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RESEARCH ARTICLE

Molecular characterisation of hemagglutinin and neuraminidase genes of the first highly pathogenic Avian Influenza H5N1 2.3.2.1c virus isolated from Sabah, Malaysia

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ABSTRACT

Highly Pathogenic Avian Influenza (HPAI) is a highly contagious disease in poultry. The outbreaks can lead to flock mortality up to 100% in two to three days. In July 2018, high mortality in a commercial layer farm in Kauluan village, Sabah was reported. Samples were sent to Veterinary Research Institute Ipoh for diagnosis. Virus isolation and molecular detection is carried out simultaneously. The causative agent was then identified as AI H5N1 virus by real time reverse transcription-polymerase chain reaction (RT-PCR). The virus was then subjected for further nucleotide sequencing of full length hemagglutinin (HA) and neuraminidase (NA) gene. The PQRERRRKR/GLF motif at the HA cleavage site indicated that the isolate was of HPAI virus. Phylogenetic analysis of the HA gene showed that the isolate was belonged to the clade 2.3.2.1c virus. In the HA gene, besides the S133A substitution, the virus possesses conserved amino acid at most of the avian receptor binding sites including the glutamine (Q) and glycine (G) at position 222 and 224 respectively, indicating that the virus retains the avian-type receptor binding preference. As such, the zoonotic potential of the virus was relatively low. On the other hand, though the N154D and T156A substitution were detected in the same gene, the pandemic potential of this Sabah 2.3.2.1c virus is low in the absence of the Q222L, G224S, H103Y, N220K and T315I. A typical 20 amino acid deletion with loss of four corresponding glycosylation sites in the NA stalk region was visible. Though three NA resistance markers were detected, the virus was predicted to be sensitive to NA inhibitor. This is the first HPAI H5N1 outbreak in Sabah. The introduction of this virus into East Malaysia for the first time raised an alert alarm of the future epidemic potential. Strict farm biosecurity, continuous surveillance programme in poultry, wild birds, migratory birds; molecular epidemiology as well as risk assessment for the virus with pandemic potential are needed in dealing with emergence of new influenza virus in the country.

Keywords: Sabah; H5N1; clade 2.3.2.1c; hemagglutinin; neuraminidase.

INTRODUCTION

Avian influenza (AI) is caused by the type A influenza virus which belongs to genus *Influenzavirus A* in the *Orthomyxoviridae* family. There are eight segments of negative sense and single stranded RNA in the viral genome which code for ten proteins. These proteins are hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix 1 (M1), matrix 2 (M2), non-structural 1 (NS1), non-structural 2 (NS2), PB1, PB2 and PA (Swayne & Halvorson, 2008). AI viruses can be classified into subtypes based on the antigenic difference in the surface glycoproteins, the HA and NA (Swayne & Halvorson, 2008; OIE, 2021). To date, there are 16 HA subtypes (H1-H16) and nine NA subtypes (N1-N9) were identified. New subtypes H17, H18 (OIE, 2021) and N10, N11 (Suttie *et al*., 2019) have only been detected in bats. Most of the AI circulating in the domestic poultry as low pathogenic AI (LPAI) causes no or mild disease (Suttie *et al*., 2019). Naturally occurring highly pathogenic AI (HPAI) that causes disease in chicken, turkey and other birds of economic importance is of the H5 or H7 subtypes (OIE, 2021). Low pathogenicity H5 and H7 occur widely in poultry and aquatic wild birds (OIE, 2021). Sporadic zoonotic infections have been caused by some AI viruses particularly of H5, H7 and H9 subtypes and the potential pandemic risk of these three subtypes have been highlighted (OIE, 2021).

The first HPAI H5N1 virus was detected in China in geese in 1996. The viral progeny of A/goose/Guangdong/1/1996 (A/Gs/Gd/1/96) has undergone various genetic reassortments and spread to more than 60 countries, infecting wild birds or domestic poultry with sporadic zoonotic human infections (FAO, 2013; El-Shesheny *et al*., 2014; Suttie *et al*., 2019). The disease causes huge economic impact to the country not only losses due to high mortality in the poultry, but also the cost used in control and containment of the virus including constraints of poultry movement, interruption of trade and hazard to

food security in low-income countries (FAO, 2013). As the HA gene of HPAI H5N1 has continued to rapidly evolve, a clade classification system was set up and updated based on the basis of phylogenetic analysis by WHO/OIE/FAO H5N1 evolution working group (Smith *et al.*, 2015). Eleven H5 clades were identified and the clades is further divided into subclades of the second-, third-, fourth- and fifth-order groups (Smith *et al.*, 2015).

The HA protein binds the virus particle to host sialic acid (SA) receptors and fuses the viral envelope with host cell membrane (Steinhauer, 1999; Chen *et al*., 2012). The HA is synthesised as a precursor (HA_0) which can be cleaved by protease into subunits HA1 and HA2. The cleavage will activates the membrane fusion potential of the HA which is crucial for virus infectivity (Steinhauer, 1999). The HA cleavage site and the distribution of infectivity-activating protease in the host are important in tissue tropism and pathogenicity (Steinhauer, 1999). The HA of AI preferentially binds to SA receptor linked to galactose (Gal) through an α-2,3 linkage (SA α-2,3 Gal) whereas the HA of human influenza was preferentially binds to α-2,6 linkage (Gutiérrez *et al*., 2009; Suttie *et al*., 2019). Mutation of certain amino acid in the HA gene such as Q222L and G224S (H5 numbering) is associated with the change of receptor binding preference from avian-type α-2,3 to human-type α-2,6 receptors. These mutations increase the risk of AI transmissibility in human, alarming the pandemic potential of H5N1 virus (Gutiérrez *et al*., 2009; Suttie *et al*., 2019).

