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GENETIC ANALYSIS OF H9N2 AVIAN INFLUENZA VIRUSES ISOLATED FROM CHICKENS IN MALAYSIA FROM 2015- 2018

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ABSTRACT. Avian influenza (AI) H9N2 has become a major problem in the poultry industry in many countries. Although H9N2 viruses are considered as low pathogenic avian influenza (LPAI), they pose a significant threat to public health as they can potentially be pandemic. This study presents molecular characterisation of eleven (11) H9N2 AI isolates from case investigations and field outbreaks in poultry that occurred in Malaysia from 2015 to 2018. Complete haemagglutinin (HA) gene was amplified by RT-PCR, then directly sequenced. Seven isolates showed 98% nucleotide similarities with Indonesian and Vietnam strains while four isolates had 96% to 97% similarities with Korean strains. Phylogenetic analysis revealed that H9N2 isolates in this study belonged to two distinct lineages — Korean and Y280, indicating that they have different sources of origin. A special note to point out is that at position 226 for isolates from Y280 lineage, there is an amino acid exchange from glutamine to leucine (Q->L) at the receptor binding site indicating that these isolates have the potential to infect mammals including humans. This is the first report of isolation of H9N2 from chickens in Malaysia. The disease outbreak in 2018 suggests that the H9N2 isolates in Malaysia can cause major losses to the poultry industry. Findings from this study revealed that these isolates are potentially infectious to humans. This highlights the necessity of implementing farm biosecurity, continuous and thorough surveillance paired with risk-assessment of the circulation of H9N2 influenza viruses.

Keywords: LPAI, H9N2, poultry, Malaysia

INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease affecting several species including birds. AI viruses are classified into highly pathogenic avian influenza (HPAI) virus and low pathogenic avian influenza (LPAI) virus, depending on the severity of the disease in susceptible birds (Peacock T.P. *et al.*, 2018).

Among the LPAIs, H9N2 is considered the most prevalent LPAI in the world. H9N2 are endemic across much of Asia, the Middle East, and north and west Africa. It had caused severe economic losses to the poultry industry through reduced broiler growth rates, drops in egg production, moderate to high morbidity and mortality (Jonas M. *et al.*, 2018), pathologic lesions and egg production. Confirmation was made using virus isolation and reverse transcriptase PCR (RT-PCR) (Amer M.M., 2018; Pusch E.A. *et al.*,

2018; Peacock T.P. *et al.,* 2018; Butt A.M. *et al.*, 2010).

Although it is categorised as LPAI, H9N2 is a possible threat to human health. Previous studies revealed that H9N2 influenza viruses from poultry could occasionally be transmitted from poultry to mammalian species, including pigs and humans (Pusch E.A. *et al.*, 2018; Ge F. *et al.*, 2009). It was reported that H9N2 may be significant donors of genetic material to emerging human pathogen. Thus, H9N2 viruses are considered one of the most likely candidates to cause a new influenza pandemic in humans (Shen H.Q. *et al.*, 2015; Jiang W. *et al.*, 2012).

Avian influenza viruses affiliates to the genus of type A influenza virus in the *Orthomyxoviridae* family, packaged with eight negative-sense and single-strand RNA segments. Among the segments, haemagglutinin (HA) is an important surface protein that plays a vital role when influenza viruses are introduced into host cells. (Zhu R. *et al.*, 2018). In addition to that, it has been well documented that the receptor binding site motif of HA is critical for cellular receptor specificity and determining virus host range (Butt A.M. *et al.*, 2010). It has been shown that in HA protein, amino acids at position 226 is critical for determining the affinity for α2,3 or α2,6 sialic acid binding with glutamine (Q) for avian adapted virus and leucine (L) for mammals/human adapted virus (Pusch E.A. *et al.,* 2018; Wan H. *et al.,* 2007).

According to epidemiological and genetic studies, the HA genes in H9N2 viruses can be divided into 2 lineages: north American and Eurasian (Guo Y.J. *et al.*, 2000). The H9N2 viruses of the Eurasian lineage are categorised into three major sublineages: the G1 lineage, represented by A/Quail/Hong Kong/GA/Quail/Hong Kong/G1/97 (G1-like); the Y280 lineage, represented by three prototype viruses A/duck/Hong Kong/Y280/97 (Y280-like), A/Chicken/ Beijing/1/94 (BJ94-like), and A/Chicken/Hong Kong/G9/ 97 (G9-like) and the Korean lineage, represented by A/chicken/Korea/38349-p96323/96 (Korean-like) and A/duck/Hong Kong/Y439/97 (Y439-like) (Butt A.M. *et al.,* 2010).

