UNIVERSITI PUTRA MALAYSIA

JAPANESE ENCEPHALITIS ANTIBODY DETECTION FROM BLOOD SAMPLES OF DOMESTIC DOGS AND CATS IN PENINSULAR MALAYSIA

PALLIYAGE DON HESHINI ERANDIKA PERERA

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PALLIYAGE DON HESHINI ERANDIKA PERERA

A project paper submitted to the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Japanese Encephalitis Antibody Detection From Blood Samples of Domestic Dogs and Cats in Peninsular Malaysia", by Heshini Erandika Perera 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.

DR. GAYATHRI THEVI SELVARAJAH
DVM (UPM), PhD. (Netherlands)
Senior lecturer
Faculty of Veterinary Medicine
University Putra Malaysia
(Supervisor)

ASSOC. PROF. DR. SITI SURI ARSHAD
DVM (UPM), M.Sc. (UPM), PhD. (London)
Deputy Dean of Academic and Student Affairs
Faculty of Veterinary Medicine
University Putra Malaysia
(Co-Supervisor)

DR. OOI PECK TOUNG
DVM (UPM), PhD. (Glasgow)
Senior lecturer
Faculty of Veterinary Medicine
University Putra Malaysia
(Co-Supervisor)

DEDICATIONS

This project paper is dedicated to my dearest family,

Grandmother

Mother

Father

Sister

Sham Pei Ni

Yong Li Hui

Tan Ying Yi

& Tai Shen Rong

And to my teachers who have guided many through the path of education, including myself.

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I would like to express my sincere gratitude to every single person who has contributed to this project;

Firstly, to my dearest supervisors, Dr. Gayathri, Dr. Siti Suri and Dr. Ooi for their endless support and guidance.

To the shelters and University Veterinary Hospital for all their contributions, without which this project would not be possible.

To all the helping hands, Mr. Maniam, Kiven, Mira, Hidaya, Husna, Shing Wei and all other classmates who contributed to this project.

To my family without which I would not be the person I am today and to my close friends Pei Ni, Stephanie and Raquel, as well as my partner Shen Rong for staying with me throughout the process.

And finally, to DVM 2011/2016.

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LIST OF ABBREVIATIONS

%	Percent
µl	Microliter
CSF	Cerebro-spinal Fluid
ELISA	Enzyme Linked Immunosorbent Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
JE	Japanese Encephalitis
JEV	Japanese Encephalitis Virus
MAC ELISA	Immunoglobulin M Antibody Capture Enzyme Linked Immunosorbent Assay
MVEV	Murray Valley Encephalitis Virus
nm	Nanometer
No.	Number
PCR	Polymerase Chain Reaction
^{o}C	Degree Celsius
SLEV	St. Louis Encephalitis Virus
SPSS	Statistical Package for the Social Sciences
™	Trademark
v	Version
VNT	Virus Neutralisation Test

WHO	World Health Organisation
WNV	West Nile Virus

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD4999- Final Year Project.

JAPANESE ENCEPHALITIS ANTIBODY DETECTION FROM BLOOD SAMPLES OF DOMESTIC DOGS AND CATS IN PENINSULAR MALAYSIA.

By

Palliyage Don Heshini Erandika Perera

2016

Supervisor : Dr. Gayathri Thevi Selvarajah

Co-Supervisor: Assoc. Prof. Dr. Siti Suri Arshad, Dr. Ooi Peck Toung

Introduction

Japanese Encephalitis Virus (JEV), of the *Flaviviridae* family, is a known cause of acute encephalitis in humans throughout South East Asia. It is transmitted through mosquito vector, with *Culex tritaeniorhynchus* being the vector most associated with spread of the disease. It has been identified in various animals, including in cats and dogs, however, there has been no study done in Malaysia investigating JEV in cats and dogs. The purpose of this study is to identify the presence of JEV antibodies in cats and dogs in Malaysia, using Enzyme Linked Immunosorbent Assay (ELISA).

Methods

Two to five ml of blood was collected from shelter cats and dogs and two ml of

serum was collected from diagnostic samples of cat patients to University Veterinary Hospital, with consent. Information collected for each animal included age, sex, health status, management and environment through observation and patient records. Three ELISA assays were performed, following protocol provided by the manufacturer (SunRed Biotechnology Cat JE IgG ELISA kit and MyBioSource Dog JE IgG ELISA kit). The tests were carried out with all samples in duplicate and the positive and negative samples were identified by calculating the critical value as instructed by the manufacturer.

