks12/2011 | A | K | A | H | Y | K | I | A | F |
| Bali/Bangli1/2011 | A | K | A | H | Y | K | I | A | F |
| Bali/Bangli9/2011 | A | K | A | H | Y | K | I | A | F |
| Indonesian H5N1 Indonesia origin from birds/poultry | T | R | T | Q | C | R | S | V | C |
| Most of Indonesian H5N1 human viruses | A | K | A | H | Y | K | I | A | F |
| Indonesian H5N1 viruses (2011) | A | K | A | H | Y | K | I | A | F |

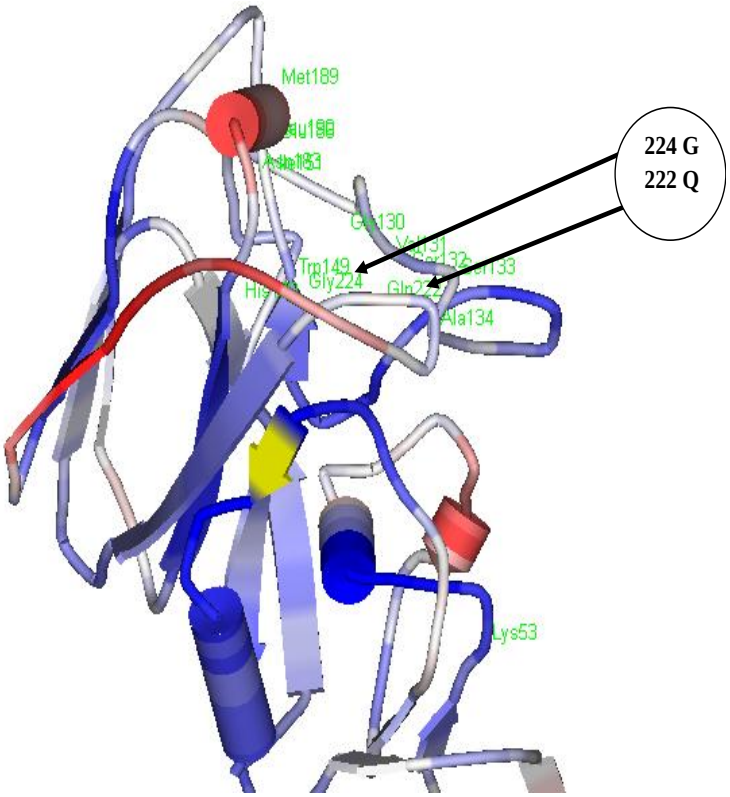


**Figure 2.** Prediction of three Dimension of HA protein of Bali virus. The viruses still recognize avian receptor. The template highest homology is obtained by using the BLAST search (DS server). HA1 template of H5N1 in this study is 21BX_A that is equal to 96%.

and Bali/2011 viruses have specific amino acid substitutions in the Matrix protein. This result had same result with Dharmayanti et al (2011a) findings. Phylogenetic analysis of M1 (Figure 3) showed that Bekasi and Bali viruses were in one group with H5N1 2011 viruses group; human H5N1 viruses and also avian viruses were isolated from human H5N1 in 2007 (Bks2/2007).

In the analysis of the NS protein level, there were substitutions in R44K; M79K and has a PDZ binding motif as ESEV (avian origin). This means there is a significant mutation at amino acid position 79 where Methionine (M) replaced the basic amino acid lysine (K) and had the possibility of affecting binding protein of the virus. Phylogenetic analysis of NS1 also showed that H5N1 Bekasi and Bali viruses are in one group with human H5N1 viruses and avian viruses.

Analysis of the PB2 protein showed that there is no mutation at position E627 and D701 amino acids. The phylogenetic of PB2 gene showed that the viruses used in this study close relationship with Indonesian human H5N1 viruses (Figure 4).