Based on the cases in Veterinary Research Institute (VRI) Ipoh, H9N2 has been isolated from chickens in 2015 and 2017. In the last quarter of 2018, epidemic of H9N2 occurred in Peninsular Malaysia not only in village chickens but also in commercial poultry farms. Outbreaks were continuously reported with high morbidity, drop in egg production and reduced weight gain. Due to this disease, Malaysia had faced a shortage of eggs during the outbreaks as it affects layer chickens (Abdul R. Omar, 2018).

There has not been any report detailing on the H9N2 isolates from chickens specifically focusing on molecular perspective in Malaysia. Therefore in the present study, the HA genes of H9N2 viruses identified in Malaysia during 2015-2018 were sequenced and analysed the relationships between the Malaysia isolates and viruses isolated in the other countries or areas.

METHODOLOGY

Virus Isolation

Eleven H9N2 isolates were detected positive by real time RT-PCR and isolated

by egg inoculation in year 2015 to 2018 at the Avian Virology Laboratory, Veterinary Research Institute (VRI), Ipoh. The isolates were used in this study (Table 1). The H9N2 viruses were isolated from chickens suspected with AI observed with clinical symtoms and drop in egg production. The isolates were propagated in the allantoic cavity of 9 to 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs in accordance with the instructions of the World Organization for Animal Health (OIE) manual and identified by haemagglutination (HA) and haemagglutination inhibition (HI) tests. Infected allantoic fluid samples were centrifuged at 2500 × *g* for 10 minutes at 4 °C and supernatant was stored at -80 °C for later analysis.

RNA Extractionand Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Viral RNA extraction was carried out on infected allantoic fluid using QIAamp cador Pathogen kit (Qiagen N.V., Netherlands) in accordance with the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) was carried out using SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). Primers 5'-CTCAGGGAGCAAAAGCAGGGG-3' (forward) and 5'-GTATTAGTAGAAACAAGGGT-GTTTT-3' (reverse), covering the entire length of the HA gene were used (Hoffmann E. *et al.,* 2001).

RT was carried out at 48 °C for 30 min. The reaction mix was then subjected to 94 °C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 68 °C for 2 min with a final extension for 10 min at 68 °C.

Upon RT-PCR completion, the RT-PCR reaction mixture was loaded into 1.0% agarose gel containing SyBr Safe (Invitrogen, USA) for electrophoresis and visualized by UV transilluminator.

Sequencing, Analysis of Nucleotide and Amino Acid Sequences and Phylogenetic Analysis

PCR products were excised from agarose gel and purified using QIAQuick Gel Extraction Kit (Qiagen N.V., Netherlands) prior to Sanger sequencing. Sequencing was performed by First BASE Laboratories Sdn Bhd, Malaysia. The primers used for sequence analysis were the same as those used for RT-PCR amplification. The raw sequences were manually edited and assembled using SeqMan Pro software (DNAStar Lasergene, USA). The sequences were compared with sequences accessible in the NCBI GenBank® database (Clark K. *et al.*, 2016) and subjected to the online BLAST search at www.blast.ncbi.nlm.nih. gov/Blast.cgi (Camacho C. *et al.,* 2009).

Nucleotide and amino acid sequences were then aligned with Clustal W multiple alignment method and analysed using BioEdit program version 7.2.5. The isolates in this study together with other 29 haemagglutinin (HA) gene of H9N2 sequences from GenBank® were included for phylogenetic analysis. Phylogenetic tree was constructed with MEGA v6.06 using neighbour joining Kimura 2 parameter

Table 1. List of isolates in this study including the HA cleavage motif, BLAST results and the amino acid residues at the receptor binding site (RBS) at position 226 (H3 numbering) or 234 (H9 numbering). Legend; Q: Glutamine; L: Leucine

