UNIVERSITI PUTRA MALAYSIA

SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS (JEV) IN LONG-TAILED MACAQUE (MACACA FASCICULARIS) IN PENINSULAR MALAYSIA

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SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS (JEV) IN LONG-TAILED MACAQUE (*Macaca fascicularis)* IN PENINSULAR MALAYSIA

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Faculty of Veterinary Medicine, Universiti Putra Malaysia
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Seroprevalence of Japanese Encephalitis Virus (JEV) in Long-tailed Macaque (*Macaca fascicularis*) in Peninsular Malaysia", by Norsuzana Binti Hashim and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project

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DEDICATION

The love of my life, my parents

(Hashim Bin Ali & Tuan Yam Binti Tuan Hassan),

My siblings

(Nik Siti Rahami, Nik Siti Rashidah, Siti Riyani,

Umirah, Nor Suraya and Nik Aiman Hakimi),

My friends,

My teachers,

&

Virology and Wildlife Enthusiasts.

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LIST OF ABBREVIATIONS

%	Percentage
C	Capsid
CDC	Centres for Disease Control and Prevention
CF	Complement Fixation
CSF	Cerebrospinal fluid
DVS	Department of Veterinary Service
E	Envelope
ELISA	Enzyme-Linked Immunosorbent Assay
G	gravity/g-force
HI	Hemagglutination Inhibition
IgG	Immunoglobulin G
IUCN	International Union Conservation of Nature
JE	Japanese Encephalitis
JEAb-IgG	Japanese Encephalitis Antibody Immuno Globulin
JEAb-IgG-HRP	Japanese Encephalitis Antibody Immuno Globulin horseradish peroxidase
JEV	Japanese Encephalitis Virus
Kb	Kilobase
Ml	Millilitre
Nm	Nanometre
NS1	Non-structural protein- 1
NS2A	Non-structural protein- 2A
NS2B	Non-structural protein- 2B

NS3	Non-structural protein- 3
NS4A	Non-structural protein- 4A
NS4B	Non-structural protein- 4B
NS5	Non-structural protein- 5
ºC	Degree Celsius
PCR	Polymerase Chain Reaction
PrM	Pre-Membrane
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
VN	Viral Neutralisation
WHO	World Health Organization
WNV	West Nile Virus
µl	Microliter

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

SEROPREVALENS JAPANESE ENCEPHALITIS VIRUS DALAM KALANGAN KERA (*Macaca fascicularis*) DI SEMENANJUNG MALAYSIA

Oleh

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2016

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Japanese Encephalitis Virus (JEV) adalah diantara viral encephalitis terpenting di Asia yang disebabkan oleh positif sense RNA virus yang bersarung daripada genus *Flaviviridae* dan dari famili *Flavivirus*. JEV dikekalkan dalam kitaran zoonotik dimana nyamuk sebagai vektor pembawa penyakit terutamanya spesies *Culex tritaeniorhychus*. Babi dan burung akuatik merupakan perumah pengganda virus JE manakala manusia, kuda, haiwan domestik dan hidupan liar adalah host terakhir kepada virus ini. Menurut WHO, JEV adalah endemik di negara Asia Tenggara dan Pasifik Barat termasuklah Malaysia. Namun

begitu, tiada kajian dijalankan terhadap prevalens antibodi JEV dalam populasi kera di Malaysia. Justeru itu, kajian ini dijalankan unuk mengetahui JEV seroprevalens didalam populasi kera di Semenanjung Malaysia. Sejumlah 44 sampel serum dari kera (*Macaca fascicularis*) diperoleh daripada Makmal Parasitologi, Fakulti Perubatan Veterinar, Universiti Putra Malaysia. Sampel tersebut diperoleh daripada Kelantan, Pahang dan Perlis dan dari pelbagai jenis umur, jantina dan habitat. Ujian serologi dijalankan mengunakan kit ELISA Monkey Japanese Encephalitis Antibody IgG. Hasil ELISA mendapati, 9 daripada 44 sampel kera adalah positif terhadap antibodi JEV (20.45%) dan Kelantan menunjukkan prevalens yang paling tinggi berbanding negeri-negeri lain. Kesimpulannya, kajian ini mendapati kera di Malaysia pernah mengalami infeksi terhadap JEV. Kajian pada masa hadapan perlu ditekankan terhadap pengesanan genotype yang terdapat dikalangan kera melalui kajian molecular.

Kata kunci: Japanese Encephalitis Virus, kompetitif ELISA, kera, *Macaca fascicularis*, seropositif, prevalens.

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project.

Seroprevalence of Japanese Encephalitis Virus Infection in Long-Tailed Macaque (*Macaca fascicularis*) in Peninsular Malaysia

By Norsuzana Binti Hashim

2016

Supervisor: Assoc. Prof. Dr Siti Suri Arshad

Co-supervisors: Dr Reuben Sunil Kumar Sharma

Dr Nor Yasmin Abd Rahaman

Japanese Encephalitis Virus (JEV) is among the most important viral encephalitis in Asia caused by an envelope single stranded positive sense RNA virus genus of *Flaviviridae* and from family *Flavivirus*. JEV is maintained in zoonotic cycle with mosquito as vector, principally *Culex tritaeniorhychus*. Pig and aquatic bird are the reservoir and amplifying host whereas human, horse, wild and domestic mammals are the dead end hosts. According to WHO, JEV is widely endemic in the Southeast Asia and Western Pacific regions including Malaysia. However, there is no studies have been carried out to determine the prevalence of JEV antibody in macaque in Malaysia. Thus, this study was conducted to determine the seroprevalence of JEV in macaque in Peninsular

Malaysia. A total of 44 serum samples of long-tailed macaque (*Macaca fascicularis*) collected from Pahang, Kelantan and Perlis and from different ages, sex and habitats were analyzed using quantitative competitive ELISA test. The result revealed that 9 of 44 monkeys (20.45%) were seropositive for JEV with highest prevalence in Kelantan (33.33%) compared to others state. As a conclusion, this study revealed that long tailed macaque in Peninsular Malaysia have been exposed to JEV infection. Further study should determine the genotype of the virus circulating on the macaque population by molecular studies.

Keywords: Japanese Encephalitis Virus, competitive ELISA, long-tailed macaque, *Macaca fascicularis*, seropositive, prevalence.

1.0 INTRODUCTION

Japanese Encephalitis Virus (JEV) is a single-stranded RNA virus serogroup of the genus *Flavivirus*, family *Flaviviridae.* The virus is transmitted between vertebrae hosts by mosquitoes vector, principally *Culex tritaeniorhynchus* although other species of genera *Culex, Monsonia, Aedes* and *Anopheles* have yielded isolates of JEV. The vertebrae hosts of JEV are humans and domestic animals with aquatic birds playing a major amplifying role. Other JE serogroup of the genus flaviviruses include West Nile virus (WNV), St. Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV), and together they form the mosquito-borne encephalitis complex (Zuckerman et al., 2009).