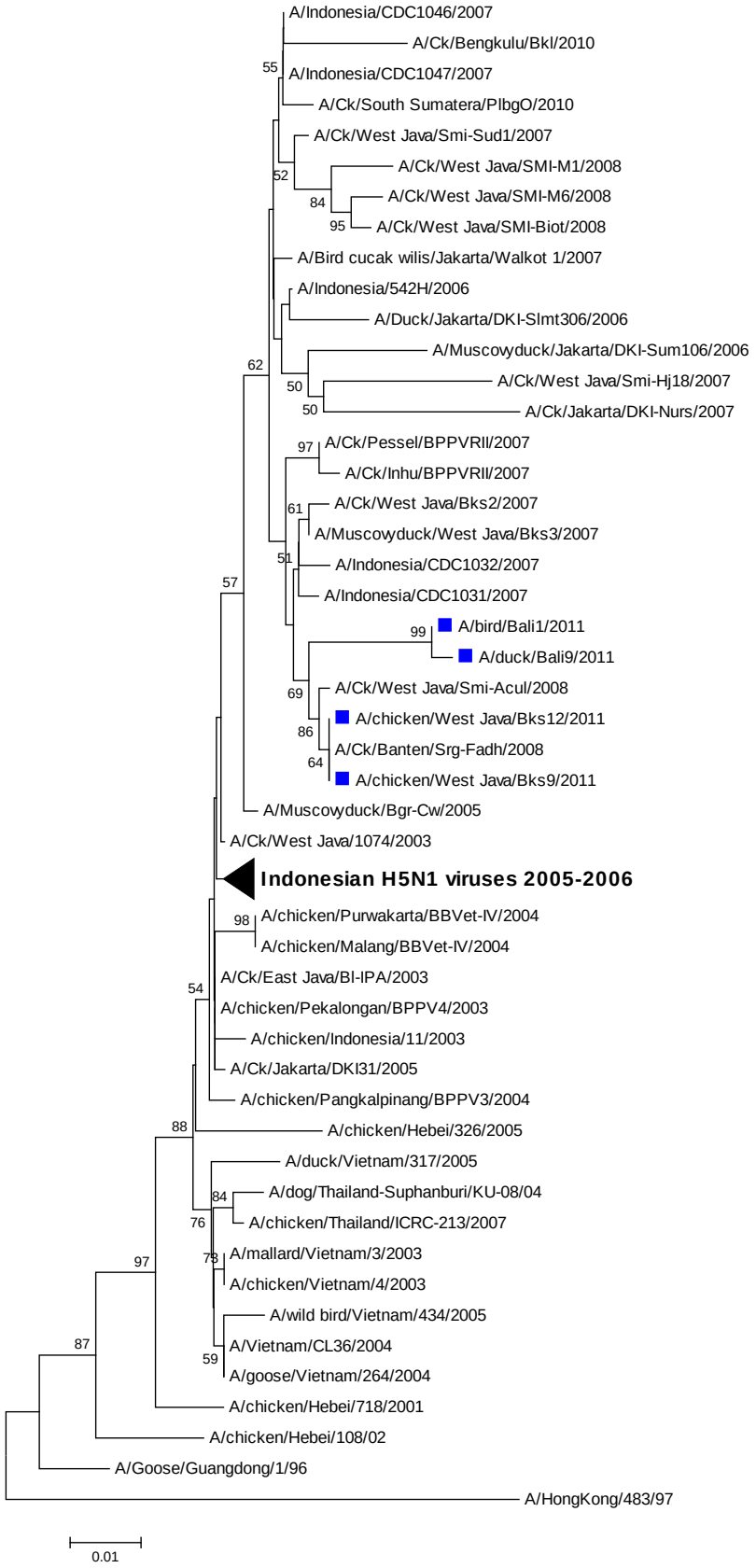


**Figure 3.** Phylogenetic of M1 of H5N1 viruses.

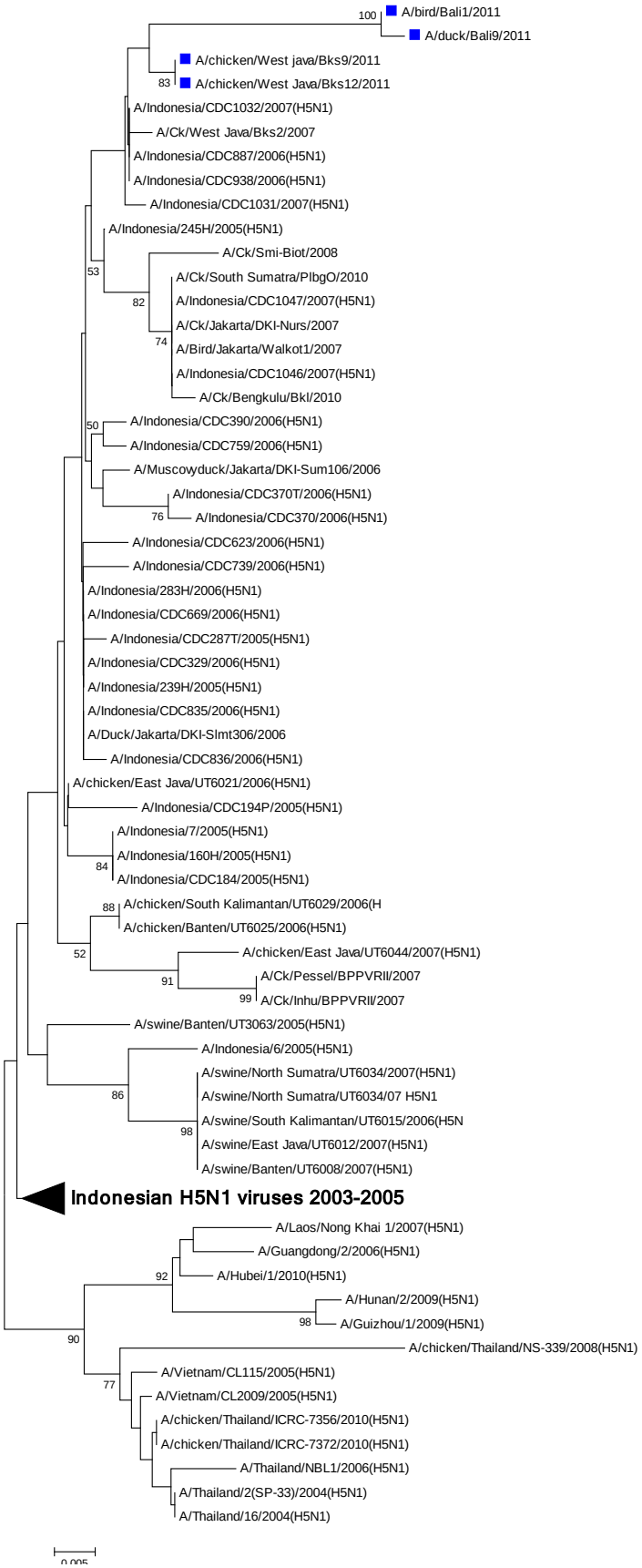


**Figure 4.** Phylogenetic of PB2 gene of H5N1 viruses.

## DISCUSSION

We did a surveillance and collected specimen from birds/environment of victims' house to identify the risk factor and source of the viruses that infected human, especially in Bekasi 2011 and Bali 2011. We got four isolates of H5N1 viruses: two viruses from Bekasi and two isolates from Bali. The genetic characteristic showed that there is no mutation on 182 and 192 of HA protein. Mutations at positions 182 and 192 that change HA protein of H5N1 avian receptor are known to recognize human receptors. Such mutations are Asn182Lys and Gln192Arg that change the specificity of the receptor, because these two residues play a role in stabilizing the interaction between sugar and the bond sialic acid α2, 6 (Yamada et al., 2006). Herfst et al. (2012), in their study, also showed that four amino acid substitutions in the hemagglutinin (HA) protein and one in the polymerase complex protein basic polymarase 2 (PB2) in A/H5N1 influenza virus can have ability to do airborne transmission between mammals without adapting to an intermadiate host; and furthermore can become a risky influenza pandemic. Bekasi 2011 and Bali 2011 viruses used in this study have Asn and Gln at positions 182 and 192 respectively so that the bond is still not able to recognize α2, 6. In addition to the isolates, avian receptors were still recognized because they have residue 222 (Q) and 224 (G) (Stevens et al., 2006). Gao et al. (2009) also showed that substitution of amino acid in the HA protein (Thr160Ala) resulting in the loss of glycosilation at 156-160 was responsible for binding to sialylated glycans and was critical for H5N1 virus transmission in guinea pigs.

