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Original Article

Assessing pathogenicity potential of H5N1 avian influenza virus strains isolated from Indonesian waterfowl in chickens and Balb-c mice

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Abstract

In Indonesia, the outbreaks of highly pathogenic avian influenza (HPAI) have been including in domestic poultry since 2006. In this study, the pathogenicity of two isolates of H5N1 (A/goose/Bojonggenteng/IPB2-RS and A/duck/Parung/IPB8-RS), which had been isolated from waterfowls, was assessed in chickens and Balb-c mice. The egg infectious dose EID_{50} results were high in chickens. The virus killed the chickens within 24 hours after post infection and it showed maximum IVPI value. The virus antigens were detected in chicken internal organs. Further assessment using a biological test in Balb-c mice was performed to determine the infection effectiveness and pathogenicity phenotype. Two virus isolates were highly pathogenic and the viruses were detected in the mice core organs. This result indicated that the virus was able to replicate in mammals. Moreover, the avian influenza viruses were found genetically and biologically pathogenic.

Keywords: avian influenza virus, H5N1, pathogenicity, waterfowl

1. Introduction

Historically, the lethality level of poultry, especially chicken, has been correlated with the pathogenicity of avian influenza virus (AIV) (Swayne and Pantin-Jackwood, 2006). Highly pathogenic avian influenza (HPAI) virus causes systemic diseases associated with high mortality and morbidity in chickens and turkeys (OIE, 2005). Typically, high mortality in chickens and other galliformes caused by HPAI does not profoundly influence waterfowl (Swayne and Pantin-Jackwood, 2006). The waterfowl are considered to be a natural reservoir for AIV; they can carry various subtypes of AIV with little or no impact on their health (Webster *et al.*, 1992). The HPAI viruses have rarely been isolated from waterfowl even on farms experiencing HPAI outbreaks in poultry (Swayne and Suarez, 2000). However, the outbreaks of H5N1 in Hong Kong in 2002 recorded that there were several numbers of waterfowl deaths (Sturm-Ramirez *et al.*, 2004). In addition, Sakoda *et al.* (2010) successfully isolated the HPAI subtype H5N1 from dead wild waterfowl found in Mongolia. Furthermore, the isolates were inoculated into ducks, and several neurological signs were observed. The ducks died in 4 to 9 days post-inoculation and viruses were found in each of the tested tissues of dead ducks.

The characterization of avian influenza viruses to be classified as highly pathogenic depends on specific pathogenicity tests and the amino acid sequence of hemagglutinin cleavage site (Alexander, 2000). The pathogenicity analysis comprises molecular and biological approaches. Using molecular technique (sequencing method), the viral pathogenicity could be defined by gene encoding in the amino acid sequences of hemagglutinin cleavage site (Susanti *et al.*, 2008). For example, the PQRERRRKKR cleavage site was a typical cause of death of poultry outbreaks in Hong Kong (1997) and Asian countries (2003-2007) (Guan *et al.*, 2004; Smith *et al.*, 2006; Stevens *et al.*, 2006). The pattern of QRESRRKKR was typical in H5N1 that caused human death in Indonesia from 2005 to 2007 (CDC, 2007). In present study,

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the viruses were isolated from local waterfowl, and then were antigenically and genetically characterized. Pathogenicity of the isolated H5N1 viruses was investigated by experimental infection studies in mice. The present results strongly help to understand that even though avian influenza virus infects waterfowl and shows asymptomaticity on their health, it is necessary to give some awareness on them. The present study demonstrated that AIV isolated from waterfowl was considered as highly pathogenic and could potentially be transferred to the mammalian body.

2. Materials and Methods

2.1 Materials

The virus isolates used in this study were obtained from the previous study by Susanti *et al.* (2008). The research was conducted in the Laboratory of Medical Veterinary, Bogor Agricultural Institute (IPB, Indonesia). The RNA was isolated using Trizol®LS Reagent (Invitrogen). The staining reagent was obtained from Temasek Laboratory, Singapore. The RT-PCR was conducted using SuperscriptTM III One-step reverse-transcription polymerase chain reaction.

