GENOME CHARACTERISATION AND PATHOGENICITY STUDY OF LOW PATHOGENIC AVIAN INFLUENZA VIRUS SUBTYPE H9N2 ISOLATED IN MALAYSIA

By

GUNASEKARA NAMBIKALU ARACHCHIGE DONA ERANDI MAHESHIKA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2022

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DEDICATION

This thesis is dedicated
To my two daughters
Tamasha and Ginuki.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : ProfessorAbdul Rahman Omar, DVM, PhD
Institute : Bioscience

Low pathogenic avian influenza (LPAI) virus subtype H9N2 has spread widely in the Asian region and posed a significant threat to the poultry industry. Besides, the virus is potentially zoonotic, where it can directly infect mammals and donate its gene segments to create deadly novel subtypes of pandemic concern. Therefore, identifying the genetic evolution, pathogenesis, and host adaptation of the H9N2 virus is of paramount importance. In late 2017, Malaysia reported repeated incidences of H9N2 in commercial birds, including in the breeder and layer flocks. However, the full genome and pathogenicity of the H9N2 viruses have not been studied. Two H9N2 viruses, A/chicken/Malaysia/Negeri Sembilan/UPM994/2018 and A/chicken/ Malaysia/Johore/UPM2033/2019 were isolated from breeder and layer flocks in Peninsular Malaysia, respectively, were molecularly characterized and pathogenic nature in SPF chicken was identified in this study.

Phylogenetic analysis revealed that both viruses were multiple genotypes reassortant strains with genes originated from Y280-like (HA gene), F/98-like (NS, NP, PA), G1-like (M and PB2) and Korean-like (PB1), indicating the viruses belong to a novel genotype of a divergent from G57 lineage of Chinese origin. The seven genes namely NS, M, HA, NA, NP, PA and PB2 are closely related to viruses isolated from Indonesia, Taiwan, Japan, and Cambodia. However, the PB1 genes of both viruses are different from other H9N2 viruses detected in the region. They have a 97% similarity with the Korean H9N2 strain A/chicken/Korea/1310/2001. In addition, the virus strain has been developed and used as an inactivated vaccine in Korea and other countries.

Deduced amino acids mutations were analysed and compared with previously reported mutation markers that were confirmed by reverse genetic and cell culture experiments. Both Malaysian H9N2 isolates in this study contained the hemagglutinin (HA) Q226L

substitution, which favors mammalian receptor type binding. The molecular and deduced amino acid analysis revealed that both viruses possess a dibasic cleavage site at the position 333-PSRSSR-GLF-341 of their respective HA gene cleavage sites. Furthermore, the polymerase complex genes have mammalian adaptation mutation (550L in PA gene) and enhanced pathogenicity markers (A588V in PB2 gene). Both H9N2 viruses were susceptible to the neuraminidase inhibitor antivirals; however, their M2 genes have an S31N substitution which associated with amantadine resistance.

Pathogenicity study of one of the isolates, UPM994/2018 was performed in one-week-old specific-pathogen-free (SPF) chickens following inoculation of the virus at 10^7 EID_{50} via the oro-nasal route. Clinical signs such as ruffled feathers, mild tracheal rales, gasping, facial edema, sero-nasal discharge and diarrhoea were observed from 6 to 10 days post-inoculation (pi). However, no mortality was recorded. Based on real-time PCR results, the viruses can be detected in the lungs, tracheas, and kidneys of the inoculated chickens on the second day and increased until day 10, then declined at day 16 pi. However, swab samples collected from the oropharyngeal and cloacal remain positive from day 2 to day 14 pi, with the highest viral load detected at day 10 pi.

In conclusion, the characterised Malaysian H9N2 virus is a Y280-like virus resembling H9N2 isolated from Indonesia, Taiwan, Japan and Cambodia. However, the virus is a novel genotype of a divergent from G57 lineage of Chinese origin with PB1 gene originated from Korean lineages H9N2 virus. Although the virus is an LPAI, it is pathogenic in SPF chickens causing respiratory, gastrointestinal and renal-associated illnesses.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN GENOM DAN KAJIAN KEPATOGENAN VIRUS SELESEMA BURUNG PATOGEN RENDAH SUBTIP H9N2 YANG DIASINGKAN DI MALAYSIA