JEV is distributed in temperate and tropical areas of eastern and southern Asia. Its geographic range extends from eastern Asia (China, Japan, Korea maritime Siberia, Taiwan, the Philippines and Vietnam), to Southeast Asia and northern Australasia (Cambodia, Indonesia, Laos, Malaysia, Papua New Guinea, Thailand, and the Torres Straits islands) Van den Hurk et al. (2009). There are two epidemiological patterns of JEV which are epidemic activity in temperate and subtropical regions which sporadic cases of encephalitis occur throughout the year and endemic activity in tropical regions which occur throughout the year. Based on nucleotide sequencing, four genotypes of virus have been identified. According to Solomon et al. (2003), genotypes I and III were isolates from epidemic area include northern Thailand, Cambodia, Korea, Japan, China, Taiwan and Philippines. Genotypes II and IV were isolates from endemic area include southern Thailand, Malaysia, Indonesia, Northern Australia and Indonesia.

It estimated that approximately 67 900 JE cases typically occur annually in the 24 JE endemic countries and exposing more than 3 billion people to risks of infection reported by WHO (2014). In Malaysia, outbreaks of JE occurred in Langkawi in 1974 with 10 cases and two deaths, Penang in 1988 with nine cases and four deaths, and in the Serian district of Sarawak in 1992 with nine cases and four deaths (Ismail, 2014). According to the Health Ministry, a total of 16 JE cases have been reported nationwide in 2014, including four deaths.

There are several studies had been done on JEV in animals especially macaque. According to Myint et al. (1999), rhesus macaques are highly susceptible to a lethal infection that resembles fatal human disease by intranasal inoculation with JEV. Furthermore, study conducted in Philippines revealed that *Macaca fascicularis* can be naturally infected by JEV with consistent antibody prevalence rate of 35.2% using indirect IgG ELISA (Inoue et al., 2003). In addition, a serosurvey of JEV in Japanese macaque *(Macaca fuscata)* in Japan revealed that 44% were seropositive for JEV by using ELISA (Shimoda et al., 2014). Besides that, according to Nakgoi et al. (2014), revealed that 9 out of 38 Captive Monkey (*Macaca nemestrina*) colonies in Northern Thailand, were seropositive against Japanese Encephalitis Virus by using plaque reduction neutralisation test (PNRT) method.

However, there is lack of study of JEV in Malaysia. In addition to that, the prevalence of JEV in primates has not been documented in Malaysia. Thus, justifications for this study are JEV is a zoonotic disease which is endemic in Malaysia and the prevalence of JEV antibodies in macaque has not been documented in Peninsular

Thus, the objectives for this study includes:

1. To determine the seroprevalence of Japanese Encephalitis in macaque in Peninsular Malaysia.

The hypotheses for this study was:

1. There is prevalence of seroconversion against JEV in Peninsular Malaysia.

2.0 LITERATURE REVIEW

2.1 Japanese Encephalitis

2.1.1 Japanese Encephalitis Virus

Japanese Encephalitis Virus (JEV) is a spherical, enveloped virus about 50 nm in diameter containing a single-stranded positive-sense RNA genome of approximately 11 kb in length single-stranded RNA virus, serogroup of the family *Flaviviridae* and genus *Flavivirus*. The viral genome encodes three structural proteins (capsid [C], precursor membrane [prM], and envelope [E] proteins) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Murphy et al,. 1999) Figure 1.

Figure 1: Japanese encephalitis genome organization (Shailendra et al., 2013)

Flaviviridae belongs to Arbovirus together with *Togaviridae, Bunyaviridae, Rhabdoviridae, Reoviridae, Orthomyxoviridae* and *Iridoviridae* families (Go et al., 2014; Mohammed et al., 2011). By definition, arthropod borne viruses (Arbovirus), have a transmission cycle, which always involves a hematophagous arthropod vector, such as mosquitoes or ticks among others. The virus is replicate in the vectors and the vectors can transmit the virus from one vertebrate host to another (Meltzer, 2012).

2.1.2 Transmission cycle

JEV is maintained in zoonotic (enzootic) cycle and transmitted between vertebrae hosts by mosquitoes vector, principally *Culex tritaeniorhynchus*. This mosquito vector mainly hatches in rice fields in Asian countries, with multiplication increasing up to 50% in fertilized fields. Other species of genera *Culex, Monsonia, Aedes* and *Anopheles* also have yielded isolates of JEV (Zuckerman et al., 2009). Aquatic bird and pig are the efficient amplifying host and reservoir hosts of JEV that develop high-titered viraemia providing a source of infection for mosquitos. According to Rosen (1986), pigs and waterbirds increase the risk transmission to humans and equines, especially in agricultural settings such as rice cultivation areas. This give an epidemiological advantage of JEV in rice crops and pig farming combination. The dead-end host of JEV is human and horses since they do not develop sufficient level of viraemia for mosquito transmission and they succumb to encephalitis after infection (Go et al., 2014). The transmission cycle of JEV can be illustrated in Figure 2.

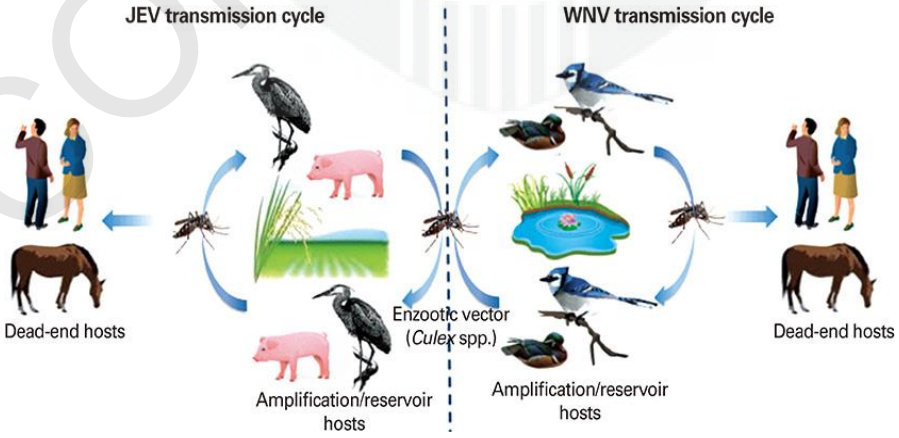


Figure 2: Enzootic and epizootic/epidemic transmission cycles of Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Go et al., 2014).

