EVALUATION OF HEMAGGLUTININ, NEURAMINIDASE AND MATRIX GENES OF MALAYSIAN LOW PATHOGENIC AVIAN INFLUENZA VIRUS STRAIN H5N2 THROUGH COMPREHENSIVE SEQUENCE ANALYSIS

By

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December 2022

Chair : Mariatulqabtiah Abdul Razak, PhD
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The avian influenza viruses (AIV) of the H5 subtype have the ability to spontaneously mutate from low pathogenic (LPAI) to highly pathogenic (HPAI), which can cause high mortality in poultry. In 2004, H5N2 is an example of an LPAI strain that circulated in Malaysia. The sporadic activity of this strain was still observed in other countries, with HPAI H5N2 strain has been reported in Minnesota U.S. in 2015. Little is known about the pathogenic switching, apart from the mutation at the hemagglutinin cleavage site which significantly contribute to the phenomenon. Therefore, any other markers that could potentially confer pathogenic switching would be important to determine the pathogenicity of the virus. This study aims to propagate and determine infectivity of the Malaysian LPAI H5N2 virus, to amplify the hemagglutinin (HA), neuraminidase (NA), and matrix (M) genes, and to use bioinformatics software to analyse the obtained sequences. Upon H5N2 strain A/Duck/Malaysia/8443/2004 virus propagation in SPF eggs, the allantoic fluid was harvested and subjected to hemagglutination assay. Viral RNAs were extracted and amplified by RT-PCR prior to sequencing. Results showed successful amplifications of HA (1732 bp), NA (1431 bp) and M (1019 bp) genes, whereby the multibasic amino acid (aa) sequence at the HA cleavage site was not observed. Host-associated analysis detected one human-associated mutation Q78K at the M2 protein. The HA protein showed identical aa with HPAI reference strain at position 113 which is known to affect pathogenicity, but no D94N and N182K/D mutations, which influence the binding of human influenza receptor. The virus also showed less glycosylation sites, but similar aa sequence at the NA active sites. Known motifs that decrease the susceptibility of NA or M2 inhibitor drugs were not found. The phylogenetic tree showed that the virus was grouped within the Eurasian H5 lineage, and clustered with other AIV subtypes. These data demonstrate diverse characteristics of the Malaysian LPAI H5N2, compared to HPAI H5N2. Although it may be difficult to predict the LPAI to HPAI pathogenic switching effectively, the studied AIV exhibited no considerable

markers that would signal a prospective transition to HPAI. This comprehensive sequence analysis will aid epidemiological research of the dynamics and evolution of circulating AIV in poultry.

Keywords: Avian influenza virus, hemagglutinin, neuraminidase, matrix gene, H5N2

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

PENILAIAN GENETIK PATOGENIK RENDAH INFLUENZA H5N2 MALAYSIA HEMAGGLUTININ, NEURAMINIDASE DAN MATRIK GEN MELALUI ANALISIS JURUTAN YANG KOMPREHENSIF

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Virus avian influenza (AIV) subjenis H5 mempunyai keupayaan untuk bermutasi secara spontan daripada patogenik rendah (LPAI) kepada sangat patogenik (HPAI), yang boleh menyebabkan kematian yang tinggi dalam ayam. H5N2 ialah contoh strain LPAI yang telah beredar di Malaysia pada tahun 2004. Aktiviti sporadis strain ini masih diperhatikan di negara lain, di mana strain HPAI H5N2 telah dilaporkan di Minnesota A.S. pada tahun 2015. Sedikit yang diketahui tentang perubahan patogen, selain daripada mutasi di tapak belahan hemagglutinin yang menyumbang dengan ketara kepada fenomena tersebut. Oleh itu, sebarang penanda lain yang berpotensi memberikan pertukaran patogenik adalah penting untuk menentukan kepatogenan virus. Objektif kajian ini adalah untuk membiakkan dan mengkuantisasi virus LPAI H5N2 Malaysia, untuk memperbanyakkan gen hemagglutinin (HA), neuraminidase (NA) dan matriks (M), dan untuk menganalisis jujukan yang diperoleh menggunakan perisian bioinformatik. Selepas pembiakan virus H5N2 strain A/Duck/Malaysia/8443/2004 ke dalam telur SPF, cecair alantoik telah dituai dan ujian haemagglutinasi telah dilakukan. RNA virus telah diekstrak dan diperbanyakkan oleh RT-PCR sebelum penjujukan. Keputusan menunjukkan gen HA (1732 bp), NA (1431 bp) dan M (1019 bp) yang berjaya, di mana jujukan asid amino berbilang asas di tapak pembelahan HA tidak ditemukan. Analisis berkaitan hos, mengesan satu mutasi berkaitan manusia Q78K pada protein M2. Protein HA menunjukkan asid amino yang sama dengan strain rujukan HPAI pada kedudukan 113 yang diketahui memberi kesan kepada patogenik, tetapi tiada mutasi D94N dan N182K/D, yang mempengaruhi pengikatan reseptor influenza manusia. Virus ini juga menunjukkan kurang tapak glikosilasi, tetapi urutan aa serupa di tapak aktif NA. Motif yang diketahui yang mengurangkan kerentanan dadah perencat NA atau M2 tidak dijumpai. Pokok filogenetik menunjukkan bahawa virus ini dikelompokkan didalam keturunan Eurasia H5,