Oleh

GUNASEKARA NAMBIKALU ARACHCHIGE DONA ERANDI MAHESHIKA

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Virus selesema burung patogen rendah (LPAI) H9N2 telah merebak secara meluas di rantau Asia dan menimbulkan ancaman besar bagi industri pertenakkan. Selain itu, virus ini berpotensi menjadi zoonotik, di mana ia secara langsung dapat menjangkiti mamalia dan menimbulkan kerisauan kerana mampu menyumbangkan segmen gennya untuk membuat subjenis novel yang merbahaya. Oleh itu, dengan cara mengenal pasti evolusi genetik, patogenesis, dan penyesuaian perumah H9N2 adalah sangat penting. Pada akhir 2017, Malaysia telah melaporkan kejadian berulang wabak H9N2 pada burung komersial, termasuk pada ayam daging dan ayam telur. Namun, kajian genom dan kepatogenan virus H9N2 masih belum dibuat. Dua virus H9N2 yang telah dikaji dalam kajian ini adalah, A/ayam/Malaysia/NegeriSembilan/UPM994/2018 dan A/ayam/Malaysia/Johore/UPM2033/2019 yang masing-masing telah diisolasi dari ayam daging dan ayam telur di Semenanjung Malaysia.

Analisis filogenetik menunjukkan bahawa kedua-dua virus itu adalah strain genotip pelbagai dan percantuman antara gen yang berasal dari Y280-seperti (gen HA), F/98-seperti (NS, NP, PA), seperti G1 (M dan PB2) dan seperti Korea (PB1), ini bermaksud bahawa virus itu adalah termasuk dalam genotip novel yang berubah dari susur galur G57 yang berasal dari China. Tujuh gen iaitu, NS, M, HA, NA, NP, PA dan PB2 adalah berkait rapat dengan virus yang dijumpai dari Indonesia, Taiwan, Jepun, dan Kemboja. Walau bagaimanapun, gen PB1 kedua-dua virus ini berbeza dengan virus H9N2 lain yang telah dikesan di rantau ini. Mereka mempunyai 97% persamaan dengan strain H9N2 Korea strain ayam/Korea/01310/2001. Tambahan lagi, virus ini telah dikaji dan digunakan sebagai vaksin yang tidak aktif di Korea dan negara lain.

Analisis dan perbandingan mutasi dibuat berdasarkan kepada mutasi yang dikesan sebelum ini berdasarkan ujikaji genetik berbalik dan sel kultur. Analisis molekul dan

asid amino menunjukkan bahawa kedua-dua virus tersebut mempunyai tapak pembelahan 'dibasic' pada kedudukan 333-PSRSSR-GLF-341 dari lokasi pembelahan gen HA masing-masing. Virus yang diisolasi dari H9N2 Malaysia dalam kajian ini mengandungi penggantian hemagglutinin (HA) Q226L, yang menyokong pengikatan jenis reseptor mamalia. Selanjutnya, gen kompleks polimerase mempunyai mutasi penyesuaian mamalia (550L dalam gen PA) dan penanda patogenik yang dipertingkatkan (A588V dalam gen PB2). Kedua-dua strain Malaysia terdedah kepada antivirus perencat neuraminidase; namun, gen M2 mereka mempunyai penggantian S31N yang dikaitkan dengan ketahanan terhadap amantadine.

Kajian patogenan pada salah satu isolasi, UPM994/2018 dilakukan pada ayam bebas-patogen-spesifik (SPF) berusia satu minggu menggunakan inokulasi virus pada kepekatan 107 EID50 melalui laluan oral. Tanda-tanda klinikal seperti bulu yang gugur, bunyi penafasan trakea ringan, tercungap-cungap, edema di muka, lelehan sero nasal dan cirit-birit diperhatikan dari hari ke-6 hingga hari yang ke-10 selepas inokulasi (PI). Walau bagaimanapun, tidak ada kematian yang dilaporkan. Berdasarkan hasil PCR masa-nyata, virus dapat dikesan di paru-paru, trakea, dan ginjal ayam yang diinokulasi pada hari kedua dan meningkat hingga hari ke-10, kemudian menurun pada hari ke-16 selepas inokulasi. Walau bagaimanapun, sampel swab yang dikumpulkan dari laluan penafasan dan cloaca tetap positif dari hari ke-2 hingga hari ke-14, dengan kandungan virus tertinggi dikesan pada hari ke-10 PI.