DI:	Date received	State	Area	Species	Breed	Age	Gender	HA Cleavage Site 335-339	BLAST		HA RBS at position 226 (H3 Numbering)
									Highest Nucleotide Homology	Identity (%)	
A/chicken/Malaysia/Perak/2061/2015	3/3/2015	Perak	Bukit Merah, Perak	Chicken	Village (Commercial)	75 days	Mixed	RSKR↓G	Korea	97	Q
A/chicken/Malaysia/Penang/1373/2017	13/2/2017	Pulau Pinang	Nibong Tebal	Chicken	CP Brown	72 days (Layer)	Mixed	RSKR↓G	Korea	97	Q
A/chicken/Malaysia/Penang/3174/2017	17/3/2017	Pulau Pinang	Nibong Tebal	Chicken	Isa Brown	Adult (Layer)	Female	RSKR↓G	Korea	97	Q
A/chicken/Malaysia/NegeriSembilan/7896/2018	6/10/2018	Negeri Sembilan	Rantau	Chicken	Hisex	Adult (Layer)	Female	RSSR↓G	Indonesia	98	L
A/chicken/Malaysia/NegeriSembilan/7895/2018	8/10/2018	Negeri Sembilan	Rantau	Chicken	Cobb	Adult (Breeder)	Mixed	RSSR↓G	Indonesia	98	L
A/chicken/Malaysia/Penang/8231/2018	15/10/2018	Pulau Pinang	Seberang Jaya	Chicken	Village	2 months (Adult)	Mixed	RSKR↓G	Korea	96	Q
A/chicken/Malaysia/Penang/8232/2018	15/10/2018	Pulau Pinang	Bukit Mertajam	Chicken	Ross 308	32 days (Broiler)	Mixed	RSSR↓G	Indonesia	98	L
A/chicken/Malaysia/Penang/9541/2018	28/11/2018	Pulau Pinang	Bukit Mertajam,	Chicken	Village	Chick	Mixed	RSSR↓G	Indonesia	98	L
A/chicken/Malaysia/Perak/9745/2018	4/12/2018	Perak	Bidor	Chicken	Hisex Lohmann	Pullet (Layer)	Female	RSSR↓G	Indonesia	98	L
A/chicken/Malaysia/Melaka/10291/2018	20/12/2018	Melaka	Kuala Sg Baru,	Chicken	Lohmann	Adult (Layer)	Female	RSSR↓G	Indonesia	98	L
A/chicken/Malaysia/Melaka/10298/2018	20/12/2018	Melaka	Kuala Sg Baru,	Chicken	Lohmann	Adult (Layer)	Female	RSSR↓G	Indonesia	98	L