The NA protein cleaves the HA from host SA at the final stage of virus infection, promotes the release of the progeny virions from host cell surface and enables the subsequent spreading of the virus to other cells (Gutiérrez *et al*., 2009; Chen *et al*., 2012). Besides, NA also facilitates the entry of the virus into host cell via the complementary specificity of HA receptor binding (Chen *et al*., 2012).

N-Glycosylation is important in the folding, maturation, transport or degradation and biological functioning of protein (Chen *et al*., 2012; Suttie *et al*., 2019). The potential glycosites of the HA and NA protein can provide identical glycans (similar to the host's glycans) to shield the antigenic site to escape detection by the host immune system (Chen *et al*., 2012; Suttie *et al*., 2019). Besides that, glycosylation also influences the HA receptor binding preference, protection of stalk domain and cleavage site, and the temperature sensitivity of HA protein (Chen *et al*., 2012; Suttie *et al*., 2019).

Malaysia has gone through four waves of HPAI H5N1 outbreak. The first outbreak of H5N1 was reported in Kelantan in 2004. Second was in 2006 in Wilayah Persekutuan, Perak and Penang state. In 2007, the third outbreak occurred in Selangor and the fourth wave was reported again in Kelantan in 2017 (Nur Adibah *et al*., 2017; Wan Norulhuda & Tariq, 2018). The end of July 2018, a report of high mortality in a commercial layer farm in Kauluan village, Tamparuli, Tuaran district, Sabah was received by Sabah Department of Veterinary Services. Disease investigation and sampling was carried out by the authority immediately. Virus isolation was carried out by the Kota Kinabalu veterinary diagnostic laboratory. Allantoic fluid was harvested and tested positive for AI by rapid test kit. The allantoic fluids were then sent to Veterinary Research Institute (VRI) Ipoh for AI confirmation and subtyping. As such, this study aims to molecular characterise the HA and NA gene of the Sabah isolate.

MATERIALS AND METHODS

Virus isolation and HA subtype identification by hemagglutination-inhibition (HI) assay

In VRI, from early August to early September 2018, ten cases which comprised of total 18 allantoic fluid samples were received from Kota Kinabalu veterinary diagnostic laboratory for AI confirmation and subtyping. The received allantoic fluid samples were filtered prior to virus isolation. The samples were propagated in 9 to 11 days old Specific Pathogen Free (SPF) embryonated chicken eggs via the intra-allantoic route and incubated at 37°C for three days. The infected allantoic fluid was harvested and subjected to hemagglutination test for hemagglutination activity detection (OIE, 2021). Virus identification through HI test is then carried out on the HA positive virus (OIE, 2021). The H subtype of the virus is determined by reacting the virus with antiserum specific to AI subtype H5. The antiserum specific to Newcastle disease (ND) virus is also used to rule out the presence of ND virus.

Ribonucleic acid (RNA) extraction and real-time reverse transcription - polymerase chain reaction (RT-PCR)

The 18 allantoic fluid samples received from Sabah were also subjected for molecular detection for AI. The samples were first screened for AI by one-step Taqman real-time RT-PCR assay using primers and probe specific for influenza type A (matrix gene) (AAHL, 2015). The antigenic subtyping by real-time RT-PCR using the H and N subtype specific primers and probe is then carried out to identify the subtype of the AI virus (AAHL, 2015). The real-time assay was performed in the QuantStudio 3 real-time PCR system (Applied Biosystems, USA). One isolate is chosen from the positive cases for further nucleotide sequencing of full length HA and NA gene of the AI virus.

Gene sequencing

The viral RNA was extracted from the infected allantoic fluid by IndiSpin Pathogen Kit (Indical Bioscience). RT-PCR was carried out using the reagent SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA). The primer sets (AAHL, 2004) used in the amplification of full length HA gene are shown in Table 1. The amplification was performed in T100 Thermal Cycler (Bio-Rad, USA). In the amplification, the RT was carried out at 48°C for 30 min. The reaction mixture was then subjected to 94°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 1 min with a final extension for 10 min at 68°C.

Table 1. Primer sets used in the amplification of full length HA gene

Name	Sequences 5' – 3'	Size of amplicon (bp)
HA-10F	TGT AAA ACG ACG GCC AGT AGC AAA AGC AGG GGT	350
HA-10R	GAA ACA GCT ATG ACC CTT CTC CAC TAT GTA AGA CCA TTC CG	
HA-20F	TGT AAA ACG ACG GCC AGT GTT ACA CAT GCC CAA GAC ATA CTG	750
HA-20R	CAG GAA ACA GCT ATG ACC TGA GTC CCC TTT CTT GAC AAT TTT GT	
HA-30F	TGT AAA ACG ACG GCC AGT TTT CCG TTG GGA CAT CAA CAC TAA	750
HA-30R	CAG GAA ACA GCT ATG ACC CCA TGA GAA CCA GAA GTT CAG CA	
HA-40F	TGT AAA ACG ACG GCC AGT GGA TGG CAG GGA ATG GTA GAT G	750
HA-40R	CAG GAA ACA GCT ATG ACC AGT AGA AAC AAG GGT GTT	

The full length NA gene was generated by primer set of Ba-NA-1/Ba-NA-1413R as described by Hoffmann *et al*. (2001) which generates an amplicon of 1 442bp. The amplification thermal profile for NA gene is same as the full length HA gene except the annealing temperature is set at 58°C and the extension at 68°C is carried out for 3 min.