dan berkelompok dengan subjenis AIV yang lain. Data ini menunjukkan pelbagai ciri LPAI H5N2 Malaysia, berbanding HPAI H5N2. Walaupun mungkin sukar untuk meramalkan perubahan patogenik LPAI kepada HPAI secara berkesan, AIV yang dikaji tidak menunjukkan penanda yang besar yang akan menandakan peralihan prospektif kepada HPAI. Analisis jujukan komprehensif ini akan membantu penyelidikan epidemiologi tentang dinamik dan evolusi AIV yang beredar dalam ayam.

Keywords: Virus avian influenza, hemagglutinin, neuraminidase, matrix gen, H5N2

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LIST OF ABBREVIATIONS

aa	Amino acid
AI	Avian influenza
AIVs	Avian Influenza Viruses
BLAST	Basic Local Alignment Search Tool
°C	Degree Celsius
cDNA	Complementary deoxyribonucleic acid
ECE	Embryonated chicken eggs
EDTA	Ethylenediamine tetraacetic acid
HA	Hemagglutinin
HPAIV	High pathogenic avian influenza virus
LPAIV	Low pathogenic avian influenza virus
M	Matrix
MEGA	Molecular Evolutionary Genetics Analysis
NA	Neuraminidase
OIE	World Organization for Animal Health (Office International des Epizooties)
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
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
µL	Microliter
mM	Millimolar
µm	Micrometre
&	And
nt	Nucleotide

CHAPTER 1

INTRODUCTION

Influenza viruses are composed of antisense, single stranded and segmented RNA and belongs to *Orthomyxoviridae* family. Type A, B, C, and D are the four types of significant of influenza virus. Type A viruses have infected numerous birds and mammals, whereas the other types of viruses have a more restricted host range. These viruses can infect domestic poultry and other animal species and occur naturally among aquatic birds worldwide. Generally, Avian influenza virus (AIV) is incapable of infecting humans. However, there have been isolated incidences of human infection with avian flu viruses. In addition, several countries, such as Asia, North Africa, and the Middle East, have been affected by AIV epidemics over the past few decades. As a result, various countries have begun developing AIV vaccines using multiple methodologies and strategies.

The combination of the surface glycoproteins hemagglutinin and neuraminidase determines the subtypes of influenza A viruses, each of which contains 18 (H1–H18) and 11 (N1–N11) antigens respectively, according to the Centers for Disease Control and Prevention (CDC) (2021). The virus genome is approximately 13.5 kb in length in total and divided into eight segments that encode ten or eleven proteins, depending on the virus strain (Hutchinson et al., 2014). The genomic segments ranging in size from 890 to 2341 nucleotides for examples Polymerase base subunit one (PB1) have approximate 2.3 kbp, Polymerase base subunit two (PB2) have approximate 2.3 kbp, polymerase acidic (PA) have approximate 2.2 kbp, hemagglutinin (HA) have 1.7 kbp, nucleoprotein (NP) have 1.5 kbp, neuraminidase (NA) 1.4 kbp, matrix proteins (M) 1 kbp, and non-structural protein (NS) 800 bp (Jiao et al., 2012).

