GENETIC CHARACTERISTICS OF AVIAN INFLUENZA VIRUSES ISOLATED FROM DUCKS IN JAVA ISLAND, INDONESIA YEAR 2015-2017

Mrs. Lestari -

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ลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดนกที่แยกได้จากเป็ดในเขตจาวา ประเทศอินโดนิเซีย ระหว่างปี 2558-2560

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Thesis Title	GENETIC CHARACTERISTICS OF AVIAN INFLUENZA VIRUSES ISOLATED FROM DUCKS IN JAVA ISLAND, INDONESIA YEAR 2015-2017
By	Mrs. Lestari -
Field of Study	Veterinary Science and technology
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เลสตารี - : ลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดนกที่แยกได้จากเป็ดในเขตจาวา ประเทศอินโดนิเซีย ระหว่างปี 2558-2560. (GENETIC CHARACTERISTICS OF AVIAN INFLUENZA VIRUSES ISOLATED FROM DUCKS IN JAVA ISLAND, INDONESIA YEAR 2015-2017) อ.ที่ปรึกษาหลัก : อลงกร อมรศิลป์

โรคไข้หวัดนกเป็นโรคติดต่อสำคัญทางสาธารณสุขที่ส่งผลกระทบต่อสุขภาพของทั้งคนและสัตว์ การศึกษานี้มีวัตถุประสงค์ที่จะระบุและแยกเชื้อไวรัสไข้หวัดนกจากเป็ดที่ถูกเลี้ยงบนเกาะชวา ประเทศอินโดนีเซีย ช่วงปี ค.ศ. 2015 ถึง 2017 ในครั้งนี้ผู้ทำการศึกษาได้รับตัวอย่างจากศูนย์สืบสวนโรคระบาด (Disease Investigation Center: DIC) แห่งเมืองยกยาการ์ตา ประเทศอินโดนีเซีย จำนวน 50 ตัวอย่าง ตัวอย่างเหล่านี้ถูกเก็บในช่วงการระบาดของโรคไข้หวัดนกและจากการเฝ้าระวังโรคไข้หวัดนกใน 3 จังหวัด ได้แก่ ชวาตะวันออกจำนวน 26 ตัวอย่าง ชวากลางจำนวน 16 ตัวอย่างและ ยกยาการ์ตาจำนวน 8 ตัวอย่าง ตัวอย่างทั้งหมดถูกตรวจสอบด้วยวิธีการ realtime RT-PCR โดยใช้ไพรเมอร์ที่มีความจำเพาะกับ M gene ผลการศึกษาพบว่า 46 จาก 50 ตัวอย่าง (92%) ให้ผลบวกกับการตรวจไวรัสไข้หวัดนกชนิด A จากนั้นตัวอย่างที่แสดงผลบวกทั้งหมดถูกนำไปทำวิธีการ virus isolation และวิธีการ hemagglutination (HA) พบว่า 7 จาก 46 ตัวอย่างให้ผลบวกกับการตรวจยืนยันไวรัสไข้หวัดนกชนิด A โดยวิธีการ multi-segment reverse transcription (M-RTPCR) จากนั้นตัวอย่างที่ให้ผลบวกทั้งหมดถูกส่งเพื่อทำ next generation sequencing จากผลการศึกษาพบว่า 6 ตัวอย่างเป็นสายพันธุ์ H5N1 และอีกหนึ่งตัวอย่างเป็นสายพันธุ์ H9N2 ผลจากการทำ Phylogenetic analysis พบว่าตัวอย่างสายพันธุ์ H5N1 จำนวน 6 ตัวอย่างถูกจัดอยู่ใน clade 2.3.2.1c และตัวอย่างสายพันธุ์ H9N2 จำนวน 1 ตัวอย่างถูกจัดอยู่ในกลุ่ม Y280-like ผลการวิเคราะห์ phylogenetic tree พบว่าหนึ่งในตัวอย่างสายพันธุ์ H5N1 clade 2.3.2.1c ได้รับ internal gene มาจาก M gene ของไวรัสไข้หวัดนกสายพันธุ์ H5N1 clade 2.3.2 ในขณะที่ตัวอย่างสายพันธุ์ H9N2 ได้รับ internal gene มาจากไวรัสไข้หวัดใหญ่สายพันธุ์ H7N9 ของคนซึ่งแยกได้จากประเทศจีน จากการวิเคราะห์ทางพันธุกรรมพบว่าไวรัสไข้หวัดนกสายพันธุ์ H5N1 และ H9N2 ถูกจัดหมวดหมู่ให้อยู่ในประเภทก่อโรครุนแรง HPAI และก่อโรคไม่รุนแรง LPAI ตามลำดับ กล่าวโดยสรุป ไวรัสไข้หวัดนกทั้งสายพันธุ์ H5N1 และ H9N2 สามารถพบได้ในเป็ดที่ถูกเลี้ยงบนเกาะชวา ประเทศอินโดนีเซีย ดังนั้นการเฝ้าระวังโรคอย่างสม่ำเสมอจึงมีความสำคัญต่อการควบคุมและป้องกันโรคไข้หวัดนกซึ่งมีโอกาสในการเกิดไวรัสสายพันธุ์ใหม่เป็นอย่างมาก

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Lestari - : GENETIC CHARACTERISTICS OF AVIAN INFLUENZA VIRUSES ISOLATED FROM DUCKS IN JAVA ISLAND, INDONESIA YEAR 2015-2017. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Avian influenza becomes public health concern over years due to the serious impacts in both animal and human health. This study aimed to identify and characterize avian influenza viruses isolated from ducks in Java Indonesia during 2015-2017. Total 50 samples of previously identified as avian influenza viruses were acquired from the virus/culture collections of Disease Investigation Center (DIC) Wates Yogyakarta, Indonesia. The samples were recovered from either the avian influenza surveillance or avian influenza outbreaks in ducks in 3 provinces, East Java (n=26), Central Java (n=16), and Yogyakarta (n=8). Then the samples were processed for influenza A virus screening by realtime RT-PCR using M gene specific primers. The results showed that 46 out of 50 samples (92%) were positive for influenza A virus screening. The positive samples were then subjected for influenza A virus isolation. Seven out of 46 samples were positive for influenza A virus confirmation by using multi-segment reverse transcription PCR (M-RTPCR). All 7 avian influenza viruses were subjected to whole genome sequencing by next generation sequencing. The viruses were then identified as avian influenza subtype H5N1 (n=6) and H9N2 (n=1). Phylogenetic analysis showed that the avian influenza viruses were clustered into H5N1 virus of clade 2.3.2.1c (n=6) and H9N2 viruses of Y280-like group (n=1). Phylogenetic analysis of internal genes indicated that among the H5N1 of clade 2.3.2.1c (n=1), the evidence of inter-lineage reassortment by acquiring the M gene from H5N1 of clade 2.1.3.2 could be observed. On the other hand, phylogenetic analysis of H9N2 showed that all internal genes of H9N2 were closely related to the human H7N9 virus isolated from China. Genetic analysis showed that the H5N1 and H9N2 viruses were categorized as HPAI and LPAI, respectively. In conclusion, our results suggested that both HPAI-H5N1 and LPAI-H9N2 were circulated in ducks in Java, Indonesia. Routine surveillance and genetic characterization are important to monitor the dynamic and genetic diversity of avian influenza viruses and possibility of novel reassortment strains.

Field of Study:	Veterinary Science and technology	Student's Signature .....
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LIST OF ABBREVIATIONS

AI	Avian Influenza
BLAST	Basic Local Alignment Search Tool
Ct	Cycle threshold
DIC	Disease Investigation Center
HA	Hemagglutinin
HPAI	Highly Pathogenic Avian Influenza
IVPI	Intravenous pathogenicity index
LPAI	Low Pathogenic Avian Influenza
M	Matrix
M-RTPCR	Multi-segment reverse transcription Polymerase Chain Reaction
NA	Neuraminidase
NEP	Nuclear export protein
NGS	Next generation sequencing
NP	Nucleoprotein
NS	Non-structural
OIE	World Organization for Animal Health
PA	Polymerase acid
PB1	Polymerase basic 1
PB2	Polymerase basic 2
RT-PCR	Reverse Transcription Polymerase Chain Reaction
vRNPs	viral Ribonucleoproteins
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Avian influenza (AI) is a contagious viral disease and becomes public health concern over years. Avian influenza has gained the attention of the international communities due to the serious impact, both in domestic birds and human (Chmielewski and Swayne, 2011). Avian influenza affects not only to the economic losses and trade in poultry industry, but also to the public health. Since the first report of Highly Pathogenic Avian Influenza subtype H5N1 (HPAI-H5N1) in geese in Guangdong, China, 1996 and re-emerged in poultry in 2003, the HPAI-H5N1 has spread around the world (Webster and Govorkova, 2006). World organization for animal health (OIE) has updated that the HPAI has been circulated in domestic birds in 68 countries during January 2013-August 2018 (OIE 2018). AS of February 2019, World health organization (WHO) has reported a total of 860 human cases of HPAI-H5N1 infection (with 454 death) from 16 countries since January 2003 (WHO, 2019).

In Indonesia, the first outbreak of HPAI-H5N1 was reported in February 2004, resulting in 2.4 million poultry deaths (OIE, 2004). Since then, the HPAI-H5N1 has spread and become endemic in most provinces in Indonesia (Stoops et al., 2009). Due to the high number of human cases with HPAI-H5N1 infection, Indonesia has faced serious public health problem. As of 2019, 200 human cases with 168 death have been reported in Indonesia (WHO, 2019). Predominantly clade of HPAI-H5N1 viruses circulating in poultry and human was HPAI-H5N1 clade 2.1. The HPAI-H5N1 clade 2.1 has subsequently evolved into sub clade 2.1.1, 2.1.2 and 2.1.3. Since 2008, clade 2.1.3 has further evolved into the sub clade 2.1.3.1, 2.1.3.2 and 2.1.3.3 (WHO, 2012). It has been known that HPAI-H5N1 Infection in poultry, especially layer, broiler and native chicken cause severe disease with high mortality, but low pathogenic in aquatic birds

such as ducks (Bingham et al., 2009; Wibawa et al., 2014). In contrast, by the end of 2012, the outbreaks of HPAI-H5N1 with high mortality in ducks were observed in Central Java, Yogyakarta and East Java, Indonesia. Genetic analysis of the viruses shows that HPAI-H5N1 clade 2.3.2.1 were responsible for these outbreaks suggesting new clade of HPAI-H5N1 circulating in the country (Wibawa et al., 2012). HPAI-H5N1 clade 2.3.2.1 could also be found in environmental samples from live birds market during the outbreaks (Dharmayanti et al., 2014). In addition, the low pathogenic avian influenza (LPAI) subtype H9N2 was introduced in Indonesia since 2016. The LPAI H9N2 virus has caused the decreased egg production and the increased mortality of chicken in commercial poultry farm of sector 1, 2 and 3. (Jonas et al., 2018).

Avian influenza virus has ability to easily spread and rapidly changes due to its nature of RNA virus (Horimoto and Kawaoka, 2001; Chen et al., 2006). Thus, the surveillance and genetic monitoring of avian influenza is important for understanding the persistence, transmission, and evolution of the viruses. In Indonesia, active surveillances program on avian influenza has been intensively conducted to determine and monitor the occurrence of avian influenza viruses. The active surveillance is not only focused on gallinaceous birds such as chicken but also in aquatic birds such as ducks. However, the information about genetic characteristics of avian influenza viruses isolated from ducks is still limited. Therefore, this thesis aimed to identify, characterize and determine genetic diversity of avian influenza viruses isolated from ducks in Indonesia during year 2015-2017. The information gained from this study is useful to support prevention and control strategies of avian influenza in Indonesia.

Question of study

1. What are the subtypes of avian influenza viruses isolated from ducks in Java Island, Indonesia during year 2015-2017?
2. What are the genetic characteristics and diversity of avian influenza viruses isolated from ducks in Java Island, Indonesia during year 2015-2017?

Objectives of study

1. To identify subtypes of avian influenza viruses isolated from ducks in Java Island, Indonesia during 2015-2017
2. To characterize and determine genetic diversity of avian influenza viruses isolated from ducks in Java Island, Indonesia during 2015-2017.

CHAPTER II

LITERATURE REVIEW

2.1 Influenza A virus

Influenza type A virus is a negative sense, single-stranded and segmented RNA virus. Influenza A virus belongs to the family *Orthomyxoviridae* (Knipe et al., 2007). Recently, members of the family *Orthommyxoviridae* were renamed as *Alphainfluenzavirus* (influenza type A), *Betainfluenzavirus* (influenza type B), *Gammainfluenzavirus* (influenza type C) and *Deltainfluenzavirus* (influenza type D) (Van Regenmortel et al., 2010; ICTV, 2018). Influenza type A is the most important type due to the viruses can infect a large number of avian and mammalian species (Suarez, 2008). Influenza type B and type C primarily infect to human, while the new proposed of influenza type D have been isolated from cattle and pig (Taubenberger and Morens, 2008; Hause et al., 2014). Influenza A virus contains eight genomic segments including Polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M) and non-structural (NS) that encoding nine structural proteins; PB2, PB1, PB1-F2, PA, HA, NA, NP, M1 and M2, and two non-structural proteins; NS1 and NS2 (Lamb, 1983). The HA, NA and M2 proteins form the lipid bilayer which are located on the envelope of the virus, whereas the M1 protein lies just underneath the envelope. The core of the influenza virus contains the viral ribonucleoproteins (vRNPs) complex, which covered by NP and a small amount of nuclear export protein (NEP). The polymerase subunit, PB1, PB2, PA and NP, are located on the tips of the vRNPs (Knipe et al., 2007). Based on antigenic properties of the surface glycoprotein HA and NA, influenza A viruses can be classified into the numerous of subtypes. Recently, there are H1-H16 and N1-N9 subtypes of

influenza A viruses from birds and mammals and H17-H18 and N10-N11 subtypes of influenza A-like viruses from bats (Alexander, 2008; Tong et al., 2013).

2.2 Classification of avian influenza virus

Avian influenza is a contagious infectious disease infecting birds and mammals. Based on the pathogenicity of avian influenza viruses in poultry, the viruses can be classified into two pathotypes, Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI). According to the OIE (2015), the virus that has intravenous pathogenicity index (IVPI) greater than 1.2 when infect to the 6-week-old chicken or the virus cause mortality at least 75% in 4 to 8 week-old chicken is classified as HPAI. Most of HPAI viruses have the multiple basic amino acids at HA cleavage site. HPAI include the viruses of H5 and H7 subtypes, however not all of H5 and H7 subtypes are HPAI (Tsukamoto et al., 2008). HPAI typically cause high morbidity and mortality rate in poultry with severe systemic infection. HPAI is extremely infectious, whereas LPAI usually cause only few or no clinical sign (Swayne and Suarez, 2000).

2.3 Influenza virus life cycle

The influenza virus replication could be described into the following steps: binding and entry into the host cell, transcription and replication of the viral genome, and assembly and budding of progeny virus into the host cell plasma membrane. The entry of influenza virus into the host cell is initiated by the attachment of its HA protein to the neuraminic acid (sialic acid) receptor (SA) of host at in the surface of host cell membrane (Knipe et al., 2007). Thus, the specificity of HA virus plays an important role to bind to the sialic acid (SA) host receptors. Viruses isolated from human preferentially bind to the N-acetylneuraminic acid which are present on the galactose sugar by an α2,6 linkage (SA α2,6 Gal), while avian and equine viruses mostly bind to the SA α2,3 Gal. However, it should be noted that the viral HA binding specificity is not absolute

due to the SA α2,3 Gal and SA α2,6 Gal present in human and avian species (Skehel and Wiley, 2000; Costa et al., 2012). The next step of virus replication is the receptor-mediated endocytosis and entry of virus that would occur after the receptor binding to the host cell. To induce the virus fusion, the HA0 precursor must be cleaved into two subunits, HA1 and HA2, by intracellular protease of host. The low pH in endosome induces a conformational change in HA0, causing the HA1 receptor binding domain could be maintained and the N-terminus of HA2 subunit could be inserted into the endosome membranes for fusion (Knipe et al., 2007). The low pH condition allows the opening of the M2 ion channel; as a result the vRNPs could be released into the cytoplasm of host. The nuclear localization signals (NLSs) contained in the NP, PB1 and PB2, which are comprised the vRNPs, bind to the nuclear import machinery, thus, the viral protein are imported in to the nucleus (Pinto and Lamb, 2006). The transcription and replication of virus that are catalyzed by the viral polymerase subunit proteins occur in the nucleus of host. The initial of transcription require the cap snatching process, which is the viral endonuclease cut the 5' methylgluanosine cap of RNA that will be used as a primer to initiate the transcription. The translation of six viral segments generates six viral proteins, and two other segments generate two proteins in each, M1 and M2 from M gene segment and NS1 and NEP from NS gene segment due to splicing. After release from the nucleus to the cytoplasm, the HA, NA and M protein are transported to the plasma membrane followed by eight RNA segment for virus assembly and budding. Finally, the neuraminidase cleavage the sialic acid receptor on the membrane, thus allowing the progeny virus is released from the host cell (Lamb, 2001; Samji, 2009).

2.4 Evolution of influenza virus

To maintain the ability of influenza virus to interact and survive in the host cell, influenza virus must have the mechanism to escape immune recognition from

the host, including mutation (antigenic drift) and re-assortment (antigenic shift). Thus, through these mechanisms, the influenza viruses have evolutionary changes on their genetic material. Antigenic drift happens continually over time in a small change of gene during the RNA replication. This mechanism usually present at the antibody binding sites of HA and NA proteins due to the immune system of the host, either through previous infection or immunization. Thus, the immune system unable to recognize the newly genetic changed virus (Carrat and Flahault, 2007). Antigenic shift is a major change in the HA and NA proteins of influenza virus. As a consequence, the new strains and / or subtypes are produced due to the re-assortment of different influenza virus gene segments. This mechanism occurs when more than one genotype of influenza viruses infect in a host cell. The novel reassortant viruses could cause pandemic influenza due to the lack of immunity to recognize the novel strain of virus (Reid and Taubenberger, 2003; Kilbourne, 2006).