The amplicons were loaded on 1.5% or 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen). Gel electrophoresis is then performed to separate and analyse the amplicon. The results are viewed under UV transilluminator. The amplified PCR products were cut from the gel and sent for Sanger sequencing (Apical Scientifics (M) Sdn Bhd). Primers used in the Sanger sequencing are same as those used in the RT-PCR amplification.

Phylogenetic analysis and molecular characterisation

SeqMan Pro software (DNA Star Lasergene, USA) was used in the assembly of the nucleotide sequences. The alignment and comparison of the HA and NA gene of the Sabah isolate and other published sequences was performed by the BioEdit Sequence Alignment Editor version 7.1.9 (Hall, 1999). Phylogenetic tree was constructed by Maximum Likelihood statistical method based on the Kimura 2-parameter model and setting bootstrap 1,000 replicates using MEGA version 6.06 (Tamura *et al*., 2013). The genetic relationship of the HA and NA gene between the Sabah isolate and other respective strains in the world were examined through the phylogram. In the genetic analysis of the HA and NA gene, the NetNGlyc 1.0 Server programme (Chen *et al*., 2012) was used to predict the potential glycosites. To assess the presence of amino acid substitutions in the HA gene that cause the phenotypic difference of the virus, an online HA subtype numbering conversion tool is used to renumber the HA sequence (Burke & Smith, 2014) before the analysis of amino acid residues within subtypes is made. An online antiviral resistance risk assessment was performed through the analysis tool in the Influenza Research Database to determine the susceptibility of the virus against antiviral drugs. The nucleotide sequences of the HA and NA gene for the Sabah isolate were deposited to GenBank and their accession numbers are OM070364 and OM070365, respectively.

RESULTS

Virus isolation and HI assay

Samples received from Sabah were successfully isolated in the SPF embryonated chicken eggs. The harvested allantoic fluid samples were tested positive for HA test with the HA titre range from 16 to 128. In the HI assay, the viruses were neutralised by antiserum specific to AI subtype H5 with the HI titre range from 8 to 64 indicating the viruses were of AI subtype H5. The samples were ruled out as ND virus as all the samples did not react with antiserum specific to ND virus.

Real-time RT-PCR

In the Taqman real-time RT-PCR assay, all the 18 samples received from Sabah were given ct value range from 11.00 to 18.67 for influenza type A assay. These samples were then identified as subtype H5N1 virus by subtype H5 and subtype N1 assay with ct value range from 11.86 to 18.54 and 13.01 to 17.91 respectively.

Gene sequencing

As the first confirmed case of H5N1 virus from the samples received, the isolate that designated as A/chicken/Malaysia (Sabah)/6123/2018 was chosen from the positive cases for further molecular characterisation on the full length HA and NA gene. The sample was successfully amplified by both HA and NA gene specific primers (Figure 1).

Phylogenetic analysis

Nucleotide sequences of 1701bp and 1350bp for HA and NA gene respectively were used in the sequence comparison with other published sequences retrieved from GenBank database. Based on the A (H5) HA clade nomenclature (Smith *et al.,* 2015), the phylogenetic analysis revealed that the Sabah isolate belongs to clade 2.3.2.1c and grouped together with other viruses from South East Asia countries including Vietnam, Indonesia, Laos; China, Hong Kong, Mongolia, Nepal, Japan, Korea and other countries (Figure 2). On the other hand, the phylogenetic analysis of the NA gene

