PHYLOGENETIC ANALYSIS OF RABIES VIRUSES DETECTED IN MALAYSIA

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ABSTRACT. Rabies is a major fatal zoonotic disease among dog population in some parts of Malaysia and the risk of human rabies is thought to be steadily high in this region. This study was conducted to determine the recent dynamics of rabies virus (RABV) in Peninsular Malaysia and Sarawak. cDNA from 17 rabies viruses were selected and amplified by nested PCR, followed by sequencing and phylogenetic analysis on the N protein. Based on the partial sequences of N gene (460 bp), all 17 viruses shared almost 99% similarity with each other. BLAST analysis showed that nucleotides of the N gene was more than 98% similar to the Sulawesi and Kalimantan rabies viruses. Among these 17 positive cases, one from Perak (VRI 7687/2017) showed some differences between nucleotides probably due to adaptation and survival activities. Based on the phylogenetic analysis constructed, all these positive cases detected in Malaysia were clustered under the lineage of rabies viruses from Indonesia. In comparison, reported cases detected in 2016 were under the South-east Asia (SEA) lineage. The differences between rabies cases of 2016 and 2017 showed that there are different lineages of viruses circulating in the dog population in Malaysia. The detection of the Indonesian lineage in peninsular Malaysia for the first time raises an alarm about the potential of the viruses becoming an epidemic if uncontrolled. Activities like vaccination of target population, post-antibody screening programmes, rapid diagnostics capabilities, risk assessments and awareness programmes for the public, are needed to prevent the virus from spreading to other parts of the country.

Keywords: rabies, phylogenetic analysis, zoonotic, dog, virus

INTRODUCTION

The rabies virus (RABV) belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*. It was enzootic throughout most of the world. Domestic dog acts as the principle vector but according to Childs and Real (2007), a range of mammalian carnivores also can act as a host. Rabies remains the only disease which can cause 100% mortality and, thus, millions of animals are killed each year in disease control programmes (Knobel *et al.*, 2005). The susceptibility to rabies infection depends on the viral strain, genetic makeup of the host, concentration of the neurotransmitter receptors at the bite site, inoculum size and the most important is the proximity of the bite to the central nervous system. Rabies is a neurological disease but

the rabies virus has been found in several organs outside the central nervous system (CNS). The rabies virus antigens or its RNA has been identified from salivary glands, lungs, kidneys, heart and liver (Luiz F. P. Viera *et al*., 2011). Salivary glands can be infectious depending on the centrifugal neural spread of the virus from the CNS and its transfer from axons to glandular epithelial cells.

In Malaysia, rabies is a public health problem, mainly along the Malaysian-Thailand and Malaysian (Sarawak)-Indonesian (Kalimantan) borders (Ganesan, 1993). The disease, along both these borders has been periodically introduced through infected dogs crossing over from neighbouring countries. Rabies is still being reported in a few states in Malaysia. At the time of this study, there has been a total of 15 human deaths recorded since the start of rabies outbreaks in Malaysia. According to the Director-General of Health Malaysia (kpkesihatan.com, 2018), rabies infections in Sarawak has spread to 54 areas, and two in Peninsular Malaysia, that is, in Bukit Gantang and Selama.

The rabies virus has a non-segmented single-stranded RNA genome approximately 12 kb in size (Tordo *et al*., 1986). It encodes five types of proteins which are nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA polymerase or large protein (L). According to Nyoman Dibia *et al. (*2015), the nucleotide sequence of nucleoprotein (N) gene fragment has been studied extensively and is used as a tool to clarify the lineage of rabies viruses studied. In this study, the N gene has been chosen because of its highly conserved nature which allows the viral strains to be accurately differentiated by analysing genetic differences that are present within the gene (Johnson *et al*., 2002). Many molecular epidemiological studies have been performed by targeting either the selected region of the N gene or the entire N gene of the rabies virus (Yamagata *et al*., 2007).

Considered as gold standard methods for rabies virus diagnosis, the fluorescence antibody test (FAT) (Dean, 1973) and the mouse inoculation test (MIT) (Koprowski, 1973) are time-consuming and have variable specificity and sensitivity. Thus, reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of rabies virus were developed and proposed by Heaton *et al.* (1997) and various researchers. Due to its higher sensitivity compared to FAT (Heaton *et al*., 1997), RT-PCR can be used to detect the presence of rabies virus from naturally decomposed samples (David *et al.,* 2002). Moreover, the RT-PCR allows genetic characterisation of the virus which are very important for epidemiological studies. With such information, the origin of the virus can be studied and future plans for disease control and surveillance by the authorities could be taken to prevent the virus from spreading to other areas. In this study, a total of 17 samples collected in 2017, were tested positive for rabies virus, and were then characterised to provide information on its outbreak in Malaysia. It was then compared with reported cases of 2016 in ASEAN countries.

