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RESEARCH ARTICLE

Surveillance of Avian Influenza Virus of H5N1 Subtype in Backyard Animals and Its Introduction in Bali, Indonesia

GN Mahardika*, AAAM Adi, NK Besung, NS Dharmawan, GAY Kencana, ALT Rompis, P Sampurna, LE Setiasih, W Suardana, IBK Suardana, GK Suarjana, N Suartha, GAA Suartini, NK Suwiti and IH Utama

The Animal Biomedical and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University, Jl. PB Sudirman, Denpasar 80225, Bali, Indonesia
*Corresponding author: gnmahardika@unud.ac.id

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ABSTRACT

Epidemiological studies of the highly pathogenic avian influenza virus H5N1 (HPAIV-H5N1) in pet and backyard animals are limited. Here we provide serological and virological evidences of infection in various animals in households in Bali, Indonesia in 2005 and 2006. Serum and swab samples from poultry, pigs, dogs, and cats were collected using a stratified random sampling design. Antibodies against HPAIV-H5 were detected in sera using the standard hemagglutination inhibition assay, and the presence of HPAIV-H5N1 in swabs was confirmed by using egg inoculation technique, a hemagglutination assay, and molecular methods. The phylogeny and virus dispersal were inferred using BEAST and SPREAD software. The results showed that the seroprevalence of village chickens to waterfowl, poultry to pigs, and year of study varied significantly in the province ($P<0.001$). The seroprevalences in dogs and cats were 1.85% and 7.50%, respectively. Moreover, HPAIV-H5N1 was isolated in all species except cats. The isolation rates varied between species and between the years of surveillance, too. Virus dispersal analysis showed that isolates from Bali grouped into two major clades with good statistical support. In light of these findings, surveillance of HPAIV should be extended to all poultry and mammalian species present in backyard environment.

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INTRODUCTION

Following pan-zoonotic spread since early 2000, highly pathogenic avian influenza virus of subtype H5N1 (HPAIV-H5N1) has become enzootic in various countries including Indonesia (Daniels *et al*., 2013). Although it cannot be efficiently transmitted from birds to humans, or from human to human (Guan and Smith, 2013), this long-term endemicity is a continuous threat for the poultry industry as well as human health. As available at The World Health Organization website (www.who.int) on Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2015, this influenza virus subtype is also responsible for human fatalities, with Indonesia having the highest fatality rate in the world in this regard. Human infection is believed to result from transmission of virus from infected poultry (Harfoot and Webby, 2017). Although few human clusters have been reported in various countries, the data still indicate that the virus is more efficiently to be transmitted from bird to bird than bird to human and human-to-human transmission.

The main challenge for controlling HPAI in developing countries is the animal production system, which mostly involves backyard farming (FAO, 2011). Up to 80% of the poultry in Africa and Asia is kept in backyard-type systems. In such settings, various poultry species are roaming freely and animals feed mostly by scavenging or eating household leftovers. As a consequence, biosecurity is at its minimum level and vaccination is almost impossible (FAO, 2011; Conan *et al*., 2012). Besides poultry, household mammals such as pigs, dogs, and cats, which are also susceptible to HPAIV-H5N1, as reported by natural cases and experimental

infections (Sims and Narrod, 2008). This so-called sector 4 in poultry production (FAO, 2013) might contribute less in viral evolution compare to sector 1, 2, and 3 facilities. Intensive poultry production is a huge industry worldwide and will play a critical role in the spread and enhancement of the pathogenicity of HPAIVs (Olsen *et al*., 2006). An intensive poultry farm with a high degree of genetic uniformity between birds provides the optimum conditions for viral mutation and transmission.

The report on epidemiological pattern of HPAI-H5N1 infection in birds and other contact animal in backyard setting is not conducted widely, while the role of commercial poultry has been published elsewhere. By nature, animal density at this setting will be much lower than in the industry, and therefore it poses less threat for the transmission of the virus (Alders *et al*., 2014). However, backyard settings provide an opportunity for the virus to silently perpetuate, then potentially transfer to livestock or infect humans. Backyard systems therefore represent an ecological niche (Hogerwerf *et al*., 2010) for the persistence of the virus in the environment.