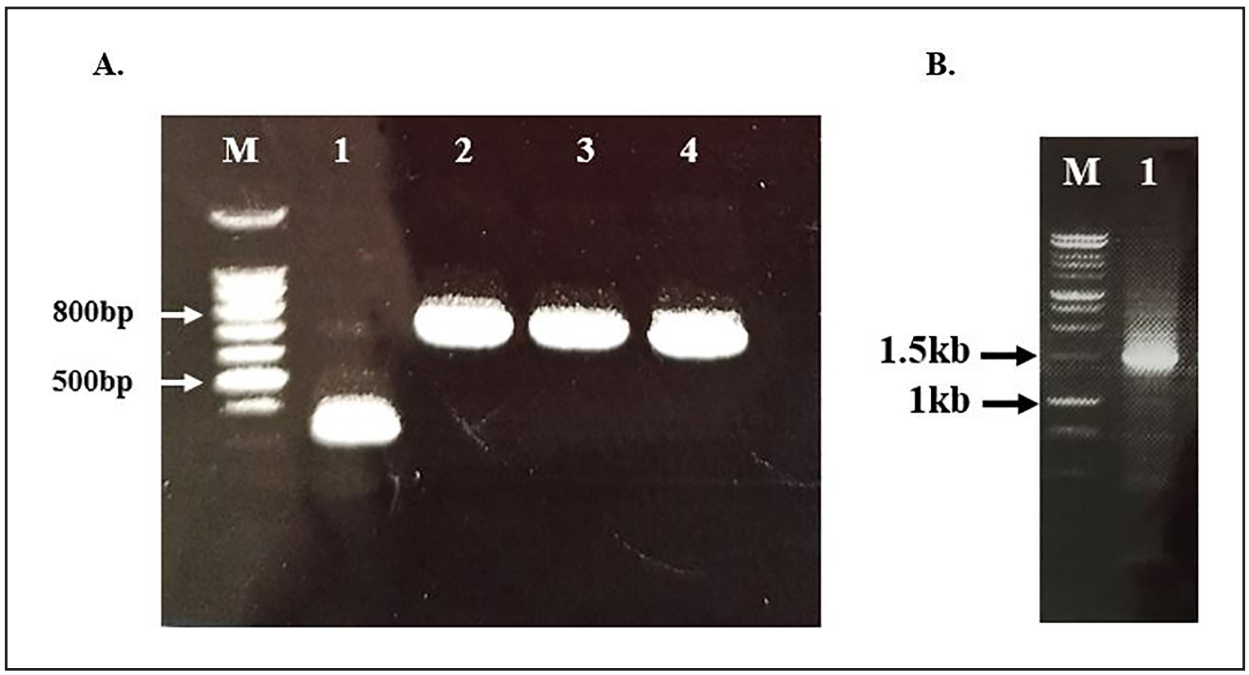

Figure 1. RT-PCR amplification of full length HA and NA gene of A/chicken/Malaysia (Sabah)/6123/2018. (A) Amplification products of full length HA gene. Lane 1 to 4: Amplicons of primer sets of HA-10F/HA-10R, HA-20F/HA-20R, HA-30F/HA-30R and HA-40F/HA-40R respectively. M: 100bp DNA ladder marker. (B) Amplification products of full length NA gene. M: 1kb DNA ladder marker.

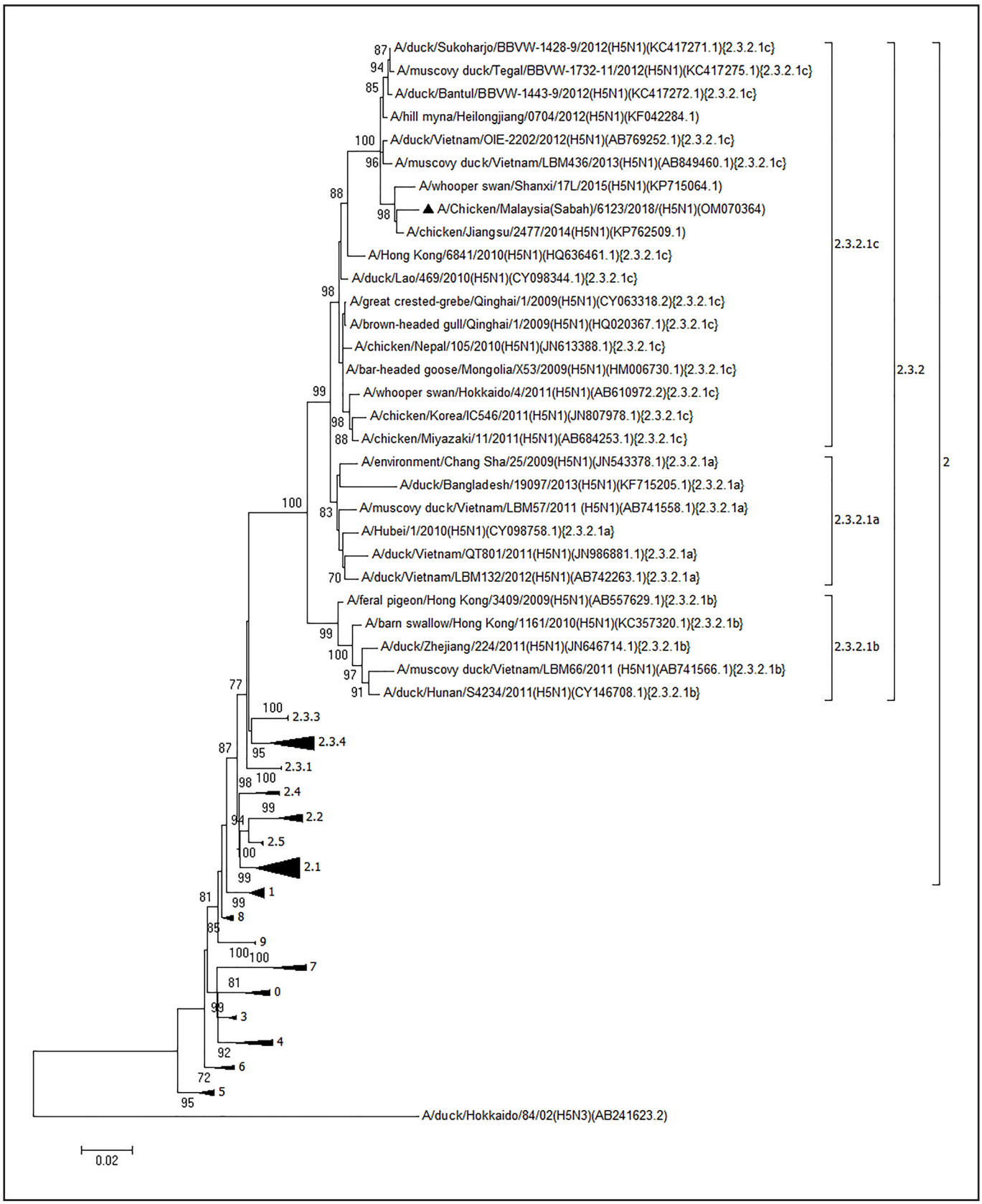

Figure 2. Phylogenetic tree of the Sabah HPAI H5N1 2.3.2.1c virus (A/chicken/Malaysia (Sabah)/6123/2018) and 85 reference H5N1 strains based on the full length HA gene. The evolutionary history was inferred with the Maximum Likelihood statistical method based on the Kimura 2-parameter model and setting bootstrap 1,000 replicates using MEGA version 6.06. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. A H5N3 virus was used as outgroup. Nodes with bootstrap values more than 70% are shown. The black triangle (▲) represents the Sabah H5N1 virus.

