

HOST-RESTRICTED RANGE OF H5N1 AVIAN INFLUENZA VIRUSES ASSOCIATED WITH CHARACTERS OF POLYMERASE COMPLEX OF PB2 AND PB1-F2 PROTEINS

Keterbatasan Adaptasi Virus Avian Influenza H5N1 pada Inang Berasosiasi dengan Karakter Protein Polimerase Kompleks PB2 dan PB1-F2

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ABSTRACT

Epidemiological studies on H5N1 avian influenza viruses indicated that the viruses do not transmit efficiently from human to human. Transmissibility of viruses among human population is very complex and polygenic. Studies on molecular determinants facilitating interspecies transmission of the viruses suggested that two polymerase complex proteins such as PB2 and PB1-F2 are important. PB2 is critical in determining the host specificity, whereas mutations in PB1-F2 increase the viral virulence. The study aimed to characterize the polymerase complex of PB2 and PB1-F2 proteins of H5N1 avian influenza viruses isolated from Indonesia. The DNA samples encoding the PB2 and PB1-F2 complex proteins of several H5N1 isolates were sequenced and analyzed. Pathogenicity of the viruses was studied in both avian and mammal models. The sequencing results showed that there was no mutation in both proteins of PB2 and PB1-F2 of the avian influenza virus isolates. The molecular character for host specificity was consistent with the animal experiment results. The H5N1 virus isolates were only infectious and pathogenic in chickens, but not in BALB/C mice as the mammal model. The study suggests that host range of H5N1 virus isolates of Indonesia is restricted to poultry and not transmissible to mammal model used in this study.

[Keywords: H5N1 avian influenza virus, PB2 protein, PB1-F2 protein, polymerase activity, Indonesia]

ABSTRAK

Studi epidemiologi menunjukkan virus influenza H5N1 tidak efisien ditularkan dari manusia ke manusia. Penularan di antara manusia bersifat kompleks dan poligenik. Studi penentuan marka molekuler untuk mengidentifikasi determinan molekuler transmisi virus menunjukkan pentingnya peran protein polimerase kompleks virus H5N1, yaitu protein PB2 dan PB1-F2. Protein PB2 berperan penting dalam menentukan spesifisitas inang, sementara itu mutasi pada PB1-F2 meningkatkan virulensi virus. Penelitian ini bertujuan untuk mengetahui karakter dua protein polimerase kompleks yaitu protein PB2 dan PB1-F2 terhadap patogenesis

virus avian influenza sub tipe H5N1 asal Indonesia. DNA yang mengkode protein kompleks PB2 dan PB1-F2 dari beberapa isolat virus H5N1 disekuensing dan patogenesisnya dianalisis pada model mamalia dan unggas. Hasil penelitian menunjukkan bahwa secara molekuler protein PB2 dan PB1-F2 virus avian influenza yang digunakan dalam penelitian tidak menunjukkan adanya mutasi. Karakter molekuler spesifisitas inang konsisten dengan hasil studi patogenesis. Virus H5N1 hanya infeksius dan patogen pada ayam, tetapi tidak pada model mamalia yaitu mencit BALB/C. Studi ini memperkirakan bahwa rentang inang virus H5N1 di Indonesia yang digunakan pada studi ini terbatas pada unggas dan tidak ditransmisikan pada mencit BALB/C.

[Kata kunci: Virus avian influenza H5N1, protein PB2, protein PB1-F2, aktivitas polimerase, Indonesia]

INTRODUCTION

Influenza virus belonging to Orthomyxoviridae family has a negative-stranded RNA genome that consists of eight segments (Palese and Shaw 2007; Wise *et al.* 2009). Influenza viruses in avian species are classified into three types: A, B and C; however, only influenza A viruses have a large genetic variation and a wide host range (Webster *et al.* 1992; Ozawa and Kawaoka 2011). Subsequently, influenza A viruses are divided into several subtypes according to the outside glycoprotein properties, including the hemagglutinin (HA) and neuraminidase (NA). Up to now, 16 types of HA and 9 types of NA have been identified in avian species (Webster *et al.* 1992; Fouchier *et al.* 2005), while two types of HA17 and 18 were recently discovered in fruit bat species (Tong *et al.* 2012; 2013). Most of influenza A viruses are not pathogenic and often asymptomatic in wild birds and waterfowls that serve as a natural reservoir for influenza A viruses (Webster *et al.* 1992). Moreover, analysis of

phylogenetic viral gene indicated that all influenza A viruses in mammals was currently derived from influenza virus in avian species (Webster *et al.* 1992).

