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Penelitian

Seroprevalence and Detection of H5N1 Avian Influenza Virus in Local Chickens in Tabanan Regency, Bali, Indonesia

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ABSTRACT

Avian Influenza (AI) is a zoonotic disease that causes death in poultry and humans. Monitoring the virus needs to be carried out continuously to prevent outbreaks of the disease. Seroprevalence and detection of H5N1 and H9N2 AI virus antigen were intended to monitor the presence of viruses in local chickens in Tabanan, a Regency of the Indonesian island Province of Bali. The research aims were to detect the presence of H5N1 AI virus, and measure the seroprevalence of this virus in Tabanan Regency of Bali Province. Research located in six districts of Tabanan regency namely Baturiti, Penebel, Marga, Kediri, Tabanan and Kerambitan. A total of 1,398 local chickens that never been vaccinated with AI were randomly sampled in this study. The samples collected were serum, cloacal and tracheal swabs. Serum samples were tested with hemagglutination inhibition (HI) assay. While samples of cloacal and tracheal swabs were isolated in 9-day-old germinated chicken eggs, followed by hemagglutination assay and RT-PCR test using H5N1 primer. AI seroprevalence in local chickens in Tabanan Regency was 1% with the distribution in each district as follows; Penebel 1.6%, Kerambitan 1.2%, Marga 1%, while Tabanan, Kediri, and Baturiti 0.7% each. H5N1 AI virus was detected in 11 samples, i.e. five in Marga district and three in Penebel district, two in Kediri, and one in Tabanan, while the H9N2 AI virus was not detected. These results indicate that H5N1 AI virus may still circulate in local chickens in Tabanan Regency of Bali Province, with 1% of prevalence.

Keywords: Avian influenza, Bali, H5N1, local chicken, seroprevalence

ABSTRAK

Avian Influenza (AI) adalah penyakit zoonosis yang dapat menyebabkan kematian baik pada unggas maupun manusia. Pengawasan virus ini harus terus dilakukan secara berkelanjutan untuk mencegah terjadinya wabah penyakit AI. Seroprevalensi dan deteksi antigen virus AI H5N1 dan H9N2 ditujukan untuk memonitor keberadaan virus pada ayam kampung di Kabupaten Tabanan, salah satu wilayah di Pulau Bali, Indonesia. Tujuan dari penelitian ini adalah untuk mengukur seroprevalensi serta mendeteksi antigen virus di Kabupaten Tabanan Provinsi Bali. Lokasi penelitian dilakukan di enam Kecamatan yang terdapat di Kabupaten Tabanan, yaitu Baturiti, Penebel, Marga, Kediri, Tabanan dan Kerambitan. Sebanyak 1.398 sampel ayam kampung yang belum pernah mendapatkan vaksinasi AI dikumpulkan secara acak pada penelitian ini. Sampel yang diambil berupa serum, swab kloaka dan trakea. Sampel serum diuji menggunakan metode hambatan hemaglutinasi (HI). Sementara, sampel swab diinokulasi pada telur ayam bertunas (usia 9 hari), selanjutnya diuji hemaglutinasi, dan sampel positif dilakukan pemeriksaan RT-PCR dengan primer H5N1. Seroprevalensi AI pada ayam kampung di Kabupaten Tabanan diketahui sebanyak 1% yang terdistribusi keseluruh Kecamatan yaitu masing-masing Penebel 1.6%, Kerambitan 1.2%, Marga 1%, while Tabanan, Kediri, dan Baturiti 0.7%. Virus H5N1 juga terdeteksi pada sebanyak 11 sampel di empat Kecamatan, yaitu, lima di Marga, tiga di Penebel, dua di Kediri, dan satu di Tabanan. Sementara antigen H9N2 tidak ditemukan. Hasil ini mengindikasikan bahwa virus AI H5N1 masih bersirkulasi pada ayam kampung di Kabupaten Tabanan, Provinsi Bali dengan prevalensi 1%.

Kata kunci : Avian influenza, Bali, H5N1, ayam kampung, seroprevalensi

INTRODUCTION

Long-term and ongoing monitoring of avian influenza (AI) viruses is needed (Jonas *et al.,* 2018; Li *et al.,* 2004; Machalaba *et al.,* 2015). Since 2002, the AI virus has spread to almost all parts of the world (Alexander, 2007; Chen *et al.*, 2005; Ellis *et al.,* 2004; Pantin-Jackwood and Swayne, 2007; Sturm-Ramirez *et al.,* 2004) including Indonesia (Kandun *et al.,* 2006). AI viruses are known to be endemic in some wild birds and infect domestic birds (Capua and Alexander, 2006). In Indonesia, this disease is classified as one of some infectious diseases in animals that is prioritized to be controlled (Santhia *et al.,* 2009).