2.1.3 Geographical range and Epidemiology patterns

JEV was first documented as viral encephalitis in the 1870s and isolated in 1935 from the brain of a fatal human encephalitis case in the Tokyo, Japan (Burke and Leake, 1988). JEV is distributed in temperate and tropical areas of eastern and southern Asia. Its geographic range extends from eastern Asia (China, Japan, Korea maritime Siberia, Taiwan, the Philippines and Vietnam), to Southeast Asia and northern Australasia (Cambodia, Indonesia, Laos, Malaysia, Papua New Guinea, Thailand, and the Torres Straits islands) (Van den Hurk et al., 2009) as in Figure 3. There are two epidemiological patterns of JEV which are epidemic activity in temperate and subtropical regions which sporadic cases of encephalitis occur throughout the year and endemic activity in tropical regions which occur throughout the year. Based on nucleotide sequencing, five genotypes of virus have been identified. According to Solomon et al. (2003), genotypes I and III were isolates from epidemic area include northern Thailand, Cambodia, Korea, Japan, China, Taiwan and Phillippines. Genotypes II and IV were isolates from endemic area include southern Thailand, Malaysia, Indonesia, Northern Australia and Indonesia. Recently, Genotype V has been isolate from Muar region, Malaysia (Mohammed et al., 2011).

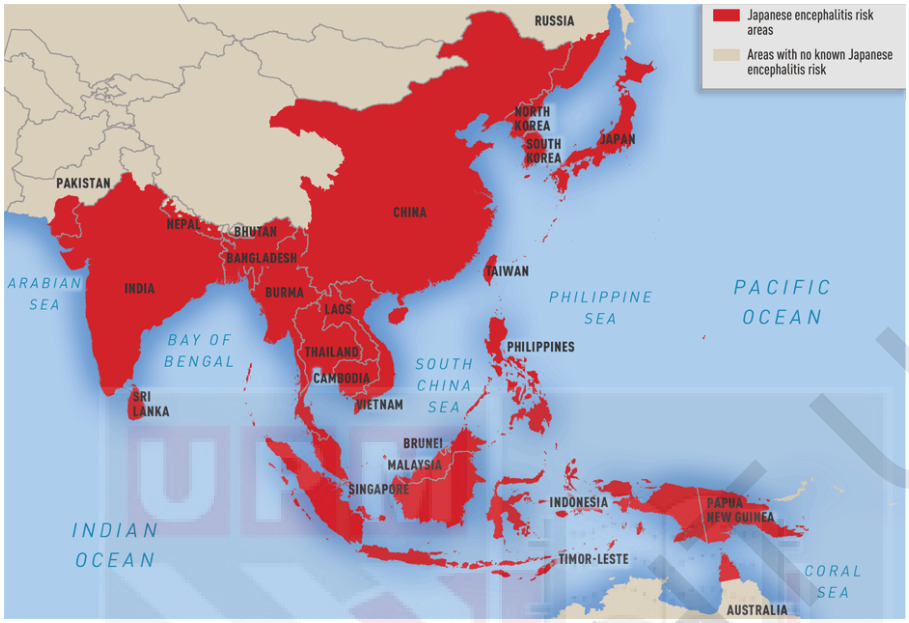


Figure 3: Geographic distribution of Japanese encephalitis (CDC, 2016)

2.1.4 Clinical signs

It estimated that approximately 67 900 JE cases typically occur annually in the 24 JE endemic countries and exposing more than 3 billion people to risks of infection reported by WHO (2014). In Malaysia, first JEV cases was reported in 1952 and first outbreaks of JE occurred in Langkawi in 1974 with 10 cases and two deaths, Penang in 1988 with nine cases and four deaths, and in the Serian district of Sarawak in 1992 with nine cases and four deaths as claimed by Ismail, 2014. According to the Ministry of Health, a total of 16 JE cases have been reported nationwide in 2014, including four deaths. Most of the patients were adult males who worked on pig farms (Wang & Liang, 2015).

JE is mostly a disease of children and young adults. Rates of infection in the 3 to 15 year age group are five to ten times higher than in older individuals, because of

high background immunity in older individuals (Tiwari et al., 2012). The majority of human infections with encephalitic flaviviruses are asymptomatic or give rise to only a mild febrile (Shailendra et al., 2013). On average, only one in 300 cases produce clinical symptoms. The first signs of infection appear after an incubation period between six and 14 days. It usually starts with a fever above 38°C, chills, muscle pain, and meningitis-type headaches accompanied by vomiting. These may include confusion, paralysis, Parkinsonian movement disorders, abnormal posturing, seizures, and coma. A proportion of patients with JE have an acute flaccid paralysis that is easily mistaken for poliomyelitis, but the majority present with a reduced level of consciousness, often heralded by generalized convulsions. Fatality is observed in 20 to 30% of the cases, with signs of acute cerebral edema or severe respiratory distress from pulmonary edema. The duration of the coma is associated with repetitive seizures, peduncular damage, or intracranial hypertension, which are considered poor prognostic factors, leading to fatality. Many survivors of JE acquire neuropsychiatric sequelae with learning difficulties, behavioural problems and language impairment (Tiwari et al., 2012).

2.1.5 Diagnosis of JEV

There are several diagnostic method to diagnose JEV such as cell/tissue culture, antigen detection, and antibody detection. According to WHO, 2007, isolation of JEV must take account of the risk and require laboratory biosafety level 3 (BSL-3), and staff should be vaccinated against JE. JEV can be isolated from CSF or from brain tissue samples of fatal cases. After isolation, virus can be confirmed and identified using: appropriate polyclonal or monoclonal antibodies, by indirect

immunofluorescence, by RT-PCR using JEV specific primers, or by nucleotide sequencing.

For serological testing, viral neutralisation (VN) test, hemagglutination inhibition (HI) assay, complement fixation (CF) and enzyme-linked immunosorbent assay (ELISA) are used in laboratory. There is serological cross reactivity with other flaviviruses, such as West Nile Virus, which can confuse the diagnosis (OIE, 2010). However, VN test is considered as "gold standard" for serological differentiation of flavivirus infections (Maeda et al., 2013). VN test requires a special facility, high level of technical skill and large volume of serum samples. In contrast, ELISA does not require the use of live virus and procedure is simple and requires only a small amount of serum samples.