Since 1997, avian influenza virus from H5N1 subtype has caused sporadic and fatal cases for human beings. The data from WHO on May 1st, 2015 showed about 840 human infection cases of H5N1 virus from 2003 to 2015 with 447 deaths. There were 167 fatal cases of 199 cases happened in Indonesia (WHO 2015). Nevertheless, the H5N1 influenza virus is still believed not efficiently transmitted from human to human. According to the previous cases and studies, several viral factors are needed to support transmission between different hosts. Since the species barrier crossing and human-to-human transmission of H5N1 influenza virus have great consequences for influenza pandemic, numerous significant efforts have been expended to identify the molecular determinants of the virus transmission (de Jong *et al.* 2006).

Transmissibility of virus among humans is complex and polygenic (Maines *et al.* 2006; Neumann *et al.* 2007). Virus affinity of HA protein residues -2, 6 linked sialic acid has been proved required (Tumpey *et al.* 2007), but it is not sufficient to support transmission between ferret (Maines *et al.* 2006; Yen *et al.* 2007). Even though it has not been proved yet, the viral polymerase complex is assumed as other factor that plays an important role in the transmission (Hatta *et al.* 2007; Neumann *et al.* 2007).

The PB2 protein has a role in determining the host range specificity and pathogenicity (Almond 1977; Naffakh *et al.* 2000; Manzoor *et al.* 2009). For instance, previous studies suggested that the origin of avian influenza virus can replicate in mammalian cells through changes in the amino acid at position 627 (Subbarao *et al.* 1993). Most of PB2 protein at position 627 is lysine (K) for human influenza isolates and glutamic acid (E) from avian influenza isolates (Graef *et al.* 2010). Another polymerase protein is PB1 located in the segment 2 of mRNA influenza virus genome. PB1 protein has two overlapping open reading frames (ORFs) including PB1 and PB1-F2 (Chen *et al.* 2001). PB1-F2 protein becomes an important determinant because it is associated to the death cell induction and contributes to the pathogenesis on mice (Chen *et al.* 2001; Zamarin *et al.* 2006). PB1-F2 is encoded by most of influenza A viruses isolated from a wide variety of hosts, including human and birds.

The size of PB1-F2 protein varies ranging between 57-101 amino acids (Zell *et al.* 2007). Mutation of asparagine into serine at position 66 (N66S) in PB1-F2

protein was reported increasing virus virulence and delaying immune response (Conenello *et al.* 2007; Conenello *et al.* 2011). Due to the importance of acknowledging the character of both polymerase protein complexes towards transmission and pathogenicity of avian influenza viruses, this study aimed to characterize the polymerase complex of PB2 and PB1-F2 proteins of H5N1 avian influenza viruses isolated from Indonesia.

MATERIALS AND METHODS

H5N1 Avian Influenza Virus

Six H5N1 viruses belonging to the Indonesian Research Center for Veterinary Science were used in this study. These H5N1 viruses have already been identified as avian influenza virus, namely A/chicken/WestJava/Smi-udn/2011, A/chicken/WestJava/Smi-amn/2011, A/bird/Bali/2011, A/chicken/WestJava/Smi-M6/2008, A/chicken/EastJava/BwiI2/2010, and A/chicken/Bengkulu/Blk/2010.