Here we report intensive sero-epidemiological and virological surveillance data for HPAI-H5N1 in the post-epizootic and early enzootic phases, from a densely populated area in Bali, Indonesia. Our study focused on backyard settings, sampling backyard birds, pigs, dogs, and cats. The dissemination of HPAIV-H5N1 within Bali, and into and out of Bali, was also reconstructed.

MATERIALS AND METHODS

Virus and antibody standards: Standard virus HPAI H5N1 and chicken anti-H5 antibody were provided by the Veterinary Research Institute Bogor (Bogor, Indonesia).

Oligonucleotide primers: Standard primer sets for detecting the matrix, H5 and N1 gene fragments were employed (WHO, 2002, 2005). For sequencing the complete hemagglutinin (HA) and neuraminidase (NA) genes of the selected isolates, previously published primer sets (Salzberg *et al*., 2007) along with M13-primer linkers were employed.

Study design and population target: The study was designed as a cross sectional active surveillance. Samples were collected from sub-districts in 2005 and 2006. Of the total 57 sub-districts throughout the province, 50 percent were randomly selected. The total number of selected sub-districts was 29. Villages were further sampled which the minimum number was 50% of the total villages in the sub-districts. In 2005, the main targets for the study were free-roaming village chickens and waterfowl in household backyards, whereas in 2006, the main targets were backyard pigs. Minimum sample size was 20 sera and 40 swabs per village, which was determined following standard epidemiological methods (Thrusfield, 2005) with an estimation of sero and virus-prevalence was 5% and 2.5%, respectively, with 95% confidence limit.

Sample treatment: Samples comprised serum and cloaca swabs from birds and serum and nasal/pharyngeal swabs from mammals. Serum samples were collected, transported and preserved following standard procedures (WHO, 2002).

Anti-H5 antibody detection: All sera were diluted five times with phosphate-buffered saline (PBS), heated at 56°C for 30 minutes, and adsorbed 1:1 with 0.5% purified chicken red blood cells. Therefore, the starting dilution of the sera was 10 times. All mammalian-positive sera were further treated with receptor destroying enzymes (University of Hong Kong) according to a standard protocol (WHO, 2002). A hemagglutination inhibition (HI) assay was performed according to a standard protocol (WHO, 2002).

Virus isolation: Five swab samples were pooled and clarified by centrifugation at 1000 *g* for 5 minutes. The supernatant was then diluted 10 times with PBS containing antibiotics and an antifungal agent. To reduce the risk of laboratory contamination to the laboratory workers, every four pools from a household were re-pooled. All procedures using potentially active virus were conducted in restricted areas in a biosafety cabinet with class III laminar flow. Each pooled sample was inoculated into the allantois cavity of two specific-pathogen-free (SPF) eggs. The eggs were incubated at 39°C and harvested when the embryo showed decreased activity or on the third day after inoculation. Allantois fluid was harvested using a syringe without opening the eggshell to avoid contamination. All eggs were dipped in a high concentration of calcium chloride solution and then burned. Upon positive virus detection, every sample in the respective pool was individually tested.

RNA isolation: Total RNA was isolated from the allantois fluid of eggs using proteinase K digestion followed by Trizol (Invitrogen, Carlsbad, CA, USA) extraction according to the manufacturer's guidelines.

Reverse transcriptase polymerase chain reaction (RT-PCR): RT-PCR was conducted using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA polymerase (Invitrogen) following manufacturer's guideline.

Sequencing: The RT-PCR products were purified with the QIA quick PCR Purification Kit (Qiagen GmbH, D-40724, Hilden, Germany). The purified products were sequenced directly using the same primers used for RT-PCR, or M13 primer when appropriate. A cycle sequencing reaction was performed with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) at the Eijkman Institute (Jakarta, Indonesia). The sequence data were aligned using the Clustal W program in the MEGA6 software (Tamura *et al*., 2013) and H5 and N1 sequence identification was confirmed using BLAST analysis.