2.2 LONG-TAILED MACAQUE

2.2.1 Long-tailed macaque (*Macaca fascicularis)*

Macaca fascicularis also known as crab-eating Macaque, long-tailed Macaque or cynomolgus Monkey is in the orders Primates and family of Cercopithecidae. This species is listed as Least Concern in view of its wide distribution, presumed large population, tolerance of a broad range of habitats, occurrence in a number of protected areas, and because it is unlikely to be declining at nearly the rate required to qualify for listing in a threatened category (IUCN, 2008). This macaques were extinguished by extraordinary long tail length, light brown or grayish to brown fur and present of cheek whiskers (Groves, 2005). Although it is under heavy hunting pressure for meat, sport and trophies, this is not considered a major threat to the species overall. This species is native in Bangladesh,

Brunei Darussalam, Cambodia, India, Indonesia, Myanmar, Philippines, Singapore, Thailand, Timor-Leste, Viet Nam and Malaysia as in Figure 4. According to Karimullah & Shahrul, (2012), this species is the largest distribution of all macaque species in Malaysia. The species is extremely tolerant of a range of habitats, including mangrove and swamp forests, and can be found in agricultural areas near forest. This species is semi-terrestrial, diurnal, and omnivorous (Ong et al., 2008).

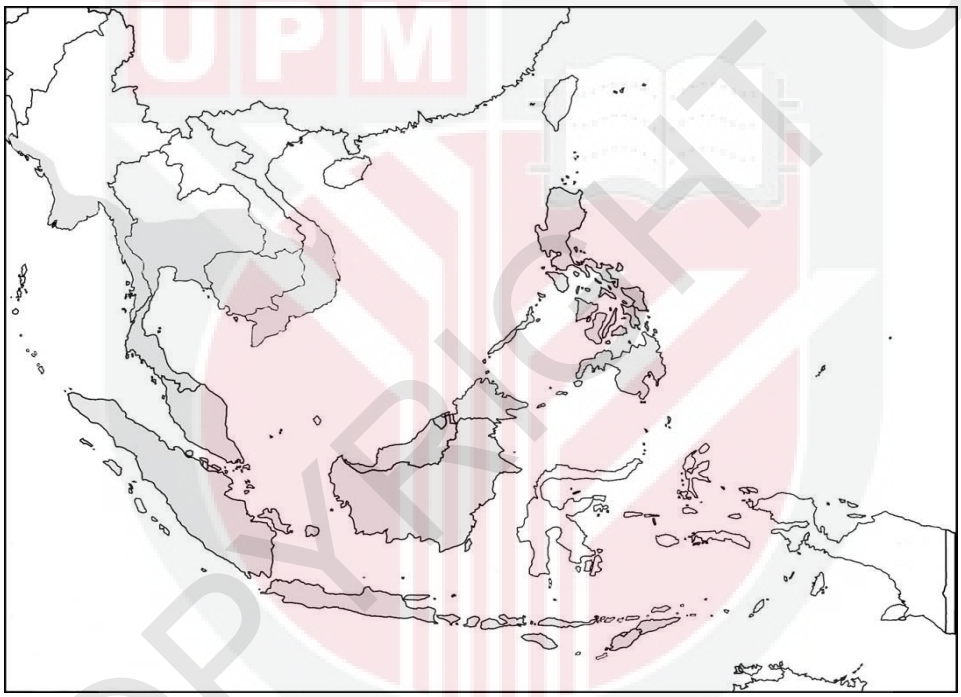


Figure 4: Distribution of long tailed macaque in Asia (Gumert et al., 2011)

2.2.2 Serological prevalence of JEV in macaque

There are several studies had been done on JEV in animals especially macaque. According to (Myint et al., 1999), rhesus macaques are highly susceptible to a lethal infection that resembles fatal human disease by intranasal inoculation with JEV.

Study conducted in Philippines revealed that *Macaca fascicularis* can be naturally infected by JEV with consistent antibody prevalence rate of 35.2% using indirect

IgG ELISA (Inoue et al., 2003). In addition, a serosurvey of JEV in Japanese macaque *(Macaca fuscata)* in Japan revealed that 146 of 332 monkeys (44%) were seropositive for JEV using ELISA (Shimoda et al., 2014). Besides that, according to Nakgoi et al. (2014), revealed that 9 out of 38 Captive Monkey (*Macaca nemestrina*) colonies in Northern Thailand, were seropositive against Japanese Encephalitis Virus by using plaque reduction neutralisation test (PRNT).

4.0 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

According to Aydin (2015), there are several types of ELISA. In direct ELISA, the surface of the plate is coated directly with the antibody or antigen. An enzyme tagged antibody or antigen enables the measurement. Incubation is followed by washing which removes the unbound antigens or antibodies from the medium. Then the appropriate substrate is added to the medium to produce a signal through coloration. The signal is measured to determine the amount of the antigen or antibody.

In indirect ELISA, the diseased serum is added to the antigen-coated wells and the plates are incubated. During this incubation, the antibodies formed against the

antigens in the diseased serum plaque produce an antigen–antibody complex. In order to render the antigen–antibody complex visible, a secondary antibody that recognizes the antibody in the serum and that is tagged with the enzyme is added. Then the substrate of the enzyme is added to the medium to produce color and the concentration is determined. This method is called the indirect method is because secondary antibody that is placed in the medium was measured and not the primary antibody.

In sandwich ELISA, the wells are coated with a capture anti-body and blocked. The sample is added to the microplate wells coated with the antibody; then, the plate is incubated for allocate time and washed. Following the washing step, antibodies that are tagged with the enzyme specific to the antigen are added and incubated. In order to reveal the enzyme activity, enzyme substrate is added to the medium and coloration is ensured. As the relevant protein is stuck between two antibody molecules, this method is called Sandwich ELISA.

In competitive ELISA method, the surface of the wells is coated with the antigen-specific antibody or antibody-specific antigen. The sample to be measured and the enzyme-tagged antigen or antibody are placed into the well simultaneously. The tagged and untagged antigen (patient antigen) or antibody molecules compete with each other to bind to the antibody or antigen in the wells. After the wells are washed and enzyme substrate is added, the resulting coloration enables quantifying the concentration. The advantage and differences of ELISA tests are presented in Table 1.

The concentration is determined when the substrate of the enzyme is added to the medium to produce colour. Coloration shows a positive result, while lack of coloration indicates lack of enzymes, or a negative result. There is an inverse proportion in competitive ELISA where the amount of the antigen or the antibody analysed in the serum is low, high absorbance is obtained, while greater quantities produce low absorbance.