Propagation of Avian Influenza Viruses

Avian influenza virus stocks in allantois fluid which are stored in cryotube ampoule in freeze about -20°C were thawed and inoculated into 9-11 days specific pathogen free (SPF) embryonated chicken eggs. Subsequently, the RNA isolation was conducted using QIAmp RNA mini Kit (Qiagen) from the harvested allantoic fluid and as a template for reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The RT-PCR was performed using Superscript III One Step RT-PCR System (Invitrogen). The RNA template was used for re-confirmation of the viruses using a set of specific primers for H5 and N1 (Wright *et al.* 1995; Lee *et al.* 2001).

DNA Sequencing

DNA sequencing was done using RNA of the viruses positively H5N1. Sequencing method and the set of primers for amplifying fragments of PB2 and PB1-F2 gene were performed according to Dharmayanti *et al.* (2014). The primers can be provided by request. DNA sequencing of fragments of PB2 and PB1-F2 gene was conducted using the GENETYX Analyzer 3130 machine (Applied Biosystems, USA). The sequencing results were verified and analyzed with the Bioedit

Software Version 7 (<http://www.mbio.ncsu.edu/BioEdit>). A phylogenetic tree of the H5N1 virus isolates studied amongst the international references was constructed with the Maximum Likelihood Tree (1,000 replicates) in the MEGA Software Version 5.2 (<http://www.megasoftware.net>).

Ethical Clearance of Animal Study

The use of animal in the study was approved by the Animal Care Committee of the Indonesian Research Center for Veterinary Science No. BBVA012012. In this study, three H5N1 avian influenza viruses (i.e. BwiI2/2010, Smi-M6/2008 and Bali/ 2011) were used. These isolates were selected based on their molecular characters of the PB2 protein. These viruses were characterized for their phenotype, pathogenicity and virulence in the 4 week-old SPF chickens and the 3 month-old BALB/C mice as the avian and mammal models, respectively.

Briefly, in the chicken model, the SPF chickens were divided into four different groups (i.e. three groups for three different H5N1 viruses and one group as the uninoculated negative control). Each group consisted of four SPF chickens. Each virus isolate was infected into these three chickens, whereas one chicken as the contact animal was kept together with other three infected chickens. Similarly, mice were used as the animal model in the study. Mice were infected via nasal route with viruses, where the clinical symptoms after the infection were observed regularly until the infected mice died. The viral shedding was identified from the tracheal and cloacal swab of the infected chicken using RT-PCR test. Meanwhile, the viral shedding in the infected mice was detected in the nasal wash. All of the animals were kept in the Biosafety Level 3 (BSL-3) facilities.

RESULTS

Analysis of PB2 Gene and Protein of H5N1 Avian Influenza Virus

Sequencing analysis of the PB2 gene was successfully conducted for the six avian influenza virus isolates, i.e. A/chicken/WestJava/Smi-udn/2011, A/chicken/WestJava/Smi-amn/2011, A/bird/Bali/2011, A/chicken/WestJava/Smi-M6/2008, A/chicken/EastJava/BwiI2/2010, and A/chicken/Bengkulu/Blk/2010. The phylogenetic analysis based on PB2 gene showed

that these avian influenza virus isolates were in the same group with H5N1 viruses from human in Indonesia (Fig. 1). PB2 protein of these viruses showed no mutation (Fig. 2).

Analysis of multiple sequence alignment of PB2 protein showed that all the six viruses used in this study encoded a glutamic acid (E) at the position 627, in contrast to the three mutated H5N1 viruses from the NCBI Genbank database, such as A/Indonesia/6/2005, A/Indonesia/CDC579/2006 and A/Indonesia/CDC1031/2007, which display mutation at the position 627 (E627K) (Fig. 2). Moreover, another important identity marker was the gene encoding PB2 protein for mutation of aspartic acid (D) to asparagine (N) at position 701. The recent study revealed that all the six viruses used did not show any mutation in the PB2 gene for amino acid, compared with the international reference of H5N1 viruses.

