J I T A A
Journal of the Indonesian Tropical Animal Agriculture
Accredited by Ditjen Penguatan Risbang No. 60/E/KPT/2016

J. Indonesian Trop. Anim. Agric.
pISSN 2087-8273 eISSN 2460-6278
http://ejournal.undip.ac.id/index.php/jitaa
42(2):72-80, June 2017
DOI: 10.14710/jitaa.42.2.72-80

The pathogenecity of H5N1 highly pathogenic Avian Influenza (HPAI) virus clade 2.3.2. in Indonesian indigenous chicken by contact tranmission with infected duck

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Received September 23, 2016; Accepted November 18, 2016

ABSTRAK

Penelitian penularan buatan dilakukan pada sembilan ekor ayam kampung sehat yang dipelihara bersama dengan dua ekor itik umur 30 hari yang diinfeksi secara buatan dengan virus H5N1 HPAI clade 2.3.2 pada fasilitas Biosafety Laboratory Level 3 (BSL-3). Tujuan penelitian adalah untuk mengetahui patogenitas virus H5N1 HPAI clade 2.3.2 pada ayam kampung. Hasil penelitian menunjukkan bahwa ayam kampung dalam waktu 24 jam menunjukkan gejala klinis ringan seperti bulu berdiri dan lemah dan setelah 48 jam kesembilan ayam mati, sedangkan kedua itik tetap hidup dengan gejala klinis sangat berat. Virus HPAI H5N1 berhasil diisolasi dari preparat usap (swab) yang berasal dari ayam dan itik tersebut, menandakan bahwa mereka terinfeksi virus tersebut. Secara mikroskopik, ayam kampung menunjukkan ensefalitis, trakheitis, miokarditis, pneumonia interstitialis, hepatitis, proventrikulitis, enteritis, pankreatitis, nefritis dan bursitis yang kesemuanya bersifat peradangan non-supuratif. Lesi dominan lainnya yaitu nekrosis pada limpa dan pankreas. Virus H5N1 HPAI clade 2.3.2 dideteksi dengan metode imunohistokimia dan ditemukan pada hampir semua organ visceral yang terserang. Hasil ini menunjukkan bahwa ayam kampung asal Indonesia tergolong peka terhadap infeksi virus HPAI H5N1 clade 2.3.2 dan virus tersebut dapat ditransmisikan dengan mudah melalui penularan kontak dari itik yang terinfeksi ke ayam kampung.

Kata kunci: Patogenitas, virus H5N1 HPAI clade 2.3.2, ayam kampung

ABSTRACT

An experimental transmission study was conducted using nine healthy Indonesian indigenous chickens placed together with two 30 days old ducks which were experimentally infected with H5N1 HPAI clade 2.3.2 virus in the Biosafety Laboratory Level 3 (BSL-3) facilities. The aim of the study was to find out the pathogenicity of H5N1 HPAI virus clade 2.3.2 in Indonesian indigenous chickens. The study showed that within twenty four hours rearing, the chickens were exhibited mild clinical signs and by 48 hours, all of the chickens died, whereas the ducks survived but with severe clinical signs. The H5N1 HPAI virus has been successfully isolated from chickens and ducks swabs, confirming that those animals were infected by the virus. Histologically, the infected chicken encountered with severe inflammation reaction namely non suppuratives encephalitis, tracheitis, myocarditis, interstitial pneumonia, hepatitis, proventriculitis, enteritis, pancreatitis, nephritis and bursitis. Necrotizing spleen and pancreas were also prominent. Viral antigen was detected by immunohistochemistry staining in various affected visceral organs. This suggests that Indonesian indigenous chickens were susceptible to H5N1 HPAI virus clade 2.3.2 and it can be transmitted easily to Indonesian indigenous chickens by contact transmission with infected ducks.