2.5 Host range of influenza virus

Influenza A viruses have been reported to infect a wide variety of avian species (such as wild and domesticated birds) and mammals species (such as pigs, horses, cats, seals, mink, and whales). Birds are the reservoirs host of influenza A viruses. All of the 16 HA (H1-H16) and 9 NA (N1-N9) subtypes of influenza A viruses have been isolated from various species of birds, including anseriformes (ducks, geese, swan), charadriiformes (gulls, terns, suffbirds, sanpipers), and domestic species (chickens, turkeys, quails, pheasants, gooses, ducks). The disease in birds shows typically subclinical with mild infection of respiratory disease and /or dropping in eggs production. However, some of avian influenza viruses cause high morbidity and mortality up to 100% in birds with severe clinical signs, such as neurological sign, depression, lethargie, anorexia and mucoid diarrhea. The viruses that cause high

pathogenicity up to date have been identified from either H5 or H7 subtype (OIE, 2015).

Although the influenza A virus usually infect birds, the number of human infection caused by these viruses have been reported (Sandrock and Kelly, 2007; Hammond et al., 2017). The transmission of influenza virus from animals to human have been associated with direct contact of infected animals or contaminated environment (Sandrock and Kelly, 2007). The clinical sign of influenza virus infection are vary from mild to severe, including influenza-like illness with fever, cough, pharyngitis, muscle aches and headache, sometimes abdominal pain, diarrhea, vomiting, pneumonia and respiratory failure. Pathological findings are mostly presented in the respiratory tract (Taubenberger and Morens, 2008; Chmielewski and Swayne, 2011).

Pigs can also be infected with influenza A virus. Many studies have suggested that pigs play a role in the emergence of pandemic influenza due to pigs can be the mixing vessel which allowing viral reassortment and generating new strain of virus. Pig epithelial cells contain both human and avian receptors, SA α2,3 Gal and SA α2,6 Gal. For example, the reassortant viruses from swine, avian and human influenza viruses have been found to infect human and cause pandemic outbreaks with some fatal in human (Claas et al., 1994; Ito et al., 1998).

2.6 Global HPAI Outbreaks

The HPAI-H5N1 was firstly identified in 1996 in Guangdong, China. Then the outbreaks of HPAI-H5N1 were reported during 2003-2004. Since then the HPAI-H5N1 virus has spread extensively and have affected poultry in East Asia (including South Korea and Japan) and South East Asia (including Malaysia, Laos, Cambodia, Thailand, Vietnam and Indonesia). In April 2005, the HPAI-H5N1 was reported in wild migratory

birds in Qinghai Lake, western China and spread to Kazakhstan, Mongolia and Russia by migratory birds in August 2005. The HPAI-H5N1 virus continuously spread to migratory birds and backyard poultry in Romania, Turkey, Croatia and Kuwait in late of 2005. In late 2006, the H5N1 viruses spread to Middle East and Europe (Sonnberg et al., 2013). During 2007-2012, the outbreaks continuously occurred in some of countries in Asia, Middle East, and Europe. As of January 2013-August 2018, total 68 countries across Asia, Europe, America, Africa and Oceania was affected by HPAI, including H5N1, H5N2, H5N3, H5N5 H5N6, H5N8, H5N9, H7N2, H7N3, H7N7, H7N8, and H7N9 (OIE, 2018).

2.7 HPAI and LPAI in Indonesia

The HPAI-H5N1 was firstly reported in Indonesia in 2004. The virus was confirmed to cause the outbreak in poultry farms. The outbreak was firstly started in Java and subsequently spread to other provinces across Indonesia (Lam et al., 2008; Stoops et al., 2009; Njoto et al., 2018). At least 2.4 million poultry was death or destroyed during the first outbreak (OIE, 2004). In 2005, the HPAI-H5N1 virus continued to spread and caused human infection in West Java (Kandun et al., 2006). Since then, the human cases caused by HPAI-H5N1 have continued been reported. To date, total 200 human cases with 168 death have been reported in Indonesia (WHO, 2018).

Based on the molecular analysis, the HPAI-H5N1 circulating in Indonesia was mainly belonged to clade 2.1; with the three sub clades: 2.1.1, 2.1.2 and 2.1.3. subsequently, the sub clade 2.1.3 has further evolved into sub clades 2.1.3.1, 2.1.3.2 and 2.1.3.3 (WHO, 2012). According to the nomenclature system updated in 2015 (Smith et al., 2015), the Indonesian sub clade of 2.1.3.2 was evolved into sub clades 2.1.3.2a and 2.1.3.2b. All of these HPAI-H5N1 virus clades were circulating in Indonesia causing high mortality in poultry, especially in layer, broiler and native chicken. However, since 2012, HPAI-H5N1 virus of clade 2.3.2.1 was reported with high mortality

in duck farms in Central Java, Yogyakarta and East Java Provinces (Dharmayanti et al., 2014). To date, the HPAI-H5N1 of clade 2.3.2.1c virus has become predominantly clade circulating in Indonesia (Dharmayanti et al., 2018; Wibawa et al., 2018)

Beside the HPAI-H5N1 virus, Indonesia has faced challenges in the LPAI-H9N2 virus which was introduced in the country in 2016. The LPAI-H9N2 has caused egg production drop up to 70% in commercial poultry farms of sector 1, 2 and 3. This virus affected Layer chicken and broiler in many provinces in Indonesia. The clinical signs of LPAI-H9N2 infection includes swollen head, discharge, torticollis, snoring, thicker egg shell and decreased body weight. Post mortem showed the exudates in nasal cavity and respiratory tract, focal hemorrhages in digestives tract, congested brain and diffuse hemorrhagic in ovary (Jonas et al., 2018).

2.8 Influenza A virus in ducks in Indonesia

Influenza virus has long been in state of evolutionary static in aquatic birds, including ducks. Therefore, ducks are the natural reservoir of influenza A virus. The influenza A virus infection of ducks, either by naturally or experimentally infection, are typically asymptomatic without any clinical signs. The viruses usually replicate in the epithelial cells of digestive tract and only in a few of respiratory tract of aquatic birds (Alexander et al., 1986; Webster et al., 1992). However, the recent studies have shown that the long standing equilibrium of influenza viruses in aquatics birds may not exactly true. The outbreak of H5N1 in Hong Kong in 2002 caused death of many aquatics birds, including ducks (Ellis et al., 2004). Further experimental study showed that the H5N1 had replicated and transmitted efficiently in ducks. The viral infection showed asymptomatic to severe clinical signs and death. The virus titer was high in respiratory tract than in cloacal, indicating the viruses could replicate efficiently in respiratory tract of ducks (Sturm-Ramirez et al., 2005; Brown et al., 2006; Wibawa et al., 2014). In 2012,

the outbreaks of avian influenza in Indonesia with high mortality in ducks have been reported, indicating that the ducks is not only the natural reservoir but also susceptible for avian influenza virus infection.

2.9 Duck raising system in Indonesia

Duck is one of important poultry commodity for providing eggs and meat in Indonesia. Based on the statistics data, the population of ducks in 2017 was raised up to 50 million. West Java, Central Java, East Java and South Sulawesi Provinces were the main ducks raising areas in the country (Diarmita K, 2017). There are three types of duck farming system in Indonesia, including traditional system (scavenging system), semi-intensive system and intensive system. The traditional system (scavenging) or known as extensive system is the herding system with the feed mostly from natural environment. Usually the ducks are allowed to search the food in harvested rice field or other areas for enough feeding. When the feed supply is inadequate, the supplemental feed sometimes is given to the duck flocks. Ducks are kept only in simple confinement. Biosafety and biosecurity of this system are usually low and without vaccination. The second system is semi-intensive system where ducks are kept in a confinement with a pond around the confinement for resting and swimming. This system is very common applied in Indonesia due to suitable system and lower cost compared with intensive system. The intensive system applied by kept the individual duck in confinement with 100% of industrial feed as layer system. The biosafety and biosecurity are applied with vaccination and medication intensively (CIVAS, 2006).

2.10 Prevention and control strategies of avian influenza in Indonesia

To minimize the economic loss in poultry industry and risk to public health, the Indonesian government has established the strategies for prevention and control of avian influenza outbreaks in Indonesia. Based on the regulation of Ministry of

Agriculture Republic of Indonesia (No. 30034/PK.320/F4/01/2017: The increasing of awareness and control strategies of contagious animal diseases), there are main action points for control strategies of avian influenza in Indonesia, including the community awareness to rapidly report the cases of avian influenza, implement the effective biosecurity in the poultry farm and poultry market chain, application of the appropriate vaccination and correct vaccination schedule, improvement of avian influenza virus surveillance and monitoring, control movement of poultry and poultry products and improvement of the hygienic and healthy lifestyle.

CHAPTER III

MATERIALS AND METHODS

This study consisted of 3 phases; Phase 1: Acquisition of avian influenza viruses (AIVs) from the Disease Investigation Center (DIC) Wates Yogyakarta, Indonesia; phase 2: Identification, isolation, and confirmation of avian influenza viruses; phase 3: Whole genome sequencing and genetic characterization of avian influenza viruses. The conceptual framework of this study is shown in figure 1.

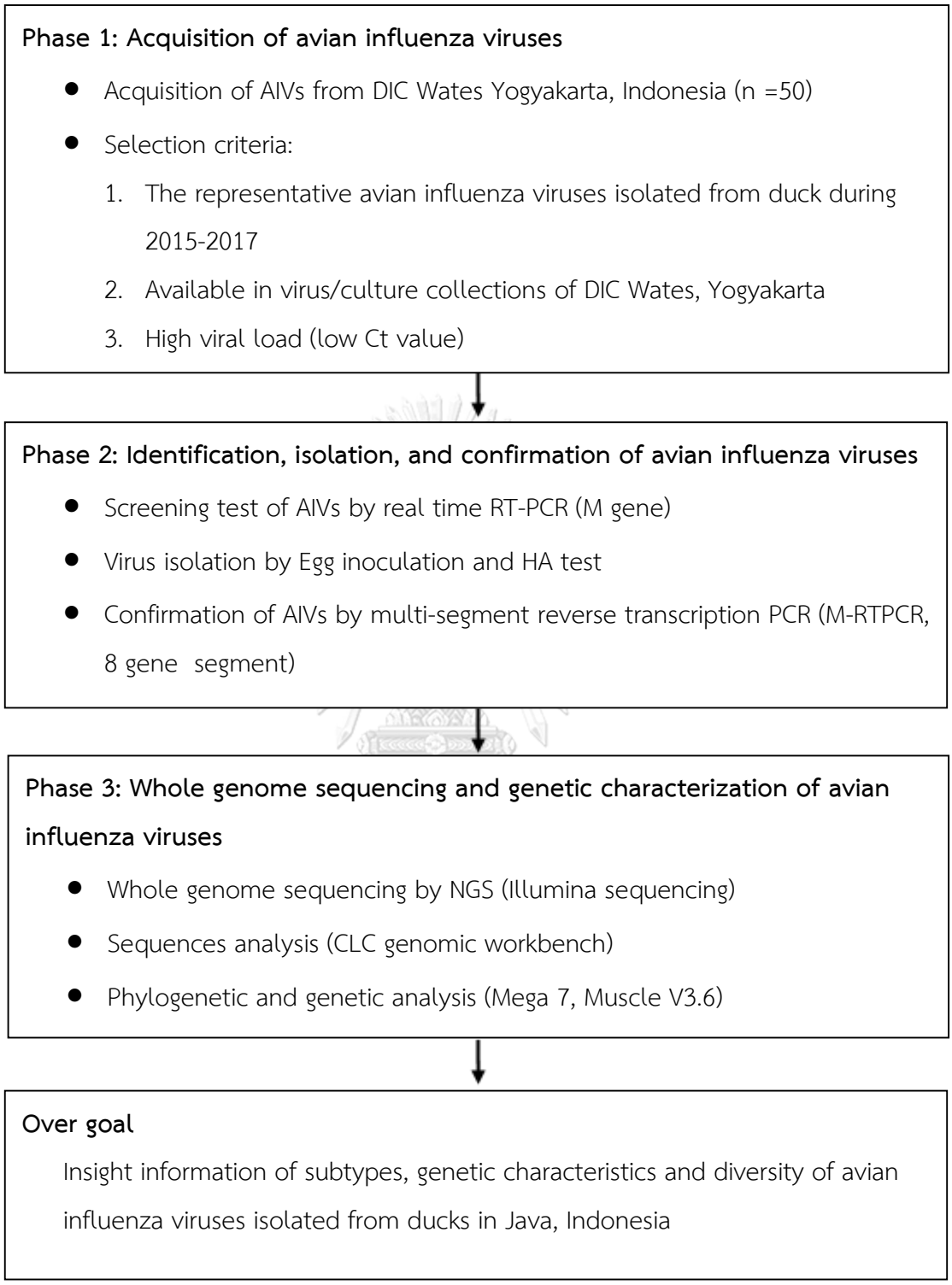

Figure 1 Conceptual framework of this study

Phase 1: Acquisition of avian influenza viruses

In this study, total 50 samples were acquired from Disease Investigation Center (DIC) Wates Yogyakarta, Indonesia. The samples were recovered either from the national program of avian influenza surveillance in ducks in Java, Indonesia or the outbreaks of avian influenza in ducks in Java, Indonesia during 2015-2017. The samples were selected based on the criteria including 1) the representative avian influenza viruses isolated from ducks during 2015-2017, 2) available in virus/culture collections of DIC, Yogyakarta, 3) high viral load (low Ct value). The detail information of samples in this study is shown in Table 1 and Figure 2.

1.1 Samples from avian influenza surveillance in ducks

The active surveillance program for avian influenza in ducks in Java, Indonesia was conducted during 2015-2017. In this surveillance program, the samples were collected from duck farms in Central Java, East Java and Yogyakarta Provinces. Duck farming systems included both traditional/scavenging system and semi-intensive system. Of 3,694 samples tested in the national surveillance program, 16.13% were positive for avian influenza (M gene) and 1.54% were positive for H5 subtype (H5 gene). In this study, 47 samples from the national surveillance program of avian influenza in ducks in Java during 2015-2017 were acquired from the samples collection of the DIC Wates Yogyakarta for further identification, isolation and characterization in phase 2. The samples were recovered from ducks in East Java (n=25), Central Java (n=16), and Yogyakarta (n=6) Provinces during 2015-2017 (Table1, Figure 2).

1.2 Samples from avian influenza outbreaks in ducks

Since 2003, several avian influenza outbreaks had occurred in Indonesia, including the outbreaks of avian influenza in Java in 2016. The first AI outbreak in Java was occurred in duck farms in Bantul, Yogyakarta in July 2016, with approximately

1,000 ducks died and culled. The infected ducks showed torticollis, anorexia, lethargy, incoordination, nasal discharge and cornea opacity. The rapid influenza test indicated avian influenza virus positive. The other avian influenza outbreak was reported in duck farms in Lamongan, East Java, in November 2016. The cause of outbreaks has been confirmed as avian influenza virus subtype H5 and H9 by real time RT-PCR at the DIC Wates Yogyakarta. In this study, 3 samples from 2016 avian influenza outbreaks were acquired from the culture collection of the DIC Wates Yogyakarta for further identification, isolation and characterization in phase 2. Two samples were recovered from ducks in Bantul, Yogyakarta in 2016. Another sample was recovered from ducks in Lamongan, East Java in 2016 (Table 1, figure 2).

Table 1 List of samples acquired in this study

Source of sample	Location	Number of samples
AI surveillance (2015-2017)	East Java	25
	Central Java	16
	Yogyakarta	6
AI outbreak (2016)	East Java	1
	Yogyakarta	2
Total		50

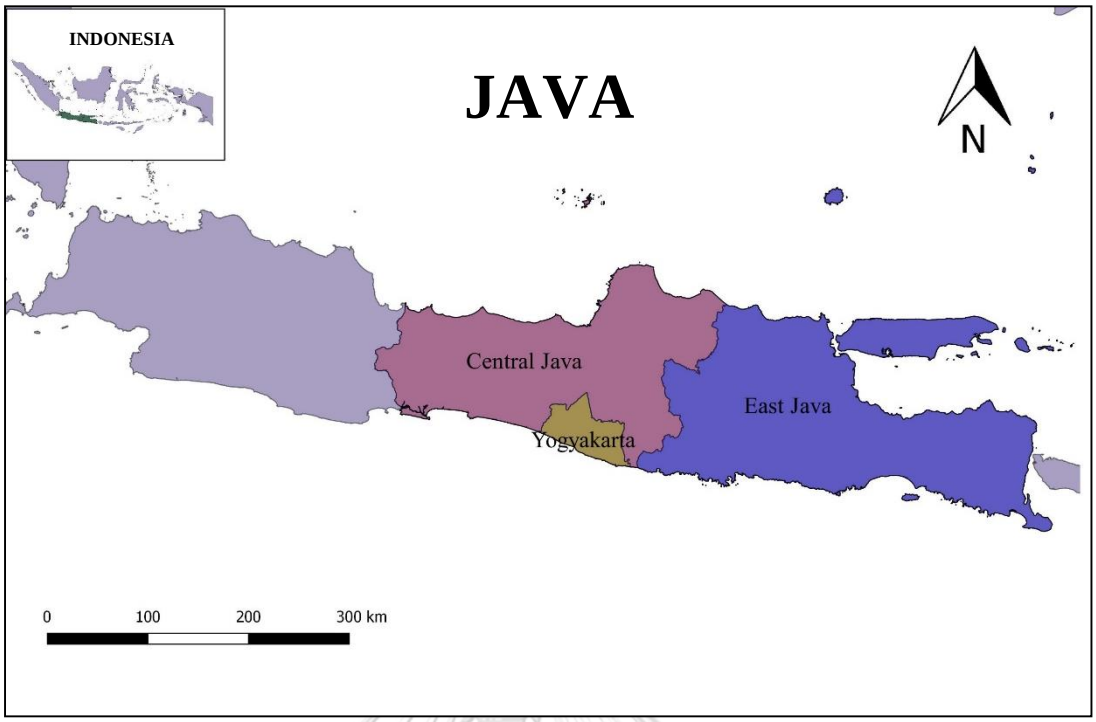

Figure 2 Map of Java Island, Indonesia and the provinces where the samples were selected in this study

Phase 2: Identification, isolation, and confirmation of avian influenza viruses

2.1 Avian influenza virus screening

All 50 samples were screened for influenza A virus by realtime RT-PCR. To identify the influenza A virus, viral RNAs were extracted from the samples by using QiaAmp viral RNA Minikit (Qiagen, Germany). Then RNAs were subjected to influenza A identification by using real time RT-PCR with M gene specific primers. The one step realtime RT-PCR was performed by using AgPath-ID One-Step RT-PCR kit (Ambion®, Life Technologies, USA) under 7500 Real-time PCR System (Applied Biosystem, Foster City CA USA). In brief, one set of specific primer/probe for M gene of influenza virus were used in the realtime RT-PCR reaction. Total 25 ul reaction contained a mixtures of 12.5ul of 2x RT-PCR Buffer, 1ul of 25x RT-PCR enzyme mix, 3.5ul of primer/probe mix (20uM and 5uM), 3ul of RNase free water and 5ul of viral RNA. The realtime RT-

PCR condition was 45^0 C for 10 min for reverse transcription; 95^0 C for 10 min for enzyme activation/initial denaturation and 45 cycles of 95^0 C for 15 sec and 60^0 C for 45 sec for PCR amplification. The results were interpreted as Ct-value < 40 (positive), Ct value of 40-45 (suspected) and Ct-value of > 45 (negative). The samples with positive influenza A virus screening were then submitted for virus isolation/re-isolation (phase 2.2).