Types	Screening	Disadvantage	Advantages
Direct	Antibody	False positive	Low sensitive
Indirect	Antigen/Antibody	Immobilization non-specific	High sensitive
Sandwich	Antigen	-	Very high sensitive
Competitive	Antibody	-	High sensitive

Table 1: Interpretation of the features and the differences between ELISA types (Aydin, 2015)

3.0 MATERIALS AND METHODS

3.1 Sample and data collection

A total of 44 serum samples of long-tailed macaque (*Macaca fascicularis*) were obtained from archived samples in Parasitology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The samples were collected from the year of 2015 to 2016. The samples consisted of 21 males and 23 females from juvenile, subadult and adult age group and were collected from three different states which namely Pahang, Kelantan and Perlis and were subdivided to different habitats which are suburban, urban, plantation and secondary forest. Immediately after blood collection, the blood was transferred into the plain tube (BD Vacutainer®, USA) and allowed to clot for 2 hours in standing position. The tubes were centrifuged at 1000 x *g* for 10 minutes, and the serum was extracted using a micropipette and placed in Eppendorf tube. The serum samples were labelled and stored at -20^{o}C (Sanyo, Japan).

3.2 Enzyme-linked Immunosorbent assay (ELISA)

The presence of JEV-specific IgG antibodies in the monkey serum samples were tested using the commercial Monkey Japanese Encephalitis Antibody IgG (JEAb-IgG) ELISA test kit according to the manufacturer instructions (MyBiosource, USA). Briefly, the assay uses the quantitative competitive enzyme immunoassay technique where the micro-titre plate was pre-coated with anti-JEAb-IgG antibody. The standards and samples were incubated together with JEAb-IgG-HRP conjugate. Since the number of sites is limited, JEAb-IgG from the samples and JEAb-IgG–HRP conjugate will compete for the anti-JEAb-IgG antibody binding sites. After the incubation period, the wells were decanted and washed five times.

Next, the wells were then incubated with substrate horseradish peroxidase (HRP) enzyme. The product of the enzyme-substrate reactions will forms a blue coloured complex. A stop solution is added to stop the reaction, which will then turn into yellow solution and measured in a microplate reader. The intensity of the colour is inversely proportional to the JEAb-IgG concentration.

All kit components and samples were brought into room temperature (20-25°C) before use. 100µl of standard A (0µg/ml), standard B (0.5µg/ml), standard C (1.0µg/ml), Standard D (2.5µg/ml), Standard E (5.0µg/ml), Standard F (10µg/ml) were pipetted into the respective well. Samples were pipetting in duplicate to the microtiter plate. The arrangement of blank, standard and sample were arranged as in Figure 5. A total of 50 µL of Conjugate was added in each well except blank control well and were mixed well. The micro titre plate was covered and incubate for 1 hour at 37°C (Binder, Germany). The content of the wells were washed using automated washer for five times with 350µL diluted wash solution/well/wash and soak for 10 seconds and shake for 5 seconds between each wash (Bio-Rad, USA). Then, the plate was blot dry.

About 50 µL Substrate A and 50µL of Substrate B were added to each well including the blank control well accordingly. The micro plate was covered and incubate for 15 minutes at 37°C. Then, Stop solution was added to each well and mixed well. The Optical Density (O.D) at 450nm was determined using a microplate reader (Tecan, Switzerland).

For result interpretation, standard curve was constructed by plotting mean absorbance for each standard on the x-axis against the concentration on the y-axis and best fit curve was draw through the points on the graph using CurveExpert 1.4.

3.3 Statistical Analysis

Statistical Package for the Social Science (SPSS) 22 was used for statistical evaluation. The categorical data were assessed in cross tabulation analyses using the Chi-squared test and *P* values of <0.05 were considered statistically significant. Odd ratio was calculated using Binary Logistic Regression to study the association of risk factor and the JEV antibodies.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	1	5	9	13	17	21	25	29	33	37	41
B	Blank	1	5	9	13	17	21	25	29	33	37	41
C	Standard A	2	6	10	14	18	22	26	30	34	38	42
D	Standard B	2	6	10	14	18	22	26	30	34	38	42
E	Standard C	3	7	11	15	19	23	27	31	35	39	43
F	Standard D	3	7	11	15	19	23	27	31	35	39	43
G	Standard E	4	8	12	16	20	24	28	32	36	40	44
H	Standard F	4	8	12	16	20	24	28	32	36	40	44

Figure 5: Illustration of sample arrangement on ELISA plate.

Legend:

Blank sample

Standard A, Standard B, Standard C, Standard D, Standard E and Standard F

Macaque serum from Pahang

Macaque serum from Kelantan

Macaque serum from Perlis

4.0 RESULTS

4.1 Determination of the cut off value

To determine the antibodies concentration of the sample, the standard curve was constructed by plotting mean absorbance for each standard (0, 0.5, 1.0, 2.5, 5.0, 10µg/ml) on the x-axis against the concentration on y-axis. Then, best fit curve was draw through the points on the graph using CurveExpert 1.4 giving a significant correlation coefficient (R^2> 0.9868) and y=1/(a+bx^c). Coefficient Data:

a = -1.94722876416E+000

b = 6.66826019477E+000

c = 1.24709175045E+000

Each sample was tested twice and mean absorbance value was calculated with the reference from the standard curve. The positive sample was range between 0.5µg/ml to 10µg/ml according to manufacture protocol.

4.2 Seroprevalence of JEV among macaque.

The results from ELISA revealed that 9 of 44 monkeys (20.45%) were seropositive for JEV. Analysis by state indicate that Kelantan had the highest prevalence of JEV antibodies (33.33%) followed by Perlis (31.58%) and Pahang which is 5.26%. The study also revealed that there is significance association between JEV antibody prevalence and state (Chi-square test, P=0.0443). Odd ratio revealed that Kelantan has 9 times greater risk of seroconversion compared to sample from Pahang whereas Perlis has 8.3 times greater risk than Pahang.

The prevalence of JEV antibodies is highest in juvenile group with 29.31% compared to subadult (21.43%) and adult group (7.69%). Juvenile has 5 times greater risk than adult whereas subadult has 3.2 times risk compared to adult. However, there is no significant associations of between age and the risk of JEV antibody (P= 0.1473).

The prevalence of JEV antibodies is highest in plantation area (40%) compared to urban area (36.36%), secondary forest (16.67%) and suburban area (9.09%). Plantation, urban and secondary forest have 8.8, 7.3, 2.2 times greater to have JEV seroconversion compared to suburban area respectively. There is no association between habitat and the risk of JEV antibody since the P-value is more than 0.05 (P value: 0.2913).

Male group (34.78%) has higher prevalence than female macaque (4.76%). P value is 0.0227 which is lower than 0.05 which indicate there is significant association between JEV antibodies based on sex. Male has 10 times greater risk to have JEV antibodies compared to female.