Wild ducks are a reservoir for HPAI and LPAI viruses (Perkins & Swayne,2003; Kishida et al., 2005). However, they rarely exhibit clinical symptoms following HPAI infection. Depending on the age and genetic background of the ducks, however, certain HPAI H5N1 strains may be lethal to ducks (Cagle et al., 2011; Wasilenko et al., 2011). The vast majority of HPAI H5N1 virus strains frequently result in 100 percent mortality in chickens, turkeys, and quails within two to three days. Due to their intrinsic resistance, ducks also enhance the genetic reassortment of influenza viruses, facilitating the generation of genetically diverse AIVs, such as HPAI H5N1 viruses (Kuchipudi et al., 2014).

Low pathogenic avian influenza viruses (LPAIVs) of the H5 and H7 subtypes typically carry mutation at the HA cleavage site to become HPAI viruses, causing infection in humans. Outbreaks of avian influenza virus have enormous effects on both human health and the economy. Multiple independent LPAI virus (LPAIV) to HPAI conversion events have been documented since 1959, when the first H5 HPAI virus was found in chicken farms in Scotland (Dhingra et al., 2018; Alexander & Brown, 2009).

According to Cornelissen et al., (2013), the chicken's cytokine-mediated inflammatory response is overly delayed. Consequently, an insufficient cellular immune response may contribute to illness. Unlike ducks, the pattern recognition receptors (Toll-Like receptors 7, RIG-like receptors, and melanoma differentiation-associated protein 5) will induce a rapid cytokine production and sustained cellular response. Due to the absence of RIG-I, chickens may be more susceptible to the influenza virus than ducks. This RNA sensor is essential for IFN-mediated antiviral responses (Barber et al., 2010).

Despite the fact that AIV continues to circulate globally in humans due to population connectedness (Lemey et al., 2014), the majority of variety is found in avian species, and the reservoir population is comprised of birds (Fouchier & Guan, 2013). Understanding the mutation patterns of AIV epidemiology in birds would therefore contribute in clarifying the origins of prior pandemics and may aid in the prediction of future ones.

Malaysia experienced an outbreak of the A/Malaysia/Duck/8443/04 (H5N2) virus which involve ducks at Ipoh, Perak in 2004. As this is the only Malaysian H5N2 isolate, several studies have been performed to characterize this LPAI AIV strain. Despite the research, there's a gap in understanding the specific sequence analysis of the virus strain of interest, whereby the viral natural host or reservoir was a duck as opposed to the experiments performed using chickens. Therefore our study focuses on a detailed analysis of haemagglutinin (HA), neuraminidase (NA), and matrix (M) genes of the AIV Malaysian isolate upon direct sequencing through primer walking, whereby the comparison with human AIV amino acid signatures was also included in the analysis to compare the inter-species relation, zoonotic potentials and antiviral resistance. The purpose of focusing on these two genes, HA and NA, is because of their significant roles in viral replication and as "antigenic determinants", which make them the most relevant for studies of evolution and disease, as well as for categorization purposes. While the M gene is known as a is highly conserved gene among influenza A viruses.

The objectives of this research were:

1. To propagate and determine infectivity of the LPAI H5N2 virus isolated from duck in Specified-Pathogen-Free (SPF) chicken eggs.
2. To amplify the HA, NA and M genes of AIV by using different sets of gene-specific primer and polymerase chain reaction approaches.
3. To characterize and analyse the sequences obtained through molecular analysis and phylogenetic investigation.

REFERENCES

Abed, Y., & Boivin, G. (2017). A review of Clinical Influenza A and B infections with reduced susceptibility to both oseltamivir and Zanamivir. *Open Forum Infectious Diseases*, *4*(3). https://doi.org/10.1093/ofid/ofx105

Alexander, D. J., & Brown, I. H. (2009). History of highly pathogenic avian influenza. *Revue Scientifique Et Technique De L'OIE*, *28*(1), 19–38. https://doi.org/10.20506/rst.28.1.1856

Auld, D. S. (2013). Zinc-binding sites in proteins. *Encyclopedia of Metalloproteins*, 2554–2559. https://doi.org/10.1007/978-1-4614-1533-6_182

Barber, M. R., Aldridge, J. R., Webster, R. G., & Magor, K. E. (2010). Association of Rig-I with innate immunity of ducks to influenza. *Proceedings of the National Academy of Sciences*, *107*(13), 5913–5918. https://doi.org/10.1073/pnas.1001755107

Beerens, N., Heutink, R., & Peeters, B. (2020). Emergence and selection of a highly pathogenic avian influenza H7N3 virus. *Viruses 2020—Novel Concepts in Virology*. https://doi.org/10.3390/proceedings2020050046