2.2 Avian influenza virus isolation

All positive avian influenza viruses by realtime RT-PCR were proceeded for virus isolation in class II type B2 Biosafety cabinet (BSC) following to the OIE protocol (OIE, 2015). For virus isolation, the embryonated chicken eggs (9-11 days old) were used (3 eggs per sample). For sample preparation, 1/10 volume of concentrated antibiotics were added into the sample supernatant. In brief, 200ul of prepared samples were inoculated into the allantoic cavity of specific antibody negative (SAN) embryonated chicken egg. The eggs were then incubated at 37^0 C for 72 hours. During incubation period, the inoculation eggs were monitored every 24 hours. Before harvesting, the eggs were chilled in 4^0 C for 24 hours. The allantoic fluids were collected and tested for the hemagglutination activity by hemagglutination (HA) test. The HA test was performed by using 1% chicken red blood cells (RBCs). The HA positive isolates were then subjected for avian influenza virus confirmation by using multi-segment genomic detection (multi-segment reverse transcription PCR: M-RTPCR).

2.3 Avian influenza virus confirmation by multi-segment reverse transcription PCR (M-RTPCR)

The multi-segment reverse transcription PCR (M-RTPCR) was conducted for the avian influenza virus confirmation. Briefly, the viral RNAs were extracted by using QiaAmp viral RNA Minikit (Qiagen, Germany). The M-RTPCR was performed by using the

Superscript III one step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen; California, USA) with specific primers MBTuni 12 and MBTuni 13 to amplify all eight gene segments of avian influenza virus (Zhou et al., 2009). In detail, 10 ul RNA template was added into the mixture of 25ul of 2x Reaction mix, 2ul of Superscript III RT/Platinum Taq Mix, and 20uM of working concentration of each primer and RNase free water up to 50ul. The viral RNA segments were amplified under the M-RTPCR condition, including 42°C for 60 min; 94°C for 2 min, followed by 5 cycles (94°C for 30 sec; 45°C for 45 sec; and 68°C for 3 min), 35 cycles (94°C for 30 sec; 57°C for 45 sec, and 68°C for 3 min) and 1 cycle of 68°C for 5 min. The PCR products of each gene (2.3kb for PB2 and PB1, 2.2kb for PA, 1.8kb for HA, 1.6kb for NP, 1.4kb for NA, 1.0kb for M and 0.9kb for NS) were analyzed by electrophoresis in 1.5% agarose gel. The purification of PCR products were performed by DNA Clean and ConcentratorTM-5 (Zimo Research Corp.) followed manufacture's instruction. The purified products were then subjected for whole genome sequencing.

Phase 3: Whole genome sequencing and genetic characterization of avian influenza viruses

3.1 Whole Genome Sequencing

The purified products of M-RTPCR were quantified and qualified by using QubitTM dsDNA HS assay kits (Invitrogen, USA) with QubitTM Fluorometer (Invitrogen, Life Technologies). The whole genome sequencing was performed by using Next Generation Sequencer (Illumina, MiSeq). The DNA libraries generation were prepared and sequenced by using Nextera XT DNA kit (Illumina Inc., CA, USA) and MiSeq Reagent Kit v2 (Illumina), respectively. Firstly, total 2ng of input DNA was processed for tagmentation by using the Nextera tagmentation reaction mix containing Nextera transposome enzyme and adapter sequences to tagment and taq the DNA. The

tagmented DNAs were mixed with the indexes and full adapter sequences required for cluster formation. The mixed reaction were then subsequently amplified using a limited-cycle PCR program condition: 72^0 C for 3 min and 95^0 C for 30 sec, followed by 12 cycles of 95^0 C for 30 sec, 55^0 C for 30 sec and 72^0 C for 30 sec and then 1 cycle of 72^0 C for 5 min with holding temperature 10^0 C. The libraries were cleaned up by using Ampure XP Bead (Beckman Coulter, Life Science) with re-suspension buffer to purify the library DNA and remove short library fragments. The purified DNA libraries were then normalized by using Library normalized reagent which normalizes the quantity of each library to allow the library representation in sample more equal. The 2nM library was then prepared and subsequently combined with Phix control (12.5pM) contained in Miseq Reagent Kit V2 (Illumina). The library mix was loaded into the cartridge for sequencing following Miseq System's recommendation. The sequencing products were analyzed with CLC software for nucleotide sequences validation.

3.2 NGS Sequences analysis

The nucleotide sequences validation, assembly and NGS sequences analysis were performed by using CLC Genomics workbench (CLC Bio, Denmark). First, the paired reads (forward/R1.fastq and reverse/R2.fastq) of data sequences were imported into the CLC Genomic workbench. Each nucleotide sequence reads were trimmed to remove adaptors. The Nextera adapter list was selected and used for adaptor trimming. The parameter default was set up during processing of adapter trimming. The trimmed sequences were then assembled by de-novo assembly. In this step, the minimum contiq length of 500 was set up and the contigs below this length were not used. The contigs from de-novo assembly were compared by using BLAST to the sequences database through website https://www.ncbi.nlm.nih.gov to obtain the sequences reference of influenza virus. The reference sequences of influenza virus were downloaded from the GenBank database and were then imported into CLC

genomics workbench. The trimmed sequences were mapped to the selected reference sequences of influenza viruses. The consensus sequences were extracted and the parameter of insert 'N' for ambiguity symbols in the workbench was selected for simply add Ns for each base in the low coverage region. Finally, the influenza virus sequences were extracted in FASTA format from CLC Genomic workbench.

3.3 Phylogenetic and genetic analysis

Phylogenetic and genetic analyses were conducted by comparing each gene segment of the avian influenza viruses with those of reference viruses available in the GenBank database. The reference nucleotide sequences representing vary locations, years and clades of the viruses were downloaded from NCBI (https://www.ncbi.nlm.nih.gov) and Influenza Research Database (https://www.fludb.org). The nucleotide sequences of avian influenza viruses from this study and the reference viruses were then included for phylogenetic and genetics analysis. Nucleotide sequence alignments were performed by using Muscle program v3.6 (Edgar, 2004). The phylogenetic trees were constructed by using MEGA 7 software using neighbor-joining with Kimura-2 parameter and 1,000 bootstrap replications (Kumar et al., 2016). For genetic analysis, the deduced amino acids of each gene segments were aligned by using Muscle program v3.6 in Mega 7. Genetic characteristics and virulence determinants of the viruses, such as pathogenicity (HA cleavage site), receptor binding site, and anti-viral drug resistance determinants were determined.

CHAPTER IV

RESULTS

In this study**,** fifty samples were selected and acquired for screening, virus isolation and genetic characterization of avian influenza viruses. Then the samples were processed for screening of influenza A virus by realtime RT-PCR using M gene specific primers. The positive samples were then subjected for influenza A virus isolation/re-isolation and hemagglutination (HA) test. The multi-segment reverse transcription PCR (M-RTPCR) was performed for influenza A virus confirmation. Whole genome sequencing of avian influenza viruses was performed by Next Generation Sequencing (NGS). Phylogenetic analysis and genetic characterization of whole genome sequences of avian influenza viruses recovered from ducks in Java were performed.

4.1 Acquisition of avian influenza viruses

In this study, fifty samples of previously identified avian influenza viruses were selected and acquired for virus screening, isolation and genetic characterization. The 50 samples were retrieved from Disease Investigation Center (DIC) Wates Yogyakarta, Indonesia. The samples included 47 samples from the avian influenza surveillance program in ducks in Java during 2015- 2017 and 3 samples from the avian influenza outbreaks during 2016. In detail, the samples were collected from ducks from 7 districts (Trenggalek, Tulungagung, Lamongan, Blitar, Situbondo, Sidoarjo, and Malang) in East Java Province (n=26), 4 districts (Kebumen, Banyumas, Brebes and Purworejo) in Central Java Province (n=16), and 2 districts (Bantul and Sleman) in Yogyakarta Province (n=8). Detail description of samples retrieved for avian influenza virus screening is shown in Table 2.

Table 2 Detail description of samples acquired for avian influenza virus screening and isolation in this study

Province	District	Year of collection	Number of sample	Influenza A virus identification*		
				IAV	IAV subtype H5	IAV subtype H9
East Java (total n=26)	Trenggalek	2015	1	1	1	0
	Tulungagung	2015&2017	8	8	0	0
	Lamongan	2016	1	1	1	0
	Blitar	2016	6	6	0	0
	Situbondo	2016	2	2	0	0
	Sidoarjo	2016	2	2	0	0
	Malang	2017	6	6	1	0
Central Java (total n=16)	Kebumen	2015	10	10	10	0
	Banyumas	2017	3	3	0	0
	Brebes	2017	2	2	0	0
	Purworejo	2017	1	1	1	0
Yogyakarta (total n=8)	Bantul	2016&2017	4	1	2	1
	Sleman	2017	4	4	0	0
Total			50	50	16	1

*****Influenza A and/or subtype; previously identified by DIC

4.2 Avian influenza virus screening, isolation and confirmation

4.2.1 Avian influenza virus screening and identification

All 50 samples were screened for influenza A virus by realtime RT-PCR using M gene specific primers. In this step, total 46 samples from all provinces and all districts showed positive results for influenza A virus (Ct value< 40). This results indicated that

92% (46/50) of the retrieved samples were positive for influenza A viruses and suitable for virus isolation/re-isolation (Table 3).

4.2.2 Avian influenza virus isolation/re-isolation

The influenza A virus positive samples were processed for virus isolation by eggs inoculation. The allantoic fluids were then proceeded for Hemagglutination (HA) test. Of 46 isolates, 4 isolates showed positive hemagglutination activity, including 2 isolates from East Java (Trenggalek and Lamongan) and 2 isolates from Yogyakarta (Bantul) (Table 3). Forty two isolates were negative for hemagglutination (HA) test.

Table 3 Detail description of avian influenza virus screening and isolation

Province	District	Number of sample	Virus screening	Virus isolation
			Realtime RT-PCR positive	HA test positive
East Java	Trenggalek	1	1	1
	Tulungagung	8	8	0
	Lamongan	1	1	1
	Blitar	6	6	0
	Situbondo	2	1	0
	Sidoarjo	2	2	0
	Malang	6	6	0
Central Java	Kebumen	10	7	0
	Banyumas	3	3	0
	Brebes	2	2	0
	Purworejo	1	1	0
Yogyakarta	Bantul	4	4	2
	Sleman	4	4	0
Total		50	46	4

4.2.3 Avian influenza virus confirmation by multi-segment genomic detection (M-RTPCR)

Initially, based on virus isolation/re-isolation and HA test results, the samples were HA positive (n=4) and HA negative samples (n=42). In detail, one positive sample (A/duck/Trenggalek/04150329-17/2015=Tr/17/15) was recovered from ducks in East Java from the national surveillance program of avian influenza in Java during 2015, and the two positive samples (A/duck/Bantul/04161291-OP/2016= Bt/1291-OP/16 and A/duck/Bantul/04161291-OP/2016= Bt/1291-OR/16) and one positive sample (A/duck/Lamongan/04161958/2016= Lm/1958/16) were recovered from ducks in Yogyakarta and East Java, respectively, during avian influenza outbreaks in 2016. In addition, the samples that have low Ct value by realtime RT-PCR (but negative for HA test) (n=3) were also included and subjected for avian influenza virus confirmation by M-RTPCR. In detail, the three samples were recovered from ducks in Java from the national surveillance program of avian influenza during 2015-2017. The one sample (A/duck/Malang/04170342-MO46/2017= Ml/46/17) was recovered from ducks in East Java and two samples (A/duck/Kebumen/04150777-33/2015=Kb/33/15 and A/duck/Purworejo/04171160-111/2017= Pw/111/17) were recovered from ducks in Central Java (Figure 3).

After M-RTPCR, all samples (n=7) were confirmed as avian influenza A virus. The confirmed avian influenza virus were then subjected for whole genome sequencing and genetic characterization. Detail description of samples positive for avian influenza virus confirmation and selected for genetic characterization is shown in Table 4.

Table 4 Detail description of samples positive for avian influenza virus by M-RTPCR and selected for genetic characterization

Sample	Province	District	Collection date	Source of sample	Type of sample
Tr/17/15	East Java	Trenggalek	Mar, 2015	AI surveillance	Allantoic fluid
Lm/1958/16	East Java	Lamongan	Nov, 2016	AI outbreak	Allantoic fluid
Ml/46/17	East Java	Malang	Mar, 2017	AI surveillance	Oropharyngeal swab
Kb/33/15	Central Java	Kebumen	Apr, 2015	AI surveillance	Oropharyngeal & cloacal swab
Pw/111/17	Central Java	Purworejo	May, 2017	AI surveillance	Tissues
Bt/1291-OR/16	Yogyakarta	Bantul	Jul, 2016	AI outbreak	Allantoic fluid
Bt/1291-OP/16	Yogyakarta	Bantul	Jul, 2016	AI outbreak	Allantoic fluid

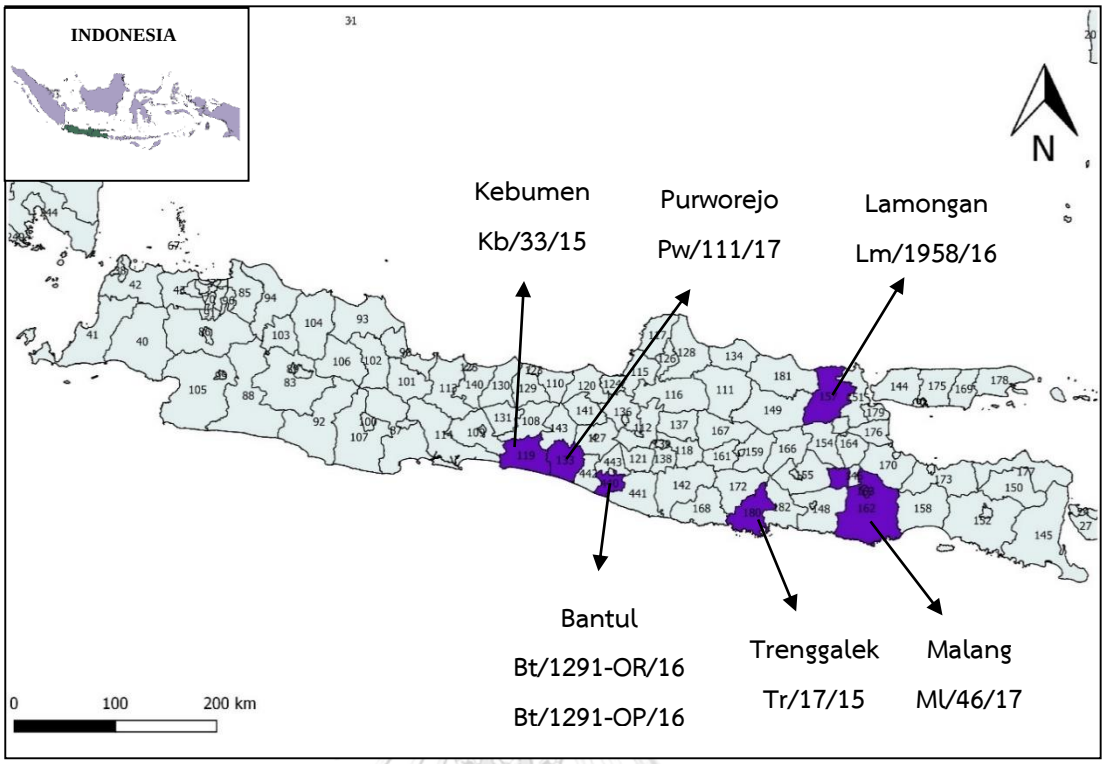

Figure 3 Map of Java island, Indonesia and the location of seven avian influenza viruses selected for whole genome sequencing and genetic characterization.

4.3 Whole genome sequencing and genetic characterization of avian influenza viruses

4.3.1. Whole genome sequencing by next generation sequencing (NGS)

The seven avian influenza viruses were subjected to whole genome sequencing by next generation sequencing using Illumina sequencing. Whole genome sequences of all seven avian influenza viruses were successfully determined and the results is shown in Table 5. It is noted that only partial PB1 gene of the virus Pw/111/2017 was available. Subtyping of avian influenza viruses were also successfully determined by comparing the available HA and NA genes by using BLAST analysis. Based on BLAST analysis, the results revealed that the avian influenza viruses characterized in this study were influenza A subtype H5N1 (n=6) and H9N2 (n=1).

Table 5 Detail of whole genome sequences of avian influenza viruses by next generation sequencing in this study

Virus	Sub-type	Length of gene segments (bp)							
		PB2	PB1	PA	HA	NP	NA	M	NS
Tr/17/15	H5N1	2232	2341	2223	1773	1565	1398	1027	870
Lm/1958/16	H5N1	2322	2298	2223	1775	1565	1399	1027	875
Ml/46/17	H5N1	2307	2341	2151	1775	1565	1399	1027	875
Kb/33/15	H5N1	2280	2341	2233	1775	1497	1398	982	875
Pw/111/17	H5N1	2315	1136*	2233	1775	1565	1398	1027	875
Bt/1291-OR/16	H5N1	2341	2341	2223	1773	1542	1398	1027	870
Bt/1291-OP/16	H9N2	2328	2341	2233	1742	1563	1465	1027	890

*Partial gene sequence

4.3.2 Whole genome sequences comparison of avian influenza viruses

Whole genome sequences of all seven avian influenza viruses were compared with the virus references. The virus references included in the sequences comparison were the H5N1 virus of clade 0 (A/goose/Guangdong/1/1996), the H5N1 viruses of clade 2.3.2.1c (A/quail/Sukoharjo/04152003/2015 and A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain)), the H5N1 virus of clade 2.1.3.2 (A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain)), the H9N2 viruses (A/turkey/Wisconsin/1/1966 and A/chicken/Vietnam/NCVD-15A13/2015) and the H7N9 viruses (A/Hangzhou/1/2013 and A/Shanghai/2/2013).