2.2. Sampling method

Samples was taken in the previous study, in two districts of West Java, Indonesia, namely Bogor and Sukabumi (Susanti *et al.*, 2008). A total amount of 0.07% of waterfowl population was taken from each area, 267 samples were from Bogor and 139 samples were from Sukabumi. From the result of polybasic amino acid analysis, twenty isolates were shown having QRERRRKKR, whereas one isolate had QRESRRKKR sequences in its hemagglutinin cleavage site. Two out of twenty strains (A/goose/Bojonggenteng/IPB2-RS and 1014/ml A/duck/Parung/IPB8-RS) were used for further analysis in the present study.

2.3 Screening of avian influenza virus using the haemagglutination analysis

Virus propagation was performed by using the method established by the World Health Organization (WHO). The allantoic fluid of chicken embryos that died on the fourth day was harvested for further screening of its ability to agglutinate red blood cells (WHO, 2002). The virus isolates were investigated for their purity and their possibility of bacterial contamination using blood agar plate test. A bacterial contamination in the virus cultures was cleaned by filtration using the 0.45 µm ultrafiltration membrane.

2.4 Identification of influenza virus isolate subtypes using RT-PCR Mmethod

Haemagglutination test was performed to know the virus infection. Subsequently, the viral RNA was extracted from the allantoic fluids of chicken embryos based on the results of haemagglutination test. The procedure of Trizol® LS Reagent (Invitrogen) was performed prior to RNA isolation. The viral RNA was applied in the thermal reaction using SuperscriptTM III One-step reverse-transcription polymerase chain reaction (RT-PCR) system. Viral subtype was analysed according to the method of Payungporn *et al.* (2004).

2.5 Analysis of Egg Infectious Dose 50 (EID_{50})

The determination of EID_{50} was performed in triplicate virus propagation in chicken embryos ranging from 10^{-1} to 10^{-20}. EID_{50} dose was calculated based on the formula of Reed and Munch (WHO, 2002).

2.6 Analysis of Intra Venous Pathogenicity Index (IVPI)

The 8-week-old chickens were inoculated with shed virus having haemagglutinin (HA) titer more than 2^4. The IVPI index was calculated according to OIE standards (OIE, 2000).

2.7 Experimental infection of Balb-c mice with H5N1 isolates

Regarding the highly pathogenic avian influenza isolates, each virus was inoculated into mice (*Mus musculus*). Ten µl of each H5N1 isolate containing $10^{7.0}$ EID_{50} was inoculated orally into 8-week-old female Balb-c mice. In order to determine the possibility of viral transmission between animals, six non-inoculated mice were put into the cage 1 h after the inoculation. As the control, 12 mice were placed in a separate cage. Daily observation was carried out for 14 days for clinical symptoms and death. Three mice were euthanized on the 4th day of post-inoculation. Then, lungs, liver, brain, kidneys, heart and spleen were collected aseptically. The remaining mice were observed continually for 14 days. The organs received from dead mice were investigated to record the presence of the virus using chicken embryos, RT-PCR and immunohistochemistry analysis. On day 4, 8 and 12, nasal and anal swabs of mice were collected and then analysed by propagation in the chicken embryos. Next, viral RNA was extracted and further performed in RT-PCR (Maines, 2005).

2.8 Virus distribution in mice organs by immunohistochemistry analysis

Chicken and mice organs were cut 0.5 cm in size and then fixed in 10% formalin buffer solution for 24 h. Subsequently, they were processed into paraffin blocks and then it was cut into 3-4 µm. The sections were affixed to the gelatine-coated object glass, and then deparaffinised using Xylol solutions for 3 min. Later, it was rehydrated with alcohol concentrations ranging from 96% to 70%, for 3 min, respectively. Subsequently, the slides were washed off using distilled water for 3-5 min followed by PBS solution for 5-

10 min. The staining method from Temasek Laboratory, Singapore, was accomplished to detect avian influenza. The slides were subjected to the incubation in monoclonal antibodies using H5N1. After the slide staining procedures, observation was made to perceiving presence of brown-stained antigen.

3. Results and Discussion

In this study, HPAI viruses isolated from ducks and geese (Anseriformes) might determine the role of waterfowl as a reservoir of AIV. This result was reliable according to the other species of waterfowl (Anseriformes and Charadriiformes) known as the reservoir of influenza viruses (Fouchier *et al.*, 2007; Webster *et al.*, 2007). It is also supported by the research of Pantin-Jackwood and Swayne (2009), Passerines and Columbiformes have demonstrated inefficiency of contact transmission and could act as a reservoir of the virus, while Anseriformes might act as a reservoir.