The H5N1of AI virus epidemic in Indonesia began on Java in August 2003 which attacked domestic and commercial chickens (Santhia *et al.,* 2009; Wiyono *et al.,* 2004). Then the outbreak occurred in the Province of Bali began in October 2003 (Santhia *et al.,* 2009). This case was first reported in Karangasem Regency, which was allegedly due to the entry of sick birds from Java. The same outbreak also occurred in Tabanan Regency and then spread rapidly to other districts in the island (Santhia *et al.,* 2009).

From October 2003 to September 2004, the highest percentage of AI infected villages in Bali was found in Bangli and Jembrana districts at 39.1% and 29.4% respectively. While the average outbreak rate was 20.4%, where the highest in Tabanan and Karangasem districts at 48.4% and 30.2% respectively (Santhia and Putra, 2004). More detail, Mahardika *et al.* (2018) reported that all districts in Bali Province have been infected with AI H5N1. AI antibodies as an indication of poultry have been infected with AI viruses detected from local chickens, ducks, thugs, geese and pigeons. It was also reported that seroprevalence of H5N1 AI virus infection in each district in Bali varied from 1.23% to 6.09% with the proportion of seroprevalence in local chickens (2.69%), waterfowl (9%), and various other poultry (8.06%).

AI outbreaks in Tabanan Regency caused fatalities and economic losses. In 2007, a resident who worked as a collector of poultry from Kediri District died because of AI infection (Lestari, 2009). In addition, due to the AI outbreak in 2012 hundreds of chickens died suddenly in Marga Subdistrict (Karminiasih *et al.*, 2014). The AI-H5N1 virus is very detrimental, including the reduced number of breeders, decreased income of poultry farmers, decreased supply, import and export of DOC for both broiler and layer, and the price of input and output of the poultry business.

As a result of the AI outbreak in Indonesia since 2004 ± 2008 caused loss of Rp. 4.3 trillion, excluding losses from lost job opportunities and reduced public protein consumption. FAO estimated that there were AI virus mutations in Indonesia that may cause a pandemic (Basuno, 2008).

Various factors are known associated with the H5N1 AI virus to be sustained in Tabanan Regency, namely poultry trade traffic between regions, buying and selling of poultry in traditional markets, unhygienic processing of poultry meat and the habit of people throwing dead chicken carcasses into rivers (Suartha *et al.,* 2010). Seroprevalence and AI antigen detection study in local chickens in Tabanan are still limited, therefore the study of AI on household scale farms in the area is needed.

MATERIALS AND METHODS

Ethical Clearance

This research was approved by the Ethical Commission for the Use of Animals in Research and Education of the Faculty of Veterinary Medicine, Udayana University, Indonesia with Ref. No. 0034a/UN14.2.9/PD/2019.

Sample

The research samples were taken randomly from the Tabanan Regency of the Bali Province (Table 1). Sampling locations in six districts namely Kediri, Penebel, Baturiti, Marga, Kerambitan and Tabanan. Eight villages from each subdistrict were sampled, 3 sub-villages from each village, and from each sub-village 7-10 local chickens were sampled (Thrusfield, 2007). Samples consisted of serums, cloaca and tracheal swabs from the sampled chickens that were free to roam and had never vaccinated against AI virus (Sarker *et al.*, 2017). A total of 1,398 samples used in this research.

The sampling location was determined based on the purposive sampling method. Meanwhile, in taking the sample used the Stratified Random Sampling method based on the data from reported by the Bali Livestock Service stated that the most cases of avian influenza occurred in Tabanan Regency, as many as 34 sub-village in 29 villages had contracted the virus (Lestari, 2009). Nine villages were selected in each district, and every village selected 3 sub-villages. Each sub-village sampled 10 chickens from the residents. The residents who being sampled must have more than 10 chickens which have not AI vaccinated record and the chickens were allowed to free roam. Three chicken were sampled in

Table 1 Sample distribution in Tabanan Regency

No	District name	Number of villages	Number of sub-villages	Number of samples per sub-village	Total sample
1	Penebel	8	3	10	240
2	Baturiti	6	4	12	288
3	Marga	8	3	12	288
4	Kediri	9	3	10	270
5	Tabanan	6	3	8	144
6	Kerambitan	8	3	7	168
				Total sample	1,398

each resident. Sampling was considering the age of the chickens, which was above 3 months to eliminate the influence of maternal antibodies but ignoring gender, and body weight.