For H5N1 virus analysis, our results showed that H5N1 viruses from this study had high percentage of nucleotide identities to H5N1 virus of clade 2.3.2.1c (A/quail/Sukoharjo/04152003/2015) with ranging from 98.03-99.56% (PB2); 98.45-99.12% (PB1); 98.28-98.88% (PA); 97.11- 99.77% (HA); 98.73-98.40% (NP); 98.54-99.54% (NA); 93.58- 99.80% (M) and 99.16-99.52% (NS) (Table 6). In contrast, most H5N1 viruses

showed low nucleotide identities to H5N1 viruses of clade 2.1.3.2 (A/chicken/West Java/Sbg-29/2007). However, it is interesting to note that the M gene from one H5N1 virus (Pw/111/17) had higher nucleotide identity (95.62%) to H5N1 virus clade 2.1.3.2 comparing with H5N1 virus clade 2.3.2.1c (93.58%).

For H9N2 virus analysis, the virus (Bt/1291-OP/16) had high percentage of nucleotide identities to H9N2 virus recovered from Vietnam (A/chicken/Vietnam/NCVD-15A13/2015) with 93.03% (PB2); 97.43% (PB1); 98.56% (PA); 98.51% (HA); 97.72% (NP), 98.16% (NA); 99.29% (M) and 94.55 (NS). In addition, the internal genes of H9N2 virus (Bt/1291-OP/16) had high nucleotide identity to human H7N9 viruses from China (A/Shanghai/2/2013) with the percentage of nucleotide identities of 97.06% (PB2); 95.95% (PB1); 96.55% (PA); 95.02% (NP), 97.06% (M) and 98.45% (NS) (Table 7).

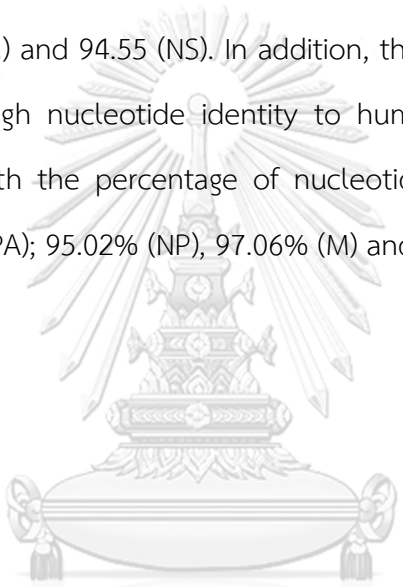

Table 6 Nucleotides identity of H5N1 viruses in this study compared to the virus references available in the GenBank database

Reference Viruses	Subtype/clade	% nucleotide identities (Tr/17/15, Lm/1958/16, Ml/46/17, Kb/33/15, Pw/111/17, Bt/1291-OR/16)							
		PB2	PB1	PA	HA	NP	NA	M	NS
gs/Gd/1/96[a]	H5N1 (clade 0)	91.05-91.27	90.51-91.08	90.66-91.07	89.94-90.86	90.65-91.58	91.93-92.67	93.48-95.32	70.69-71.29
ck/Sb/29/07[b]	H5N1 (clade 2.1.3.2)	92.46-93.76	93.76-94.42	89.21-89.63	89.44-90.26	94.82-95.66	91.78-92.74	95.03-96.03	94.45-94.93
qa/Sk/04152003/15[c]	H5N1 (clade 2.3.2.1c)	98.03-99.56	98.45-99.12	98.28-98.88	97.11-99.77	98.73-98.40	98.54-99.54	93.58-99.80	99.16-99.52
dk/Sk/1428-9/12[d]	H5N1 (clade 2.3.2.1c)	97.68-98.33	97.89-98.38	97.95-98.40	97.57-98.61	98.13-99.06	97.85-98.96	93.79-99.68	98.16-99.26
ck/Vn/15A13/15[e]	H9N2	84.95-85.45	86.70-87.39	87.40-87.82	55.86-56.15	92.72-93.59	51.20-51.72	88.80-89.61	86.73-87.21
tk/Ws/1/66[f]	H9N2	83.20-83.60	86.69-87.14	85.08-85.40	56.12-56.71	88.38-88.84	50.75-51.57	89.92-91.14	90.83-91.07

[a]A/goose/Guangdong/1/1996
[b]A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain of H5N1 clade 2.1.3.2)
[c]A/quail/Sukoharjo/04152003/2015
[d]A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain of H5N1 clade 2.3.2.1c)
[e]A/chicken/Vietnam/NCVD-15A13/2015
[f]A/turkey/Wisconsin/1/1966

Table 7 Nucleotides identity of H9N2 virus in this study compared to the virus references available in the GenBank database

Reference Viruses	Subtype/clade	% nucleotide identities							
		Bt/1291-OP/16							
		PB2	PB1	PA	HA	NP	NA	M	NS
gs/Gd/1/96[a]	H5N1 (clade 0)	86.62	90.78	89.35	56.44	90.71	92.3	90.94	68.72
ck/Sb/29/07[b]	H5N1 (clade 2.1.3.2)	85.7	89.2	87.26	56.07	93.92	51.85	89.31	87.09
qa/Sk/04152003/15[c]	H5N1 (clade 2.3.2.1c)	84.3	87.73	87.54	61.32	92.99	52.09	89.61	85.77
dk/Sk/1428-9/12[d]	H5N1 (clade 2.3.2.1c)	84.3	87.88	87.87	56.5	93.45	51.87	89.61	85.29
ck/Vn/15A13/15[e]	H9N2	93.03	97.43	98.56	98.51	97.72	98.16	99.29	94.55
tk/Ws/1/66[f]	H9N2	83.25	87.35	86.15	80.82	87.78	84.25	89.41	88.15
A/Hz/1/13[g]	H7N9	96.97	95.82	96.47	42.76	95.06	52.73	97.05	98.33
A/Sh/**2**/13[h]	H7N9	97.06	95.95	96.55	52.71	95.02	52.66	97.06	98.45

[a]A/goose/Guangdong/1/1996

[b]A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain of H5N1 clade 2.1.3.2)

[c]A/quail/Sukoharjo/04152003/2015

[d]A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain of H5N1 clade 2.3.2.1c)

[e]A/chicken/Vietnam/NCVD-15A13/2015

[f]A/turkey/Wisconsin/1/1966

[g]A/Hangzhou/1/2013 (H7N9)

[h]A/Shanghai/2/2013 ((H7N9)

4.3.3 Phylogenetic analysis of avian influenza viruses

The phylogenetic analyses of each gene segment of avian influenza viruses were performed. The phylogenetic analyses included the viruses from this study and the virus references retrieved from the GenBank database. The avian influenza virus references were selected to represent different hosts, locations and clades of the viruses including Indonesia, Southeast Asia, Asia, Europe and Africa.

4.3.3.1 Phylogenetic analysis of H5N1 viruses

In this study, our results showed that the HA genes of H5N1 viruses recovered from ducks in Java Indonesia were clustered into H5N1 virus of clade 2.3.2.1c. In detail, 6 H5N1 viruses (Tr/17/15, Lm/1958/16, Ml/46/17, Pw/111/17, Kb/33/15, and Bt/1291-OR/16) were grouped among them and grouped together with H5N1 viruses of clade 2.3.2.1c from Indonesia and Vietnam. It is noted that, these H5N1 viruses were also clustered in the same group with Indonesia vaccine strain (A/duck/Sukoharjo/BBVW-1428-9/2012) (clade 2.3.2.1c) (Figure 4). The phylogenetic tree of HA gene of H5N1 viruses was rooted with Goose/Guangdong/1/1996 (clade 0).

Phylogenetic analysis of NA gene of H5N1 viruses showed that all of H5N1 viruses were clustered into H5N1 virus of clade 2.3.2.1c. Similar to the HA gene, the NA gene were grouped together with other H5N1 viruses of clade 2.3.2.1c from Indonesia and Vietnam (Figure 5).

4.3.3.2 Phylogenetic analysis of H9N2 viruses

In this study, the phylogenetic tree showed that the HA gene of H9N2 virus (Bt/1291-OP/16) recovered from duck in Java Indonesia was clustered with H9N2 viruses of Y280-like group. This H9N2 virus was grouped together with the other Indonesian H9N2 viruses and was closely related with the Vietnam H9N2 viruses. It

should be noted that this virus was isolated from the same district and the same duck farm with the H5N1 virus (Bt/1291-OR/16) (Figure 6). The phylogenetic tree of HA gene of H9N2 viruses was rooted with Turkey/Wisconsin/1/1966.

Phylogenetic analysis of NA gene of H9N2 viruses revealed that the H9N2 virus (Bt/1291-OP/16) was grouped together with other Indonesia and Vietnam H9N2 viruses, differed from other H9N2 groups (Y280 like, G1-like and Y439-like) (Figure 7).

4.3.3.3 Phylogenetic analysis of internal genes of H5N1 viruses

The phylogenetic tree of 6 internal genes of H5N1 viruses (Tr/17/15, Lm/1958/16, Ml/46/17, Pw/111/17, Kb/33/15, and Bt/1291-OR/16) were constructed. The Phylogenetic analysis of PB2 of H5N1 viruses revealed that all of H5N1 viruses were grouped into H5N1 viruses of clade 2.3.2.1c (Figure 8). Similar to PB2 gene, the PB1, PA, NP, and NS genes of H5N1 viruses in this study were clustered with H5N1 viruses of clade 2.3.2.1c from Indonesia and other countries (Figure 9, 10, 11 and 13). In contrast, the M gene of one H5N1 virus (Pw/111/17) was grouped together with H5N1 viruses of clade 2.1.3.2, differed with other five H5N1 viruses (Tr/17/15, Lm/1958/16, Ml/46/17, Kb/33/15, and Bt/1291-OR/16) which clustered into H5N1 viruses of clade 2.3.2.1c (Figure 12)

4.3.3.4 Phylogenetic analysis of internal genes of H9N2 viruses

Different from the HA and NA genes of H9N2 viruses, the phylogenetic analysis of all internal genes (PB2, PB1, PA, NP, M and NS) of H9N2 virus (Bt/1291-OP/16) were grouped together with H7N9-like viruses. The Bt/1291- OP/16 was closely related to the Vietnam H9N2 viruses and human isolates of H7N9 virus from China (Figure 8, 9, 10, 11, 12 and 13).

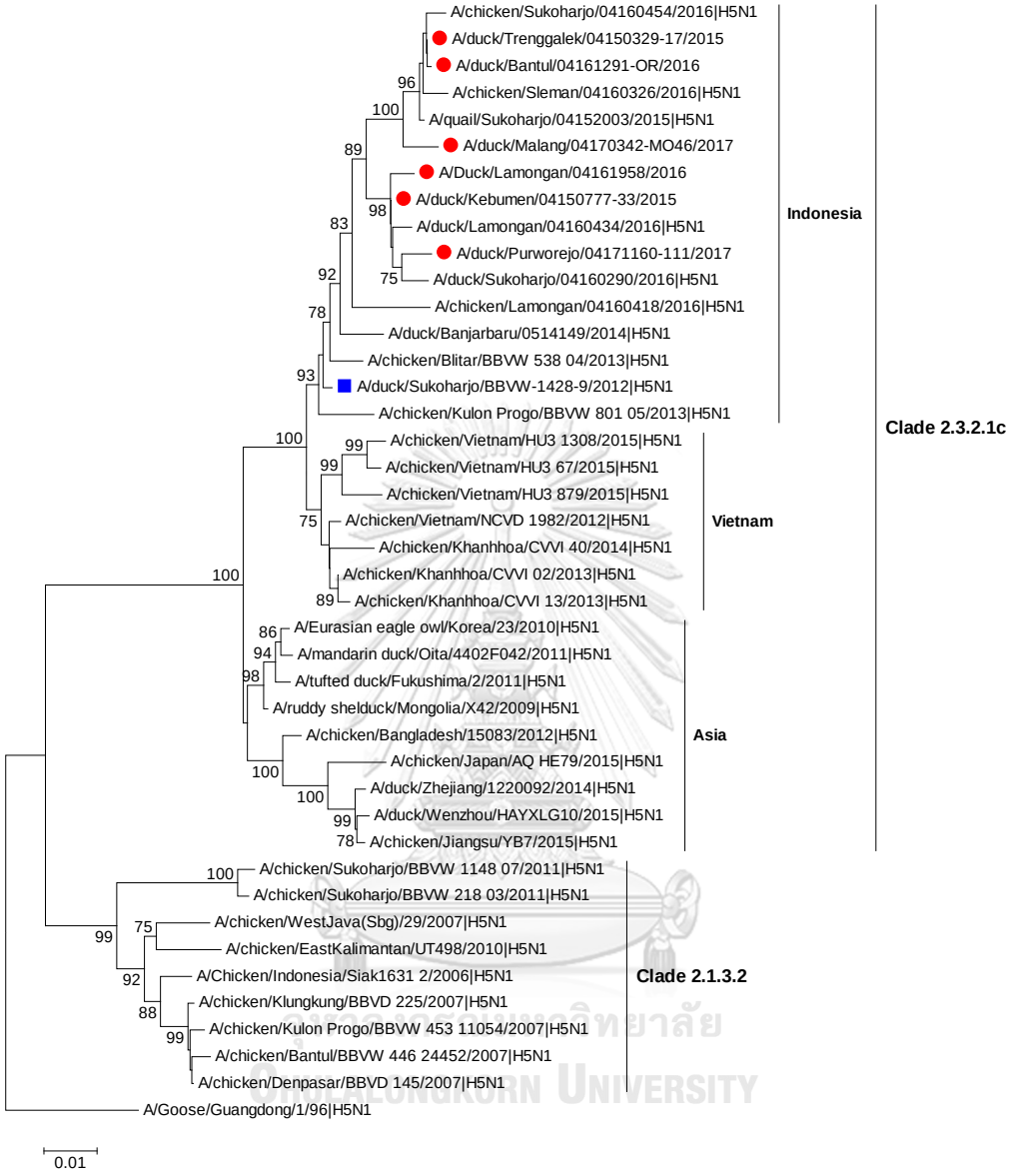

Figure 4 Phylogenetic analysis of HA5. Circle and square labels indicate the H5N1 viruses in this study and the H5N1 Indonesia vaccine virus, respectively. The scale bar indicates the distance matrix between sequence pairs. The tree was rooted to A/goose/Guangdong/1/1966.

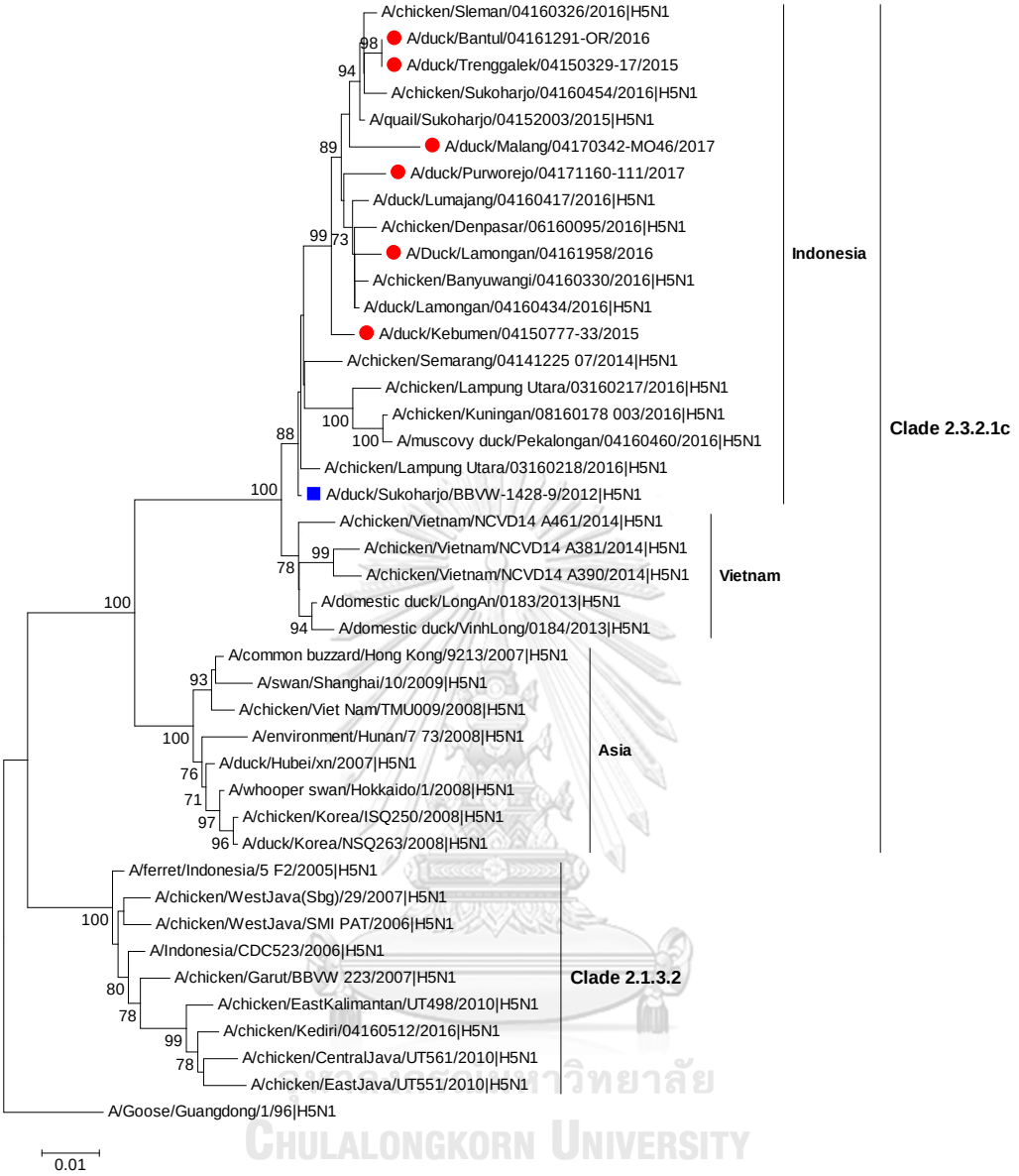

Figure 5 Phylogenetic analysis of NA1. Circle and square labels indicate the H5N1 viruses in this study and the H5N1 Indonesia vaccine virus, respectively. The scale bar indicates the distance matrix between sequence pairs. The tree was rooted to A/goose/Guangdong/1/1966.