Virus isolation and propagation results showed that hemagglutinin HA titer were high in two waterfowl samples, i.e. A/goose/Bojonggenteng/IPB2-RS (2^9) and A/duck/Parung/IPB8-RS (2^{12}). However, after the isolates from allantoic fluid inoculated on blood agar medium, some bacterial species were detected, i.e. Gram-negative *bacilli* and *cocci*. The contaminated isolates were filtered to remove contamination using 0.45 µm ultrafiltration systems. The results of EID_{50} of on these two isolates were $10^{15.64}$/ml and 10^{14}/ml, respectively (Table 1).

In the present study, two H5N1 viruses (A/goose/Bojonggenteng/IPB2-RS and A/duck/Parung/IPB8-RS) were categorized as HPAI based on the QRERRRKKR amino acid sequence of hemagglutinin cleavage site. The results of the present study were in line with the previous research report. Two HPAI H5N1 viruses isolated from healthy geese in Vietnam poultry markets were indicated having the PRIERRRKKR sequence at the cleavage site. The pattern of amino acid sequences QRERRRKKR was a typical cause of death of poultry; e.g. the evidence in Hong Kong (1997) and Asian countries (2003-2007) (Smith *et al.*, 2006; Stevens *et al.*, 2006; Guan *et al.*, 2004). Another study showed that the virus isolate A/chicken/Texas/298313/04 (TX/04) (H5N2) was categorized as having low pathogenic virus based on its mild clinical symptoms. The molecular analysis showed that this virus had polybasic acid sequence at its haemagglutinin cleavage site. In addition, the pathogenicity of this virus increased in vitro as well as in vivo by adding the amino acid at its haemagglutinin cleavage site (Lee *et al.*, 2005). In fact, the virus spread through the vascular system ending in the tissue blood junction and then spread to various organs (Kuiken *et al.*, 2006). In addition, the genetic characterization was performed to see the sequence regions of haemagglutinin cleavage site of the virus isolated from chickens (Susanti *et al.*, 2008). As revealed by the previous study, the haemagglutinin polybasic sequence allowed the proteolytic activities, that is performed by proteases such as fruit and proprotein convertase 6 (PC6) in the Golgi apparatus of all cells. AIV with polybasic cleavage had an unlimited network distribution and could cause a fatal systemic infection (Chen *et al.*, 2004).

The pathogenicity of H5N1 isolates in chickens was assessed by inoculation of each of A/goose/Bojonggenteng/IPB2-RS and A/duck/Parung/IPB8-RS in chickens. The IVPI values of the viruses killed chickens within 24 h post infection with IVPI value of 3.0. This result showed that the two isolates of the virus should be categorized as high pathogenicity phenotype (HPAI). The HPAI virus isolates was detected in chicken tissues i.e. trachea, lung, kidney, liver, pancreas, brain, and bursa of Fabricius as illustrated in Figure 1. Some genotypes of HPAI AIV subtype H5N1 that was isolated in 1997-2001 in Hong Kong killed chickens within 24 h after intravenous inoculation (Guan *et al.*, 2004). In addition, two HPAI virus isolates from India were tested for IVPI and resulted in 2.96 and 2.95, respectively (Shankar *et al.*, 2009). As listed in OIE guideline (OIE, 2000), the virus isolates that have the IVPI more than 1.2 was classified as HPAI. According to WHO (2002), AIV is classified as highly pathogenic virus when an infection in 4-8-week-old chicken causes 75% mortality within eight days.

The detection of the virus in various organs strengthens the IVPI analysis results in both isolates. As mentioned in a previous study, the level of pathogenicity was in line with high titers of virus replication, particularly in certain tissues such as the brain and heart. It was reported that HPAI viruses could replicate efficiently in vascular endothelial cells as well as in perivascular parenchymal cells. For this reason, the virus could be detected in various internal organs and blood vessels (Horimoto and Kawaoka 2005). According to the study results of Shankar *et al.* (2009), HPAI caused microscopic lesions in the organs of chickens mainly in the trachea, lung, kidney and spleen. In gallinaceous poultry, HPAI virus cause high morbidity and mortality, also a systemic disease with necrosis and inflammation in several visceral organs, nervous systems, cardiovascular systems, as well as the integument (Pantin-Jackwood and Swayne, 2009).