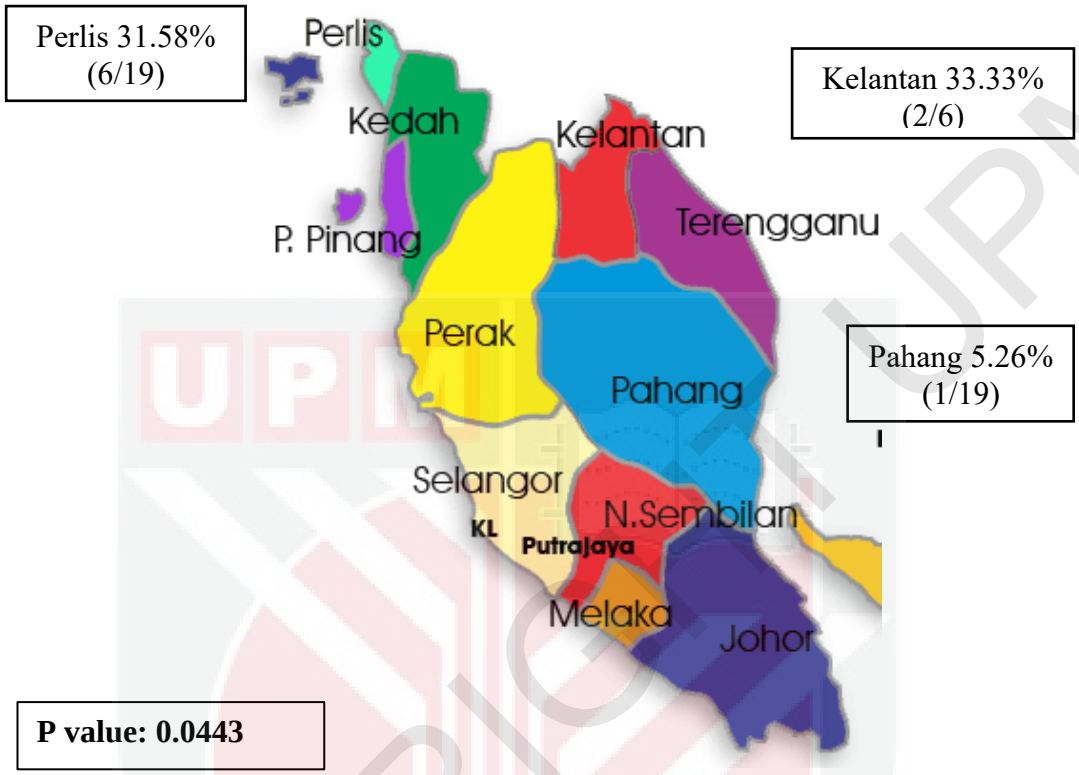
	Age			**State**		
	Juvenile	**Subadult**	**Adult**	**Pahang**	**Kelantan**	**Perlis**
No. of monkey examined	17	14	13	19	6	19
No. of positive monkey	5	3	1	1	2	6
% of positive monkeys	29.41%	21.43%	7.69%	5.26%	33.33%	31.58%

Table 2: Seroprevalence of JEV in long-tailed macaque based on age and state

	Habitat				Sex	
	Suburban	**Urban**	**Plantation**	**Secondary forest**	**Female**	**Male**
No. of monkey examined	22	11	5	6	21	23
No. of positive monkey	2	4	2	1	1	8
% of positive monkeys	9.09%	36.36%	40.00%	16.67%	4.76%	34.78%

Table 3: Seroprevalence of JEV in long-tailed macaque based on habitat and sex

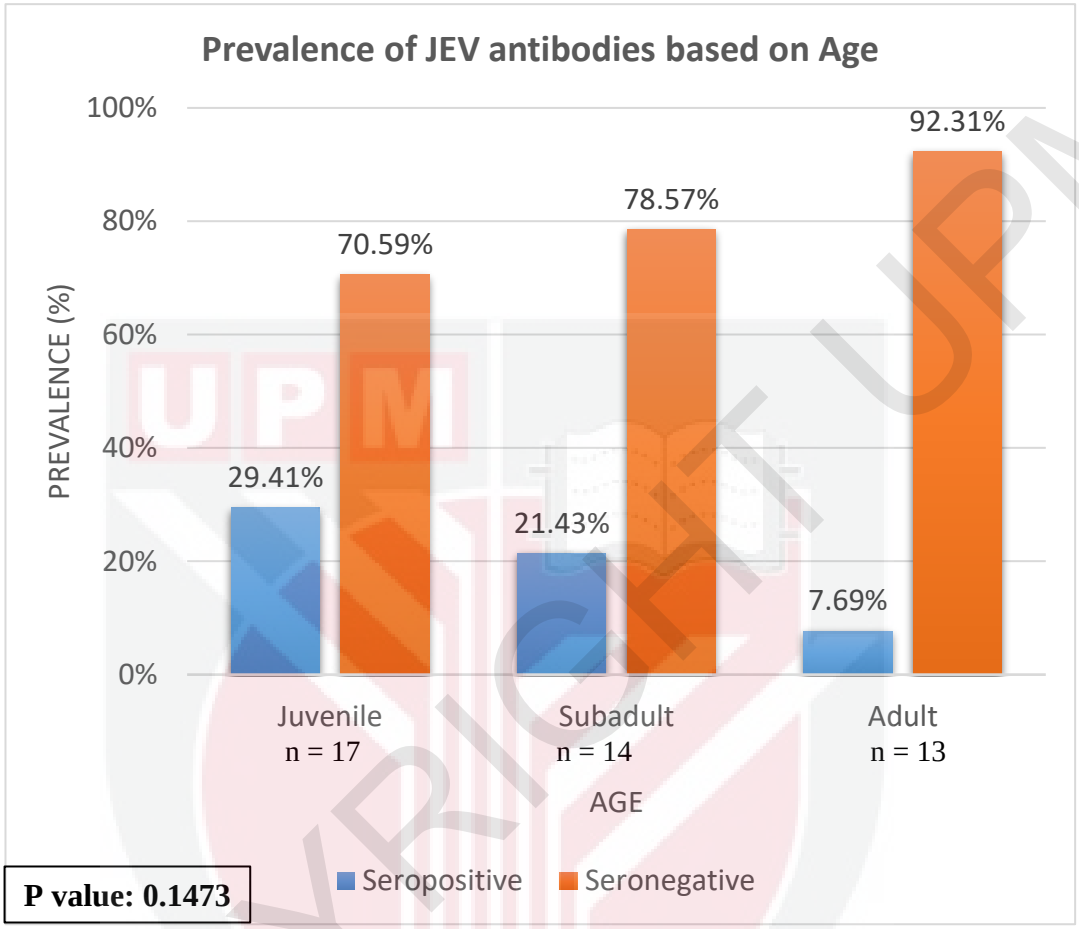
Figure 6: Prevalence of JEV antibodies based on state



