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Epidemiologie Van Hoogpathogeen Vogelgriepvirus H5N1 In Indonesisch Pluimvee

(with a summary in English / met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. AVIAN INFLUENZA

Avian Influenza is a highly transmissible disease caused by type A Influenza viruses of the family Orthomyxoviridae. Avian Influenza viruses are negative sense, single stranded RNA viruses, pleomorphic and enveloped. The viruses have eight segments of RNA that encode for 11 proteins: Polymerase A, B1 and B2; Haemagglutinin (HA), Nucleoprotein (NP); Neuraminidase (NA), Matrix (M1 and M2) and Non-Structural proteins (NS1 and NS2) [1-3]. Haemagglutinin is a major surface protein of avian influenza virus that manifests in 16 different subtypes in birds (H1-H16). This protein is responsible for viral binding and envelope fusion with the host cell. Viruses of the subtypes H5 and H7 can have low or high pathogenicity in poultry depending on the sequence of amino acids at cleavage site. Neuraminidase is the other surface protein used to subtype viruses (N1-9 in birds).

Low pathogenicity avian influenza (LPAI) is characterized by a mild clinical manifestation, and/or production losses [4-6]. Infection occurs locally e.g., in the gut and respiratory tract. In contrast, highly pathogenic avian influenza (HPAI) viruses can replicate systemically in the body causing very severe disease and high mortality in gallinaceous poultry. In other bird species the course of disease may be milder [7-10]. Water birds are considered the indigenous host to Influenza A viruses. Infections in poultry have their origin in water birds but are nowadays mostly the result of transmission among poultry. Knowledge of the evolution, phylodynamic, and reassortment of highly pathogenic avian influenza viruses will aid in understanding the transmission of highly pathogenic avian influenza viruses in Indonesian poultry.

2. POULTRY IN INDONESIA

With an average annual consumption of 8 kg per person, poultry is the most important source of animal protein in Indonesia. Broiler meat and native chicken meat are 53% and 10% of all meat production in Indonesia, respectively. [11, 12].

The Indonesian poultry industry comprises of both modern and traditional elements. Modern corporations function as integrators, have large farms of their own, and also engage smallholders in a contract farming system. Charoen Pokphand Indonesia and Japfa Comfeed, the two major integrators in Indonesia, dominate the market for Day Old Chicks and feed supplies for independent small and medium-sized farmers. [11]. In contrast, traditional poultry farming uses

traditional housing (bamboo, slatted floors, natural ventilation) with one or more homes containing a limited number of birds [11]. Traditional poultry also includes backyard, indigenous, and small scale semi intensive poultry raised by a family or household in rural or peri-urban areas [13].

Based on the level of biosecurity categorized by FAO, poultry production in Indonesia is divided into four sectors; 1: industrial; 2 and 3: commercial; 4: backyard. Sector 1 farms produce according to an industrial integrated system, commercial and have a high level of biosecurity. Sector 2 and 3 farms are equally commercial; however, their biosecurity levels range from moderate to high to negligible. Sector 4 farms are small flocks of chicken for local consumption with minimal biosecurity [13, 14]. Sector 1 is comprised of integrators and large corporations, sectors 2 and 3 are comprised of smaller holding farmers or contractors, and sector 4 contains backyard, native, and village birds (chickens or ducks).

Backyard poultry is kept in small flocks, usually with various species, breeds and ages mixed. The flocks mostly have close contact with humans in the same household, as well as with wild birds and other livestock [13]. The lack of biosecurity and free-range housing increases the introduction and transmission of Avian Influenza viruses inside a flock, as well as their subsequent spread to other flocks.

Flocks near wetlands and flocks of itinerant ducks have a significant risk of exposure to the AI virus from wild birds. Prior research has demonstrated that ponds and canals enhance the spread of HPAI in rural areas. The pathogen can then spread to other sectors despite the application of biosecurity measures. In nations with a substantial backyard population, it is more difficult to prevent the spread of HPAI virus. Understanding the incidence of HPAI in Indonesia will therefore improve risk management for the prevention and control of HPAI [13, 15-17]. In this study, we unraveled HPAI transmission using molecular epidemiology and classical epidemiological techniques.

3. H5N1 IN INDONESIA

HPAI caused by H5N1 virus was first observed in Indonesia in August 2003 [18-20] in the Banten province, Java Island [18-22]. The disease not only caused large economic losses in poultry across Indonesia, but also resulted in human infections [19, 21, 22]. The HPAI H5N1 viruses circulating in Indonesia were classified as clade 2.1 and likely originated from Hunan province in China [23, 24]. In 2004, the disease spread among poultry in 15 of 30 provinces and caused the loss of

approximately 10–16.2 million birds due to disease and culling. The direct economic losses were estimated at US$171 million [20, 25]. In 2005, the first human cases of HPAIv A(H5N1) were reported and 168 cases from a total of 200 cases. In 2004, the disease spread among poultry in 15 of 30 provinces and caused the loss of approximately 10 – 16.2 million birds due to disease and culling. The direct economic losses were estimated at US$171 million [20, 25]. In 2005, the first human cases of HPAIv A(H5N1) were reported and 168 cases from a total of 200 cases. or 84%, were fatal [26-29]. Since 2006, the disease has been officially declared endemic in Indonesia and outbreaks have occurred frequently on the Java, Bali, Sulawesi, and Sumatra islands. HA clade 2.1 was the predominant subtype [30], until HA clade 2.3 was detected in duck farms and live bird markets in Central Java in 2012 [31].

After the first HPAIv A(H5N1) notification, measures were implemented to control the outbreaks. Active surveillance was carried out in 2004 to 2005 to detect, report and eliminate HPAIv A(H5N1) [21]. In January 2006, National Strategic Plans were issued for the comprehensive handling of HPAIv A(H5N1) both in the animals and human healthcare. Animal disease control, human cases management, high-risk groups protection, epidemiological surveillance integration, poultry industry reconstruction, risk communication, education and public awareness, law regulation and enforcement, research and development, capacity building, monitoring and evaluation [32] were all included in guidelines for the measurement and mitigation control of HPAIv A(H5N1) in Indonesia. These comprehensive strategic protocols were carried out by a ministerial-level committee, the National Commission for Avian Influenza Control and Pandemic Preparedness (NCAICPP) or Komnas FBPI, which was established in all 33 provinces and more than 490 districts in Indonesia [33].

In 2004, active surveillance was carried out for early detection of outbreaks. International agencies supported these active surveillances in the four poultry production sectors because of the limitations of the capacities of the Indonesian veterinary services and laboratories. This active surveillance was initiated as a joint corporation program between the Ministry of Agriculture of the Republic of Indonesia and the Food and Agriculture Organization (FAO), World Organization for Animal Health (OIE), and the World Health Organization (WHO) [40]. However, due to the labour and high costs associated with active surveillance, in 2006 active surveillance was replaced by passive surveillance, which relied of the farmers reporting outbreaks to be investigated by the National Veterinary services [34]. To enhance passive surveillance Participatory Disease Surveillance and Response (PDSR) was established, in which the rural community and farmer were stimulated to report HPAIv A(H5N1) suspicions

before the national veterinary services officer went to the field to investigate the outbreak and collect the samples for further confirmation test [35-37] and epidemiological data. These participatory reports were supported by online reporting system for influenza virus monitoring (IVM online) and analysed through National Animal Health Information System (ISIKHNAS) for the further policy making [34, 38-40].

Upon the introduction of HPAIv A(H5N1) in Indonesia various control measures have been implemented, such as stamping out and depopulation, biosecurity, animal movement bans, quarantines, tracing infections and vaccination [32]. However, despite these efforts the virus circulation continued and infection is endemic in Indonesia [41]. Vaccination without adequate surveillance and biosecurity has resulted in the silent spread of HPAIv A(H5N1) in Indonesia. This silent spread and cocirculation of viruses in endemic regions pose a threat to the emergence of new reassortant viruses. Before meaningful actions can be taken in this situation it is crucial to better understand the transmission of the virus in this endemic situation.

In 2003, HPAIv A(H5N1) clade 2.1 was first detected in Indonesia, and it subsequently was found in many different regions of the country. The virus most likely spread through poultry movements. Afterwards various clades were identified, clade 2.1.2 was detected in Sumatra and Java in 2004. This clade caused outbreaks not only in animals but also in human and was observed from 2004 to 2007. Another clade, A(H5N1) clade 2.1.3 was also identified in humans and in animals and evolved to clades 2.1.3.1, 2.1.3.3 and 2.1.3.2. Clade 2.1.3.2a became predominant throughout Indonesia between 2009 and 2012. By then clade 2.3.2.1 was discovered in duck farms and live birds market outbreak in Central Java [31]. Nowadays clade 2.1.3.2c is predominant in Indonesia [31].

4. SCOPE OF THE THESIS

Silent spread of Highly Pathogenic Avian Influenza virus (HPAIv) A(H5N1) causes a global concern due to the continuing evolution of the viruses. Even though many attempts have been made to stop the infection by biosecurity and vaccination the disease is endemic to large parts of Asia. The ability of viruses to mutate and re-assort with other subtypes enables them to escape immunity and makes viral eradication difficult in these countries. In addition, wild birds are known as AI viral reservoir that can bring new LPAI viruses to poultry. More importantly, poultry movements, live bird trades and markets, free range duck rearing system, minimal biosecurity particularly in semi intensive poultry production are factors driving of AI virus circulation in South East countries [42].

Restructuring the poultry sector is needed to reduce HPAIv transmission and for that understanding the viral transmission in Indonesian poultry is crucial. Such knowledge will be useful to Indonesia, but also to Southeast Asian countries that have similar production systems. Transmission pathways and viral dynamics should be analysed and unravelled using temporal, geographical and genetic data. Molecular epidemiology can be helpful to show the genetic diversity in the endemic region giving valuable insights of avian influenza disease dynamic and the probabilities of infection in different poultry farms in in Indonesia [43]. This study aims to understand AI viral evolution and phylodynamics and to unravel the associated risk factors for the transmission in poultry sectors in Indonesia. For that reason, outbreaks were studied by collecting epidemiological data and by sequencing the viruses. Also, contact structure between and within poultry was analyzed to see the route of transmission.

5. OBJECTIVES

The study objectives are to gain insight in the Highly pathogenic avian influenza virus transmission in Indonesia using epidemiological analysis and molecular epidemiology, more specifically,

1) To explore the contact facilitating transmission of HPAIv A(H5N1) between poutry farms in West Java, to estimate the rate of contacts that could potentially transfer HPAIv 1) between farms within the same poultry production chain (broilers, layers, ducks, backyard), 2) between farms in different poultry production chains and 3) to and within poultry collector houses and live bid markets (Chapter 2).
2) To explore Highly Pathogenic Avian Influenza A(H5N1) Outbreaks in West Java Indonesia 2015–2016, particularly in Clinical Manifestation and Associated Risk Factors (Chapter 3)
3) To create a collection of isolates of HPAIv coming from different components of the Indonesian poultry industry and perform a full genome sequencing of all isolates and to perform genetic analysis of the isolates and study the Phylogenetic tree and Reassortments among Avian Influenza A(H5N1) Viruses Circulating in Indonesia, 2015–2016 (Chapter 4)
4) To explore the phylodynamics of Highly Pathogenic Avian Influenza A(H5N1) in Indonesian poultry (Chapter 5).

The thesis is concluded by a general discussion.

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CHAPTER 2

Exploring contacts facilitating transmission of influenza A(H5N1) virus between poultry farms in West Java, Indonesia: A major role for backyard farms?

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Exploring contacts facilitating transmission of influenza A(H5N1) virus between poultry farms in West Java, Indonesia: A major role for backyard farms?

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A B S T R A C T

Highly pathogenic avian influenza virus (HPAIV) H5N1 has been reported in Asia, including Indonesia since 2003. Although several risk factors related to the HPAI outbreaks in poultry in Indonesia have been identified, little is known of the contact structure of farms of different poultry production types (backyard chickens, broilers, layers, and ducks). This study aims to quantify the contact rates associated with the movement of people, and movements of live birds and products and equipment that affect the risk of HPAIV H5N1 transmission between poultry farms in Indonesia. On 124 poultry farms in 6 districts in West Java, logbooks were distributed to record the movements of farmers/staff and visitors and their poultry contacts. Most movements in backyard chicken, commercial native chicken, broiler and duck farms were visits to and from other poultry farms, whilst in layer farms visits to and from poultry companies, visits to egg collection houses and visit from other poultry farms were most frequent. Over 75% of persons visiting backyard chicken and duck farms had previously visited other poultry farms on the same day. Visitors of backyard chicken farms had the highest average contact rate, either direct contact with poultry on other farms before the visits (1.35 contact/day) or contact during their visits in the farms (10.03 contact/day). These results suggest that backyard chicken farms are most at risk for transmission of HPAIV

compared to farms of the other poultry production types. Since visits of farm-to-farm were high, backyard farms could also be a potential source for HPAIV transmission to commercial poultry farms.

1. Introduction

The emergence of multiple highly pathogenic avian influenza virus (HPAIV) H5N1 sub lineages in China between 2000 and 2002 was followed by rapid and widespread virus dissemination resulting in disease outbreaks in poultry and wild birds across Asia, the Middle East, Europe, and Africa between 2003 and 2005 (Kilpatrick et al., 2006;Vijaykrishna et al., 2008). Indonesia is one of the countries severely affected by HPAIV H5N1 infection with poultry outbreaks first reported in late 2003 (Vijaykrishna et al., 2008). The economic losses were estimated at least U$ 330 million during 2004–2008 due to culling of poultry, decreasing demand of poultry products, and the costs of disease control (Basuno, 2008). Moreover, H5N1 virus infection in humanshas been associated with a high case fatality rate (84%, 168 deaths from 200 confirmed cases in Indonesia up to September 2017) (WHO, 2017). Several measures to control HPAIV have been implemented by the Government of Indonesia resulting in a reduction of disease outbreaks in poultry since 2012 (FAO, 2012) and human H5N1 cases have decreased substantially since 2013 (WHO, 2017). However, HPAIV H5N1 continues to pose a threat to public health as evidenced by reports of outbreaks in poultry to date (DGLAHS, 2017) and a report of a fatal case in humans in September 2017 (WHO, 2017).

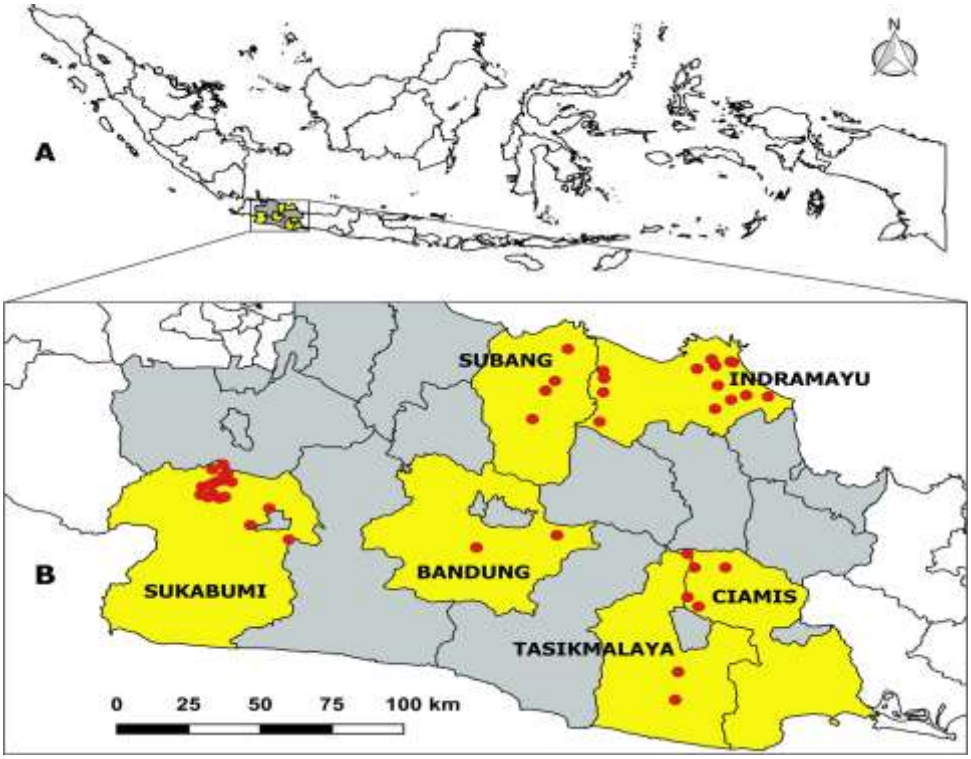

Fig. 1. The geographical map of the Republic of Indonesia and the study area. (A) Provincial boundaries of Indonesia with the West Java Province are highlighted. (B) The West Java Province showing the district boundaries with approximate locations of poultry farms are shown. Districs where enquiries were conducted are yellow coloured and farms are represented as red-round dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The poultry production sector in Indonesia is highly diverse. It includes backyard poultry farms with minimal biosecurity and a small number of birds per farm for local consumption (Sector 4); small- to medium-scale commercial poultry farms, housing broilers, layers, or ducks with low biosecurity and birds/products are usually sold through live bird markets (Sector 3); medium- to large-scale commercial poultry farms, mainly housing broilers or layers with moderate to high biosecurity and birds/products are sold through slaughterhouses or poultry markets (Sector 2); and large industrial integrated poultry farms with high biosecurity and bird/products are always marketed commercially (Sector 1) (FAO, 2004; Azhar et al., 2010). Poor biosecurity practices, for instance uncontrolled movements of live poultry, have been associated with HPAIV virus transmission within and between poultry farms

and spread between regions (Sims et al., 2005; Soares Magalhaes et al.,2010; Ssematimba et al., 2013). The difficulty in controlling HPAIV H5N1 spread through poultry movements within and between production sectors and related marketing chains, including trade in live bird markets, is considered as an important reason why the poultry sector in Indonesia is still confronted with outbreaks (McLeod et al., 2009; Millar et al., 2015). Quantitative knowledge of the contact structure of poultry farms is required to improve HPAIV control in Indonesia. For instance, such knowledge could help the Indonesian Government to educate farmers to make them aware of the frequency of contacts in order to become more critical on who they admit to their farm, and if whether they can have access to poultry and where they will pay visits themselves.

Mathematical modelling has been extensively used to increase insight in understanding disease dynamics and interpreting epidemiological data. Moreover, it can be used to support decisions on measures to control infectious diseases (Dorigatti et al., 2010; Stegeman et al., 2010; Patyk et al., 2013) and knowledge of the contact structure and contact rates is important for such modeling (Ortiz-Pelaez et al., 2006; Dent et al., 2008; Park and Bolker, 2017). In relation to HPAIV transmission, there are only a few published studies on the contact structure of poultry farms in Indonesia (de Glanville et al., 2010; Henning et al., 2016; Kurscheid et al., 2017). Whilst de Glanville et al. (2010) performed a quantitative risk assessment of HPAIV transmission amongst smallholder broiler farms in West Java, Henning et al. (2016) and Kurscheid et al. (2017) used a network analysis to investigate the patterns of duck movements in Central Java and poultry movements on/off live bird markets in Bali and Lombok, respectively. In the present study, we aimed to quantify the rate of poultry contacts from different poultry sectors and production types in West Java. Moreover, we determined whether there was direct contact with poultry and which type of contact that most likely contributed to HPAIV virus transmission to poultry farms. Quantitative knowledge of these poultry contacts is important to understand the endemicity of HPAIV H5N1 in Indonesia and may indicate possibilities to reduce the contact rate and HPAIV transmission between farms.

2. Material and methods

2.1. Data collection and management

The study was conducted in West Java Province, the province that produces most poultry within Indonesia (DGLAHS, 2016a) and has repeatedly reported HPAIV

H5N1outbreaks (DGLAHS, 2016b). The target population is the poultry farms in West Java Province. We then selected six districts within this province (Bandung, Ciamis, Indramayu, Subang, Sukabumi, and Tasikmalaya) (Fig. 1) because of their: (1) high poultry density, (2) various poultry production systems and (3) HPAIV H5N1 outbreaks reported between 2013 and 2015 (CENTRAS, 2015).The source population consisted of poultry farms of different production types (broilers, layers, ducks, native or local chickens) and different sectors (Sector 1–4) in those six districts.

A farm logbook was developed based on the literature (Ssematimba et al., 2013) as well as interviews from local experts (veterinarians, poultry technical services, and senior animal health officers of district livestock agencies) to collect general data of the poultry farms, contact types, and certain risks factors. It contained a combination of filling sections and questions to describe specific poultry contacts, including details of farmer, location, poultry population, and period of recording and description of HPAI history (Supplementary Material). In addition, farmers and staff of farms were asked to record their movements outside the farm ("outbound visit"), including visits to: other farms, traditional markets, poultry companies, poultry slaughterhouses, egg collection houses, and other places. The farmers were also asked to register the movements of visitors into the farm ("inbound visit"), as well as to record their intention to visit the farm and whether they had been incontact with poultry on the day before arriving at the farm ("off-farm contact") and contact to poultry during their visit on the farm ("on-farm contact"). For the inbound visit, the same places as outbound visit were considered as possible sites where they had been on that same day before visiting the farm. The farm owners and staff were trained how to fill out the logbook before starting the recording of the logbook. The farm logbooks were distributed in June 2015 and the farmers were asked to fill out their logbook for one month. To ensure that the farmers have properly recorded the visits in the logbook, the officials of district livestock agency checked the logbook, at least, once during the period of study.

Traditional markets in Indonesia trade both poultry products (mainly meat and eggs) and live birds for trade or for slaughter. Therefore, these markets are considered and referred to herein, as live bird markets as previously described (Indriani et al., 2010). Poultry companies in this study include core companies (Sector 1 that have agribusiness contracts with commercial farms of Sector 3 for raising poultry, particularly broilers), feed or pharmaceutical companies (for broiler and layer farms), and poultry-product shops and retailers (for duck, commercial and backyard chicken).

To determine whether different poultry production types have different characteristics of visit purposes, the inbound visit was further analysed by examining the intention of visitors to come to the farms. This includes visits with the purpose of: (a) observing poultry (to get compliance whether or not purchasing poultry), (b) buying or collecting poultry and related products (harvest ready-to-slaughter broilers, purchase/collect dead birds or manure in broiler and layer farms, purchase eggs in layer, ducks and commercial native chicken farms, or purchase live birds for restocking or consumption in backyard chickens, commercial native, or ducks), (c) selling or offering poultry and related products (promote information of poultry [e.g. day old chicken/duck/ pullet], feed or pharmaceutical products like poultry vaccines/drugs/ vitamins), (d) transporting feed, poultry or equipment, (e) inspecting farm or poultry health, (f) vaccination or health treatment, (g) other work at the farm (e.g. renovation of farm premises or poultry houses, service for farm equipment, and so on), and (h) social relationships.

Random selection of poultry farms was not possible, because there is no poultry farm database comprising farmers from all poultry sectors (sector 1–4) in the study area. As the next best option, livestock officials with expertise of local poultry productions selected farms in the districts aiming for a representative sample of the major poultry producing farms in the region. But, they were depending on the willingness of farmers to participate in the study, resulting in underrepresentation of sector 1 and sector 2 farms. As a results, the logbooks were distributed to 150 different poultry farms representing sector 3-commercial farms and sector 4-backyard farms. Poultry collector houses and live bird markets were not included in the study, as they have been studied previously (Kurscheid et al., 2017).

From 150 distributed logbooks, 146 were collected and the data were compiled and entered in a Microsoft Excel file after excluding 21 farm logbooks because of incomplete or inconsistent data. Data from quail farms were not included in the analysis since only one good quality logbook was available. Thus, a dataset from 124 farm logbooks were analysed. This dataset was imported into an open-source integrated R environment for statistical computing and graphics (version 3.3.3, https://www.r-project.org).

2.2. Data analysis

An open-source geographic information system QGIS (version 2.18 Las Palmas, https://www.qgis.org) was used to map the locations of poultry farms. The proportion of outbound visits by farmers/staff and inbound visits by visitors were calculated. Differences in proportions of visits based on the

purpose of visitors coming to the farm were also examined. The rates of off-farm and on-farm poultry contacts by visitors were estimated using a *generalized linear mixed-effect model* (GLMM) with Poisson distribution and a log link using *lme4* package for R (https:// cran.r-project.org/package=lme4). Furthermore, the risk ratio (RR) of poultry contact per day was calculated. Total number of off-farm or on- farm contacts in the one-month period represented the respective dependent variables; whereas district, poultry sector, farming system, poultry production types, farm size and HPAI history were added as independent categorical-fixed effect-variables and observed poultry farm was added as a random effect. Since the length of the period farmers recorded the logbook varied from 22 to 45 days (median: 30 days), a natural logarithm (*ln*) of these observation times was included as offset variable. To build the model, a univariable logistic regression analysis using GLMM was firstly carried out to examine the association of each independent variable with the dependent variable. Then, a full multi-variable logistic regression analysis was performed using all the independent variables with a *p*-value less than 0.25 from the univariable analysis. After backward stepwise procedures applied, poultry sector and subsequently farming system were excluded because the multivariable analyses indicated a rank deficient matrix due to collinearity (O'Brien, 2012); whereas all the other independent variables were maintained in the final multivariable analysis. We used Akaike's information criterion (AIC) to test goodness of fit of the model, selecting the one with lowest AIC (Motulsky and Christopoulos, 2004). All analyses were performed in R (version 3.3.3, https://www.r-project.org).

3. Results

3.1. Descriptive analysis

We collected data from 114 commercial and 10 backyard poultry farms in the study area (Fig. 1) and poultry types including broilers, layers, ducks and native chickens. Three different native chickens were found: the Indonesian-local village chickens that are raised traditionally in household backyard (hereafter referred as "backyard chicken"), the male of local village chickens and the crossbreed chickens between the Belgian-Braekel chickens and the local village chickens that both are housed in a fenced area within the farmyard. The last two types of native chickens were raised for commercial purposes (hereafter referred as "commercial native chicken"), particularly for meat and egg production, respectively.

The number and type of poultry production included in the study varied between the districts: Subang (23 farms: all broiler farms), Bandung (16 farms: 5 backyard chicken, 6 commercial native chicken and 5 duck farms), Ciamis (14 farms: all broiler farms), Indramayu (34 farms: 5 backyard chicken, 28 duck and 1 layer farms), Sukabumi (24 farms: 7 commercial native chicken, 10 broiler and 7 layer farms), and Tasikmalaya (13 farms: 6 broiler and 7 layer farms) (Fig. 2). Backyard chicken farms raised 20–100 birds/farm and flock sizes observed in commercial native chicken farms ranged from 60–30,000 birds/farm. The numbers of birds housed on layer farms varied from 1000 to 60,000 birds, whereas most broiler farms housed between 1000 and 10,000 birds and most duck farms housed between 100 and 1000 birds. A small proportion of poultry farms in this study had a size of more than 30,000 birds. The proportion of poultry farms reporting a previous HPAIV history in backyard chickens and in commercial native chickens were more or less similar (approximately 60%). Few HPAIV outbreaks were reported from layer farms (7%) and broiler farms (20%), whereas most duck farms (85%) had experienced HPAIV outbreaks in the past.

3.2. Outbound and inbound visits

Amongst poultry production types, visits to and from other poultry farms were most frequent (Fig. 3). Over 75% of persons visiting backyard chicken and duck farms had previously visited other poultry farms on the same day. Higher proportions of visits to live bird markets by farmers/staff of backyard chicken, commercial native chicken and broiler farms were observed (24–31%), when compared with farmers/staff of layer and duck farms that showed higher proportions of visits to poultry companies (35% and 27%, respectively) and to egg collection houses (39% and 20%, respectively). Broiler farms had more outbound visits to live bird markets (30%) than to poultry slaughterhouses (5%); on the other hand, they had only few inbound visits from live bird markets (1%). In addition, the proportion of visits from poultry companies to broiler and layer farms were higher (23% and 36%, respectively) than to farms of the other poultry types (3–8%).

A high proportion of visits had the objective to buy/collect poultry and related products (19–30%) (Fig. 4). Only a small proportion of inbound visits had the aim to sell/offer poultry and related products (1–7%), to vaccinate or treat poultry (0–4%), or to have other work at the farm (2–10%). Backyard chicken and duck farms showed a higher proportion of visits to observe poultry (20–21%), than visits to transport feed, poultry or equipment (8–14%). Commercial native chicken, broiler and layer farms had more visits to transport feed, poultry or equipment (15–37%) than to inspect farm and poultry health (13–23%).

Visits related to social relationships were commonly found (14–39%) in all poultry production types. Additionally, we observed that the frequency of visits to transport feed, poultry or equipment and to inspect farm and poultry health was associated with an increase in the farm size; while there was a negative association between the farm size and the frequency of visits for observing poultry, purchasing poultry or products, and social visits (Fig. 3).

3.3. Contacts of visitors to poultry

The rate of off-farm and on-farm contacts differed significantly per district, production type and farm size categories (Table 1). The probability of off-farm contact per day amongst poultry farms in Ciamis and in Tasikmalaya were 2–3 times higher (RR: 2.15 [95% CI:1.05–4.44] and (RR: 2.91 [95% CI:1.26–6.78], respectively) than that of poultry farms in the reference district (Subang). Also, their on-farm contact RR was higher although this difference was not significant. On the other hand, on-farm contacts amongst poultry farms in Bandung and in Indramayu were very low (RR: 0.03 [95% CI: 0.00–0.29]). Backyard chicken farms had significantly higher off-farm contact rate (1.35 contact/day on average) and on-farm contact rate (10.03 contact/day) than the other poultry farm types. There were no significant differences in the contact rate of poultry farms with small (1,001–10,000 birds/farm) and with moderate population sizes and 10,001–30,000 birds/farm). However, poultry farms with the biggest population (30,001–60,000 birds/farm at 1 broiler and 3-layer farms) showed a significant increase in off-farm poultry contact (RR: 23.56 [95% CI:4.06–137.75]). At last, although there was no significant difference on the contact rate between farms which had experienced HPAIV outbreak and those that did not, their on-farm contact rate was about 7 times higher than off-farm contact rate (Table 1).