Analysis of PB1 Gene and Protein of H5N1 Avian Influenza Virus

The phylogenetic PB1 gene analysis of these four avian influenza viruses (A/bird/Bali/2011, A/chicken/EastJava/BwiI2/2010, A/chicken/WestJava/Smi-udn/2011 and A/West Java/Smi-amn/2011) showed a genetic proximity with H5N1 viruses isolated from Indonesia in 2010-2011. A/bird/Bali/2011 isolate had a very high genetic proximity with A/Indonesia/NHIRD11767/2011, which is an avian influenza virus of subtype H5N1 from the human case. Moreover, other three viruses also had high proximity with human H5N1 viruses in Indonesia (Fig. 3).

In this study, the multiple sequence alignment analysis indicated that four avian influenza viruses used in this study had the same PB1-F2 protein profile with other H5N1 avian influenza viruses published in the NCBI (Fig. 4). However, the length of amino acid protein of PB1-F2 varied. Most avian influenza viruses used in the analysis had a length in about 90 amino acids. The isolate of A/bird/Bali/2011 had 89 amino acid, A/chicken/EastJava/BwiI2/2010 had 90 amino acids. Meanwhile the isolate A/chicken/West Java/Smi-udn/2011 which was similar with A/Bali/U8661/2009 had 87 amino acids with variation in 12 amino acid positions (Fig. 5). Moreover, the analysis showed no mutation of amino acid asparagine into serine at position 66 (N66S). All viruses analyzed in this study had asparagine at position 66.