Table 1. The result of HA titer, EID50 and IVPI of two isolates HPAI virus subtype H5N1 from waterfowl

No	Virus Isolate	HA Titer	EID_{50}	IVPI
1	A/goose/Bojonggenteng/IPB2-RS (H5N1)	2^{12}	$10^{15,64}$/ml	3.0
2	A/duck/Parung/IPB8-RS (H5N1)	2^{9}	$10^{14,00}$/ml	3.0

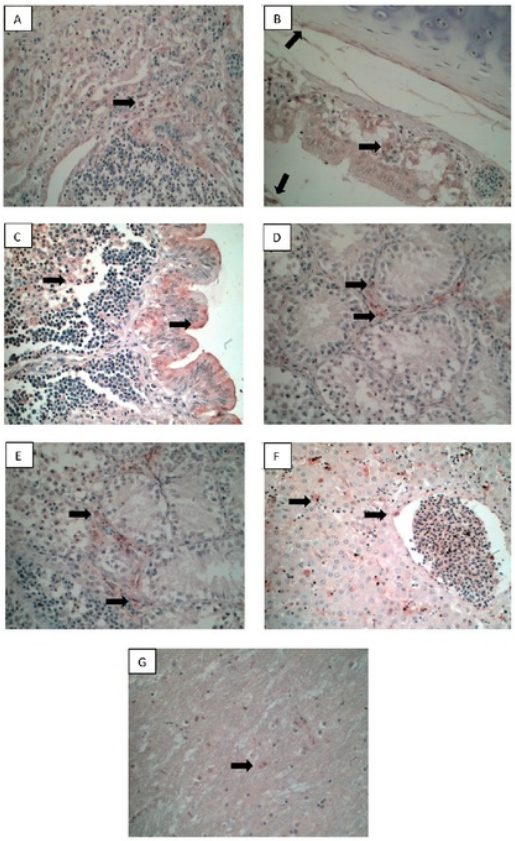

Figure 1. HPAI H5N1 virus antigen stained in chicken organs: (A) pulmonary air capillary, (B) mucosale pithelial cells, lamina propria cells, vascular endothelium and necrotic debris trachea (inset), (C) epithelium and follicle of Fabricius bursa, (D) interstitial tissue cells and renal tubules, (E) Langerhans islet cells and pancreatic acini cells, (F) hepatocytes and blood vessels of the liver endothelial cells, (G) brain neuronal cells.

Although waterfowl serve as the reservoir, it is always necessary to be cautious about the possibility of the evolution of the virus to become pathogenic in waterfowl. As mentioned by Swayne and Pantin-Jackwood (2006), the HPAI virus of Asian lineage has evolved, and some virus strains have caused inconsistent respiratory infections in ducks. In addition, they tended to be highly pathogenic in the internal organs and the brain. Similar result reported that HPAI H5N1 virus infections in duck cau systemic damage. The damage occurred in the brain, trachea, lung, liver, pancreas, rectum, spleen, bursa of Fabricius, heart, and kidney (Songserm *et al.*, 006). Seven of the eight White Pekin Duck (weeks-old) inoculated with A/Egret/HK/757.2/02 showed acute disea including severe neurological dysfunction and death. A/Vietnam/1203/04 and A/Crow/Thailand/04 caused high mortality with microscopic lesions and AI viral antigen in the nasal cavity, brain, heart, adrenal gland, and pancreas. Moreover, A/ThailandPB/6231/04 killed three of the eight ducks; however, it did not induce neurological signs (P n-Jackwood and Swayne, 2009). In this study, although the H5N1 isolates were classified as highly pathogenic (HPAI) virus based on molecular and biological results, in fact, they had low pathogenic phenotypes. H5N1 isolates were obtained from healthy and unvaccinated waterfowl; however, it allowed waterfowl act as the reservoir and spreading media of HPAI virus into the environment. This characteristic was in line with the previous study that stated the waterfowl as a potential reservoi H5N1 virus was shown by H5N1 virus Asian lineage (Webster *et al.*, 2007; Pantin-Jackwood and Swayne, 2009). HPAI H5N1 Asian lineage viruses do not cause the morbidity and mortality of waterfowl (ua and Marangon, 2007). HPAI virus subtypes H5 and H7 in the United States and Europe were the result of the evolution of LPAI viruses found in aquatic birds (Senne, 2007).