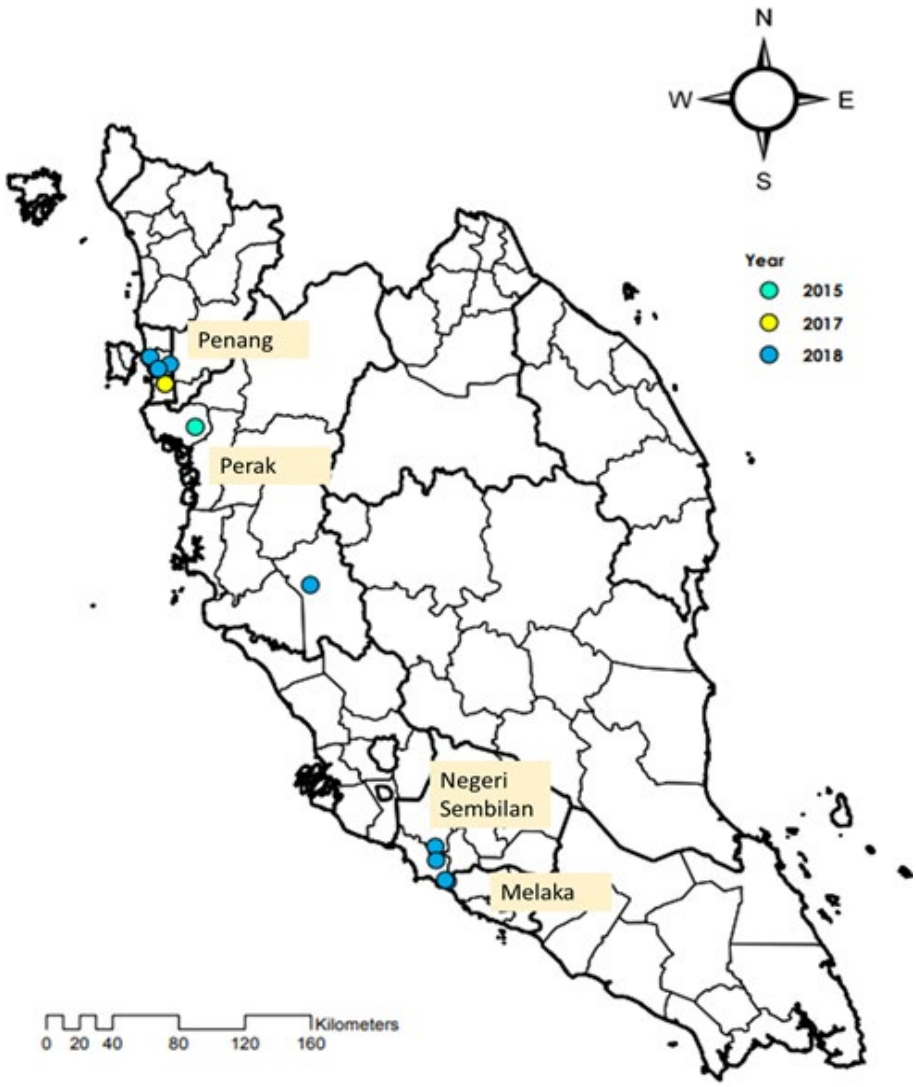


Figure 1. Map of Peninsular Malaysia showing the locations of H9N2 positive cases in various states during 2015-2018. Google earth was used to map out the distribution of H9N2 cases according to decimal degree coordinate of the locations with World Geodetic System (WGS).

model with 1,000 bootstrapped replications (Tamura K. *et al.*, 2013). Phylogenetic analysis of the H9N2 isolates was generated based on complete HA gene from nucleotide 1 to 1631.

RESULTS

All eleven isolates were successfully amplified and sequenced. Table 1 shows the list and details of isolates. From 2015 to 2018, there were eleven H9N2 viruses isolated. One case was isolated in Perak in 2015 from commercial village chickens. Two cases were isolated in 2017 in Penang. In 2018, a total of eight isolates were found in many states of Peninsular Malaysia, in Perak, Penang, Negeri Sembilan and Melaka. Figure 1 shows the locations of these cases in the various states.

The BLAST results revealed that based on the HA encoding gene, four isolates from the northern region (Perak and Penang) had 96% to 97% nucleotide homology with Korean isolate A/chicken/Korea/01310/2001 while the remaining seven isolates from various parts of Peninsular Malaysia (Penang, Perak, Melaka and Negeri Sembilan) had 98% nucleotide identities with the Indonesian strain A/chicken/North Sumatera/M92_22/2017 (Table 1).

Percentage similarities of nucleotide sequences of H9N2 HA gene are shown in Table 2. The isolates in this study shared 79.7% to 100% nucleotide similarities. An isolate of 2018, isolate A/chicken/Malaysia/Penang/8231/2018 had high similarities with isolates from 2015 and 2017 in the range of 97.5% to 97.8%. The isolates had high similarities with the Korean strain A/chicken/Korea/01310/2001 between 96.0% to 97.1%.

The remaining seven isolates of 2018 in this study had high nucleotide similarities 96.9% to 100%. Two isolates, one from Perak and another from Penang, was 99.2% similar to each other but slightly different from the other five isolates similarities at 96.9% to 97.8%. Compared with other circulating H9N2 in the region, these seven isolates showed high similarities with isolates from Indonesia A/chicken/North Sumatera/M92_22/2017 and Vietnam A/chicken/Vietnam/H7F-LC4-371/2014 at 97.8% to 98.8% and 97.3% to 97.8%, respectively.

Based on the phylogenetic tree (Figure 2), H9N2 isolates can be divided into three major lineages, Y280, G1 and Korean.

Isolates from 2015, 2017 and one isolate from Penang (A/chicken/Malaysia/Penang/8231/2018) were clustered under the Korean lineage. The remaining seven isolates were grouped in Y280 lineage. The Korean lineage, is branched out into Korea-like and Y439-like sublineages. For the Malaysian isolates, they were subclustered under Korea-like forming a distinct group having closely related strains from Korea isolated in 2001.

In the Y280 lineage, five isolates were clustered together with Indonesian strain while two isolates A/chicken/Malaysia/Penang/9541/2018 and A/chicken/Malaysia/Perak/9745/2018 formed a distinct cluster related with the Vietnam strain.

The amino acid composition of the cleavage site of Malaysian H9N2 isolates is described in Table 1. According to the cleavage site amino acid motifs, all isolates can be distributed into two groups. The first group with the cleavage site motif RSKR included isolates from 2015, 2017 and one

Table 2. Similarity analysis of nucleotide sequence (%) of the HA genes of the isolates in this study to those of H9N2 reference strains.