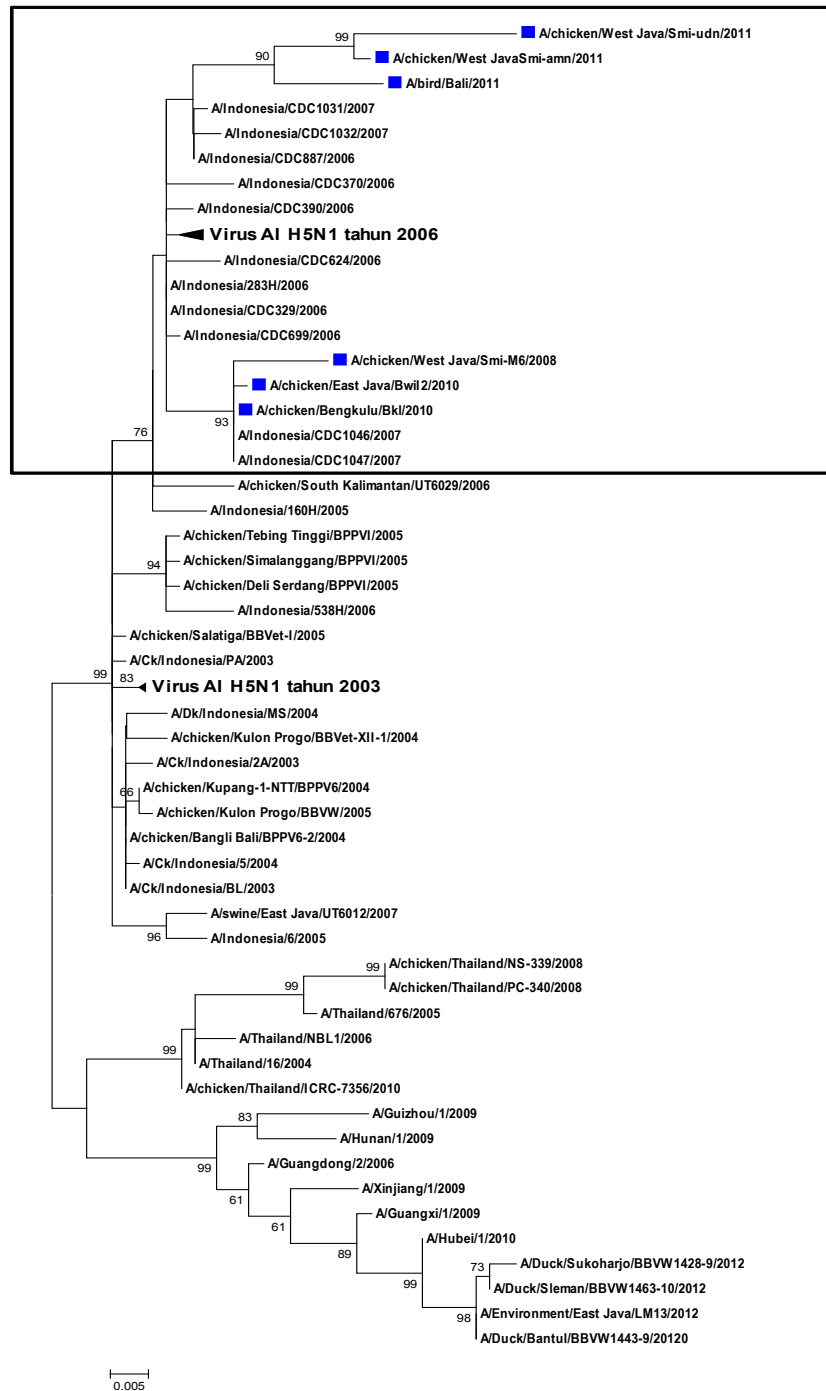


Fig. 1. Phylogenetic tree of H5N1 avian influenza viruses from Indonesia amongst the international references based on the genes encoding PB2 protein. Viruses used in this study (in the blue color) have close relationship with the Indonesian H5N1 human viruses.

Results of Pathogenicity Tests in Chickens and Mice

The pathogenicity tests of three avian influenza viruses showed that chickens were very sensitive towards these H5N1 viruses. This fact is confirmed by the death of chickens after the intranasal inoculation (Table 1). Meanwhile groups of chickens infected by

A/chicken/East Java/BwiI2/2010 and *A/chicken/WestJava/Smi-M6* isolates died on the second day post-inoculation (dpi). Chickens infected with *A/bird/Bali/2011* died in between of 2-4 days post-inoculation. The died chickens developed the clinical symptoms similar with avian influenza disease symptoms (Fig. 5). Moreover, the shedding of virus in chickens was detected on the 2-4 days by the RT-PCR