Bialy, D., & Shelton, H. (2020). Functional neuraminidase inhibitor resistance motifs in avian influenza A(H5NX) viruses. *Antiviral Research*, *182*, 104886. https://doi.org/10.1016/j.antiviral.2020.104886

Castro-Sanguinetti, G. R., Marques Simas, P. V., Apaza-Chiara, A. P., Callupe-Leyva, J. A., Rondon-Espinoza, J. A., Gavidia, C. M., More-Bayona, J. A., Gonzalez Veliz, R. I., Vakharia, V. N., & Icochea, M. E. (2022). Genetic subtyping and phylogenetic analysis of HA and NA from avian influenza virus in wild birds from Peru reveals unique features among circulating strains in America. *PLOS ONE*, *17*(6). https://doi.org/10.1371/journal.pone.0268957

Center of Disease control and Prevention (2021). Type of influenza virus. Retrieved from https://www.cdc.gov/flu/about/viruses/types.htm

Chan, P. K. (2002). Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. *Clinical Infectious Diseases*, *34*(Supplement_2). https://doi.org/10.1086/338820

Chander, Y., Jindal, N., Sreevatsan, S., Stallknecht, D. E., & Goyal, S. M. (2012). Molecular and phylogenetic analysis of matrix gene of avian influenza viruses isolated from wild birds and live bird markets in the USA. *Influenza and Other Respiratory Viruses*, *7*(4), 513–520. https://doi.org/10.1111/irv.12003

Chen, L., Zhu, F., Xiong, C., Zhang, Z., Jiang, L., Chen, Y., Zhao, G., & Jiang, Q. (2015). Could a deletion in neuraminidase stalk strengthen human tropism of the novel avian influenza virus H7N9 in China, 2013? *International Journal of Environmental Research and Public Health*, *12*(1), 1020–1028. https://doi.org/10.3390/ijerph120101020

Chou, J. J., Pielak, R. M., Schnell, J. R., & Wang, J. (2010). Structure and mechanism of influenza proton channels. *Biophysical Journal*, *98*(3). https://doi.org/10.1016/j.bpj.2009.12.1059

Continued evolution of highly pathogenic avian influenza A (H5N1): Updated nomenclature. (2011). *Influenza and Other Respiratory Viruses*, *6*(1), 1–5. https://doi.org/10.1111/j.1750-2659.2011.00298.x

Cornelissen, J. B., Vervelde, L., Post, J., & Rebel, J. M. (2013). Differences in highly pathogenic avian influenza viral pathogenesis and associated early inflammatory response in chickens and ducks. *Avian Pathology*, *42*(4), 347–364. https://doi.org/10.1080/03079457.2013.807325

Dhingra, M. S., Artois, J., Dellicour, S., Lemey, P., Dauphin, G., Von Dobschuetz, S., Van Boeckel, T. P., Castellan, D. M., Morzaria, S., & Gilbert, M. (2018). Geographical and historical patterns in the emergences of novel highly pathogenic avian influenza (Hpai) H5 and H7 viruses in Poultry. *Frontiers in Veterinary Science*, *5*. https://doi.org/10.3389/fvets.2018.00084

Dong, G., Peng, C., Luo, J., Wang, C., Han, L., Wu, B., Ji, G., & He, H. (2015). Adamantane-resistant influenza A viruses in the world (1902–2013): Frequency and distribution of M2 gene mutations. *PLOS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0119115

Du, Y., Chen, M., Yang, J., Jia, Y., Han, S., Holmes, E. C., & Cui, J. (2017). Molecular evolution and emergence of H5N6 avian influenza virus in central China. *Journal of Virology*, *91*(12). https://doi.org/10.1128/jvi.00143-17

ElHefnawi, M., AlAidi, O., Mohamed, N., Kamar, M., El-Azab, I., Zada, S., & Siam, R. (2011). Identification of novel conserved functional motifs across most influenza a viral strains. *Virology Journal*, *8*(1). https://doi.org/10.1186/1743-422x-8-44

Evseev, D., & Magor, K. (2019). Innate immune responses to avian influenza viruses in ducks and chickens. *Veterinary Sciences*, *6*(1), 5. https://doi.org/10.3390/vetsci6010005