	H9N2 isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	A/chicken/ Korea/01310/2001														
2	A/chicken/ North Sumatera/ M92_22/2017	81.1													
3	A/chicken/Vietnam/ H7F-LC4-371/2014	81.5	98.2												
4	A/chicken/Malaysia/ Perak/2061/2015	97.1	80.4	81.0											
5	A/chicken/Malaysia/ Penang/1373/2017	96.8	80.1	80.7	99.3										
6	A/chicken/Malaysia/ Penang/3174/2017	96.6	80.2	80.8	99.4	99.8									
7	A/chicken/Malaysia/ NegeriSembilan/ 7896/2018	81.1	98.6	97.6	80.4	80.1	80.2								
8	A/chicken/Malaysia/ NegeriSembilan/ 7895/2018	81.3	98.8	97.8	80.6	80.2	80.3	99.8							
9	A/chicken/Malaysia/ Penang/8231/2018	96.0	80.0	80.7	97.8	97.7	97.5	79.8	80.0						
10	A/chicken/Malaysia/ Penang/8232/2018	81.0	98.7	97.5	80.2	79.9	80.0	99.1	99.2	79.8					
11	A/chicken/Malaysia/ Penang/9541/2018	81.1	98.0	97.4	80.2	80.0	80.0	97.3	97.5	80.0	97.8				
12	A/chicken/Malaysia/ Perak/9745/2018	81.3	97.8	97.3	80.5	80.2	80.3	97.2	97.3	80.2	97.1	99.2			
13	A/chicken/Malaysia/ Melaka/10291/2018	81.0	98.3	97.4	80.3	80.0	80.0	99.6	99.5	79.7	98.9	97.1	96.9		
14	A/chicken/Malaysia/ Melaka/10298/2018	81.0	98.3	97.4	80.3	80.0	80.0	99.6	99.5	79.7	98.9	97.1	96.9	100.0	

isolate from Penang in 2018 (A/chicken/Malaysia/Penang/8231/2018).

The remaining isolates from 2018 were included in the second group with the motif of RSSR.

Based on the amino acid analysis, the differences in their receptor binding site is shown in Table 1. Four of the isolates (2015, 2017 and one isolate from Penang in 2018 (A/chicken/Malaysia/Penang/8231/2018)) possessed a glutamine (Q) at position 234 (H3 numbering: 226) of the HA receptor binding site, However, seven isolates from 2018 had amino acid exchange from glutamine to leucine (Q226L) at the receptor binding site.

DISCUSSION

Avian Influenza H9N2 viruses were first isolated in 1966 in turkeys. It later emerged in several terrestrial avian species and is one of the major subtypes endemic in chickens (Naguib M.M. *et al.,* 2019; Ali M. *et al.,* 2018). H9N2 from chicken has not been reported previously in Malaysia. In 2000, Banks J. *et al.* (2000) reported that H9N2 has been found in Malaysia from Pekin ducks in 1998 and this isolate is similar with Hong Kong strains from 1979. H9N2, also from duck, was isolated in 2001 (Nucleotide [Internet], 2011).

Based on the results of this study, H9N2 from village chicken breed in 2015 were reared commercially in north Perak. In 2017, two isolates were obtained from commercial layers in Penang.

In 2018, Malaysia had major H9N2 outbreaks which involved mainly commercial layers and breeders. The locations of the outbreaks were reported in the west coast of Malaysia (Negeri Sembilan, Melaka, Perak and Penang). It is consistent with the list of farms in Malaysia where most of commercial poultry farms are located (DVS, 2017). Based on the results, isolates in this study had wide range of similarities of 79.7-100%. These findings suggest that these isolates came from two different introduction or sources. It is consistent with the phylogenetic analysis that showed that isolates from 2015 to 2018 can be divided into two lineages, Korean and Y280. The results suggested that isolates related to Korean lineage were only circulating in Northern part of Peninsular Malaysia. Whereas isolates that were clustered under Y280 are more diverse where this strain has affected many commercial poultry farms in West Coast region.

One of the cases from Penang in 2018 that was clustered under Y280 lineage, A/chicken/Malaysia/Penang/9541/2018 was not isolated from commercial chicken but village chicken. It is noteworthy to mention that this case was located 1 km away from the previous case A/chicken/Malaysia/Penang/8232/2018 of a virus isolated from commercial layer chickens. Therefore, it indicates that the source of the former isolate was originated from the latter. Based on personal communication, this commercial farm (A/chicken/Malaysia/Penang/8232/2018) from Penang obtained the breed from one of the farms in Negeri Sembilan. It is therefore likely that H9N2 outbreaks in Penang were originated from Negeri Sembilan as it was the state when H9N2 outbreaks were first reported, in 2018.

