Tropical Biomedicine 41(2): 183-189 (2024)
https://doi.org/10.47665/tb.41.2.008

RESEARCH ARTICLE

Sequence analysis and molecular characterization of low pathogenic avian influenza H9N2 virus isolated from chickens in Sabah

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ARTICLE HISTORY

Received: 14 December 2023
Revised: 14 March 2024
Accepted: 15 March 2024
Published: 30 June 2024

ABSTRACT

Low pathogenic avian influenza (LPAI) subtype H9N2 is a causative agent that has raised increasing concern about its impact on poultry and potential public health threats. Even though H9N2 is endemic in Peninsular Malaysia, it was first reported in Sabah in August 2022, after an outbreak associated with high mortality in broiler chickens. In the present study, based on the hemagglutinin (HA) gene, we report the genetic variations and phylogenetic analysis of a H9N2 virus isolated from broiler chickens in Sabah. The sequence analysis of the HA gene revealed a 98% similarity to the H9N2 virus recently isolated from China in 2018. The amino acids in the HA cleavage site displayed a characteristic LPAI motif (PARSSR/GLF). Notably, at position 226, the isolate had amino acid Leucine (L) demonstrating its ability to bind to the receptor of mammals, resulting in the potential risk of transmission to humans. In addition, the H9N2 isolate harboured seven potential N-glycosylation sites. The phylogenetic analysis revealed that the isolate belonged to clade h9.4.2.5 in the Y280 lineage, similar to previously reported in Malaysia. However, we observed that the isolate in this study falls in a different cluster compared with previous Malaysian isolates, suggesting different source of H9N2 introduction into the country. This prompts us to propose continuous and thorough surveillance of poultry across the country and the necessity of implementing farm biosecurity to minimize economic losses and potential threats to public health.

Keywords: LPAI; H9N2; hemagglutinin; chickens; Sabah.

INTRODUCTION

Influenza virus type A, a member of the genus Influenzavirus and family *Orthomyxoviridae*, is the source of avian influenza (AI) (Peacock *et al*., 2018). The virus genome consists of approximately 13.2 kb of single-stranded, negative-sense RNA with eight segmented genes that encode 10 proteins including hemagglutinin (HA), matrix (M1 and M2), neuraminidase (NA), polymerase acidic (PA), non-structural (NS1 and NS2), nucleoprotein (NP) and polymerase base (PB1 and PB2) (Wise *et al*., 2012; Gao *et al*., 2021). According to Peacock *et al*. (2018), HA is a crucial surface protein that functions as the membrane fusion glycoprotein and receptor-binding of AI virus (AIV), playing a key role in inducing a neutralizing antibody in response to viral infection. Influenza A virus subtypes are identified by the combination of the surface glycoproteins HA and NA, with 18 (H1–H18) and 11 (N1–N11) subtypes, respectively (Kosik & Yewdell, 2019). AIVs can be categorized as highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) based on the amino acid composition of the HA protein at the cleavage site (Peacock *et al*., 2018).

Of the various LPAIs, H9N2, is widely distributed worldwide and seems to be responsible for endemic outbreaks with varying degrees of mortality and pathogenicity in the poultry industry (Lee *et al*., 2012; Syamsiah *et al*., 2019). H9N2 virus infections in poultry typically only cause mild respiratory symptoms, however the widespread distribution of H9N2 could result in substantial losses to the economy due to lower growth rates, a decline in egg production, high mortality caused by coinfection with other respiratory pathogens and a higher risk of immunosuppression (Gao *et al*., 2021). H9N2 has been isolated not only from domestic poultry and wild birds but also from a wide range of hosts, including horses, pigs, dogs, ferrets, minks and humans (Liu *et al*., 2016; Liu *et al*., 2023).