Fereidouni, S. R., Starick, E., Grund, C., Globig, A., Mettenleiter, T. C., Beer, M., & Harder, T. (2009). Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. *Veterinary Microbiology*, *135*(3-4), 253–260. https://doi.org/10.1016/j.vetmic.2008.09.077

Fouchier, R. A. M., & Guan, Y. (2013). Ecology and evolution of influenza viruses in wild and domestic birds. *Textbook of Influenza*, 173–189. https://doi.org/10.1002/9781118636817.ch11

Furuse, Y., Suzuki, A., Kamigaki, T., & Oshitani, H. (2009). Evolution of the M gene of the influenza A virus in different host species: Large-scale sequence analysis. *Virology Journal*, *6*(1). https://doi.org/10.1186/1743-422x-6-67

Ge, Z., Xu, L., Hu, X., Zhu, S., Zhao, Y., Li, Y., Liu, K., Gao, R., Wang, X., Hu, J., Liu, X., Hu, S., Peng, D., Gu, M., & Liu, X. (2022). Phylogenetic and phenotypic characterization of two novel clade 2.3.2.1 H5N2 subtype avian influenza viruses from chickens in China. *Infection, Genetics and Evolution*, *98*, 105205. https://doi.org/10.1016/j.meegid.2022.105205

Gholami-Ahangaran, M., Basiratpour, A., Pourmahdi, O., Khorrami, P., Ostadpoor, M., Mirbagheri, M. J., & Ahmadi-Dastgerdi, A. (2022). The sequence analysis of M2 gene for identification of amantadine resistance in avian influenza virus (H9N2 subtype), detected from broiler chickens with respiratory syndrome during 2016-2018, in Isfahan-Iran. *Acta Scientiarum. Animal Sciences*, *44*. https://doi.org/10.4025/actascianimsci.v44i1.54894

Gholami-Ahangaran, M., Basiratpour, A., Pourmahdi, O., Khorrami, P., Ostadpoor, M., Mirbagheri, M. J., & Ahmadi-Dastgerdi, A. (2022). The sequence analysis of M2 gene for identification of amantadine resistance in avian influenza virus (H9N2 subtype), detected from broiler chickens with respiratory syndrome during 2016-2018, in Isfahan-Iran. *Acta Scientiarum. Animal Sciences*, *44*. https://doi.org/10.4025/actascianimsci.v44i1.54894

Gong, J., Xu, W., & Zhang, J. (2007). Structure and functions of influenza virus neuraminidase. *Current Medicinal Chemistry*, *14*(1), 113–122. https://doi.org/10.2174/092986707779313444

Hervé, P.-L., Lorin, V., Jouvion, G., Da Costa, B., & Escriou, N. (2015). Addition of N-glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity. *Virology*, *486*, 134–145. https://doi.org/10.1016/j.virol.2015.08.033

Hoffmann, E., Stech, J., Guan, Y., Webster, R. G., & Perez, D. R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. *Archives of Virology*, *146*(12), 2275–2289. https://doi.org/10.1007/s007050170002

Hutchinson, E. C., Charles, P. D., Hester, S. S., Thomas, B., Trudgian, D., Martínez-Alonso, M., & Fodor, E. (2014). Conserved and host-specific

features of influenza virion architecture. *Nature Communications*, *5*(1). https://doi.org/10.1038/ncomms5816

Hutchinson, E. C., Charles, P. D., Hester, S. S., Thomas, B., Trudgian, D., Martínez-Alonso, M., & Fodor, E. (2014). Conserved and host-specific features of influenza virion architecture. *Nature Communications*, *5*(1). https://doi.org/10.1038/ncomms5816

Kim, H.-R., Park, C.-K., Oem, J.-K., Bae, Y.-C., Choi, J.-G., Lee, O.-S., & Lee, Y.-J. (2010). Characterization of H5N2 influenza viruses isolated in South Korea and their influence on the emergence of a novel H9N2 influenza virus. *Journal of General Virology*, *91*(8), 1978–1983. https://doi.org/10.1099/vir.0.021238-0

Kim, P., Jang, Y., Kwon, S., Lee, C., Han, G., & Seong, B. (2018). Glycosylation of hemagglutinin and neuraminidase of influenza A virus as signature for ecological spillover and adaptation among influenza reservoirs. *Viruses*, *10*(4), 183. https://doi.org/10.3390/v10040183

Klenk, H.-D., & Rott, R. (1988). The molecular biology of influenza virus pathogenicity. *Advances in Virus Research*, 247–281. https://doi.org/10.1016/s0065-3527(08)60520-5