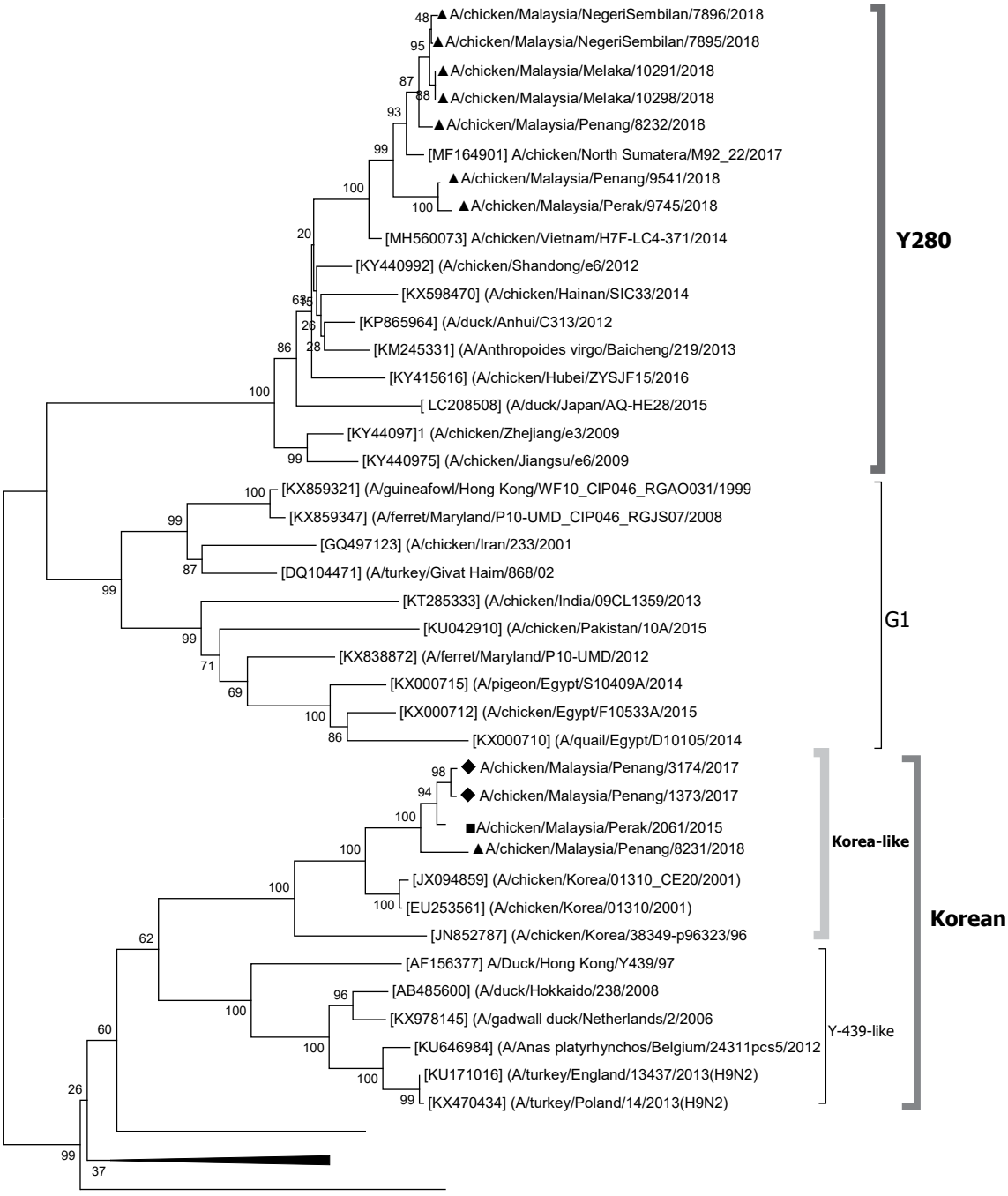
Four isolates that were clustered under the Korean lineage had high similarities with the 2001 isolate A/chicken/

Korea/01310/2001 (Choi J.G. *et al.,* 2008). Korea has been endemic with H9N2 virus since 1996. Interestingly, since 2007, Korean veterinary authority has permitted the use of inactivated oil adjuvant H9N2 LPAI vaccine derived from this strain in commercial layer and broiler breeder chickens (Lee D. *et al.,* 2016). It was also reported that this isolate falls within clade A in the phylogenetic tree that contains only Korean isolates from 2001 to 2006 (Lee D. *et al.,* 2016). After the introduction of the vaccine, the virus from this clade has not reappeared in the field. The disappearance of this genetic group may be explained due to the fact that the vaccine strain belongs to this clade.