State	Odd Ratio (OR)	95% Confidence Interval	P value
Kelantan	9	0.64 to 125.4	0.1326
Perlis	8.3	0.88 to 77.61	0.0897
Pahang	1		

Table 4: Association between state and the risk of JEV antibodies

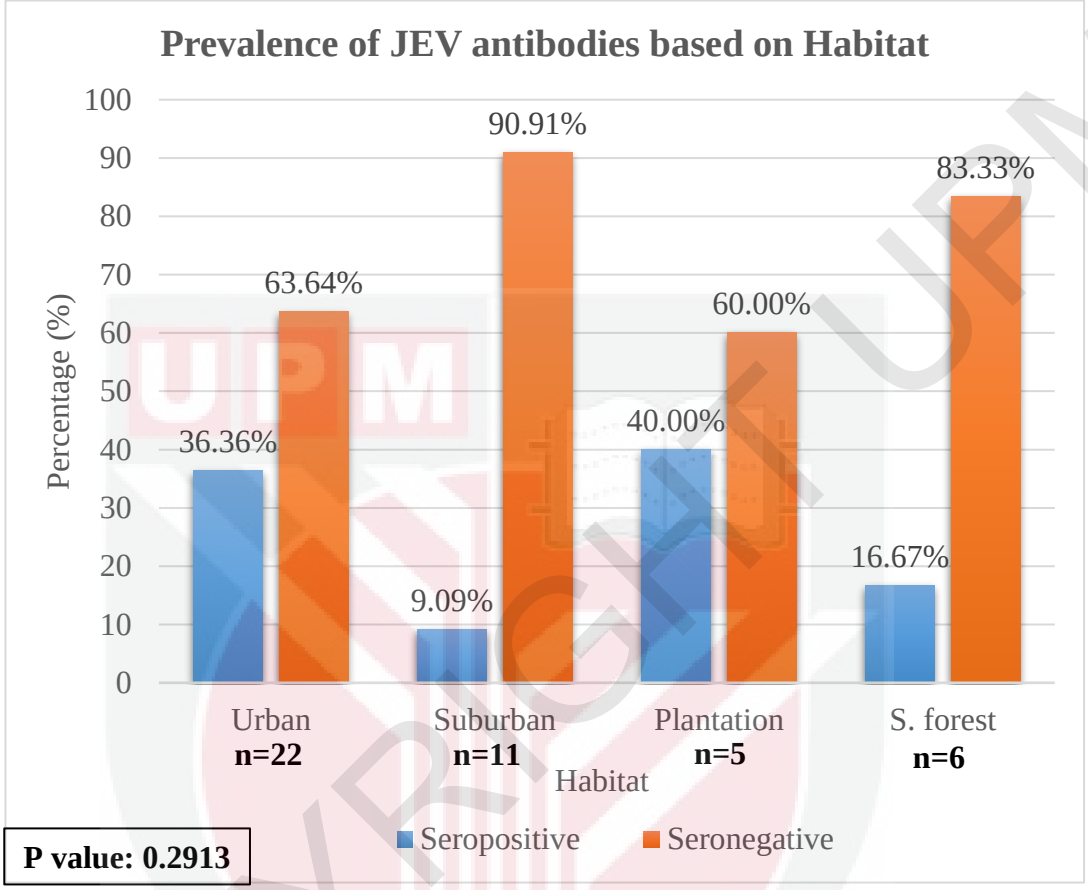
Figure 7: Prevalence of JEV antibodies based on age



Age	Odd Ratio (OR)	95% Confidence Interval	P value
Juvenile	5	0.50 to 49.97	0.169
Subadult	3.2	0.29 to 35.33	0.334
Adult	1		

Table 5: Association between age and the risk of JEV antibodies

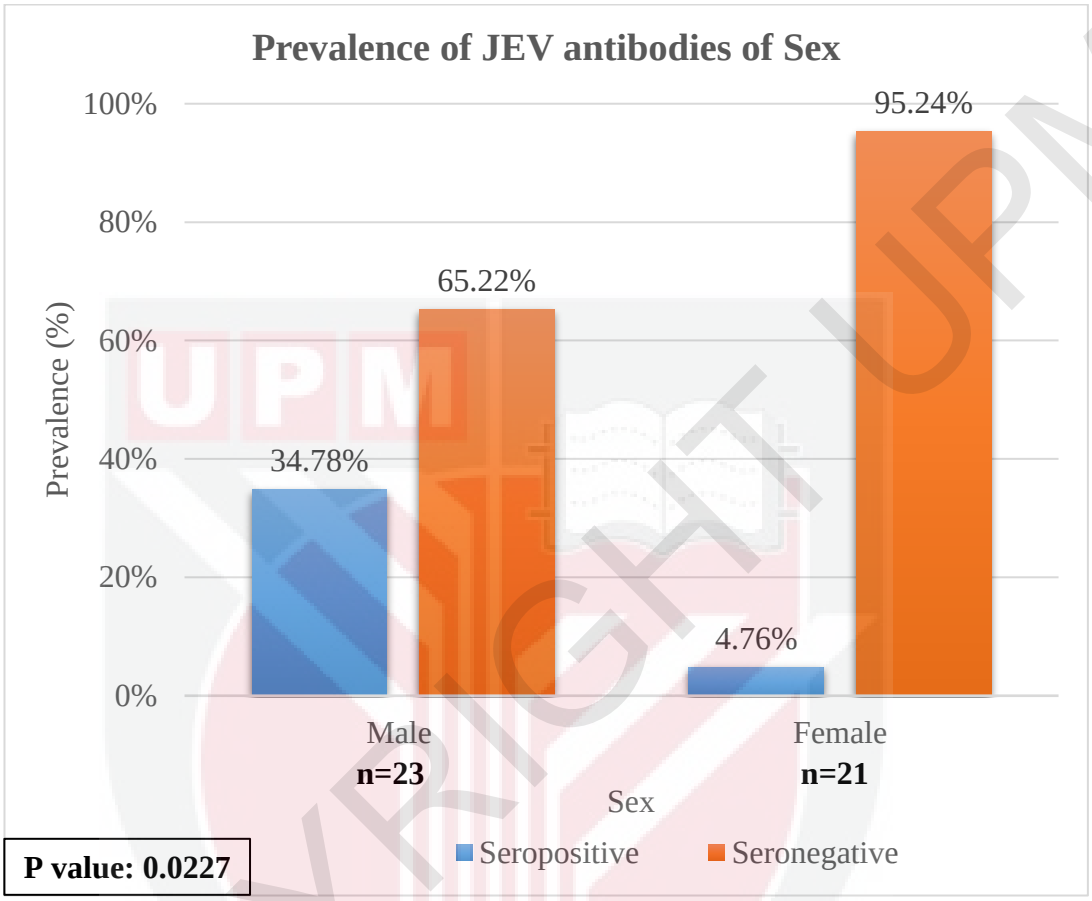
Figure 8: Prevalence of JEV antibodies based on habitat



