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RESEARCH ARTICLE

Phylogenetic analysis of nucleoprotein gene of Rabies virus in Malaysia from 2015 to 2018

Leow, B.L.[1*], Khoo, C.K.[1], Syamsiah Aini, S.[1], Roslina, H.[1], Faizah Hanim, M.S.[1]

[1]Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia
*Corresponding author: leowbl@hotmail.com, leow@dvs.gov.my

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ABSTRACT

Rabies is a fatal zoonotic disease caused by rabies virus (RABV) and remains a public health problem in Malaysia. Malaysia was declared rabies-free in 2012, however rabies outbreaks occurred at few states in Peninsular Malaysia three years later; and for the first time, in Sarawak (East Malaysia) in 2017 which has caused more than 20 human deaths. This study describes the phylogenetic analysis of the complete nucleoprotein (*N*) gene of RABV from animal samples in Malaysia from year 2015 to 2018. The *N* gene of 17 RABVs from Perlis, Kedah and Sarawak were amplified and sequenced. The nucleotide and deduced amino acid similarities of *N* gene analysis indicated that there is high similarity among the local RABVs. Phylogenetic analysis of the *N* gene revealed that all Malaysia RABVs belonged to the Asian clade. Among these, RABVs from Peninsular Malaysia were clustered together with RABVs from Thailand, Vietnam and other Southeast Asia countries except Indonesia. However, RABVs from Sarawak were grouped together with Indonesian strains from Kalimantan. Our study provides baseline genetic information of the potential origins of the circulating RABVs in Malaysia. This crucial information helped the authority in policies making and strategies to be taken in outbreak control. Continuous surveillance program to monitor the disease trend, strict border control, vaccination of dog and cat population and public awareness are important steps to control the spread of the RABV.

Keywords: Rabies virus; *N* gene; Sarawak; Malaysia.

INTRODUCTION

Rabies, a fatal encephalitis disease, is caused by rabies virus (RABV) and is transmitted by the bite of rabid animal (Zhang *et al.,* 2006). RABV belongs to the genus Lyssavirus in the family *Rhabdoviridae* (Bourhy *et al.,* 1993, 2008; Dibia *et al.,* 2015). The virus is a single-stranded, negative sense RNA virus with genome of approximately 12 kb that encodes for nucleoprotein (*N*), phosphoprotein (*P*), matrix protein (*M*), glycoprotein (*G*) and RNA-dependent RNA polymerase or large protein (*L*) (Zhang *et al.,* 2006; Susetya *et al.,* 2008; Dibia *et al.,* 2015). Nucleoprotein plays an important role in inducing immunity especially against infection with heterologous Lyssaviruses (Bourhy *et al.,* 1993). *N* gene is highly conserved and therefore allows the genotyping of Lyssavirus based on nucleotide sequences (Bourhy *et al.,* 1993).

Although all mammals can be infected by RABV, only few species of animals can transmit RABV to humans. Among them are several canid, feline and chiropteran species (Susetya *et al.,* 2008) with more than 99% human cases are caused by dog-mediated rabies (Bourhy *et al.,* 2008). Rabies is also an important fatal zoonotic disease in many countries. Fifty nine thousand human deaths annually around the world have been estimated to be due to dog-mediated rabies (WHO, 2018). The majority of deaths have occurred in Asia (59.6%) and Africa (36.4%) (WHO, 2018).

In Malaysia, rabies is a public health problem especially along the Malaysia and Thailand border. Dog is the primary reservoir of rabies infection in the country (Ganesan & Sinniah, 1993). The success of the National Rabies Control Programme implemented in 1952 led Malaysia to declared as rabies-free country in 1954 (Ganesan & Sinniah, 1993; Loke *et al.,* 1998). However, sporadic rabies cases were still reported in Perlis, Kedah and Kelantan where these states share a common border with rabies endemic area in Thailand (Loke *et al.,* 1998). The establishment of 50-80 km "immune belt" program in 1955 covering Perlis, Kedah, Kelantan and northern Perak, bordering Thailand and strict regulation of dog importation has successfully prevented the transmission of rabies from the north (Ganesan & Sinniah, 1993). The success can be seen by only 1.3 cases per year were reported from 1955 to 1986, where most cases occurred at the border area and only 6 cases reported in other states among these years (Loke *et al.,* 1998). Malaysia was again declared as rabies-

free in July 2013 with the last case occurred in 1999 (OIE, 2013). However, three years after the declaration, rabies outbreaks reoccurred in Perlis, Kedah and Pulau Pinang (Navanithakumar *et al.,* 2019). In 2017, rabies was reported for the first time in Sarawak, East Malaysia, causing 28 human deaths (Kpkesihatan.com**,** 2020). Until today, rabies remains a public health threat in our country.