Based on this study, we cannot conclude that the four Malaysian Korea-like H9N2 viruses were derived from Korea vaccine as the HA cleavage motif is not the same. The motif for Korea isolate is TSGR while Malaysia isolates is RSKR. Interestingly this RSKR motif has been initially found only in Israel isolates where it had caused H9N2 outbreaks in Israel poultry population between 2001 and 2003 (Tse L.V. *et al.,* 2013; Perk S. *et al.,* 2006). In addition to that, the Israel isolates were clustered in a different lineage (G1 lineage) compared to the Korean isolates. Furthermore, this motif was also found in chickens infected with H9N2 in Netherlands during the year 2010 and it is closely related to H9N8 viruses isolated from waterfowls in Italy (Verhagen J.H. *et al.*, 2017). Therefore, the source of introduction of these H9N2 strains in Malaysia remains unknown. It is possible that these isolates had undergone active reassortment of internal and surface protein genes (Lee C.H. *et al.,* 2012). Whole genome sequencing of these isolates is highly recommended for a better understanding.

The remaining isolates in this study were grouped under Y280 lineage. The lineage considered as extremely diverse and is also the predominant progenitor of H9N2 virus lineages currently circulating in China and Southeast Asia (Pusch E.A. *et al.,* 2018; Thuy D.M. *et al.*, 2016; Oo S.M. and Win M.M., 2017). Based on the BLAST and phylogenetic analysis results, these isolates were related to the Indonesian isolates in 2017. The Indonesian strain was isolated from outbreaks that occurred in Indonesia during 2016-2017 (Jonas M. *et al.,* 2018), The virus affected layers, breeders and broiler farms in Indonesia, similar to outbreaks in Malaysia in 2018 (Table 1). The Indonesian strain is believed to be related to isolates from Vietnam as it had 98% nucleotide similarities of HA encoding gene with the Vietnam strain that was isolated in 2014 (Jonas M. *et al.,* 2018). These results were also consistent with findings in this study in that the HA cleavage site motif of the Indonesia and Vietnam strain were RSSR (Jonas M. *et al.,* 2018). Therefore, it may suggest that the outbreak source in Malaysia came from neighbouring countries.

The site of HA cleavage plays a key role in the pathogenicity of AI virus as the sequence of amino acids at this HA domain is the observed primary molecular marker for pathogenicity determinant. Another key region of the HA protein is the receptor binding site (RBS) responsible for recognition and attachment to cell receptors. Amino acids in particular positions of HA RBS determine the affinity to human or avian-like sialic acid receptors. Position 226 according


▲A/chicken/Malaysia/NegeriSembilan/7896/2018
▲A/chicken/Malaysia/NegeriSembilan/7895/2018
▲A/chicken/Malaysia/Melaka/10291/2018
▲A/chicken/Malaysia/Melaka/10298/2018
▲A/chicken/Malaysia/Penang/8232/2018
[MF164901] A/chicken/North Sumatera/M92_22/2017
▲A/chicken/Malaysia/Penang/9541/2018
▲A/chicken/Malaysia/Perak/9745/2018
[MH560073] A/chicken/Vietnam/H7F-LC4-371/2014
[KY440992] (A/chicken/Shandong/e6/2012
[KX598470] (A/chicken/Hainan/SIC33/2014
[KP865964] (A/duck/Anhui/C313/2012
[KM245331] (A/Anthropoides virgo/Baicheng/219/2013
[KY415616] (A/chicken/Hubei/ZYSJF15/2016
[LC208508] (A/duck/Japan/AQ-HE28/2015
[KY44097]1 (A/chicken/Zhejiang/e3/2009
[KY440975] (A/chicken/Jiangsu/e6/2009
Y280
[KX859321] (A/guineafowl/Hong Kong/WF10_CIP046_RGAO031/1999
[KX859347] (A/ferret/Maryland/P10-UMD_CIP046_RGJS07/2008
[GQ497123] (A/chicken/Iran/233/2001
[DQ104471] (A/turkey/Givat Haim/868/02
[KT285333] (A/chicken/India/09CL1359/2013
[KU042910] (A/chicken/Pakistan/10A/2015
[KX838872] (A/ferret/Maryland/P10-UMD/2012
[KX000715] (A/pigeon/Egypt/S10409A/2014
[KX000712] (A/chicken/Egypt/F10533A/2015
[KX000710] (A/quail/Egypt/D10105/2014
G1
◆ A/chicken/Malaysia/Penang/3174/2017
◆ A/chicken/Malaysia/Penang/1373/2017
■A/chicken/Malaysia/Perak/2061/2015
▲A/chicken/Malaysia/Penang/8231/2018
Korea-like
[JX094859] (A/chicken/Korea/01310_CE20/2001)
[EU253561] (A/chicken/Korea/01310/2001)
[JN852787] (A/chicken/Korea/38349-p96323/96
Korean
[AF156377] A/Duck/Hong Kong/Y439/97
[AB485600] (A/duck/Hokkaido/238/2008
[KX978145] (A/gadwall duck/Netherlands/2/2006
[KU646984] (A/Anas platyrhynchos/Belgium/24311pcs5/2012
[KU171016] (A/turkey/England/13437/2013(H9N2)
[KX470434] (A/turkey/Poland/14/2013(H9N2)
Y-439-like