Despite being classified as an LPAI, H9N2 has the potential to cause a pandemic in humans due to biological features such as affinity for the human receptor-binding profile and transmission across species (Kimble *et al*., 2010; Pusch & Suarez, 2018). In general, based on the HA gene, H9N2 viruses are widely divided into two major lineages: American and Eurasian (Kye *et al*., 2021). In poultry, the Eurasian lineage is classified into three genetic lineages: Y280, Y439/Korea and G1 (Pusch & Suarez, 2018; Carnaccini & Perez, 2020). At the same time, a similar lineage/clade nomenclature system proposed by Liu *et al.* (2009) and Chen *et al*. (2009) has also been used (Shen *et al*., 2015; Nugroho *et al*., 2021). The proposed system is similar to the WHO/OIE/FAO's H5 nomenclature system which contemplates H9 lineages globally. The numbering system divides the American lineage into 2 subclades: h9.1 and h9.2. Meanwhile, the Eurasian lineage is classified into three major sub lineages: h9.3 (Y439/Korea lineage), h9.4.1 (G1 lineage), and h9.4.2 (Y280 lineage). Subclade h9.4.2 was further classified into h9.4.2.1 to h9.4.2.6 (Liu *et al*., 2022).

Previously, in Malaysia, H9N2 was reported only from ducks in 1998 and 2001 (Banks *et al*., 2000; Syamsiah *et al*., 2019). H9N2 isolated in chickens was reported for the first time in 2015; the virus was of Korean lineage but did not cause an outbreak. It was not until 2018 that outbreaks involving commercial layers and breeders occurred in high-density poultry farms in Peninsular Malaysia. These outbreaks were caused by H9N2 viruses from the Y280 lineage (Syamsiah *et al*., 2019).

In August 2022, reports of significant mortality rates of up to 30% were received in commercial broiler flocks in Papar, Sabah. Samples were collected and disease investigations were carried out. Virus isolation was conducted by the Veterinary Diagnostic Laboratory Kota Kinabalu in Sabah. Using real-time reverse transcription-polymerase chain reaction (qRT-PCR) on the harvested allantoic fluid, AI was shown to be positive. The allantoic fluid was then referred to Veterinary Research Institute (VRI) in Ipoh for AI confirmation and its subtype. Therefore, this study aims to molecularly characterize the Sabah isolate based on the HA gene.

MATERIALS AND METHODS

Virus isolation

In August 2022, one allantoic fluid sample from Veterinary Diagnostic Laboratory Kota Kinabalu, Sabah was sent to Avian Virology Section at VRI, Ipoh for AI confirmation and subtyping. Prior to viral isolation, the received allantoic fluid sample was filtered. The sample was then propagated in the allantoic cavities of 9 to 11-day-old Specific Pathogen-Free (SPF) embryonated chicken eggs and incubated for three days at 37°C. The allantoic fluid was harvested and hemagglutination test was carried out to determine the hemagglutination activity according to OIE (2021). Subsequently, the isolate showing positive hemagglutination activity was tested for hemagglutination inhibition (HI) using specific antisera against AI subtypes H5, H7 and H9 (OIE, 2021).

Extraction of viral ribonucleic acid (RNA) and real-time reverse transcription- polymerase chain reaction (qRT-PCR)

Viral RNA extraction was also performed from the harvested allantoic fluid using IndiSpin Pathogen Kit (Indical Bioscience, Germany) in accordance with the manufacturer's instructions. The sample was screened for AIV using a one-step Taqman qRT-PCR assay (ACDP, 2022). To identify the subtype of the AIV, specific primers and probes for H and N subtyping were employed using qRT -PCR (ACDP, 2022). The QuantStudio 3 real-time PCR system (Applied Biosystems, USA) was used to conduct the real-time assays.

Amplification and sequencing of HA gene

SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA) was used to perform RT-PCR. Primers covering the whole length of the HA gene, 5'-CTCAGGGAGCAAAAGCAGGGG-3' (forward) and 5'-GTATTAGTAGAAACAAGGGTG TTTT-3' (reverse), were used (Hoffmann *et al*., 2001). RT was performed at 48°C for 30 min. After that, the reaction mix was denatured at 94°C for 5 min, then 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 at 68°C for 10 min.

After RT-PCR completed, the amplified product was subjected to agarose gel electrophoresis at 100V for 35 min and visualized by UV transilluminator. The amplicons were excised from the gel and sent for Sanger sequencing (Apical Scientifics (M) Sdn Bhd).