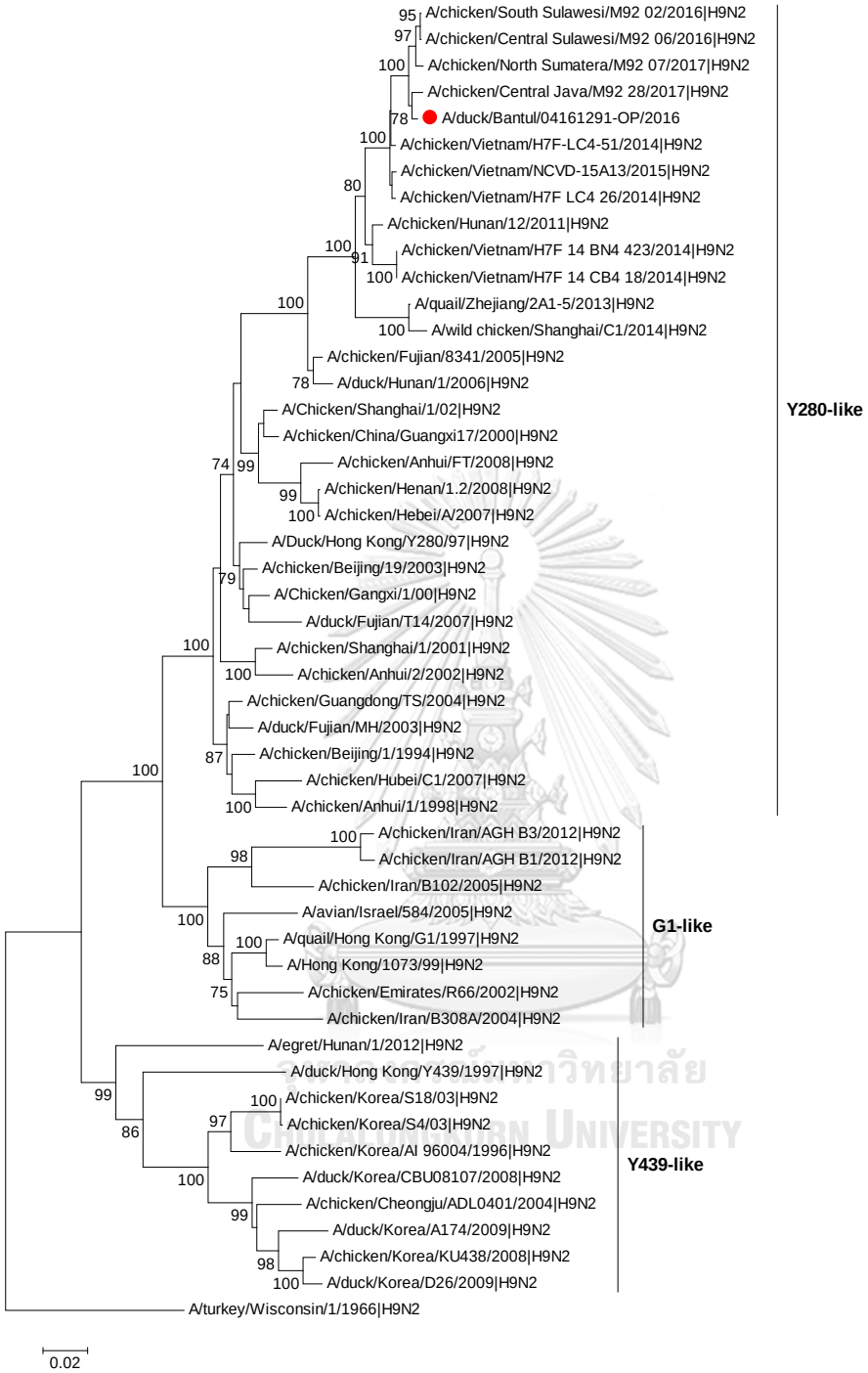

Figure 6 Phylogenetic analysis of HA9. Circle label indicates the H9N2 virus in this study. The scale bar indicates the distance matrix between sequence pairs. The tree was rooted to A/turkey/Wisconsin/1/1966.

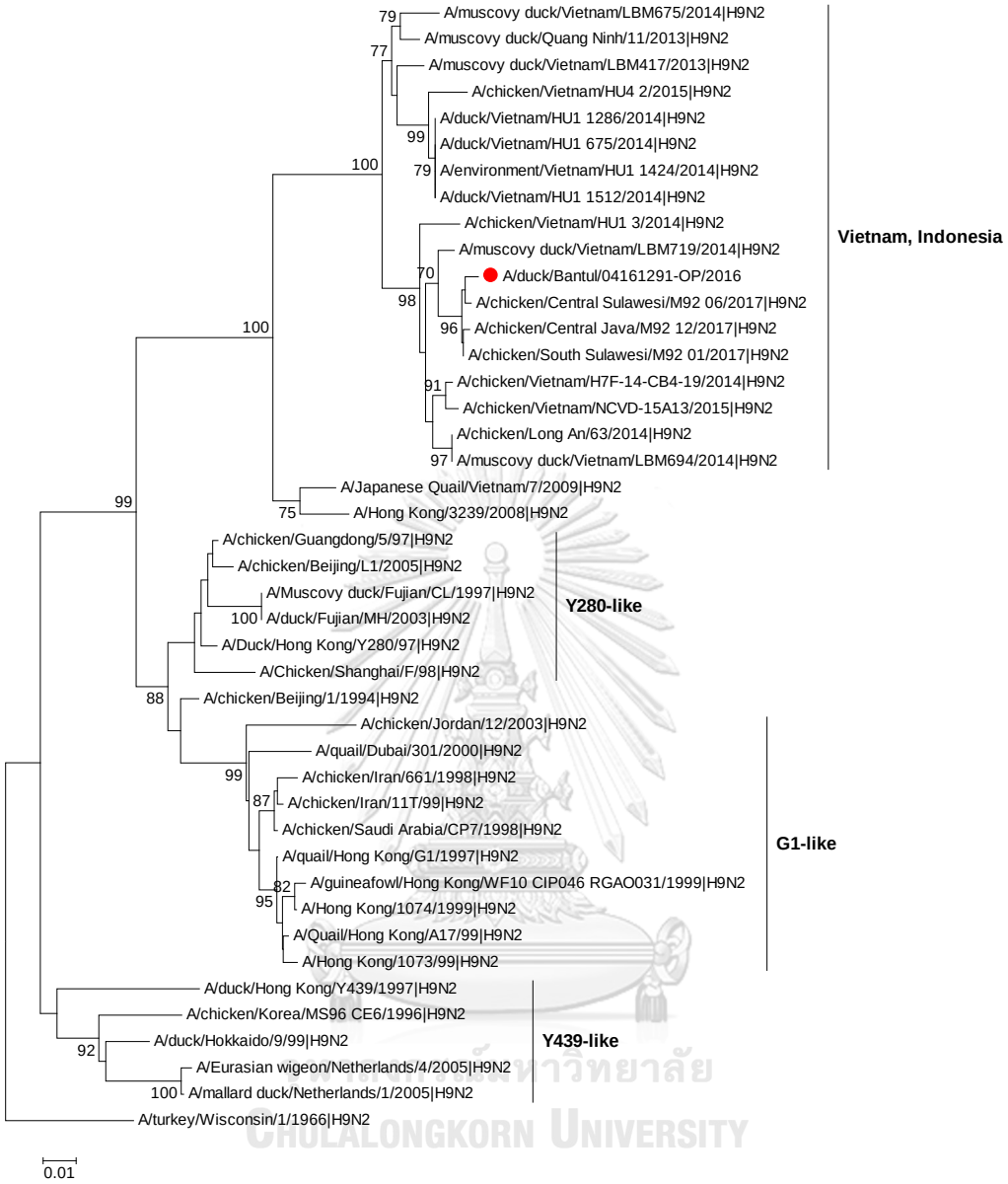

Figure 7 Phylogenetic analysis of NA2. Circle label indicates the H9N2 viruses in this study. The scale bar indicates the distance matrix between sequence pairs. The tree was rooted to A/turkey/Wisconsin/1/1966.

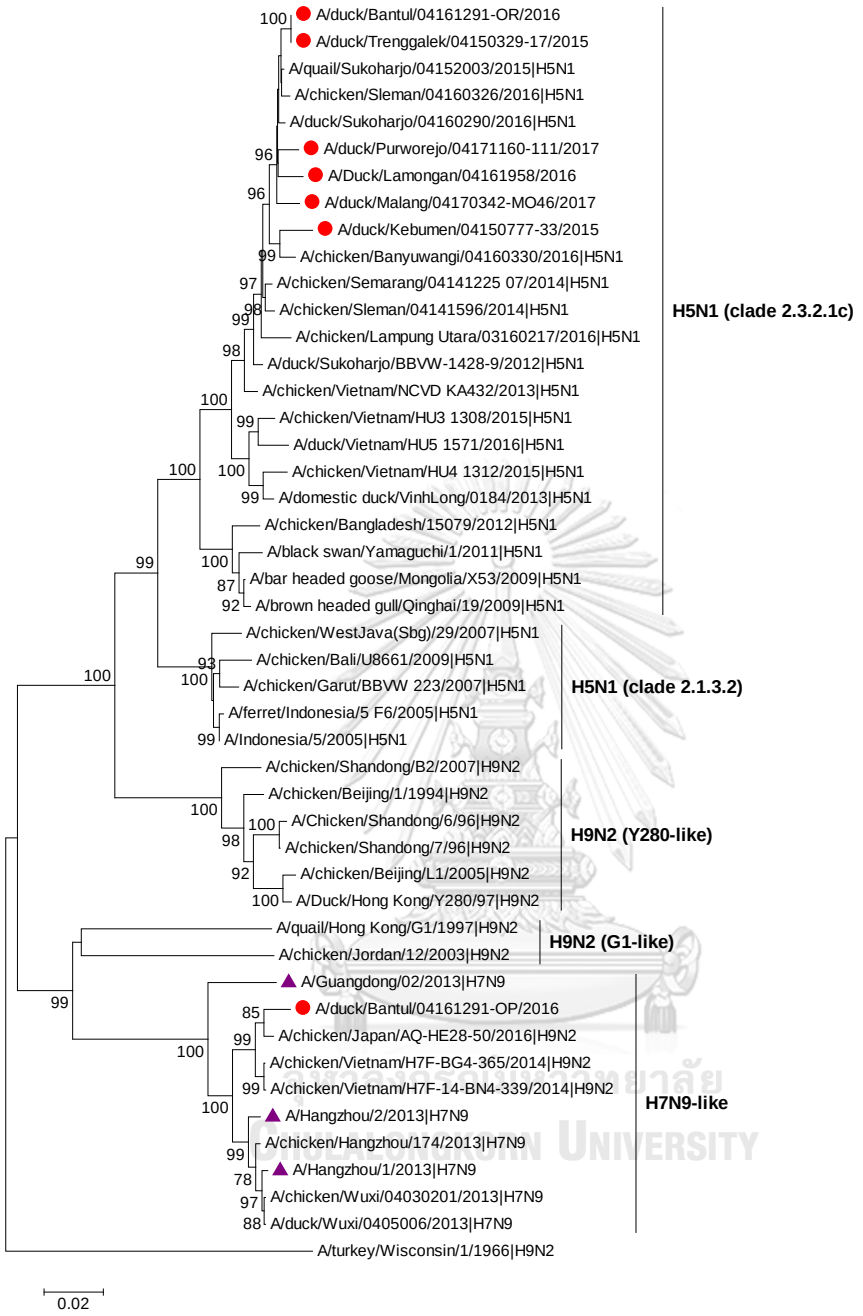

Figure 8 Phylogenetic analysis of PB2 gene. Circle label indicates the H5N1 and H9N2 viruses in this study. Triangle label indicates human H7N9 viruses from China.

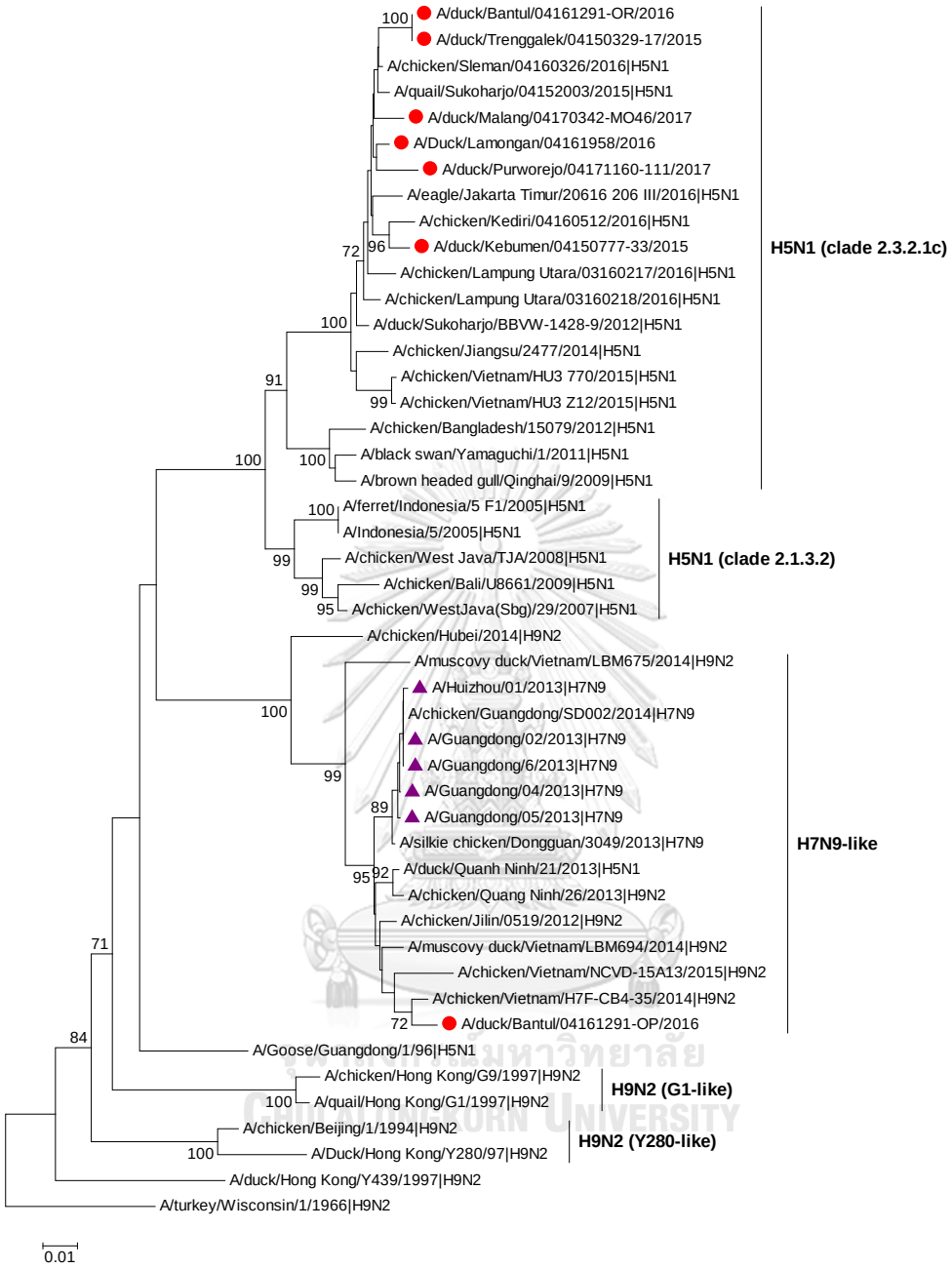

Figure 9 Phylogenetic analysis of PB1 gene. Circle label indicates the H5N1 and H9N2 viruses in this study. Triangle label indicates human H7N9 viruses from China.

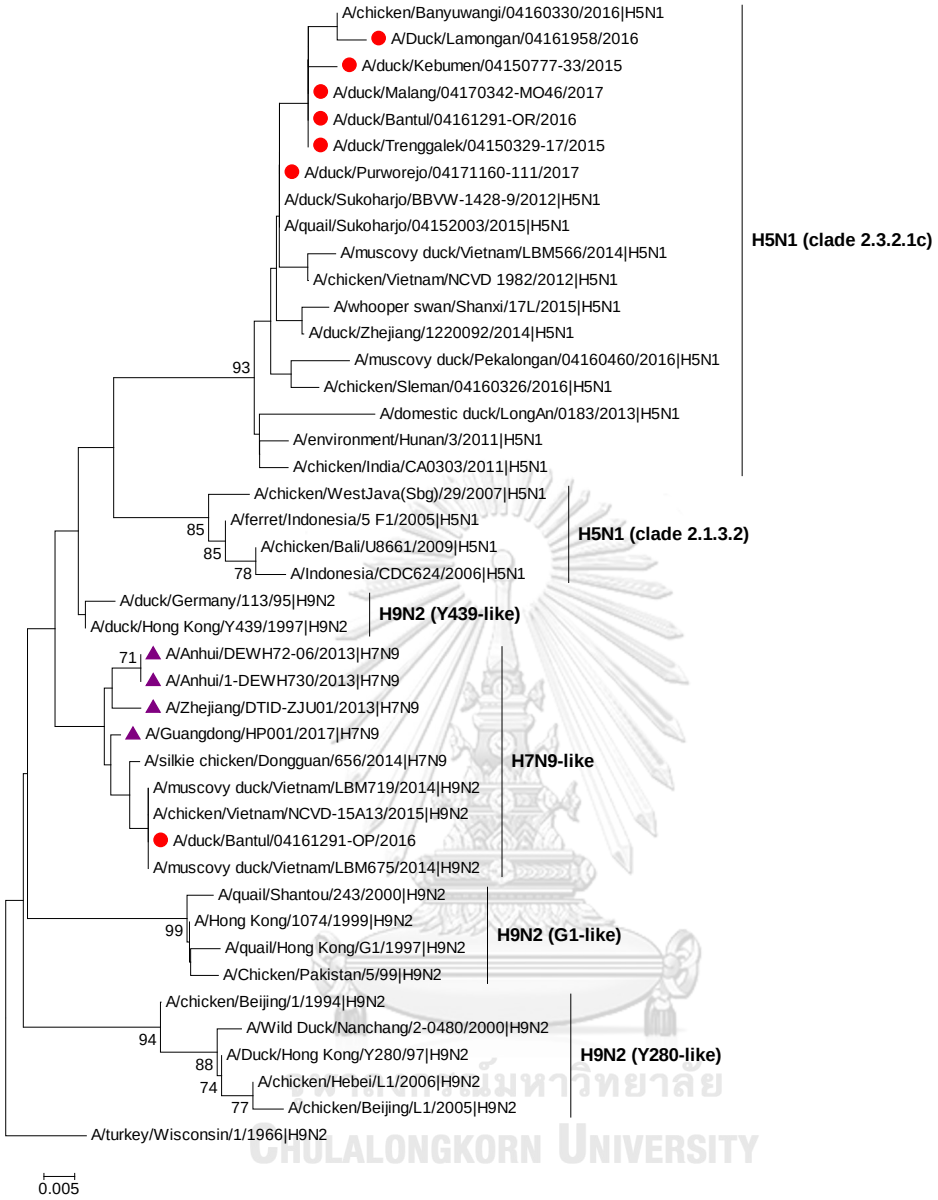

Figure 10 Phylogenetic analysis of PA gene. Circle label indicates the H5N1 and H9N2 viruses in this study. Triangle label indicates human H7N9 viruses from China.