4. Discussion

This study identified that the most frequent movements in backyard chicken, commercial native chicken, broiler and duck farms were associated with visits to and from other poultry farms, whilst in layer farms visits to egg collection houses, visits from other farms, and visits to and from poultry companies were most frequent. Risks of HPAIV exposure could arise from visits by farmers/staff or visitors to or from a farm experiencing H5N1 infection. If such a contact occurs and no adequate biosecurity protocols are in place, between farm HPAIV transmission might occur directly or indirectly (Idris et al., 2010; Fasina et al., 2011; Ssematimba et al., 2013; Durr et al., 2016). Apart from visits to other farms, farmers/staff of backyard chicken, commercial native chicken and broiler farms in this study showed more visits to live bird markets than those of the

other farm types. Visits to these sites bear a high risk of HPAIV exposure as they are considered as a source of virus (Indriani et al., 2010; Samaan et al., 2011) and facilitate HPAIV transmission and large-scale disease spread (Sims et al., 2005; Fournie et al., 2013).

The intention of visitors was evaluated to examine whether during the farm visit direct contact with poultry would occur. Visits with the aim to purchase or collect poultry and related products pose a higher risk for virus transmission since these are mostly acted by middleman who can have multiple visits and have direct contact with poultry on different farms on the same day (Idris et al., 2010; Henning et al., 2013; Durr et al., 2016). Commercial farms with a larger farm size tend to have more poultry contacts, as shown in this study where farms housing more than 30,000 birds showed substantial increase in the likelihood of poultry contacts of visitors before visiting the farm (RR: 23.56 [CI:4.06–137.75]). This might be associated with contacts via movements of visitors across different farms to supply live birds, feed and equipment, or to inspect farm or poultry health. Previous studies indicated that persons who are commonly in charge for checking flock health (poultry technical services or veterinarians) have been considered risk factors promoting virus transmission, if they do not follow proper biosecurity practices during the visit (Idris et al., 2010; Ssematimba et al., 2013; Osmani et al., 2014). In addition, layer farms with a larger population size usually have a number of flocks of different ages; thus, more frequent visits for bird replacement were observed (Durr et al., 2016). A small proportion of visits were aimed to vaccinate broiler and/or layer flocks which could be directed not only to protect against HPAIV H5N1 but also against other avian pathogens. Such visits will occur less frequently to broiler flocks as vaccination is less common because of the shorter-life span of broilers compared to layers. In addition, layer farms often can manage vaccination by own resources (layer farms in Indonesia usually have equipment and staff members who have been trained for vaccination).

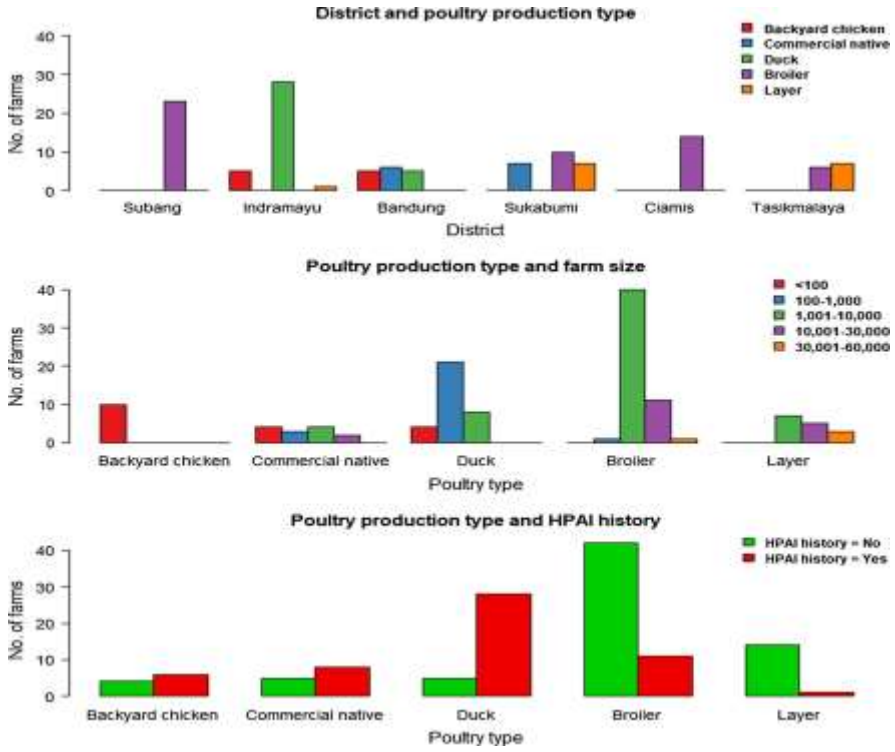

Fig. 2. Distribution of poultry farms across district, production type, farm size and HPAI history.

HPAIV outbreaks in duck farms have been frequently reported since the introduction of H5N1 Clade 2.3.2.1c in poultry in Indonesia in 2012 (Wibawa et al., 2012). The present study shows that a higher number of duck farms with HPAI history was observed in comparison to the other farm types. However, the contact rates amongst poultry farms that had or had not experienced HPAIV outbreaks in this study were not significantly different. Because the infection history may have influenced the contact structure, we could not certainly infer from this that contact structure is not influencing HPAIV transmission. In addition, incoming visitors having direct contact with poultry inside farm, either with or without HPAI histories, were seven times higher than farmers/staff having direct contact with poultry outside farms, suggesting that the majority of poultry farms in this study lacks proper biosecurity protocols for visitors.

HPAIV can be transmitted between commercial farms or between commercial farms and backyard farms through several pathways involving direct bird-to-bird contacts or indirect contacts of infected birds via environment or fomites

(de Glanville et al., 2010; Ssematimba et al., 2012). De Glanville et al. (2010) estimated that the probability of HPAIV H5N1 transmission from infected to susceptible flocks in Sector 3-broiler farms in Bogor District, West Java, were 0.032–0.130 per contact of poultry collectors and 0.029 to 0.095 per contact of animal health workers. In addition, Ssematimba et al. (2012) estimated that during the 2003 HPAIV H7N7 epidemic in the Netherlands, per-contact probability of a susceptible layer farm infected by H7N7 virus from an infectious farm were between 0.0414 and 0.308, depending on the type and purpose of visits. Although estimating the probability of virus transmission per contact was beyond the goal of this study, we could show that the average daily contact of visitors to poultry varied amongst production types; visitor-to-poultry contact in backyard chicken farms was significantly higher (10.03 contact/day) than contacts in farms of ducks (4.19), commercial native chickens (0.15), broilers (0.11) and layers (0.06). This is no surprise since many backyard chickens lack permanent confinement and poultry owners might not be familiar with basic biosecurity measures and let their chickens roam freely around the house.

A higher poultry contact in backyard poultry is likely associated with type and purpose of visits that show more visits of farm-to-farm and farm-to-live bird market with aims to observe birds or to purchase live birds and products. This indicates that Sector 4-backyard poultry in Indonesia retains the highest risk to be exposed by HPAIV from other poultry sectors as well as to be a potential source of infection, particularly towards small-scale commercial sector farms (de Glanville et al., 2010; Idris et al., 2010). It could also contribute to the course of HPAIV transmission between commercial farms through spill-over infection (Smith and Dunipace, 2011). Moreover, both Sector 4-backyard and Sector 3-commercial poultry farms have been reported to have a higher probability of HPAIV infection (Durr et al., 2016) and a higher proportion of disease outbreaks in Indonesia (DGLAHS, 2016b); hence, they are considered to play a role in maintaining the infection cycle of HPAIV in poultry within the country (Idris et al., 2010; Henning et al., 2013).

Various studies reporting risk factors for HPAIV H5N1 have been published (Biswas et al., 2009; Desvaux et al., 2011; Fasina et al., 2011; Henning et al., 2016), but to our knowledge, analytical epidemiological studies in quantifying contacts in different poultry production types are scarce, particularly for Indonesia. Nevertheless, the results of this study should be interpreted with caution because it might have been subject to selection bias since the poultry farms were not randomly selected. In the context of HPAI control strategy in Indonesia, however, we consider that the results still relevant because the selected farms represented poultry sectors where most HPAIV outbreaks are

reported. In addition, to minimise inconsistency in reporting, farmers were trained how to fill the logbook and we excluded incomplete logbooks. The poultry contacts by farmers/staff outside the farm as well as hygiene and biosecurity implementation could also be important risk factors associated with HPAIV transmission, but we were unable to evaluate these as they were not recorded.

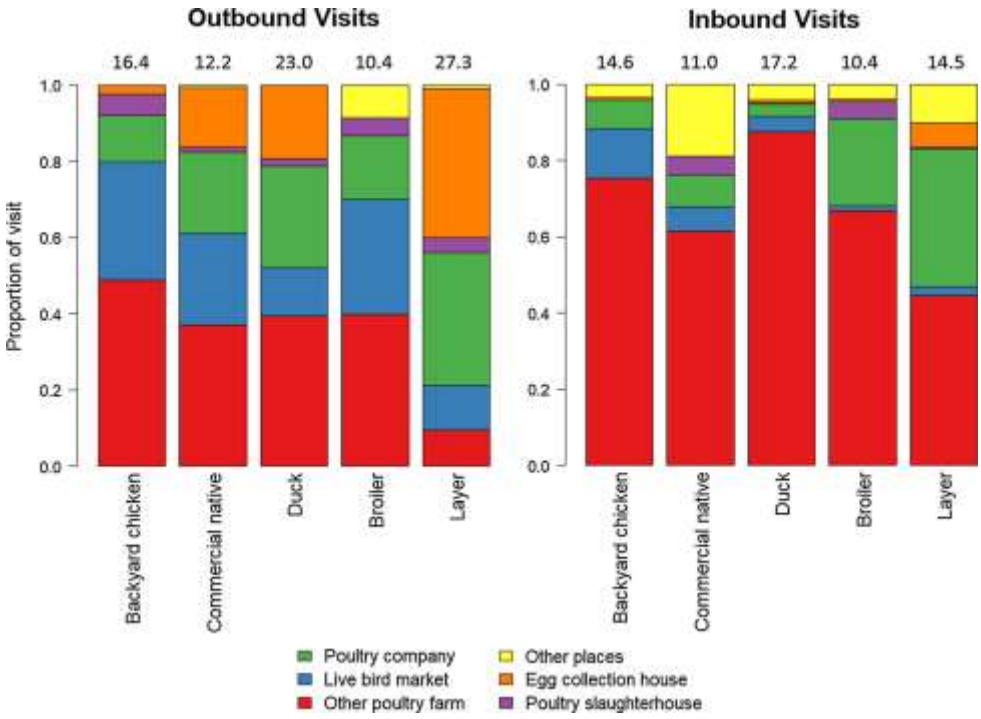

Fig. 3. Outbound and inbound visits amongst poultry production types. The proportion of each visit (e.g. visit to other farm) to the sum of all visit types per farm is shown using stacked-column barplots with the average number of visit per farm is indicated above each bar.

In conclusion, this study identified that Sector 4-backyard farms showed the highest in and outbound contact rates compared to farms of the other poultry production types in West Java, Indonesia. Since many Sector 3-commercial farms in Indonesia are situated amongst Sector 4- backyard farms (Idris et al., 2010) and both sectors are directly or indirectly connected to a bigger and complex market network involving live bird markets (McLeod et al., 2009; Kurscheid et al., 2017), between farms transmission of HPAIV H5N1 could occur at any time as the proportion visits of farm-to-farm or farm-to-live bird markets in these sectors were also high. In accordance with reports of previous

quantitative studies (Roberts and Heesterbeek, 2003; Smith and Dunipace, 2011), our study indicates that HPAIV control strategies should not only focus on one host type or one poultry sector, but also emphasizes the need for efforts eliminating potency of both backyard and commercial poultry farms as well as live bird market to become "a house" for HPAIV H5N1 circulation. This study suggests that in order to help with control and eradication of HPAIV H5N1 in Indonesia, restructuring the poultry husbandry is necessary, particularly for sector 3-commercial and sector 4-backyard farms to have an improvement on biosecurity, production and marketing practices.

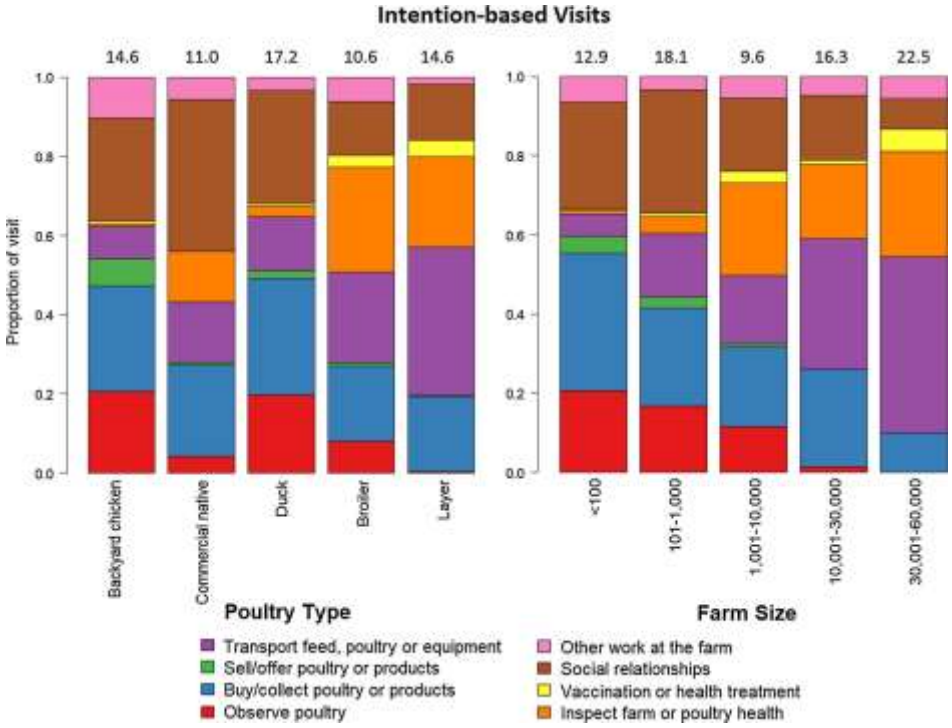

Fig. 4. The visit purposes of visitors to poultry farm based on the production type and the farm size. The proportion of each visit purpose to the sum of all visit types per farm is shown using stacked column barplots with the average number of visit per farm.

Table 1. Results of multivariable logistic regression analysis of off-farm contact rate and on-farm contact rate in West Java and its association with district, production type, farm size and HPAI history.

Independent Variable	Poultry contact by visitors (dependent variable)			
	Off-farm contact		On-farm contact	
	Contact rate	RR [95%CI]	Contact rate	RR [95%CI]
District:				
Subang	1.35	1.00	10.03	1.00
Bandung	0.24	0.18 [0.03–1.23]	0.28	0.03** [0.00–0.29]
Ciamis	2.90	2.15* [1.05–4.44]	16.79	1.67 [0.93–3.01]
Indramayu	0.22	0.16 [0.02–1.15]	0.30	0.03** [0.00–0.29]
Sukabumi	0.64	0.47 [0.17–1.31]	6.90	0.69 [0.31–1.52]]
Tasikmalaya	3.93	2.91* [1.26–6.78]	13.44	1.34 [0.67–2.68]
Poultry production:				
Backyard chicken	1.35	1.00	10.03	1.00
Broiler	0.02	0.01*** [0.00–0.10]	0.11	0.01*** [0.00–0.11]
Commercial native chicken	0.04	0.03*** [0.00–0.15]	0.15	0.01*** [0.00–0.13]
Duck	0.29	0.21** [0.07–0.66]	4.19	0.42 [0.16–1.06]
Layer	0.00	0.00*** [0.00–0.02]	0.06	0.01*** [0.00–0.06]
Farm size (No. of birds):				
<100	1.35	1.00	10.03	1.00
100–1.000	3.49	2.59 [0.86–7.86]	9.92	0.99 [0.37–2.63]
1.001–10.000	3.26	2.41 [0.70–8.45]	8.87	0.88 [0.30–2.65]
10.001–30.000	5.00	3.70 [0.85–16.28]	11.59	1.16 [0.33–4.10]
31.000–60.000	31.81	23.56*** [4.06–137.75]	24.81	2.47 [0.55–11.23]
HPAI history:				
No	1.35	1.00	10.03	1.00
Yes	1.55	1.15 [0.68–1.94]	10.73	1.07 [0.69–1.67]

Contact rate refers to the average poultry contact by visitors per day.

Risk ratio (RR) with asterisks indicate significant differences (p-values: ***p < 0.001, **p < 0.01, *p < 0.05) followed by 95% confidence intervals. Categorical factor that was set as reference in multivariable analysis is italicised.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2018.04. 008.

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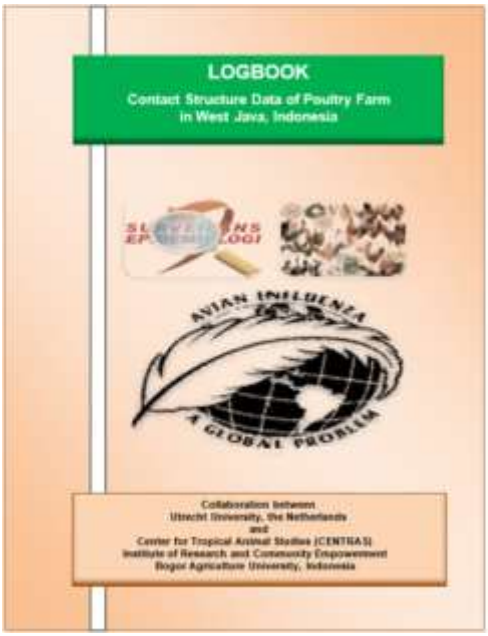
Supplementary Material. Overview of farm logbook used in this study



LOGBOOK
Data collection for contact structure of poultry farm in West Java, Indonesia

Name of owner :
Address/Location/GPS :
Phone No. :
Type of poultry farm :
Number of poultry stock :
Date of recording start :
Date of recording end :

I. History of avian influenza on the farm
Please describe briefly

II. Movements of owner and staff from the farm
Please record movement of farm owner and staff to the following places

Date	Places					
	Other Farm	Traditional market (incl. livestock market)	Poultry slaughter-house	Poultry/feed Company	Egg Collection House	Other Places

III. Movements of visitors to the farm
Please record movement of visitors with the following questions and place the answers in the box provided below:
1. Date and time of visit?
2. Name of visitor?
3. What is/are the purpose(s) of visit?
4. Where did the visitor come from before arriving at the farm?
5. Has the visitor been in contact with poultry before coming to the farm?
6. Has the visitor actually been in contact with poultry on the farm during the visit?

Answers:

1	2	3	4	5	6

CHAPTER 3

Highly Pathogenic Avian Influenza A(H5N1) Outbreaks in West Java Indonesia 2015–2016: Clinical Manifestation and Associated Risk Factors

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Highly Pathogenic Avian Influenza A(H5N1) Outbreaks in West Java Indonesia 2015–2016: ClinicalManifestation and Associated Risk Factors

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Abstract: Knowledge of outbreaks and associated risk factors is helpful to improve control ofthe Highly Pathogenic Avian Influenza A(H5N1) virus (HPAI) in Indonesia. This study was conducted to detect outbreaks of HPAI H5N1 in endemically infected regions by enhanced passive surveillance, to describe the clinical manifestation of these outbreaks and identify associated risk factors. From November 2015 to November 2016, HPAI outbreak investigations were conducted in seven districts of West Java. In total 64 outbreaks were confirmed out of 75 reported suspicions and outbreak characteristics were recorded. The highest mortality was reported in backyard chickens (average 59%, $CI_{95\%}$: 49–69%). Dermal apoptosis and lesions (64%, $CI_{95\%}$: 52–76%) and respiratory signs (39%, $CI_{95\%}$: 27–51%) were the clinical signs observed overall most frequently, while neurological signs were most frequently observed in ducks (68%, $CI_{95\%}$: 47–90%). In comparison with 60 non-infected control farms, the rate of visitor contacts onto a farm was associated with the odds of HPAI infection. Moreover, duck farms had higher odds of being infected

than backyard farms, and larger farms had lower odds than small farms. Results indicate that better external biosecurity isneeded to reduce transmission of HPAI A(H5N1) in Indonesia.

Keywords: HPAI (H5N1); risk factors; West Java; outbreak investigation; case-control

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1. Introduction

Ever since its first notification in Hong Kong in the late 90s [1,2], Highly Pathogenic Avian Influenza A (HPAI) viruses of subtype H5N1 have caused numerous outbreaks in poultry worldwideand hundreds of (mostly fatal) cases in humans [3,4]. In addition, because the virus became endemicto the poultry populations of several countries in Asia and in Egypt there was ample opportunity for reassortment with other AI viruses, which has resulted in a multitude of HPAI virus clades that have subsequently circulated globally [5–9]. As long as there are endemically infected regions, new reassortants will arise and pose a threat to global poultry and wild bird populations and to humans and other mammals such as pigs and cats.

To control the spread of HPAI among poultry flocks, biosecurity, early detection and depopulation of infected flocks are all of crucial importance. In addition, if vaccination is applied, (i) high-quality vaccines should be used based on viruses that are antigenically closely related to circulating field strains; (ii) vaccination coverage should be high; and (iii) vaccination should be accompanied by adequate surveillance to not result in silent spread of the virus [10]. Information on risk factors associated withoutbreaks in the countries endemically infected with HPAI may be helpful to support control programs to eliminate the virus from such regions in the long run [11,12].

Since 2003, HPAI A(H5N1) infections have been endemic to the Indonesian poultry population [13]. Collector houses and live bird markets play an important role in the spread of infection [14–16]. Nevertheless, knowledge on risk factors for HPAI on poultry farms is also important for the control of the infection. Studies into factors associated with HPAI H5N1 outbreaks in Indonesian poultry have been previously reported, but they used secondary data, defined outbreaks on clinical diagnosisor the outcome of non-validated rapid tests only or did not use controls [17–19]. In addition, to our knowledge no risk factor studies concerning the currently circulating virus clade 2.3.2.1c [20,21] have been published.

Consequently, a study describing the characteristics of outbreaks caused by currently circulating HPAI A(H5N1) and quantifying associated risk factors is valuable, in particular because the Indonesian poultry sector is expanding due to the growing demand for poultry products.

West Java is the province in Indonesia which has been affected most severely by HPAI H5N1 outbreaks, because it has the highest poultry density, intensive poultry trading and a poultry sector consisting of various types of poultry, including ducks. The aims of this study were to detect outbreaks of HPAI H5N1 in an endemically infected region by enhanced passive surveillance, describe the mortality, morbidity and clinical manifestation of these outbreaks and identify associated risk factors.

2. Materials and Methods

2.1. Poultry Farming in Indonesia

According to the classification used by the Food and Agricultural Organization of the United Nations (FAO), the poultry production in Indonesia can be classified in four sectors [22,23]. Sector 1 farms are associated with high biosecurity and industrial integrated production. The chickens are brought to slaughterhouses and products are commercially marketed. Sector 2 farms have moderate to high biosecurity with medium to large scale production. Birds or products are sold via poultry markets or slaughterhouses. Sector 3 farms are of low biosecurity and small to medium size. Birds and products are usually sold through live bird markets. Backyard chicken farming is classified as poultry sector 4and includes the majority of poultry farms in Indonesia. It is commonly practiced in village areas with minimal biosecurity. The poultry products of these chickens are mostly for domestic consumption.

2.2. Description of Outbreaks

From November 2015 to October 2016, outbreak investigations were conducted in seven districts of the West Java province: Bandung, Ciamis, Indramayu, Subang, Sukabumi, Purwakarta and Tasikmalaya (Figure 1). The study was preceded by a pilot study to explore the feasibility of collectingthe samples from clinical outbreaks, processing and testing them properly and collecting the information.The selected districts were selected based on HPAI H5N1 history, poultry density, and presence of various types of poultry and poultry production systems. The investigations were conducted in collaboration with

the Animal Health Agencies in the districts, who received a financial incentive for confirmed outbreaks.

Poultry farmers reported suspected outbreaks to the district veterinary officers, who subsequently visited the farm. They inspected the poultry for the presence of HPAI associated clinical signs, such as cyanosis and edema of the head, comb, or wattle, respiratory and neurological signs, and lethargy. In addition, the number of sick and dead birds was recorded. In the case that the district veterinary officer considered the farm HPAI suspect, samples were collected. Oropharyngeal samples and cloacal samples of five sick birds were pooled and stored separately in brain heart infusion broth containingantibiotics according to the L237/8 Official Journal of European Union EN 31.8.2006 under chilled conditions. The samples were delivered by the veterinary officers to the local Animal Health Laboratory Cikole of West Java Province, or District Investigation Centre Subang of the Directorate General of Livestock and Animal Health Services, at maximum within 48 hours after they had been collected.

Figure 1. The location of outbreaks and control farms.

2.3. Testing of the Samples

The samples were tested in the Animal Health Laboratory Cikole and District Investigation Center Subang using real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) targeting the M gene detecting any Influenza

A viruses from different hosts including avian, equine and other species. Samples with CT values less than 40 were considered positive for (HP)AIV and used for subtyping by using H5 specific RT-PCRs [24–26]. Samples with a CT value < 30 were subjected to Sanger Sequencing at the Eijkman Institute for Molecular Biology as described previously [27].

2.4. Collection of Epidemiological Data

The location of each poultry farm was recorded at the village level (Figure S2). Poultry type, poultry sector according to Food and Agriculture Organization (FAO) [23,28], open or closed housing, the purpose of raising poultry (commercial/non-commercial) and the number of birds were also recorded during the visit. Visitors to the poultry farms were also documented. Furthermore, the date of the first onset of disease according to the farmer, clinical signs presented by the birds and morbidity and mortality were recorded. In addition, the vaccination status was recorded (Figure S2 and Table S1).

Five additional outbreaks detected in the same period in the study region were included; two insector 4 farms in Sukabumi obtained via the Animal Health Laboratory Cikole and three in farms inBogor and Sukabumi obtained from ProLab Diagnostic Laboratory, a poultry industry laboratory, to also include sector 1 in the study.

2.5. Description of Control Farms

As control farms we selected farms (Table S1) in the study region (to ensure similar exposure toHPAI A(H5N1)) without a history of HPAI from the collected questionnaire (Figure S1). The absence of a poultry farm database comprising farmers from all poultry sectors in West Java made random selectionof control farms not possible. To ensure a representative sample of HPAI free farms in the study region local animal health officials with expertise of poultry production in the districts selected farms aimingfor a representative sample of the major poultry producing farms in their region. In addition, farms were selected from Sierad Produce to also include sector 1 farms from the study region, as they could not be selected by the local animal health officials [29]. From the selected farms we included a total of 60 farms without any history of HPAI A(H5N1) infection.

2.6. Data Analysis

Seven categorized factors: District, Poultry Type, Housing System, Sector, Farm Size, Purpose of raising the poultry and number of incoming contacts were

included as dependent variables in the data analysis. To prevent imbalance due to small categories districts were grouped in three, namely Indramayu, Subang and Rest. Poultry Type was classified as backyard chickens, ducks and others (broiler breeders and commercial broilers, laying hens, native chickens, quails, and turkeys). The poultry Housing System was grouped as open or closed house; in an open house bird are confined, but the poultry house does not have a closed wall. The purpose of raising was household or commercial and farm size was categorized as less than 1000 birds per farm and 1000 or more birds per farm. The number of visitors were categorized as below or above 10 in the preceding 14 days.

The dataset was compiled in a Microsoft Excel file and transferred to an open-source integrated R environment for statistical analysis and graphics, RStudio (version version 1.1.463, https://www. rstudio.com/).

The association between potential risk factors and HPAI status was determined by logistic regression analysis using outbreak (yes/no) as dependent variable and the potential risk factors mentioned above as independent variables. Data were analysed using a logistic regression model. First, each independent variable was subject to univariate analyses and, subsequently, a full model was made including all independent variables, taking into account collinearity. Next, the variables with the highest p-value were removed from the model stepwise, until the model with the lowest value of Akaike's information criterion (AIC) was obtained (final model). R package "Stats and MASS, was used for logistic regression and the odd ratio's (OR's) were calculated using MASS package and confirmed using mfx package.

3. Results

3.1. Outbreaks Description

A total of 75 HPAI suspected outbreaks were reported by farmers during the period of this study. In 64 of these suspicions (85%) samples turned out positive in real-time M RT-PCR, indicating a high specificity of the reporting. With the additional five positive farms, as mentioned in the Materials and Methods, a total of 69 outbreak farms are displayed in Figure 1. From 39 of these samples a full genome sequence was obtained as decribed previously [27].

Most outbreaks were observed in Indramayu (30 farms) and Subang (27 farms). The rest of the outbreaks were located in Purwakarta (four farms), Sukabumi (four farms), Tasikmalaya (two farms),Bandung (one farm), Bogor (one farm).

Descriptive results are presented in Figure 2. Backyard chickens (35 farms) and ducks (19 farms) were the dominant poultry types on the outbreak farms. The other affected poultry types were breeders (two farms), commercial broilers (one farm), layers (two farms)and native chickens and quails (10 farms) as displayed in Table S1.