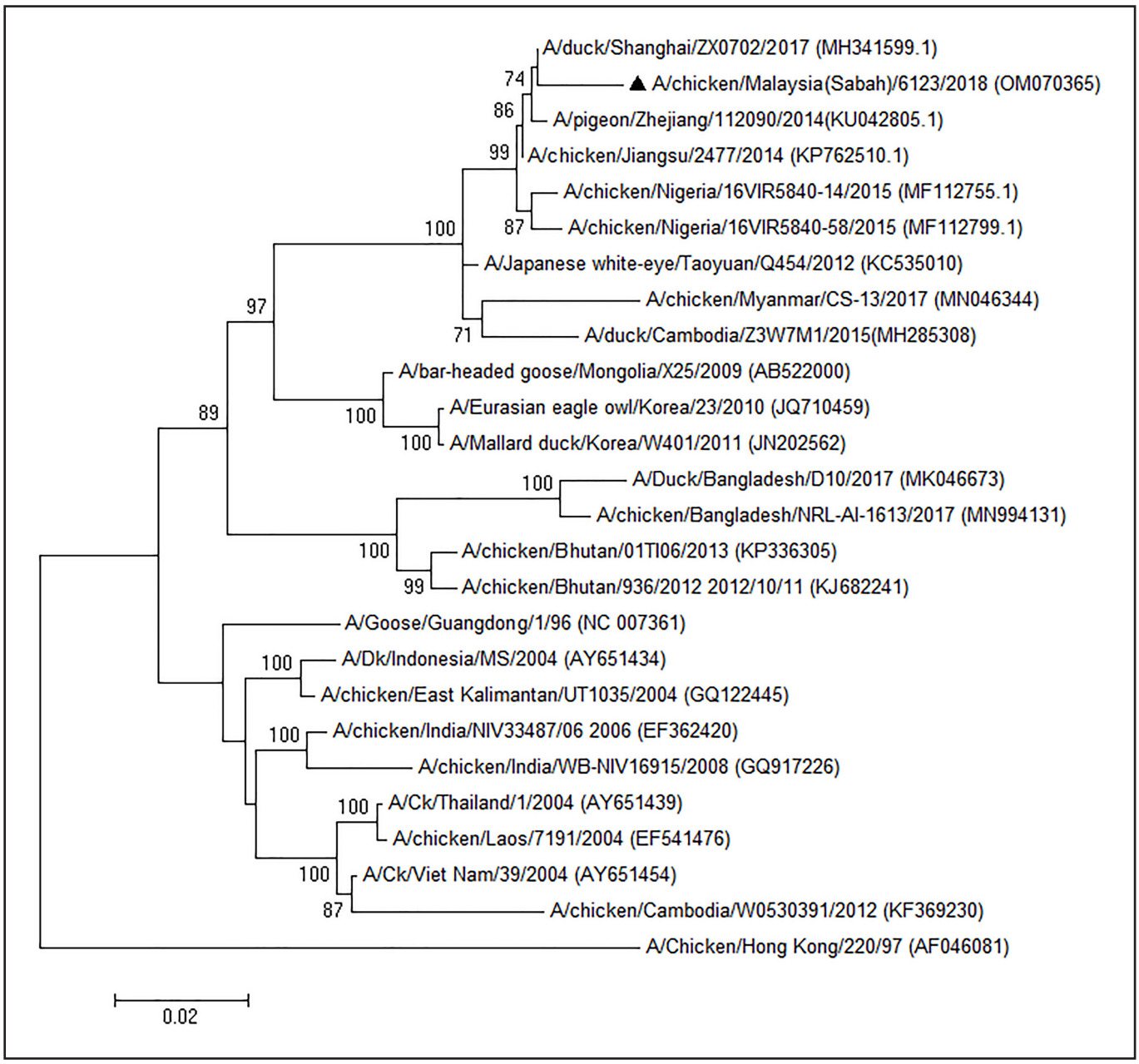

Figure 3. Phylogenetic tree of the Sabah HPAI H5N1 2.3.2.1c virus (A/chicken/Malaysia (Sabah)/6123/2018) and 24 reference H5N1 strains based on the full length NA gene. The evolutionary history was inferred with the Maximum Likelihood statistical method based on the Kimura 2-parameter model and setting bootstrap 1,000 replicates using MEGA version 6.06. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Nodes with bootstrap values more than 70 % are shown. The black triangle (▲) represents the Sabah H5N1 virus.

showed that the Sabah isolate was grouped together with viruses from China (Figure 3).

Molecular characterisation of the full length HA and NA gene

The presence of multi basic cleavage site, the PQRERRRKR/GLF in the HA gene characterised the Sabah 2.3.2.1c virus was of HPAI strain. The presence of amino acid substitutions which is associated with the change in the virus phenotype in the HA gene of the virus is analysed according to Burke & Smith (2014) (Table 2). The virus possessed a conserved glutamine (Q) at position 222 and glycine (G) at position 224 (H5 numbering) indicating the preferential binding of the virus to avian α-2,3 receptor. However, there is a substitution of serine (S) to alanine (A) in position 133 in the HA gene which is associated with the increased preferentially binding of the virus to mammalian α-2,6 receptor. The N154D and T156A found in the virus may indicate the loss of N-glycosylation and increase the binding and transmission ability of the virus in mammals such as ferrets (Herfst *et al*., 2012; Imai *et al*., 2012). Insertion of arginine (R) in position 326 indicates the virus possessed a polybasic cleavage site where the pathogenicity of the virus in chicken is increased. Interestingly, few amino acid residues at the positions 129, 384, 440, 443 and 493 were found to be different compared to amino acid residues described by Burke & Smith (2014). Six N-linked glycosylation sites were predicted from the HA gene. They are 27NST, 39NVT, 181NNT, 302NSS, 499NGT and 558NGS (Table 3A).