Kesimpulannya, H9N2 Malaysia adalah virus seperti Y280 yang menyerupai H9N2 yang diisolasi dari Indonesia, Taiwan, Jepun dan Kemboja. Walau bagaimanapun, virus ini adalah genotip baru yang menyimpang dari susur galur G57 yang berasal dari Cina dengan gen PB1 yang berasal dari virus dari Korea. Walaupun virus itu adalah LPAI, ia adalah patogenik pada ayam SPF yang menyebabkan penyakit berkaitan pernafasan, gastrointestinal dan buah pinggang.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of the Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS AND ACRONYMS

AI	Avian influenza
AIV	Avian influenza viruses
AF	Allantoic fluid
BLAST	Basic Local Alignment Search Tool
cRNA	Complementary ribonucleic acid
℃	Degree celsius
ECE	Embryonated chicken egg
EID	Embryo infectious dose
HA	Hemagglutinin
HPAIV	High pathogenic avian influenza virus
H&E	Haematoxylin and eosin
LPAIV	Low pathogenic avian influenza virus
M	Matrix
MEGA	Molecular Evolutionary Genetics Analysis
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non-structural
NLS	Nuclear localization signal
OIE	World Organization for Animal Health (Office International des Episooties)
PB1	Polymerase basic 1

PB2	Polymerase basic 2
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKR	dsRNA-dependent protein kinase
qRT-PCR	Quantitative real-time RT-PCR
RBCs	Red blood cells
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Sialic acid
SPF	Specific-pathogen-free
vRNPs	Viral ribonucleo proteins
vRNA	Viral ribonucleic acid
WHO	World Health Organization
%	Percentage
&	And
aa	Amino acid
nt	Nucleotide
Rm	Room temperature
pi	post infection
min	Minute
h	Hour

CHAPTER 1

INTRODUCTION

Avian influenza virus (AIV) is an enveloped RNA virus which belongs to the genus avian influenza A of the family Orthomyxoviridae (Bouvier & Palese, 2008). The virus genome comprises approximately 13.2 kb of negative-sense, single-stranded and eight segmented genes encode 10 or 11 proteins, depending on their virus strain (Bouvier & Palese, 2008). The genome consists of 8 genes, polymerase base subunit two (PB2, 2,341 bp), polymerase base subunit one (PB1, 2341bp), polymerase acidic (PA, 2,233 bp), hemagglutinin (HA, 1,728 bp), nucleoprotein (NP, 1,565 bp), neuraminidase (NA, 1,460 bp), matrix proteins (M, 1,027 bp) and non-structural protein (NS, 890 bp) (Jiao et al., 2012a). The replication cycle of the virus takes place in the nucleus (Dou et al., 2018).

AIV, which belongs to type A can affect a wide variety of species of wild birds (Webster et al., 1992). Wild birds, namely waterfowls and migratory birds serve as reservoirs of all influenza A, viruses over a long period and have been the source of infection in domestic poultry and mammals. Transmission of AIV to domestic poultry is introduced primarily through direct or indirect contact with infected birds (Harder et al., 2016).

AIV is classified into various subtypes, depending on the surface glycoproteins (HA and NA). However, the virus is classified as highly pathogenic avian influenza virus (HPAIV) or low pathogenic avian influenza virus (LPAIV) based on their pathogenicity in chicken and molecular marker of cleavage site motif of the HA gene (Fouchier et al., 2005).

LPAI of subtype H9N2 was first reported in 1960 in the US (Homme et al., 1970). Presently, the virus has been reported in major poultry-producing countries in Asia, the Middle East and Africa (Peacock et al., 2019). Although the virus has been classified as LPAIV, it sometimes causes high mortality and manifests with a drop in egg production in complicated H9N2 infection due to concurrent infections with other pathogens, hence, resulting in significant losses in the poultry industry (Brown et al., 2006). In addition, H9N2 viruses are potentially zoonotic, infecting humans causing mild respiratory symptoms (Zou et al., 2019).