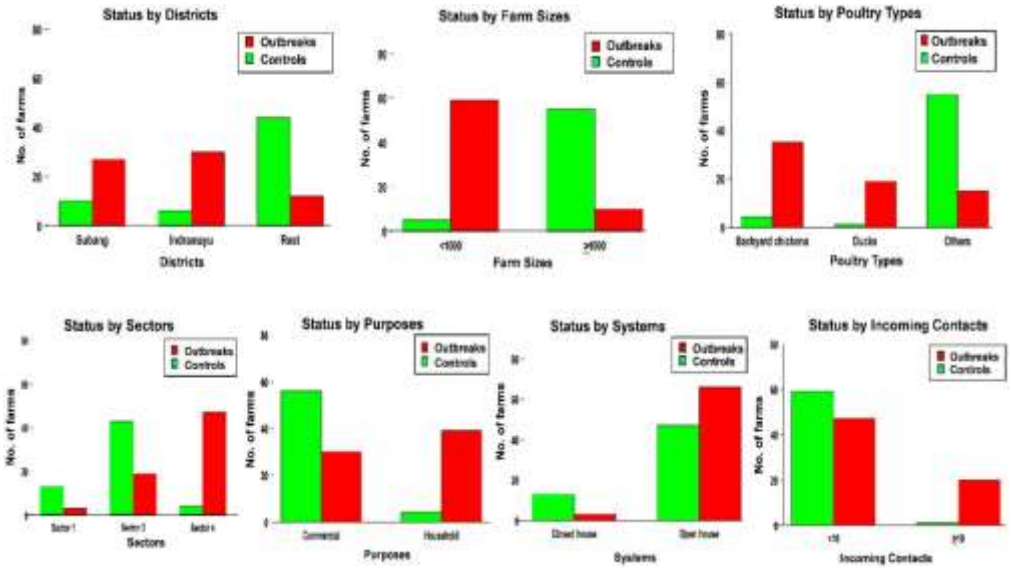

Figure 2. Distribution of outbreak and control farms across districts, farm size, poultry type, sector,purpose, housing system and incoming contacts.

Most HPAI outbreaks were detected on small farms (59 farms housed less than 1000 birds) andmost farms had an open poultry house and produced poultry for household purposes. From all totaloutbreaks, 47 farms were categorized as sector 4, while 19 farms were categorized as sector 3 and three farms belonged to sector 1. Twenty outbreak farms had more than 10 visitors to the farm in the preceding fortnight.

The recorded mortality and morbidity are shown in Figure 3. The highest mortality was reported in backyard chickens (average 59%, $CI_{95\%}$: 49–69%), compared to ducks (average 32%, $CI_{95\%}$: 19–45%) and others (average 28%, $CI_{95\%}$: 16–40%). Observed morbidity was also highest in backyard chickens, with average morbidity of 44% ($CI_{95\%}$: 32–54%), whereas it was 28% in ducks ($CI_{95\%}$: 17–38%) and 23% in others ($CI_{95\%}$: 9–37%). Higher mortality than morbidity indicates that a proportion of the birds were found dead before the farmers noticed clinical signs. On four farms poultry had been vaccinated, two farms with broiler breeders, a duck and a quail farm. The observed mortality was 30% on each of

the two broiler breeder farms, 50% on the duck and 26% on the quail farm.

The distribution of observed clinical signs over the poultry categories is shown in Figure 3. Dermal apoptosis and lesions (64%, $CI_{95\%}$: 52–76%) and respiratory signs (39%, $CI_{95\%}$: 27–51%) were overall observed most frequently. Neurological signs were most frequently observed in ducks (68%, $CI_{95\%}$: 47–90%). With respect to virus type, H5N1 clade 2.3.2.1c A was associated with more neurological signs than the other viruses, whereas H5N1 clade 2.3.2.1c B showed more fever lethargyand depression.

Also H5N1 clade 2.3.2.1c A was predominantly in Duck farms with proportion 60%, ($CI_{95\%}$: 14.6–94.7%) while H5N1 clade 2.3.2.1c B (63%, $CI_{95\%}$: 40.6–81.2%) and reassorted H5N1 (67%, $CI_{95\%}$: 29.9–92.5%) were mostly discovered in backyard chicken farms as displayed in Figure 4.Interestingly the observed clinical signs showed that the neurological signs were also predominantly in Ducks.

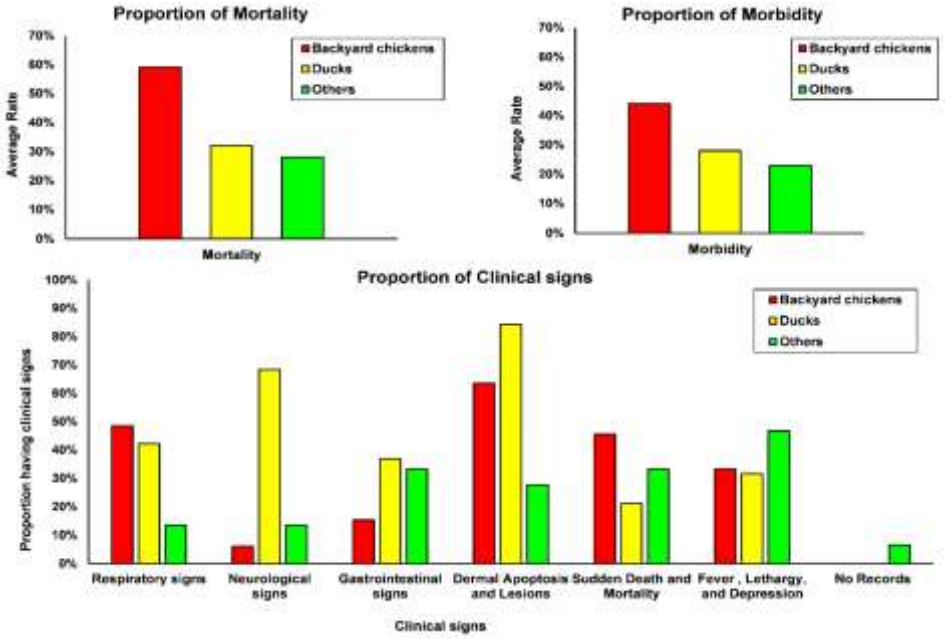

Figure 3. The average mortality and morbidity per farm in the different poultry types and the morbidity associated clinical signs.

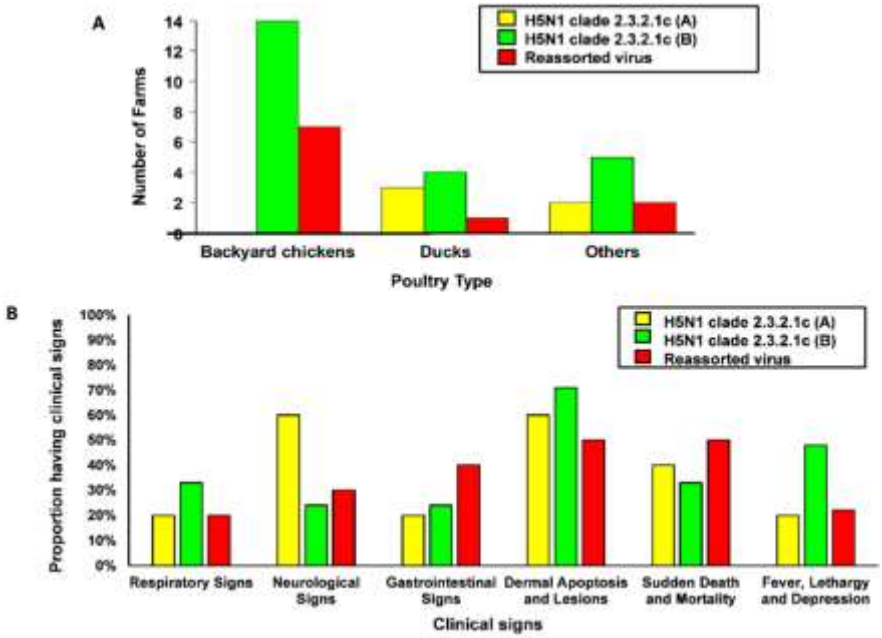

Figure 4. The number of H5N1 clade 2.3.2.1c A and B and reassorted H5N1 viruses in different poultrytypes (**A**) and the morbidity associated clinical signs in different subgroup H5N1 clade 2.3.2.1c A and B and reassorted H5N1 viruses (**B**) as mentioned in a parallel study [27].

3.2. Description of Controls

A total of 60 control farms were included in the study (Figure 1) Distribution of potential risk factors across the control farms is shown in Figure 2. The control farms included commercial broilers (36 farms), broiler breeders (seven farms), layers (seven farms), backyard chickens (four farms), ducks (one farm) and native chickens and quails (five farms). Most control farms housed more than 1000 birds (55 farms) and kept poultry for commercial purposes (56 farms) and used an open house system (47 farms) as displayed in Table S1.

3.3. Factors Associated with HPAI Outbreaks

In univariate logistic regression all factors were significantly associated with the odds of an HPAIoutbreak (Table 1). Multivariate analysis showed, however, that only Poultry Type, Farm Size and incoming contacts were significantly associated with HPAI outbreak. The analysis unveiled that ducks had 15.4 times higher odds of an HPAI outbreak ($CI_{95\%}$: 1.12–706) compared to backyard

chickens, while other poultry types had a similar odd compared to backyard chickens. Furthermore, larger farms had a much lower odds to get infected than small farms (OR: 0.019, $CI_{95\%}$: 0.00095–0.128). Also, farms having at least 10 visitors per fortnight had 13.5 times higher odds ($CI_{95\%}$: 1.7–300) than those visited less frequently (Table 1).

Table 1. The odds ratio of Outbreak and Control farms with statistical significance univariate and multivariate analysis.

	Factors	**Categories**	**Odds Ratio**		
			Estimate	**Std.Err**	***p*-Value**
Univariate Analysis	District	Indramayu	Ref (1)		
		Subang	0.54	0.16–1.65	0.28851
		Rest	0.054	0.017–0.15	1.46×10^{-7} ***
	Poultry Type	Backyard Chickens	Ref (1)		
		Ducks	2.71	0.295–44.103	0.502
		Others	0.031	0.008–0.0923	8.75×10^{-9} ***
	Farm Size	<1000	Ref (1)		
		≥1000	0.015	0.0044–0.044	5.67×10^{-13} ***
	Housing System	Closed house	Ref (1)		
		Open (shed) house	6.085	1.84–27.62	0.00689 **
	Purpose	Household	Ref (1)		
		Commercials	0.054	0.015–0.152	3.86×10^{-7} ***
	Sector	Sector 4	Ref (1)		
		Sector 3	0.038	0.0102–0.1082	2.58×10^{-8} ***
		Sector 1	0.019	0.003–0.086	1.93×10^{-6} ***
	Inbound contacts	<10	Ref (1)		
		≥10	25.106	4.95–458.76	0.002 **
Multivariate Analysis	Poultry Type	Backyard Chickens	Ref		
		Ducks	15.442	1.12–706	0.082084.
		Others	1.222	0.14–26.58	0.869206
	Farm Size	<1000	Ref		
		≥1000	0.0190	0.00095–0.12	0.000503 ***
	Incoming Contacts	<10	Ref		
		≥10	13.536	1.65–299.88	0.033523 *

Discussion

The aims of this study were to detect outbreaks of HPAI H5N1 in an endemically infected region, describe their characteristics and identify risk factors associated with these outbreaks. A total of 64 outbreaks were detected, indicating that HPAI A(H5N1) is still endemically circulating in this part of Indonesia, mostly in backyard chickens and ducks. New findings of the study arethe association between incoming contacts and the odds of an HPAI A(H5N1) outbreak and a comprehensive description of the clinical manifestation of outbreaks in various poultry types of the currently circulating virus clade 2.3.2.1c.

In this study, 75 suspected HPAI outbreaks were reported and 64 of these were confirmed (85.3%). This indicates a high specificity of field diagnosis by district animal health officers and community-based reporting. Participatory disease surveillance programs may have increased the ability of poultry farmers to recognize HPAI outbreaks. The higher specificity of clinical diagnosis in this study (85%) than previously observed (29%) [30] also underpins the capability of the district animal health officers to collect samples from clinically suspected animals and it indicates that the samples have been handled and stored correctly before testing. This shows the potential of an HPAI outbreak investigation in Indonesia. In that respect it is notable that the number of detected outbreaks in the study period in the seven districts was much higher than during the years before and after (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail). This may have been due to the use of incentives in this study. On the other hand, results of genetic analyses of the samples suggested many undetected outbreaks during the study period [27], indicating that the sensitivity of passive surveillance during the study period was still limited. Vaccination may have contributed to reduced sensitivity of passive surveillance, as it can lead to silent spread of the virus [10]. Nevertheless, the four vaccinated outbreak farms had a clear clinical manifestation of the infection, which could have been due to a mismatch between vaccine and circulating virus or improper vaccination of the flocks.

Overall morbidity and mortality were higher in backyard chickens than in ducks, which may be due to better adaptation of the virus to ducks [31–33]. Nevertheless, clinical signs of HPAI observed in the ducks were more prominent than previously reported [31,32]. Neurological signs in particular were observed frequently. Also, dermal apoptosis and lesions were observed frequently in ducks, but also in backyard chicken. The results suggest that clade

2.3.2.1c of HPAI A(H5N1) is more virulent to ducks than the previously circulating clade 2.1.3. Our observations are in line with those observedin ducks after experimental inoculation of this virus clade [34], but the background for the difference in clinical manifestations of the two different subgroup of A(H5N1) clade 2.3.2.1c (A and B) is not clear yet. Previous pathobiological study of A(H5N1) infections showed different clinical manifestation of Indonesian A(H5N1) viruses representing clades 2.1.1 and 2.1.3 in ducks and chickens [35]. The clinical manifestation upon experimental infection with A(H5N1) viruses representing different groups in clade 2.3.2.1c has not been reported yet.

Ducks have a higher risk of HPAI than other poultry types, which is in agreement with previous studies in Indonesia [36–38]. Free ranging ducks in the post-harvest paddy fields can easily encounter AI viruses from wild bird populations or other free ranging ducks. Also, contacts between duck flocks support the transmission of HPAI between duck farms in Indonesia [39].

Farm size was significantly associated with HPAI in this study. The lower risk of HPAI in largerfarm size (≥1000 birds per farm) is in contrast with previous studies in Indonesia and a study in Thailand [18,40]. Most likely flock size acts as a confounder in this study for better management practices, better vaccination programs and better biosecurity in large farms than in small ones in West Java [17]. To our knowledge this is the first study showing that an increasing rate of persons visitingthe farm is associated with an increased risk of HPAI infection. Consequently, advising farmers to minimize admittance of visitors to their poultry and ensure visitors follow biosecurity protocols are potential measures to reduce HPAI infection. Although Figure 1 shows a clustering of outbreaks across the districts, the factor district was deleted in the stepwise backward selection to get to the final statistical model, because it was not significantly associated with HPAI outbreak. This implies that the significant factors in the final model "poultry type, farms size and incoming contacts" were not evenly distributed across the districts and from that the model concludes that it is actually these factors that explain the difference between outbreak and control farms, and not the districts.

This study has its limitations. Vaccination data of control farms were unavailable so the odds of vaccination as risk factor could not be quantified. The presence of four vaccinated farms among the outbreaks shows, however, that vaccination does not prevent all outbreaks. Participation of farms in the study depended on the willingness of poultry farmers and industry to cooperate, which may have introduced bias. Nevertheless, this study is unique, because it was based on enhanced passive surveillance and structured investigation of suspected outbreaks in a restricted area and time period,which has not been

performed in Indonesia before.

Results of this study indicate that the HPAI A(H5N1) virus is still endemically circulating in West Java. Also vaccinated flocks can be affected by HPAI, indicating poor match between circulating virus and vaccine, or too low vaccination coverage. Further change of the Indonesian poultry production system to bigger farms able to invest in proper biosecurity and cold chain slaughter systems might improve the HPAI situation on the long term [41–45]. Until then, to reduce virus circulation and lower the risk of human exposure increasing vaccination coverage combined with outbreak detection in backyard production systems is required, in addition to reducing the rate of visitors in contact with poultry as demonstrated by this study.

4. Conclusions

HPAI A(H5N1) was still endemically circulating in West Java 2015-2016, in particular in ducks and in backyard chickens. Despite the endemic circulation, morbidity and mortality observed in outbreaks are still high, and in ducks neurological signs frequently occur. The rate of persons visiting a farm was associated with the odds of HPAI infection. Results indicate that better external biosecurity might reduce transmission of HPAI A(H5N1) in Indonesia.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of a potential conflict of interest.

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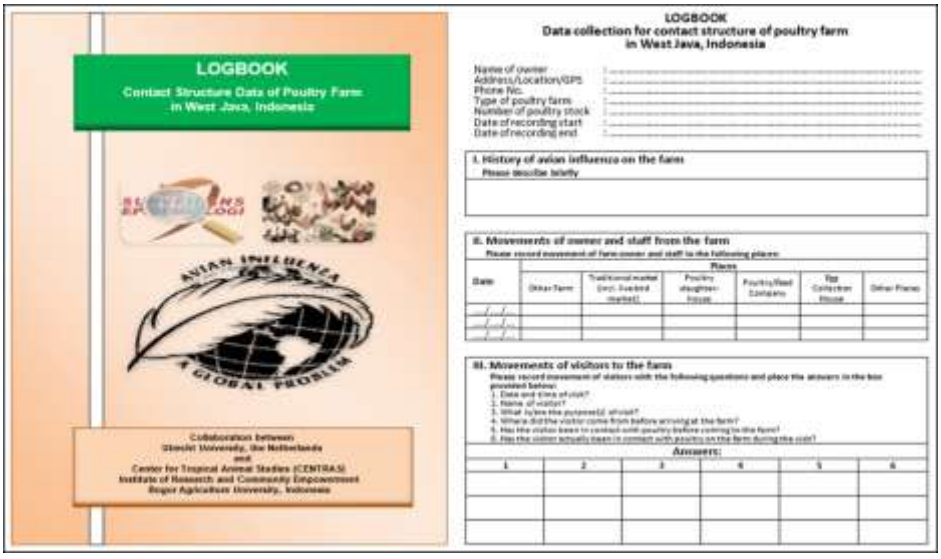

Supplementary Figure S1 Logbook for epidemiological data of normal farms.

Supplementary Figure S2. A few parts of outbreak questionnaire

CHAPTER 4

Reassortments among Avian Influenza A(H5N1) Viruses Circulating in Indonesia, 2015-2016

Desniwaty Karo-karo, Rogier Bodewes, Hendra Wibawa, I Made Artika, Eko Sugeng Pribadi, D. Diyantoro, Widya Pratomo, Agus Sugama, Nani Hendrayani, Iin Indasari, Michael Haryadi Wibowo, David Handojo Muljono, Jan Arend Stegeman, Guus Koch

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Highly pathogenic avian influenza (HPAI) A(H5N1) viruses have been circulating since 2003 in Indonesia, with major impacts on poultry health, severe economic losses, and 168 fatal laboratory-confirmed human cases. We performed phylogenetic analysis on 39 full-genome H5N1 virus samples collected during outbreaks among poultry in 2015–2016 in West Java and compared them with recentlypublished sequences from Indonesia. Phylogenetic analysis revealed that the hemagglutinin gene of all samples belonged to 2 genetic groups in clade 2.3.2.1c. We also observed these groups for the neuraminidase, nucleoprotein, polymerase, and polymerase basic 1 genes. Matrix, nonstructural protein, and polymerase basic 2 genes of some HPAI were most closely related to clade 2.1.3 instead of clade 2.3.2.1c, and a polymerase basic 2 gene was most closely related to Eurasian low pathogenicity avian influenza. Our results detected a total of 13 reassortment types among HPAI in Indonesia, mostly in backyard chickens in Indramayu.

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1. Introduction

Highly pathogenic avian influenza viruses (HPAI) continue to be a major global problem for both animal and human health. Since the first outbreak of HPAI A(H5N1)in Guangdong, China, in 1996, these viruses have causedoutbreaks in various species of birds globally. HPAIH5N1 is endemic in multiple countries and causes a major impact on poultry health and severe economic losses. In addition, >860 laboratory-confirmed human cases of HPAI H5N1 have been reported to the World Health Organization(WHO). In Indonesia, 200 laboratory-

confirmed human cases of avian influenza A(H5N1) have been reported, with a case-fatality rate of 84%, which is higher than the currentglobal case-fatality rate of 53% (*1*). The zoonotic potentialof HPAI is a global public health concern, particularly in preventing a potential pandemic (*2*,*3*).

In Indonesia, HPAI H5N1 has been endemic in poultrysince 2003 and continues to cause major economic losses to both poultry industry and backyard farms. The disease has been reported in 32/34 provinces, resulting in the deathof millions of birds (*4*,*5*) and the closure of many farmsin high incidence areas (*6*). While HPAI H5N1 viruses continuously circulated among poultry in Indonesia during2003–2010, the hemagglutinin (HA) genes evolved from clade 2.1 into multiple subclades, according to the unified nomenclature system for the HA gene of HPAI H5N1 virus (7). In 2012, a new virus classified as clade 2.3.2.1 was detected in ducks, suggesting a new incursion of HPAI H5N1viruses in Indonesia from other parts of Southeast Asia (*7*–*9*). Vaccination programs have been applied to control the spread of HPAI H5N1 but have not prevented it because oflow vaccination coverage and use of unlicensed vaccines. These problems have led to the emergence of antigenicallydistinct HPAI H5N1 virus clades in Indonesia (*10*). In addition to the continuous circulation of HPAI H5N1 virusesin poultry, transmission to humans has been reported in Indonesia since 2005 (*1*).

Clarifying the epidemiology of HPAI H5N1 requires more intense monitoring of outbreaks of HPAI in Indonesia and performing genetic and phylogenetic analysis on viruses detected during these outbreaks. However, recent information on the genetic divergence of HA, and in particular on other gene segments, is very limited (*8*,*11*–*13*), and samples are often collected in a nonsystematic way. Therefore, the aim of this study was to perform genetic and phylogenetic analysis on recent HPAI H5N1 viruses that were obtained from poultry during active searches for outbreaks in West Java, a province of Indonesia. West Java was selected for this study because it has a high poultry density, multiple different farming systems and live-bird markets, and several environmental components that all form risk factors for HPAI H5N1 virus transmission. Moreover, because a high percentage of the land in this region is paddy fields and water sources, free-ranging ducks and chickens undermine the effectiveness of prevention and control measures, resulting in the continuous circulation ofthe virus (*14*,*15*).

2. Materials and Methods

2.1. Sample Collection

During April 2015–October 2016, district animal health officers of the West Java Animal Health Authority collected samples from birds in 6 districts of West Java Province: Subang, Indramayu, Tasikmalaya, Purwakarta, Sukabumi,and Bandung (Figure 1). The districts were chosen on the basis of the history and reoccurrence of HPAI outbreaks. In addition, these districts have multiple sectors of poultryfarms using various production systems and a high densityof poultry farms that have ≥50 birds/farm (*4*,*16*).

The samples were collected after detection of clinical signs in or increased mortality of birds. The criteriafor increased mortality were set at >5% of the population in birds vaccinated against H5N1 and 10% in those unvaccinated for 2 consecutive days. When the criteria were met, oropharyngeal and cloacal samples were collected from 5 sick birds and pooled into viral transport medium containing brain–heart infusion broth and antimicrobial drugs according to European Union instructions (http:// extwprlegs1.fao.org/docs/pdf/eur65757.pdf). The specimens were kept chilled and shipped by overnight courierto the 2 collaborating veterinary laboratories, Disease Investigation Center (DIC) Subang and West Java Animal Health Laboratory Cikole.

2.2. Sample Screening

We tested the collected samples in veterinary laboratories using a national protocol for influenza A screening developed from a real-time reverse transcription PCR (RT-PCR) targeting the matrix gene. Specimens with a cycle threshold value <30 were inactivated using binding buffer of High Pure Viral RNA kit (Roche Applied Science, http://www.roche.com), and transported to the Eijkman Institutefor Molecular Biology in Jakarta for Sanger sequencing. Two additional HPAI H5N1–positive samples, collectedin 2016 and obtained from the Animal Health Laboratory (AHL) Cikole of West Java, were also inactivated and included in this study for Sanger sequencing.

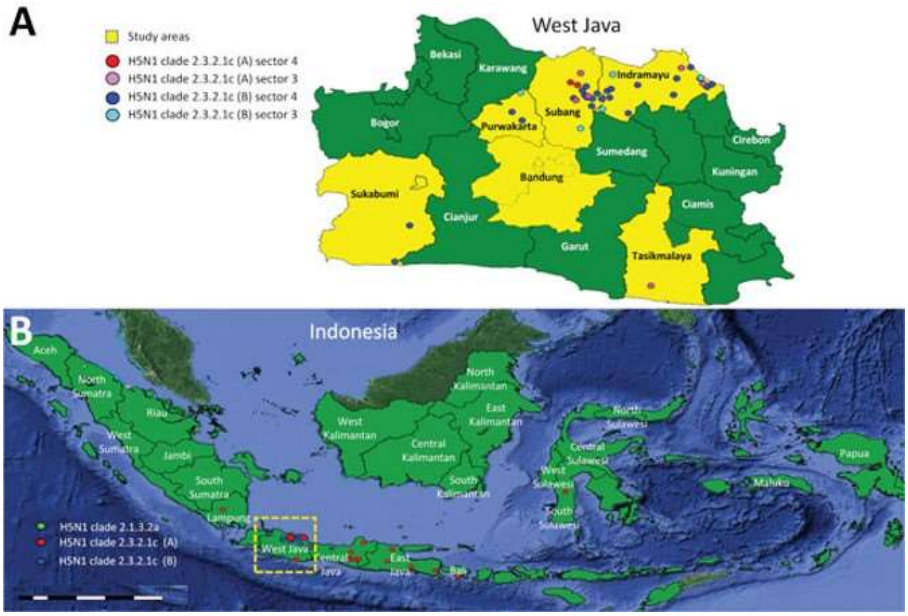

Figure 1. Locations of sampling areas and of different hemagglutinin (HA) clades in study of avian influenza A(H5N1) viruses circulating in Indonesia, 2015–2016. A) West Java Province; B) location of province in Indonesia (box). Data were compiled from this study and additional sequence data of Directorate General for Livestock Services, the Indonesian Ministry of Agriculture, and submitted to GenBank (accession nos. EPI1009273–463).

2.3. *Sequencing*

At the Eijkman Institute, we rescreened the specimens and extracted RNA in accordance with the protocol of the manufacturer and synthesized cDNA by Invitrogen Super Script III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, http://www.thermofisher.com) with Uni12 primer (*17*). On specimens that tested positive in this PCR, we performed additional PCRs to amplify other gene segments present in the samples. We performed amplification of the full genomes of HPAI H5N1 viruses using a 2-step RT-PCR TaKaRa Z-Taq DNA Polymerase (Takara Bio, http://www.takarabio.com) or Toyobo KOD FX Neo (Toyobo, http://www.toyobo-global.com) if the genomes were not successfully amplified using the Takara product.

The primers used were primarily designed by Wageningen Bioveterinary Research. We obtained additional primer sequences from the Australian Animal Health Laboratory and from scientific literature (*17*,*18*) and applied them to

unsuccessfully sequenced gene fragments that could not be amplified by standard primers.We purified the amplified PCR products with Roche High Pure PCR Product Purification Kit (Roche) or Zymoclean Gel DNA Recovery Kit (Zymo Research, https://www.zymoresearch.com) for PCR products for which gel separation was necessary, and subsequently sequenced them using a BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (both from Thermo Fisher).

2.4. *Genetic and Phylogenetic Analysis*

We assembled and edited sequences with Lasergene SeqMan Pro version 12 (DNASTAR, http://www. dnastar.com) and aligned them by using MUSCLE (*19*). We initially determined HA clade of sequenced HPAIH5N1 viruses using the Highly Pathogenic H5N1 Clade Classification Tool of the Influenza Research Database (https://www.fludb.org) and confirmed results through further phylogenetic analysis (*20*). We estimated phylogenetic inference using the maximum-likelihood method with 1,000 bootstrap replicates (Figure 2; Appendix 1 Figure 1, https://wwwnc.cdc.gov/EID/article/25/3/18-0167-App1.pdf). We chose the most suitable substitution rates and pattern model based on the lowest Akaike information criterion for each alignment. Evolutionary distances were computed using average pairwise distance (APD) between and within sequence groups. Evolutionary analyses and APD were conducted in MEGA6 (*21*).

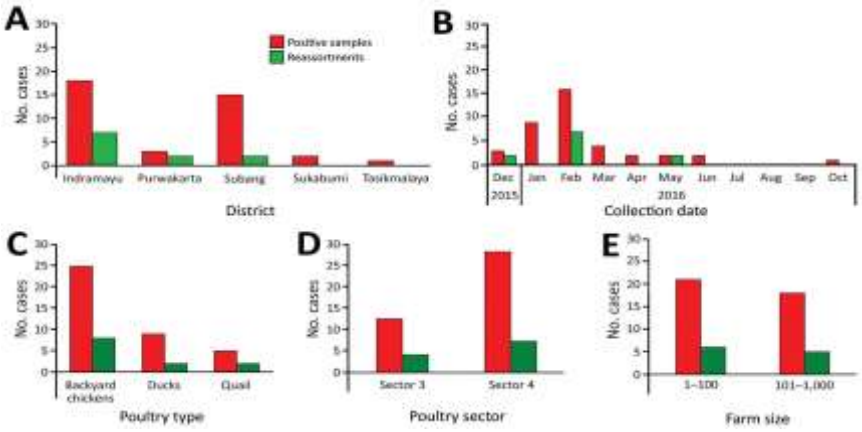

Figure 2. Number of samples in study of avian influenza A(H5N1) viruses circulating in Indonesia, 2015–2016, by district (A), time (B), poultry type (C), poultry sector (D), and farm size (E) from which the complete HPAIV H5N1 genome could be obtained.