Statistical and phylogenetic analysis: Statistical analysis was conducted on the seroprevalence data from birds and pigs using Statistical Product and Service Solution (SPSS) Ver. 22 Software. Pearson's chi-square test was conducted to evaluate the significance different of seroprevalence between species, districts and the year of surveillance. The seroprevalence of dogs and cats, and the virus isolation rate, were not statistically analyzed, as the samples were not distributed from all districts.

The sequence data obtained in this study were aligned with the data for HPAIV-H5N1 isolated from Indonesian poultry up to 2006 that are available in the GenBank database. The HPAIV Goose/Guangdong/1996 was co-analyzed as the origin of HPAIV-H5N1 (Wan, 2012). The list and origin of GenBank secondary sequence data is available upon inquiries. The maximum clade credibility and discrete location annotated phylogenies were inferred using BEAST software package (Drummond *et al*., 2012) with the BEAGLE library (Ayres *et al*., 2012). The best model selection and tree estimation validity check were conducted using the Tracer program (http://beast.bio.ed.ac.uk/Tracer). A Keyhole Markup Language file from discrete phylogeny and visualization in Google Earth were generated using SPREAD software (http://www.phylogeography.org/SPREAD.html).

RESULTS

The number of serum samples based on poultry type and household mammalian species, district, and year of study, as well as the result of statistical analysis of the seroprevalence are summarized in Table 1. The results indicated that the overall seroprevalence based on poultry type (village chickens and waterfowl) and poultry to pigs varied significantly in the province (P<0.001). Moreover, seroprevalence variations were observed between species and between the years of study for all districts. The rate of seroprevalence in dogs and cats were 1.85 and 7.50%, respectively.

The summary of virus isolation rates of HPAIV-H5N1 from backyard birds and various animals in all districts of Bali, Indonesia, in 2005 and 2006 are presented in table 2. The results confirmed that HPAIV-H5N1 was isolated in all species except cats. The isolation rates varied between species and between the years of surveillance. The number of confirmed isolates and the isolation rates were 3 (0.007%) and 8 (1.25%) for village chickens in 2005 and 2006 respectively, compared with 7 (0.76%) and 7 (2.24%) for waterfowl in the same years. The number of isolates from pigs and dogs was 10 and three, with isolation rates of 0.56 and 2.56%, respectively. The pigs positive for HPAIV-H5N1 were from six out of nine districts tested, whereas the dogs were from two out of seven districts. None of the 41 cat swabs tested were found to be positive for HPAIV-H5N1.

All of the confirmed sequences obtained in this study have been deposited in the GenBank database. The GenBank Accession numbers of the sequences are DQ644955, DQ644959, DQ644957, EU812564-EU812566 and KR987691-KR987722. Only sequences that covered the HA-1 fragment are included in the phylogenetic reconstruction. The phylogenetic reconstruction of the HA-1 fragment of HPAIV-H5N1, together with avian Indonesian isolates sampled from 2003-2006 is presented in Fig. 1. The results showed that isolates from Bali grouped into two major clades with good statistical support (posterior probability values of 1). The bigger clade was further splitting into some sub-clades with very week support (posterior probability <0.50). The sequences of Bali's viruses are located in three sub-clades of this group. Estimations of virus dispersal from other provinces to various districts in Bali (Fig. 2 & 3) indicated that HPAI-H5N1 has been introduced multiple times into Bali from other provinces in Indonesia (West Java and East Java), and they have been spread from Bali to other provinces. Inter-district dispersal dispersals have occurred. Fig. 2 and 3 also provides geographic location of provinces in Indonesia as well as districts in Bali.