Keywords: Pathogenicity, H5N1 HPAI virus clade 2.3.2, Indonesian indigenous chickens

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) H5N1 was firstly reported in 1996 in Guangdong Province, China (Xu *et al*., 1999). This disease has spread widely around the world, and caused significant impact in affected countries (Webster and Govorkova, 2006), including in Indonesia (Wiyono *et al.,* 2004). The virus of H5N1 HPAI is very contagious in poultry and generates high mortality up to 100% (Swayne and Halvorson, 2008). Aetiological agent of HPAI belongs to *orthomyxoviridae* family and genus of *influenza A virus* (The International Committee on Taxonomy of Viruses 2012). Genus of *influenza A virus* have broad host species, from animals to human (Swayne and Halvorson 2008). Based on its surface glycoproteins: hemmaglutinin (HA) and neuraminidase (NA), influenza virus classified into 18 hemaglutinin subtypes (H1-18) and 11 neuraminidase subtypes (N1-11) had been recognized (Tong *et al.,* 2012; 2013). H5N1 HPAI virus infected systematically and produced damaged in cardiovascular, nervous system (Swayne and Halvorson 2008), integument and skeleton (Swayne and Pantin-Jackwood, 2008). Inflammation and necrosis in various visceral organs were detected (Swayne and Pantin-Jackwood, 2008).

In Indonesia, HPAI cases were first reported in the end of 2003 affecting not only commercial poultry farms but also Indonesian indegenous chicken (Wiyono *et al.,* 2004). This viruses were majority affecting chicken in Indonesia (Wiyono *et al.,* 2004). However, in late 2012, there were outbreaks causing high mortality in ducks in three provinces i.e. Central and East Java and Yogyakarta, and further characterization showed that these outbreaks caused by a new clade 2.3.2 virus (Wibawa, *et al.,* 2012; Dharmayanti *et al.,* 2013). Since it has been reported to be isolated from dead migratory birds in Qinghai Lake region, China in 2009 (Hu *et al.,* 2011; Zhao *et al.,* 2012) H5N1 HPAI virus clade 2.3.2 has been reported to circulate in several countries not only affecting wild birds (including ducks) but also chicken (Kang *et al.,* 2011; Reid *et al.,* 2011; Marinova-Petkova *et al.,* 2012; Nagarajan *et al.,* 2012; Nemeth *et al.,* 2013).

In Indonesia, even though clade 2.3.2 has been reported to cause high mortality in domestic ducks, there was limited information on the possibility of infected duck to spread the disease to chickens, and the pathogenicity mechanism of this virus in chickens, particularly in Indonesian indigenous chicken. In natural cases, sub-clinical or less severe infection of H5N1 HPAI is sometimes reported to occur in Indonesian indigenous chicken. Hypothetically, this phenomenon is probably due to the resistance of chicken to the disease (Sartika *et al*., 2011). In order to reveal the circumstances, the study was aimed to find out the pathogenicity of the new clade 2.3.2 in Indonesian indigenous chickens.

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MATERIALS AND METHODS

H5N1 HPAI virus strain

The H5N1 HPAI virus used in this study was A/duck/Sukoharjo/BBVW-1428-9/2012 belonging to clade 2.3.2 (Wibawa *et al.,* 2012). Virus was isolated from duck in Central Java Province, Indonesia (Wibawa *et al.,* 2012).

Experimental Animals

The use of materials in this study has been approved by Animal Ethics Committee for Using Animal and Scientific Procedures in Indonesian Research Center for Veterinary Science, Bogor. Nine birds of 20 weeks old Indonesian indigenous chickens (*Gallus-gallus bankiva*) and 30 days old of two Alabio ducks (*Anas platyrhinchos*), which were clinically healthy were used in this study. The chickens and ducks were obtained from a farm in Bogor, West Java, Indonesia. Chickens and ducks were housed in isolation unit (*Montair Andersen B.V.* HM 1500, Sevenum, The Netherlands) which were ventilated with HEPA-filtered air, and equipped with continuous lighting. Commercial pellets and water were provided *ad libitum*. Numbered leg bands were used to identify the birds individually.

Experimental Transmission

Two ducks were inoculated by intra-nasal, oral and ocular routes with a total of 0.2 mL of diluted infective allantoic fluid containing a total of $10^{6.8}$ ELD_{50}. Three days after ducks being inoculated and exhibited clinical signs, nine Indonesian indigenous chickens were placed in the same isolator unit as the ducks. Chickens and ducks were monitored daily for clinical signs and mortalities.

Samples Collection

Oropharyngeal and cloacal swabs were collected using sterile cotton swabs prior to

challenge, at 48 hours and 3 days post-challenge for chickens and ducks. They were put in a tube of transport medium containing MEM medium, 2% fetal calf sera (FCS) and antibiotics. These were stored at –70ºC before subjected to virus isolation. Blood samples were collected from brachial veins of each chicken and duck prior to challenge according to animal welfare standard for serological test. Tissue samples of brain, trachea, lung, liver, heart, skeletal muscle, intestine, kidney and spleen from chickens and ducks that euthanized or died were collected for histopathological examination .