MATERIAL AND METHODS

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction

Viral RNA was extracted from 200 µl of each processed brain sample using QIAamp Cador Pathogen Kit (Qiagen, USA), according to the manufacturer's instructions. The RNA was subjected to cDNA synthesis using primer RabN1 (30 pmol/µl) and subjected to 65 °C for 10 minutes and was later snap-cooled on ice and briefly spun down. cDNA synthesis was done using iScript™ cDNA Synthesis Kit (BioRad, USA). Reverse transcriptase mix was prepared and subjected to 25 °C for 5 minutes for priming process, 46 °C for 20 minutes (reverse transcription), 95 °C for 1 minute (RT inactivation) and 4 °C in the thermal cycler (Biorad T100).

Nested RT-PCR was carried out using GoTaq® Green Master Mix (Promega). Specific primer pairs Rab N1, Rab N5, RabNfor and RabNrev as described by Nadin-Davis (1998) were used. Upon PCR completion, the PCR reaction mixture was loaded into 1.5% agarose gel containing SyBr Safe (Invitrogen) for electrophoresis and visualised with a UV transilluminator.

Nucleotide Sequencing

PCR products were excised from agarose gel and purified using QIAQuick Gel Extraction Kit (Qiagen) prior to Sanger sequencing. Sequencing was performed by First Base Laboratories (First Base, Malaysia). The primers used for sequence analysis were the same as those used for PCR amplification. The raw sequences were manually edited and assembled using Seqman (DNAStar Lasergene, USA). The sequences were compared with sequences accessible in the GenBank® database (International Nucleotide Sequence Database Collaboration, 2019) using Basic Local Alignment Search Tool (BLAST) algorithm (National Center for Biotechnology Information).

Phylogenetic and sequence similarity analysis

Nucleotide sequences were then analysed using software BioEdit 7.2.5 (Tom Hall/Ibis Therapeutics, USA) and Clustal W multiple alignment method (Thomson J.D., Higgins D.G. and Gibson T.J., 1994). The isolates in this study, together with other rabies sequences from GenBank®, were included for phylogenetic analysis. The phylogenetic tree was constructed with MEGA v6.06 using neighbour joining Kimura 2 parameter model with 1,000 bootstrapped replications (Tamura K. *et. al*; 2013). Phylogenetic analysis was generated based on N gene (470 bp). The similarities of the nucleotide and amino acid sequences were calculated using BioEdit 7.2.5.

RESULTS AND DISCUSSION

The N gene of 17 brains samples collected in 2017 from endemic areas in Malaysia were successfully amplified using nested RT-PCR. A total of 16 samples were obtained from Sarawak and one from Perak (north of Peninsular Malaysia). The length of readable cDNA sequences of the 17 RABVs was 460 bp. Based on partial sequence analysis of the N

Table 1. Differences of amino acid between 2016 and 2017 rabies virus detected in Malaysia

Amino acid position	2016 rabies virus	2017 rabies virus
84	Serine	Threonine
119	Asparagine	Serine
128	Valine	Leucine
135	Alanine	Serine
375	Methionine	Threonine
426	Alanine	Serine
443	Serine	Asparagine

gene, it was found that all 17 viruses were identical with 99% similarity to each other.

Using BLAST analysis, 16 of 17 RABVs viruses detected in Sarawak shared 99% homology similarities with Indonesian rabies viruses (SW01-11) from Sulawesi, Indonesia, while the positive RABVs from Perak showed 98% similarities with (KL177-09) from Kalimantan, Indonesia. There were some differences of nucleotide from positive cases from Perak compared with the rest from Sarawak probably due to the virus adaptation and survival activities.

The phylogenetic tree of the 17 RABVs is shown in Figure 1. All 17 samples were clustered under the same group located under the same lineage with the Indonesia subgroup. In comparison, the rabies viruses detected in Penang in 2016 were clustered under another subgroup called South-east Asian (SEA) lineage. Both lineages were identical with 77% similarity.

Rabies virus N gene consists of 1500 bp of amino acids. Based on a partial N gene amino acid sequence, the 2016 RABV showed some differences compared to the 2017 RABV (Table 1).

The difference of multiple amino acids in different positions showed that both 2016 and 2017 rabies viruses were in separate lineages. As shown in Figure 1, the 2016 RABV were under the SEA lineage and the 2017 viruses were clustered under the Indonesian lineage. Both lineage has 77% similarity of amino acids in their partial N gene. The difference was estimated around 23% indicating the different areas of origin and distribution.

Interestingly, the 2017 rabies virus detected in Perak was similar to the 2017 rabies viruses detected in Sarawak. The case was detected during the Sarawak rabies outbreak. Based on the geographical situation, the chances of the Sarawak outbreak spreading to Perak was very minimum. Once the Perak case was confirmed as rabies, a group of investigators and the health unit of the Department of Veterinary Services (DVS) carried out a surveillance sampling of the infected area with negative results for rabies. On further investigation, the infection was deduced to be from a direct source, the interaction of the dog with an infected Indonesian dog on a shared fishing vessel during a joint