Table 1: Summary of anti-H5 antibody testing and statistical analysis of seroprevalence between districts and animal as well as year of study in the whole province in Bali, Indonesia, in 2005 and 2006

Year	Animal	Number of positive samples/Total samples (Prevalence %)	P value*
2005	Chicken	110/2266 (4.85)	<0.001
	Waterfowl	70/717 (9.76)	0.008
2006	Chicken	35/619 (5.65)	0.004
	Waterfowl	37/287 (12.89)	0.036
	Pig	11/1786 (0.62)	0.271
	Dog	2/108 (1.85)	NA
	Cat	3/40 (7.50)	NA
P value village chicken and waterfowl 2005			<0.001
P value village chicken and waterfowl 2006			<0.001
P value 2005 and 2006 (total village chicken and waterfowl)			<0.001
P value pig vs chicken+ water fowl 2006			<0.001

Note: The values for Chi-square analysis between animal species for each district are shown. *p value of Chi-square analysis of the corresponding animal species between districts. NA=Not Applicable

Table 2: Summary of virus isolation rate of HPAIV H5N1 in backyard birds and various animals in all districts in Bali, Indonesia (2005-2006)

Year	Animal	Number of positive samples/ Total samples (prevalence %)
2005	Village chicken	3/4218 (0.07)
	Waterfowl	7/916 (0.76)
2006	Village chicken	8/638 (1.25)
	Waterfowl	7/312 (2.24)
	Pig	10/1772 (0.56)
	Dog	3/117 (2.56)
	Cat	0/41 (0.00)

DISCUSSION

Backyard farming is a major part of the poultry industry, especially in developing countries in Asia and Africa. Furthermore, this poultry raising system has become a social security system, with each household owning tens of mixed free-roaming poultry. In non-Muslim societies, people also keep pigs and have free-roaming pet animals, mostly dogs and cats. Adult pigs are usually kept in a simple stall or leashed under a tree or house canopy. Households also often keep various pet birds. This type of environment allows for direct contact between people, poultry, pigs, and pet animals.

The findings of our study provide evidence that various backyard animals have contracted HPAIV-H5N1. The anti-H5 antibody and HPAIV-H5N1 were detected in a range of animals, with the exception of cats. The serological prevalence in waterfowl was significantly higher than in village chickens. The anti-H5 prevalence in pigs was significantly lower than in poultry in 2006. The virus isolation rates in chickens and waterfowl in 2006 were higher than in 2005. All samples that were anti-H5 positive (268 samples) were HPAIV-H5N1 negative. (Data not shown).