Kuchipudi, S. V., Nelli, R., & White, G. A. (2009). Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. *Journal of Molecular and Genetic Medicine*, *03*(01). https://doi.org/10.4172/1747-0862.1000026

Kuchipudi, S. V., Tellabati, M., Sebastian, S., Londt, B. Z., Jansen, C., Vervelde, L., Brookes, S. M., Brown, I. H., Dunham, S. P., & Chang, K.-C. (2014). Highly pathogenic avian influenza virus infection in chickens but not ducks is associated with elevated host immune and pro-inflammatory responses. *Veterinary Research*, *45*(1). https://doi.org/10.1186/s13567-014-0118-3

Kuiken, T. (2013). Is low pathogenic avian influenza virus virulent for wild waterbirds? *Proceedings of the Royal Society B: Biological Sciences*, *280*(1763), 20130990. https://doi.org/10.1098/rspb.2013.0990

Laleye, A. T., & Abolnik, C. (2020). Emergence of highly pathogenic H5N2 and H7N1 influenza a viruses from low pathogenic precursors by serial passage in ovo. *PLOS ONE*, *15*(10). https://doi.org/10.1371/journal.pone.0240290

Lang, V., Rinder, M., Hafner-Marx, A., Rabl, S., Bogner, K. H., Neubauer-Juric, A., & Büttner, M. (2010). Avian Influenza A virus monitoring in wild birds in bavaria: Occurrence and heterogeneity of H5 and N1 encoding genes. *Zoonoses and Public Health*, *57*(7-8). https://doi.org/10.1111/j.1863-2378.2010.01326.x

Lee, C.-C. D., Zhu, H., Huang, P.-Y., Peng, L., Chang, Y.-C., Yip, C.-H., Li, Y.-T., Cheung, C.-L., Compans, R., Yang, C., Smith, D. K., Lam, T. T.-Y., King, C.-C., & Guan, Y. (2014). Emergence and evolution of avian H5N2 influenza viruses in chickens in Taiwan. *Journal of Virology*, *88*(10), 5677–5686. https://doi.org/10.1128/jvi.00139-14

Lee, M.-S., Chang, P.-C., Shien, J.-H., Cheng, M.-C., & Shieh, H. K. (2001). Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *Journal of Virological Methods*, *97*(1-2), 13–22. https://doi.org/10.1016/s0166-0934(01)00301-9

Lee, Y.-N., Lee, D.-H., Cheon, S.-H., Park, Y.-R., Baek, Y.-G., Si, Y.-J., Kye, S.-J., Lee, E.-K., Heo, G.-B., Bae, Y.-C., Lee, M.-H., & Lee, Y.-J. (2020). Genetic characteristics and pathogenesis of H5 low pathogenic avian influenza viruses from wild birds and domestic ducks in South Korea. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-68720-w

Lemey, P., Rambaut, A., Bedford, T., Faria, N., Bielejec, F., Baele, G., Russell, C. A., Smith, D. J., Pybus, O. G., Brockmann, D., & Suchard, M. A. (2014). Unifying viral genetics and human transportation data to predict the global transmission dynamics of human influenza H3N2. *PLoS Pathogens*, *10*(2). https://doi.org/10.1371/journal.ppat.1003932

Li, K. S., Guan, Y., Wang, J., Smith, G. J., Xu, K. M., Duan, L., Rahardjo, A. P., Puthavathana, P., Buranathai, C., Nguyen, T. D., Estoepangestie, A. T., Chaisingh, A., Auewarakul, P., Long, H. T., Hanh, N. T., Webby, R. J., Poon, L. L., Chen, H., Shortridge, K. F., … Peiris, J. S. (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in Eastern Asia. *Nature*, *430*(6996), 209–213. https://doi.org/10.1038/nature02746

Li, Y., Liu, D., Wang, Y., Su, W., Liu, G., & Dong, W. (2021). The importance of glycans of viral and host proteins in enveloped virus infection. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.638573

Lim, K.-L., Jazayeri, S. D., Yeap, S. K., Alitheen, N. B., Bejo, M. H., Ideris, A., & Omar, A. R. (2012). Co-administration of avian influenza virus H5 plasmid DNA with chicken IL-15 and IL-18 enhanced chickens immune responses. *BMC Veterinary Research*, *8*(1). https://doi.org/10.1186/1746-6148-8-132