The *N* gene sequence data of RABVs is now widely used in the rabies molecular epidemiological studies as well as the origination, genetic diversity, distribution and the transmission pattern of the disease (Kissi *et al.,* 1995; Zhang *et al.,* 2006; Bourhy *et al.,* 2008; Susetya *et al.,* 2008; Dibia *et al*., 2015). For better management and control of rabies outbreak, sequencing data also enables various downstream molecular applications such as laboratory diagnosis and vaccine development. However, to date, there is no report on the complete *N* gene nucleotide sequence of the RABV detected in Malaysia. Therefore, this study is to genetically characterize and phylogenetically analyze the complete *N* gene of the RABVs detected in several states in Malaysia from 2015 to 2018.

MATERIALS AND METHODS

Samples

Brain samples (n=17) in this study were from various animals in different states in Malaysia that were received at Veterinary Research Institute (VRI), Ipoh for rabies diagnosis. These samples were collected from 2015 to 2018 either for diagnostic purpose or from surveillance activities. All samples were subjected to direct fluorescent antibody (DFA) test (OIE, 2018) prior to molecular detection. Rabies cases detected in Kedah and Pulau Pinang in 2015, and in Perak in 2017 are not included in this study due to the deterioration or insufficient sample for molecular detection.

Primers and Reverse transcription polymerase chain reaction (RT-PCR)

Samples that were positive for rabies by DFA test were subjected to RT-PCR. The viral RNA was extracted from the brain sample by phenol chloroform method using TRIzol LS Reagent (Invitrogen, USA) followed the manufacturer's instruction. Subsequently, RT-PCR was carried out using SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA). The primer sets used in this study were shown in Table 1. The amplification was performed in T100 Thermal Cycler (Bio-Rad, USA). The reaction mix was then subjected to 1 cycle of reverse transcription at 48°C for 30 min and 94°C for 5 min for initial denaturation; 10 cycles of amplification at 94°C for 1 min, 37°C for 1 min and 68°C for 3 min; 20 cycles of amplification at 94°C for 1 min, 52°C for 1 min and 68°C for 3 min and the final extension was carried out at 68°C for 10 min (M. Chris, personal communication, November 4, 2015). Primer set NF (forward): ACGCTTAACAACAAAAYCA DAGAAG and NR (reverse): GGRTTGACGAARATCTTGCTCAT designed by Feng *et al*. (2019) was also used in the study. This primer set can amplify approximately 1,536 bp which covers the entire *N* gene of the RABV. In brief, the 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 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 68°C for 2 min with a final extension for 10 min at 68°C. The amplicons were then analyzed by electrophoresis on 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, USA).

Gene sequencing, nucleotide/amino acid similarity analysis and phylogenetic analysis

The amplicons were cut from the gel and sent for Sanger sequencing (Apical Scientifics (M) Sdn Bhd). Primers used for DNA sequencing are corresponding to those used in the RT-PCR amplification. Nucleotide sequences were assembled using SeqMan Pro software (DNAStar Lasergene, USA). BioEdit Sequence Alignment Editor version 7.1.9 (Hall, 1999) was used in the alignment and comparison of the *N* gene nucleotide sequences of the 17 local samples and other published sequences (Table 2). Similarities of nucleotide and deduced amino acid of the *N* gene among the local samples were analyzed. A phylogenetic tree was constructed by Neighbor-Joining statistical method with model number of differences and setting bootstrap 1,000 replicates using MEGA version 6.06 (Tamura *et al*., 2013). The genetic relationship of the *N* gene between the local samples and other respective strain in the world were examined through the phylogram.