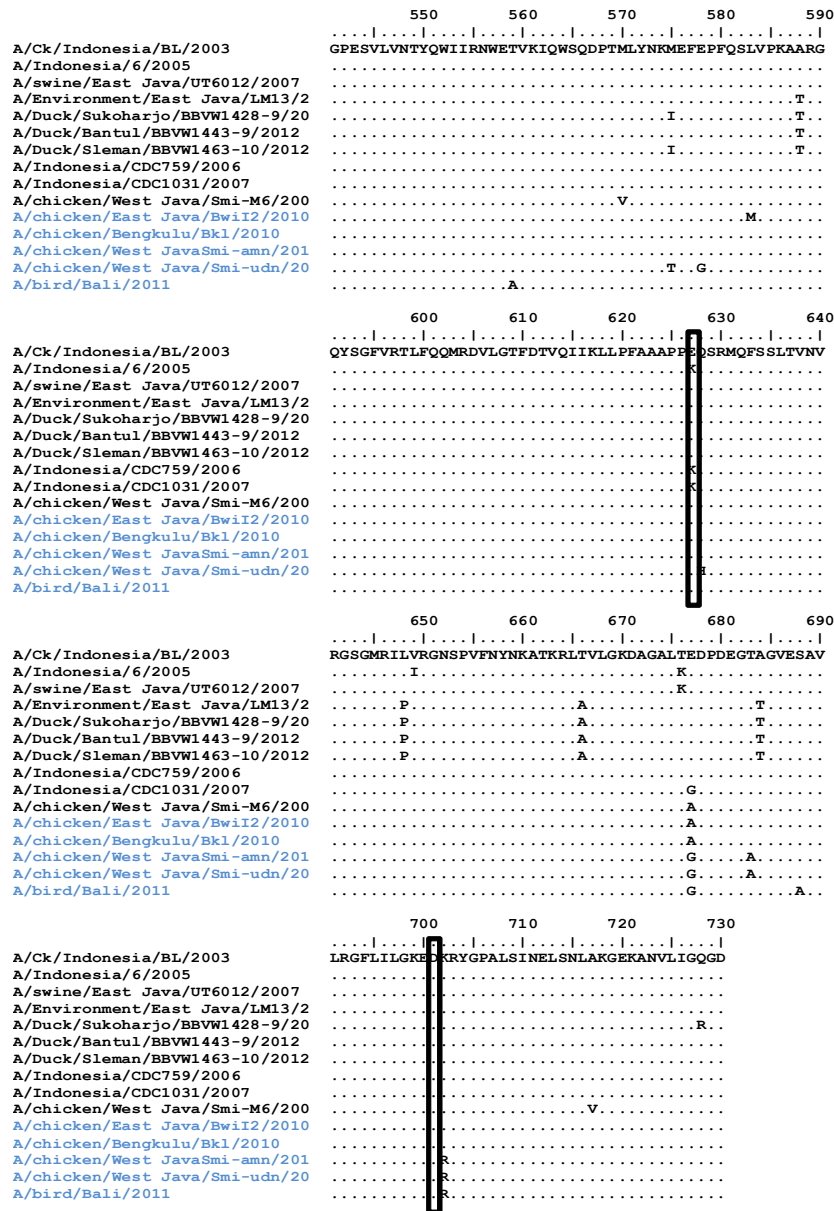


Fig. 2. Multiple sequence alignment of PB2 protein of H5N1 avian influenza viruses from Indonesia. Viruses used in this study are shown by blue color. The amino acid position at the 627 and 701 of PB2 protein is shown in the boxes.

test specific to H5 subtype. In addition, the negative control group that is not infected with the viruses remained alive and healthy without any clinical symptoms until the end of the experiment (Fig. 6).

On the other hand, the pathogenicity tests in mice had different results with the tests in chickens. The group of mice showed no clinical symptoms, temperature measurements as well as body weight significantly. Groups of mice infected the virus remained alive until the end of the experiment as well as the negative control group that is not infected by the viruses (Table 2).

DISCUSSION

The polymerase complexes are associated with pathogenic variation of H5N1 avian influenza viruses either in the experimental laboratory or in the field cases (Solomon *et al.* 2006). One of molecular determinants is amino acid mutation at position 627 from glutamic acid (E) into lysine (K) in the PB2 protein that increases virulence and adaptation to mammalian infection (Subbarao and Katz 2000). Other significant mutation is the change from aspartic acid (D) at position 701 to asparagine (N) that may