Genetic analysis of the NA gene revealed 20 amino acid deletion at residue 49-68 (N1 numbering) in the stalk region when compared to the N1 NA of A/Gs/Gd/1/96 virus. There are three potential glycosites found in the NA gene, the 68NSS, 126NGT and 215NGS (Table 3B). An antiviral resistance risk assessment was also carried

Table 2. Summary of amino acid substitutions in HA gene of the A/chicken/Malaysia (Sabah)/6123/2018 virus associated with changes in virus phenotype based on Burke and Smith (2014)

Mutation	Phenotype	Amino acid position based on H5 strain	A/chicken/Malaysia (Sabah)/6123/2018
Ala → △	Increased virus binding to α2-6 glycans	129	Leu*
Ser → Ala	Increased virus binding to α2-6 glycans	133	Ala*
Asn → Asp	Loss of N-glycosylation, increased binding and transmission	154	Asp*
Thr → Ala	Loss of N-glycosylation, increased binding and transmission	156	Ala*
Insertion of Arg or Lys	Poly-basic cleavage, increased pathogenicity	326	Insertion of Arg
Lys → Ile	Increase in fusion pH, increased stability	384	Ser*
Asn → Lys	Increase in fusion pH, decreased stability	440	Phe*
Asn → Asp	Increase in fusion pH	443	Ser*
Arg → Lys	Increased virus binding to α2-6 glycans	493	Ser*

△: A deletion correlated to other AI subtypes.
* : Amino acid substitution detected in A/chicken/Malaysia (Sabah)/6123/2018 virus.

Table 3. N-linked glycosylation analysis of A/chicken/Malaysia (Sabah)/6123/2018 virus

A. N-linked glycosylation analysis of HA protein

(Threshold=0.5)

SeqName	Position	Potential	Jury Agreement	*N-Glyc result	
OM070364	26 NNST	0.3708	(9/9)	−−	
OM070364	27 NSTE	0.7756	(9/9)	+++	
OM070364	39 NVTV	0.7100	(9/9)	++	
OM070364	156 NSSF	0.4608	(6/9)	−	
OM070364	181 NNTN	0.6184	(8/9)	+	
OM070364	209 NPTA	0.7082	(9/9)	++	WARNING: PRO-X1.
OM070364	302 NSSM	0.5420	(6/9)	+	
OM070364	499 NGTY	0.5801	(6/9)	+	
OM070364	558 NGSL	0.6827	(9/9)	++	

B. N-linked glycosylation analysis of NA protein

(Threshold=0.5)

SeqName	Position	Potential	Jury Agreement	*N-Glyc result
OM070365	68 NSSL	0.7661	(9/9)	+++
OM070365	126 NGTV	0.6929	(9/9)	++
OM070365	215 NGSC	0.6821	(9/9)	++

The number in red indicates the highest score of the jury agreement
***−, −−:** non-glycosylated sites; **+, ++, +++:** glycosylated sites.

out and the susceptibility of the virus to antiviral drugs is showed in Table 4.

DISCUSSIONS

Over the past two decades, Malaysia has experienced four HPAI H5N1 outbreaks where all were occurred in Peninsular Malaysia. This is the first time where HPAI H5N1 was detected in East Malaysia, Sabah. The previous four waves of H5N1 were reported in August 2004, February 2006, June 2007 and February 2017, respectively. One year later, again, it struck Malaysia, marking the first case of H5N1 in Sabah (OIE, 2019). The outbreaks were occurred in July 2018. From the time of outbreaks occurrence, it seems difficult to predict the next H5N1 outbreak. Nonetheless, from the observation of author, the disease seems to be occurred at certain months only. As Malaysia is located within the East Asian-Australasian flyway of the wild migratory birds, many birds migrate to Malaysia from Northern Asia annually. A report by Payne & Parish (1985) mentioned that a bird sanctuary in Sabah, the Kota Belud Bird Sanctuary recorded the highest number of migrant birds from October to March. Phillipps and Phillipps (2011) reported that October is the peak migration season where the northern migrants arrive the

Table 4. The antiviral resistance risk assessment of A/chicken/Malaysia (Sabah)/6123/2018 compared to reference sequence, the A/California/07/2009(H1N1) (ACQ63272.1)