RESULTS

Seventeen brain samples from 14 dogs and 3 cats collected from different area in several states which include Perlis, Kedah and Sarawak from 2015 to 2018 were used in the study (Table 3). All 17 samples were successfully amplified with *N* gene specific primers. Nucleotide sequence of 1,353 bp was used in the sequence comparison and phylogenetic analysis. The *N* gene sequences obtained in this study have been submitted to GenBank under the accession numbers listed in Table 3. Sequences comparison revealed that 88.3 to 100% nucleotide similarity and 97.5 to 100% deduced amino acid similarity was detected among the Malaysia RABVs (Table 4). Among these, the nucleotide and deduced amino acid similarity of Kedah and Perlis RABVs was 99.1 to 100% and 99.3 to 100%, respectively. On the other hand, nucleotide and deduced amino acid similarity of 99.8 to 100% and 99.7 to 100%, respectively, was observed for the Sarawak RABVs.

The Malaysia RABVs were then compared with other rabies strains in the world. A total of 48 worldwide RABVs *N* gene sequences retrieved from GenBank database were used in the sequence alignment and phylogenetic analysis. Phylogenetic analysis showed that all 17 Malaysia RABVs belongs to Asian clade (Figure 1). All seven RABVs that were detected in Perlis and Kedah were grouped in the Asian II lineage, whereas the ten RABVs collected from Sarawak were clustered in Asian I lineage. The Peninsular Malaysia RABVs

Table 1. Primer sets used in the amplification of complete *N* gene (Bourhy *et al.,* 1993)

Name	Sequences 5' – 3'	Position in rabies virus PV strain	Size of amplicon (bp)
Rabies-N7	ATG TAA CAC CTC TAC AAT G	55-73	1,531
Rabies-N8	AGT TTC TTC AGC CAT CTC	1,585-1,568	
Rabies-N7	ATG TAA CAC CTC TAC AAT G	55-73	591
Rabies-N14	TTG TGA GTA GTC ATT A	645-630	
Rabies-N5	GAA GGC AAT TGG GCT C	419-434	612
Rabies-N2	CCC ATA TAG CAT CCT AC	1,030-1,013	
Rabies-N17	TTC TTC CAC AAG AAC TTT G	848-866	738
Rabies-N8	AGT TTC TTC AGC CAT CTC	1,585-1,568	
Rabies-N1	TTTGAGACAGCCCCTTTTG	587-605	999
Rabies-N8	AGT TTC TTC AGC CAT CTC	1,585-1,568	