Sample Collection

All samples were taken according to the FAO procedure (FAO, 2014). Blood was drawn through the brachial vein using a 3 ml syringe. The serum was collected by centrifugation at a speed of 10,000 rpm for 5 minutes. The serum samples were stored at -18C before being tested using Hemagglutination inhibition assay (HI) (Pedersen, 2014). The cloaca and trachea swabs were taken using cotton swabs and directly inserted into the transport media (which contains PBS + Penicillin and Streptomycin). The suspension of the cloacal and tracheal swabs was then made up to 10% inoculum, 5000 IU of Penicillin and 5 µg/ml of Streptomycin added. 0.1 ml of inoculum of each sample was isolated in 10-day-old hatched chicken eggs through the allantois chamber. The eggs were then incubated for 3 days at 37°C, and were observed every day.

All dead eggs were removed from the incubator, then put them into the 4°C refrigerator overnight. Allantois fluid was harvested for tested by a Hemagglutination assay (HA) (Killian, 2014) and confirmed by a molecular test using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (FAO, 2014; OIE, 2008).

DNA Isolation

DNA isolation process was done according to the Qiagen® DNeasy KIT. A total of 25 ml samples of each dead egg was extracted.

Antigen and Primers

Antigen and standard serum for H5N1 and H9N2 AI viruses used in this research were originated from Pusvetma Surabaya. While the primers sequence used were H5-1B: 5'-GCCATTCCACAACATACACCC-3', H5-3B: 5'-CTCCCCTGCTCATTGCTATG-3', N1-Fwd: 5'-TAGACTGCATGAGGCCTTGCTTCTG-3', and N1-Rev: 5'-CACCGTCTGGCCAAGACCAACCTA-3'(Network, 2005).

Polymerase Chain Reaction

The PCR mixture consisted of 2,5 µl (each) deoxynucleoside triphosphates (8 µM), 2,5 µl 10 X PCR buffer, 2.0 µl (1.5 mM) $MgCl_2$, 0,125 µl PE Amplitaq (5unit/ µl), 1,215 µl of forward and reverse primers (10 mM), 1 µl DNA sample and 14.5 ul dH_2O in a total 25 µl reaction mixture.

Amplification was performed with predenaturation condition at 95°C for seven minutes, followed by 39 cycles with the following reaction conditions: dena-turation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, and polymerization at 72°C for one minute. At the end of the polymerase was added at 72°C for seven minutes (Mahardika *et al.*, 2018). All PCR product run in the gel electrophoresis to find positive sample with 200 bp result.

Data Analysis

Data of serology and antigen test results were calculated statistically with SPSS versions 13 using the crosstabulation method, and continued with the Chi Square test (Arkkelin, 2014).

RESULTS AND DISCUSSION

Analysis of hemagglutination inhibition assay results on 1,398 samples of local chicken serums against the AI virus in Tabanan Regency is presented in Table 2. Almost all sampled regions in Tabanan Regency detected antibodies against AI virus in the chickens, and Penebel.

The latest research on AI seroprevalence in Bali found that the seroprevalence of AI virus infection in local chickens in Tabanan Regency was 1.79% averaged from the seroprevalence in Marga District 11.63%, and Penebel 1.59%, while other districts were negative (Mahardika, 2005). The span of nearly 13 years proves that until now Tabanan Regency is still suspected of having AI virus cycle, although the percentage of seroprevalence is lower than in 2005. The decrease in seroprevalence is probably due to the socialization from the government on prevention of AI in poultry to the public through a good vaccination and biosecurity program. Public support in the form of awareness for a good poultry raising system is also believed to contribute to the decrease of the infection, as its implemented by the Government in Turkey (Edirne *et al.*, 2011).

Even if the seroprevalence is decreased, when it compared to the previous data (Mahardika, 2005), the AI virus is still suspected spreading out in Tabanan Regency. This is presumably because local free roam chickens can easily come in contact with wild birds, and contaminated their feed by feces or secretions that may contain the virus (Spackman, 2009). Elfidasari *et al.* (2015) stated that chickens around the Serang Nature Reserve area of Banten were infected with AI virus due to the drinking water in the area was also consumed by wild water birds which may shed the virus in the water.