Virus Isolation in Embryonated Chicken Eggs (ECE's)

Virus isolation in embryonated chicken egg Specific Pathogen Free (SPF) and Specific Antibody Negative (SAN) was conducted according to World Organization for Animal Health (OIE, 2012). Briefly, 0.2 mL sample was inoculated into allantoic fluid of 9-11 days old eggs. Inoculated eggs were incubated at 37ºC for 5 days. Survived, dying and dead eggs were stored at 4ºC for at least 12 hours. The eggs allantoic fluids were tested with rapid agglutination test (HA) by adding 25 μL of 10% chicken red blood cells (RBC) into 25 μL of allantoic fluids. Allantoic fluids were declared as positive if there is agglutination.

Serological Test

Serum samples were tested using Hemaglutination test followed the procedure from World Organization for Animal Health (OIE) with minor modification (OIE, 2012) to determine the antibody titer of the chickens and ducks vefore experiment. Formaldehide-inactivated antigen generated from A/duck/Sukoharjo/BBVW-1428-9/2012 virus was used in this test. HI titer were reported as log2 titers. Briefly, sera were inactivated at 56^0 C for 30 minutes. Sera were treated with 0.5% chicken red blood cells (RBC) to remove nonspecific reaction. Sera were tested with HI test by using AI antigen and 0.5% RBC.

Post-mortum Examination

The birds dying were sacrificed according to the animal welfare standard. Sacrificed birds were necropsied and examined for gross pathological lesions. Several organs were sampled, processed and stained with Hematoxillin and eosin (H&E) using standard method (Drury and Wallington, 1980). The descriptive lesions were scored according to the degree of severity for each organ. No specific lesion (NSL) or mild, moderate and severe lesions were marked as +, ++ and +++ respectively.

Immunohistochemistry Assay

Immunohistochemistry was conducted according to Damayanti *et al.* (2004a) to detect the viral antigens. The slides were treated with proteolytic enzyme to unmask the aldehyde linkage (Shi and Taylor 2013). Briefly, slides were added with primary antisera, followed by 3% hydrogen peroxides, biotinylated secondary antibody (DAKO, Denmark) and avidin biotin peroxsidase (DAKO). Visualization of the antigen was achieved by adding with substrate called 3-3-Diamino benzidine (DAB) (DAKO, Denmark). Samples were determined as positive if viral antigens were detected as brown color and microscopically (10x20 magnification/field) was scored as - if there was no antigen at all, few (1-5 antigens/cell), moderate (6-10 antigens/cell) and huge (more than 10 antigens/cell).

Data Analysis

Data from serological test, virus isolation, clinical signs, post-mortum examination, and immunohistochemistry assay were analysed descriptively.

RESULTS AND DISCUSSIONS

As indicated in Table 1, prior to infection, chickens and ducks were clinically healthy and their sera were negative after tested by HI assay and all swabs tested were negative by virus isolation in eggs. This indicates that chickens and ducks were not previously exposed with H5N1 HPAI. After infection, HI test shows that all the chickens and ducks were serologically negative for influenza A virus subtype H5 prior to challenge as shown in Tabel 1. These features were also reported by Wibawa *et al.* (2013).

In acute stage the chickens developed severe clinical sign and succumbed to death within 48 hours post infection (Figure 1). This is in accordance with previous study where H5N1 HPAI clade 2.3.2 isolated from healthy mallard was highly pathogenic in chickens generating mortality in all chickens within 24 hours post infection (Kim *et al.,* 2011). In this study the chickens were dead faster than the ducks that were infected earlier. This means that Indonesian H5N1 HPAI virus clade 2.3.2 was more

Table 1. Clinical Signs, Serology and Virus Isolation Detected in Ducks and Chickens Before and After Infection.