Habitat	Odd Ratio (OR)	95% Confidence Interval	P value
Plantation	8.8	1.24 to 62.19	0.1444
Urban	7.3	0.73 to 73.24	0.1458
Secondary Forest	2.2	0.85 to 38.34	0.5299
Suburban	1		

Table 6: Association between habitat and the risk of JEV antibodies

Figure 9: Prevalence of JEV antibodies based on sex



Sex	Odd Ratio (OR)	95% Confidence Interval	P value
Male	10	1.2 to 94.79	0.0227
Female	1		

Table 7: Association between sex and the risk of JEV antibodies

DISCUSSION

This study shown that seroprevalence of JEV in monkey was found to be 20.45%. This positive detection rate is consistent with other publications on detection of JEV antibodies. Based on the previous similar studies done in Manila, *Macaca fascicularis* can be naturally infected by JEV with consistent antibody prevalence rate of 35.2% (Inoue et al., 2003). Moreover, a serosurvey of JEV in Japanese macaque (*Macaca fuscata*) revealed that 44% were seropositive for JEV (Shimoda et al., 2014). Besides that, according to Nakgoi et al. (2014) revealed that 9 out of 38 Captive Monkey (*Macaca nemestrina*) colonies in Northern Thailand, were seropositive against Japanese Encephalitis Virus. These results indicate that JEV infection also had occurred among long tailed macaque in Peninsular Malaysia.

Based on this study, Kelantan had shown the highest prevalence of JE which is 33.33% compared to Pahang and Perlis. According to Upadhyay, (2015), JEV outbreak is associated with climate favours epidemic. Most of JEV incidence occur in rainy season due to formation of enlarged surface water covers that supports mass breeding of mosquito vector. In fact, Kelantan had experienced big flood in 2015 which may increase the risk of JE transmission. With increasing humidity, mosquitoes survive longer and disperse further, which increases the chance of transmitting the virus to humans or other susceptible hosts (Wang et al., 2014).

In this study, juvenile group has highest JEV antibodies compared to subadult and adult group. In contrast with others study shown seroprevalence of JEV increased with the age of the monkey. This correlate with the period of exposure to JEV-infected mosquitoes (Shimoda et al., 2014).

In this study, plantation has highest JEV antibodies prevalence may due to present of vegetation, bushes and trees and irrigation hole. This correspond to study conducted by Chen (2009) revealed that ecology comprising mostly of vegetation, bushes and trees which are typical breeding foci of *Aedes albopictus* which is competence to harbour JEV infections.

In addition, there are several factors that can distributed to JEV infections in macaque. As JEV is an arbovirus, it need blood-sucking arthropod to complete their life cycle and to transmit the virus. Beside *Culex tritaeniorhynchus* that can be found in Malaysia, others JEV mosquito vectors such as *Culex gelidus, Culex fuscocephala, Aedes hutleri, Culex quinquefasciatus, Aedes lineatopennis,and Aedes (Cancraedes) sp.* can also be found in Malaysia (Vythilingam et al., 1997). These vectors breed in rice fields, irrigation canals and water pools filled with stagnant water and in standing puddles, open sewers, fish ponds (Shailendra al., 2013). The highest prevalence JEV occurs in rural or agricultural areas, especially associated with irrigated rice agriculture (Inoue et al., 2003). Furthermore, according to FAO, rice cultivation is the major food crop in Malaysia. Thus, it served as a conducive environment for the vector breeding and this eventually increased the mosquito's population in Malaysia. Thus increase the vector population and lead to higher JEV transmission to macaque.

Besides that, increasing vertebrate host population also lead to JEV prevalence and spreading (Upadhyay, 2015). Pig and ardeid birds are the reservoir of JEV and they have high JEV viraemia that become outstanding source of infection for JEV-free mosquitoes to susceptible hosts (Mackenzie et al., 2007). There are

over 90 wild and domestic bird species have been observed to have viremia and/or seroconversion to JEV such as Black-crowned night heron (*Nycticorax nycticorax*), Plumed egrets (*Egretta intermedia*), and Little egrets (*Egretta garzetta*) (Mackenzie et al., 2007). According to Khoon (2016), the species of black-crowned night heron and little egrets are widespread and breeding well in Malaysia. They can be reservoir for JEV introductions in macaque.

Besides that, the standing pig population of Malaysia is estimated at 1.842 million heads in 2013 (DVS, 2015). This population does not include wild boars population where wild boars also harbours the JEV as 4.8% of JEV RNA positivity was detected in wild boar in Selangor (Chiet Wei, 2012). Hence, JEV is possibly transferred to macaque from pig or wild boars during macaque-swine interface as both species live in wild. All of these factors have contributed to increased mosquito and vertebrate host populations and increased risk of JEV in macaque.

There is no specific treatment or anti-viral agent for JE infection and treatment is mainly supportive. Thus, prevention methods are very important for minimizing JE infection. The most effective method of preventing JEV is by vaccination in amplifiers host (pig) and dead end host (human and horse) (Zuckerman et al., 2009). As reported by Hennessy et al, (1996), immunization of humans with JE can control the disease with efficacies of up to 98%. Similarly, JEV vaccine have been used in pigs and believed to decrease the amplification of the virus, and help protect horses and humans (Rosen, 1986). However, pig vaccination is not practical and sustainable because of the rapid turnover in pigs, the relative cost of vaccines, and not necessarily effective in piglets (Igarashi, 2002). Besides that, the

prevention of vector-man contact is very good preventive method by eliminating potential mosquito breeding areas, environmental sanitation and waste water management (Shailendra et al, 2013).

CONCLUSIONS

In conclusions, this study revealed that long tailed macaque in Peninsular Malaysia have been exposed to JEV infection with the overall prevalence of 20.45%. Therefore, there may be a risk of JEV transmission to humans by JEV-infected mosquitoes as several JEV mosquitoes vector display similar feeding pattern between human and non-human primates. Environmental and ecological factors are responsible for the spread of JEV.

RECOMMENDATIONS

As the samples taken only from three state of Malaysia, therefore a wider sampling area could be done so that a better serological prevalence in a larger monkey population in Malaysia. Further study should determine the genotype of the virus circulating on the monkey population by molecular studies.

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