Clinical symptoms appearing in Balb-c mice inoculated with 10 µl virus at a dose of 10^7 EID_{50} included dull hair, weakness and diarrhea. Until 14 post-inoculation days, no mice died. However, the virus spread systemically via the od circulation to the brain (neurotropism). Evidently, this virus was detected in the liver, brain, kidney, intestine, lung, and spleen (Figure 2), indicating that the virus replicated in

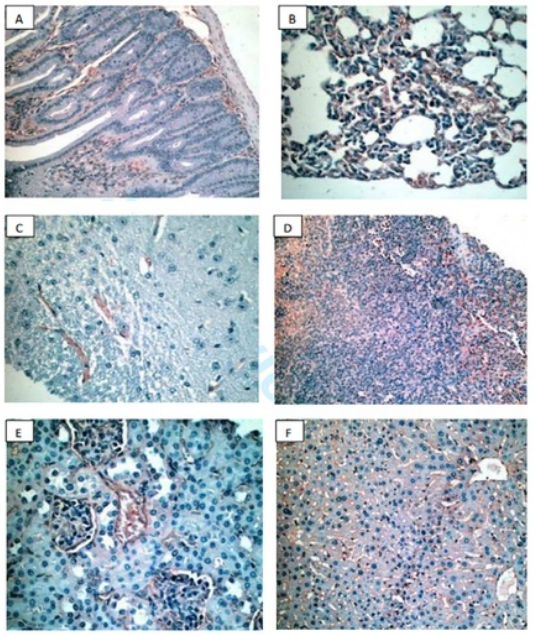

Figure 2. HPAI H5N1 virus antigen stained in Balb-c mice organs: (A) intestine cells, 11 (B) lung cells, (C) brain neuronal cells, (D) spleen cells, (E) kidney cells (F) hepatocytes 12 cells

mammals (Balb-c mice) without prior adaptation. The healthy mice placed as stable mates with the infected mice also showed the same symptoms.

There is a shortage of information about biological and molecular components involved in the virulence of HPAI H5N1 in humans. The study on mammalian models such as nonhuman primates, ferrets, and mice is required to reveal the virulence mechanism of HPAI H5N1. Nonhuman primate *Macaca fascicularis* have been reported as an animal model of H5N1, but is not much used for practical reasons being less ethical and economic costly (Maines, 2005). Squirrel (*Saimiri sciureus*) can be used model of influenza virus, because the virus may develop in the upper respiratory tract. However, influenza virus infections in these squirrels not cause fever and illness after trans-tracheal infection (Zitzow *et al.*, 2002).

Ferrets (*Mustela putorius* furo) are widely used as an animal model of VAI pathogenicity in humans/mammals (Maines *et al.*, 2005, Zitzow *et al.*, 2002). Ferrets are natural hosts of the human influenza virus A and B and the disease resembled human flu; thus, these animals are widely used as a model study of pathogenicity and immunity. Ferrets are also sensitive to the avian flu virus. However, only human and swine viruses induce fever and disease. It is further reported that ferrets are very effective to evaluate the virulence of the virus and test the safety and efficacy of a vaccine candidate (Zitzow *et al.*, 2002). Balb-c mice are commonly as an animal model of viral infection from native birds to mammals. The results showed a balanced level of virulence of H5N1 in ferrets and Balb-c mice. However, ferrets better reflect the increased mortality among human isolates from 2004 to 2005 (Maines *et al.*, 2005).

The human, geese, and chickens virus isolates can replicate efficiently in the lungs of mice without requiring prior adaptation. Neurotropic pathogenic variants were isolated from the brains of mice after a single passage in the lungs. Neurological symptoms (paralysis) appeared in mice; however, they did not appear in mice that inoculated with human viruses. The mortality rate of a human virus in mice was 90%, whereas in the avian virus was 30%. Moreover, the morbidity of human viruses was associated with a weight decreased to 40% before death, whereas the morbidity of goose virus infection decreased to 10% followed by a rapid recovery. Both viruses were found replicating in the lungs of mice, whereas the human virus was 62% higher than the goose virus. These results indicated that human isolates were more pathogenic in mice than avian isolates (Guan *et al.*, 2004).

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