Sequence and phylogenetic analysis

The sequences were edited and compiled using SeqMan Pro software (DNAStar Lasergene, USA). BioEdit Sequence Alignment Editor version 7.1.9 was used to align and compare the sequences in this study with other published sequences (Hall, 1999). Amino acid sequences were obtained by converting the nucleotide sequences to amino acids, which were then compared with the sequences of the HA gene of six published H9N2 isolates from different states in Peninsular Malaysia, along with reference viruses, to identify potential mutations in the gene as described by Lai *et al*. (2021). The sequences were compared to other sequences in the GenBank NCBI database using the Basic Local Alignment Search Tool (BLAST) (NCBI, 2016). The potential N-glycosylation sites in the HA genes were identified using the online software NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and compared with other H9N2 isolates as described by Lai *et al*. (2021). The phylogenetic tree was constructed using MEGA 6.06 software, applying the maximum likelihood method and the Kimura 2-parameter model with 1000 bootstrap replicates (Tamura *et al*., 2013). Nucleotide sequence in this study was submitted to the GenBank database under accession number OR946212.

RESULTS

Virus isolation and qRT-PCR

The Sabah sample was successfully isolated in SPF embryonated eggs. Concurrently, the isolate tested positive for AIV and was further identified as H9N2 using TaqMan qRT-PCR assay.

Sequence and phylogenetic analysis

The 1631 bp nucleotide sequence of the HA gene from the isolate was compared to six other published Malaysian H9N2 sequences and reference strains. The virus isolate was designated as A/chicken/Malaysia/5394/2022. Sequence homology between this isolate and the Malaysian isolate A/chicken/Perak/2061-2015, as well as other Malaysian H9N2 isolates from 2018 and 2019 (from various states of Malaysia) was 81.1% and between 90.6% to 90.9% respectively (Table 1). BLAST results indicated that the isolate shared 98%

Table 1. Sequence homology comparison of nucleotide sequence (%) of the HA gene of the isolate in this study with other Malaysian H9N2 isolates and H9N2 reference strains

	H9N2 isolates	1	2	3	4	5	6	7	8	9	10
1	A/chicken/Malaysia/5394/2022										
2	A/chicken/UPM/994/2018 (H9N2)(Negeri Sembilan)	90.8									
3	A/chicken/UPM/2033/2019 (H9N2)(Johor)	90.9	99.8								
4	A/chicken/Melaka/10291-2018	90.7	99.8	99.6							
5	A/chicken/Perak/9745-2018	90.6	97.1	97.1	97.1						
6	A/chicken/Penang/9541-2018	90.7	97.3	97.3	97.2	99.2					
7	A/chicken/Perak/2061-2015	81.1	80.9	80.9	80.8	81.0	80.8				
8	A/Duck/Hong_Kong/Y280/97	85.1	86.3	86.3	86.3	86.1	86.2	79.5			
9	A/Duck/Hong_Kong/Y439/97	75.5	75.7	75.7	75.7	75.6	75.5	82.8	82.3		
10	A/Pigeon/Guangdong/G2174/2018	98.0	91.6	91.7	91.6	91.5	91.6	81.4	86.0	75.5	

nucleotide homology with strain A/Pigeon/Guangdong/G2174/2018 based on the HA gene.

Phylogenetic analysis revealed that the H9N2 virus isolated in this study belonged to clade h9.4.2.5 of the Y280 lineage, similar to previous H9N2 isolates in Malaysia. However, A/chicken/Malaysia/5394/2022 was grouped in a different cluster where it formed a distinct cluster with isolates from China (Figure 1).

Molecular characterization

The PSRSSRGLF motif was found at the HA cleavage site of A/chicken/Malaysia/5394/2022, which is characteristic of typical low pathogenic H9N2 viruses (Table 2). The isolate in this study conserved residues at P109, W161, T163, Y203, and G234 in the receptor-binding pocket of the HA gene, comparable to other Malaysian Y280 H9N2 isolates. However, the Sabah isolate has a unique point mutation at position T198. There were two substitution mutations at positions 149 and 150 at the right edge binding pocket: K149S and A150T. The amino acid sequence, 232NGLMGR237 in the left edge of the receptor binding pocket was conserved among Malaysian H9N2 isolates including the Sabah isolate.