Pfeiffer *et al.* (2011) argues that the high frequency of AI virus transmission in several East and Southeast Asian countries is due to the high density of terrestrial and waterfowl populations supported by commercialalivestock breeding and trade in poultry, which triggers antigenic drift. The presence of poultry slaughtering facilities in Marga subdistrict was suspected to be an important factor of the spread and propagation of the AI virus. Slaughterhouse owners tend to combine various types of poultry in one place (Suartha *et al.,* 2010). While the slaughter process was also carried out without good biosecurity (Lohiniva *et al.,* 2013). The spread of AI viruses tends to increase during the rainy season due to the migration of wild birds that occur in July to November (Halvorson *et al.*, 1985).

Local chicken antibody titers against AI viruses detected in this study were classified as low (2^2-2^4 HI Unit) and its serologically unprotected against the virus. This can be caused by local chickens that never been vaccinated against AI which lead to the presence of very low antibody titers. Alternatively, it is likely due to natural infections from AI-contaminated environments. In this condition, the chickens will be susceptible to a virulent AI virus, with mortality can be reached up to 100% (Swayne and Suarez, 2000). This antibody variation titers can be influenced by several conditions including the health of chickens, the type and amount of virus that infects, as well as the difference period or the phase of infection when blood samples are taken (Darmawi *et al.,* 2012).

From the swab samples tested for hemagglutination, we found the highest AI seroprevalence was in Marga subdistrict at 2%, Penebel and Tabanan was the same at 1% and other districts were negative (Table 3).

Table 2 Tabulation of seroprevalence antibodies against AI virus based on hemagglutination inhibition assay results from local chicken serums in Tabanan Regency

Sample origin		HI test results				Total
		Positive	%	Negative	%	
District	Penebel	4	1.6	236	98.4	240
	Tabanan	1	0.7	143	99.3	144
	Kediri	2	0.7	268	99.3	270
	Baturiti	2	0.7	286	99.3	288
	Marga	3	1.0	285	99.0	288
	Kerambitan	2	1.2	166	98.8	168
Total		14	1	1,384	99	1,398

Table 3 The seroprevalence antibodies against H5N1 AI virus in local chickens in Tabanan regency based on hemagglutination assay

Sample origin		HA test results				Total
		Positive	%	Negative	%	
District	Penebel	3	1	237	99	240
	Tabanan	1	1	143	99	144
	Kediri	2	0.74	270	100	270
	Baturiti	0	0	288	100	288
	Marga	5	2	283	98	288
	Kerambitan	0	0	168	100	168
Total		11	0.79	1,389	99.4	1,398

Table 4 RT-PCR results for AI virus in local chicken samples in Tabanan Regency

Sample origin		Number of samples	AI-H5N1 (+)	AI-H9N2 (+)
District	Penebel	240	3	0
	Kediri	270	2	0
	Marga	288	5	0
	Tabanan	144	1	0
	Baturiti	288	0	0
	Kerambitan	168	0	0
Total		1.398	11	0

We found that 11 samples were positive of the H5N1 virus based on RT-PCR test (Table 4). These results indicate that the virus spread in Tabanan and it may be in accordance with previous reports where the Avian Influenza H5N1 subtype virus is still circulating in traditional poultry markets and farms (Dharmayanti *et al.*, 2016; Hewajuli *et al.*, 2017; Mahardika *et al.*, 2018) in which it has been detected as AI virus subtype H5N1 clade 2.1.3 and clade 2.3.2 (Kusumastuti *et al.*, 2015). Identification of the Avian Influenza virus using RT-PCR is very important to be conducted to assess the genetic mutations of various viral genomes, especially for them that can cause an annual epidemic or even occasional pandemic (Shao *et al.*, 2017). In addition, the effect of the virus mutation to the pathogenic avian influenza can result in economic losses due to high morbidity and mortality in both poultry and humans (El-Shesheny *et al.*, 2014).

Seroprevalence of AI virus in local chickens in Tabanan found at 1% spread out in all subdistricts in the regency. As much as 11/1,398 of H5N1 avian influenza virus in Tabanan regency observed where it spread out in the four districts, while no sample was detected for H9N2.

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"The authors declare that they have not competing interests".

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