Results

The results revealed that 15% of 40 pet cats, 17.7% of shelter cats and 80% of shelter dogs were positive for JEV antibodies, with shelter dogs being four times more likely to be seropositive than shelter cats. Fisher's Exact Test ($p < 0.05$) was used to compare results and possible factors affecting the result, from patient information, revealing that there appeared to be no significant relation between sex, health, management, age and location.

Conclusion

Dogs and cats in Malaysia are seropositive for JEV antibodies and can be used as sentinels.

Keywords: *Japanese Encephalitis, Dog, Cat, ELISA, IgG*

ABSTRAK

Abstrak kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar sebagai memenuhi sebahagian daripada kursus VPD4999- Projek Tahun Akhir.

PENGESANAN ANTIBODI JAPANESE ENCEPHALITIS DARIPADA SAMPEL DARAH ANJING DAN KUCING DOMESTIK DI SEMENANJUNG MALAYSIA.

oleh

Palliyage Don Heshini Erandika Perera

2016

Penyelia: Dr. Gayathri Thevi Selvarajah

Penyelia bersama: Prof. Madya Dr. Siti Suri Arshad, Dr. Ooi Peck Toung

Pengenalan

Virus Japanese Encephalitis (JEV), keluarga Flaviviridae, adalah punca yang diketahui ensefalitis akut pada manusia di seluruh Asia Tenggara. Virus ini disebarkan melalui nyamuk, dan Culex tritaeniorhynchus menjadi vektor utama yang dikaitkan dengan penyebaran penyakit ini. Virus ini telah dikenal pasti dalam pelbagai jenis haiwan, termasuk pada kucing dan anjing; walau bagaimanapun, tiada kajian yang dilakukan di Malaysia untuk mengkaji JEV pada kucing dan anjing. Tujuan kajian ini adalah untuk mengenal pasti kehadiran antibodi JEV pada kucing dan anjing di Malaysia, dengan menggunakan Enzim Berkaitan imunoserapan asai (ELISA).

Kaedah

Dua hingga lima ml darah telah dikumpul daripada kucing dari pusat perlindungan

dan anjing, dan dua ml serum dikumpulkan daripada sampel diagnostik kucing dari Hospital Veterinar Universiti, dengan keizinan pemilik haiwan tersebut. Maklumat yang dikumpul bagi setiap haiwan termasuk umur, jantina, status kesihatan, pengurusan dan persekitaran berdasarkan pemerhatian dan rekod pesakit. Tiga asai ELISA telah dilakukan berdasarkan protokol yang disediakan oleh pengeluar (kit SunRed Bioteknologi Cat JE IgG ELISA dan kit MyBioSource Dog JE IgG ELISA). Ujian telah dijalankan terhadap semua sampel dalam dua salina. Sampel positif dan negatif telah dikenal pasti dengan mengira nilai kritikal seperti yang diarahkan oleh pengeluar.

Keputusan

Hasil kajian menunjukkan bahawa 15% daripada 40 kucing haiwan peliharaan, 17.7% daripada kucing dari pusat perlindungan dan 80% daripada anjing dari pusat perlindungan adalah positif untuk antibodi JEV, dengan anjing dari pusat perlindungan yang empat kali lebih cenderung seropositive berbanding kucing dari pusat perlindungan. Fishers' Exact Test ($p < 0.05$) digunakan untuk membandingkan keputusan dan faktor-faktor yang mungkin mempengaruhi keputusan. Berdasarkan maklumat pesakit, terdapat hubungkait yang signifikan di antara jantina, kesihatan, pengurusan, umur dan lokasi.

Kesimpulan

Anjing dan kucing di Malaysia adalah seropositif untuk antibodi JEV dan boleh digunakan sebagai sentinel.

Kata kunci: *Japanese Encephalitis, Dog, Cat, ELISA, IgG*

1.0 INTRODUCTION

1.1 Japanese Encephalitis Virus

Japanese Encephalitis Virus (JEV) of the *Flaviviridae* family, originally known as Japanese B Encephalitis, is a known cause of acute encephalitis in humans throughout South East Asia and has been discovered in various animals including racoon dogs (Ohno *et al.*, 2009), buffaloes (Mall *et al.*, 1995), pigs and water birds, with pigs acting as an amplifying host and humans being dead-end hosts. (Solomon, 2006). The virus was suspected to have come to light in the Malay Archipelago and various genotypes have evolved from it, all of which can be found in Malaysia (Solomon *et al.*, 2002), from genotype I to V and the latest genotype V that was discovered in a person originating from Muar, Malaysia in 1952 (Manal *et al.*, 2011).