Sequence Feature Name	Variant Type	Number of Variant Types	Position in Reference Sequence	Amino Acid in Reference Sequence	Position in A/chicken/ Malaysia (Sabah)/ 6123/2018	Amino Acid in A/chicken/ Malaysia (Sabah)/ 6123/2018	Phenotype(s) in A/chicken/Malaysia (Sabah)/6123/2018
Influenza A_N1_antiviral-response_117(1)	VT-1	11	117	I	97	I	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_149(1)	VT-2	15	149	V	129	V	Sensitive to Zanamivir
Influenza A_N1_antiviral-response_156(1)	VT-1	10	156	R	136	R	Sensitive to Peramivir Sensitive to Oseltamivir Sensitive to Zanamivir
Influenza A_N1_antiviral-response_199(1)	VT-1	10	199	D	179	D	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_278(1)	VT-1	8	278	E	258	E	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_223(1)	VT-1	12	223	I	203	I	Sensitive to Oseltamivir Sensitive to Zanamivir
Influenza A_N1_antiviral-response_275(1)	VT-1	8	275	H	255	H	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_119(1)	VT-1	10	119	E	99	E	Sensitive to Oseltamivir Sensitive to Zanamivir Sensitive to Peramivir
Influenza A_N1_antiviral-response_136(1)	VT-1	8	136	Q	116	Q	Sensitive to Oseltamivir Sensitive to Zanamivir
Influenza A_N1_antiviral-response_119(1)	VT-1	10	119	E	99	E	Sensitive to Zanamivir Sensitive to Oseltamivir Sensitive to Peramivir
Influenza A_N1_antiviral-response_136(1)	VT-1	8	136	Q	116	Q	Sensitive to Peramivir Sensitive to Zanamivir
Influenza A_N1_antiviral-response_116(1)	VT-1	9	116	V	96	V	Sensitive to Oseltamivir Sensitive to Zanamivir
Influenza A_N1_antiviral-response_247(1)	VT-1	9	247	S	227	S	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_295(1)	VT-1	10	295	N	275	N	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_223(2)	VT-1	17	223,297	IH	203,277	IH	Sensitive to Oseltamivir Sensitive to Zanamivir Sensitive to Peramivir
Influenza A_N1_antiviral-response_223(2)	VT-1	17	223,297	IH	203,277	IH	Sensitive to Oseltamivir Sensitive to Peramivir
Influenza A_N1_antiviral-response_223(2)	VT-1	17	223,297	IH	203,277	IH	Sensitive to Peramivir Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_223(1)	VT-1	12	223	I	203	I	Sensitive to Oseltamivir
Influenza A_N1_antiviral-response_223(1)	VT-1	12	223	I	203	I	Sensitive to Oseltamivir

north-western coast of Borneo. Considering the migratory birds may serves as one of the possibility for the source of the outbreak, more surveillances on migratory birds should be carried out. By having better understanding of the migratory birds including their wintering and stopover sites; best ecology condition and habitat for feeding, breeding and raising their young, we can have an early and better readiness and preparedness for the outbreak risk.

H5N1 clade 1, 2.3, and 2.3.4 viruses were the aetiological agent for the previous outbreaks in Malaysia (Nur Adibah *et al*., 2017). Phylogenetic analysis revealed that the virus detected in Sabah was of H5N1 clade 2.3.2.1c virus. H5N1 clade 2.3.2.1c virus was first detected in Malaysia in Kelantan in 2017 (OIE, 2020). A year later, this clade is detected in East Malaysia, Sabah for the first time. Clade 2.3.2.1 virus is originated from clade 2.3.2 virus which is enzootic in China and has become the predominant clade causing the HPAI H5N1 outbreak in poultry and wild birds in 14 countries in East, South and Southeast Asia from 2010 to 2012 (FAO, 2013). Besides China, clade 2.3.2.1c virus is also now detected in Southeast Asia, Japan, Korea, Nepal, Mongolia, Russia, the Middle East such as Iran and even Europe (Creanga *et al*., 2013; FAO, 2013). Based on the genetic clusters, clade 2.3.2.1 virus can be further divided into subclade 2.3.2.1a (A/Hubei/1/2010-like) virus, subclade 2.3.2.1b (A/barn-swallow/Hong Kong/1161/2010-like) virus and subclade 2.3.2.1c (A/Hong Kong/6841/2010-like) virus (Smith *et al.,* 2015). FAO (2013) has warned that countries in the Asian region whether endemic or not for AI, remain at risk of introduction of clade 2.3.2.1 viruses as the virus continue to circulate and new strains continue to merge from regional hotspots. Hence, it is not surprising that Sabah was struck by clade 2.3.2.1c virus for the first HPAI H5N1 outbreak.

The multi basic amino acid sequence in the HA cleavage site of the Sabah 2.3.2.1c virus was in agreement with the characteristic of HPAI strain (OFFLU, 2020). Based on the two key residues at position 222 and 224 that determine the receptor binding preference, the virus retained avian-type receptor binding signature. The virus does not contain amino acid substitutions that are associated with mammalian adaptation except the S133A substitution. Despite the S133A substitution, the virus retained the conserved amino acid at most of the avian receptor binding preference sites based on Burke & Smith (2014). In short, the ability of this virus to transmit to humans is relatively low. On the other hand, the binding and transmission ability of the Sabah 2.3.2.1c virus may also increase as N154D and T156A substitution were seen. Study reported that amino acid substitutions of the Q222L, G224S, T156A and H103Y in the HA protein; and E627K in the PB2 protein allow the transmission of H5N1 virus via respiratory droplets between the ferrets (Herfst *et al*., 2012). Also, Imai *et al*. (2012) demonstrated that substitutions of the N220K, Q222L, N154D and T315I in the HA permits the droplet transmission of a reassortant H5 HA/H1N1 virus in the same animal model. In this study, the Sabah 2.3.2.1c virus possess conserved histidine (H) at position 103, asparagine (N) at position 220 and threonine (T) at position 315, respectively. Taken together, though the N154D and T156A substitution were detected, the pandemic potential of this Sabah 2.3.2.1c virus is low in the absence of the Q222L, G224S, H103Y, N220K and T315I.

There were differences of amino acid residues at position 129, 384, 440, 443 and 493 of the Sabah 2.3.2.1c virus compared to Burke & Smith (2014). However, when aligned with the reference sequence, the A/Vietnam/1203/2004 in the HA subtype numbering conversion tool, the amino acid residues of the Sabah virus at these positions are the same as the reference sequence and there was no mutation found. The differences of the amino acid as compared to Burke & Smith (2014) may be due to the equivalent amino acid residues numbering described by the above mentioned authors are not only meant for H5 subtype but also across all influenza A subtypes. Therefore, differences in the amino acid at certain position in HA gene is expected.