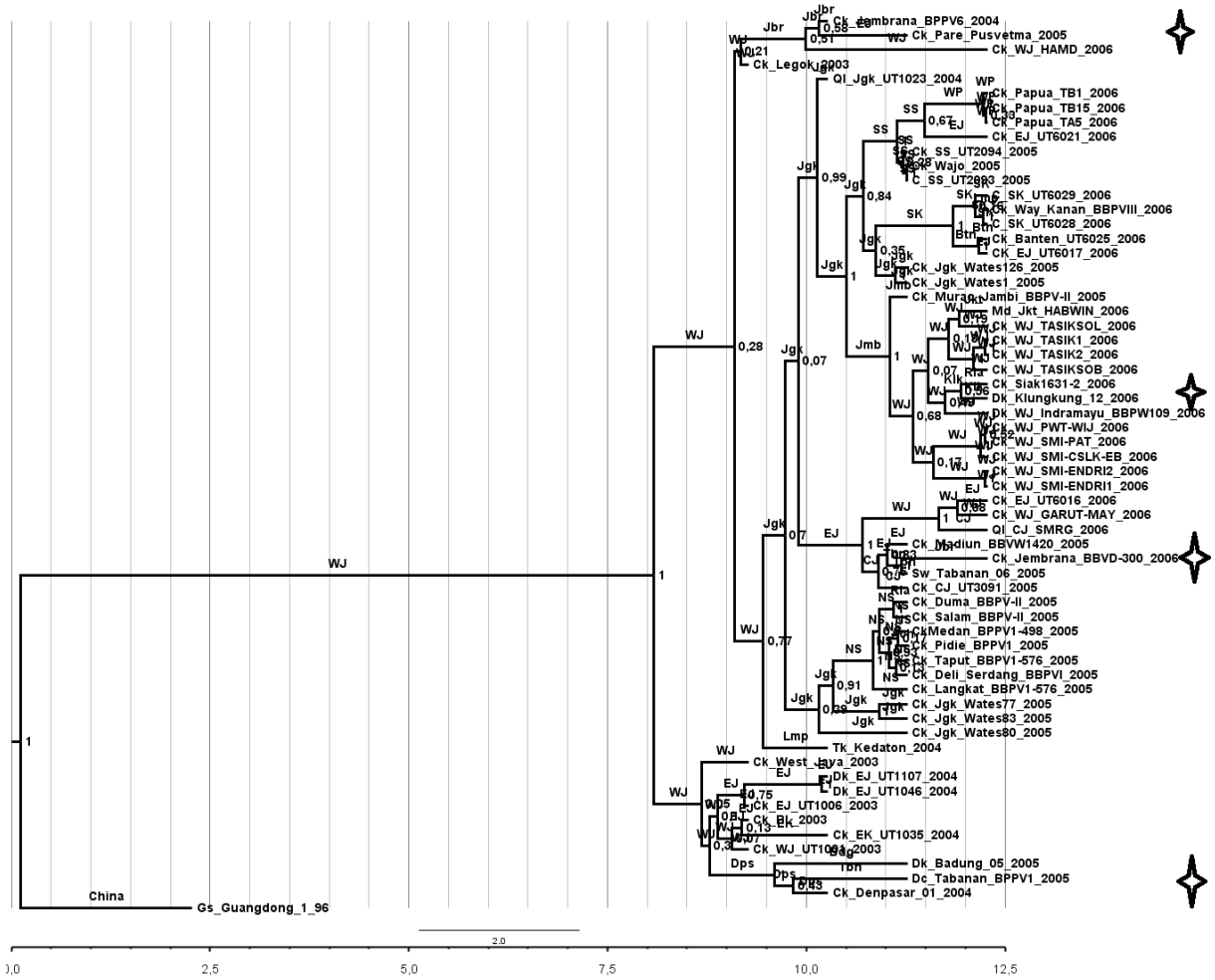

Fig. 1: The location-annotated-maximum clade credibility (MCC) phylogeny of HA-1 of HPAIV-H5N1 sequences from isolates sampled in Indonesia from 2003–2006 available in the GenBank database or reported in this study. Goose/Guandong/1996 as the origin of HPAIV-H5N1 (Wan, 2012) was used as the tree root. Branch's posterior probability values are shown. The highest location probabilities are shown above of the branches and the posterior probability values are shown next to the node. The province and Bali's district names are abbreviated: Ach (Nanggroe Aceh Darusalam), NS (North Sumatra), Jmb (Jambi), Ria (Riau), Lmp (Lampung), Btn (Banten), Jkt (Jakarta), WJ (West Java), CJ (East Java), EJ (East Java), Jgj (Jogjakarta), SK (South Kalimantan), EK (East Kalimantan), SS (South Sulawesi), and WP (West Papua).

The low isolation rate reported in this study is in agreement with similar surveillance performed in China around the same time. In the Chinese study, the virus isolation rate in 2006 was 2.4%, with the highest prevalence in geese and ducks, while native chickens showed lower rates (Smith *et al.*, 2006). A study in backyard pigs and poultry in Cambodia (Osbjer *et al.*, 2017) also found low AIV isolation rate in pig, chicken and duck. The HPAIV-H5N1 was negative.

Virus prevalence patterns were dynamic for every species, geographical area, and sampling time tested. Our findings showed that overall seroprevalence rates between poultry types, and poultry and pigs, varied in Bali (Table 1). Moreover, we detected seroprevalence dynamics between species and the year of study in all districts. This should be taken into account when conducting longitudinal surveillance since dynamicity will be encountered owing to animal movement, animal age, and adaptability of the agent.