Table 2. Reference RABV strains retrieved from GenBank database

Lyssavirus genotype	Country	Identification number/strain	Species	Year	Accession number	Reference
1	Afghanistan	04027AFG	Dog	1996	EU086162.1	Bourhy *et al.,* 2008
1	Saudi Arabia	8706ARS	Fox	1987	EU086163.1	Bourhy *et al.,* 2008
1	Myanmar	9909BIR	Dog	1999	EU086164.1	Bourhy *et al.,* 2008
1	Myanmar	9915BIR	Dog	1999	EU086166.1	Bourhy *et al.,* 2008
1	Cambodia	9914CBG	Dog	1997	EU086170.1	Bourhy *et al.,* 2008
1	Cambodia	02006CBG	Dog	1998	EU086172.1	Bourhy *et al.,* 2008
1	China	9811CHI	Dog	1998	EU086173.1	Bourhy *et al.,* 2008
1	China	05009CHI	Dog	2005	EU086190.1	Bourhy *et al.,* 2008
1	India	9702INDI	Human	1997	EU086191.1	Bourhy *et al.,* 2008
1	Indonesia	03003INDO	Dog	2003	EU086192.1	Bourhy *et al.,* 2008
1	Laos	9910LAO	Dog	1999	EU086193.1	Bourhy *et al.,* 2008
1	Nepal	9901NEP	Dog	1998	EU086196.1	Bourhy *et al.,* 2008
1	Nepal	9902NEP	Goat	1998	EU086197.1	Bourhy *et al.,* 2008
1	Philippines	94273PHI	Dog	1994	EU086201.1	Bourhy *et al.,* 2008
1	Philippines	03006PHI	Human	2000	EU086203.1	Bourhy *et al.,* 2008
1	Thailand	8743THA	Human	1983	EU086207.1	Bourhy *et al.,* 2008
1	Thailand	8738THA	Human	1983	EU086208.1	Bourhy *et al.,* 2008
1	Vietnam	01016VNM	Dog	2001	EU086209.1	Bourhy *et al.,* 2008
1	Vietnam	01017VNM	Dog	2001	EU086210.1	Bourhy *et al.,* 2008
1	Guinea	9024GUI	Dog	1990	U22641.1	Bourhy *et al.,* 1995
1	South Africa	1500AFS	Yellow Mongoose	1987	U22628.1	Bourhy *et al.,* 1995
1	Sri Lanka	1294	Dog	1986	AY138549.1	Nanayakkara *et al.,* 2003
1	Iran	8681IRA	Dog	1986	U22482.1	Kissi *et al.,* 1995
1	Poland	8618POL	Raccoon Dog	1985	U22840.1	Kissi *et al.,* 1995
1	China, Fujian	FJ003	Dog	2008	FJ561728.1	Feng *et al.,* 2016
1	China, Fujian	FJ008	Dog	2008	FJ866835.1	Feng *et al.,* 2016
1	Inner Mongolia	CNM1104D	Dog	2011	KC252634.1	Feng *et al.,* 2016
1	China, Shanxi	SXDTD13	Dog	2013	KT894584.1	Feng *et al.,* 2016
1	China, Guizhou	A103	Dog	2005	DQ666290.1	Feng *et al.,* 2016
1	South Korea	KRC5-04	Dog	2004	AY730597.1	Park *et al.,* 2005
1	China	HuNDN16	Dog	–	DQ515993.1	GenBank
1	Mongolia	MGL-2	Cattle	2005	AB570995.1	Boldbaatar *et al.,* 2010
1	Mongolia	MGL-22	Dog	2007	AB571004.1	Boldbaatar *et al.,* 2010
1	Bangladesh	BDR7	Cattle	2010	AB699219.1	Jamil *et al.,* 2012
1	Indonesia, Bali	BL659-09	Dog	2009	JX462623.1	Dibia *et al.,* 2015
1	Pakistan	Pk 23	Cattle	2010	HE802675.1	GenBank
1	Pakistan	Pk 24	Cattle	2007	HE802676.1	GenBank
1	Bhutan	Btn115	Cattle	2010	AB910532.1	Jamil *et al.,* 2012
1	China	CQWSD01	Dog	2005	KT221095.1	GenBank
1	India	IAP-R91	Buffalo	2002	KX434489.1	GenBank
1	Indonesia, Kalimantan	KL00-18	Dog	2000	AB154221.1	Susetya *et al.,* 2008
1	Indonesia, Kalimantan	KL97-03	Dog	1997	AB154223.1	Susetya *et al.,* 2008
1	China	FJDRV	Dog	2008	JN609295.1	GenBank
1	Malaysia, Serian	RB03	Human	2017	MF620996.1	GenBank
1	Laos	Lao11	Dog	2012	AB981671.1	Ahmed *et al.,* 2015
1	Thailand	D23	Dog	–	AY218996.1	Hemachudha *et al.,* 2003
Vaccine strain	–	AVO1	–	–	X13357.1	Poch *et al.,* 1988
5	France	03002FRA	Bat	2003	EU293109.1	Dolmes *et al.,* 2008

Table 3. Rabies samples collected from different area in several states in Malaysia from year 2015 to 2018