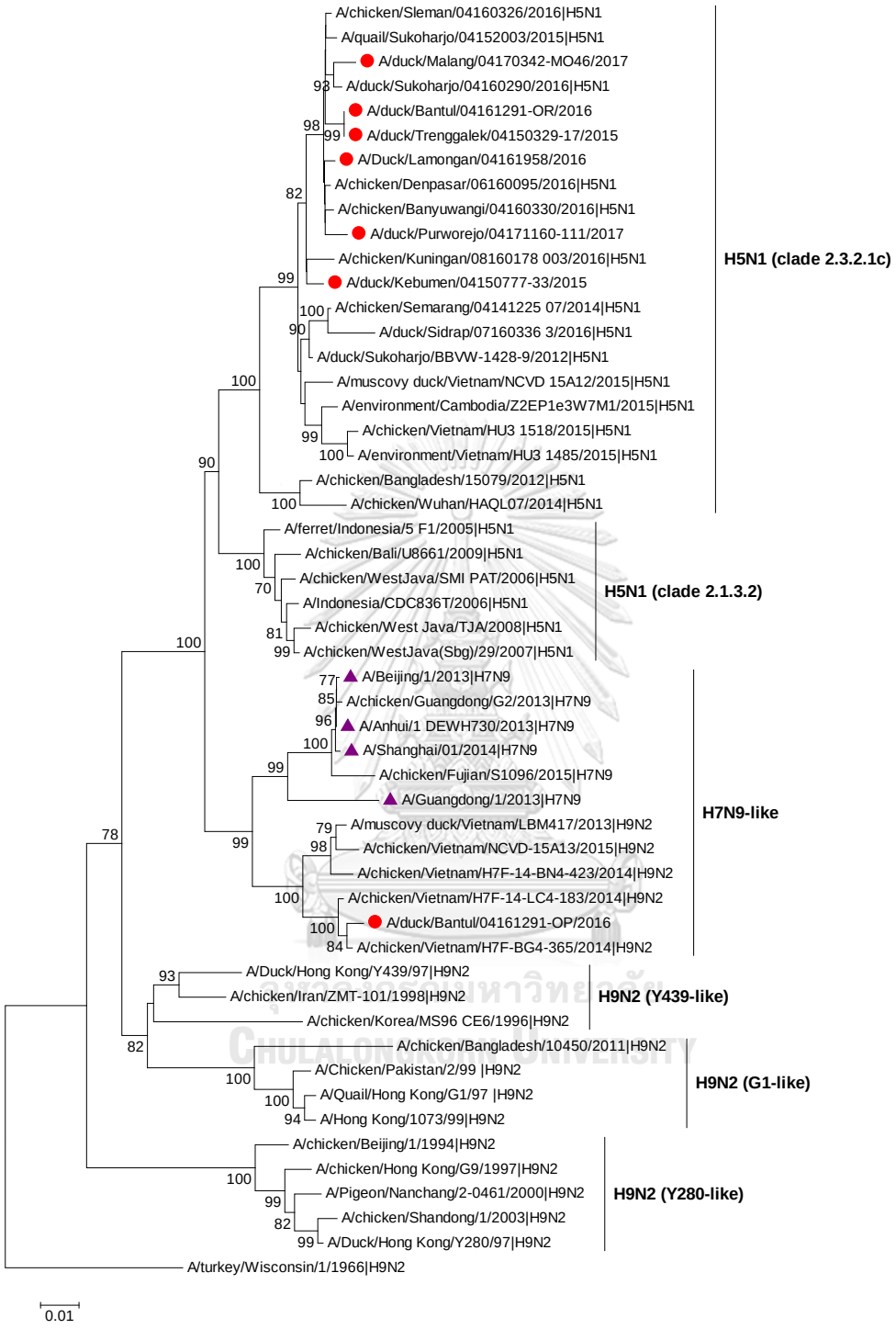

Figure 11 Phylogenetic analysis of NP gene. Circle label indicates the H5N1 and H9N2 viruses in this study. Triangle label indicates human H7N9 viruses from China.

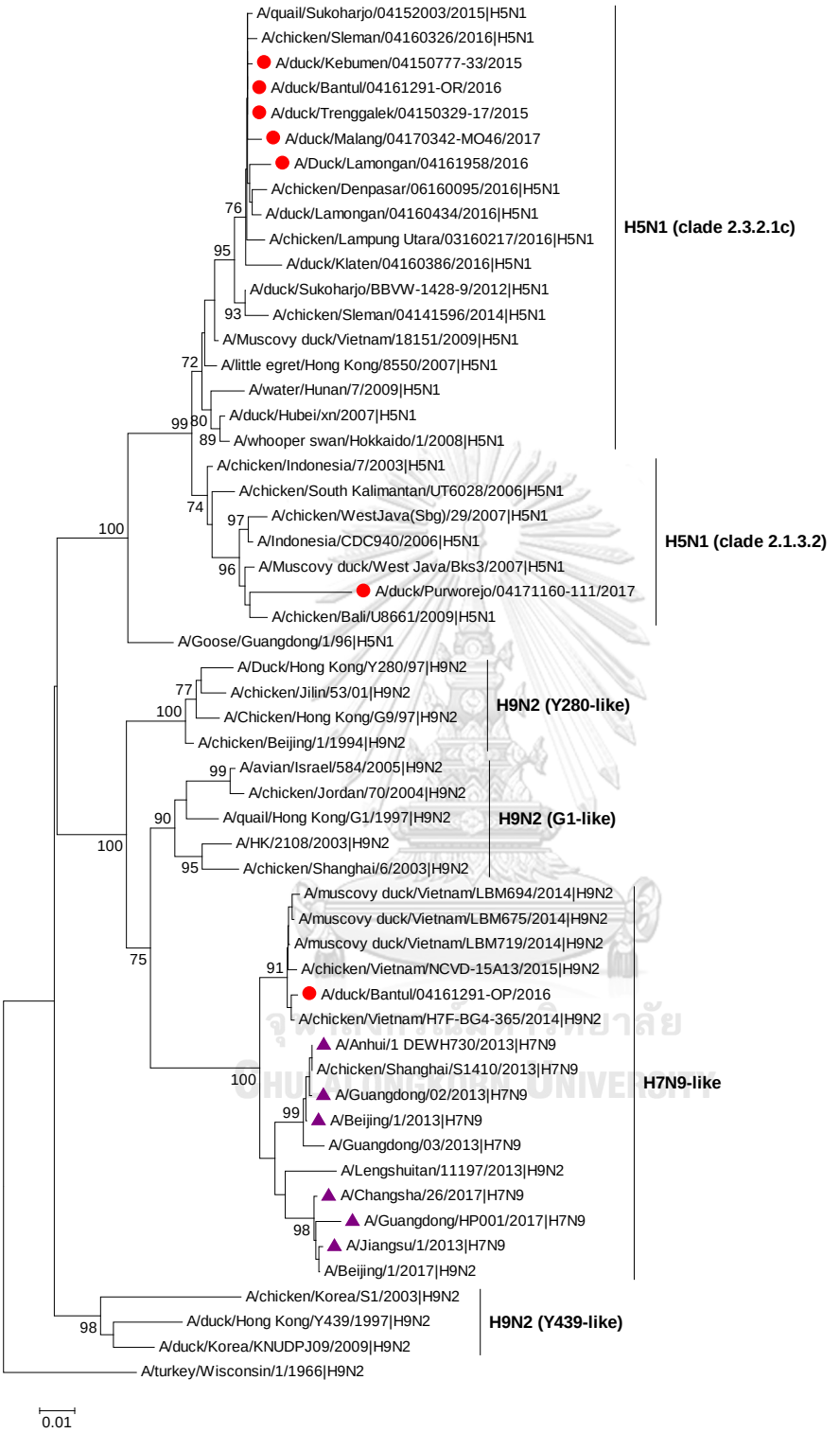

Figure 12 Phylogenetic analysis of M gene. Circle label indicates the H5N1 and H9N2 viruses in this study. Triangle label indicates human H7N9 viruses from China.

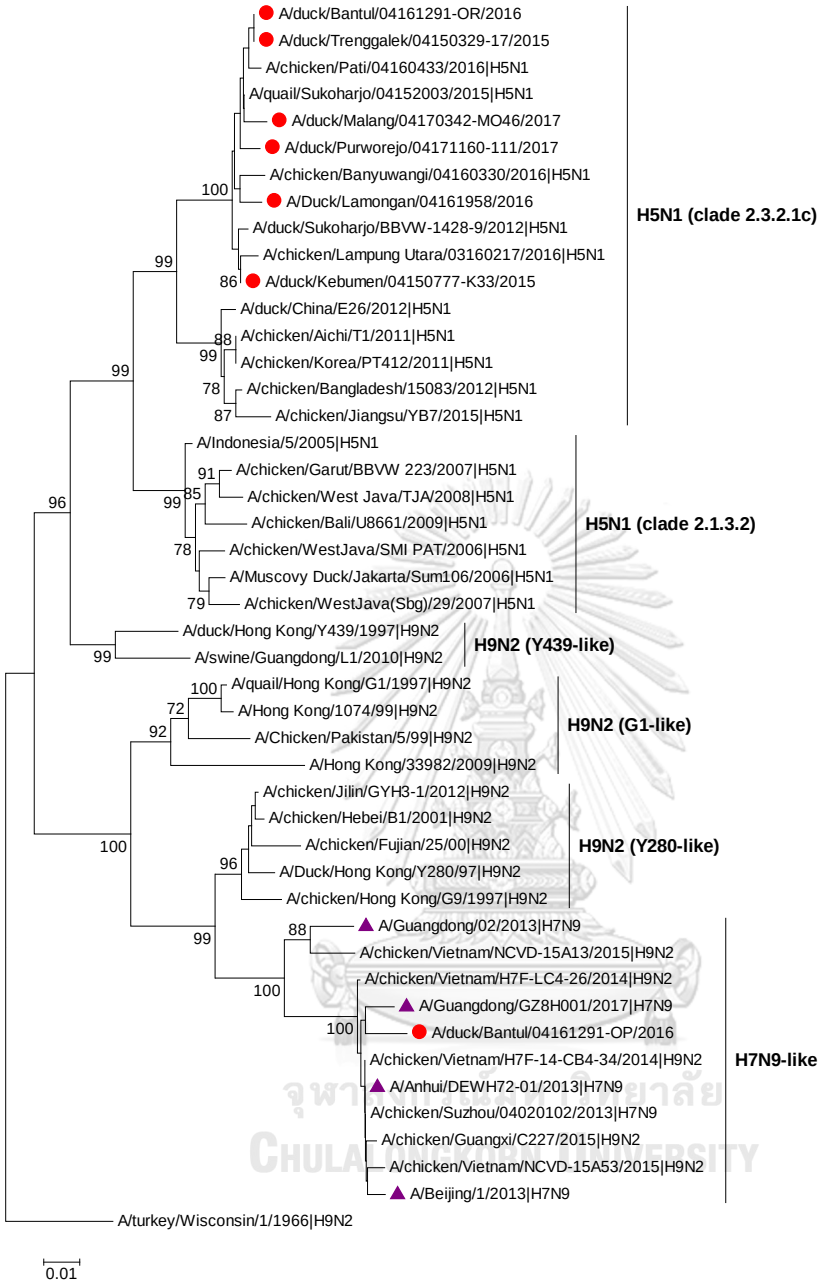

Figure 13 Phylogenetic analysis of NS gene. Circle label indicates the H5N1 and H9N2 viruses in this study. Triangle label indicates human H7N9 viruses from China.

4.3.4 Genetic analysis of H5N1 viruses

Genetic analysis of HA gene of H5N1 viruses was conducted by comparing deduced amino acid sequences amongst the viruses. Our results showed that H5N1 viruses recovered from ducks in Indonesia possess multiple basic amino acids at the HA cleavage site indicating Highly Pathogenic Avian Influenza (HPAI) characteristics. The multiple basic amino acids 'RERRRKR' motif of the H5N1 viruses in this study were similar to those of the reference H5N1 virus (A/quail/Sukoharjo/04152003/2015) and the Indonesia reference vaccine H5N1 virus of clade 2.3.2.1c. The H5N1 viruses also harbored amino acids Glutamine (Q) at position 222 (Q222) and Glycine (G) at position 224 (G224) indicating the avian specific receptor binding site. The H5N1 viruses contained N-link glycosylation site (N-N-T) at position 165-167 suggesting increase virulence of the viruses due to masking of antigenic epitope of HA1 globular head. The 165-167 N-link glycosylation motif was also observed in H5N1 viruses of clade 2.3.2.1c (Table 8).

Genetic analysis of NA gene of H5N1 viruses for antiviral resistance markers showed the amino acids at position 119 Glutamic acid (E), 275 Histidine (H,) 293 Arginine (R) and 295 Asparagine (N), indicating that these virus were sensitive to antiviral drugs (oseltamivir). In addition, the deletion of 20 amino acids at NA stalk region was observed in all H5N1 viruses isolated from Indonesia and the virus references of clade 2.3.2.1c. While progeny H5N1 virus of clade 0 (A/goose/Guangdong/1/96), no 20 amino acid deletion at NA stalk region was identified (Table 9).

4.3.5 Genetic analysis of H9N2 viruses

Genetic analysis of HA gene of H9N2 viruses showed that H9N2 virus recovered from duck in Indonesia (Bt/1291-OP/16) possess amino acids at the HA cleavage site as 'SRSSR' suggesting Low Pathogenic Avian Influenza (LPAI) characteristics. At receptor

binding site, the virus harbored 155-Threonine (T), 183-asparagine (N) and 226-Leucine (L) amino acids indicating the human specific receptor binding site. Our results showed that the HA cleavage site and receptor binding site at HA9 gene of the virus (Bt/1291-OP/16) was similar to the H9N2 virus isolated from Vietnam (A/Chicken/Vietnam/NCVD-15A13/2015) (Table 10).

Genetic analysis of NA gene of H9N2 virus showed that the H9N2 virus (Bt/1291-OP/16) possess Glutamic acid (E) at position 119, indicating sensitive to oseltamivir. No amino acid deletion at position 63-65 was observed in NA gene of H9N2 virus suggesting low viral virulence in chicken and mammals. The detail of genetic analysis of HA9 and NA2 genes of H9N2 virus is shown in Table 10.

Table 8 Genetic analysis of HA5 gene of amino acid of H5N1 viruses in this study

Virus	HA			
	Clade	HA cleavage site	Receptor binding site		N-Link Glycocylation site
		323-332*	222*	224*	165-167*
Tr/17/15	2.3.2.1c	RERRRKR/G	Q	G	NNT
Kb/33/15	2.3.2.1c	RERRRKR/G	Q	G	NNT
Bt/1291-OR/16	2.3.2.1c	RERRRKR/G	Q	G	NNT
Lm/1958/16	2.3.2.1c	RERRRKR/G	Q	G	NNT
Ml/46/17	2.3.2.1c	RERRRKR/G	Q	G	NNT
Pw/111/17	2.3.2.1c	RERRRKR/G	Q	G	NNT
qa/Sk/04152003/15[a]	2.3.2.1c	RERRRKR/G	Q	G	NNT
dk/Sk/1428-9/12[b]	2.3.2.1c	RERRRKR/G	Q	G	NNT
ck/Sb/29/07[c]	2.1.3.2	RESRRKKR/G	Q	G	NNT
gs/Gd/1/96[d]	0	RERRRKKR/G	Q	G	NNT

*H3 numbering

[a]A/quail/Sukoharjo/04152003/2015

[b]A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain of H5N1 clade 2.3.2.1c)

[c]A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain of H5N1 clade 2.1.3.2)

[d]A/goose/Guangdong/1/1996

Table 9 Genetic analysis of NA1 gene amino acid of H5N1 viruses in this study

Virus	NA					
	Clade	Antiviral resistance marker				NA stalk region
		119	275	293	295	49-68 (20 aa deletion)
Tr/17/15	2.3.2.1c	E	H	R	N	Yes
Kb/33/15	2.3.2.1c	E	H	R	N	Yes
Bt/1291-OR/16	2.3.2.1c	E	H	R	N	Yes
Lm/1958/16	2.3.2.1c	E	H	R	N	Yes
Ml/46/17	2.3.2.1c	E	H	R	N	Yes
Pw/111/17	2.3.2.1c	E	H	R	N	Yes
qa/Sk/04152003/15[a]	2.3.2.1c	E	H	R	N	Yes
dk/Sk/1428-9/12[b]	2.3.2.1c	E	H	R	N	Yes
ck/Sb/29/07[c]	2.1.3.2	E	H	R	N	Yes
gs/Gd/1/96[d]	0	E	H	R	N	No

[a]A/quail/Sukoharjo/04152003/2015

[b]A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain of H5N1 clade 2.3.2.1c)

[c]A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain of H5N1 clade 2.1.3.2)

[d]A/goose/Guangdong/1/1996

Table 10 Genetic analysis of HA9 and NA2 genes amino acid of H9N2 viruses in this study

Virus	HA				NA	
	HA cleavage site	Receptor binding site			Antiviral resistance marker	Deletion amino acid
	334-339*	155*	183*	226*	119	63-65
Bt/1291-OP/16	SRSSR	T	N	L	E	No
ck/Vn/15A13/15[a]	SRSSR	T	N	L	E	No
ck/Bj/1/94[b]	ARSSR	T	N	Q	E	No
tk/Ws/1/66f[c]	AVSSR	T	H	Q	E	No

*H3 numbering

[a]A/chicken/Vietnam/NCVD-15A13/2015

[b]A/chicken/Beijing/1/1994

[c]A/turkey/Wisconsin/1/1966

4.3.6 Genetic analysis of internal genes of H5N1 viruses

The internal genes (PB2, PB1, PA, NP, M and NS) of H5N1 viruses recovered from ducks in Indonesia were compared with the virus references. The genetic analysis of PB2 gene showed that all H5N1 viruses in this study possess Glutamic acid (E) at position 627 and aspartic acid (D) at position 701, which known as less virulence in mammals. In addition, the characteristics of less virulence virus were also detected in PB1-F2 which harbor amino acid asparagine (N) at position 66 instead of serine (S) and PA genes which harbor Serine (S) at position 224 instead of Proline (P). However, the genetic analysis of the amino acids that were demonstrated to enhance virulence in mammals species could be detected in PB1 gene with Proline (P) at position 13. In addition, the characteristics to enhance virulence of the viruses in duck was detected

at PA gene with Aspartic acid (D) at position 383 and Threonine (T) at position 515. Moreover, the Aspartic acid (D) at position 30 and Alanine (A) at position 215 of the M1 gene were identified indicating increase the virulence of virus in mice. In NS1 gene contained Serine (S) at position 42 and Alanine (A) at position 149, similar to the reference H5N1 virus. Deletion of amino acids at position 80-84 at NS1 of all H5N1 viruses also observed indicating enhance viral virulence in chicken and mice, differed from progeny of H5N1 virus (A/goose/Guangdong/1/1996). The PDF binding motif ligand 'ESEV' at c-terminal of NS1 was identified in all of H5N1 viruses suggesting enhance viral virulence and pathogenesis. NP gene of the of H5N1 viruses carried amino acid Leucine (L) at position 136, suggesting avian-like characteristics, differing with the progeny of H5N1 virus A/goose/Guangdong/1/1996 which containing Methionine (M) at that position. For M2 gene, amantadine resistance markers were observed in some of H5N1 viruses in this study with alanine (A) at position 27 (Pw/111/17) and asparagine (N) at position 31 (Tr/17/15 and Pw/111/17). However, the Leucine (L) at position 26 and Alanine (A) at position 30 of M2 gene still could be identified from all of H5N1 viruses indicating sensitive to antiviral drugs (Table 11 and 12).