to H3 numbering (234 in H9 numbering) is the most significant as it was shown that the presence of L226 results has a better affinity for human/ mammalian-type receptors, whereas Q226 determines avian-like receptor specificity (Świętoń E. *et al.*, 2017). Based on the amino acid analysis, all Malaysian isolates clustered under Korean lineage possessed a glutamine (Q) while, those isolates that fall within Y280 lineage had leucine (L) at the same position. These results are consistent with Pusch E.A. *et al.*, (2018) who stated that Korean lineage has maintained glutamine at 226 position for over 16 years and 90% of recent viruses Y280 or the G1 lineage have leucine at similar position. Results in this study showed that isolates from Y280 lineage is a greater risk to public health compared to the Korean lineage. Since almost all the H9N2 outbreaks in 2018 were caused by Y280 lineage, it is recommended that consistent monitoring of H9N2 in poultry is necessary to better understand the prospective risk to human health. In addition, H9N2 influenza virus has been recognised to reassort with multiple other subtypes including the H5N1, H7N9, and H10N8 viruses, which can infect humans, causing a potential threat to public health (Zhu R. *et al.,* 2018).

Migratory birds potentially have contributed to the introduction and the circulation of H9N2 in Malaysia. The ability of wild birds, in particular waterfowl – to replicate a high diversity of AI and to be highly mobile, facilitates the emergence of newly introduced form of AI virus (Naguib M.M. *et al.,* 2019). This is in agreement with Hu M. *et al.* (2017), where he concluded that wild birds' migration has contributed much to the long-distance global spread of the H9N2 virus. He also reported that in the process of H9N2 virus global transmission, United States is the origin of the H9N2 virus and mainland China, Hong Kong SAR, Japan, and Korea were important transfer centres. The fact that the transmission routes of the virus coincided with the main flyways of the wild birds, further supported this assumption. Once introduced and adapted to poultry, viruses are able to keep circulating among domestic bird populations. Movement of infected domestic poultry from one area to another, lack of farm biosecurity and people's lifestyle (backyard farming) might have spread the virus in short distance transmission (Naguib M.M. *et al.,* 2019).

Unlike H5 and H7, H9N2 is not listed as notifiable disease hence they are not subject to specific international control measures (Bonfante F. *et al.*, 2018). In many countries, vaccination is the major measure used to control infection by H9N2 (Abid M. *et al.*, 2017; Lee D. *et al.*, 2016; Shen H.Q. *et al.,* 2015). However, AIV vaccine usage is not permitted in Malaysia, including H9N2. But this policy was made before Malaysia had major H9N2 outbreaks in poultry. It cannot be denied that, although H9N2 is LPAI, it has cause enormous economic burden for the poultry sector. Since H9N2 has been endemic in Malaysia, control measures such as test and slaughter or stamping out approaches may not be sustainable in the long run because of high economic cost. If Malaysia would like to implement vaccination against H9N2 in poultry, it is very crucial to select the right candidate strain to closely match with the current circulating field viruses and it is essential to routinely update the vaccine

strain. Nevertheless, vaccination cannot be used alone for the control of H9N2 and must be accompanied by other control measures including implementation of monitoring programs, good biosecurity at farms, quarantines, controlled depopulation and increased surveillance (Pusch E.A. *et al.,* 2018; Fiala S.M. *et al.,* 2018).

CONCLUSION

In conclusion, this is the first report of H9N2 LPAI in Malaysia isolated from chickens. In summary, H9N2 isolates in this study belonged to two distinct lineages – Korean and Y280, indicating that they have different sources of origin. Isolates from Korean lineage were circulating only in northern Peninsular Malaysia while isolates from Y280 lineage were more diverse. The disease outbreak in 2018 shows that H9N2 isolates in Malaysia can cause major losses to the poultry industry. Findings from this study revealed that these isolates are potentially infectious to humans. Thus, there is a need for routine, continuous and thorough surveillance, paired with risk-assessment of circulating H9N2 viruses in Malaysia to identify the evolution and adaptation of viruses to humans and to be better prepared for potential future outbreaks. This study has also demonstrated the necessity of planning for an applied policy and prevention strategies aimed at controlling and managing H9N2 infections in Malaysia. Finally, determination of the complete genome sequences of the Malaysian H9N2 AI viruses may be helpful towards understanding the epidemiology of H9N2 in the field and the region.

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