In addition, similar to other Malaysian H9N2 isolates, A/chicken/Malaysia/5394/2022 possessed leucine (L) instead of glutamine (Q) at position 234 at the receptor binding site indicating its ability to preferentially bind to α-2,6 linked sialic acid, a mammalian species receptor. Analysis in this study revealed that the H9N2 Sabah isolate has seven potential glycosylation sites at position 29 (NSTE), 82 (NPSC), 141 (NVSY), 298 (NTTL), 305 (NVSK), 313 (NCSK) and 492 (NGTY) (Table 3).

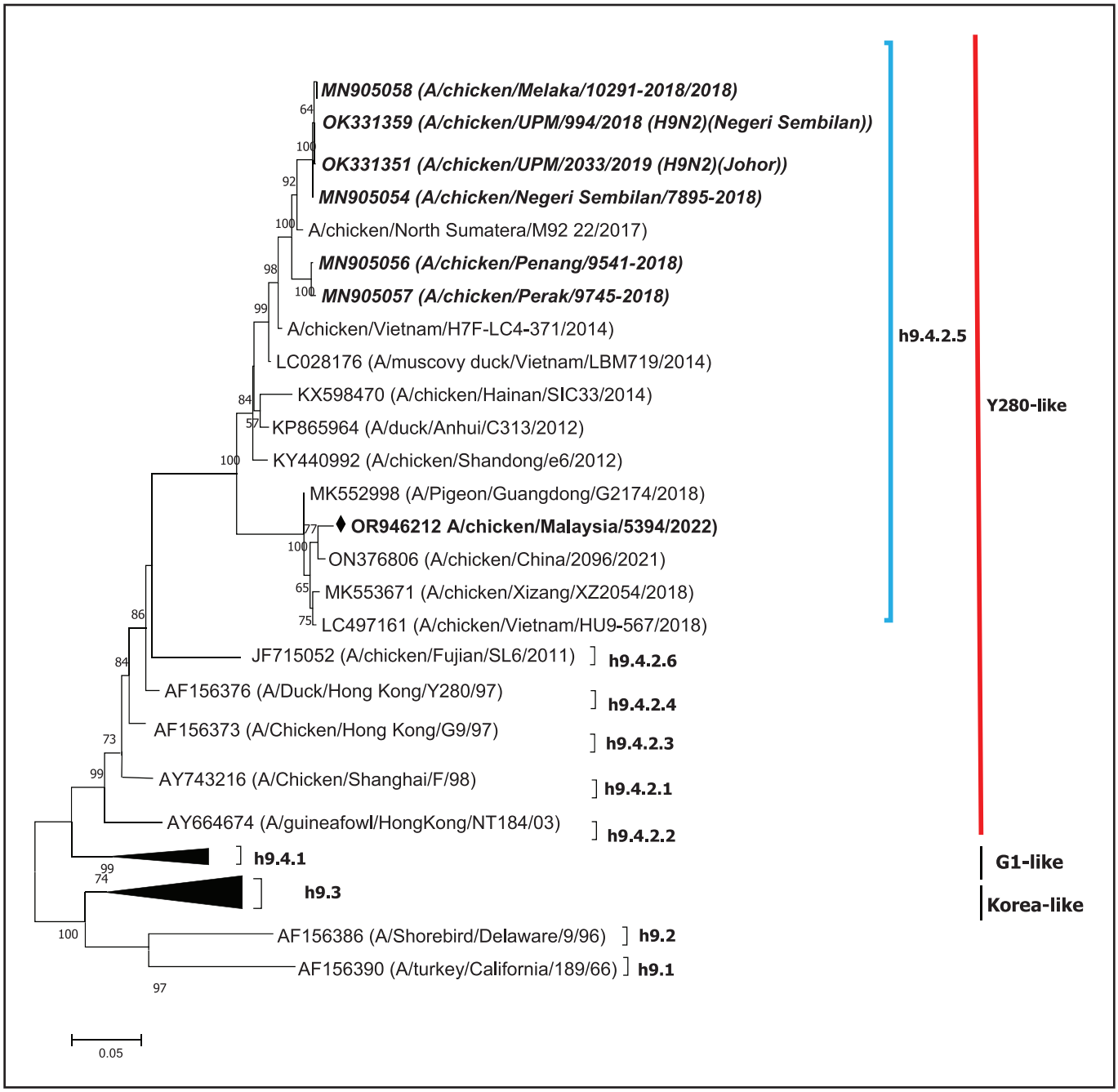

Figure 1. Phylogenetic tree of the Sabah LPAI H9N2, Y280 lineage, clade h9.4.2.5 virus (A/chicken/Malaysia/5394/2022) with 43 other H9N2 reference strains based on 1631bp of HA gene. The evolutionary history was inferred using the Maximum Likelihood statistical method based on the Kimura 2-parameter model and setting bootstrap 1,000 replicates using MEGA version 6.06. The bold rotated square represents the Sabah H9N2 virus, while the bold italic represents previous Malaysian H9N2 isolates.