Identification no	Species	Year	Area	State	GenBank accession number
VRI-8687-2015/dog/2015/Malaysia/Perlis	Dog	2015	Pekan Kaki Bukit	Perlis	MW690139
VRI-9027-2015/dog/2015/Malaysia/Perlis	Dog	2015	Kangar	Perlis	MW690140
VRI-3020-2016/dog/2016/Malaysia/Perlis	Dog	2016	Chuping	Perlis	MW690141
VRI-1370-2017/dog/2017/Malaysia/Kedah	Dog	2017	Padang Terap	Kedah	MW690142
VRI-7308-2017/cat/2017/Malaysia/Sarawak	Cat	2017	Serian	Sarawak	MW690143
VRI-7541-2017/dog/2017/Malaysia/Sarawak	Dog	2017	Serian	Sarawak	MW690144
VRI-7611-2017/dog/2017/Malaysia/Sarawak	Dog	2017	Serian	Sarawak	MW690145
VRI-7619-2017/dog/2017/Malaysia/Sarawak	Dog	2017	Serian	Sarawak	MW690146
VRI-10080-2017/dog/2017/Malaysia/Sarawak	Dog	2017	Kuching	Sarawak	MW690147
VRI-14552-2017/dog/2017/Malaysia/Sarawak	Dog	2017	Bau	Sarawak	MW690148
VRI-351-2018/dog/2018/Malaysia/Sarawak	Dog	2018	Kota Samarahan	Sarawak	MW690149
VRI-625-2018/cat/2018/Malaysia/Sarawak	Cat	2018	Kuching	Sarawak	MW690150
VRI-2246-2018/dog/2018/Malaysia/Sarawak	Dog	2018	Kota Samarahan	Sarawak	MW690151
VRI-4212-2018/dog/2018/Malaysia/Perlis	Dog	2018	Kangar	Perlis	MW690152
VRI-7449-2018/dog/2018/Malaysia/Sarawak	Dog	2018	Sri Aman	Sarawak	MW690153
VRI-8872-2018/cat/2018/Malaysia/Kedah	Cat	2018	KubangPasu	Kedah	MW690154
VRI-8988-2018/dog/2018/Malaysia/Kedah	Dog	2018	Jitra	Kedah	MW690155

Table 4. Nucleotide and amino acid similarities of the *N* gene among the local RABVs

	Nucleoprotein (N) sequences Nucleotide identity (%)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	VRI-8687-2015/dog/2015/Malaysia/Perlis		1	0.999	0.992	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.887	0.889	0.998	0.889	0.998	0.998
2	VRI-9027-2015/dog/2015/Malaysia/Perlis	1		0.999	0.992	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.887	0.889	0.998	0.889	0.998	0.998
3	VRI-3020-2016/dog/2016/Malaysia/Perlis	1	1		0.991	0.888	0.888	0.888	0.888	0.888	0.888	0.888	0.886	0.888	0.997	0.888	0.997	0.997
4	VRI-1370-2017/dog/2017/Malaysia/Kedah	0.995	0.995	0.995		0.885	0.885	0.885	0.885	0.885	0.885	0.885	0.883	0.885	0.992	0.885	0.992	0.992
5	VRI-7308-2017/cat/2017/Malaysia/Sarawak	0.982	0.982	0.982	0.977		1	1	1	1	1	1	0.998	1	0.889	1	0.889	0.889
6	VRI-7541-2017/dog/2017/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1		1	1	1	1	1	0.998	1	0.889	1	0.889	0.889
7	VRI-7611-2017/dog/2017/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1		1	1	1	1	0.998	1	0.889	1	0.889	0.889
8	VRI-7619-2017/dog/2017/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1	1		1	1	1	0.998	1	0.889	1	0.889	0.889
9	VRI-10080-2017/dog/2017/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1	1	1		1	1	0.998	1	0.889	1	0.889	0.889
10	VRI-14552-2017/dog/2017/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1	1	1	1		1	0.998	1	0.889	1	0.889	0.889
11	VRI-351-2018/dog/2018/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1	1	1	1	1		0.998	1	0.889	1	0.889	0.889
12	VRI-625-2018/cat/2018/Malaysia/Sarawak	0.98	0.98	0.98	0.975	0.997	0.997	0.997	0.997	0.997	0.997	0.997		0.998	0.887	0.998	0.887	0.887
13	VRI-2246-2018/dog/2018/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1	1	1	1	1	1	0.997		0.889	1	0.889	0.889
14	VRI-4212-2018/dog/2018/Malaysia/Perlis	0.997	0.997	0.997	0.993	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.977	0.98		0.889	0.998	0.998
15	VRI-7449-2018/dog/2018/Malaysia/Sarawak	0.982	0.982	0.982	0.977	1	1	1	1	1	1	1	0.997	1	0.98		0.889	0.889
16	VRI-8872-2018/cat/2018/Malaysia/Kedah	1	1	1	0.995	0.982	0.982	0.982	0.982	0.982	0.982	0.982	0.98	0.982	0.997	0.982		1
17	VRI-8988-2018/dog/2018/Malaysia/Kedah	1	1	1	0.995	0.982	0.982	0.982	0.982	0.982	0.982	0.982	0.98	0.982	0.997	0.982	1	
	Amino acid identity (%)																	