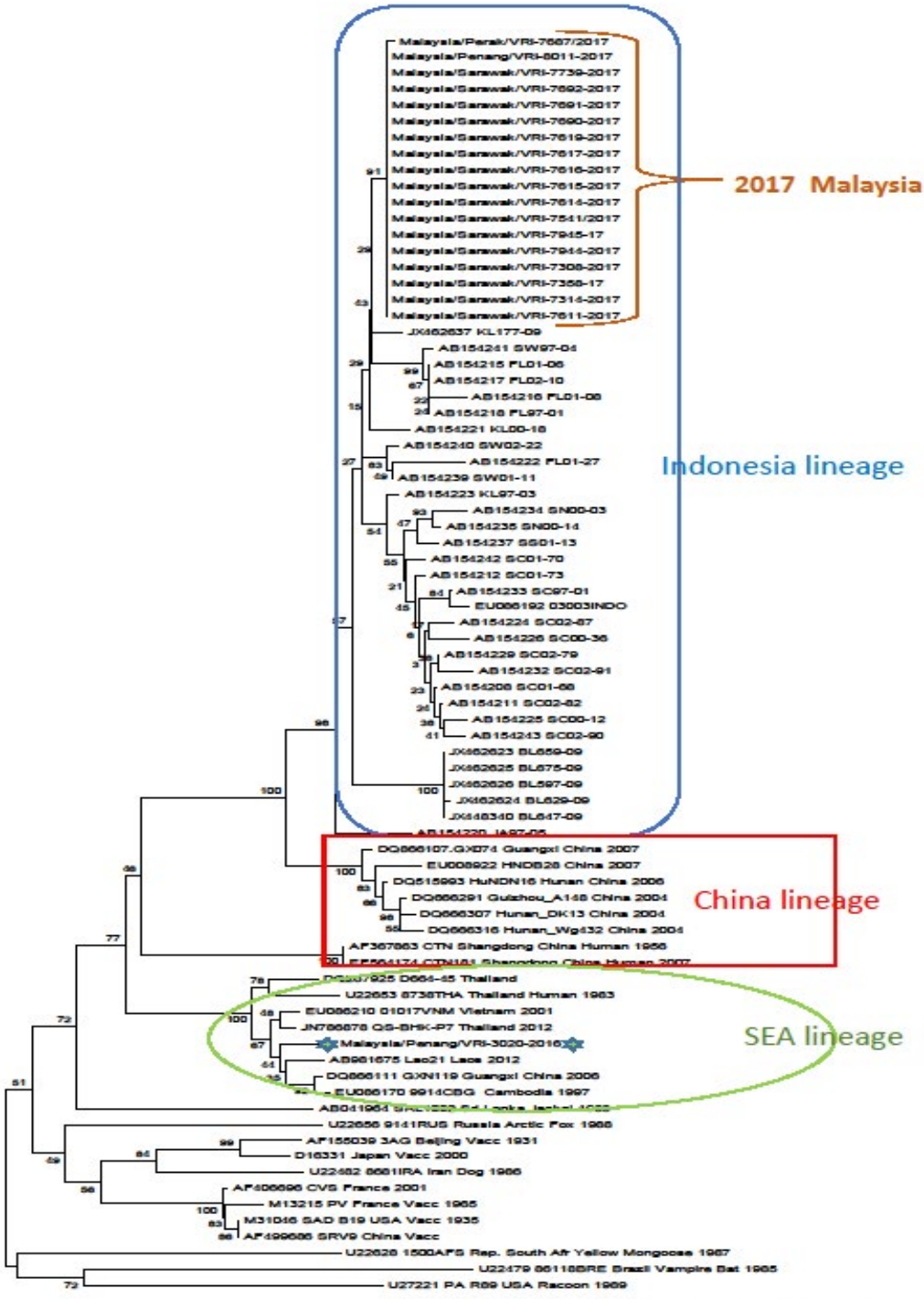

Figure 1. Phylogenetic tree constructed based on 460 bp long of nucleotide sequences of N gene of Malaysia rabies viruses and other rabies isolates of several countries.

fishing activity in the Straits of Malacca. This explains why the virus belongs to the Indonesian strain instead of the SEA strain of the cases in 2016.

Sarawak outbreak was caused by the interaction between stray dogs and pets with infected dogs from rabies endemic area in Kalimantan, Indonesia. It is believed that the spreading of rabies viruses in stray dog populations and pets in the villagers occurred near the international borders of Malaysia and Indonesia. Based on the geographical proximity, there is no barrier preventing the dogs from crossing over the border. Dogs and other animals freely travel across the countries, possibly carrying and spreading viruses into other villages. Active surveillance and vaccination programmes for pets and stray dogs were conducted by DVS to prevent the spread of rabies virus to new areas.

CONCLUSION

In conclusion, this study has found that the 2017 RABVs infection in dog population in Malaysia originated from Indonesia unlike RABV infections in 2016 which were found to be under the SEA lineage. The introduction of the Indonesian lineage in Peninsular Malaysia for the first time raised an alarm on the potential of the viruses becoming an epidemic if uncontrolled. Activities like vaccination of targeted population, post-antibody screening programmes, rapid diagnostics capabilities, risk assessments and awareness programmes for the public are needed to prevent the virus from spreading to other parts of the country. It was also found that the immune belt strategy can be used not only for pets, but also for other stray mammalians to minimise the chances of being infected with the rabies virus.

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