We aligned the sequences of HPAI H5N1 gene segments collected during this study with reference sequences from GenBank (Appendix 1 Figures 1–8) using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We included in the analysis sequences obtained from viruses detected during other recent outbreaks in Indonesia (2014–2016). These viruses had been collected via passive outbreak surveillance by the Disease Investigation Centres (DIC) in Medan, Sumatra; Wates, Central Java; and Denpasar, Bali, under the Directorate General for Livestock and Animal Health Services and the Indonesian Ministry of Agriculture (DGLAHS-MoA). Viruses were submitted by DIC Wates of DGLAHS-MoA to GenBank, and then downloaded to GISAID (https://www.gisaid.org; accession nos. EPI1009273–463) (Appendix 2 Table 1, https://wwwnc. cdc.gov/EID/article/25/3/18-0167-App2.xls). For sequencing, we used mainly viruses from original material, as well as some isolates obtained after 1–2 passages in embryonated chicken eggs. We deduced reassortment events on the basis of deviant location of sequences in maximum- likelihood trees of different gene segments.

We used deduced HA amino acid sequences to calculate estimated antigenic distances of viruses based on 27 aa residues in HA, as described previously (22). We measured the antigenic distances with 3 HPAI H5N1 strains that are or were routinely used to vaccinate poultry in Indonesia: A/chicken/Legok/2003 (clade 2.1.1); A/chicken/West Java/ PWT-WIJ/2006 (clade 2.1.3.2); and A/duck/Sukoharjo/ BBVW-1428–9/2012 (clade 2.3.2.1c). We used a *t*-test to estimate the significance of the comparison between the 2 averages of antigenic distances.

3. Results

3.1. Detection and Sequencing of HPAI Viruses

A total of 76 pooled samples were collected from various districts of West Java, Indonesia (Figure 1). We observed the highest number of outbreaks in Indramayu in February2016. During April 2015–October 2016, a total of 56 of the samples tested positive for influenza A virus by real-time RT-PCR with a cycle threshold value <30. We obtained the complete genome from 37 oropharyngeal samples and 2 swab specimens of the 55 samples and used these sequences in the analysis. Positive samples with complete genomes were mostly collected in Indramayu (46.15%, 95% CI 30.5%–61.8%) and Subang (38.46%, 95% CI %–53.7%); the highest peak came in February 2016 (41.3%, 95% CI 25.6%–56.5%), and most positive samples came from backyard chickens (69.23%, 95% CI 54.74%–83.71%). The positive samples were primarily from sector4 (69.23%, 95% CI 52.4%–83%), from farms with <100 birds/farm (53.85%, 95% CI 37.2%–70%)

(Figure 2). Sequences comprising the whole genome were submitted to GISAID (Appendix 2 Table 1).

3.2. Phylogenetic Analysis of HPAI H5N1 Genes

Analysis of obtained hemagglutinin (HA) and neuraminidase (NA) nucleotide and deduced amino acid sequence data confirmed that viruses in our samples were HPAI H5N1 with polybasic cleavage motif (Q-R-E-R-R-R-K- R-G-L-F) and (Q-R-E-K-R-R-K-R-G-L-F). Phylogenetic analysis showed that the HA genes of the HPAI H5N1 viruses in our study samples all belong to clade 2.3.2.1c. In-depth analysis revealed that Indonesia 2015–2016 HPAI H5N1 clade 2.3.2.1c has evolved into 2 putative new subgroups, A and B. The APD between the 2 subgroups within clade 2.3.2.1c was >1.5% (3.3% ± 0.4%); the bootstrap value was >60%; and the APDs within the 2 groups withinclade 2.3.2.1c were <1.5% (0.9% ± 0.1% for subgroup A and 1.9% ± 0.2% for subgroup B). One sample collected by DIC Medan in 2016 from Sumatra Island was identified as clade 2.1.3.2a (Appendix 1 Figure 1).

We observed the evolution of clade 2.3.2.1c of Indonesia 2015–2016 HPAI H5N1 viruses into putative new subgroups (A and B) for the polymerase basic 1 (PB1), polymerase (PA), nucleoprotein (NP), and neuraminidase (NA) genes, as became apparent from comparing respective phylogenetic trees of these genes (Appendix 1 Figures 2–5). The APDs of the PB1, PA, NP, and NA genes were computed, although APD for these genes has not been usedyet for HPAI nomenclature. The APD between the 2 different subgroups A and B within clade 2.3.2.1c viruses was 2.3% ±0.3% for PB1, 2.4% ±0.3% for PA, 2.1% ±0.3% for NP, and 3.4% ±0.3% for NA; and the APDs within the 2 different subgroups of clade 2.3.2.1c were 0.8% ±0.1% (A)and 1.6% ±0.2% (B) for PB1, 0.7% ±0.1% (A) and 1.3% ±0.1% (B) for PA, 0.6% ±0.1% (A) and 1.1% ±0.1% (B) for NP, and 0.7% ±0.1% (A) and 1.9% ±0.2% (B) for NA.

We identified 4 different variants of PB2 in HPAI H5N1 cases from Indonesia in 2015–2016, whereas MP and NS consisted of 3 variants. One of the 4 variants in thePB2 gene of HPAI H5N1 viruses collected by DIC from poultry outbreaks in Central and East Java in 2016 was similar to PB2 of LPAI from Asia (Appendix 1 Figures 1, 7, 8).

3.3. Detection of Possible Reassortments

Analysis of obtained sequence data by the maximum-likelihood method revealed the presence of multiple reassortments of HPAI H5N1 virus gene segments of different viruses circulating in Indonesia, using viruses of clade

2.3.2.1c, 2.1.3.2a, and Asia LPAI as parent strains (Figure 3). Based on the complete genome sequences of 37 positivesamples, we identified the district with the most reassorted viruses as Indramayu (20.5%, CI 95% 9.3%–36.5%). The month with the largest proportion of infections was February 2016 (18%, 95% CI 7.5%–33.5%), and the type of poultry with the largest proportion of infections was backyard chickens (15.4%, 95% CI 5.9%–30.5%). We identified ≈18% (95% CI 7.5%–33.5%) of reassorted viruses in poultry sector 4; 15.4% (95% CI 5.95%–30.5%) were in farms with ≤100 birds/farm (Figure 3).

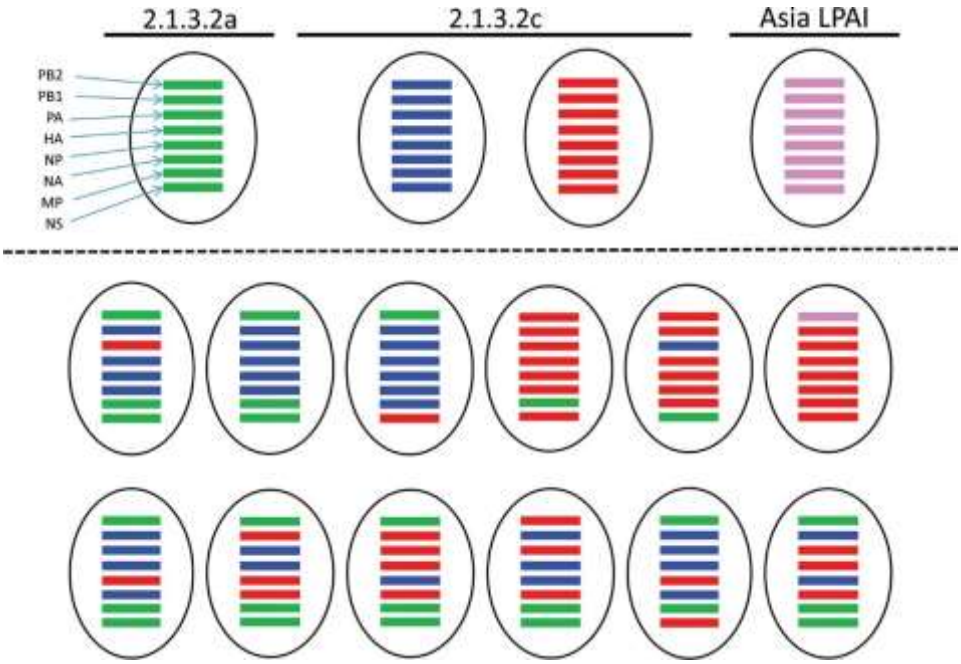

Figure 3. Reassortment eventsof avian influenza A(H5N1) viruses in samples from Indonesia, 2015–2016, some of which were confirmed using maximum-likelihood analysis withparent strains clade 2.3.2.1c, 2005-11 (clade 2.1.3.2a), and Asia low pathogenicity avian influenza virus. Parent strains appear above the dotted line and 13 detected reassortmenttypes below the dotted line. HA, hemagglutinin; LPAI, lowpathogenicity avian influenzavirus; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase; PB1, polymerase basic 1; PB2; polymerase basic 2. Green bars indicate clade 2.1.3.1a; blue, clade 2.1.3.2c subgroup A; red, clade 2.1.3.2c subgroup B; violet,Asia LPAI.

3.4. Antigenic Distance Based on Genetic Distance

It has been demonstrated recently that genetic distances in 27 selected amino acid residues of the HA of HPAI H5 viruses correlate with antigenic distances (*22*). These 27 positions correlate closely with antigenicity and are close to receptor binding sites (*23*,*24*). We observed amino acid changes in the HA of the HPAI H5N1 viruses analyzed in our study at 19/27 selected residues: N72D, D97N, Q115H,S129L, S133A, P136S/P136L, L138Q, S140N, P141S, N154D/N154S, R162K, S163G/S163N/S163T, D183N, E184G, A185G, T188I, K189R/K189M, R212K, M226I (Appendix 2 Table 4).

Results show that the estimated average antigenic distance of HPAI H5N1 viruses from subgroup A was slightlysmaller than from subgroup B to the most recent seed virus vaccine, A/duck/Sukoharjo/BBVW-1428-9/2012. Not surprisingly, these average antigenic distances were lower than to older seed vaccine strains of different clades (A/ chicken/Legok/2003, A/chicken/West clade 2.1, and Java/ PWT-WIJ/2006 clade 2.1.3.2). In all cases, the distance difference between subgroup A or B and the 3 seed vaccinestrains was significant ($p<0.05$) (Appendix 2 Tables 4, 5).

4. Discussion

We performed genetic and phylogenetic analysis on 39 complete genomes of HPAI H5N1 viruses obtained from recentoutbreaks in West Java, Indonesia. The results of genetic analyses of the samples indicated that H5N1 clade 2.3.2.1c viruses are currently circulating predominantly in West Java and Sumatra. The finding of a single clade 2.1.3.2a virus, however, showed that this clade is still present in Indonesia. More systemic surveillance is required to confirm the prevalence of HA clade 2.1.3.2a viruses in Sumatra andJava. Of interest, we detected 2 new subgroups HA within clade 2.3.2.1c. These subgroups are candidate subclades; they share a common node, monophyletic grouping with bootstraps values ≥60, and APD between groups of >1.5%and within groups of <1.5%, fulfilling the criteria designedby the World Health Organization/World Organization for Animal Health/Food and Agriculture Organization (WHO/ OIE/FAO) H5 Evolution Working Group (*7*).

The diversity we detected in the HA subgroups of HPAI viruses in Indonesia in 2015–2016 we also detected in gene segments PB1, PA, NP, and NA, as was apparent by determination of the APD. However, although the APDbetween the groups was >2%, not all bootstrap values were >60. At the least, the calculated APD of PB1, PA, NP, and NAsuggests that genetic variation of these genes is similarto that in HA.

The antigenic distances we deduced of the differencesof 27 aa that determine antigenicity vaccination effectiveness of West_Java/PWT-Wij/2006 vaccines are expected tobe lower against clade 2.3.2.1c than against clade 2.1.3.2a. Whether immunity induced by routine vaccination practices actually, did facilitate (*25*–*28*) the replacement of 2.1.3.2a viruses by clade 2.3.2.1c after its incursion in Indonesia in2012 needs further investigation. Whether vaccination alsoplayed a role in the emergence of subgroup B viruses is lesslikely; the difference in the antigenic distance between sub- group B and the vaccine virus A/duck/Sukoharjo/BBVW-128-9/2012, which came into use after 2012, is rather smalland only just significant. Additional studies of other variables that might have affected the evolution of H5N1 virusin Indonesia, such as transmission efficiency of the virusesin different hosts, are required to prove or reject a possiblerole of vaccination. In all cases, the observed genetic variation combined with its effect on antigenicity illustrates the need for continued intense surveillance and prompt genetic characterization. Calculating antigenic distances based onthe 27 aa of HA could greatly speed up the process of seed virus selection because serologic analyses, antigenic cartography, and experimental vaccination-challenge experiments are time-consuming and costly processes. However,such studies are still crucial to confirm the validity and reliability of this antigenic distance method for seed selection.

We observed the evolution of clade 2.3.2.1c into 2 subgroups in 2 different locations. One subgroup withinthis clade (A) was observed mostly in West Java, whereasanother subgroup (B) was seen in diverse regions of Indonesia (Figure 1; Appendix 1). Additional studies are neededto confirm that there are indeed geographic differences between subgroups A and B and to elucidate possible causes,such as differences in vaccination strategies and differences in trade connections (*29*).

We identified reassortment events in West Java, mostly in backyard chickens in Indramayu. The high poultry density, the presence of different poultry types, and the frequent contacts between poultry farms and between domestic poultry and wild birds may have led to reassortmentin West Java (*14*). A parallel study on contacts of different poultry sectors revealed that backyard chicken farms have the highest contact rate (*30*), which may have facilitated reassortment in West Java. Of interest, a recent study described reassortant HPAI H5N1 viruses in samples collected from live-bird markets associated with suspected human HPAI H5N1 cases in Indonesia (*13*). More intense surveillance programs are required to confirm the prevalence and distribution of the clade 2.1.3.2a and 2.3.2.1c subgroups and its reassortments and to be able to unveil the transmission of HPAI from different sectors, vaccination practices, and regions.

Reassortments between influenza viruses can only occur when a host cell is infected by ≥2 viruses with discretegenomes and when mixing within the host cells produces a hybrid genotype from segments of different parental strains. Because such events are dependent on simultaneous infections with multiple viruses, reassortments are more likely to occur at hotspots such as live- bird markets where different types of birds originating from many different farms, and potentially infected with different viruses, come together (*29*,*31*). Some computational methods have recently been developed to identify a putative reassortment event (*32*,*33*). In this study, the events were identified by maximum-likelihood phylogeny and genetic distance-based methods; we reconfirmed selected reassortments by Graph Incompatibility based on Reassortment Finder usingMarkov chain Monte Carlo computational methods (data not shown).

Phylogenetic analysis of PB2, M, and NS indicated reassortment between viruses circulating in Indonesia. The detection of 3 different variants of M and NS, and 4 different variants of PB2 suggests that reassortment occurs frequently in HPAI viruses in West Java, Indonesia. Of interest, 1 variant of PB2 was highly similar to LPAI from nearby countries: Malaysia (H5N2), Korea (H7N7, H3N8), Japan (H1N1), and Mongolia (H7N1); viruses that until recently had not been detected in Indonesia (*31*). A similar PB2 and putative reassortants with other LPAI viruses were recently reported (*13*). These results suggest that many more LPAI viruses are likely to circulate in Indonesia but are not detected because active surveillance in wild birds or poultry is not performed. Also, diagnostic procedures that solely focus on the detection of H5N1 viruses may contribute to missing influenza viruses ofother subtypes.

The presence of multiple reassortants of HPAI virusesshould be an alert to the regional and international community to strengthen mitigation action plans to prevent thefurther reassortment and genetic drift of the viruses. Preventing virus transmission between poultry flocks, stringent biosecurity measure in (wild) bird markets and keeping poultry separated from wild birds will help to prevent introduction, adaptation, and reassortment of LPAI viruses to a possibly novel zoonotic HPAI virus as currently observed in China and other countries (*18*,*34*,*35*).

Structured, active surveillance in combination with genetic and phylogenetic analysis are urgently needed to reveal these viruses' mutations and potential zoonotic effects, as the viruses rapidly and continually evolve with frequent reassortment (*36*). Also, adequate interventions atlive poultry markets, such as separate markets for different poultry types with higher biosecurity and restructuring of the poultry chain, are crucial to prevent further loss from novel

reassortant HPAI H5N1 viruses (*29*,*37*,*38*).

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APPENDIX

Appendix Captions

Appendix 1 Figure 1. Phylogenetic trees of HA segment of influenza A(H5N1) virus. Evolutionary historywas inferred using the maximum-likelihood method based on the GTR+G model (*1*). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (*2*). Blue indicates subgroup B;red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1.

Appendix 1 Figure 2. Phylogenetic trees of PB1 segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The treewith the highest log likelihood is shown. The percentage of replicate trees in which the associated taxaclustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (*2*). Blueindicates subgroup B; red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1; pink, Eurasian LPAI viruses.

Appendix 1 Figure 3. Phylogenetic trees of PA segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (*2*). Blue indicates subgroup B;red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1.

Appendix 1 Figure 4. Phylogenetic trees of NP segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (2). Blue indicates subgroup B; red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1.

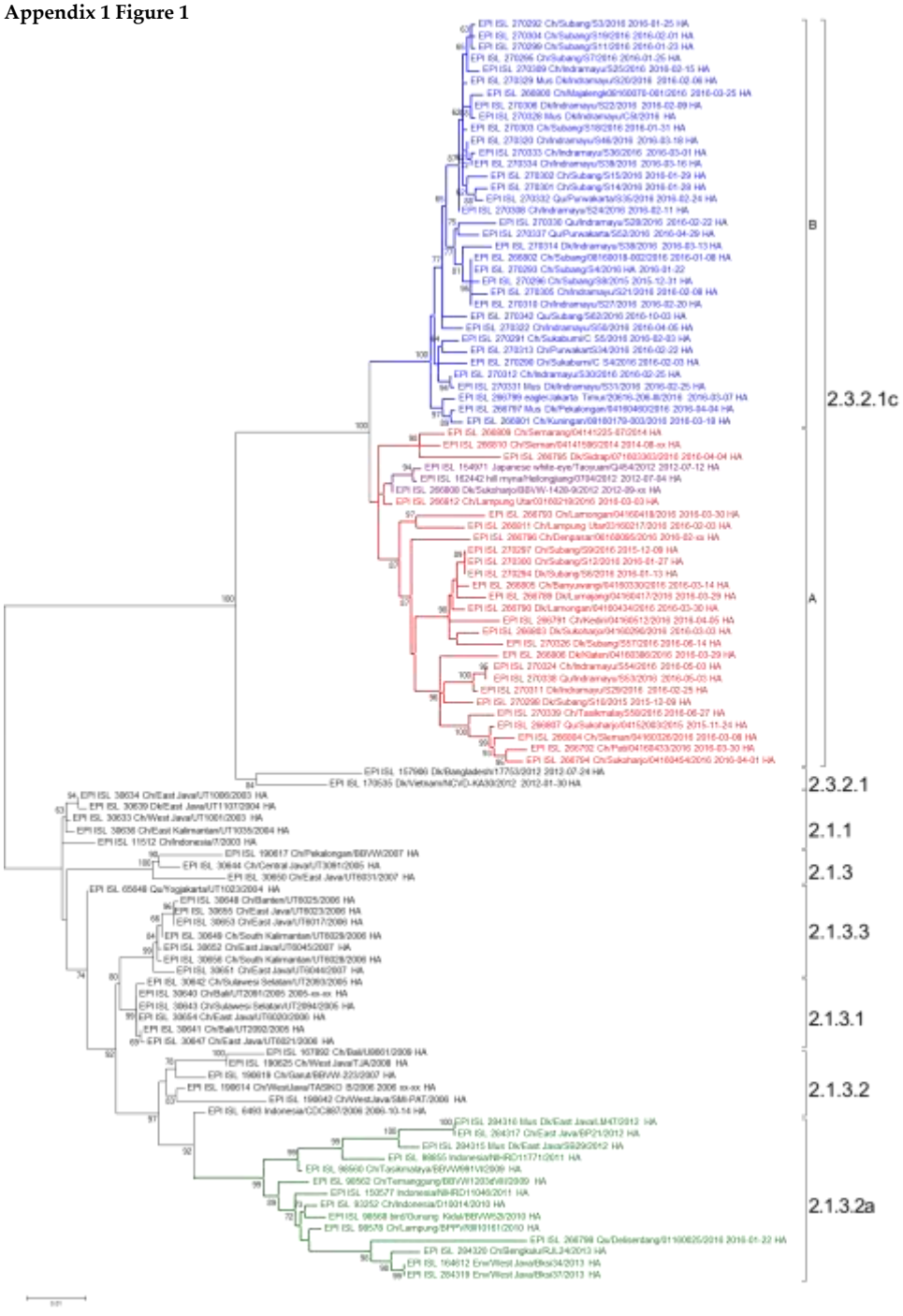
Appendix 1 Figure 5. Phylogenetic trees of NA segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (*2*). Blue indicates subgroup B;red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1.

Appendix 1 Figure 6. Phylogenetic trees of PB2 segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (2). Blue indicates subgroup B; red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1; pink, Eurasian LPAI viruses.

Appendix 1 Figure 7. Phylogenetic trees of MP segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (2). Blue indicates subgroup B;red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1.

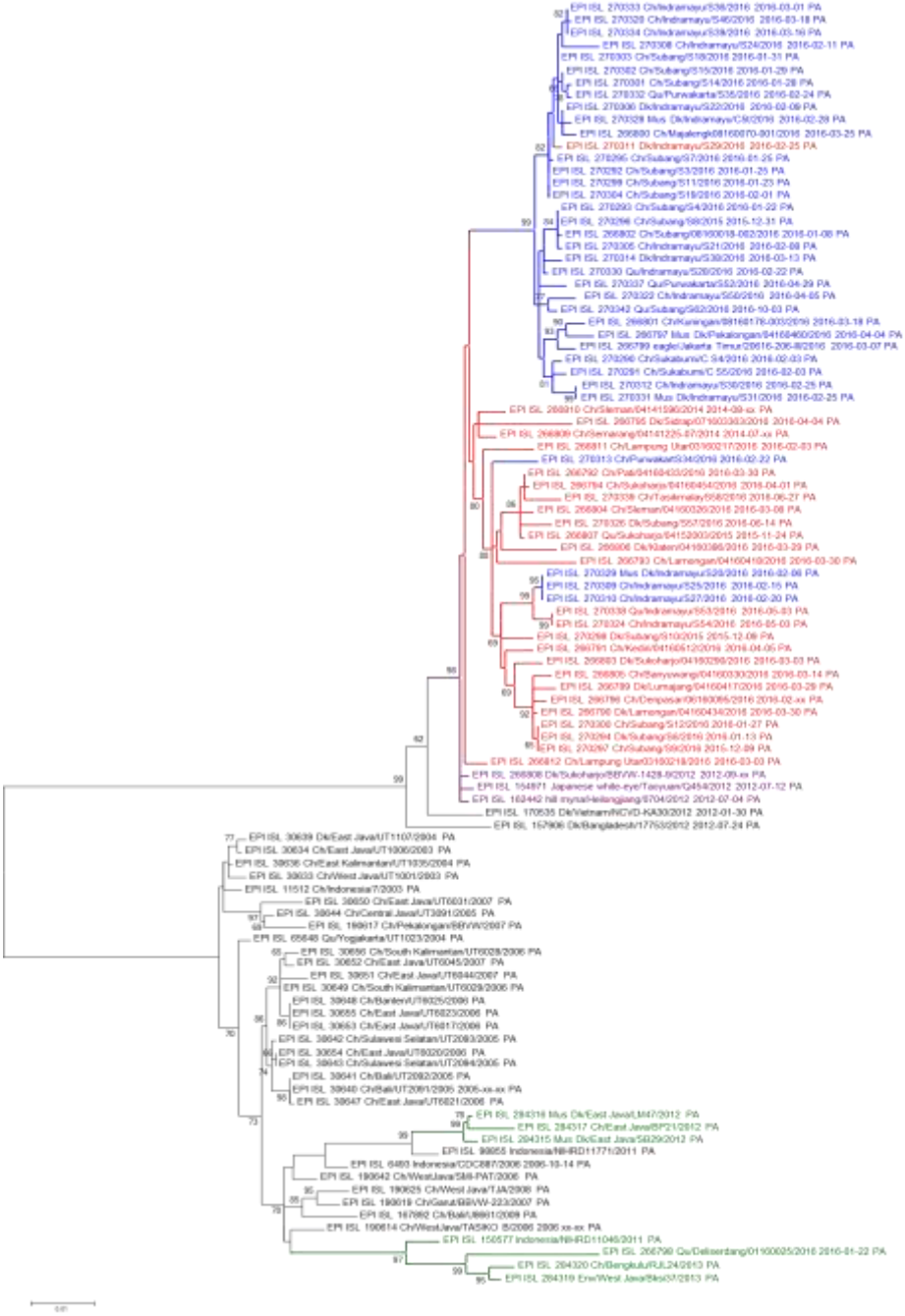
Appendix 1 Figure 8. Phylogenetic trees of NS segment of influenza A(H5N1) virus. Evolutionary history was inferred using the maximum-likelihood method based on the GTR+G+I model (1). The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the nodes. Scale bar represents number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (2). Blue indicates subgroup B;red, subgroup A; purple, strains of clade 2.3.2.1c; green, clade 2.1.3.2a H5N1; pink, Eurasian LPAI viruses

Appendix 1 Figure 1

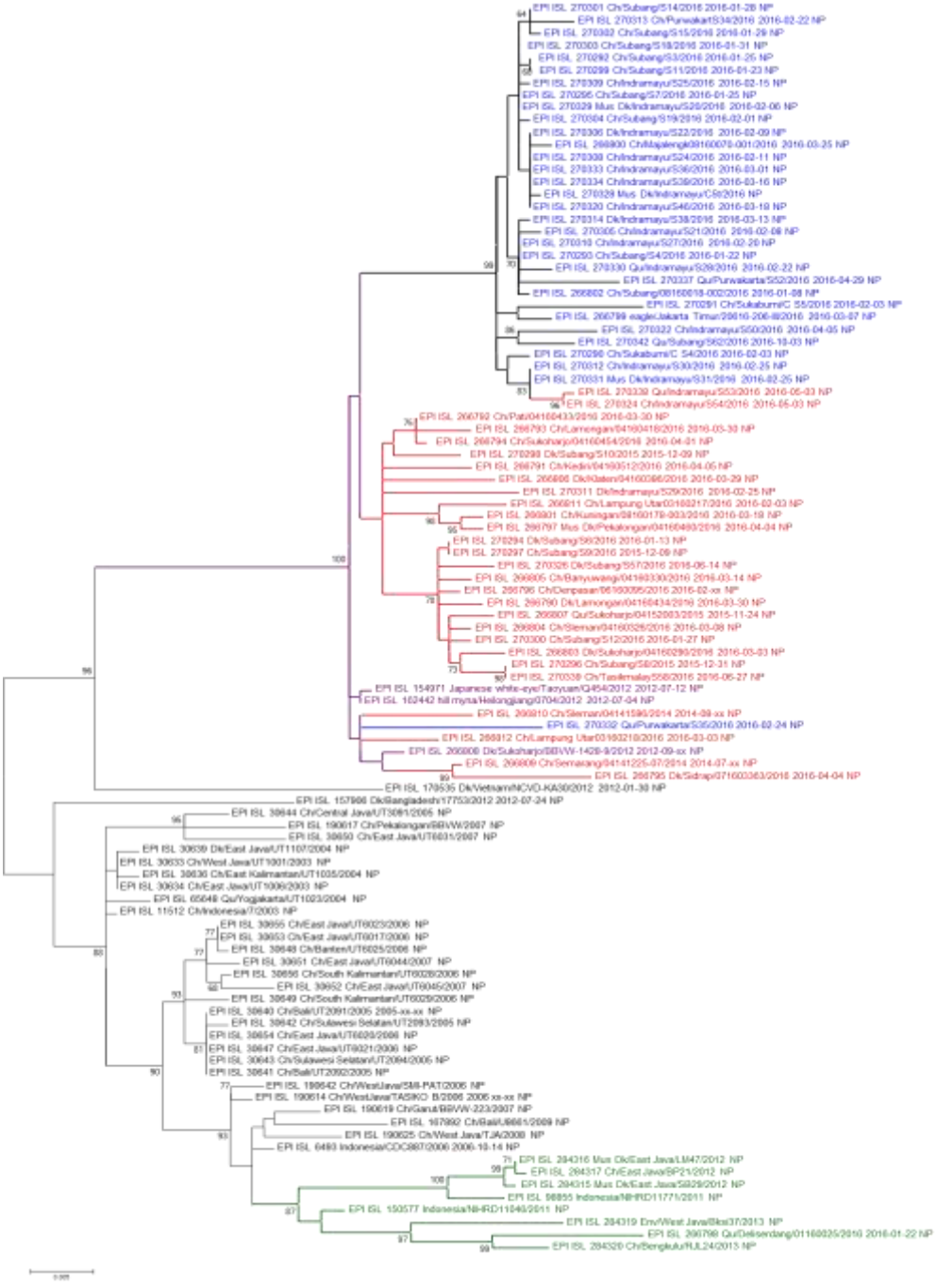


Appendix 1 Figure 2

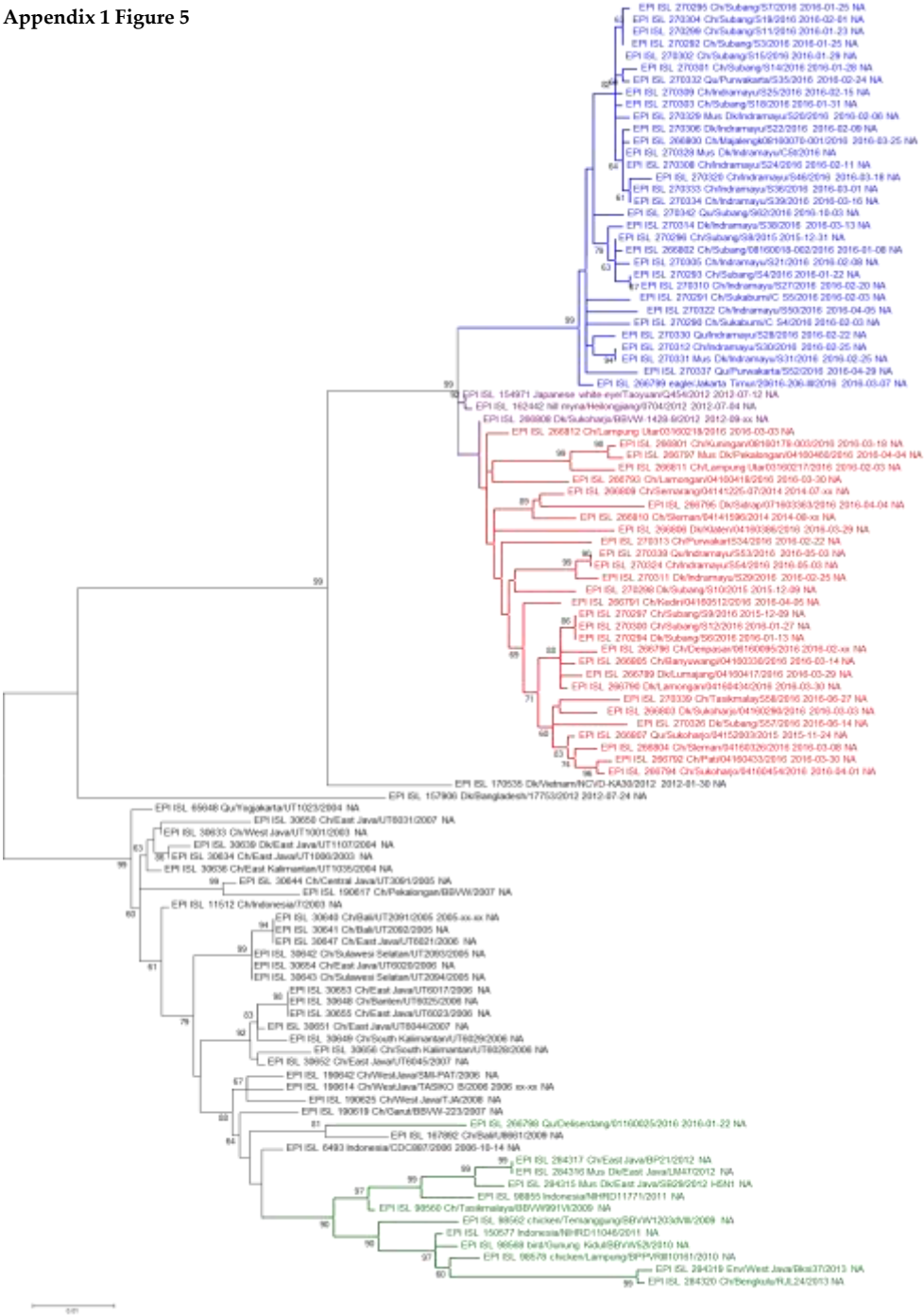
Appendix 1 Figure 3



Appendix 1 Figure 4.