The first reported cases of Japanese Encephalitis (JE) occurred in the 1870's in Japan, and the virus was isolated in the 1930's from the brain of a human (Solomon, 2006). The main vectors for this disease, mosquitoes (Culex tritaeniorhyncus), are rampant within South East Asia, including Malaysia and have been effective at transmitting the virus from infected animals to humans. Thus, this became a disease of increasing importance due to its zoonotic characteristics, with infected humans showing signs of seizures and coma, especially without the existence of any effective antiviral treatment and lack of knowledge on the pathophysiological workings of the infection (Solomon *et al.*, 2002).

1.2 Japanese Encephalitis in Cats and Dogs

A study carried out in Japan (Shimoda *et al.*, 2010), explored the seroprevalence of Japanese Encephalitis within dogs and cats in the country. The purpose of the study was to identify whether the animals proved to act as good sentinals for the virus and the study concluded that dogs appeared to be so, more than cats. Another study, (Shimoda *et al.*, 2011) showed that dogs experimentally infected with the virus, did not appear to display any clinical signs. This leads us to question, whether these cats and dogs can act as silent carriers of impending infection to humans or whether they may be used in order to monitor the spread of the disease during an outbreak.

As of now there are no studies covering the seroprevalence of Japanese Encephalitis in Malaysian dogs and cats which would be an important piece of information considering the endemicity of the disease. Therefore, this study aims to detect the antibody against JEV in serum samples from domestic dogs and cats from both pet and shelter/ stray animal populations.

The hypothesis for this study is that domestic cats and dogs are seropositive for Japanese Encephalitis.

2.0 LITERATURE REVIEW

2.1 Disease Hosts

The most important mammal involved in the transmission of JEV are pigs, that act as amplifying hosts and maintenance host in areas that are endemic (Simpson *et al.*, 1976). In addition to that, a study published in 2012 relays the correlation between the disease vector and presence of pigs. There was a significant increase in *Culex tritaeniorhynchus* near pigs, as compared to humans and large ruminants (Lindahl *et al.*, 2012).
The primary enzootic hosts of the JEV are also thought to include wading birds belonging to the Ardeidae family as they are known to take part in the role of virus amplifiers in certain locations (Rodrigues *et al.*, 1981), though viremia does occur in birds of various different avian families, both domestic and wild.

However, regardless of presence of amplifying hosts, it is the amount of contact between said host and vector that affects the JEV transmission cycle. The mosquito vectors feed based on opportunity presented, therefore feeding patterns are affected based on availability of the host (Takken & Verhulst, 2013). However, in a study carried out in 1998, cattle appeared to attract a larger number of the disease vector in general as compared to pigs (Mwandawiro *et al.*, 1999), though there appeared to be no difference between the two in attracting *Culex tritaeniorhynchus*.

As for humans, only 5% of the vectors blood meals are from a human (Van Den Hurk *et al.*, 2009).

2.2 Disease in Humans Worldwide

The JEV serological complex consists of eight species and two subtypes, causing considerable mortality as well as morbidity; West Nile Virus (WNV), St. Louis Encephalitis Virus (SLEV), Murray Valley Encephalitis Virus (MVEV), Japanese Encephalitis Virus (JEV), Kunjin,Yaounde, Alfuy, Cacipacore, Ustusu and Koutango viruses (King *et al.*, 2011).

About 67,900 cases of Japanese Encephalitis are said to occur annually, with only 10% being reported to the World Health Organisation (WHO). Almost 50% of the cases appear to occur in China (Campbell *et al.*, 2011). The Phillippines reported 16% to 40% of clinical encephalitis cases to have resulted from a JEV inection. Samples tested from 2011 to 2014 revealed, of 497 suspected JE cases, 15% actually resulted from the virus infection (Lopez *et al.*, 2015). Between 2001 to 2012, Taiwan reported an incidence rate of 0.052 to 0.167 JE cases per 100,000 of its population (Hsu *et al.*, 2014).