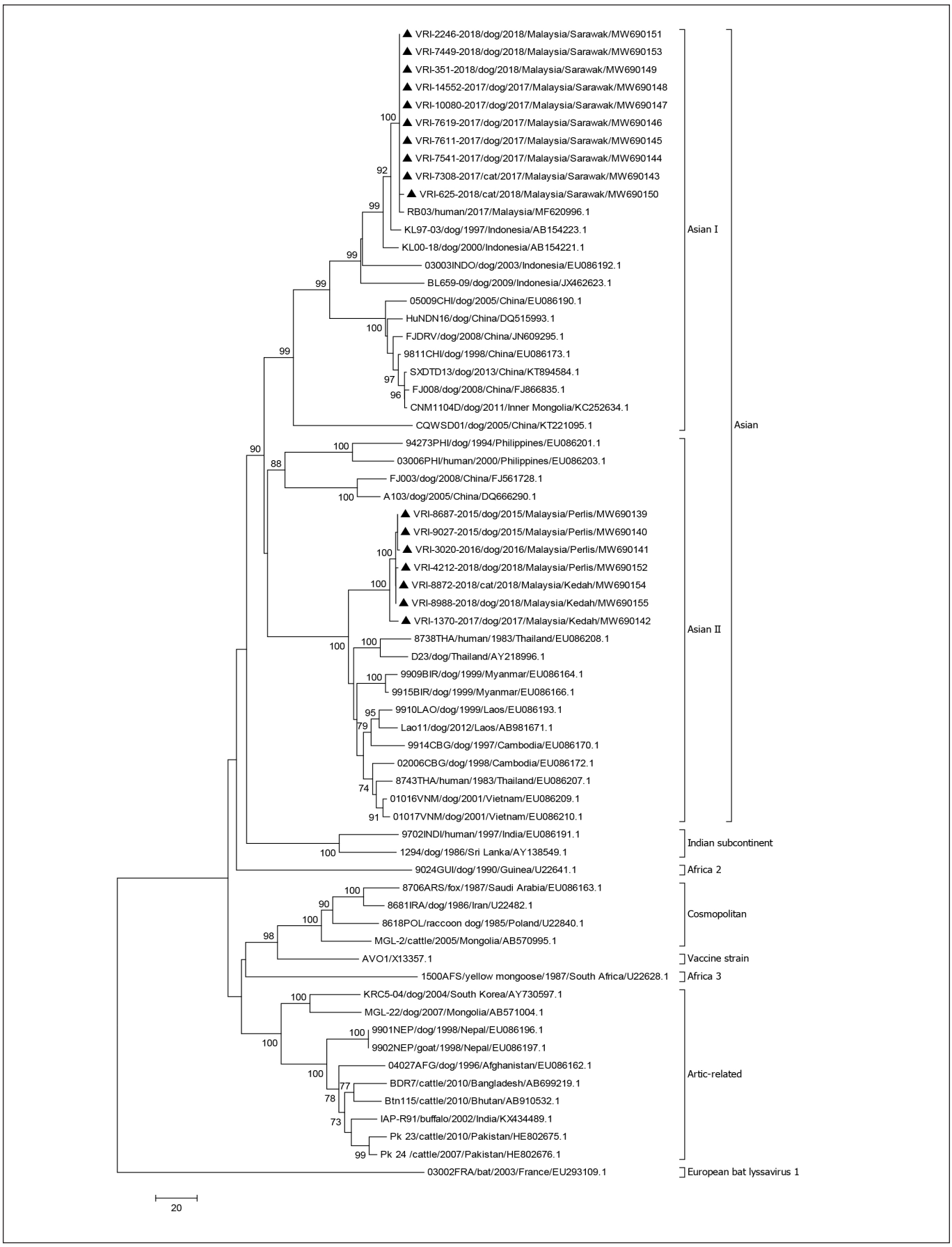

Figure 1. Phylogenetic tree of 17 local RABVs and 48 reference rabies strains based on complete *N* gene of RABV. Tree was constructed using MEGA version 6.06 by Neighbour-Joining statistical method with model number of differences and setting bootstrap 1,000 replicates. European bat Lyssavirus 1 was used as outgroup. Nodes with bootstrap values more than 70% are shown. The black triangles (▲) represent the Malaysian RABVs. The local strain name is suffixed by VRI disease investigation number / species / year detected / country / state /GenBank accession number.

formed a distinct cluster in the phylogenetic analysis despite the difference in the years and location where the virus was detected. Based on the phylogenetic analysis, these viruses belonged to the same group of Asian II lineage RABVs from Thailand, Vietnam and other Southeast Asia countries excluding Indonesia. Conversely, the RABVs detected in Sarawak formed a unique cluster not only among themselves but also with human RABV detected in Serian, Sarawak. The RABVs from Sarawak is grouped together and closely related to RABVs isolated in Kalimantan (Indonesia), China and InnerMongolia that belongs to Asian I lineage.

DISCUSSION

RABV can be classified into six groups, the Africa 2, Africa 3, Artic-related, Asian, Cosmopolitan and Indian subcontinent clades based on the phylogenetic analysis of the *N* gene (Bourhy *et al.,* 2008, Puyati *et al*, 2016). In this study, all Malaysia RABVs were grouped in Asian clade, indicating close relationship with the RABVs circulating in Asia. This is in agreement with Kissi *et al*. (1995) and Puyati *et al*. (2016) that RABV found in Malaysia, Thailand, Cambodia, Laos, Myanmar, Indonesia, Philippines and Vietnam are clustered in the Asia clade.