Table 2. Cleavage site and receptor-binding pocket on the HA encoding gene of H9N2 field isolates from Malaysia and other reference strains

Virus	Cleavage site	Right edge of binding pocket	Receptor binding site							Left edge of binding pocket
	333–341	146-150	109	161	163	191	198	202	203	232–237
A/chicken/Malaysia/5394/2022	P**S**RSSR↓GLF	GTSST	P	W	T	N	T	L	Y	NGLMGR
A/chicken/UPM/994/2018(H9N2)(Negeri Sembilan)	P**S**RSSR↓GLF	GTSKA	P	W	T	N	V	L	Y	NGLMGR
A/chicken/UPM/2033/2019 (H9N2)(Johor)	P**S**RSSR↓GLF	GTSKA	P	W	T	N	V	L	Y	NGLMGR
A/chicken/Melaka/10291–2018	P**S**RSSR↓GLF	GTSKA	P	W	T	N	V	L	Y	NGLMGR
A/chicken/Perak/9745–2018	P**S**RSSR↓GLF	GTSKA	P	W	T	N	V	L	Y	NGLMGR
A/chicken/Penang/9541–2018	P**S**RSSR↓GLF	GTSKA	P	W	T	N	A	L	Y	NGLMGR
A/chicken/Perak/2061–2015	PARSKR↓GLF	GTSRA	P	W	T	H	E	L	Y	NGQQGR
A/Duck/Hong_Kong/Y280/97	PARSSR↓GLF	GTSKA	P	W	T	N	T	L	Y	NGLQGR
A/Duck/Hong_Kong/Y439/97	PAASNR↓GLF	GTSRA	P	W	T	H	E	L	Y	NDQQGR
A/Pigeon/Guangdong/G2174/2018	P**S**RSSR↓GLF	GTSNT	P	W	T	N	T	L	Y	NGLMGR

Note: Underline indicates A334S substitution which is associated with increased of H9N2 virulence in chickens.

Table 3. N-linked glycosylation analysis of the HA protein

Virus	Potential N-glycosylation sites in the HA region								
Amino acid positions (H9 numbering)	29-32	82-85	141-144	145-148	218-220	298-300	305-308	313-316	492-495
A/chicken/Malaysia/5394/2022	NSTE	NPSC	NVSY	–	–	NTTL	NVSK	NCSK	NGTY
A/chicken/UPM/994/2018 (H9N2)(Negeri Sembilan)	NSTE	NPSC	NVSY	–	–	NTTL	NVSR	NCSK	NGTY
A/chicken/UPM/2033/2019 (H9N2)(Johor)	NSTE	NPSC	NVSY	–	–	NTTL	NVSR	NCSK	NGTY
A/chicken/Melaka/10291–2018	NSTE	NPSC	NVSY	–	–	NTTL	NVSR	NCSK	NGTY
A/chicken/Perak/9745–2018	NSTE	NPSC	NVSY	–	NRTF	NTTL	NVSK	NCSK	NGTY
A/chicken/Penang/9541–2018	NSTE	NPSC	NVSY	–	NRTF	NTTL	NVSK	NCSK	NGTY
A/chicken/Perak/2061–2015	NSTE	NPSC	NVTF	NGTS	NRTF	NCTI	NVSK	–	NGTY
A/Duck/Hong_Kong/Y280/97	NSTE	NPSC	NVSY	–	NRTF	NTTL	NVSK	–	NGTY
A/Duck/Hong_Kong/Y439/97	NSTE	NPSC	NVTY	–	NRTF	NTTL	NVSK	–	NGTY
A/Pigeon/Guangdong/G2174/2018	NSTE	NPSC	NVSY	–	–	NTTL	NVSK	NCSK	NGTY

DISCUSSION

H9N2 continues to inflict substantial economic losses on the domestic poultry industry globally and has become the most common enzootic subtype in chicken farms not only in Asia but also in North African and Middle Eastern countries (Lee *et al*., 2012; Peacock *et al*., 2018). This is consistent with the results by Syamsiah *et al*. (2019) where in 2018, H9N2 caused major outbreaks in commercial chicken farms including layer chickens in different states on the West Coast of Peninsular Malaysia. Due to these outbreaks, Malaysia experienced a shortage of egg supply (Abdul, 2018).