Fever, disorientation and seizures are a few of the wide variety of clinical symptoms displayed in a human infection of Japanese Encephalitis (World Health Organisation, 2015). However, only 1 in 50-1000 infections will result in encephalitis, with most infections being asymptomatic (Vaughn & Hoke, 1992).

Majority of infections result in a mortality rate between 20% to 40% (Kono & Kim, 1969), however at the highest point, it may be a little above 66%, with 22% of survivors suffering from neurological complications and 10% with functional deficiencies (Ding *et al.*, 2007).

2.3 Disease in Malaysia

Japanese Encephalitis is not a new disease to Malaysia, with the first report of Japanese Encephalitis being made in 1952 (Paterson *et al.*, 1952) and a World Health Organisation report in 1999 claimed that yearly, nine to ninety-one cases are reported in the country. Malaysia had a rich history with the disease; with outbreaks occuring in Langkawi in 1974, Penang in 1988 and in 1992 in Sarawak (World Health Organisation, 1999). A study published in 1995, studying cases of viral encephalitis in children in Penang, reported that 38.5% of viral encephalitis in children was a result of JEV (Cardosa *et al.*, 1995).

In fact, a newspaper article published in 2014 reported 16 cases by the month of June, with four cases resulting in death (Ismail, 2014). This endemic occurrence of Japanese Encephalitis is further propagated by Malaysia's vector population. A study in 1997 reveal *Culex tritaeniorhynchus* forming the majority of a sample of 81,889 mosquitoes collected in Sepang, Malaysia, with fluctuating population depending on rainfall patterns (Vythilingam *et al.*, 1997).

2.4 Disease Diagnosis

Diagnosis of JE is typically done using serum or cerebro-spinal fluid (CSF) to detect virus specific Immunoglobulin M antibodies. The antibodies tend to be detectable for a period of 1 to 3 months or more after formation at 5 to 6 days post-infection; therefore, a positive result does not necessarily indicate an ongoing infection (Centres for Disease Control and Prevention, 2015).

Various methods for antibody detection exist, including detection using a nitrocellulose membrane-based immunoglobulin M (IgM) capture dot enzyme immunoassay, that allows for rapid diagnosis of JE, especially useful for field studies (Solomon *et al.*, 1998).

The usage of Enzyme Linked Immunosorbent Assay (ELISA) is also a valuable method, though cross reactivity between antibodies to agents of the same family such as West Nile Virus, can be a barrier in diagnosing the disease. However, Immunoglobulin M antibody capture ELISA (MAC ELISA) is capable of distinguishing the flaviviruses. The only drawback to the ELISA is the expense that goes into the equipment required, which may not be feasible in diagnosing Japanese Encephalitis in endemic areas that tend to be rural locations that cannot afford the cost (Solomon *et al.*, 1998).

3.0 MATERIALS AND METHODS

3.1 Serological Sample Collection

A total of 90 cat sera and 45 dog sera were sampled for this project. Of the 90 cats, 45 were from shelters in Malaysia and 45 were public owned pet animals presented to the University Veterinary Hospital, Universiti Putra Malaysia for health check up. Of the dogs sampled, all 45 were from shelters in Malaysia. None of the public owned pet dog samples were evaluated in this study. The reasearch project was approved by the Institutional Animal Care and Use Committee (IACUC), with reference number: UPM/IACUC/AUP – R008/2015 (Appendix A). Written consents were obtained from the animal shelters and owners of patients of University Veterinary Hospital.

3.2 Sample and data collection

The dogs and cats were restrained physically and blood samples were obtained from the jugular, saphenous or cephalic vein, which ever possible, and transferred into a sterile-plain tube. The information on condition of the animal in terms of health and the animals environment was recorded such as any nearby bodies of water, if the animals were housed indoors or outdoors, as well as the presence of other animals within the vicinity.

Serum samples of the pet animals were obtained from blood samples submitted to a veterinary clinic laboratory for patient complete blood count and biochemistry evaluation. The basic signalment of each cat and dog was recorded: age, sex, neuter status (where available) and breed.

3.3 Sample Processing and Storage

The tubes were labelled with the animals' identification and were transported to the Virology Laboratory of Universiti Putra Malaysia in boxes containing dry ice (cool temmperature). The red tube containing serum from the shelter animals was left for two hours at room temperature (approximately 25 ^{o}C) for the blood to clot and the serum to form. The tube was then centrifuged for ten minutes at 4000 revolutions per minute and the serum was transferred with a micropipette into an Eppendorf tube, labelled and stored at -30^{o}C. The serum samples from the pet animals were also stored in similar conditions.