The findings of this study showed that there are two different genetic groups of RABVs circulating in Malaysia. RABVs detected in Perlis and Kedah were grouped in Asian II lineage, which is associated with the virus from Thailand, Myanmar, Laos, Cambodia and Vietnam (Feng *et al.,* 2019), indicates potential sharing of a common ancestor. From the geographical point of view, the Peninsular Malaysia is connected to Thailand, and further up with Myanmar, Cambodia, Laos, Vietnam and other countries. Thus, it is not surprising that the RABVs detected in Kedah and Perlis are closely related to the RABVs that were isolated in these countries. In addition, from 2015 to 2018, there were no rabies cases reported in other states in the Peninsular indicating that rabies is confined to the northern states of Peninsular Malaysia that bordering Thailand, highlighting the importance of the 'immune belt'. The immune belt serves as a buffer zone to allow the permanent rabies control measures to be continuously implemented (Navanithakumar *et al.,* 2019). In the immune belt area, compulsory dog licensing and vaccination program are carried out. Unlicensed and stray dogs will be put down humanely. Public education is conducted not only to the public but also school children, to deliver the knowledge and enhance rabies awareness among the community (Navanithakumar *et al.,* 2019).

The same goes to the Sarawak state where the virus detected is highly similar to those in the Kalimantan (Indonesia). These findings are in accordance with reports by Bourhy *et al*. (2008) and Dibia *et al.* (2015) that RABV is grouped according to their geographical origin. Sarawak is four times larger than the northern region of Peninsular Malaysia (Perlis, Kedah, Pulau Pinang and Perak) in total land area, yet the RABV detected in these areas were distinct and confined to their respective geographical area. This observation was further evidenced whereby the RABVs detected in Malaysia grouped in their respective cluster despite different in the year and location within the same state.