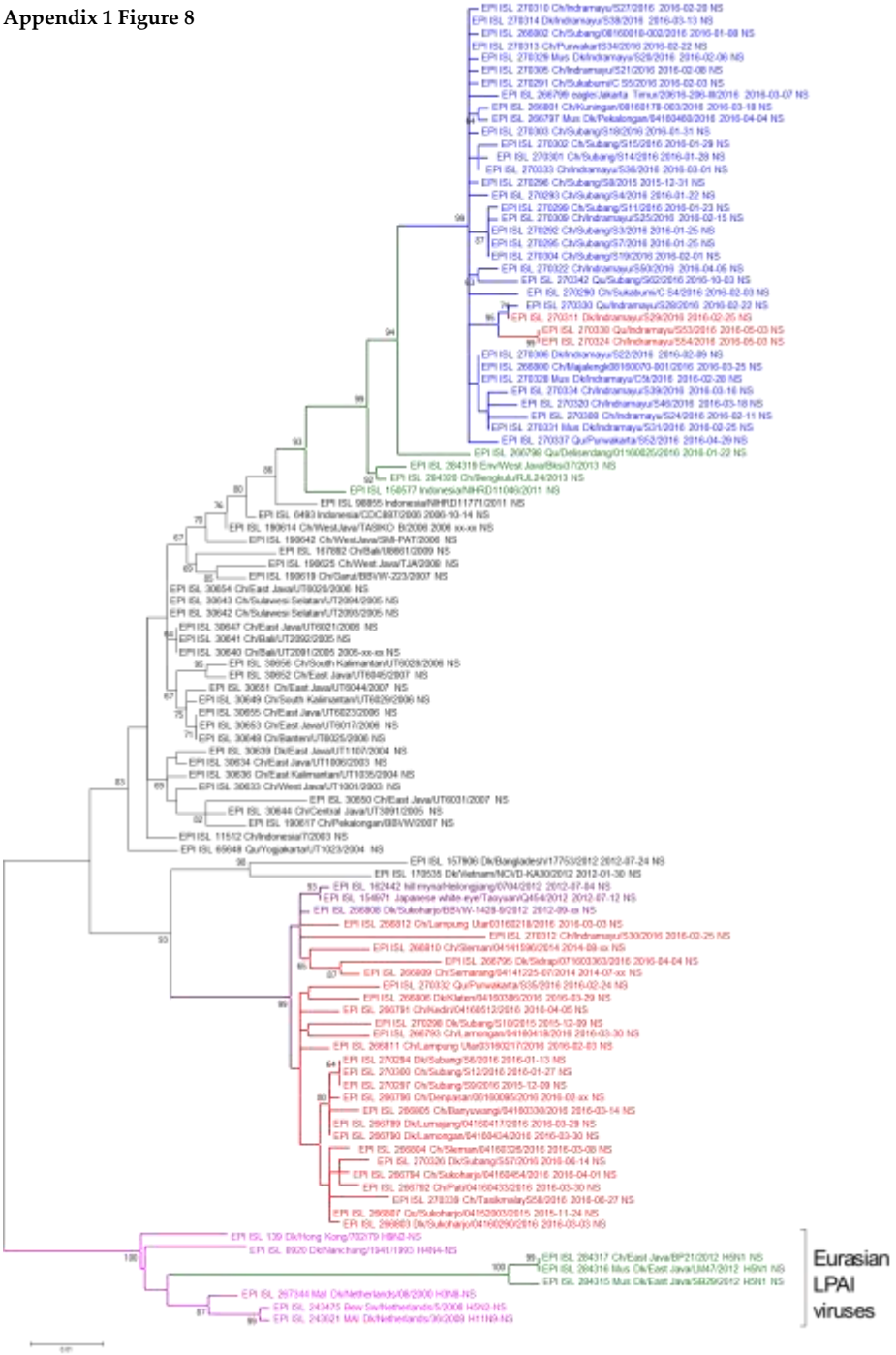
Appendix 1 Figure 5



Appendix 1 Figure 6

Appendix 1 Figure 7

Appendix 1 Figure 8



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CHAPTER 5

Phylodynamics of Highly Pathogenic Avian Influenza A(H5N1) Virus Circulating in Indonesian Poultry

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Phylodynamics of Highly Pathogenic Avian Influenza A(H5N1) Virus Circulating in Indonesian Poultry

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Abstract: After its first detection in 1996, the highly pathogenic avian influenza A(H5Nx) virus hasspread extensively worldwide. HPAIv A(H5N1) was first detected in Indonesia in 2003 and has been endemic in poultry in this country ever since. However, Indonesia has limited information related to the phylodynamics of HPAIv A(H5N1) in poultry. The present study aimed to increase the understanding of the evolution and temporal dynamics of HPAIv H5N1 in Indonesian poultry between 2003 and 2016. To this end, HPAIv A(H5N1) hemagglutinin sequences of viruses collectedfrom 2003 to 2016 were analyzed using Bayesian evolutionary analysis sampling trees. Results indicated that the common ancestor of Indonesian poultry HPAIv H5N1 arose approximately five years after the common ancestor worldwide of HPAI A(H5Nx). In addition, this study indicated that only two introductions of HPAIv A(H5N1) occurred, after which these viruses continued to evolve due to extensive spread among poultry. Furthermore, this study revealed the

divergence of H5N1 clade 2.3.2.1c from H5N1 clade 2.3.2.1b. Both clades 2.3.2.1c and 2.3.2.1b share a common ancestor, clade 1, suggesting that clade 2.3.2.1 originated and diverged from China and other Asiancountries. Since there was limited sequence and surveillance data for the HPAIv A(H5N1) from wild birds in Indonesia, the exact role of wild birds in the spread of HPAIv in Indonesia is currently unknown. The evolutionary dynamics of the Indonesian HPAIv A(H5N1) highlight the importance of continuing and improved genomic surveillance and adequate control measures in the different regions of both the poultry and wild birds. Spatial genomic surveillance is useful to take adequate control measures. Therefore, it will help to prevent the future evolution of HPAI A(H5N1) and pandemic threats.

Keywords: HPAI; H5N1; Indonesia; phylodynamic; Bayesian evolutionary analysis

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1. Introduction

In 1996, the first outbreak of highly pathogenic avian influenza virus (HPAIv) A(H5N1) occurred in China. Subsequently, this virus from the goose/Guangdong (Gs/Gd) lineage spread to multiple other countries. Nowadays, outbreaks of HPAIv A(H5N1) and related HPAIv have caused economic losses due to the deaths and culling of millions of chickens and other poultry worldwide. In addition, 865 human cases of HPAIv A(H5N1) infections were reported with a case-fatality rate of 53% from 2003 to 2022 [1].

The HPAIv A(H5N1) virus was first reported in Indonesia in 2003 and became endemic in multiple regions afterward. The introduction to and spread of HPAIv A(H5N1) within Indonesia was facilitated by several factors [2]. First, Indonesia is located at the crossroads of international trade between two continents (Asia and Australia) and two oceans (Peace, the Indian Oceans). Second, two wild bird migratory flyways, the East Asian–Australasian (EAAF) and the West Pacific (WPF) flyways include Indonesia. Third,the high contact rate between poultry from different locations [3] and between domestic ducks and wild birds due to poor biosecurity, particularly for backyard and moving or scavenging ducks [4]. Virus transmission between farms was facilitated by poultry trade and live bird markets and by human–animal interaction from inbound and outbound visits to poultry farms and live bird markets. Humans, via contact with poultry, could act as a vector of HPAIv A(H5N1) and facilitate transmission between poultry flocks [3,5].

Molecular surveillance is an important tool to support the control of HPAIv A(H5N1). HPAI genome sequence data obtained from avian and human cases

can be used to understand transmission pathways [6], identify molecular markers for disease [7,8], expand host coverage [9], and detect variants associated with vaccine escape [10]. Molecular surveillance can also help to identify possible genetic drift and reassortments of HPAIv A(H5N1) with other influenza A viruses that may result in newly emerging viruses with possible increased transmission in poultry and wild birds, different pathogenicity which may also result in a wider host range [11,12].

Based on the global analysis of genomic data of HPAIv A(H5N1) detected in Indonesia, HPAIv A (H5N1) were classified into various clades, starting with clade 2.1, which subsequently branched into clades 2.1.1, 2.1.2, 2.1.3.2, and 2.1.3.2a; most clades havebeen reported to affect poultry [13–15]. In 2012, a new clade, 2.3.2.1c, was isolated from aduck farm and live bird markets in Java with high mortality among duck and amino changes such as a Ser deletion at position 325 in the multibasic amino acid cleavage site, and a K328R substitution [16]. The detection of HPAIv from this new clade was thought to be the result of a new incursion from other parts of Southeast Asia to Indonesia [11,17], as the clades 2.3.2.1, 2.3.2.1a, and 2.3.2.1b have been reported in other South-East countriessuch as China, Vietnam and Bangladesh [13,14,18,19]. Clade 2.3.2.1c subsequently circulated in poultry, while HPAIv from clade 2.1.3.2a was only detected in Sumatra [11,16,20–22]. A molecular study of HPAIv A(H5N1) carried out in 2015 and 2016 suggested that this new clade had diverged into two putative subgroups, clades 2.3.2.1c (A) and 2.3.2.1c (B) [11].

Although the major clades of HPAIv A(H5N1) in Indonesia are known, there is limited understanding of the evolution of HPAIv A(H5N1) in Indonesia. This knowledge can be useful to help focus surveillance and strengthen control measures aiming to reduce future reassortments and transmission of HPAIv among poultry and humans. The presentstudy aimed to increase the knowledge of HPAIv A(H5N1) evolution in Indonesia from 2003–2016, with a particular focus on the HA gene segment and the jump of the clades ofH5N1v.

To this end, we analyzed the available sequences of hemagglutinin (HA) in the genome database to improve the understanding of the phylodynamics of HPAIv A(H5N1)in Indonesia.

2. Materials and Methods

2.1. Dataset Preparation

Complete sequences of HA genes obtained from HPAIV A(H5Nx) detected in poultry in Indonesia from 2003 to 2016 were downloaded from the genome database, GISAID, and GENBANK and compiled as Indonesian H5N1 (HA).

Another data compilation was downloaded from all available global sequences including Indonesia from 1966 to 2022 and separated as Global H5 (HA). Additional separated data for clades 2.3.2.1c, 2.3.2.1a, and 2.3.2.1 were also downloaded from the database. The HA gene was chosen because the HA protein is located on the outer surface of the virus particle, has a role in the virus–host cell interaction and is the main target for the protective antibody response [23]. Additionally, HA genes are published most frequently in the genome database, indicating that a worldwide phylodynamic analysis of H5N1v using HA geneswill provide the most information.

The sequences were then aligned using MUSCLE [24] and the HA clades of the virus were phylogenetically analyzed using MEGA 7 [25] as described in a previous study [11]. The clade of HA was confirmed using the Highly Pathogenic H5N1 Clade Classification Tool of the Influenza Research Database (https://www.fludb.org/brc/home.spg?decorator=influenza, last accessed on 13 September 2022).

2.2. Clustering HA Gene Segments

The dataset of HA genome sequences was processed with cd-hit-est software of the CD-HIT Suite (http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit-est, last accessed on 13 September 2022) to cluster sequences that shared 100% nucleotideidentity [26–28]. The CD-HIT_EST test was performed on the globally available 12,018 HA genome sequences (1966–2022) irrespective of the accompanying NA. To condense the global taxa of the full genomes of HA genes, 80 to 99% identity thresholds were examined to obtain the cluster representative sequences. Maximum-likelihood analysis with bootstrapping was performed at different thresholds, and clusters of representativetaxa were selected from taxa that share a larger identity than 98%. The representative sequences were used as a dataset for time-scale phylogeny analysis and demography reconstruction.

2.3. Time-Scale Phylogeny of Indonesian HPAIv A(H5N1) Sequences

Divergence times and evolutionary analysis were estimated simultaneously with Bayesian phylogenetic inference (BI) implemented in BEAST v.2.6.7 [29] (http://www.beast2.org/). The optimal substitution model was selected by the BEAST-ModelTest (bModelTest) v.1.2.1 package implemented in BEAST using transdimensionalMarkov chain Monte Carlo (MCMC) methods [30]. The best substitution model from bModelTest was also compared to the best substitution model selected by the Modeltest in the phangorn package implemented in the R (version R-4.0.3) environment for statistical analysis. bModelTest was also used to infer the gamma-distributed rate of heterogeneity, invariable site proportions, and unequal base frequencies [30].

The tree and clock priors were set on a coalescent Bayesian skyline tree and a relaxed molecular clock (assuming an uncorrelated lognormal distribution clock model) which was calibrated by using the sample collection dates. The Bayesian MCMC analysis was performed for 150–300 million generations sampled every 1000–3000 generations.

The parameter convergences were viewed and evaluated using Tracer v.1.7.1 [31] (http://tree.bio.ed.ac.uk/software/tracer/). The maximum clade credibility (MCC) phylogenetic trees were constructed by TreeAnnotator v.2.6.7 (BEAST package) by removing the initial 10–25% (burn-in) trees (burn-in settings depend on convergence). Then, phylogenetic trees were visualized by using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). The calendar date of origin of tMRCA of Indonesian HPAIv A(H5N1) estimated in the BEAST analysis was converted using the lubridate package (https://lubridate.tidyverse.org/) implemented in R (version R-4.0.3).

The first step of BEAST was to analyze the Indonesian HA HPAIv A(H5N1) gene segments from viruses collected from 2010 to 2016. Then, HA gene segment analysis wasperformed separately on viral sequences of three different HA clades, clades 2.1.3.2, 2.1.3.2a, and 2.3.2.1c, collected from 2005 to 2016. To confirm the evolution in 2010–2016, MCMC analysis (BEAST) of the Indonesian HA HPAIv A(H5N1) gene was performed over the extended period of 2003–2016. The BEAST analysis over the period 2003–2016 isdisplayed in the results of Indonesian HA (H5).

In the final stage, we performed BEAST to analyze the worldwide HA of all availableavian influenza viral sequences (2005–2021). The full length 12,018 HA sequences were downloaded from GISAID and clustered using CD-HIT-EST as described above. the Reference sequences closely related to Indonesian HPAIv H5N1 according to the maximum likelihood tree were selected and aligned using MEGA 7 [25] before proceedingto the BEAST analysis.

3. Results

3.1. Bayesian Evolutionary Analysis of HA of Indonesian H5N1

The number of taxa used for the BEAST analysis performed on HA sequences from Indonesia and worldwide with different times of collection and the number of sites is presented in Table 1.

Time-measured phylogenetic analysis of 1707 sites from 94 taxa using the substitution model TIM1 + Γ + I showed the evolution of various clades HPAIv

A(H5N1)in Indonesia (Table 1).

Time-measured phylogenetic analysis (Figures 1 and A1) estimated that HPAIv A(H5N1) clade 2.1-like, 2.1.1, 2.1.2, 2.1.3, 2.1.3.1, 2.1.3.3, 2.1.3.2 and 2.1.3.2a evolved from a common ancestor in the year 2002. In addition, the analysis indicated that some of the HA clades 2.1.1 shared a common ancestor with 2.3.2.1c in the year 2001. Subsequently, the HPAIv A(H5N1) clade 2.1.1 in 2003 diverged into HPAIv A(H5N1) HA clade 2.3.2.1c, which was detected mostly in 2015/16. A significant divergence in 2011 was also observed between HA subgroup 2.3.2.1c (A) and 2.3.2.1c (B) (Figure A1) in the phylogeneticanalysis, with posterior values of more than 0.7 (Figure A2).

In 2015–2016, HPAIv A(H5N1) clade 2.1.3.2a was still detected. In contrast, the 2.1-like 2.1.2, 2.1.3, 2.1.3.1, 2.1.3.2, and 2.1.3.3 were no longer detected in Indonesia after 2012. Detection of clades of HPAIv A (H5N1) in Indonesian poultry varied between years. From 2005 to 2007 (2 years), the HA clades 2.1.1, 2.1.2, and 2.1.3 were detected. Between 2005 and 2012 (7 years), the HPAIv H5N1 clade 2.1.3.1 was detected. From 2005 to 2010 (5years), the virus HA sequences were classified as clades 2.1.3.2 and 2.1.3.3. Of the subclades of HPAIv A(H5N1) clade 2.1, only 2.1.3.2a was still detected in 2016, while subclade 2.3.2.1c was mostly detected after 2010.

Spatiotemporal analysis indicated that various HA clades of Indonesian HPAIv A(H5N1) were detected in different areas (Figure 2). Most viruses were detected on Java Island. HA clades 2.1.3.2 and 2.1.3.2a were detected in most of the regions of Indonesia, while some clades were only detected in specific regions. For example, HPAIv A(H5N1) clade 2.1-like viruses were only detected in Jakarta and Yogyakarta, and HPAIv (AH5N1) clade 2.1.3 was only detected in Central Java, East Java, Yogyakarta, and Bali.

The BEAST analysis estimated that the mean nucleotide substitution rate of HA was 0.0042 substitution/site/year (s/s/y) (95% Interval, 0.0038–0.0046) over the course of 13 years. The mean substitution rate of clade 2.1.3.2a was 0.0042 (s/s/y) (95% Interval, 0.0031– 0.0054) over an 8-year period, not statistically significant different than that of clade 2.3.2.1c (0.0036 s/s/y; 95% Interval, 0.0026–0.0041) over 4 years (Table 1).

Table 1. The substitution rates of Indonesian HPAIv A(H5N1) 2003–2016 and representativeworldwide HA of H5N1v with the estimation of root height.

Hemagglutinin Gene	Collection Time (year)	Taxa (n)	Sites (Character)	Evolutionary Models	Substitution Rate (10^{-3} s/s/y)		Number of Substituted Sequences (Subst/Genome/Year)
					Mean	95% HPD Interval	
Geographical Zone							
Indonesian H5N1 (HA)	2003–2016	502	1559	TVM + Γ + I	0.0042	0.0038–0.0046	6
Global H5 (HA)	2005–2021	284	1723	TVM+ Γ + I	0.0065	0.0061–0.0070	11
HA clade (Indonesian HA (H5N1))							
Clade 2.1.3.2	2005–2010	203	1559	TIM1 + Γ + I	0.0046	0.0036–0.0056	7
Clade 2.1.3.2a	2008–2016	73	1730	TIM1 + Γ + I	0.0049	0.0032–0.0069	7
Clade 2.3.2.1c	2012–2016	94	1707	TIM1 + Γ + I	0.0036	0.0026–0.0046	6

The mean of rates is posteriorly estimated based on Bayesian MCMC analysis using evolutionary models. The number of sequences is labeled as a taxon (taxa). The character of the number of differed sites is normalized from the length of a sequence to get the proportion of differences between two sequences [24]. Abbreviations: TVM (transversion model), TIM (transition model), Γ (gamma), I (Invariant), bp (base pair), s/s/y (substitution/site/years).

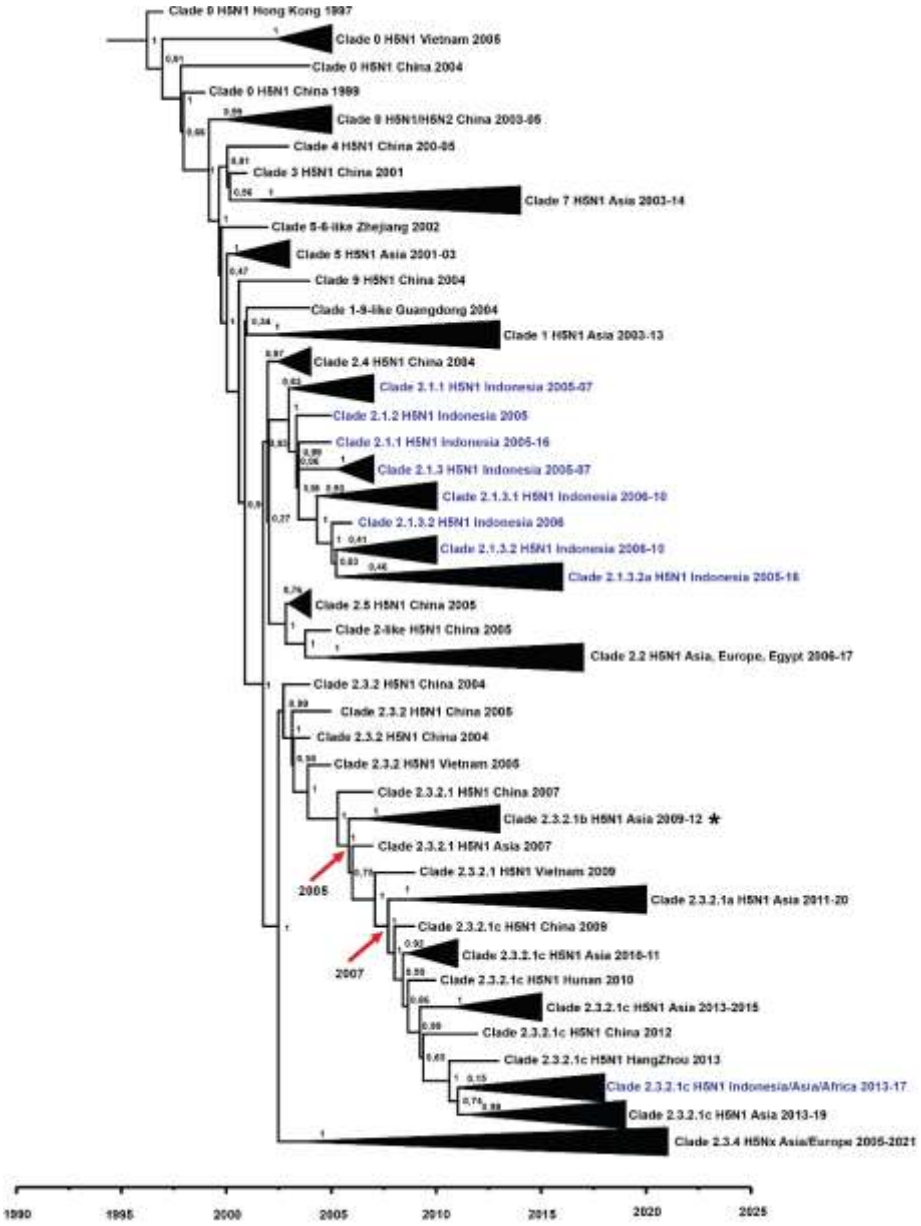

Figure 1. Time-scale phylogeny of selected worldwide HA of H5N1v. The estimated origin of thedivergence of the HA 2.3.2.1c clade is highlighted in the asterisk symbol. The tMRCA of HA clades 2.3.2.1b and 2.2.3.1a are pointed out by the arrow. The blue colour highlights the HA of H5N1v from Indonesia. The node labels display the posterior value. The original sequences (GISAID ID) for worldwide HA of H5N1v phylogeny are displayed in Supplementary Table S1.

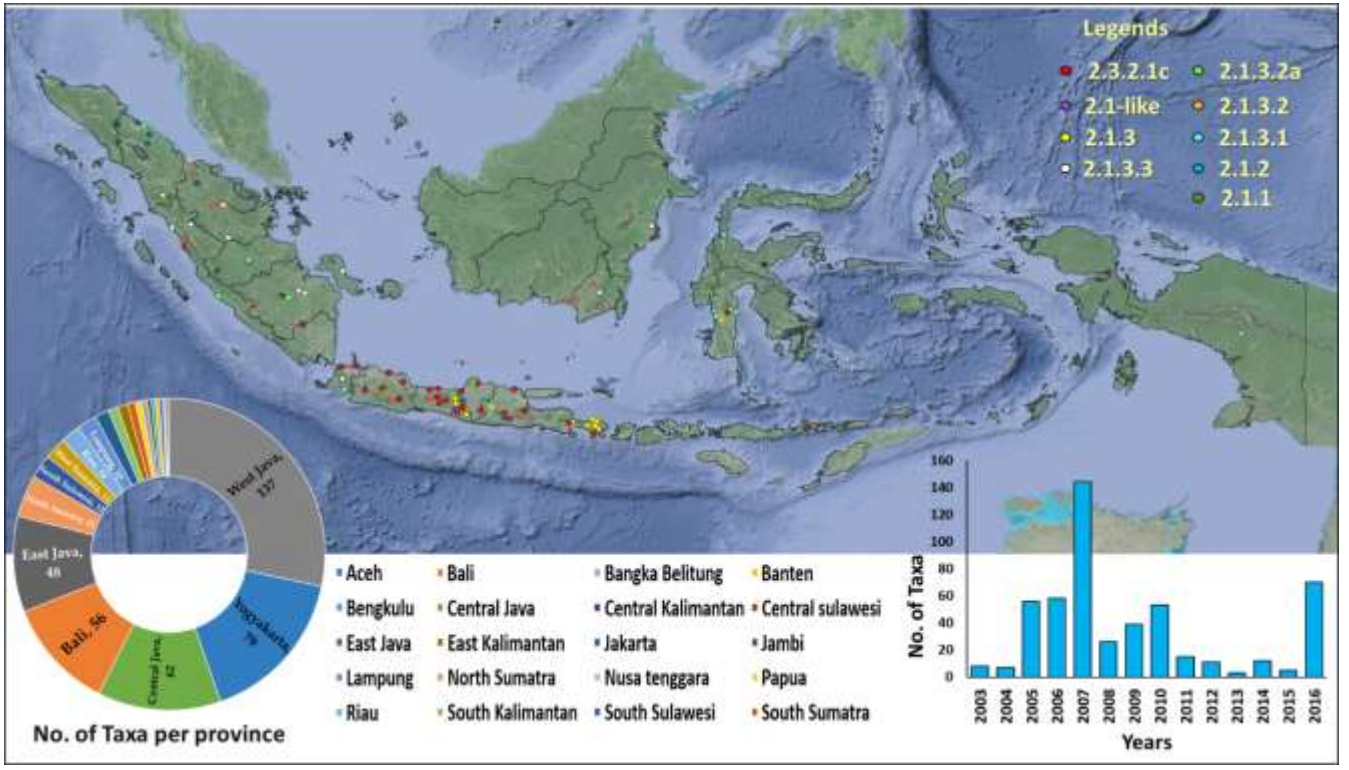

Figure 2. The distribution of HA of HPAIv A(H5N1) was detected in poultry based on different clades and different provinces of Indonesia. The number of taxaper province and per years are displayed in Supplementary Tables S2 and S3.

3.2. Indonesian Viruses in the Phylodynamic of Indonesian Worldwide Avian Influenza H5N1Virus (AI H5N1v)

The phylogeny of the worldwide HA including the Indonesian viruses is depicted in Figure 1. Based on analysis of representative sequences, H5N1v clade 2.1 and its subcladeswere only detected in Indonesia, while clade 2.3 viruses were detected in multiple countries in Asia, Europe, and Africa, including Indonesia, since 2009. Other subclades of clade 2, such as 2.2, 2.4, and 2.5, were circulating in multiple countries, such as China, Egypt, Germany, India, and Japan, but were not detected in Indonesia. These different geographic distributions of viruses also indicate geographic imbalances in virus spread and geographic leaps of multiple viruses from various clades.

The BEAST analysis estimated that the mean nucleotide substitution rate of global HA was 0.0065 substitution/site/year (s/s/y) over the course of 16 years (95% Interval, 0.0061–0.0070) (Table 1).