Luczo, J. M., Stambas, J., Durr, P. A., Michalski, W. P., & Bingham, J. (2015). Molecular pathogenesis of H5 highly pathogenic avian influenza: The role of the haemagglutinin cleavage site motif. *Reviews in Medical Virology*, *25*(6), 406–430. https://doi.org/10.1002/rmv.1846

Lv, J., Wei, L., Yang, Y., Wang, B., Liang, W., Gao, Y., Xia, X., Gao, L., Cai, Y., Hou, P., Yang, H., Wang, A., Huang, R., Gao, J., & Chai, T. (2015). Amino acid substitutions in the neuraminidase protein of an H9N2 avian influenza

virus affect its airborne transmission in chickens. *Veterinary Research*, *46*(1). https://doi.org/10.1186/s13567-014-0142-3

Mariatulqabtiah, A. R., Nor Majid, N., Giotis, E. S., Omar, A. R., & Skinner, M. A. (2019). Inoculation of fowlpox viruses coexpressing avian influenza H5 and chicken IL-15 cytokine gene stimulates diverse host immune responses. *Asia Pacific Journal of Molecular Biology and Biotechnology*, 84–94. https://doi.org/10.35118/apjmbb.2019.027.1.09

Martins, N. R. (2012). An overview on avian influenza. *Revista Brasileira De Ciência Avícola*, *14*(2), 71–87. https://doi.org/10.1590/s1516-635x2012000200001

Matrosovich, M. N., Gambaryan, A. S., Teneberg, S., Piskarev, V. E., Yamnikova, S. S., Lvov, D. K., Robertson, J. S., & Karlsson, K.-A. (1997). Avian Influenza A viruses differ from human viruses by recognition of Sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology*, *233*(1), 224–234. https://doi.org/10.1006/viro.1997.8580

Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A., & Klenk, H.-D. (2004). Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proceedings of the National Academy of Sciences*, *101*(13), 4620–4624. https://doi.org/10.1073/pnas.0308001101

McAuley, J. L., Gilbertson, B. P., Trifkovic, S., Brown, L. E., & McKimm-Breschkin, J. L. (2019). Influenza virus neuraminidase structure and functions. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00039

Munier, S., Larcher, T., Cormier-Aline, F., Soubieux, D., Su, B., Guigand, L., Labrosse, B., Cherel, Y., Quéré P., Marc, D., & Naffakh, N. (2010). A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. *Journal of Virology*, *84*(2), 940–952. https://doi.org/10.1128/jvi.01581-09

Muraina, I. A., Meseko, C. A., & Fasina, F. O. (2020). The potential of putative zinc-binding motifs of haemagglutinin (ha) protein for categorization and prediction of pathogenicity of H5 subtypes of avian influenza virus. *Medical Hypotheses*, *144*, 109925. https://doi.org/10.1016/j.mehy.2020.109925

Nao, N., Kajihara, M., Manzoor, R., Maruyama, J., Yoshida, R., Muramatsu, M., Miyamoto, H., Igarashi, M., Eguchi, N., Sato, M., Kondoh, T., Okamatsu, M., Sakoda, Y., Kida, H., & Takada, A. (2015). A single amino acid in the M1 protein responsible for the different pathogenic potentials of H5N1 highly pathogenic avian influenza virus strains. *PLOS ONE*, *10*(9). https://doi.org/10.1371/journal.pone.0137989

Nguyen, A. T., Hoang, V. T., Sung, H. W., Yeo, S.-J., & Park, H. (2021). Genetic characterization and pathogenesis of three novel reassortant H5N2 viruses in South Korea, 2018. *Viruses*, *13*(11), 2192. https://doi.org/10.3390/v13112192

Ogata, T., Yamazaki, Y., Okabe, N., Nakamura, Y., Tashiro, M., Nagata, N., Itamura, S., Yasui, Y., Nakashima, K., Doi, M., Izumi, Y., Fujieda, T., Yamato, S., & Kawada, Y. (2008). Human H5N2 avian influenza infection in Japan and the factors associated with high H5N2-neutralizing antibody titer. *Journal of Epidemiology*, *18*(4), 160–166. https://doi.org/10.2188/jea.je2007446