The silent spreading of H9N2 infection is becoming a serious problem in endemic poultry countries. The economic impact of the disease is being ignored compared to H5 and H7 viruses that belong to the highly pathogenic AIV. However, evidence suggests that they might play a vital role in the next flu pandemic, directly as a subtype of the H9N2 virus or by transferring its internal genes to emerging novel virulent AIV (Zhang et al., 2008; Peacock et al., 2019). The proteolytic cleavage site of the HA protein motif is vital and essential to enable the virus to enter the cells, transmit infection across different tissues, and determine the pathogenicity of the virus (Klenk & Garten, 1994; Homme et al., 1970). However, all encoded protein segments of the genome, including accessory peptides, are also functionally engaged with determining the pathogenicity of the H9N2 virus (Iqbal et al., 2009). The LPAIV consists of a monobasic cleavage site

cleaved by trypsin-like proteases located only in the respiratory and intestinal cells. Conversely, LPAIV strains have been isolated from a limited number of other tissues, including the pancreas, kidneys and oviduct (Umar et al., 2017). In addition, Post et al. (2012) also observed viable viruses isolated from brain tissue of chickens inoculated H7N1 LPAIV (Post et al., 2012). Systemic distribution of mRNA of LPAIV strains, H7N1, and H9N2 were detected by PCR diagnostic approach (Steinhauer, 1999). Previously reported H9N2 viruses with dibasic cleavage motif like RSSR had been resulted systemic infections in poultry (Baron et al., 2013).

In the field, various factors such as host species, virus strain, and the presence of secondary respiratory pathogens can influence the outcome of H9N2 infections in chickens (Umar et al., 2017). H9N2 infected chickens showed swelling of sinuses, nasal and ocular discharge, severe respiratory signs and low mortality rate. In some experiments, birds infected with H9N2 showed slight hyperaemia and congestion in the trachea and lungs. However, renal disease-associated lesions have also been reported (Alexander, 1982; Nili & Asasi, 2002; Subtain et al., 2011).

Depending on the geographical areas, different AIV lineages are circulating and causing H9N2 infection in chickens. For instance, the viruses that circulate in America are genetically grouped as the American lineages, whereas Asia and European viruses clustered into Eurasian lineages of AIV. AI H9N2 viruses in Eurasians have been associated in mainly three well-defined poultry lineages, as the G1, Y280 and Y439 (Korean-like) lineages, respectively (Guan et al., 2000; Matrosovich et al., 2001). It has been well reported that human infection is associated primarily with the G1 and Y280 lineages of H9N2 viruses (Pusch and Suarez, 2018). Previous research has shown that new genotypes have been evolved due to the reassortment of multiple genes with the different subtypes of the virus with different lineages (Guo et al., 2000). Currently, 117 genotypes have been identified in China (Li et al., 2017). Among them, the predominant genotype is G57, a reassorted genotype where the internal genes originate from different lineages (Pu et al., 2015).

Similarly, like other RNA viruses, the H9N2 virus has a vast genetic variability due to antigenic shift and drift mechanisms. Moreover, the high mutation rate and reassortment process of which sharing of internal gene cassettes in between different avian influenza subtype and H9N2 different lineages of viruses when co-infecting in the same host are the additional mechanisms causing high genetic diversity of the AIV genome. Aquatic host, quail, pigeon, sparrows, humans and swine facilitate the transmission of virus and reassortment process (Kandeil et al., 2017). Furthermore, it has been shown that live bird markets that house different avian species are also hotspots for the emergence of novel reassorted AIVs, including H9N2 (Negovetich et al., 2011).

Currently, there are two dominant reassorted H9N2 virus lineages circulating in the Asian poultry population. One of them is in the Indian sub-continent which has triple reassorted internal gene cassettes similar to A/chicken/Pakistan/UDL-01/2008 viruses originating from Pakistan poultry (Clements et al., 2020). While G57 genotype reassorted viruses originating from China which has donated six internal genes to H7N9 viruses in the 2013 pandemic (Chang et al., 2018; Ke et al., 2014). These viruses have

swept the genes of older H9N2 viruses and become the region's fittest and most dominant virus. A recent study has shown that the fitness of viruses is associated with changes in virus polymerase activity (Clements et al., 2020).