Bird Species	Before Infection			After Infection		
	Clinical signs	Serology	Virus isolation	Clinical signs	Serology	Virus isolation
Ducks	No clinical signs	negative	negative	Clinical signs observed	NT	positive
Chickens	No clinical signs	negative	negative	Clinical signs observed	NT	positive

NT : not tested

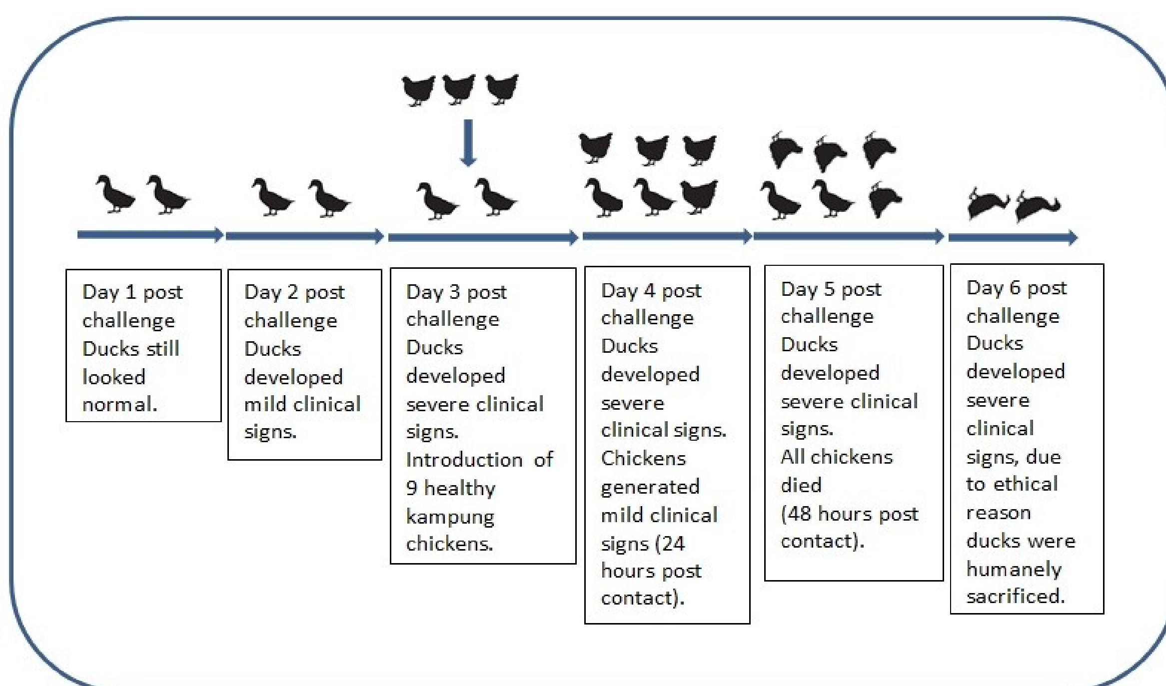

Figure 1. Diagram of the Onset of Present Study

pathogenic in Indonesian indigenous chickens than in ducks and can be transmitted easily from infected ducks particularly when those were in the stage of acute infection (days 3 post infection). According to Swayne and Pantin-Jackwood (2006), HPAI virus was more pathogenic in chickens and other galliforme birds, and usually produces no infection or mild disease in ducks, and that the severity of disease is associated with high virus replication titres in the host. In this case the chicken did not show resistance to the disease (Sartika *et al.,* 2011). This strongly suggests that Indonesian indigenous chickens should not be managed mingle with duck to minimize the transmission of H5N1 HPAI virus.

The clinical signs were characterized by slight listlessness, ruffled feathers and drowsiness started to be observed 2 days post-challenge in ducks. As seen on Figure 1, on day 3 post-challenge, ducks developed severe clinical signs such as tremors, loss of balance, paralysis and lethargy. This findings were similar to the pathogenicity study reported by Pantin-Jackwood *et al.* (2013) who stated that domestic ducks play

an important role in the epidemiology of H5N1 infection in Asia, Africa and Eastern Europe.

Table 1 indicated that swabs collected from these ducks and chickens were positive by virus isolation in eggs, suggesting that all the chickens and ducks were infected by H5N1 HPAI virus. The isolation of virus from swabs of ducks and chickens indicates the viral shedding both from infected ducks and in contact chickens. This suggests the potential of ducks and chickens to

Table 2. Degree of Severity in Various Organs of Indonesian Indigenous Chickens Infected by H5N1 Virus Clade 2.3.2.