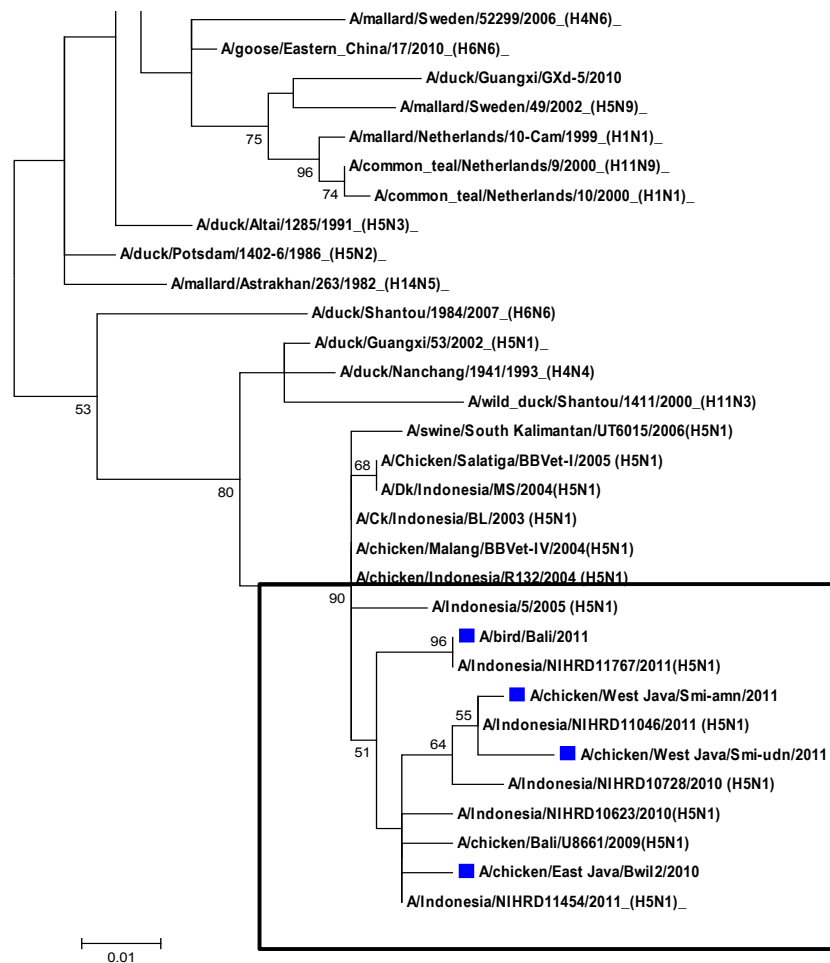


Fig. 3. Phylogenetic tree of PB1 gene of H5N1 avian influenza viruses. Four viruses used in this study are shown in the blue color. The viruses used in this study have close relationship with Indonesia H5N1 human viruses.

broaden the host range from avian like species (Li *et al.* 2005).

In this study, all investigated viruses showed no mutations in the PB2 protein of both the glutamic acid at position 627 and the aspartic acid at position 701. Therefore, the viruses were unable to replicate in mice. Mice did not show any clinical symptoms, no shedding and stayed alive until the end of the experiment in about 14 days after the infection. On the other hand, the viruses were able to kill chickens on 2-4 days and viral shedding occurred on 2-4 days after the infection. These results indicate that the viruses do not replicate in the mice and only infect the chickens. These outcomes agree with previous studies suspecting that E627K substitution is not only relate to adaptation in mammals by increasing the viral polymerase, but it is also a counterbalance of the low polymerase activity in mammal cells (Gabriel *et al.* 2005; Yamada *et al.* 2010).

The E627K residue was firstly recognized as a determinant of host range in 1993 (Subbarao *et al.*

1993). Then, it was proved to contribute to the temperature sensitivity of avian virus replication in mammalian cells (Massin *et al.* 2001). RVN1204 virus having lysine 627K grows more efficiently in the nasal turbinate of mice compared to wild type virus (Hatta *et al.* 2007). Similar to the E627K, the amino acid substitution of PB2 protein at position 701 from aspartic acid into asparagine implicates for the host range extension of H5N1 and H7N7 influenza viruses from avian-like host to other species, including mouse (Gabriel *et al.* 2005; Li *et al.* 2005) and human (de Jong *et al.* 2006). Moreover, the asparagine at position 701 of PB2 protein is a common feature of H3N2 virus (avian-like virus), which circulates in pigs in Europe. According to Steel *et al.* (2009), asparagine at position 701 can compensate the absence of residual 627K in the viral transmission in mammals.

A potential correlation between the polymorphism at position 627 and 701 in PB2 protein was observed in H5N1 viruses collected from humans in 2004-2005. De Jong *et al.* (2006) reported that among twelve



Fig. 5. Chickens infected with A/chicken/EastJava/BwiI2/2010 show weakness, listlessness and swollen eyes within two days after infection.



Fig. 6. Mice infected with A/chicken/EastJava/BwiI2/2010 show no clinical symptoms.

Table 2. The shedding of virus in the groups of mice infected with three H5N1 Indonesian avian influenza viruses detected with RT-PCR H5.