3.3. Molecular Dating of HPAIv A(H5N1)

Results of molecular dating indicated that the common ancestor of HPAIv A(H5N1)detected in Indonesia occurred in May 2001, around 5 to 7 years after the common ancestor of HPAIv A(H5N1) worldwide. The common ancestor of HPAIv A(H5N1) clades 2.1.3.2. and 2.1.3.2a occurred in the first months of 2002 according to this analysis, while the common ancestor of clade 2.3.2.1c occurred in February 2011. Results of the tMRCAs of HA of HPAIv A(H5N1) detected in Indonesian poultry and worldwide, determined by using a relaxed clock, with 95% HPD and posterior values, are displayed in Table 2.

4. Discussion

4.1. Temporal Dynamic of Indonesian HPAIv A(H5N1): Time-Measured Phylogenetic Analysis

In the present study, a time-measured phylogenetic analysis was performed to increase the understanding of the HPAIv A(H5N1) detected in Indonesia from 2003–2016. While phylogenetic analysis of HPAIv A(H5N1) was the focus of a number of studies already [11,22,32], a study including all available Indonesian virus sequences has, to our knowledge, not been performed previously. Posterior analysis of Indonesian HPAIv A(H5N1) 2003–2016 estimated that the

Table 2. tMRCA of HA of HPAIv (H5N1) Indonesian poultry and worldwide H5, determined by using a relaxed clock, with 95% HPD and posterior values.

HA Gene	tMRCA	95% HPD interval		Posterior
		Begin	End	
Geographical zone				
Indonesian H5N1 (HA)	27 May 2001	13 September 1999	02 July 2002	1.00
Global H5 (HA)	04 April 1996	27 May 1995	28 December 1996	1.00
HA clade (Indonesian HA (H5N1))				
Clade 2.1.3.2	08 January 2002	27 May 1997	13 September 2004	1.00
Clade 2.1.3.2a	15 March 2002	02 July 1997	01 January 2006	1.00
Clade 2.3.2.1c	06 February 2011	13 September 2009	13 September 2011	1.00

HPAIv A(H5N1) clade 2.3.2.1 evolved from the HA clade 2.1.1. In addition, the posterior analyses using BEAST with bModeltest, instead of the maximum likelihood approach, which is used as a criterion in a unified nomenclature system for HPAIv, confirmed the finding of our previous study [11] that HA clade 2.3.2.1c consists of two different clusters [13–15]. The time-measured analysis also showed that after 2012, mainly HPAIv A(H5N1) viruses classified as clade 2.3.2.1c were detected. The observed evolution of HPAIv A(H5N1) viruses, the emergence of new clades, and the emergence of reassortments may have been caused by biosecurity gaps leading to reassortment and limited vaccine efficacy and poor vaccination coverage, although we cannot exclude circulation of these viruses in wild birds due to the very limited surveillance of avian influenza in wild birds in Indonesia. [11,33–35].

The substitution rate of avian influenza viruses worldwide has been studied extensively [18,36,37]. A previous study [38] estimated viral RNA substitution rates in the range of 0.01 to 0.001 s/s/y. Additionally, the rapid evolutionary dynamics of avian influenza viruses were estimated by a previous study with a substitution rate range of 0.0018–0.0084s/s/y [39]. The estimated substitution rate in this study showed the fast substitution rate of Indonesian poultry HPAIv A(H5N1) and HA of worldwide H5, which was in line with previous reports by Duffy et al. (10^{-2} to 10^{-5} subs/site/year) and Chen et al. (1.8 to 8.4 × 10^{-3} subs/site/year) [38,39], but different from those reported by Ducatez et al. (3.32 ± 0.05 × 10^{-3} subs/site/year) [40]. The variation in the substitution rates between the HPAIv A(H5N1) genes can be caused by many factors, such as the differences in viral biologies such as viral genome architecture, replication speeds within-host and viral polymerase enzyme fidelities [41], and environmental selectivity related to the host factors such as species [38], vaccination status [37], contact rate, and age of infection, epidemic, and endemic status in a region during infection [41]. Positive selection pressures related to environmental selectivity have been identified at several antigenic sites of the HA gene in the previous study [22]. Meanwhile, the mean substitution rate of global HA was higher than in Indonesian poultry HPAIv A(H5N1); this observation might, however, be biased by sampling differences

The phylogenetic analysis estimated that HA clades 2.3.2.1a and 2.3.2.1c shared a common ancestor and were rooted in the clade 2.3.2.1b. The H5N1v clade 2.3.2.1c and 2.3.2.1a diverged from clade 2.3.2.1b in agreement with a previous study [13,15]. A gap in the H5N1v clades in Indonesia is indicated by the lack of report of clade 2.3.2.1b, the cladethat has been reported in Vietnam and Hong Kong [15,42]. This clade gap was assumed based on the finding in Indonesia that the HPAIv A(H5N1) clade 2.3.2.1c was rooted in HA clade 2.1.1. Bird migration and/or poultry trade could have driven the transmission and

evolution of the H5N1v clade 2.3.2.1a to clade 2.3.2.1c. Additionally, unrecognized clinical signs in poultry and the reluctance of farmers to report the H5N1 outbreaks, particularly in sector 1 farms, might have contributed to the absence of some clades of H5N1vin the data set. This gap shows the need for regular and intensive surveillance to control the evolution of H5N1v, not only in poultry but also in wild birds.

The most recent ancestor of the H5N1 influenza virus in Indonesia has been previously studied [22,32]. The first study [22] estimated the tMRCA of Indonesian H5N1 HPAIV in June 2003 (November 2002 and October 2003) and the second study [32] estimated the tMRCA of reassortant H5N1v in July 2005. This study revealed that the common ancestor of Indonesian poultry HPAIv H5N1 was introduced into Indonesia 5–7 years (2001; 95% Interval: 1999–2002) after the original ancestor of HPAI A(H5Nx) arose worldwide (1996; 95% Interval: 1995–1996). The introduction of HPAIv A(H5N1) 5–7 years after worldwide outbreaks suggested the importance of sustainability of surveillance and control measures in around 5–7 years before the new introduction of new emerging and re-emerging HPAIv into Indonesia, either from outside Indonesia via wildbirds or poultry trading of the virus, evolves within themselves in Indonesia.

4.2. Limitations and Benefits of the Study

We acknowledge several limitations in this study. First, the limited data, particularlythe number of taxa or samples, may have affected the inferences of evolutionary analysis. Surveillance data and avian influenza virus sequences in wild birds in Indonesia are very limited or absent. All avian influenza sequence data in public genome databases were obtained from domesticated birds. Differences in sampling over time and space may affect the outcomes of the analysis. Therefore, improved surveillance with good competency for clinical and laboratory diagnosis and collection of metadata, as well as the willingness to share the information, is crucial to raise the number of viral genomes in the public database. Surveillance in wild birds is also crucial to reveal the clade gap and study the evolution of the avian influenza virus. Furthermore, additional studies are needed to identify key amino acid changes and evaluate their impact on the viral phenotype, and also on therelationship with the possible role of vaccination programs on the observed evolution of HPAIV A(H5N1).

This study is of importance not only for virus identification but also for studying virus evolution in Indonesia. This study shows that probably only two introductions occurred, after which HPAIv A(H5N1) continued to circulate among poultry in Indonesia. Continuous surveillance of poultry farms in all

sectors and live bird markets in Indonesiawith global support and collaboration are essential to take adequate measures and prevent further evolution of the virus. In addition, compartmentalization, inspection, and certification [43,44] of poultry farms are also important to control the evolution of HPAIvA(H5N1) in Indonesia. Estimation of temporal characteristics of HPAIv A(H5N1) across Indonesia in association with the viral dynamics is essential in conducting prevention controls such as quarantine, movement restriction, diagnostic tools, surveillance systems, andvaccine development [45,46] for future outbreaks. The discovery of different clades in only a few regions and the fact that some Indonesian HPAIv A(H5N1) clades were not detected in other countries indicates the importance of area- and country-specific preventivemeasures for HPAI outbreaks [45]. The Indonesian archipelago, with the ocean as a geographical barrier between islands and between continents, can be an advantage for the country and region-specific preventive measures, as well as reconstructions of intensive poultry farming locations and mapping of wild bird captive areas. In parallel, capacity building is of great importance for each country, and an agreed consensus between countries is a necessity in studying the viral phylodynamics, combined with regular genomic surveillance, to prevent future HPAIv pandemics.

5. Conclusions

This study demonstrated that introductions of HPAIv A(H5N1) into Indonesia are infrequent and most of the observed changes in the virus originate from within Indonesia.The lack of detection of H5N1v clade 2.3.2.1b and the limited Indonesian HPAIv A(H5N1)genomic sequences in the database indicate that there is room for improvement in molecular surveillance of HPAIv in Indonesia. Furthermore, the evolutionary dynamics of the Indonesian HPAIv A(H5N1) highlight the need for continuing genomic surveillance and adequate control measures to prevent viral introduction and evolution, within and between farm transmission in different regions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14102216/s1, Table S1: The GISAID ID of the HA sequences(taxa) for the Phylodynamic analysis in this study; Table S2: The number of Indonesian H5N1 taxa per location; Table S3: The number of Indonesian H5N1 taxa per year.

Author Contributions: D.K.-k.: Conceptualization, data curation, investigation, methodology, formal analysis, project administration, visualization, writing—original draft; R.B.: Supervision, methodology, validation, writing—review, and editing; R.R.: Formal analysis, methodology, validation, writing—review and editing; A.B.: Formal analysis, methodology, validation, writing—review and

editing; A.A.: Formal analysis, methodology, validation, writing—review and editing; G.K.: Methodology, formal analysis, validation, funding acquisition, project administration, supervision, writ ing—review and editing; J.A.S.: Funding acquisition, project administration, resources, supervision, writing—review and editing; D.H.M.: Methodology, formal analysis, validation, supervision, project administration, writing—review and editing. All authors have read and agreed to the publishedversion of the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of potential conflicts of interest.

Appendix A

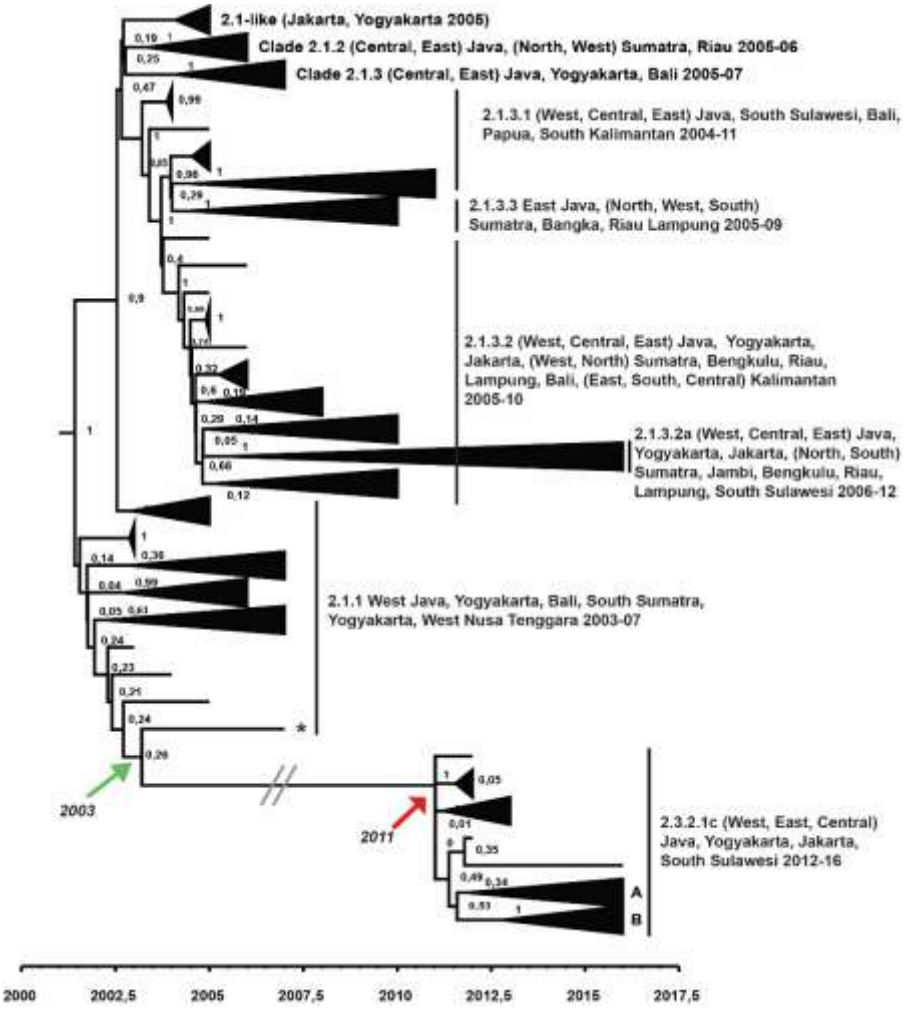

Figure A1. Time-measured phylogeny of HA genes of Indonesian poultry HPAIv A(H5N1) 2003– 2016. The estimated root of HA clade 2.3.2.1c was highlighted by asterisk symbol, the tMRCA of HAclade 2.3.2.1c is pointed out by the red arrow, and the tMRCA between HA clade 2.1.1 and 2.3.2.1c is pointed out by the green arrow. The HPAIv A(H5N1) clade 2.3.2.1c diverged into subgroups (A and B). The node labels display the posterior value. The two gray lines between the clades 2.1.1 and 2.3.2.1c represent the presence of multiple viruses between these two clades as presented in Figure 1. The original sequences (GISAID ID) of HA genes of Indonesian poultry HPAIv A(H5N1) 2003– 2016 phylogeny is displayed in Supplementary Table S1.

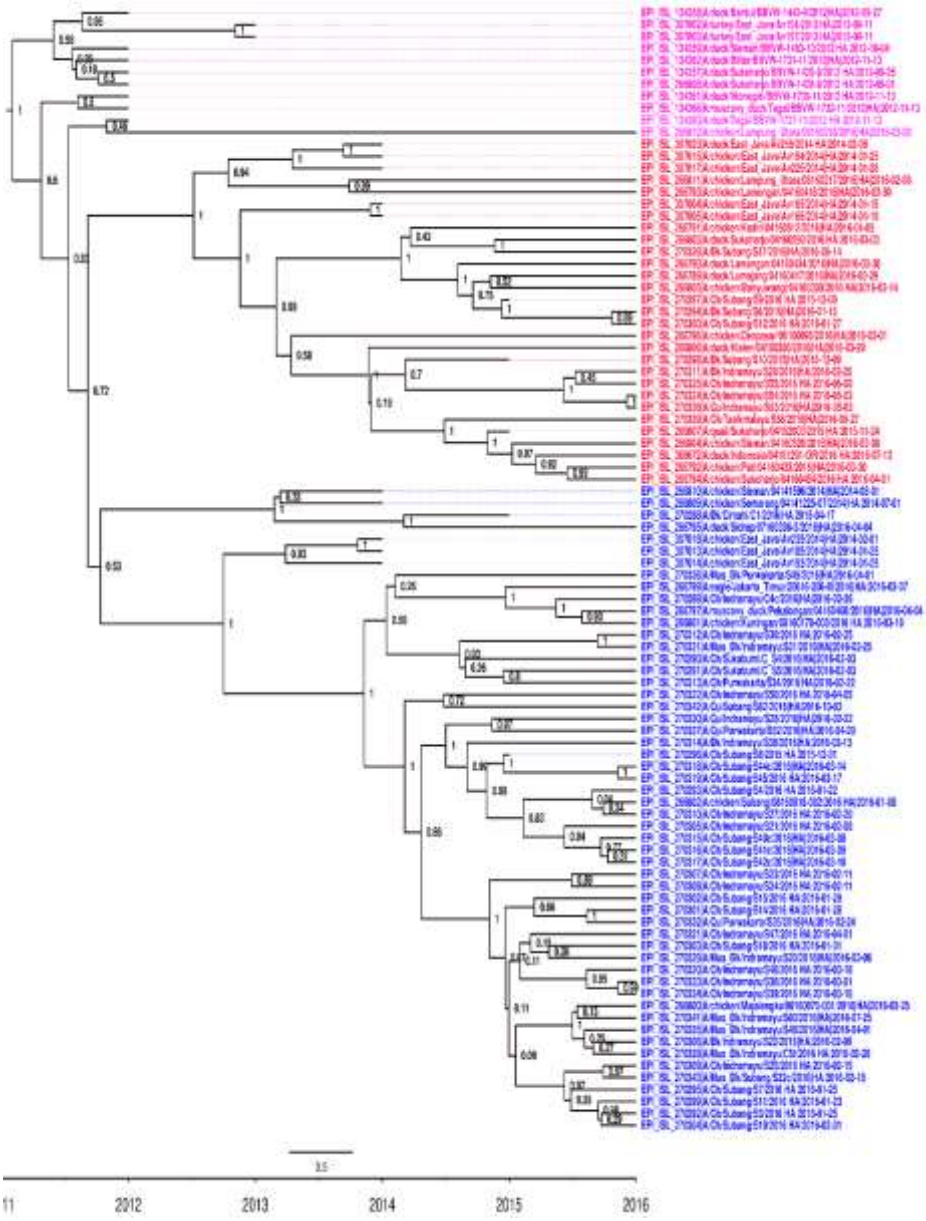

Figure A2. Time-scale phylogeny of Indonesian HA of H5N1v of HA genes of Indonesian poultry HPAIv A(H5N1) clade 2.3.2.1c. The clade 2.3.2.1c subgroup A is highlighted in red and clade 2.3.2.1csubgroup B is highlighted in blue. Another clade, 2.3.2.1c, is highlighted in pink. The node labels display the posterior value.

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Supplementary Materials for Phylodynamics of Highly Pathogenic Avian Influenza A(H5N1) virus circulating in Indonesian poultry

Table S1. The GISAID ID of the HA sequences (taxa) for the Phylodynamic analysis in this study

Sequences	GISAID ID
Indonesian HA	EPI_ISL_98578; EPI_ISL_98577; EPI_ISL_98576; EPI_ISL_98575; EPI_ISL_98574; EPI_ISL_98573; EPI_ISL_98572; EPI_ISL_98571; EPI_ISL_98570; EPI_ISL_98569; EPI_ISL_98568; EPI_ISL_98567; EPI_ISL_98566; EPI_ISL_98565; EPI_ISL_98564; EPI_ISL_98563; EPI_ISL_98562; EPI_ISL_98561; EPI_ISL_98560; EPI_ISL_98559; EPI_ISL_98558; EPI_ISL_98557; EPI_ISL_98556; EPI_ISL_93322; EPI_ISL_93253; EPI_ISL_93252; EPI_ISL_91624; EPI_ISL_91623; EPI_ISL_91553; EPI_ISL_91552; EPI_ISL_91551; EPI_ISL_91550; EPI_ISL_91549; EPI_ISL_91548; EPI_ISL_91547; EPI_ISL_91546; EPI_ISL_91545; EPI_ISL_91544; EPI_ISL_91543; EPI_ISL_91540; EPI_ISL_91539; EPI_ISL_91538; EPI_ISL_91537; EPI_ISL_91536; EPI_ISL_91535; EPI_ISL_91534; EPI_ISL_91533; EPI_ISL_91532; EPI_ISL_91531; EPI_ISL_91530; EPI_ISL_91529; EPI_ISL_91528; EPI_ISL_91527; EPI_ISL_91526; EPI_ISL_91524; EPI_ISL_91523; EPI_ISL_91522; EPI_ISL_91521; EPI_ISL_91520; EPI_ISL_91519; EPI_ISL_91518; EPI_ISL_91517; EPI_ISL_91516; EPI_ISL_91515; EPI_ISL_91514; EPI_ISL_91454; EPI_ISL_91453; EPI_ISL_91452; EPI_ISL_91451; EPI_ISL_91450; EPI_ISL_91449; EPI_ISL_91448; EPI_ISL_91447; EPI_ISL_91446; EPI_ISL_91445; EPI_ISL_91444; EPI_ISL_91443; EPI_ISL_91442; EPI_ISL_91441; EPI_ISL_91440; EPI_ISL_91439; EPI_ISL_91429; EPI_ISL_91428; EPI_ISL_91427; EPI_ISL_91426; EPI_ISL_91425; EPI_ISL_91424; EPI_ISL_91423; EPI_ISL_91422; EPI_ISL_91421; EPI_ISL_91420; EPI_ISL_91419; EPI_ISL_91418; EPI_ISL_91417; EPI_ISL_91416; EPI_ISL_91415; EPI_ISL_91414; EPI_ISL_91413; EPI_ISL_91412; EPI_ISL_91411; EPI_ISL_91410; EPI_ISL_91409; EPI_ISL_91408; EPI_ISL_91407; EPI_ISL_91406; EPI_ISL_91405; EPI_ISL_91404; EPI_ISL_91403; EPI_ISL_91402; EPI_ISL_91401; EPI_ISL_91400; EPI_ISL_91399; EPI_ISL_91398; EPI_ISL_91397; EPI_ISL_91396; EPI_ISL_91395; EPI_ISL_91394; EPI_ISL_91385; EPI_ISL_91384; EPI_ISL_91383; EPI_ISL_91382; EPI_ISL_91381; EPI_ISL_91380; EPI_ISL_91379; EPI_ISL_91378; EPI_ISL_91377; EPI_ISL_91376; EPI_ISL_91375; EPI_ISL_91374; EPI_ISL_91373; EPI_ISL_91372; EPI_ISL_91371;

	EPI_ISL_91370; EPI_ISL_91369; EPI_ISL_91368; EPI_ISL_91367; EPI_ISL_91366; EPI_ISL_91365; EPI_ISL_91364; EPI_ISL_91363; EPI_ISL_91362; EPI_ISL_91361; EPI_ISL_91360; EPI_ISL_91359; EPI_ISL_91358; EPI_ISL_91357; EPI_ISL_91356; EPI_ISL_91355; EPI_ISL_91354; EPI_ISL_91353; EPI_ISL_91352; EPI_ISL_91351; EPI_ISL_91350; EPI_ISL_91349; EPI_ISL_91348; EPI_ISL_91347; EPI_ISL_91346; EPI_ISL_91345; EPI_ISL_91344; EPI_ISL_91343; EPI_ISL_91342; EPI_ISL_87545; EPI_ISL_67389; EPI_ISL_67376; EPI_ISL_67375; EPI_ISL_67072; EPI_ISL_67067; EPI_ISL_67054; EPI_ISL_65649; EPI_ISL_65648; EPI_ISL_6486; EPI_ISL_64853; EPI_ISL_64842; EPI_ISL_64841; EPI_ISL_64840; EPI_ISL_64839; EPI_ISL_64837; EPI_ISL_64833; EPI_ISL_64826; EPI_ISL_64825; EPI_ISL_64824; EPI_ISL_64823; EPI_ISL_64820; EPI_ISL_64779; EPI_ISL_64631; EPI_ISL_64630; EPI_ISL_64629; EPI_ISL_64628; EPI_ISL_64627; EPI_ISL_64626; EPI_ISL_64625; EPI_ISL_64624; EPI_ISL_64623; EPI_ISL_64341; EPI_ISL_64340; EPI_ISL_64213; EPI_ISL_64174; EPI_ISL_64080; EPI_ISL_64079; EPI_ISL_64078; EPI_ISL_64077; EPI_ISL_64076; EPI_ISL_64075; EPI_ISL_64074; EPI_ISL_64073; EPI_ISL_64072; EPI_ISL_64071; EPI_ISL_64070; EPI_ISL_64069; EPI_ISL_5733; EPI_ISL_5732; EPI_ISL_368672; EPI_ISL_307023; EPI_ISL_307019; EPI_ISL_307018; EPI_ISL_307017; EPI_ISL_307015; EPI_ISL_307014; EPI_ISL_307013; EPI_ISL_307008; EPI_ISL_307005; EPI_ISL_307004; EPI_ISL_307003; EPI_ISL_307002; EPI_ISL_30656; EPI_ISL_30655; EPI_ISL_30654; EPI_ISL_30653; EPI_ISL_30652; EPI_ISL_30651; EPI_ISL_30650; EPI_ISL_30649; EPI_ISL_30648; EPI_ISL_30647; EPI_ISL_30646; EPI_ISL_30645; EPI_ISL_30644; EPI_ISL_30643; EPI_ISL_30642; EPI_ISL_30641; EPI_ISL_30640; EPI_ISL_30639; EPI_ISL_30637; EPI_ISL_30636; EPI_ISL_30634; EPI_ISL_30633; EPI_ISL_2917; EPI_ISL_284320; EPI_ISL_284317; EPI_ISL_284316; EPI_ISL_284315; EPI_ISL_270343; EPI_ISL_270342; EPI_ISL_270341; EPI_ISL_270339; EPI_ISL_270338; EPI_ISL_270337; EPI_ISL_270336; EPI_ISL_270335; EPI_ISL_270334; EPI_ISL_270333; EPI_ISL_270332; EPI_ISL_270331; EPI_ISL_270330; EPI_ISL_270329; EPI_ISL_270328; EPI_ISL_270326; EPI_ISL_270325; EPI_ISL_270324; EPI_ISL_270322; EPI_ISL_270321; EPI_ISL_270320; EPI_ISL_270319; EPI_ISL_270318; EPI_ISL_270317; EPI_ISL_270316; EPI_ISL_270315; EPI_ISL_270314; EPI_ISL_270313; EPI_ISL_270312; EPI_ISL_270311;

	EPI_ISL_270310;	EPI_ISL_270309;	EPI_ISL_270308;
	EPI_ISL_270307;	EPI_ISL_270306;	EPI_ISL_270305;
	EPI_ISL_270304;	EPI_ISL_270303;	EPI_ISL_270302;
	EPI_ISL_270301;	EPI_ISL_270300;	EPI_ISL_270299;
	EPI_ISL_270298;	EPI_ISL_270297;	EPI_ISL_270296;
	EPI_ISL_270295;	EPI_ISL_270294;	EPI_ISL_270293;
	EPI_ISL_270292;	EPI_ISL_270291;	EPI_ISL_270290;
	EPI_ISL_270289;	EPI_ISL_270288;	EPI_ISL_266812;
	EPI_ISL_266811;	EPI_ISL_266810;	EPI_ISL_266809;
	EPI_ISL_266808;	EPI_ISL_266807;	EPI_ISL_266806;
	EPI_ISL_266805;	EPI_ISL_266804;	EPI_ISL_266803;
	EPI_ISL_266802;	EPI_ISL_266801;	EPI_ISL_266800;
	EPI_ISL_266799;	EPI_ISL_266798;	EPI_ISL_266797;
	EPI_ISL_266796;	EPI_ISL_266795;	EPI_ISL_266794;
	EPI_ISL_266793;	EPI_ISL_266792;	EPI_ISL_266791;
	EPI_ISL_266790;	EPI_ISL_266789;	EPI_ISL_190642;
	EPI_ISL_190625;	EPI_ISL_190619;	EPI_ISL_190617;
	EPI_ISL_190614;	EPI_ISL_190516;	EPI_ISL_190515;
	EPI_ISL_167892;	EPI_ISL_163459;	EPI_ISL_163458;
	EPI_ISL_163457;	EPI_ISL_163456;	EPI_ISL_163455;
	EPI_ISL_163454;	EPI_ISL_163453;	EPI_ISL_163452;
	EPI_ISL_163451;	EPI_ISL_163450;	EPI_ISL_163449;
	EPI_ISL_163448;	EPI_ISL_163447;	EPI_ISL_163446;
	EPI_ISL_163444;	EPI_ISL_163443;	EPI_ISL_163442;
	EPI_ISL_163441;	EPI_ISL_163440;	EPI_ISL_163439;
	EPI_ISL_163438;	EPI_ISL_163437;	EPI_ISL_163436;
	EPI_ISL_163435;	EPI_ISL_163434;	EPI_ISL_163433;
	EPI_ISL_163432;	EPI_ISL_163431;	EPI_ISL_163430;
	EPI_ISL_163429;	EPI_ISL_163428;	EPI_ISL_163427;
	EPI_ISL_163426;	EPI_ISL_163425;	EPI_ISL_163424;
	EPI_ISL_163423;	EPI_ISL_163422;	EPI_ISL_163420;
	EPI_ISL_163419;	EPI_ISL_163418;	EPI_ISL_163417;
	EPI_ISL_163416;	EPI_ISL_163415;	EPI_ISL_163413;
	EPI_ISL_163412;	EPI_ISL_163411;	EPI_ISL_163410;
	EPI_ISL_163409;	EPI_ISL_163408;	EPI_ISL_163407;
	EPI_ISL_163406;	EPI_ISL_163405;	EPI_ISL_163404;
	EPI_ISL_163403;	EPI_ISL_163401;	EPI_ISL_163400;
	EPI_ISL_163399;	EPI_ISL_163397;	EPI_ISL_163396;
	EPI_ISL_163395;	EPI_ISL_163394;	EPI_ISL_163393;
	EPI_ISL_163392;	EPI_ISL_163391;	EPI_ISL_163390;
	EPI_ISL_163389;	EPI_ISL_163388;	EPI_ISL_163387;