In this present study, we identified the isolate from Sabah as LPAI subtype H9N2 virus. To the best of our knowledge, this is the first time H9N2 has been identified in East Malaysia. Previously in 2018, outbreaks due to HPAI subtype H5N1 were reported in Tamparuli, Sabah (Leow *et al*., 2022). The Y280 lineage was first documented in the 1990s in China. This lineage is considered extremely diverse as it has extensively spread throughout European countries and has become endemic in multiple regions including China, Korea, and Southeast Asian countries (Pusch & Suarez, 2018; Lai *et al*., 2021). Under the nomenclature system proposed by Liu *et al*. (2009) and Chen *et al*. (2009), the Y280 lineage is referred to as clade h9.4.2. The h9.4.2 lineage can be divided into six clades referred to as h9.4.2.1–h9.4.2.6. Clades h9.4.2.1 to h9.4.2.4 viruses generally belonged to H9N2 viruses isolated before 2007. While throughout 2007–2013, clades h9.4.2.4, h9.4.2.5, and h9.4.2.6 have spread in China, but among the clades, h9.4.2.5 viruses became well established in China and began spreading rapidly to other countries (Nugroho *et al*., 2021; Liu *et al*., 2022).

Based on the phylogenetic tree, the Sabah isolate falls under lineage Y280, clade h9.4.2.5 which is similar to those previously reported in Malaysia (Syamsiah *et al*., 2019). However, the isolate was not grouped in the same cluster as other previous Y280 Malaysian isolates. In 2019, it was reported that Malaysian isolates were clustered together with Vietnamese and Indonesian H9N2 viruses which have caused major outbreaks in their respective countries (Jonas *et al*., 2018; Nugroho *et al*., 2021). This sub-lineage was also referred to as CVI (Chinese-Vietnam-Indonesia) as it indicated a close relationship with H9N2 viruses in China (Jonas *et al*., 2018; Wibawa *et al*., 2020). The Sabah isolate was clustered together with isolates from China. Therefore, it is suggested that the H9N2 virus in Sabah was not introduced from Peninsular Malaysia. This hypothesis is further supported by the sequence homology where A/chicken/Malaysia/5394/2022 has lower sequence identities with Y280 Malaysian isolates compared with an isolate from China the A/Pigeon/Guangdong/G2174/2018.

Similar to previous Y280 Malaysian isolates, the isolate in this study possesses a monobasic HA cleavage site (PSRSSR/GLF), which is the typical motif of low pathogenicity H9N2 viruses. According to Parvin *et al*. (2020), HA with a monobasic cleavage site is digested by trypsin-like serine proteases limiting infection to the respiratory or intestinal tract of birds where such proteases are present. The isolate from this study and previous Y280 Malaysian H9N2 viruses remarkably harboured the A316S (H3 numbering) or A334S (H9 numbering) substitution at the cleavage site, which has been reported to increase the virulence of H9N2 in chickens. This may indicate why high mortalities were observed for this isolate and previous Malaysian H9N2 viruses (Syamsiah *et al*., 2019).