Table 11 Genetic analysis of internal genes (PB2, PB1, PA and NP) of H5N1 viruses

Viruses	Amino acid positions							
	PB2		PB1	PB1-F2	PA			NP
	E 627 K	D 701 N	L 13 P	N 66 S	S 224 P	N 383 D	A 515 T	L 136 M
Tr/17/15	E	D	P	N	S	D	T	L
Kb/33/15	E	D	P	N	S	D	T	L
Bt/1291-OR/16	E	D	P	N	S	D	T	L
Lm/1958/16	E	D	P	N	S	D	T	L
Ml/46/ 17	E	D	P	N	S	D	T	L
Pw/111/17	E	D	P	N	S	D	T	L
qa/Sk/ 2003/15[a]	E	D	P	N	S	D	T	L
dk/Sk/1428-9/12[b]	E	D	P	N	S	D	T	L
ck/Sb/29/07[c]	E	D	P	N	S	D	T	L
gs/Gd/1/96[d]	E	D	P	N	S	D	T	M
Function	virulence in mammals	virulence in mammals	virulence in mammals	viral virulence	virulence in duck	virulence in duck	virulence in duck	Avian/human like characteristics

[a]A/quail/Sukoharjo/04152003/2015

[b]A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain of H5N1 clade 2.3.2.1c)

[c]A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain of H5N1 clade 2.1.3.2)

[d]A/goose/Guangdong/1/1996

Table 12 Genetic analysis of internal genes of (M and NS) of H5N1 viruses

Viruses	Amino acid positions									
	M1		M2				NS1			
	N 30 D	T 215 A	L 26 F	V 27 A/T	A 30 T/V	S 31 N/G	C-terminal	P 42 S	V 149 A	aa del. (80-84)
Tr/17/15	D	A	L	I	A	N	ESEV	S	A	Yes
Kb/33/15	D	A	L	V	A	S	ESEV	S	A	Yes
Bt/1291-OR/16	D	A	L	I	A	S	ESEV	S	A	Yes
Lm/1958/16	D	A	L	I	A	S	ESEV	S	A	Yes
Ml/46/ 17	D	A	L	V	A	S	ESEV	S	A	Yes
Pw/111/17	D	A	L	A	A	N	ESEV	S	A	Yes
qa/Sk/ 2003/15[a]	D	A	L	I	A	S	ESEV	S	A	Yes
dk/Sk/1428-9/12[b]	D	A	L	I	A	S	ESEV	S	A	Yes
ck/Sb/29/07[c]	D	A	L	A	A	S	ESEV	S	A	Yes
gs/Gd/1/96[d]	D	A	L	V	A	S	ESEV	A	A	No
Function	virulence in mice	virulence in mice	Amantadine resistance	Amantadine resistance	Amantadine resistance	Amantadine resistance	Virulence-related	virulence in mice	virulence in chicken	virulence in chicken&mice

[a]A/quail/Sukoharjo/04152003/2015

[b]A/duck/Sukoharjo/BBVW-1428-9/2012 (Indonesia vaccine strain of H5N1 clade 2.3.2.1c)

[c]A/chicken/West Java/Sbg-29/2007 (Indonesia antigen challenge strain of H5N1 clade 2.1.3.2)

[d]A/goose/Guangdong/1/1996

4.3.7 Genetic analysis of internal genes of H9N2 viruses

In this study, the deduced amino acids of internal genes (PB2, PB1, PA, NP, M and NS) of H9N2 virus were analyzed. Differed from the PB2 gene of human H7N9 viruses from China which harbored amino acid Lysine (K) at position 627, known as the virulence virus in mammals, the H9N2 virus possess Glutamic acid (E) at this position indicating less virulence in mammals. In addition the amino acid aspartic acid (D) contained in PB2 gene at position 701, suggesting less virulence virus in mammals, similar with H9N2 virus from Vietnam. Furthermore, PB1-F2 harbors asparagine (N) at position 66 and PA gene harbors Serine (S) at position 224 which known as less virulence of virus in mammals. However, the genetic analysis of the amino acids Proline (P) at position 13 in PB2 gene that were demonstrated to enhance viral virulence in mammals species could be detected. In addition, the PA gene contained Aspartic acid (D) at position 383 and Threonine (T) at position 515, indicating enhance viral virulence in duck. Moreover, the M1 gene contained Aspartic acid (D) at position 30 and Alanine (A) at position 215 indicating increase viral virulence in mice. For the NS1 gene, Serine (S) at position 42 and Alanine (A) at position 149 were observed, indicating enhance virulence of virus in mice. None of the H9N2 viruses contained deletion of amino acid at position 80-84 in NS1 gene. The H9N2 virus harbor 'KPEV' at c-terminal of NS1 similar with H9N2 Vietnam virus and human H7N9 viruses from China. For NP gene, the H9N2 virus harbors Leucine (L) at position 136 identical to the H9N2 virus references indicating avian-like characteristics. For M2 gene, asparagine (N) at position 31 was observed which known as amantadine resistance marker. Our finding was differed with the ancestor of H9N2 virus (A/turkey/Wisconsin/1/1966) and human H7N9 viruses from China. On the other hand, M2 gene at position 26, 27 and 30 harbor Leucine (L), Valine (V) and Alanine (A), indicating sensitive to antiviral drugs

(amantadine), similar to those H9N2 virus references and human H7N9 viruses from China (Table 13 and 14).

Table 13 Genetic analysis of internal genes (PB2, PB1, PA and NP) of H9N2 viruses

Viruses	Amino acid positions							
	PB2		PB1	PB1-F2	PA			NP
	E627K	D701N	L13P	N66S	S224P	N383D	A515T	L136M
Bt/1291-OP/16	E	D	P	N	S	D	T	L
ck/Vn/ 15A13/15[a]	E	D	P	N	S	D	T	L
tk/Ws/1/66[b]	E	D	P	N	S	D	T	L
A/Hz/1/13c[c]	K	D	P	N	S	D	T	L
A/Sh/2/13d[d]	K	D	P	N	S	D	T	L
Functions	virulence in mammals	virulence in mammals	virulence in mammals	viral virulence	virulence in duck	virulence in duck	virulence in duck	Avian/human like characteristics

[a]A/chicken/Vietnam/NCVD-15A13/2015 (H9N2)

[b]A/turkey/Wisconsin/1/1966 (H9N2)

[c]A/Hangzhou/1/2013 (H7N9)

[d]A/Shanghai/2/2013 ((H7N9)

Table 14 Genetic analysis of internal genes (M and NS1) of H9N2 viruses

Viruses	Amino acid positions									
	M1		M2				NS1			
Position	N30D	T215A	L26F	V27A/T	A30T/V	S31N/G	C-terminal	P42S	V149A	aa del.(80-84)
Bt/1291-OP/16	D	A	L	V	A	N	KPEV	S	A	No
ck/Vn/ 15A13/15[a]	D	A	L	V	A	N	KPEV	S	A	No
tk/Ws/1/66[b]	D	A	L	V	A	S	ESEV	S	A	No
A/Hz/1/13c[c]	D	A	L	V	A	S	KPEV	S	A	No
A/Sh/2/13d[d]	D	A	L	V	A	S	KPEV	S	A	No
Functions	virulence in mice	virulence in mice	Amantadine resistance	Amantadine resistance	Amantadine resistance	Amantadine resistance	Virulence-related	virulence in mice	virulence in mice	virulence in chicken&mice

[a]A/chicken/Vietnam/NCVD-15A13/2015 (H9N2)

[b]A/turkey/Wisconsin/1/1966 (H9N2)

[c]A/Hangzhou/1/2013 (H7N9)

[d]A/Shanghai/2/2013 ((H7N9)

CHAPTER V

DISCUSSION

Highly pathogenic avian influenza (HPAI) subtype H5N1 had devastating impacts in animal and human health since the first introduction in Indonesia in 2003. The virus has caused recurring disease outbreaks in domestic poultry and become endemic in Indonesia, resulting in the death of millions of birds and culling due to the diseases. In addition, the low pathogenic avian influenza subtype H9N2 was first reported in Indonesia in 2016. The virus has affected in the decreased egg production in commercial poultry farms in many provinces of Indonesia. In consequence of the serious impacts of avian influenza, the active avian influenza surveillance program has been routinely conducted in Indonesia for monitoring, prevention and control of the diseases.

In this study, we selected and acquired 50 samples that previously identified as avian influenza viruses from Disease Investigation Center (DIC) Wates Yogyakarta, Indonesia. In detail, 47 samples were selected from the national surveillance program of avian influenza in ducks in Java during 2015-2017 and 3 samples were selected from ducks during avian influenza outbreaks in 2016. The samples were recovered from ducks in 3 provinces, East Java (n=26), Central Java (n=16), and Yogyakarta (n=8). All samples were subjected for influenza A screening by realtime RT-PCR to detect the influenza A virus. Realtime RT-PCR was used as diagnostic tool for influenza virus detection due to its high sensitivity, good reproducibility and high-throughput (Wang and Taubenberger, 2010). The positive influenza A (n=46) was then submitted for virus isolation by egg inoculation using embryonated chicken eggs. Influenza virus propagation in embryonated chicken eggs was recommended by WHO/OIE due to its sensitive assay and yield high viral titer stock (Brauer and Chen, 2015; OIE, 2015).

According to the OIE recommendation, the virus isolation were processed in BSC class II type B2 with HEPA filters which provide product, operator and environment protection (OIE, 2015). The four positive HA isolates were subjected for influenza virus confirmation and whole genome sequencing. The one isolate (Trenggalek: TR/17/15) was recovered from ducks in East Java from the national surveillance program of avian influenza in ducks in Java during 2015. The other three isolates were recovered from ducks in East Java (Lamongan: Lm/1958/16) and Yogyakarta (Bantul: Bt/1291-OP/16 and Bt/1291-OR/16) during avian influenza outbreaks in 2016. Several factors may could affect to the propagation of avian influenza viruses, such as repeatability of sample to freeze-thaw and length of storage time. The repeated freeze-thaw and longer of storage time of samples could decrease the viability and quality of samples (Eisfeld et al., 2014). The three primary samples from the national surveillance program of avian influenza in ducks in Java during 2015-2017 (ML/46/17 from Malang, East Java; Kb/33/15 from Kebumen, Central Java; and Pw/111/17 from Purworejo, Central Java) which have low Ct value by realtime RT-PCR (but HA negative) were selected and included for virus confirmation. In this study, confirmation of influenza A virus was performed by using multi segment reverse transcription-PCR (M-RTPCR). The single reaction of M-RTPCR is a suitable method for simultaneously amplifying the eight genomic RNA segment of influenza A virus for influenza virus confirmation and subsequently for whole genome sequencing through NGS. This convenience amplification method allow to obtain whole genome of influenza A virus at low titers (Zhou et al., 2009; Zou et al., 2016).

In this study, the nucleotide sequences of eight gene segments of seven avian influenza viruses isolated from ducks in Java Indonesia were elucidated. Based on the sequence analysis, the avian influenza subtype H5N1 (n=6) (Tr/17/15, Lm/1958/16, Ml/46/17, Pw/111/17, Kb/33/15, and Bt/1291-OR/16) and the avian influenza subtype

H9N2 (n=1) (Bt/1291-OP/16) were identified. Further sequence analysis showed that almost of all genes of 6 H5N1 viruses were closely related to those of H5N1 virus of clade 2.3.2.1c (A/quail/Sukoharjo/04152003/2015) with high percentages of nucleotide identities. In contrast, almost H5N1 viruses showed low nucleotide identities to H5N1 viruses of clade 2.1.3.2. In addition, from phylogenetic analysis reveal that almost internal genes of these H5N1 viruses were clustered with H5N1 viruses of clade 2.3.2.1c. Our results indicated that clade 2.3.2.1c of H5N1 virus was a recent predominating clade circulating in poultry ducks in Java Indonesia in 2015-2017. Similar study reported that avian influenza viruses H5N1 of clade 2.3.2.1c caused recent HPAI outbreak in poultry in Indonesia (Wibawa et al., 2018). Interestingly, in this study, the internal gene of Matrix (M gene) of one H5N1 virus (Pw/111/17) had high nucleotide identity (95.62%) with H5N1 virus of clade 2.1.3.2 (A/chicken/West Java/Sbg-29/2007) compared to H5N1 virus of clade 2.1.3.2c (A/quail/Sukoharjo/04152003/2015; 93.58%). Moreover, the phylogenetic analysis showed that the M gene of Pw/111/17 was clustered into H5N1 virus of clade 2.1.3.2. This finding indicates that this virus could acquire the internal gene segment from another virus/clade, suggesting inter-lineage reassortment among avian influenza viruses was occurred (Figure 14A). Similar finding of inter-lineage reassortment of PB2, M and NS genes of Indonesia H5N1 viruses in 2016 was also reported. This new reassortment was belonged to the Indonesian H5N1 virus of clade 2.3.2.1c genotype B, which their internal genes were generated from H5N1 virus of clade 2.1.3.2 (Wibawa et al., 2018). The multiple reassortment of the internal genes among H5N1 virus clades (clade 2.3.2.1a, 2.3.2.1b and 2.3.2.1c) have also been reported to cause the H5N1 outbreaks in quail farms in Vietnam in 2010 and 2014 (Nguyen et al., 2016). Similarly, the reassortment of avian influenza H5N1 virus of clade 1.1.2 with the internal genes from clade 2.3.2.1c had been reported and caused human death in Cambodia in 2013 (Rith et al., 2014). In Thailand, the reassortment between H5N1 viruses of clade 1.1 and 1.2 caused avian influenza outbreak in 2008

(Amonsin et al., 2010). It has been known that influenza virus reassortment can cause the genetic divergence and virulence enhancement of the viruses (de Silva et al., 2012; Wei et al., 2013).

The gene segment of H9N2 virus recovered from ducks in Java Indonesia were analyzed by comparing to the virus references available in the GenBank. Our results showed that the H9N2 virus (Bt/1291-OP/16) had high nucleotide identity to the H9N2 virus recovered from Vietnam (A/chicken/Vietnam/NCVD-15A13/2015). While, the internal genes of H9N2 virus were closely related to human H7N9 virus (A/Shanghai/2/2013). The phylogenetic analysis of HA gene showed that the H9N2 virus was grouped together with other H9N2 viruses from Indonesia and Vietnam. These viruses were clustered into the H9N2 viruses of Y280-like group. The Vietnam H9N2 viruses such as A/chicken/Vietnam/H7F-LC4-51/2014, A/chicken/Vietnam/H7F-LC4-26/2014 and A/chicken/Vietnam/H7F 14 BN4 423/2014 have been proved clustering into H9N2 viruses of Y280-like which the predominant H9 lineage found in China (Thuy et al., 2016). Similar with HA gene, the NA gene of H9N2 virus was grouped together with H9N2 viruses from Indonesia and Vietnam. However, this group was separated from other H9N2 groups (Y280 like, G1- like and Y439-like). Based on the phylogenetic analysis of HA and NA genes, the existence of multiple ancestries of H9N2 avian influenza viruses from chicken still remained in Indonesia (Jonas et al., 2018). On the other hand, the phylogenetic analysis of all internal genes (PB2, PB1, PA, NP, M and NS) showed that the H9N2 virus was grouped together with H7N9 viruses (Figure 14B). Similarly, previous study had also reported that the all internal gene segments of H9N2 viruses isolated from chicken in live bird market in Vietnam in 2016 were grouped together with human H7N9 viruses (Thuy et al., 2016). Our results were in agreement with this previous study that all internal genes are likely derived from H7N9 viruses. Other studies had reported that the internal genes (PB2, PB1, NP and NS) of H7N9

viruses from human and environment in Guangdong, China were belonged to a cluster of H9N2 viruses (Lu et al., 2014). In addition, all six internal genes of the H7N9 viruses from human in China in 2013 were from avian H9N2 viruses (Gao et al., 2013).