Sampling strategy, number of samples, and time of sampling all appeared to influence the sensitivity of the epidemiological study. A previous study on HPAIV-H5N1 seroprevalence in three districts of Bali reported a different picture (Santhia *et al.*, 2009). Whereas, positive sera were found both in village and market samples, and positive swabs were detected from the market only (Santhia *et al*., 2009). There was no information regarding whether the samples included broiler and layer chickens. Our study used sample from all districts in Bali rather than just three, and the sample size of our study was much higher, involving a total of 2800 and 4800 village chickens, 1000 and 1200 waterfowl, 1786 and 1772 pig sera and swabs, respectively. Sampling in live bird markets might not reflect backyard settings as there would be mixed species present, including commercial poultry.

The likelihood of a false positive result in our study was negligible, as specimen transport, testing, and interpretation were strictly controlled. Antibody to Newcastle disease virus (NDV) and the presence of NDV were co-tested in serum and swab specimens. The seroprevalence and isolation rates of NDV were much higher than HPAIV-H5N1, and most samples that were positive for Newcastle disease virus and antibody were negative for HPAIV-H5N1 (data not shown). These findings confirmed that specimen collection, preservation, and transport were conducted in an appropriate manner.

In the current study, the standard HI assay was employed. This assay is known to have limitations, and

the results of HI testing in mammalian species, including humans, should be confirmed by a microneutralization test (Nasreen *et al*., 2013). In our study, to limit any discrepancies, all of the sera were rigorously treated by dilution, red blood cell adsorption, pre-heating, and treatment with receptor destroying enzymes, to eliminate nonspecific natural inhibitors and natural agglutinins that may be present in the sample (WHO, 2002; FAO, 2014). Sera that were interpreted to be positive showed HI titers of more than 20 HI units. This cutoff is higher than the recommended value of greater than 16 HI units (FAO, 2014). Our method of choice was simple and safe, as it uses inactivated antigen, and has been widely used to conduct antigenic analysis and antigenic cartography for HPAIV-H5N1 and other influenza virus (Beato *et al*., 2013).

Our findings confirmed that the virus has been introduced into Bali multiple times from other provinces in the country. When all of the HPAIV-H5N1 sequences obtained in this study were analyzed along with the sequences of all Indonesian avian viruses dated up to 2006 available in the GenBank database, three distinct clusters were identified (posterior probability>0.92). Virus dispersal reconstruction using discrete Bayesian analysis, visualized using Google Earth (Figure 2), indicated that quadruple introductions into Bali had occurred and provided evidence of virus dispersal in Bali, as well as from Bali to East Java and Riau. Additionally, the results showed that the ancestor of clade 2.1 'arrived' in West Java, then soon after spread to other provinces throughout Indonesia.