	EPI_ISL_163386; EPI_ISL_163385; EPI_ISL_163384; EPI_ISL_163383; EPI_ISL_163382; EPI_ISL_163381; EPI_ISL_163380; EPI_ISL_163379; EPI_ISL_163378; EPI_ISL_163377; EPI_ISL_163376; EPI_ISL_163375; EPI_ISL_163374; EPI_ISL_163373; EPI_ISL_163372; EPI_ISL_163371; EPI_ISL_163370; EPI_ISL_163366; EPI_ISL_134266; EPI_ISL_134262; EPI_ISL_134261; EPI_ISL_134260; EPI_ISL_134259; EPI_ISL_134258; EPI_ISL_134257; EPI_ISL_13390; EPI_ISL_13388; EPI_ISL_13387; EPI_ISL_13386; EPI_ISL_13385; EPI_ISL_13384; EPI_ISL_13382; EPI_ISL_13381; EPI_ISL_13380; EPI_ISL_13379; EPI_ISL_13378; EPI_ISL_13377; EPI_ISL_13376; EPI_ISL_13374; EPI_ISL_13373; EPI_ISL_13372; EPI_ISL_13366; EPI_ISL_13349; EPI_ISL_13348; EPI_ISL_13345; EPI_ISL_13344; EPI_ISL_13343; EPI_ISL_13342; EPI_ISL_13341; EPI_ISL_13339; EPI_ISL_13334; EPI_ISL_13305; EPI_ISL_13304; EPI_ISL_13294; EPI_ISL_13293; EPI_ISL_13293; EPI_ISL_13292; EPI_ISL_13284; EPI_ISL_13283; EPI_ISL_13283; EPI_ISL_13282; EPI_ISL_13281; EPI_ISL_13275; EPI_ISL_13261; EPI_ISL_13260; EPI_ISL_13259; EPI_ISL_13258; EPI_ISL_13257; EPI_ISL_13256; EPI_ISL_11513; EPI_ISL_11513; EPI_ISL_11512; EPI_ISL_106095; EPI_ISL_106094; EPI_ISL_106089; EPI_ISL_106088; EPI_ISL_106087; EPI_ISL_106086; EPI_ISL_106084; EPI_ISL_106083; EPI_ISL_106082; EPI_ISL_106081; EPI_ISL_106073; EPI_ISL_106072; EPI_ISL_106071; EPI_ISL_106070; EPI_ISL_106069; EPI_ISL_106053; EPI_ISL_104854; EPI_ISL_104853; EPI_ISL_104852; EPI_ISL_104851; EPI_ISL_104850; EPI_ISL_104849; EPI_ISL_104848; EPI_ISL_104847; EPI_ISL_104056; EPI_ISL_10338;
Worldwide HA	EPI_ISL_10055; EPI_ISL_103197; EPI_ISL_10360; EPI_ISL_10378; EPI_ISL_104408; EPI_ISL_1081369; EPI_ISL_108410; EPI_ISL_108434; EPI_ISL_11362; EPI_ISL_1152; EPI_ISL_117763; EPI_ISL_117770; EPI_ISL_11887; EPI_ISL_119388; EPI_ISL_123278; EPI_ISL_123957; EPI_ISL_12401; EPI_ISL_12657; EPI_ISL_1290867; EPI_ISL_129354; EPI_ISL_129845; EPI_ISL_129889; EPI_ISL_130237; EPI_ISL_130380; EPI_ISL_13283; EPI_ISL_13345; EPI_ISL_135217; EPI_ISL_136245; EPI_ISL_137254; EPI_ISL_137553; EPI_ISL_139383; EPI_ISL_139384; EPI_ISL_139386; EPI_ISL_139508; EPI_ISL_13989;

	EPI_ISL_141284; EPI_ISL_14129; EPI_ISL_142593; EPI_ISL_143948; EPI_ISL_146766; EPI_ISL_148124; EPI_ISL_148130; EPI_ISL_148554; EPI_ISL_15159; EPI_ISL_151695; EPI_ISL_152086; EPI_ISL_15275; EPI_ISL_153620; EPI_ISL_15681; EPI_ISL_16190; EPI_ISL_162048; EPI_ISL_162422; EPI_ISL_167178; EPI_ISL_167374; EPI_ISL_170236; EPI_ISL_170538; EPI_ISL_170548; EPI_ISL_170549; EPI_ISL_170860; EPI_ISL_172815; EPI_ISL_173156; EPI_ISL_173687; EPI_ISL_174483; EPI_ISL_175618; EPI_ISL_180659; EPI_ISL_181417; EPI_ISL_190516; EPI_ISL_190617; EPI_ISL_190641; EPI_ISL_190642; EPI_ISL_193364; EPI_ISL_195600; EPI_ISL_195602; EPI_ISL_196570; EPI_ISL_198176; EPI_ISL_198771; EPI_ISL_198776; EPI_ISL_198876; EPI_ISL_198883; EPI_ISL_198916; EPI_ISL_199148; EPI_ISL_199271; EPI_ISL_199413; EPI_ISL_199444; EPI_ISL_201792; EPI_ISL_202557; EPI_ISL_205121; EPI_ISL_205503; EPI_ISL_205964; EPI_ISL_206302; EPI_ISL_208837; EPI_ISL_208842; EPI_ISL_21032; EPI_ISL_212420; EPI_ISL_219782; EPI_ISL_219845; EPI_ISL_219874; EPI_ISL_222478; EPI_ISL_229117; EPI_ISL_23104; EPI_ISL_231747; EPI_ISL_232043; EPI_ISL_232490; EPI_ISL_234280; EPI_ISL_234326; EPI_ISL_234370; EPI_ISL_234372; EPI_ISL_238009; EPI_ISL_241757; EPI_ISL_244526; EPI_ISL_244527; EPI_ISL_244533; EPI_ISL_245160; EPI_ISL_249691; EPI_ISL_252474; EPI_ISL_255754; EPI_ISL_256289; EPI_ISL_256291; EPI_ISL_256292; EPI_ISL_257010; EPI_ISL_257021; EPI_ISL_257072; EPI_ISL_257074; EPI_ISL_257078; EPI_ISL_257097; EPI_ISL_257449; EPI_ISL_257657; EPI_ISL_260058; EPI_ISL_262477; EPI_ISL_262481; EPI_ISL_266794; EPI_ISL_266798; EPI_ISL_266801; EPI_ISL_266825; EPI_ISL_268; EPI_ISL_273845; EPI_ISL_27608; EPI_ISL_277033; EPI_ISL_277041; EPI_ISL_278025; EPI_ISL_278504; EPI_ISL_278505; EPI_ISL_284317; EPI_ISL_285512; EPI_ISL_285622; EPI_ISL_28830; EPI_ISL_2917; EPI_ISL_2918; EPI_ISL_2920; EPI_ISL_295520; EPI_ISL_295793; EPI_ISL_295821; EPI_ISL_300660; EPI_ISL_30428; EPI_ISL_307001; EPI_ISL_307019; EPI_ISL_30875; EPI_ISL_321100; EPI_ISL_321130; EPI_ISL_333630;

	EPI_ISL_336925; EPI_ISL_337275; EPI_ISL_340826; EPI_ISL_340855; EPI_ISL_344530; EPI_ISL_348263; EPI_ISL_348277; EPI_ISL_354553; EPI_ISL_366635; EPI_ISL_376086; EPI_ISL_3770721; EPI_ISL_378628; EPI_ISL_379958; EPI_ISL_388007; EPI_ISL_388019; EPI_ISL_389117; EPI_ISL_389290; EPI_ISL_3895; EPI_ISL_4032280; EPI_ISL_4032309; EPI_ISL_4032542; EPI_ISL_4036; EPI_ISL_404985; EPI_ISL_404987; EPI_ISL_4055123; EPI_ISL_4055141; EPI_ISL_4055209; EPI_ISL_4055421; EPI_ISL_4055846; EPI_ISL_4061492; EPI_ISL_4061493; EPI_ISL_4062115; EPI_ISL_4062128; EPI_ISL_4069736; EPI_ISL_4071630; EPI_ISL_418178; EPI_ISL_4396748; EPI_ISL_4531; EPI_ISL_4533; EPI_ISL_4540; EPI_ISL_4580; EPI_ISL_502395; EPI_ISL_502397; EPI_ISL_503530; EPI_ISL_504924; EPI_ISL_61638; EPI_ISL_63001; EPI_ISL_63002; EPI_ISL_63018; EPI_ISL_63441; EPI_ISL_63499; EPI_ISL_63584; EPI_ISL_64847; EPI_ISL_6491; EPI_ISL_64915; EPI_ISL_65328; EPI_ISL_67383; EPI_ISL_67398; EPI_ISL_67753; EPI_ISL_6780544; EPI_ISL_6781351; EPI_ISL_6781353; EPI_ISL_6795258; EPI_ISL_6795270; EPI_ISL_68029; EPI_ISL_681286; EPI_ISL_6829533; EPI_ISL_697835; EPI_ISL_698060; EPI_ISL_707512; EPI_ISL_73303; EPI_ISL_74794; EPI_ISL_74796; EPI_ISL_74798; EPI_ISL_74799; EPI_ISL_75197; EPI_ISL_77984; EPI_ISL_77985; EPI_ISL_77996; EPI_ISL_77997; EPI_ISL_77998; EPI_ISL_77999; EPI_ISL_78001; EPI_ISL_78006; EPI_ISL_78013; EPI_ISL_78026; EPI_ISL_79686; EPI_ISL_79698; EPI_ISL_80477; EPI_ISL_80599; EPI_ISL_80605; EPI_ISL_80669; EPI_ISL_80684; EPI_ISL_80742; EPI_ISL_80744; EPI_ISL_80749; EPI_ISL_81468; EPI_ISL_81533; EPI_ISL_81534; EPI_ISL_81582; EPI_ISL_84854; EPI_ISL_8518268; EPI_ISL_8769034; EPI_ISL_91342; EPI_ISL_91349; EPI_ISL_91373; EPI_ISL_91520; EPI_ISL_91561; EPI_ISL_93253; EPI_ISL_93322; EPI_ISL_94038; EPI_ISL_94043; EPI_ISL_95061; EPI_ISL_95830; EPI_ISL_98194; EPI_ISL_98556; EPI_ISL_98559; EPI_ISL_98567; EPI_ISL_98568; EPI_ISL_98571; EPI_ISL_98574; EPI_ISL_98576; EPI_ISL_98577; EPI_ISL_98738;

Table S2. The number of Indonesian H5N1 taxa per location

Provinces	No. of Taxa
Aceh	2
Bali	57
Bangka Belitung	3
Banten	2
Bengkulu	2
Central Java	62
Central Kalimantan	1
Central Sulawesi	1
East Java	48
East Kalimantan	5
Indonesia	17
Jakarta	7
Jambi	2
Lampung	10
North Sumatra	21
Nusa Tenggara	1
Papua	3
Riau	10
South Kalimantan	5
South Sulawesi	12
South Sumatra	4
West Java	137
West Sumatra	11
Yogyakarta	79

Table S3. The number of Indonesian H5N1 taxa per year

Years	No. of Taxa
2003	8
2004	7
2005	55
2006	58
2007	144
2008	26
2009	38
2010	50
2011	15
2012	11
2013	3
2014	12
2015	3
2016	72

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

1. Avian Influenza in Indonesian poultry

Highly Pathogenic Avian Influenza HPAI virus A(H5N1) was first detected in Indonesia in 2003. It subsequently spread rapidly spread throughout the country, causing huge losses in poultry production [1,2]. Since poultry is an important source of protein in Indonesia, the outbreaks also jeopardised food security [3]. Furthermore, poultry served as a source of HPAIv A(H5N1) virus transmission to humans [4]. By September 2022, a total of 200 human cases of HPAIv A(H5N1 infection had been reported in Indonesia, of which 168 (84%) were fatal [5] (Figure 1). A previous study revealed that high viral loads in human nasal and pharyngeal specimens and the prevalence of amantadine resistance conferring M2 mutations were associated with mortality in humans caused by clade 2.1 viruses [6]. However, the incidence of infections in humans has sharply decreased since 2013, indicating a successful reduction of exposure of humans to infected poultry or the circulation of virus variants that are less capable of infecting humans. The reduced incidence of infections in humans might also be related to underdiagnosis or underreporting of cases due to the absence of clinical signs and unrecognised symptoms of human H5N1 influenza [7,8]. Continuous surveillance of the HPAIv A(H5N1) in poultry is therefore crucial for taking adequate measures to prevent the re-emergence of fatal infections by this virus in humans.

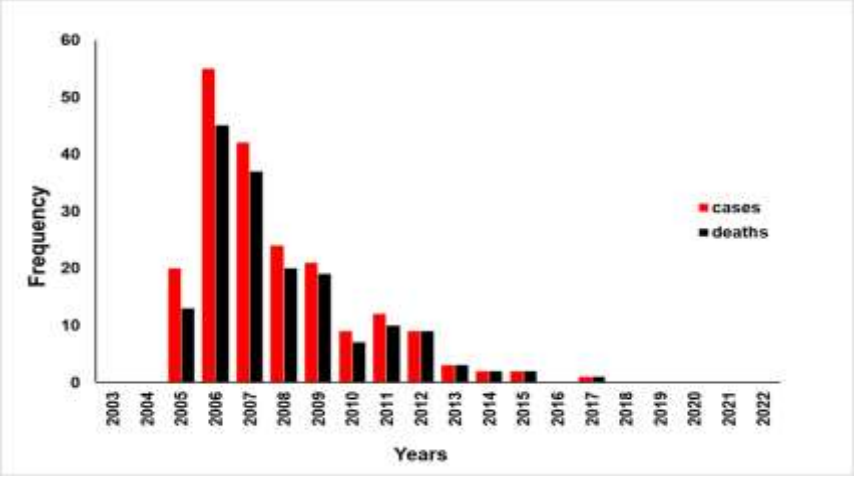

Figure 1. The cumulative number of confirmed human cases of infection with HPAIv A(H5N1) avian influenza A(H5N1) in Indonesia as reported to the WHO, 2003-2022.

Countries in which HPAIv A(H5N1) is endemic, such as Indonesia, might pose risks for global virus circulation. Endemic infections in poultry may cause spillovers to wild birds. This can give rise to new viruses through reassortment with other AI viruses and subsequent long-distance spread of the virus to other countries or even continents [9-11]. After the first introduction of HPAIv A(H5N1), the Indonesian authorities did not have the resources to implement a culling policy along with compensation for poultry farmers. Therefore, various efforts were made by the authorities and the private sector to control HPAI in Indonesian poultry in other ways. These included a surveillance and vaccination programme, biosecurity measures and animal movement control. Despite these efforts, HPAIv A(H5N1) has been endemic among Indonesian poultry [12] since 2006 [13]. Thus, understanding why HPAIv A(H5N1) is endemic among Indonesian poultry is the first step toward identifying better control options for Indonesia and other countries where HPAIV is endemic. Therefore, this study focused on the population dynamics of HPAIv A(H5N1) in Indonesian poultry in terms of molecular epidemiology and the risk factors associated with HPAIv A(H5N1).

2. The evolution of HPAIv A(H5N1) in Indonesian poultry

The evolution of HPAIv A(H5N1) has been the focus of several studies [14,15]. HPAIv A(H5N1) clade 2.1 was first detected in Indonesia in 2003. It is assumed to have evolved from the HPAIv A(H5N1) virus isolated from geese in the Guangdong province of China in 1996 [16]. After several outbreaks in South China from 1998-2003, the virus spread throughout Southeast Asia, including Thailand, Vietnam and Indonesia [15,17,18]. Two clades have so far been identified in Indonesia according to the unified nomenclature system for the HPAIv A(H5N1) virus HA gene [19], clade 2.1 and clade 2.3.

Since the first outbreak was reported in 2003 [20,21] HPAIv A(H5N1) clade 2.1 viruses have become endemic in various regions in Indonesia. Previous research has reported HPAIv H5N1 clade 2.1.1 as an early ancestor of the H5N1 clade 2.1 in Indonesia [17]. Several studies have examined the route of introduction of clade 2.1 to Indonesia [14,22-24]. One study revealed a phylogenetic relationship between Hunan and the Indonesian H5N1 virus (clade 2.1) through the movement of migratory birds and/or poultry [14], which supports various introductions that may have caused an outbreak in Indonesia in 2003. Another study focusing on HPAI phylodynamics in Indonesia suggested that the H5N1 virus originated from an introduction into East Java between November 2002 and October 2003 and then moved east and west across Java [22]. This study indicates that Java and Bali were the initial sites of HPAIv H5N1 invasion and steppingstones for its subsequent spread to other parts of Indonesia via long-distance human transport, with some of the viruses re-migrating [22].

Population dynamics, ecological analysis and phylogeographical analysis in another study indicated that the virus in Indonesia came from various introductions based on the epidemiological relationship between Indonesia and Suphanburi [23]. Different studies therefore suggest different routes of HPAIv A(H5N1) introduction into Indonesia. However, the exact identification of the ancestor of H5N1 is not yet clear, as there is insufficient information and data on viral sequences before and after the outbreak in 2003.

During the first wave of the 2003 HPAI outbreak, the H5N1 clade 2.1.1 mostly circulated in Java and then spread to Kalimantan, resulting in a relatively small number of outbreaks. The second wave of the outbreak is considered to have started between May 2004 and April 2005. Transmission of the virus outside Java began in mid-2004 and the spread of various subclades has been identified since November 2003 [22]. A different clade, clade 2.1.2, caused most of the outbreaks in Sumatra and some in Java in 2004. These occurred not just in poultry but also in humans, including a household outbreak in humans probably from the same infection source in Karo District. This clade was detected until 2007. Another clade of HPAIv A(H5N1), clade 2.1.3, was also detected throughout Indonesia and infected not only birds but also humans. Clade 2.1.3 was predominant among human isolates in West Java in 2005 [17,25,26]. Then the H5N1 clade 2.1.3 evolved into clade 2.1.3.2a and mostly infected poultry from 2009 until 2012.

Survey and characterisation of molecular and antigenic HPAIv H5N1 isolates during 2007-2010 revealed that viruses from duck farms belonged to clade 2.1 with three sub-lineages: clade 2.1.1, clade 2.1.3 and IND/6/05-like viruses (2.1 like), while from chicken farms only clade 2.1.3 was isolated. Clade 2.1.3 has been shown to cluster into three distinct groups, groups I, II and III [27]. A survey revealed that multiple sub-lineages could be detected in a single duck farm. This indicated the potential for reassortment due to multiple introductions of new genetic variants on a farm, while a single sub-lineage detected in various poultry farms indicated the mutation of existing viruses following the transmission between farms [28]. Another study also revealed that two different groups of viruses of clade 2.1.3 co-circulated in the same province (Kalimantan) during the same seasons in 2010. Yet, on the other hand, viruses in the same groups have been collected from different provinces (Sumatra and Central Java) [29]

In 2012, after reports of unusually high mortality in ducks, a new clade, entitled 2.3.2.1, of the HPAIv A(H5N1) virus was discovered on duck farms and live bird markets in Central Java [30]. However, in 2012, the "old" virus clade 2.1.3.2 was still circulating in Indonesia [30,31], while later, only clade 2.3.2.1c was detected.

Due to the lack of clarity about the evolution of HPAIv A(H5N1) clade 2.3.2.1c in recent years, we performed a genetic and phylogenetic analysis from 39 complete genomes of HPAIv A(H5N1) viruses described in Chapter 4 [12]. The complete genomes of HPAIv A(H5N1) were obtained from outbreaks in West Java from 2015-2016. This study revealed that the previously dominant clade 2.1.3.2a was only detected in North Sumatra in 2016. Also, this study identified the evolution of clade 2.3.2.1c into two putative new subgroups, A and B. The two putative subgroups share a common node and monophyletic grouping, with bootstraps value ≥ 60 and average pairwise distance (APD) between groups > 1.5% and within groups < 1.5%. Diversity was also detected in these 39 complete genomes through the identification of reassortment events. Reassortment was identified between different HPAIv A(H5N1) clades on Matrix, NS, and PB2 and between HPAI and LPAI on PB2 [12]. Attenuation of HPAIv A(H5N1) following the reassortment of the viruses in Indonesia and acquisition of genes from low pathogenicity avian influenza A virus progenitors was also identified in a previous study [4]. Reassortment was mostly identified in backyard chickens in Indramayu, West Java.

To understand the evolutionary dynamics of HPAIv A(H5N1), as described in Chapter 5 [32], we performed Bayesian Evolutionary Analysis Sampling Trees on the HA sequences of HPAIv H5N1 of viruses collected from 2003 to 2016. The analysis reveals that probably just two introductions occurred after HPAIv A(H5N1) and that these continued to circulate among poultry in Indonesia. Also, the divergence of H5N1 clade 2.3.2.1c from H5N1v clade 2.3.2.1b was revealed [32], although both clades 2.3.2.1c and 2.3.2.1b share a common recent ancestor, the H5N1v clade 1. The common ancestor of Indonesian poultry HPAIv A(H5N1) was estimated to be introduced 5-7 years after the common worldwide ancestor of HPAIv A(H5Nx) [32]. Temporal dynamic analysis also estimated the nucleotide substitution rate of HPAIv A(H5N1), which was 0.0042 substitution/site/year (s/s/y) over the course of 13 years (95% CI 0.0038–0.0046) [32]. Previous studies have also reported the substitution rate of HPAIv [33-35]. The nucleotide substitution rate in this study is in line with previous reports by Duffy et al. and Chen et al. [33,34] but different from those reported by Ducatez et al. [36]. This substitution rate variation could result from many factors, such as mutation differences and environmental selectivity [35]. The effect of positive selection pressure at several antigenic sites due to environmental selectivity was identified in a previous study [22]. Diversification of PB2-E627K, PB1-H99Y, HA-H103Y, HA-N154D, HA-T156A, HA-N220L, HA-Q222L, HA-G224S and HA-T315I has been studied as an alternative evolutionary pathway or genetic drift after relaxation of purification selection on human hosts in the absence of avian selective pressure [37]. Additional studies are needed to identify the selective pressure of the recent HPAIv A(H5N1) and the key amino acid changes so that

the impact of the environmental pressure, including the role of vaccination on the viral phenotype and evolution, can be evaluated. Also, a bias due to sampling differences might have caused the higher global mutation rate in this study (0.0065 substitution/site/year (s/s/y) over 16 years (95% CI 0.0061–0.0070)) than the substitution rate in Indonesia [32].

Genetic and antigenic changes of HPAIv A(H5N1) detected in Indonesia have been described in multiple studies [12,28,38-42]. The polymerase of influenza A virus has no proofreading activity, resulting in a relatively high mutation rate [43,44]. As a result, a proportion of the progeny viruses will have amino acid changes after infection and replication. From a virus perspective, these amino acid changes might be beneficial and can result in viruses with increased transmissibility, increased pathogenicity in certain hosts, evasion from vaccine-induced immunity (antigenic changes) or a combination of these factors.

Multiple amino acid changes in the Indonesian HPAIv A(H5N1) were already discovered in external (HA and NA) and internal genes (PB2, NS). For example, in 2003, mutations T40K, S105N, G201E, E259K and G382E were identified in the NA protein [41]. In 2006-2008 and 2008-2011, substitutions V263I, I222T/V/M, I117V [45] and N294S, S246N were identified in NA protein [39]. These mutations are related to the oseltamivir resistance [41,46,47]. The mutation of N72D, K119N, R140M, S141P, K152E, R189K, P194S, T195A, M226I, A238T and Y271H were identified in the HA [48] as well as the mutation of A90V, A160T, I217L, I316V, VAL (A90V plus I217L), ATL (A160T plus I217L) and VTI (A90V plus A160T) in HA [49]. This may be associated with an increased affinity for human SA α2,6Gal receptors and an increase in the number of glycosylation sites, and it might determine avian influenza species specificity [48,49]

In Chapter 4, various amino acid changes of external proteins were detected in Indonesian HPAIv A(H5N1) 2015-2016. The substitutions N72D, D97N, Q115H, S129L, S133A, P136S/P136L, L138Q, S140N, P141S, N154D/N154S, R162K, S163G/S163N/S163T, D183N, E184G, A185G, T188I, K189R/K189M, R212K and M226I were identified in the HA protein of HPAIv detected in unvaccinated poultry and the NA of HPAIv at positions S227N, E239G and H254Y [45]. The HA proteins with these amino acid changes had a lower mean distance with a significant difference to the latest seed virus vaccines, clade A/duck/Sukoharjo/BBVW-1428-9/2012, A/ayam/Legok/2003 and Java/PWT-WIJ/2006 as previously studied [12,50-52]. These changes might also contribute to increasing pathogenicity, and antiviral resistance [45].

In addition to amino acid substitutions in the HA and NA proteins, deletions of amino acids were observed in proteins of HPAIv A(H5N1). The deletion of

position 129 was identified in the HA protein and deletion of 20 amino acids was observed in the NA protein **[28,40]**.

Other amino acid changes were identified in the internal proteins (unpublished) of a collection of isolates of HPAIv used for Chapter 4 [12]. Analysis of the deduced amino acid sequence of the internal gene PB2 indicated amino acid changes at positions L89V, G309D, R368Q, Q391E, R477G, I495V, K526R and A676T [45]. These amino acid changes increase the polymerase activity in mammalian cell lines and increase virulence in mice [53,54]. Another remarkable amino acid change was the presence of the KSEV motif in the PDZ domain-ligand (PL) of NS1 at the C-terminal residue. Three motifs of the PDZ domain-ligand motif were ESEV, ESEI and KSEV. The PDZ domain-ligand plays a role as a potential virulence determinant [55,56]. The KSEV domain was identified in two viruses H5N1 A/chicken/Semarang/04141225-07/2014 and A/chicken/Sleman/04141596/2014 but not observed in any of the isolates collected in 2016. However, further surveillance in Central Java is required to confirm this finding. KSEV was described to be present in the 1918/1919 influenza pandemic A(H1N1) virus and is known as a mammalian virulence factor [55,56].

3. Amino acid changes in the multibasic cleavage site

Multi-base cleavage sites (MBCS) in HA are associated with a systemic spread in poultry, but the relationship with a systemic spread in mammals is less clear [57-59]. The HA of HPAIv A(H5N1) contains an MBCS and can be cleaved by ubiquitously expressed furin-like proteases. LPAI viral HA, however, can only be cleaved by trypsin-like proteases since it contains a monobasic cleavage site [57-59]. The presence of MBCS is not directly related to systematic distribution in mammals [57-60]. An MBCS has not been harboured by human pandemic or seasonal influenza viruses. Furthermore, the introduction of MBCSs to the H3N2 virus did not increase or induce systematic spread in ferrets, although in mice, MBCS was shown to be a major virulence factor [61,62]. Genetic analysis of HPAIv A(H5N1) HA genes in Indonesia indicated the presence of different motifs of the polybasic cleavage site shown in Table 1. The observed difference in amino acid sequences between animal (avian or bird) MBCSs in the latest clade 2.3.2.1c (Chapter 4) may be related to the decrease in the number of H5N1 cases in humans, as shown in Figure 1. Clade 2.3.2.1c has been identified in Indonesian poultry since 2012 but was only found in 1 human case in 2017 with a different MBCS motif (Figures 1, 6, and Table 1). As previous studies have shown that human HPAI cases are associated with poultry contact [63,64], a single amino acid deletion in animal MBCS may be related to the inability of human proteases to cleave the HA, resulting in a decrease in human HPAIv A(H5N1) cases. However, the decline in the number of human cases could also be attributed to increased public awareness, underreporting, medical infrastructure and

knowledge and standard operating procedures in recognising symptoms and treating cases of human H5N1 influenza in Indonesian hospitals [7,8,65-67].

Table 1. The multibasic cleavage site (MBCS) in the hemagglutinin (HA) of HPAIv A(H5N1) viruses in Animals and Humans in Indonesia from 2003-2017

Species	Clade	Amino Acid Position of MBCS												
		341	342	343	344	345	346	347	348	349	350	351	352	353
Animals/Human	2.1.1	P	Q	R	E	R	R	R	K	K	R	G	L	F
Animals	2.1.1	P	Q	R	E	-	R	R	**R**	K	R	G	L	F
Animals/Human	2.1.2	P	Q	R	E	R	R	R	K	K	R	G	L	F
Animals/Human	2.1.3	P	Q	R	E	R	R	R	K	K	R	G	L	F
Animals	2.1.3	P	Q	R	E	-	R	R	K	K	R	G	L	F
Animals	2.1.3.1	P	Q	R	E	R	R	R	K	K	R	G	L	F
Animals/Human	2.1.3.2	P	Q	R	E	**S**	R	R	K	K	R	G	L	F
Animals/Human	2.1.3.2a	P	Q	R	E	**S**	R	R	K	K	R	G	L	F
Human	2.1.3.2a	P	Q	R	E	**S**	R	**K**	K	K	R	G	L	F
Human	2.1.3.2a	P	Q	R	E	**S**	**K**	R	K	K	R	G	L	F
Human	2.1.3.2a	P	Q	R	E	**S**	R	R	**R**	K	R	G	L	F
Human	2.1.3.2a	P	Q	R	E	**G**	R	R	K	K	R	G	L	F
Human	2.1.3.3	P	Q	R	E	**G**	R	R	K	K	R	G	L	F
Animals	2.1.3.3	P	Q	R	E	R	R	R	K	K	R	G	L	F
Animals	2.1-like	P	Q	R	E	R	R	R	K	K	R	G	L	F
Animals	2.3.2.1c	P	Q	R	E	-	R	R	**R**	K	R	G	L	F
Animals	2.3.2.1c	P	**K**	R	E	-	R	R	**R**	K	R	G	L	F
Animals	2.3.2.1c	P	Q	R	E	-	**K**	R	**R**	K	R	G	L	F
Human	2.3.2.1c	P	Q	R	E	R	R	R	K	K	R	G	L	F

4. Reassortments

Genetic shift or reassortment as a prompt of influenza virus evolution can occur as inter- and intra-subtype reassortment. The inter-subtype is reassortment between viruses of different subtypes (e.g. H3N2 and H5N1), while the intra-subtype is reassortment between viruses of the same subtype (e.g. H5N1 and H5N1). Reassortments of HPAIv with other AI viruses have been described previously [4,68-79] and play an important role in viral diversity, host adaptation and accelerating immune escape. Reassortments of influenza A viruses have resulted in several pandemics [43]. In Chapter 4 [12], reassortment was described for both inter-and intra-reassortment. Intra-reassortment occurred between the HPAIv A(H5N1) clade 2.3.2.1c genes while inter-reassortment occurred between PB2 of LPAIv and HPAIv A(H5N1) clade 2.3.2.1c detected in 2016 [12]. Also, the

reassortment described in Chapter 4 was mostly identified from backyard chicken (Poultry Sector 4) with a small farm size (1-100 birds) **[12]**. Lack of biosecurity in backyard chicken rearing and contacts between poultry farms (as reported in Chapter 2) and with wild birds might facilitate reassortment in backyard poultry.