Six N-linked glycosylation sites were predicted in the HA protein for the Sabah 2.3.2.1c virus. The potential glycosylation sites, the 27NST, 39NVT, 181NNT and 302NSS found in the virus have all been identified in H5N1 viruses previously (Chen *et al*., 2012; Yang *et al*., 2021). On the other hand, glycosites 499NGT and 558NGS predicted in this Sabah H5 virus have not been reported by Chen *et al*. (2012). While, in 2021, Yang and his colleagues reported that 500NGT and 559 NGS are two of the conserved glycosylation sites detected in H5N1 viruses from 2004 to 2020 (Yang *et al*., 2021). In this study, the numbering of the glycosites 499NGT and 558NGS in the Sabah virus is different in one position compared to the 500NGT and 559 NGS as described by Yang *et al*. (2021). According to Chen *et al*. (2012), antigenic drift can impact on glycosites while antigenic shift can affect some adjacent glycosylation sites in the H5N1 or H1N1 viruses, leading to different numbering for the glycosites even in the same subtype. As such, it is suggested that the difference in one position in the glycosite's numbering of the Sabah 2.3.2.1c virus is due to the antigenic drift and antigenic shift in the virus.

In the phylogenetic analysis of the NA gene, 24 reference H5N1 strains from Southeast Asia countries and nearby countries were used to construct the phylogenetic tree. The phylogram revealed that the Sabah 2.3.2.1c virus was closely related to viruses from China, indicating that they may share a common ancestor. In the analysis of the NA gene, three predicted glycosites, the 68NSS, 126NGT and 215NGS are in agreement with the finding of Suttie *et al*. (2019) that these three glycosylation sites are conserved in the H5N1 virus. On the contrary to Chen *et al*. (2012) that 146N glycosylation site is conserved in all NA subtypes, this glycosite is not predicted in this Sabah 2.3.2.1c virus. In the NA stalk region, there is a typical 20 amino acid deletion at residue 49-68 which is associated with enhanced virulence in mice (Suttie *et al*., 2019). The deletion also causes the loss of four corresponding glycosites in the stalk region affecting the NA glycosylation. This finding is consistent with Gutiérrez *et al*. (2009) that there is a 20 amino acid deletion in the stalk region for clade 1 and 2 H5N1 viruses detected after 2003. The deletion will cause a reduction in enzymatic activity of the NA to release the virus from cell and may serve as virulence determinant (Li *et al*., 2014). This deletion may also influence the adaptation of the H5N1 virus from aquatic birds to poultry (Li *et al*., 2014).

Neuraminidase inhibitor (NAI) class of antiviral drugs is used as the primary treatment in human infected by H5N1 virus (Gutiérrez *et al*., 2009). Therefore, any mutation in the NA gene may interfere the sensitivity of the NAI. The Sabah 2.3.2.1c virus has an H274Y mutation which is associated with resistance to Oseltamivir (Gutiérrez *et al*., 2009). The broad usage of this antiviral drug may contribute to the increase of this H274Y mutation particularly in seasonal H1N1 and H3N2 viruses (Gutiérrez *et al*., 2009). Another two resistance markers to NAI were identified at E99V and I203M (Suttie *et al*., 2019). Despite these three NA resistance markers, there is no other amino acid substitutions seen in the NA protein including E119, H275, R293, N295, V116, I117 and K150 which is associated with reduced drug susceptibility in animal and human H5N1 infection (El-Shesheny *et al*., 2014; Lai *et al*., 2017). In order to better understand the susceptibility of the Sabah 2.3.2.1c virus to antiviral drugs, an antiviral resistance risk assessment was performed through the analysis tool in the Influenza Research Database (IRD). The analysis revealed that the virus is sensitive to Oseltamivir, Zanamivir and Peramivir. Collectively, the virus is still sensitive to NAI though three NA resistance markers were detected.

A total of seven outbreaks of HPAI H5N1 had occurred in seven villages in Tuaran district in Sabah. The reason that caused the outbreak is still undetermined though poultry smuggling is one of the suspecting factor (New Straits Times, 2018). As Malaysia is located in the East Asian-Australasian flyway of the wild migratory birds and Sabah is one of the rest place for these birds in their migratory flyway, their movement may also contribute to the outbreaks as discussed

earlier. In addition, the role of trade in wild captive birds and poultry products in spreading the disease also cannot be neglected. Thus, the cause of the outbreaks still remains elucidate.

To the best of our knowledge, there is no reported HPAI H5N1 infection in human in Malaysia. Though there is no evidence of human to human transmission of H5N1, controlling the circulation of H5N1 in the natural animal reservoir, the poultry, is very important to prevent the global pandemic threat.

CONCLUSION

As for the HPAI outbreaks in poultry in East Malaysia, Sabah was reported for the first time in 2018. The outbreak was caused by H5N1 clade 2.3.2.1c virus. Molecular characterisation of the HA gene revealed that the risk of zoonotic and pandemic potential is relatively low. Though three NA resistance markers were detected, the virus is still sensitive to NA inhibitor. This study only focused on two major surface glycoprotein, the HA and NA. Both genes were analysed and discussed from the point of amino acid substitution, receptor binding preference, glycosylation site, antiviral drug resistance and-so-on. However, the other genes of the virus worth further investigation for a better understanding of the virus. The pandemic potential of H5N1 virus should not be undervalued as the viruses continue to evolve, exhibit markers for the increment of zoonotic potential and NA resistance. Therefore ongoing surveillance of the disease in poultry, wild bird and migratory bird; monitoring antigenic changes, drug resistance, pathogenicity of the novel strains as well as molecular epidemiology of H5N1 is important in controlling and preventing the future epidemic and pandemic.

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Conflict of interest

The author declares that they have no conflict of interests.

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