Bali and Bali viruses have 7 glycosylation sites; this number is a normal amount which is owned by Indonesia H5N1 virus; so it does not have or increase the number of glycosylation sites. H5N1 viruses which have decreasing number of glycosylation sites in Indonesia have been reported previously by Dharmayanti et al (Dharmayanti et al., 2011b). Mutation causes decrease in the effectiveness of vaccines used when challenged with H5N1 virus that has seven glycosylation sites.

Matrix protein of Bekasi and Bali isolates has a specific character in protein M1 and M2 as well as other H5N1 viruses that were isolated from humans infected with H5N1 (Dharmayanti et al., 2011a). Bekasi and Bali isolates showed a deletion of amino acids at amino acid positions, 80-84. Deletion of amino acids at positions 80-84 in the NS1 protein increases the virulence of H5N1 virus (Long et al., 2008). Dharmayanti et al. (2011a) also showed that most isolates of Indonesia have these deletions. The C termini of NS1 protein of the viruses have consensus PDZ domain ligand (PL); which means a protein-protein recognition modules that recognize this protein and are bound to the C-termini are on the NS1 protein residues, 227-230 (Obenauer et al., 2006). Result of the study showed that Bekasi and Bali viruses have ESEV motif, indicating that Bekasi and Bali isolates were of avian origin. ESEV or EPEV motif was found in the origin of avian H5N1 virus (Obenauer et al., 2006). H5N1 viruses isolated from birds in H5N1 human cases have a close genetic relationship at the protein level of HA, NS, M1, M2 and PB2 of H5N1 human viruses. Our study showed that amino acid in the PB2 gene contains Glu at 627 amino acid posiition. This means there is no substitution in PB2 gene. Substitution Glu627Lys (E627K) has been associated with increased virus replication in mammalian cells (Subbarao et al., 1993). Substitution of amino acid asparagine (Asn) at 701 posiition in PB2 protein was a prerequiste for virus transmission to guinea pigs. The genetic character from the isolates used in this study has high similarity with each other and general Indonesian H5N1 viruses in 2011. Our findings show that most of H5N1 human infections were as a result of exposure of birds to H5N1 virus.