Bird ID	Lesion Severity													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	++	++	+	++	+	++	++	+	++	+++	+	+++	++	+++
2	-	+++	+	++	+	+	++	++	++	++	+++	++	++	+++
115	+	+	-	+	-	-	-	+++	++	++	++	-	++	+++
174	++	+	++	-	-	-	++	++	+	+++	-	++	++	-
181	++	-	-	-	-	-	++	-	+	-	+++	+	+++	-
191	++	+	+	+	-	-	+	+++	++	-	-	-	-	-
196	++	-	++	+	-	-	+	+	+	++	+	-	++	++

1: Brain, 2: Nasal Cavity, 3: Trachea, 4: Feather, 5: Thymus, 6: Heart, 7: Lung, 8: Proventiculus, 9: Liver, 10: Spleen, 11: Intestine, 12: Pancreas, 13: Kidney, 14: Bursa

- : No specific lesion (NSL), + : Mild lesion, ++ : Moderate lesion, +++ : Severe lesion

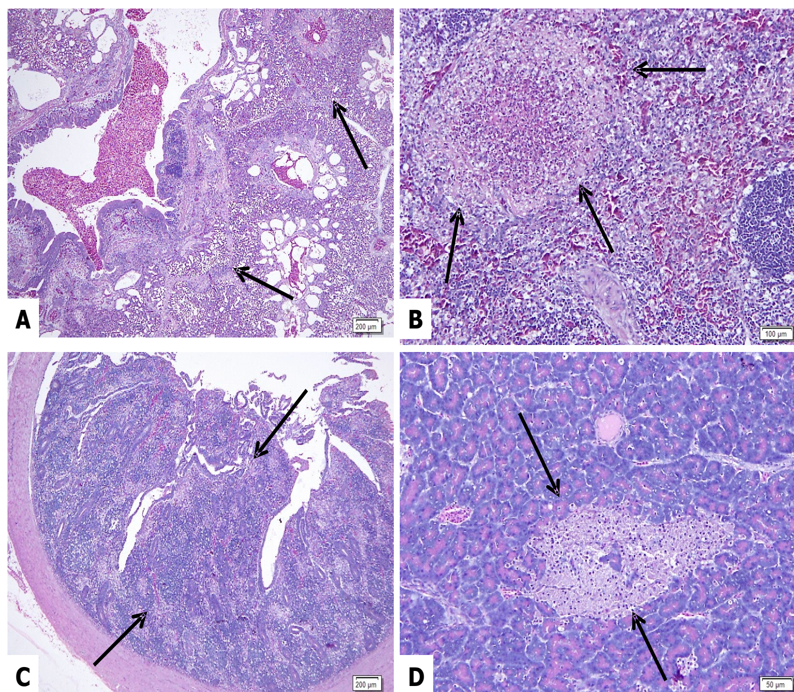

Figure 2. Interstitial Pneumonia of Lung (A); Focal Necrosis of Splenic Red Pulp (B); Enteritis (C) and Focal Necrosis of Pancreatic Islet (D)

spread H5N1 HPAI virus clade 2.3.2 to the environment. Other study showed that virus was able to be isolated from ducks following infection with clade 2.3.2 (Nemeth *et al.,* 2013).

Table 2 represents the severity degree of lesion in various organs of the chickens affected by H5N1 clade 2.3.2. The lesions had marked variation on the distribution and expression of HPAI among the tissues and avian species, as also reported by Costa *et al.* (2012). Microscopically there were multifocal degeneration to necrosis of the brain, hepatic cells, pancreatic islets, splenic

Table 3. Antigen Detection in Various Organs of Indonesian Indigenous Chickens by Immunohistochemistry

Bird ID	Antigen Detection in Various Visceral Organs													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	+++	-	+	++	+	+++	+++	++	+++	+++	++	+++	+++	+++
2	+++	++	+	+++	+++	+	+++	++	+++	+++	++	+++	+++	+++
115	+++	+	-	+++	-	-	-	++	++	+++	++	++	+++	+++
174	+++	++	++	-	-	+++	+++	++	++	+++	-	+++	+++	-
181	++	+	+	-	-	++	-	-	++	-	+++	++	++	-
191	+++	+	+	+++	-	+++	++	++	+++	+++	-	+++	+++	--
196	+++	++	++	+++	+++	+	+++	++	++	+++	+++	+++	+++	