Influenza viruses have a broad host range and are able to jump to new host species. However, the virus seldom replicates and transmits effectively enough to continue causing an infection in the new host (Pusch & Suarez, 2018). This is because the virus attaches to sialic acid as a host receptor via its surface HA protein. Many forms of sialic acid exist, and the type of sialic acid influences the relative binding affinity of the viral HA proteins, offering one mechanism that modifies host specificity. The majority of influenza viruses have a greater affinity for binding to the α2,3-linked sialic acid receptors present in birds, whereas human-adapted influenza viruses have a higher affinity for binding to the α2,6-linked sialic acid receptors found in mammals or humans (Pusch & Suarez, 2018). According to Butt *et al*. (2010), for H9N2 viruses, the glutamine (Q) at position 234 tends to bind to avian receptors, while leucine (L) at the same position has the potential to attach to human receptors. Based on our findings, the isolate in this study showed the presence of L at position 234. Additionally, this isolate also possesses other signature mutations at W161, T163, N191, T/V198, and M235 (H9 numbering) of the HA gene, indicating a higher binding affinity to mammalian host cells, highlighting the zoonotic potential (Sun & Liu, 2015). This raises a possible public health risk, as it could lead to the formation of novel influenza strains with the ability to transmit between humans. Multiple human-infecting avian influenza viruses, including H7N9, H10N8, and the recently emerged H3N8, have been linked to H9N2 viruses (Bi *et al*., 2022).

Isolate A/chicken/Malaysia/5394/2022 shared the same seven potential N-glycosylation sites motif with isolate A/Pigeon/Guangdong/G2174/2018. Compared to other Y280 Malaysian H9N2 isolates, all had seven potential N-glycosylation sites except for two isolates from Penang and Perak which had eight. For the Sabah isolate, there was a single amino acid alteration at a glycosylation site (305 NVSK &NVSR, H9 numbering). According to Ansari *et al*. (2006), N-glycosylation plays a critical role in modulating host immunological responses linked to cells, receptor binding ability, and viral infectivity. The HA protein's potential glycosites may produce glycans that are identical to or comparable to those of the host to hide the antigenic site and prevent the host's immune response (Chen *et al*., 2012; Suttie *et al*., 2019). The number of possible N-glycosylation sites on HA varies generally throughout H9N2 AIVs; nevertheless, no single particular glycosylation site has been identified to correlate with adaptation to domestic poultry (Shen *et al*., 2015).

The introduction of H9N2 to Sabah might be caused by several possible factors. Firstly, there is the spillover of the H9N2 virus from wild migratory birds into poultry. Many birds from Northern Asia migrate to Malaysia every year since the country is situated within the East Asian-Australasian flyway of wild migratory birds. According to Lahasing (2017), Sabah is one of the migratory bird migration routes that cross through the country during their seasonal migrations. Meanwhile, Wang *et al*. (2016) confirmed that in the Muscovy duck's respiratory system, the H9N2 AIV Y280 lineage could reproduce with comparatively high titers. Therefore, if the chicken-adapted H9N2 AIV replicates more effectively in ducks, it may pose a concern not only for domestic ducks but also for wild migratory ducks (Sagong *et al*., 2022). Hence, migratory birds may have vital roles in dispersing H9N2 viruses to vulnerable hosts.

The possibility of H9N2 being introduced to Sabah through trade and/or unauthorized movement of wild captive birds and poultry products cannot be ruled out (Leow *et al*., 2022). Though tenable, we lacked sufficient proof to support any of these speculations. Thus, the exact cause of the outbreaks is yet unknown.

To mitigate the impact of H9N2 in poultry, biosecurity measures, vaccination programs, and rigorous surveillance are essential. These measures help prevent the spread of the virus and minimize its effects on both chicken health and the poultry industry. Additionally, subsequent studies on the whole genome sequencing and biological characteristics of the isolate are required to further evaluate the risk of infection and to gain better awareness of the evolving nature of H9N2 and its potential risks to human and animal health.

CONCLUSION

In conclusion, this study has identified the isolate as LPAI subtype H9N2 which belongs to the Y280 lineage, clade h9.4.2.5. This is the first time H9N2 is reported in East Malaysia. Based on the HA cleavage site, phylogenetic analysis and potential N-glycosylation sites, it is suggested that this isolate originated from a different

source of introduction, not from Peninsular Malaysia. Therefore, it is recommended that the transboundary monitoring and continued surveillance be strengthened to ensure adequate disease control and prevention.

ACKNOWLEDGMENT

We would like to thank the Director General of Veterinary Services Malaysia and the Director of the Veterinary Research Division for their kind permission to publish the paper. We also extend our gratitude to the Sabah DVS and Veterinary Diagnostic Laboratory Kota Kinabalu, Sabah for providing the samples. Financial support for this research was provided by the Veterinary Research Institute, Ipoh.

Conflict of Interest

The author declares that they have no conflict of interest.

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