Mouse's code	SampleTypes	Shedding of virus					
		1 d-pre-i	2 dpi	5 dpi	8 dpi	11 dpi	14 dpi
Mouse 1 (Bwi.AE/Ms.1)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 2 (Bwi.AE/Ms.2)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 3 (Bwi.AE/Ms.3)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 4 (Bwi.AE/Ms.4)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 1 (M6.AE/Ms.1)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 2 (M6.AE/Ms.2)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 3 (M6.AE/Ms.3)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 4 (M6.AE/Ms.4)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 1 (Bali.AE2/Ms.1)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 2 (Bali.AE2/Ms.2)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 3 (Bali.AE2/Ms.3)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 4 (Bali.AE2/Ms.4)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 1 (NC.AE/Ms.1)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 2 (NC.AE/Ms.2)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 3 (NC.AE/Ms.3)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative
Mouse 4 (NC.AE/Ms.4)	Nasal Wash	Negative	Negative	Negative	Negative	Negative	Negative

d-pre-i = day pre-innoculation, dpi days post-inoculation

NC.AE is a negative control group of mice; Bwi.AE is a group of mice infected with A/chicken/EastJava/BwiI2/2010; Bali.AE is a group of mice infected with A/bird/Bali/2011; and M6.AE is a group of mice infected with A/chicken/WestJava/Smi-M6/2008.

clinical human isolates, eight have 627K while three viruses have 701N. These researchers suspected that 701N is compensated for lacking of 627K related to replication in mammalian cells. Another study acknowledged that four amino acid substitutions in the receptor binding of HA protein and one substitution in PB2 protein are consistently found in the AI virus that can be transmitted through the air on the infection experiment in ferret (Herfst *et al.* 2012).

The other important feature of polymerase complexes is PB1-F2 protein that is a short protein in about 87-90 amino acid length identified in 2011,

which is translated from +1 reading frames of the PB1 gene segment (Chen *et al.* 2001). The role of PB1-F2 protein towards characteristics of avian influenza virus in mammals is still unclear (Chen *et al.* 2001). This protein is non-functional until it is expressed. After the expression, it is rapidly degraded because it is not required for virus replication in either egg or cell cultures (Chen *et al.* 2001). PB1-F2 protein induces humoral response and T cell activation of the human immune system during the infection of H3N2 or H5N1 highly pathogenic viruses (Chen *et al.* 2001; La Gruta *et al.* 2008; Krejnosova *et al.* 2009).

In the case of influenza pandemic at the beginning of 2009 by the new reassortant H1N1 virus, the fatality rate in human was considered low with only light symptoms and low death rate despite the new virus spread widely across many countries. This fact is in contrast with the previous influenza pandemic, such as H1N1 in 1918, H2N2 in 1957 and H3N2 in 1968 that caused millions of deaths around the world (Palese 2004). The low fatality rate of this new H1N1 is presumably due to the absence of specific virulence factors such as PB1-F2 protein.

In this study, two viruses possessed PB1-F2 protein with length of 90 amino acids, while the other two viruses owned PB1-F2 protein with 87 amino acids. Moreover, all viruses had no mutation at position 66. According to Chen *et al.* (2010), the PB1-F2 protein has a variable length, amino acid sequence, cellular localization and different functions on different virus strains generating specific pathogenicity. This genetic and function diversity makes it flexible and more adaptable to produce efficiency of replication and virulence for all strains of influenza A virus.

All viruses within this study were pathogenic for chickens but not for mice. This outcome is consistent with the molecular characters of PB2 and PB1-F2 proteins, which do not undergo mutation so it is still difficult to adapt to mammals. Study by Schmolke *et al.* (2011) indicated that N66S mutation in PB1-F2 protein increases virulence and replication of recombinant H5N1 virus VN1203. Then, the artificial deletion of the ORF PB1-F2 made the virulence effect is nullified. Another data show that PB1-F2 only affects pathogenicity to mammals if there is a mutation in the N66. The results of this study consent to the previous studies about the mutation of PB1-F2 at position N66. Since no polymorphisms on N66, the viruses cannot infect mammalian species so the mice in the experiment remain healthy and stay alive until the end of the challenge infection.

CONCLUSION

The polymerase complex protein characters of PB2 and PB1-F2 in H5N1 avian influenza viruses used in the study show a restricted host range to avian-like species. There is no indication of mutation in these polymerase complex proteins. These molecular characters are in line with the animal experiment on chicken as avian model and BALB/C mice as mammalian model. The H5N1 viruses used in this study only infect the chickens, but no transmissible to BALB/C mice.

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