Richard, M., Deléage, C., Barthélémy, M., Lin, Y. P., Hay, A., Lina, B., & Ferraris, O. (2008). Impact of influenza A virus neuraminidase mutations on the stability, activity, and Sensibility of the neuraminidase to neuraminidase inhibitors. *Journal of Clinical Virology*, *41*(1), 20–24. https://doi.org/10.1016/j.jcv.2007.10.021

Runstadler, J., Hill, N., Hussein, I. T. M., Puryear, W., & Keogh, M. (2013). Connecting the study of wild influenza with the potential for pandemic disease. *Infection, Genetics and Evolution*, *17*, 162–187. https://doi.org/10.1016/j.meegid.2013.02.020

Sakurai, A., Takayama, K., Nomura, N., Munakata, T., Yamamoto, N., Tamura, T., Yamada, J., Hashimoto, M., Kuwahara, K., Sakoda, Y., Suda, Y., Kobayashi, Y., Sakaguchi, N., Kida, H., Kohara, M., & Shibasaki, F. (2013). Broad-spectrum detection of H5 subtype influenza A viruses with a new fluorescent immunochromatography system. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0076753

Sakurai, A., Takayama, K., Nomura, N., Munakata, T., Yamamoto, N., Tamura, T., Yamada, J., Hashimoto, M., Kuwahara, K., Sakoda, Y., Suda, Y., Kobayashi, Y., Sakaguchi, N., Kida, H., Kohara, M., & Shibasaki, F. (2013). Broad-spectrum detection of H5 subtype influenza A viruses with a new fluorescent immunochromatography system. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0076753

Su, W.-C., Yu, W.-Y., Huang, S.-H., & Lai, M. M. (2018). Ubiquitination of the cytoplasmic domain of influenza A virus M2 protein is crucial for production of infectious virus particles. *Journal of Virology*, *92*(4). https://doi.org/10.1128/jvi.01972-17

Suttie, A., Deng, Y.-M., Greenhill, A. R., Dussart, P., Horwood, P. F., & Karlsson, E. A. (2019). Inventory of molecular markers affecting biological characteristics of avian influenza A viruses. *Virus Genes*, *55*(6), 739–768. https://doi.org/10.1007/s11262-019-01700-z

Swayne, D. E. (2023, January 10). *Avian influenza - poultry*. MSD Veterinary Manual. Retrieved January 22, 2023, from https://www.msdvetmanual.com/poultry/avian-influenza/avian-influenza

Tada, T., Suzuki, K., Sakurai, Y., Kubo, M., Okada, H., Itoh, T., & Tsukamoto, K. (2011). NP body domain and PB2 contribute to increased virulence of H5N1 highly pathogenic avian influenza viruses in chickens. Journal of Virology, 85(4), 1834–1846. https://doi.org/10.1128/jvi.01648-10

van Riel, D., den Bakker, M. A., Leijten, L. M. E., Chutinimitkul, S., Munster, V. J., de Wit, E., Rimmelzwaan, G. F., Fouchier, R. A. M., Osterhaus, A. D. M. E., & Kuiken, T. (2010). Seasonal and pandemic human influenza viruses attach better to human upper respiratory tract epithelium than avian influenza viruses. *The American Journal of Pathology*, *176*(4), 1614–1618. https://doi.org/10.2353/ajpath.2010.090949

Venter, M., Treurnicht, F. K., Buys, A., Tempia, S., Samudzi, R., McAnerney, J., Jacobs, C. A., Thomas, J., & Blumberg, L. (2017). Risk of human infections with highly pathogenic H5N2 and low pathogenic H7N1 avian influenza strains during outbreaks in ostriches in South Africa. *The Journal of Infectious Diseases*, *216*(suppl_4). https://doi.org/10.1093/infdis/jix018

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiological Reviews*, *56*(1), 152–179. https://doi.org/10.1128/mr.56.1.152-179.1992

WHO/OIE/FAO H5N1 Evolution Working Group. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). (2008). *Emerging Infectious Diseases*, *14*(7). https://doi.org/10.3201/eid1407.071681

Yeo, D. S.-Y., Ng, S.-H., Liaw, C.-W., Ng, L.-M., Wee, E. J.-H., Lim, E. A.-S., Seah, S. L.-K., Wong, W.-K., Lim, C.-W., Sugrue, R. J., & Tan, B.-H. (2009). Molecular characterization of low pathogenic avian influenza viruses, isolated from food products imported into Singapore. *Veterinary Microbiology*, *138*(3-4), 304–317. https://doi.org/10.1016/j.vetmic.2009.04.025