1: Brain, 2: Nasal Cavity, 3: Trachea, 4: Feather, 5: Thymus, 6: Heart, 7: Lung, 8: Proventiculus, 9: Liver, 10: Spleen, 11: Intestine, 12: Pancreas, 13: Kidney, 14: Bursa

- : No antigen, + : Few (1-5 antigen/field), ++ : Moderate (6-10 antigens/field), +++ : Enormous (more than 10 antigens/field)

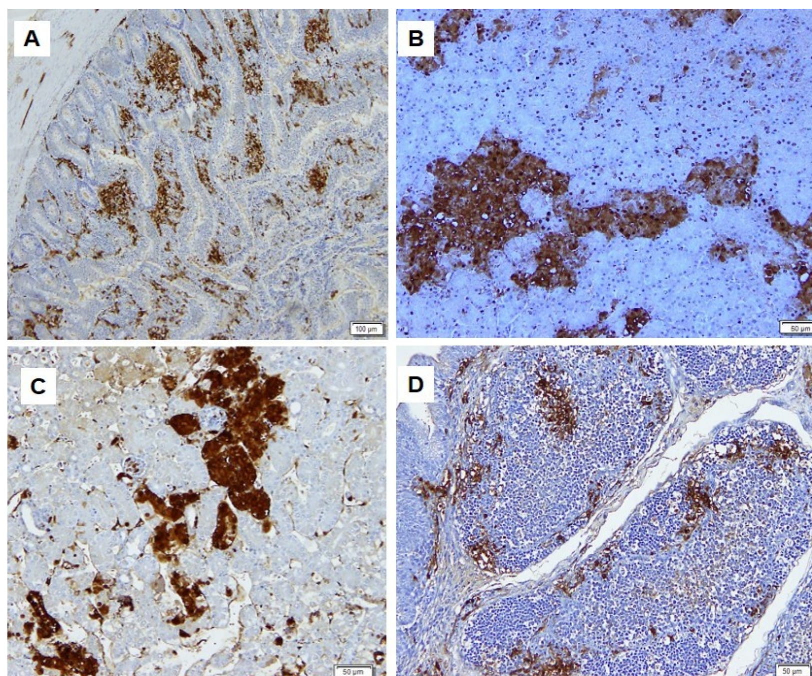

Figure 3. Antigen distribution in intestine (A), pancreas (B), kidney (C) and bursa Fabricius (D). Immunohistochemistry, DAB

red pulps, tubular area of kidneys and follicular area of bursa fabricius. Mononuclear cellular infiltration was observed in the interstitial brain tissue, tracheal mucosa, interstitial lung, heart, liver, proventriculus, intestine, pancreas and kidney. The type of lesion was classified as non suppurative. Gliosis and demyelination were also found in the brain. Necrotizing spleen and pancreas were also prominent. Figure 2 presents some of the lesions. This findings were previously reported in the first HPAI outbreak in Indonesia (Damayanti, *et al.,* 2004b) and other countries (Bröjer *et al.,* 2015).

Antigen of H5N1 was detected in high intensity in various organs: skin, feather structure and feather follicle, infra orbital and intra nasal sinuses, trachea, brain, thymus, heart, lung, liver, spleen, intestine, pancreas, kidney and bursa fabricius as shown in Table 3. This finding reflected severe clinical signs and pathogenicity of the virus. Similar results were reported by Bröjer *et al.* (2009) whereby found in visceral organs associated with viral detection in the liver, lung, adrenal glands, kidneys, and peripheral nerve ganglia by immunohistochemistry. The viral antigen in feather structure and follicle is similar to previous studies of duck and chicken infected with H5N1 HPAI virus (Nuradji *et al.,* 2015, Nuradji *et al.,* 2016, Yamamoto *et al.,* 2007).

CONCLUSION

The study shows that Indonesia H5N1 HPAI virus clade 2.3.2 is highly pathogenic for Indonesian indigenous chickens and ducks. The infected ducks can trasmit the virus to Indonesian indigenous chickens easily and generated high mortality. This suggest that ducks should not be reared with Indonesian indigenous chickens to prevent the spread of virus.

ACKNOWLEDGMENTS

This study was supported by The Indonesian Agency for Agriculture Research and Development and Indonesian Research Center for Veterinary Science. We thank Kusmaedi, Agus Winarsongko, Ani Purwani, Zulkifli, Abdul Qodir, Murniati and Yudi Mulyadi for technical assistance throughout the project.

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