Bamaiyi (2015) hypothesized that the outbreaks in Perlis and Kedah occurred in 2015 was transmitted from Thailand by land movement. The hypotheses can be further supported by the BLAST analysis of the *N* nucleotide sequences of the two viruses (VRI-8687-2015 and VRI-9027-2015) detected in Perlis in 2015, and these two RABVs have the highest nucleotide similarities with rabies strains from Thailand at 96.66% and 96.58%, respectively. Likewise, the rabies outbreak in Sarawak was believed to be spread from the neighboring region, Kalimantan, Indonesia. Rabies was identified in West Kalimantan in year 2005 and the virus is still circulating in the province though the province has been once declared as rabies-free in April 2014 (Antara Kalbar, 2018). According to Faizul *et al*. (2019), the Sarawak outbreak was caused by the interaction between the pets and strays dogs in Sarawak with the infected dogs from endemic area in Kalimantan. Due to the long border and geographical proximity between Sarawak and Kalimantan, it is difficult to prevent the dogs from crossing over the border and interacting with each other. Therefore, RABV can be easily introduced into Sarawak due to the porous border (Faizul *et al.,* 2019). In addition, with the construction of Pan Borneo Highway, and more land opening up for oil palm plantation, the foreign workers may have brought the animals with them from West Kalimantan (Vickneshwaran, 2018). This is in agreement with Bourhy *et al*. (2008) and Dibia *et al*. (2015) that human-mediated animal movements can cause introduction and transmission of rabies that has been seen in some countries including United States and Indonesia.

Rabies cases have not been reported in Sabah and Sarawak up until 2017 where the first rabies case was reported in Serian, Sarawak (Navanithakumar *et al*., 2019). To date, rabies has spread to all divisions in Sarawak except Limbang. Limbang is free from rabies so far due to its geographical inaccessibility with the only road connection to outside the division is through Brunei (Suara Sarawak, 2020). Additionally, Brunei which is rabies-free (OIE, 2012) also serves as a buffer zone for Limbang. However, this may change with the completion construction of road linking Limbang to Marudi, Baram and Miri by-passing Brunei (Suara Sarawak, 2020). Mass vaccination of dogs in Sarawak including Limbang is also being carried out (Sarawak Government, 2019). Being aware of the risk, Sabah has taken initiative to vaccinate the dog inside 30 km of Sabah along the border of Sarawak and Kalimantan (Ahmed & Ibrahim, 2019) and so far remained rabies-free.

Rabies cases in Malaysia are mainly associated with dogs, but there are also cases detected in cats as detected in this study. As mentioned by Bourhy *et al*. (2008) and Dibia *et al*. (2015), rabies are predominantly affecting dogs and spillover infection into non-reservoir hosts such as human, livestock, cats and wild animals can also happen. The RABVs detected in cats in this study are highly similar to those present in dogs with less than 1% difference of *N* gene nucleotide and deduced amino acid similarity. Importantly, the cat RABV was clustered within the same clade with dog RABV according/in to their respective geographical origin. All these may indicate potential spillover of rabies infection from dog to cat. As cats often lick their paws and like to interact with human, thus, RABV can be transmitted to human by a rabid cat through biting or scratching (Ahmed & Ibrahim, 2019). Although, there are only few cat-mediated rabies cases, the importance of spillover rabies infection into cats cannot be neglected.

To date, rabies has caused 28 human deaths in Sarawak. Though rabies is a fatal zoonotic disease, it can be preventable. Malaysia government has taken drastic measures to control the disease which involve culling the stray dogs (Bamaiyi, 2015) and mass vaccination of the animals including dogs and cats in the affected area. Strict border control should be implemented to restrict the free movement of animals and people across the border. Porous border will increase the risk of influx of RABV from neighboring countries. The

maintenance of the existing immune belt at the border of Thailand and the new establishment of an immune belt along the Sarawak-West Kalimantan border is important to control and prevent the disease. Licensing of dogs, strict regulation on importation of dogs, public awareness, and the responsibility of the pet owner also plays an essential role in controlling the disease.

CONCLUSION

This study describes the molecular characterization of rabies in Malaysia based on the phylogenetic analysis of the complete *N* gene sequences of RABV. This study indicates that Asian I and Asian II of RABVs are circulating in the country from 2015 to 2018 by analyzing the rabies positive samples from different geographical area from several states. The finding suggested that RABVs from the rabies endemic bordering countries might have been introduced to Malaysia and spread in the country. By determining the genetic characteristic and the origin of the circulating RABV, it helps the authority in policy making and strategies taken to control the disease.

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Conflict of interest

The author declares that they have no conflict of interests.

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