## ACKNOWLEDGEMENTS

We thank Nana Suryana and Teguh Suyatno for their technical assiatance, Bekasi and Bangli District Livestock Services for their field support. The study was supported by DIPA Grant from The Indonesian Agency of Agricultural Research Center (IAARC), Minsitry of Agriculture

### REFERENCES

Aditama TY, Samaan G, Kusriastuti R, Sampurno OD, Purba W, Misriyah, Santoso H, Bratasena A, Maruf A, Sariwati E, Setiawaty V, Glass K, Lokuge K, Kelly PM, Kandun IN (2012). Avian Influenza H5N1 Transmission in Households, Indonesia. PLoS ONE : 7.1:e29971. doi:10.1371/journal.pone.0029971

Dharmayanti NLP I, Samaan G, Ibrahim F, Indriani R, Darminto, Soebandrio A (2011b). The genetic drift of Indonesian avian influenza viruses A H5N1 viruses during 2003-2008. Microbiol Indones. 5. 2:68-80

Dharmayanti NLPI (2009). Perubahan Genoma Virus Avian Influenza subtipe H5N1 pada unggas di Indonesia. [Disertasi]. Jakarta : Universitas Indonesia. pp. 278

Dharmayanti NLPI, Damayanti R, Wiyono A, Indriani R, Darminto (2004). Identifikasi virus avian influenza virus isolat Indonesia dengan metode reverse transcripatese polymerase chain reaction RT-PCR. J. Ilmu Ternak dan Vet. 9(2):136-143

Dharmayanti NLPI, Ibrahim F, Darminto, Soebandrio A (2011a). Influenza H5N1 virus of birds surrounding H5N1 human cases have specific characteristic on matrix protein. Hayati J Biosci. 18(2):82-90

Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, Li Z, Guan Y, Tian G, Li Y, Shi J, Liu L, Zeng X, Bu Z, Xia X, Kawaoka Y, Chen H (2009). Identification of Amino Acids in HA and PB2 Critical for the Transmission of H5N1 Avian Influenza Viruses in a Mammalian Host. Plos Pathogens. 5. 12. e1000709.

Herfst S, Schrauwen EJA, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM (2012). Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets Science. 336:1534-1541

Hoffman E, Stech J, Guan Y, Webster RG, Perez DR **(**2001). Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146:2275-2289.

Lee MS, Chan PC, Shien JH, Cheng MC, Shieh HP (2001). Identification and subtyping of avian influenza viruses by reverse

transcription-PCR. J.Virol. Methods 97:13-22.
Long JX, Peng DC, Liu Y, YWu YT, Liu XF (2008). Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes. 36:471-478.
Obenauer JC, Denson J, Mehta PK, Su X, Mukatira S, Finkelstein DB, Xu X , Wang J, Ma J, Fan Y, Rakestraw KM, Webster RG, Hoffmann E, Krauss S, Zheng J, Zhang Z, Naeve, CW (2006). Large-scale sequence analysis of avian influenza isolates. Science 311:1576-1580.
Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Suss J, Lipkind M., Kida H, Webster RG (1996). Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis. 40:425-437
Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y (2006). Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature. 444:378-382.
Stevens A, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA (2006). Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science. 312:404-410.
Subbarao EK, London W, Murphy BR (1993). A Single Amino Acid in the PB2 Gene of Influenza A Virus Is a Determinant of Host Range. J Virol. 77. 4 : 1761-1764
Wiyono A, Indriani R, Dharmayanti NLPI, Damayanti R, Darminto (2004). Isolasi dan Karakterisasi Virus Highly Pathogenic Avian Influenza subtipe H5 dari ayam asal Wabah di Indonesia. JITV. 991:61-71.
WHO (World Health Organization, 2012). Avian influenza-Situation in Indonesia-Up date. www.who.int. Acceced on 15 March 2012.