Diagnosis of AIV in chickens is based on clinical signs, post-mortem and laboratory tests based on serological detection, and reverse-transcriptase polymerase-chain-reaction (RT-PCR) detection (OIE, 2021). Hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) are two serology assays commonly used in detecting H9N2 in chickens (OIE, 2021). Meanwhile, RT-PCR assays of H9N2 based on primers specific to HA gene have been applied in both conventional and real-time approaches (Monne et al., 2008). Several countries have adopted the vaccination program as one of the strategies to control and prevent H9N2 infection and to minimize the economic burden (Chen et al., 2017; Zhang et al., 2008). The available H9N2 vaccines are inactivated monovalent or polyvalent vaccines with NDV (Newcastle Disease Vaccine) and/or IBDV (Infectious Bursal Disease Vaccine). However, the control of disease with vaccines remains controversial due to its nature of frequent variation in antigenicity (Offeddu et al., 2016). The advancements in whole genome sequencing technology and bioinformatics analysis facilitated the discovery of several H9N2 lineages with recombination events and the evolutionary diversity of H9N2 (Chong et al., 2010); hence, facilitating the timely development of an updated H9N2 vaccine.

The poultry sector plays a vital role in the Malaysian livestock sector. It has been well established that chicken meat and eggs are the cheapest sources of proteins in the country (Isa et al., 2019). In late 2017, Malaysia reported major outbreaks of LPAI H9N2 affecting several major commercial layers and breeder poultry farms in different states in Malaysia, which led to massive economic losses. It was estimated that outbreaks of the disease were reported in 40 % and 25% of the layer and breeder farms, respectively, in Peninsular Malaysia. Since then, H9N2 has been silently spreading across Peninsular Malaysia, where the viruses have been reported in 2019 and 2020. The 2017 outbreak was not the first reported case of H9N2. The virus has been reported in waterfowls and ducks in 1998 and 2001, respectively (Shohaimi et al., 2019). In addition, at least 13 subtypes of LPAIV were isolated among avian hosts in Malaysia (Mohidem et al., 2017a). Four incidences of highly pathogenic avian influenza (HPAI) viruses of H5N1 outbreaks were reported in 2004, 2006 and 2007 and 2017, which were associated primarily with backyard village chickens. However, the recent outbreak of HPAI-H5N1 in East Malaysia in the state of Sabah in 2018 involved commercial poultry farms. As of 2021, Malaysia is free from HPAI H5N1.

Partial HA and NA gene sequence analysis of some of the Malaysian H9N2 viruses that were isolated in 2017 and 2018 have been studied by Shohaimi et al., (2019). Phylogenetically, the Malaysian H9N2 viruses belong to the Y280-like and Korean-like lineages based on the HA gene study. In addition, in the previous analysis, RSKR and RSSR di basic HA cleavage site motifs were identified (Shohaimi et al., 2019). Although H9N2 has been repeatedly detected in Malaysia, the molecular characterisation study of the H9N2 genome is lacking. Furthermore, the pathogenicity of the recently isolated H9N2 is also not well understood. Therefore, the current study focuses on determining the whole genome sequence of the H9N2 genotype by using Sanger sequencing technology and the pathogenicity of the virus in specific-pathogen-free (SPF) chickens.

Hypothesis;

1. Genome sequencing of the H9N2 viruses using the Sanger approach will provide valuable information on the genetic identity, diversity and the virus phylogeny compared to H9N2 viruses from other countries.
2. The Malaysian H9N2 virus, A/chicken/Malaysia/Negeri Sembilan/UPM994/2018 is pathogenic in SPF chickens, causing lesions namely at the respiratory and gastrointestinal tracts.

The aim of this study is to determine the complete genome characterization of the virus, including the phylogenetic analysis. Additionally, genetic analysis of the whole genome of the virus is of paramount importance in designing effective control and prevention strategy, especially in terms of the selection of vaccine strain. The pathogenicity study is an essential component in confirming the virus pathotype and the ability of the virus to damage the different tissues of the infected chickens. Therefore, the specific objectives of the study are;

1. To amplify all eight gene segments of two Malaysian H9N2 virus isolates, A/chicken/Malaysia/NegeriSembilan/UPM994/2018 and A/chicken/Malaysia/Johore/UPM2033/2019 by using different sets of gene-specific primer sets and polymerase chain reaction approaches.
2. To characterise the sequences of the amplified gene-specific PCR products based on molecular characterisation and phylogenetic analysis
3. To determine the pathogenicity of H9N2 isolate, A/chicken/Malaysia/Negeri Sembilan/UPM994/2018 in one-week-old specific-pathogen-free (SPF) chickens.

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