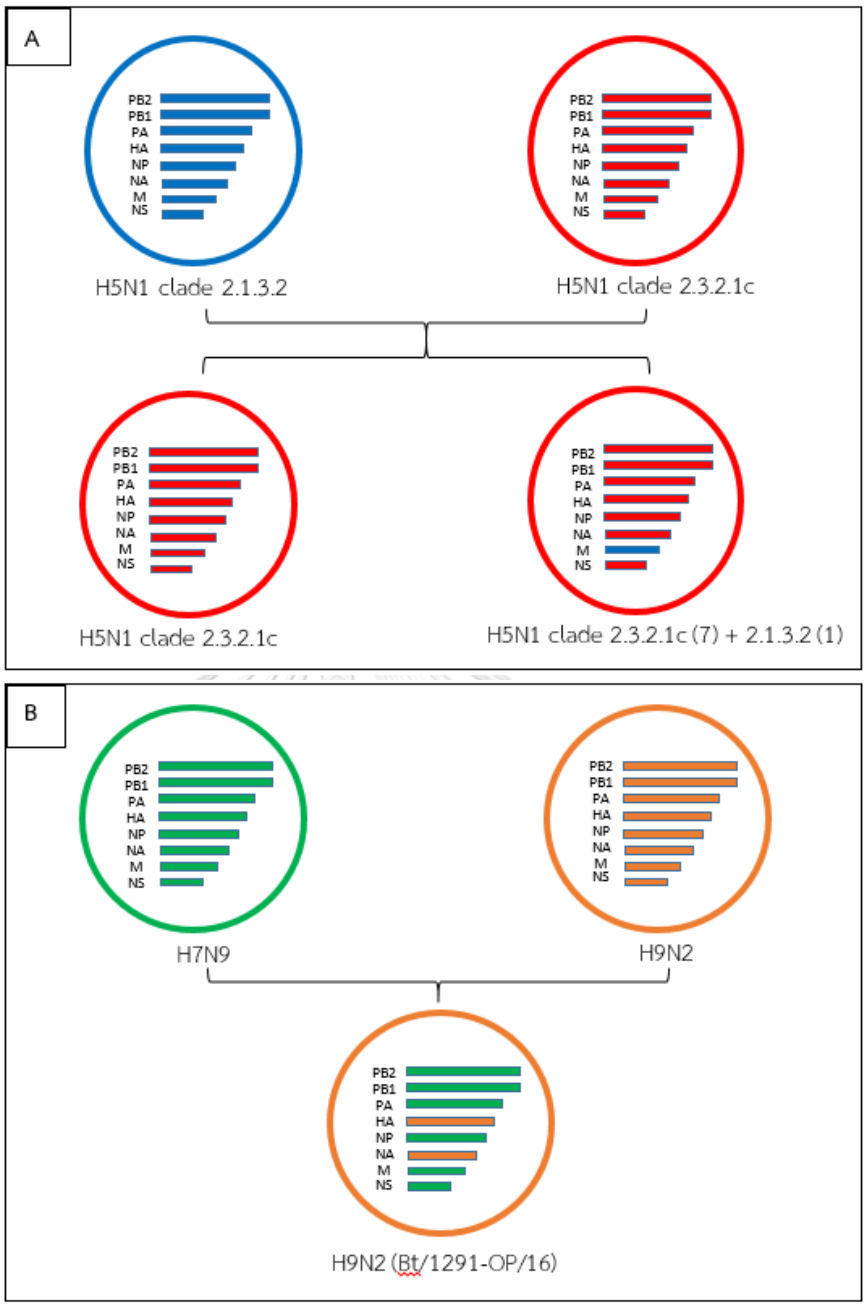

Figure 14 Genome constellation of avian influenza viruses in this study. A: The genome constellation of HPAI-H5N1 clade 2.3.2.1c (7) + 2.1.3.2 (1) (the reassortment of M gene of H5N1 virus from clade 2.1.3.2); B: The genome constellation of LPAI-H9N2, all internal genes of H9N2 virus were likely derived from H7N9 virus.

For genetic analysis, the comparison of deduced amino acid sequences amongst the viruses in this study were conducted. The HA gene analysis showed that all H5N1 viruses recovered from ducks in Indonesia contained multiple basic amino acids 'RERRRKR' at the HA cleavage site, similar to those of the H5N1 virus references and the Indonesia vaccine strain of HPAI-H5N1 clade 2.3.2.1c virus. The presence of multiple basic amino acid at HA cleavage site of avian influenza virus is known to be associated with highly pathogenic avian influenza virus (HPAI) (Senne et al., 1996; Claas et al., 1998). The amino acid related to the receptor binding site of H5N1 viruses contained Glutamine (Q) at position 222 (Q222) and Glycine (G) at position 224 (G224) indicating the preferential binding of the virus to the sialic acid α2.3 galactose (SA α2.3 Gal) receptor (avian specific receptor binding site) (Matrosovich et al., 1999; Stevens et al., 2006). All of H5N1 viruses contained N-link glycosylation site (N-N-T) at position 165-167 suggesting the enhancement of viral virulence. The attachment of an oligosaccharide to the N-glycosylation site in the globular head region contributes to escape the host immune recognition due to the interfere of antibody to bind to antigenic site of virus (Matrosovich et al., 1999; Kobayashi and Suzuki, 2012).

The genetic analysis of antiviral drug resistance markers at NA gene of H5N1 viruses revealed that all of H5N1 viruses harbored amino acids Glutamic acid (E), Histidine (H,) Arginine (R) and Asparagine (N) at position 119, 275, 293 and 295, respectively, similar to the NA gene of H5N1 virus references. The antiviral drug resistance markers at these positions do not change indicating oseltamivir sensitivity (Gubareva et al., 2000; Moscona, 2005). The deletion of 20 amino acids at NA stalk region was observed in all H5N1 viruses and the virus references of clade 2.3.2.1c, indicating the adaptation of viruses from wild birds to domestic poultry (Matrosovich et al., 1999). In contrast, the progeny H5N1 virus of clade 0 (A/goose/Guangdong/1/96) without 20 amino acid deletion at NA stalk region was observed.

For genetic analysis of HA gene of H9N2, the HA cleavage site contains single basic amino acids ‘SRSSR’ which known as the characteristics of low pathogenic avian influenza. It has been reported that the numbers of basic amino acid at the HA cleavage site may affect the pathogenesis of influenza virus due to the HA protein has to be cleaved by cellular protease enzyme of the host (Kwon et al., 2009). In this study, the H9N2 virus harbored 155-Threonine (T), 183-asparagine (N) and 226-Leucine (L) amino acids at receptor binding site indicating human specific receptor binding site. These positions were associated with binding receptor site of human-like α2.6 linked sialic acid galactose (SA α2.6 Gal) (Matrosovich et al., 2001; Wan and Perez, 2007). Similar to previous studies, the receptor binding sites of H9N2 virus from Vietnam, China and Jordan were related to binding ability to human specific receptor (Chrzastek et al., 2018; Wang et al., 2018).

The NA gene of H9N2 virus in this study contained amino acid Glutamic acid (E) at position 119 suggesting antiviral drugs sensitivity, similar to all of H9N2 virus references. The substitution of amino acid Glutamic acid (E) to Valine (V) at position 119 had indicated the oseltamivir-resistance (Oh and Hurt, 2014). All H9N2 viruses, including the H9N2 virus references had no amino acid deletion at position 63-65 in NA-stalk region, suggesting low virulence of the viruses in chicken and mammals. The deletion of 3-amino acids at position 63-65 had been observed and became dominate H9N2 viruses in China. The short deletion in NA-stalk region enhanced the virulence of H9N2 virus in chicken and mice (Sun et al., 2013).

Genetic analyses of six internal genes (PB2, PB1, PA, NP, M and NS) of H5N1 viruses in this study were conducted. For PB2 gene, H5N1 viruses in this study contained Glutamic acid (E) at position 627 and aspartic acid (D) at position 701. No amino acid substitution at PB2 which known to be related to the viral virulence in mammals were found, indicating less virulence of the viruses in mammals (Hatta et

al., 2001; Czudai-Matwich et al., 2014). The mutation in PB2 gene of H5N1 virus at position 627 of Glutamic acid (E) to Lysine (K) were associated with the outcome of infection in mice (Hatta et al., 2001). Other study revealed that the mutation of amino acid aspartic acid (D) to Asparagine (N) at position 701 in PB2 gene had indicated the adaptation of avian influenza virus to mammalian species (Czudai-Matwich et al., 2014). For PB1 gene, no substitution of amino acid Serine (S) to asparagine (N) at position 66 in PB1-F2 of H5N1 viruses, indicating the less virulence of the viruses (Conenello et al., 2007). A single mutation of amino acid Serine (S) at position 66 to asparagine (N) in PB1-F2 had contributed to increase viral virulence in mice due to the deferment of innate immune response, resulting in the uncontrollable of viral growth (Conenello et al., 2007). For PA gene, the H5N1 viruses contained amino acid Serine (S) at position 224, indicating decreased viral virulence in duck. Mutation of amino acid Serine (S) to Proline (P) at this position had indicated the enhancement of virus to replicate in duck embryo fibroblasts (Song et al., 2011).

Beside non-virulence virus characteristics, the H5N1 viruses were also harbored viral virulence characteristics. For PB1 gene, the substitution of amino acid Leucine (L) to Proline (P) at position 13, suggesting viral virulence characteristic. The mutation of amino acid (L13P) in PB1 protein had affected the virus replication in mammalian hosts due to the enhancement of polymerase activity of virus (Gabriel et al., 2005). For PA gene, the H5N1 virus possessed Aspartic acid (D) but not Asparagine (N) at position 383 and Threonine (T) but not Alanine (A) at position 515 which are related to viral virulence in duck. The 383D in PA gene had associated with the viral virulence in duck due to the enhancement of polymerase activity of virus (Song et al., 2011). The substitution of amino acid T515A had associated with high pathogenicity of H5N1 avian influenza virus in mallard duck (Hulse-Post et al., 2007). For M gene, the H5N1 viruses harbored Aspartic acid (D) at position 30 and Alanine (A) at position 215, which known

to enhance viral virulence in mice (Fan et al., 2009). For NS gene, the amino acid Serine (S) at position 42 and Alanine (A) at position 149 were observed in H5N1 viruses in this study, indicating viral virulence in mice and chicken (Li et al., 2006; Jiao et al., 2008). In addition, deletion of amino acids at position 80-84 in NS1 gene was observed, suggesting enhance viral virulence in chicken and mice (Long et al., 2008). The PDF binding motif ligand at c-terminal in NS1 gene of H5N1 viruses contained 'ESEV' suggesting viral virulence (Jackson et al., 2008). For NP gene, at amino acid position 136 contained Leucine (L) suggesting the host specific characteristics (avian-like characteristic) (Shaw et al., 2002; Reid et al., 2004). For M gene, the amantadine resistance markers were observed in some of H5N1 viruses such as alanine (A) at position 27 (Pw/111/17) and asparagine (N) at position 31 (Tr/17/15 and Pw/111/17). However, all of H5N1 viruses in this study possessed Leucine (L) at position 26 and Alanine (A) at position 30 of M2 gene indicate susceptibility to amantadine (Hay et al., 1985; Suzuki et al., 2003). The substitution of amino acid at four critical sites 26, 27, 30 and 31 in M2 gene has been identified as amantadine resistance markers (Suzuki et al., 2003).

Genetic analyses of six internal genes (PB2, PB1, PA, NP, M and NS) of H9N2 virus in this study were conducted. For PB2, the H9N2 virus possessed Glutamic acid (E) at position 627, indicating non-virulence in mammals, similar to the H9N2 viruses from Vietnam and the progeny H9N2 virus (Hatta et al., 2001; Czudai-Matwich et al., 2014). In contrast, our finding was differed from human H7N9 viruses from China, which contained amino acid Lysine (K) at position 627 indicating enhance of viral virulence in mammalian species (Gao et al., 2013). For PB2 gene, amino acid aspartic acid (D) at position 701 was observed in H9N2 virus in this study, indicating low virulence in mammals (Hatta et al., 2001). For PB1 and PA genes, the H9N2 virus harbored amino acid asparagine (N) at position 66 in PB1-F and Serine (S) at position 224 in PA gene

which associated with low-virulence in mammals (Conenello et al., 2007; Sun et al., 2011). For NS1 gene, the H9N2 virus had no amino acid deletion at position 80-84. The role of deletion of amino acids in NS1 gene of H9N2 influenza virus are not clearly identified, however this deletion was observed in H5N1 avian influenza viruses which associated with increasing viral virulence in poultry and mice (Long et al., 2008).

The characteristics of viral virulence of H9N2 virus was also observed. For PB2, at position 13 contained the amino acid Proline (P), indicating viral virulence in mammals (Gabriel et al., 2005). For PA gene, the H9N2 virus harbors Aspartic acid (D) at position 383 and Threonine (T) at position 515 which associated with enhance virulence in duck (Song et al., 2011). For M gene, the virus contained Aspartic acid (D) and Alanine (A) at position 30 and 215 which the characteristics of viral virulence in mice (Fan et al., 2009). For NS1 gene, amino acid Serine (S) at position 42 and Alanine (A) at position 149 were observed, indicating enhance viral virulence in mice (Li et al., 2006; Jiao et al., 2008). Similar with H9N2 Vietnam virus and human H7N9 viruses from China, the H9N2 virus showed PDZ binding motif 'KPEV' at c-terminal of NS1, which associated with increased viral virulence. The virus contained PDZ binding motif 'KPEV" in NS1 was observed to increase pathogenicity in mice (Jackson et al., 2008). For PA gene, the avian-like characteristics with Leucine (L) at position 136 of PA gene, similar to the reference H9N2 viruses and H5N1 viruses in this study (Reid et al., 2004). For M gene, the H9N2 virus harbors asparagine (N) at position 31 which associated with amantadine resistance. However the amino acids Leucine (L), Valine (V) and Alanine (A) at position 26, 27 and 30 of M2 gene were showed, indicating that this virus sensitive to antiviral drugs, similar to those H9N2 reference viruses and human H7N9 viruses from China (Hay et al., 1985; Suzuki et al., 2003).

Taken together, our findings revealed that two avian influenza virus subtypes (H5N1 and H9N2) were circulating in ducks in Java Indonesia during 2015-2017. The

H5N1 viruses belong to the clade 2.3.2.1c. Interestingly, the internal gene (M) of one H5N1 virus (Pw/111/17) was originated from H5N1 of clade 2.1.3.2. This finding suggested inter-lineage reassortment between avian influenza of clade 2.3.2.1c and 2.1.3.2. As previously mentioned that the inter-lineage reassortment of H5N1 had caused the poultry outbreaks and/or human cases such as reports in Vietnam, Cambodia and Thailand (Amonsin et al., 2010; Rith et al., 2014; Nguyen et al., 2016). It should be noted that the H5N1 reassortant in this study was isolated from ducks from traditional or semi-traditional duck farms. This duck farming system not only have low biosecurity but also have possibility of ducks to contact with other poultry or wild birds allowing the risk of virus transmission and co-infection, then generate reassortment (CIVAS, 2006; Lipatov et al., 2007). In addition, the H9N2 virus (Bt/1291-OP/16) was isolated from same district and same duck farm with H5N1 virus (Bt/1291-OR). From phylogenetic analysis of HA and NA genes showed that this virus was grouped into H9N2 viruses of Y280-like and Indonesia-Vietnam H9N2 virus group, respectively. However, the all of internal gene of H9N2 virus in this study were closely related with the human H7N9 virus isolated from China. It is notes that our study is the first whole genome characterization of H9N2 virus isolated in Indonesia. This findings identified the risk of the possibility of avian influenza virus reassortment between subtypes which may resulting in the generation of novel influenza viruses. The reassortant of avian influenza H5N1 with the internal genes of M or PB1 of H9N2 viruses have been reported in poultry and live bird market in Cambodia and Bangladesh (Monne et al., 2013; Suttie et al., 2018). In addition, the reassortant H5N1 viruses of clade 2.3.2.1c with the PB2 gene of H9N2 virus have reported in human in North America in 2013 (Pabbaraju et al., 2014). Many studies have reported that the H9N2 virus is not only reassorted with H5N1 virus but this virus also contributed the internal genes to other virus subtypes such as H1N1, H7N9 and H10N2, producing novel virulence strains (Sun et al., 2011; Lai et al., 2013; Chen et al., 2014).

Our findings highlight that the surveillance and genetic characterization of avian influenza viruses should be routinely conducted to monitor the dynamic and diversity of avian influenza viruses and possibility of novel reassortment strains. Moreover, the genetic analysis of the viruses indicated that the H5N1 and H9N2 avian influenza viruses in this study possess both avian-like and human-like characteristics. Further study in animal model is recommended to elucidate the pathogenicity and virulence of the viruses in difference animal species.

Conclusions and suggestions

In this study, 50 samples of ducks from 3 provinces of Java Indonesia during 2015-2017 were acquired from the collection of Disease Investigation Center (DIC) Wates Yogyakarta, Indonesia. Seven out of 50 samples were successfully confirmed as influenza A viruses. The seven viruses were then subjected to whole genome sequencing. Our findings could be concluded as follows:

1. The avian influenza viruses subtype H5N1 (n=6) and H9N2 (n=1) were circulated in ducks in Java Indonesia during 2015-2017
2. Phylogenetic analysis showed that
    a. The H5N1 viruses belonged to clade 2.3.2.1c suggesting predominant clade recently circulating in ducks in Java Indonesia during 2015-2017. Among them, the H5N1 of clade 2.3.2.1c (n=1) showed the evidence of inter-lineage reassortment by acquiring the M gene from H5N1 of clade 2.1.3.2
    b. The H9N2 virus belonged to Y280-like group. However, the internal genes of the virus were closely related to the human H7N9 virus isolated from China
3. Genetic analysis showed that
    a. The H5N1 and H9N2 viruses were characterized as highly pathogenic avian influenza (HPAI) (H5N1) and low pathogenic avian influenza (LPAI) (H9N2), respectively
    b. The receptor binding site of the H5N1 viruses possess avian binding receptor characteristic, however the H9N2 virus possess human binding receptor characteristics
    c. The amino acids associated with viral virulence characteristics showed that the H5N1 and H9N2 viruses possess both high and low viral virulence characteristics in avian and mammals species

d. The antiviral resistance markers in M2 gene was detected in H9N2 and some H5N1 viruses.

In order to further establish the avian influenza prevention and control strategies, the results from this study lead the suggestions as follows:

1. The surveillance and genetic characterization of avian influenza viruses should be routinely conducted to monitor the dynamic and genetic diversity of avian influenza viruses and possibility of the generation of novel reassortment strains
2. Further studies in animal model is recommended to better understanding the virulence and pathogenicity of the viruses in difference animal species
3. Biosecurity in duck farming system should be improved to reduce the risk of avian influenza virus infections
4. Further studies are needed for elucidating the factors correlated to the persistence and endemic of H5N1 viruses in Indonesia, such as avian influenza vaccine used, surveillance of H5N1 in wild migratory bird and/or live bird markets
5. Further studies are needed for elucidating the factors correlated to the introduction of H9N2 viruses in Indonesia, such as avian influenza surveillance in wild migratory bird and/or imported poultry products.
6. The surveillance and genetic characterization of influenza viruses in swine farms should be conducted to better understanding the dynamic, genetic diversity and possibility of influenza viruses in generating virus reassortment in pigs which known as ‘mixing vessel’.

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