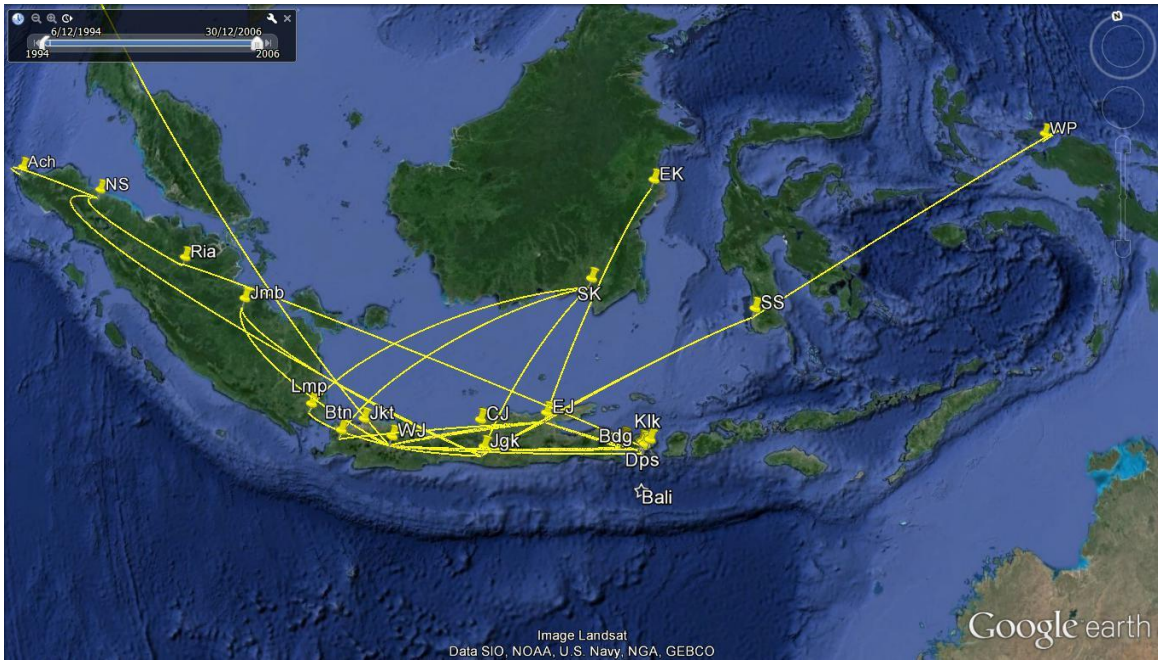

Fig. 2: Map of Indonesia and Bali Province showing HPAIV-H5N1 dispersal from 2003–2006 and the locations of provinces and districts. The geographic locations of the province capital city in Indonesia, as well as the capital cities of districts in Bali were used to draw the map. The province names are abbreviated as in Figure 1. The districts in Bali are abbreviated as Bdg, Bgl, Bll, Dps, Gia, Jbr, Kar and Klk for Badung, Bangli, Buleleng, Denpasar, Gianyar, Jembrana, Karangasem and Kelungkung, respectively.

Fig. 3: Close up of Fig. 2 showing HPAIV-H5N1 dispersal to, in, and from Bali. Abbreviations for districts in Bali are as in Fig. 1.

The geographic dispersal of HPAIV-H5N1 in Indonesia found in this analysis does not support a previous report that Indonesian HPAIV-H5N1 originated in East Java (Lam *et al*., 2012). Here, we analyzed the HA1 fragment instead of the whole HA gene, as this fragment is more polymorphic than HA2, harboring neutralizing antigenic sites, residues responsible for pathogenicity and adaptations to mammalian hosts (WHOGIPSN, 2005). The use of a different dataset and model might explain the discrepancy in results between the studies. To ensure the robustness of the analysis, the best tree model was selected after 150,000,000 runs and Bayes factor analyses applying various clock and substitution models (Li and Drummond, 2012). The other explanation seems to be the low sensitivity of HPAIV-H5N1 surveillance in Indonesia so that the data available do not represent all of the evolutionary events that affected HPAIV-H5N1. In the case of active surveillance of HPAIV-H5N1 in Egypt, the surveillance sensitivity in birds was found to be around 50% (Rabinowitz *et al*., 2012).

Our findings highlight the need for surveillance of HPAIV to be extended to household mammalian species, especially pigs, dogs, and cats. As pigs have long been regarded as a possible intermediate host ("mixing vessel") for the generation of pandemic influenza virus through reassortment (Harfoot and Webby, 2017), little is known about the potential role of domestic pets such as dogs and cats. The restricted susceptibility of these animals may allow for their use as animal models for understanding the biology and pathogenicity of HPAIV.

Conclusions: Various pet and backyard animals have contracted HPAIV-H5N1 in Bali, Indonesia, in 2005-2006. Sero- and virus-prevalence vary in species, district, and study period. The HPAIV-H5N1 has been introduced to Bali from other provinces in Indonesia in multiple events. In light of these findings, surveillance of HPAIV should be extended to all poultry and mammalian species present in backyard environment.

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Authors contribution: GNM, AAAMA and NSD conceived and designed the project. NKB, ALTR, LES, WS, GKS, and NKS collected field samples and data. GAYK, IBKS and GAAS conducted laboratory testing. NSD, PS, NS and IHU statistically analyzed the data. GNM, AAMA, GAYK, IHU conducted the phylogeography. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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