The co-circulation of HPAIv A(H5N1) viruses with reassortment events in Indonesia, as revealed in Chapter 4 [12] may also contribute to the different clinical manifestations as revealed in Chapter 3 [50]. The study in Chapter 3 showed differences in clinical manifestation of HPAIv A(H5N1) **[50]**, which might be associated with the evolution of HPAIv A(H5N1) [16,43]. HPAI disease was clinically more manifest and noticeable in backyard chickens than in ducks. The study **[50]** also revealed that the clinical manifestation of H5N1 in ducks was more prominent than previously reported, suggesting that the evolution of the viruses since 2003 from clade 2.1 to 2.3.2 contributed to the prominence of clinical manifestation **[12,28,30,80]**.

5. Antigenic drift

Antigenic drift was also described by previous studies [38-41,81,82] and is due to amino acid changes in proteins, such as HA, which play a major role in antibody-mediated protection. Between 2006 and 2008, antigenic changes in HA protein were identified in vaccinated chickens in N72K, P74Q, N84S, A86T, N109K, Q115R, S121D, N165K, P181S, D183N, A184V, A185E, T195I, N220H, E257D, P235N, I239T, N273D, I151T, K152Q, T159I, V174I and S217T. Substitution of D43N, S123P, E127T, T188K, R189S and P193S in the HA gene was also identified in vaccinated chickens in 2008. The detection of these amino acid changes in HPAIV isolated from vaccinated chickens suggests that vaccination contributed to amino acid change and antigenic drift of HPAIv A(H5N1) in Indonesia [41]. However, HPAIv A(H5N1) detected in unvaccinated chickens also underwent mutations, albeit slower [41]. Therefore, additional studies to evaluate the exact contribution of vaccination to the evolution of HPAIv A(H5N1) are crucial for effective vaccination programmes.

Results of evolutionary, genetic and antigenic studies highlight the importance of sustainable measures, such as surveillance and biosecurity, to control the circulation of HPAIv A(H5N1) in Indonesia. Reduction of virus circulation in poultry will result in a reduced risk of viruses emerging that have amino acid mutations connected with increased pathogenicity or zoonotic potential. Furthermore, with continuous molecular surveillance, the evolution of HPAIv A(H5N1) can be rapidly revealed. Additional measures can then be directly applied when viruses are detected with amino acid changes known to increase pathogenicity, reduce vaccine efficacy or increase zoonotic potential.

6. Associated risk factors of Highly Pathogenic Avian Influenza Virus H5N1 In Indonesian poultry

The risk factors of HPAIv A(H5N1) in Indonesian poultry are related to the Indonesian poultry production system. This is subdivided into four sectors (Sectors 1 to 4) based on biosecurity level and market channel in accordance with the categorisation by the FAO [83,84]. Sector 1 is an industrial and fully vertical integration system (A-type) that forms a large-scale operation that is integrated with day-old-chick (DOC) supply, hatchery eggs suppliers, feed mills, medical supply, processing plants, and abattoirs [85]. It uses sophisticated housing and equipment linked with a high biosecurity level. The birds and products of Sector 1 are marketed commercially through slaughterhouses. Poultry production in Sector 1 farms is well structured and performs at a high level of clearly defined and implemented standard operating procedures in biosecurity and health programmes.

Sector 2 also operates under high biosecurity but a centrally operated biosecurity system is not enforced across all partners in the chains. Farms in Sector 2 are usually smaller and semi-vertical integration systems (B-type), less integrated with the accompanying poultry production facilities than Sector 1 farms [83,84,86]. Sector 2 also has a commercial production system such as DOC supply and slaughterhouse but only has one contributing component between feed mills companies or drug companies, or meat processing plants [85]. Although poultry is kept indoors and has no direct contact with poultry from other farms or wild birds, the level of biosecurity is usually lower than on Sector 1 farms. Sector 3 farms are a partial vertical integration that produces commercially in small-scale units from 100 to 500 birds [83] for slaughterhouses or live bird markets. Compared to Sectors 1 and 2, Sector 3 farms have a low to minimal level of biosecurity, housing caged layers with broilers or ducks in an open shed. Sector 4 farms are backyard farms with minimal biosecurity. In Sector 4, chickens roam around the village and poultry is produced for household consumption. [84]. Within Sectors 3 and 4, HPAIv A(H5N1) viruses spread more easily, especially when the vehicles, containers and poultry-catching teams move between houses and villages [85].

The division of poultry into Sectors 1-4 was implemented for biosecurity purposes, which were correlated to the different types of integration in the broiler industries. The integration of broiler industries was divided from fully vertical to semi-vertical, partial, and non-vertical integration [87]. Broiler, breeding, and commercial layer farms are mostly classified as Sectors 1 and 2 with vertical and semi-vertical integration. Previous research showed that integration affected HPAIv A(H5N1) control [85]. The movement of large numbers of chickens from vehicles, containers and catching teams during the transport of chickens to

production units could potentially spread the HPAI virus even though biosecurity in Sectors 1 and 2 have been established in high-standard operating procedures with high to moderate biosecurity.

In Chapters 2 and 3, it was demonstrated that HPAI outbreaks were more frequent in Sectors 3 and 4 than in Sectors 1 and 2 [2,50]. The contact analysis in poultry showed a high contact rate of backyard farms with other farms through the movement of people, live birds, products, and equipment which facilitates influenza A(H5N1) virus transmission between poultry farms in West Java [2]. The lack or low level of biosecurity in Sectors 3 and 4 is presumed to cause a high number of outbreaks. However, the number of outbreaks in Sector 1 and 2 farms may be underestimated because these farms often have their own laboratory facilities, and results may not always be shared with the authorities.

The FAO and OIE define biosecurity as the implementation of measures to reduce the risk of introducing and spreading disease agents. These measures are based on bioexclusion and biocontainment principles [88,89], which aim to prevent infectious agents from entering and exiting the farms through separation barriers, cleaning, disinfection and isolation so as to minimise the spread through persons, equipment and the movement of products.

Biosecurity measures are well implemented in Sectors 1 and 2, but not in Sectors 3 and 4. The implementation is also associated with the type of business. A previous study showed that makloon contract farming (farming for a fee) has a lower biosecurity compared to independent farming. Also, the implementation of biosecurity measures depends on the incentives offered for biosecurity [90,91].

Village poultry is raised for own consumption in the backyard. Free-ranging animals and different poultry types are mixed, such as broilers, layers, native or kampong chicken and even ducks. Mixing of poultry types increases the risk for HPAIv A(H5N1) virus infection. Even though biosecurity measures, such as building fences and indoor raising, are recommended, implementing such measures is difficult. Other incentives are needed to motivate village people to apply biosecurity measures. For instance, besides reducing the entry of HPAIv, fences can prevent robbery and avoid dirty housing or bird loss.

Biosecurity is also difficult to apply in live bird markets [92,93]. Mixing species, open shed markets and the lack of sanitation enhance the probability of H5N1 virus transmission. A previous study showed that farmers even tend to sell their sick chickens in the live poultry market instead of culling these birds [94]. Therefore, community awareness and participation are required to effectively implement biosecurity measures.

In Chapter 2 [2], we explored the risk of avian influenza on Indonesian poultry by analysing contacts that facilitated the transmission of the influenza A(H5N1) virus between poultry farms in West Java, Indonesia. The risk of HPAIv exposure through the movement of backyard chickens, commercial native chickens, broilers and ducks between and within flocks associated with visits to and from poultry farms was estimated. The risk of HPAIv exposure increased with larger farm size. The average daily contact of visitors to poultry in the backyard chicken was significantly higher than contacts on farms of ducks, commercial native chickens, broilers and layers. Sector 4 backyard farms were identified as having the highest inbound and outbound contact rates compared to farms of the other poultry production types in West Java, Indonesia [2]. The study indicated that Sector 4 farms serve as a potential source of infection exposure to other poultry sectors, particularly towards small-scale commercial sector farms as a previous study revealed [95,96]. Also, the contacts could facilitate the HPAIv transmission and infection from wild birds to poultry.

Identification of the associated risk factors in Chapter 3 [50] revealed that poultry type has a significant association with HPAI outbreak. The study showed the higher odds of duck farms being infected than backyard farms [50]. As previously studied [97-99], the higher risk of duck farms was related to free-ranging ducks in post-harvest paddy fields, which might facilitate the transmission of HPAIv between duck farms via spillover from wild bird populations or other free-ranging ducks. Also, the odds of HPAI infection were associated with the rate of visitor contact with farms. This contact rate with visitors and other duck flocks supports the transmission of HPAI between duck farms in Indonesia [97]. The result also showed that farm size was significantly associated with the HPAIv A(H5N1) outbreaks as well as incoming contacts. Compared to small farms, the odds of being infected were much lower on the larger farms. This finding might be related to better management and biosecurity practices on larger farms [64]. Also, higher frequent visitors to the farm of at least 10 visitors per fortnight had 13.5 times higher odds compared to the less frequent visitors [50].

7. Sustainability of surveillance of Highly Pathogenic Avian Influenza Virus H5N1 In Indonesian Poultry

The epidemiological study of HPAIv A(H5N1) in this thesis highlights the limitations of the data for phylogeny inferences and the associated risk factor measurement. Data limitations might be caused by the limited sustainability of surveillance in Indonesian poultry.

After the first notification of HPAIv A(H5N1) on 2 February 2004, the Animal Health Service carried out active surveillance of HPAIv to strengthen the

virus elimination and control activities. HPAIv A(H5N1) in poultry production services (Sectors 1 to 4) was investigated with the support of the FAO. The number of districts reporting outbreaks in the 23 provinces increased from 74 in 2004 to 154 in 2005 [98]. This active surveillance was a component of the epidemiological disease control and eradication programmes in the 2006 National Plan and Strategy of the Ministry of Agriculture of the Republic of Indonesia [99]. However, due to the limited budget, infrastructure and human resources available, surveillance was subsequently performed using a passive scheme known as Participatory Disease Surveillance and Response (PDSR).

PDSR is an application of participatory epidemiology that uses participatory methods for epidemiological research and disease surveillance. It is a modification of passive surveillance which is also known as outbreak investigation. In an outbreak investigation, surveillance is carried out passively after farmers report the outbreak. For example, in Indonesia, outbreak investigations of HPAIv A(H5N1) are carried out by the National Veterinary Service in collaboration with local animal health officials who form the rapid response unit (URC) [100] and farmers. Outbreak investigations are conducted after notification from the farmers based on suspected clinical symptoms of HPAI observation. A sample of a dead chicken and an oropharyngeal swab from chickens in the flock areas are taken by the local veterinary authorities and sent to the laboratory to confirm the clinical diagnosis. Later, when farmers and the rural community report outbreaks, the passive surveillance is designed using PDSR. The disease surveillance is conducted in a passive outbreak investigation with participatory rural appraised methods, such as the participation of rural households, village officials and village farmer groups.

The laboratory results and investigation reports are submitted to an online influenza virus monitoring programme and analysed through the National Animal Health Information System (ISIKHNAS). Those reports comprise the number of infected animals, the population of animals, clinical signs, nearby human cases, source of infection, rapid tests, collected samples, definitive diagnosis and outbreak status. Responses to the outbreaks are also reported to ISIKHNAS, such as the treatment, stamping out, vaccination, active surveillance, farmer education and communication activities (http://wiki.isikhnas.com/w/Priority_Disease_Investigation#Powerpoints). The analysis of the data from ISIKHNAS is then used as a basis for policymaking [100-103]

The sustainability of surveillance will contribute to the continuing availability of data for epidemiological and phylogenetic studies. Surveillance priority and budget allocation for avian influenza has competition from several

prioritized infectious animal and zoonotic diseases especially in the current autonomous district governments [104], affecting the availability of data and control programmes. Limited data on vaccinated poultry, especially post-vaccination epidemiological data, affects the evaluation of vaccination as a risk factor for mutation and evolution of HPAIv A(H5N1) in Indonesia. Therefore, sustainability of surveillance of poultry farms in all sectors and live bird markets is essential for the taking of adequate measures and preventing further evolution of the virus. The active participatory method based on the spatial-temporal risk of rural community appraisal might be the best design for the sustainability of surveillance of HPAIv A(H5N1) in Indonesia. Continuing to study epidemiology and phylogenetic analysis of HPAIv A(H5N1) in Indonesian poultry via sustainability of surveillance is crucial to understand further evolution and pandemic threat of HPAIv A(H5N1).

Lack of biosecurity on the farms in Sectors 3 and 4 or free-range backyard chicken and live bird markets, could lead to exposure to wild birds and consequently a spillover of avian influenza to poultry. However, at present, only limited data and information are available concerning HPAIv in wild birds and waterfowl in Indonesia [105,106]. Continuous surveillance of HPAIv A(H5N1) in Indonesia should ideally include surveillance of both poultry and wild birds.

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Summary

Highly Pathogenic Avian Influenza (HPAIv) H5N1 virus of the A/Goose/Guandong/1/96 lineage was first reported in Asia in 1996 and has been circulating in Indonesia since 2003. Its spread continuous to have a global impact on poultry health and causes severe losses to the poultry industry. Furthermore, 200 laboratory-confirmed human cases were reported alone in Indonesia with a case-fatality rate of 84%. Surveillance and control measures have not been able to prevent HPAIv H5N1 from becoming endemic among poultry in Indonesia.

Several risk factors associated with HPAIv H5N1 have been identified previously but information about the contact structure between poultry farms was still limited. Additionally, the clinical manifestation and risk factors associated with outbreaks of the recent variant of HPAIv H5N1 in Indonesia had not extensively been studied. To fill those gaps an epidemiological study using enhanced passive surveillance of HPAIv H5N1 was conducted on West Java.

Analysis of the contact structure facilitating the transmission of HPAIv H5N1 between different types of poultry farms in West Java is described in chapter 2. Various contact types were registered by farmers in logbooks, including the in and outbound movements of people, live birds, products, and equipment. The overall contact rates were derived from distributed logbooks on 124 poultry farms in 6 districts in West Java. The poultry farms included farms with backyard chickens, commercial native chickens, broilers, and ducks. Contacts were registered with other poultry farms, from and to poultry companies, to egg collection houses and to live bird markets.

The contacts between chicken farms, free range chickens, broilers and ducks mostly came from visits to and from other poultry farms, while in layer chicken farms the most frequent movements were visits to and from poultry companies, visits to egg collecting houses and visits from other poultry farms. Over 75% of persons visiting backyard chicken and duck farms had previously visited other poultry farms on the same day. Visitors of backyard chicken farms had the highest average contact rate, either direct contact with poultry on other farms before the visits (1.35 contact/day) or contact during their visits in the farms (10.03 contact/day). These results suggest that backyard chicken farms are most at risk for transmission of HPAIv compared to farms of the other poultry production types. This also suggests that backyard farms could serve as a vector for transmitting HPAIv H5N1 to commercial poultry.

In the same six regions of West Java and one additional district passive surveillance was enhanced from November 2015 to November 2016 to detect outbreaks of HPAIv H5N1 (Chapter 3) and describe the clinical manifestation of these outbreaks and identify associated risk factors. In total 64 outbreaks were confirmed out of 75 reported suspicions and outbreak characteristics were

recorded. The highest mortality was reported in backyard chickens (average 59%, CI95%: 49–69%). Dermal apoptosis and lesions (64%, CI95%: 52–76%) and respiratory signs (39%, CI95%: 27–51%) were the clinical signs observed overall most frequently, while neurological signs were most frequently observed in ducks (68%, CI95%: 47–90%). In comparison with 60 non-infected control farms, the rate of visitor contacts onto a farm was positively associated with the odds of HPAI infection. Moreover, duck farms had higher odds of being infected than backyard farms, and larger farms had lower odds than small farms. Results indicate that better external biosecurity is needed to reduce transmission of HPAIv A(H5N1) in Indonesia.

Since 2003, several HPAIv H5N1 variants have been identified in phylogenetic studies. HPAIv H5N1 clade 2.1 with subclades 2.1.1, 2.1.2, 2.1.3, 2.1.3.2a, 2.1.3.1, 2.1.3.3 have been identified from 2003 onwards and a new subclade, 2.3.2.1c was identified from 2012 onwards. However, limited Indonesian poultry HPAIv H5N1 sequences were available since 2008 in the public database. To fill that gap, investigations were perfomed using the outbreaks described in chapter 3. Full genome sequences were obtained from samples that tested positive for HPAIv A(H5N1). The phylogenetic analyses of obtained full genomes are described in chapter 4.

Analysis of 39 full-genome HPAIv H5N1 viruses with additional reference sequences from the public genome database revealed 2 genetic clusters in clade 2.3.2.1c based on the hemagglutinin gene as well as the neuraminidase, nucleoprotein, polymerase, and polymerase basic 1 gene. The polymerase basic 2 gene had a close relation with Eurasian low pathogenic avian influenza virus (LPAIv). Also, several matrix, nonstructural protein and polymerase basic 2 genes were detected in HPAIv with higher identity with clade 2.1.3 than with clade 2.3.2.1c. The phylogenetic analysis of the samples in 2015-2016 thus identified 13 types of reassortment in HPAIv in Indonesia, mostly in native chickens in Indramayu.

The identification of reassortments in chapter 4 raised the question about the evolution of HPAIv H5N1 in Indonesia. To increase information regarding the evolution of H5N1 in Indonesia, a phylodynamic analysis of all available Indonesian poultry HPAIv A(H5N1) sequences was performed as presented in chapter 5. The Bayesian evolutionary analysis (BEAST) was carried out on sequences of Indonesian poultry HPAIv A(H5N1) collected from 2003-2016 completed with a set of sequences representating groups of global H5Nx that have identities greater than 98%. Temporal dynamic analysis revealed that most likely only two introductions of HPAIv A(H5N1) occurred in Indonesia and the common ancestor of the first introduction to Indonesian poultry HPAIv A(H5N1) emerged about five to seven years after the global common ancestor of HPAIv A(H5Nx). The divergences and common ancestor of H5N1 clade 2.3.2.1c and

clade 2.3.2.1b were described in Chapter 5. The shared common ancestor of clade 2.3.2.1c and clade 2.3.2.1 indicate that the second introduction of both HPAIv A(H5N1) into Indonesian poultry originated from viruses from the same clade as viruses. detected in China and other Asian countries.

Results of studies presented in this thesis highlight the importance of continued and improved (genomic) surveillance in both poultry, live bird markets and wild birds. Also, adequate control measures such as improving and maintaining poultry farm biosecurity as well as reconstruction of the poultry sector regarding housing systems, biosecurity controls, poultry types mixing restrictions and visitor controls are required to control the spread of HPAIv A(H5N1). To better understand the phylogeny, evolution and mechanisms of the observed reassortment events of Indonesian poultry viruses described in this thesis, additionalsequences of HPAIv and LPAIv in wild birds and poultry are needed. Capacity building and infrastructure for genomic surveillance are important to study the evolution of the HPAIv H5N1 virus in order to prevent a new emerging pandemic threat.

Hoogpathogene vogelgriep (HPAIv) H5N1-virus van de A/Goose/Guandong/1/96-lijn werd voor het eerst gemeld in Azië in 1996 en circuleert sinds 2003 in Indonesië. De voortdurende verspreiding ervan heeft een wereldwijde impact op de gezondheid van pluimvee en veroorzaakt ernstige verliezen voor de pluimvee-industrie. Bovendien werden alleen al in Indonesië 200 door laboratoriumonderzoek bevestigde gevallen bij de mens gemeld met een sterftepercentage van 84%. Surveillance en controlemaatregelen hebben niet kunnen voorkomen dat HPAIv H5N1 endemisch werd onder pluimvee in Indonesië.

Er zijn eerder verschillende risicofactoren geïdentificeerd die verband houden met HPAIv H5N1, maar informatie over de contactstructuur tussen pluimveebedrijven was nog beperkt. Bovendien waren de klinische verschijnselen en risicofactoren geassocieerd met uitbraken van de recente variant van HPAIv H5N1 in Indonesië niet uitgebreid bestudeerd. Om die kennislacunes op te vullen, werd een epidemiologische studie met versterkte passieve surveillance van HPAIv H5N1 in West-Java uitgevoerd.

Analyse van de contactstructuur die de overdracht van HPAIv H5N1 mogelijk maakt tussen verschillende soorten pluimveehouderijen in West-Java wordt beschreven in hoofdstuk 2. Verschillende contacttypen werden door pluimveehouders vastgelegd in logboeken, waaronder de in- en uitgaande bewegingen van mensen, pluimvee, producten, en materialen. De contactpercentages zijn gebaseerd op logboeken van 124 pluimveehouderijen uit 6 districten van West-Java. De pluimveebedrijven omvatten kleinschalige pluimveehouderijen voor eigen consumptie, bedrijven met commerciële inheemse kippen, vleeskuikens en eenden. Voor elk van deze bedrijven werden contacten met andere pluimveehouderijen, van en naar pluimveehouderijen, eierpakcentra en levende pluimveemarkten werden vastgelegd.

Persoonscontacten van en naar nadere pluimveehouderijen kwamen veel voor tussen kleinschalige pluimveehouderijen. Op legbedrijven kwamen dierverplaatsingen, contacten met eierpakstations veelvuldig voor. Meer dan 75% van de bezoekers van kleinschalige kippen- en eendenboerderijen had eerder op dezelfde dag andere pluimveebedrijven bezocht. Bezoekers van kleinschalige houderijen hadden het vaakst direct contact met pluimvee op andere boerderijen vóór de bezoeken (1,35 contact/dag) of indirect contact tijdens hun bezoeken aan de boerderijen (10,03 contact/dag). Deze resultaten suggereren dat kleinschalige

pluimveehouderijen een aanzienlijk risico op HPAIv-overdracht met zich meebrengen in vergelijking met andere pluimveehouderijen en dat ze een risico vormen voor commercieel pluimvee.

In dezelfde zes regio's van West-Java en één extra district werd van november 2015 tot november 2016 passieve surveillance versterkt om uitbraken van HPAIv H5N1 op te sporen (hoofdstuk 3) en om de klinische verschijnselen van deze uitbraken te beschrijven en bijbehorende risicofactoren te identificeren. Van de 75 gemelde verdenkingen van HPAIv H5N1 werden in totaal 64 uitbraken bevestigd en werden de kenmerken van de uitbraak geregistreerd. De hoogste sterfte werd gemeld bij kippen in kleinschalige houderij (gemiddeld 59%, CI95%: 49-69%). Dermale apoptose en laesies (64%, CI95%: 52-76%) en respiratoire symptomen (39%, CI95%: 27-51%) waren de meest voorkomende klinische verschijnselen, terwijl neurologische verschijnselen het meest werden waargenomen bij eenden (68%, BI95%: 47-90%). Vergeleken met 60 niet-geïnfecteerde controlebedrijven was het aantal bezoekerscontacten op een bedrijf positief geassocieerd met de kans op HPAI-infectie. Bovendien hadden eendenkwekerijen een grotere kans om besmet te raken dan boerderijen met pluimvee in de achtertuin, en hadden grotere boerderijen een lagere kans dan kleine boerderijen. De resultaten geven aan dat betere externe bioveiligheid nodig is om HPAIv A(H5N1)-overdracht in Indonesië te verminderen.

Sinds 2003 zijn er verschillende HPAIv H5N1-varianten geïdentificeerd in fylogenetische studies. HPAIv H5N1 clade 2.1 met subclades 2.1.1, 2.1.2, 2.1.3, 2.1.3.2a, 2.1.3.1, 2.1.3.3 zijn geïdentificeerd vanaf 2003 en een nieuwe subclade, 2.3.2.1c, is geïdentificeerd vanaf 2012. Vanaf 2008 waren er echter beperkte HPAIv H5N1-sequenties van Indonesisch pluimvee beschikbaar in de openbare database. Om die kennislacune op te vullen, werden studies uitgevoerd met behulp van de uitbraken beschreven in Hoofdstuk 3. Volledige genoomsequenties werden verkregen van monsters die positief testten op HPAIv A(H5N1). De fylogenetische analyses van verkregen genoomsequenties worden beschreven in hoofdstuk 4.

Analyse van 39 volledige genomen van HPAIv H5N1-virussen met aanvullende referentiesequenties uit de openbare genoomdatabase onthulde 2 genetische clusters in clade 2.3.2.1c gebaseerd op het hemagglutinine-gen en het neuraminidase, nucleoproteïne, polymerase en polymerase base 1-gen. Het polymerase basic 2-gen had een nauwe relatie met het Euraziatische laagpathogene vogelgriepvirus (LPAIv). Ook werden verschillende matrix-, niet-structurele eiwit- en polymerase basic 2-genen gedetecteerd in HPAIv met een hogere identiteit met clade 2.1.3 dan met clade 2.3.2.1c. Zo ontdekte de

fylogenetische analyse van de monsters in 2015-2016 13 soorten van reassortering in HPAIv in Indonesië, meestal in inheemse kippen in Indramayu.

De identificatie van reassorteringen in hoofdstuk 4 roept de vraag op over de evolutie van HPAIv H5N1 in Indonesië. Om de informatie over de evolutie van H5N1 in Indonesië te vergroten, werd een fylodynamische analyse van alle beschikbare HPAIv A(H5N1)-sequenties van Indonesisch pluimvee uitgevoerd, zoals gepresenteerd in hoofdstuk 5. De Bayesiaanse evolutionaire analyse (BEAST) werd uitgevoerd op sequenties van HPAIv A(H5N1) van Indonesisch gevogelte verzameld van 2003-2016 aangevuld met HPAIv uit collecties van wereldwijde H5Nx die voor meer dan 98% genetisch identiek zijn. Temporele dynamische analyse onthulde dat hoogstwaarschijnlijk slechts twee introducties van HPAIv A (H5N1) plaatsvonden in Indonesië en dat de gemeenschappelijke voorouder van de eerste introductie in Indonesisch pluimvee HPAIv A (H5N1) ongeveer vijf tot zeven jaar na de wereldwijde gemeenschappelijke voorouder van HPAIv A (H5Nx) ontstaan is. De verschillen en gemeenschappelijke voorouder van H5N1 clade 2.3.2.1c en clade 2.3.2.1b staan beschreven in Hoofdstuk 5. De gedeelde gemeenschappelijke voorouder van clade 2.3.2.1c en clade 2.3.2.1 geven aan dat de tweede introductie van beide HPAIv A(H5N1) in Indonesisch pluimvee afkomstig was van virussen uit dezelfde clade als virussen gedetecteerd in China en andere Aziatische landen.

De resultaten van het onderzoek dat in dit proefschrift wordt beschreven benadrukken het belang van continue en verbeterde (genomische) surveillance in zowel pluimvee-, op levende vogelsmarkten- als in wilde vogels. Adequate beheersmaatregelen zijn ook nodig, zoals het verbeteren van de bioveiligheid van pluimveebedrijven en de reconstructie van de pluimveesector met betrekking tot huisvestingssystemen, bioveiligheidscontroles, mengbeperkingen van pluimveesoorten en bezoekerscontroles, om de verspreiding van HPAIv A(H5N1) te beheersen. Om de fylogenie, evolutie en mechanismen van de waargenomen reassorteringen van Indonesische pluimveevirussen beter te begrijpen, zijn aanvullende sequenties van HPAIv en LPAIv in wilde vogels en pluimvee nodig. Capaciteitsopbouw en infrastructuur voor genomische surveillance zijn belangrijk om de evolutie van het HPAIv H5N1-virus te bestuderen.

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Curriculum Vitae

Desniwaty was born on December 13, 1977, in Medan, North Sumatra, Indonesia. In 2001, she obtained her Doctorate in Veterinary Medicine (DVM) from the Bogor Agricultural Institute, Bogor, Indonesia. In 2001 she joined the Makassar Animal Quarantine Agency as an apprentice and assistant to the microbiology laboratory at Hasanuddin University Makassar. In early 2003, she continued her Master's Degree in biomedical science, Hasanuddin University, Makassar. Then at the end of 2003, she was appointed as an Indonesian government employee at the Animal Quarantine Agency, Makassar, Indonesia. and started her career as a Veterinary Laboratory Diagnosticians. While undertaking postgraduate study, she applied for the Australian Development Scholarship and was awarded a scholarship in 2005 and attended an English Academic Language training in Bali.

After receiving an Australian scholarship, in 2006, she was transferred to Jakarta and temporarily served as staff at the Soekarno Hatta Agricultural Quarantine Agency, Tangerang, Indonesia, until the Indonesian Center for Agricultural Diagnostic Standards was newly established in 2007, she was appointed as a Veterinary Laboratory Diagnosticians in this center.

After moving to Jakarta and completing her Master's Degree at Hasanuddin University (UNHAS) in 2006, she continued her studies for the Master of Tropical Veterinary Science (by research) at James Cook University (JCU), North Queensland, Australia. She returned to Jakarta at the end of 2008 and continued working as a Veterinary Laboratory Diagnostician and was given the task of Manager of the Biomolecular Laboratory. She submitted her thesis after getting a month's permission to complete her thesis in Townsville in 2011 and later earned her Master's Degree at JCU.

In 2013, she received a scholarship from the Royal Netherlands Academy of Arts and Sciences (KNAW) to study PhD in the veterinary epidemiology program at the Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands. She went to the Netherlands on February 2014 to do sequencing training at Wageningen Bioveterinary Research, Wageningen University & Research (WBVR-WUR). Afterward, she started her PhD studies at Utrecht University on December 2015, under the guidance of Prof. Arjan Stegeman from the Department of Livestock Health, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, Netherlands, Prof. David Handojo Muljono from the Eijkman Institute for Molecular Biology, Jakarta, Indonesia, and Dr. Guus Koch from Wageningen Bioveterinary Research, Lelystad, The Netherlands.

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