Prior to running the ELISA test, the samples were thawed at room temperature for thirty minutes.

3.4 Cat JE IgG ELISA Procedure

SunRed Biotechnology Cat JE IgG ELISA kit, Catalogue No. SRB4502, was used for this project. It is a 96-well plate, pre-coated with anti-JEV-antibody antibody and was used to qualitatively analyse the presence of JEV IgG antibodies, through a double-antibody sandwich technique. This technique involves sandwiching of the antibody to be detected within the serum samples, between two antibodies, one which is coated in the well, the other which is attached to the conjugating protein that will allow for the detection of the JEV antibodies by producing a colour. The kit was stored at 2°C to 8°C and was thawed for 30 minutes at room temperature prior to use. Firstly, 40µl of sample diluent provided was added to each of the sample wells. Positive control was added into two positive wells (H7, H8) and the negative control was added to two negative wells (H11, H12). Two blank wells containing only distilled water (H9, H10) were left between the positive and negative wells. Then, 10µl of the cat serum was added to the respective test wells, in duplicate, so that there was two wells for each sample. The plate was shaken gently by moving the plate side to side on a flat surface and was then incubated for 30 minutes at 37°C.

After the incubation period was over, the wells were emptied by overturning the plate onto absorbent paper and then the wells were washed using provided wash solution that was diluted thirty times. The wash solution was transferred into each well using a pipette and then removed by overturning the plate onto absorbent paper. This process of washing was repeated four more times before adding in 50µl

of Horseradish Peroxidase Conjugate reagent into each well that contained the test serum samples. The plate was then, once again, shaken gently and incubated for 30 minutes at 37°C.

The washing procedure was then repeated and 50µl of Chromogen solution A and 50µl of Chromogen solution B was added to every well. The plate was shaken gently and incubated for 10 minutes at 37°C. After incubation, 50µl of stop solution was added to each well and the Optical Density of the wells was read using a Tecan infinite M200 micro-plate reader (Switzerland) with the Magellan v.6.5 software, within fifteen minutes of adding the stop solution at reference wavelength of 630nm and test reference at 450nm. The plate was read twice and average values were calculated to take into account for technical errors.

3.5 Canine JE IgG ELISA Procedure

MyBioSource Dog JE IgG ELISA kit, Catalogue No. MBS108996, was used. It is a 96-well plate, pre-coated with JEV antigen and was used to qualitatively analyse the presence of JEV IgG antibodies, through a double-antigen sandwich technique. The test kit was stored at 2°C - 8°C and was thawed at room temperature for 30 minutes prior to use. Next, 50µl of positive control was placed into two positive wells (H7, H8) and 50µl of negative control was placed into two negative wells (H11, H12). Two blank wells (H9, H10) were placed in between the positive wells and negative wells. Ten µl of the sample serum and 40µl of sample diluent was then

added into each respective test sample well. A total of 100µl of Horseradish Peroxidase Conjugate reagent was then added into each well except for the two blank wells, and the plate was incubated for 60 minutes at 37°C.

After the incubation period was over, the wells were washed using the provided wash solution, by transferring the diluted solution (1:19) into each well and emptying the wells by overturning the plate onto absorbent paper. This process was repeated three more times before adding in 50µl of Chromogen solution A and 50µl of Chromogen solution B into each well. The solutions were mixed by gently shaking the plate side to side on a flat surface and then, the plate was incubated for 20 minutes at 37°C.

After incubation, 50µl stop solution was added to each well and the Optical Density (OD) was read using a Tecan infinite M200 micro-plate reader with Magellan v.6.5 software at 450nm within 15 minutes of adding the stop solution. The plate was read twice to avoid technical errors. The OD of each well was recorded and the critical value to judge whether a well was to be considered positive or negative was calculated according to the following formula;

Critical value = (15% of OD of Positive well + OD of Negative well)

Any well with an OD value that was above the critical value was considered positive and those with an OD below the critical value was considered negative. The data was further analysed using Statistical Package for the Social Sciences (SPSS v.20, United States) at 95% confidence level, with Fisher's Exact test.

4.0 RESULTS

The following results were obtained;

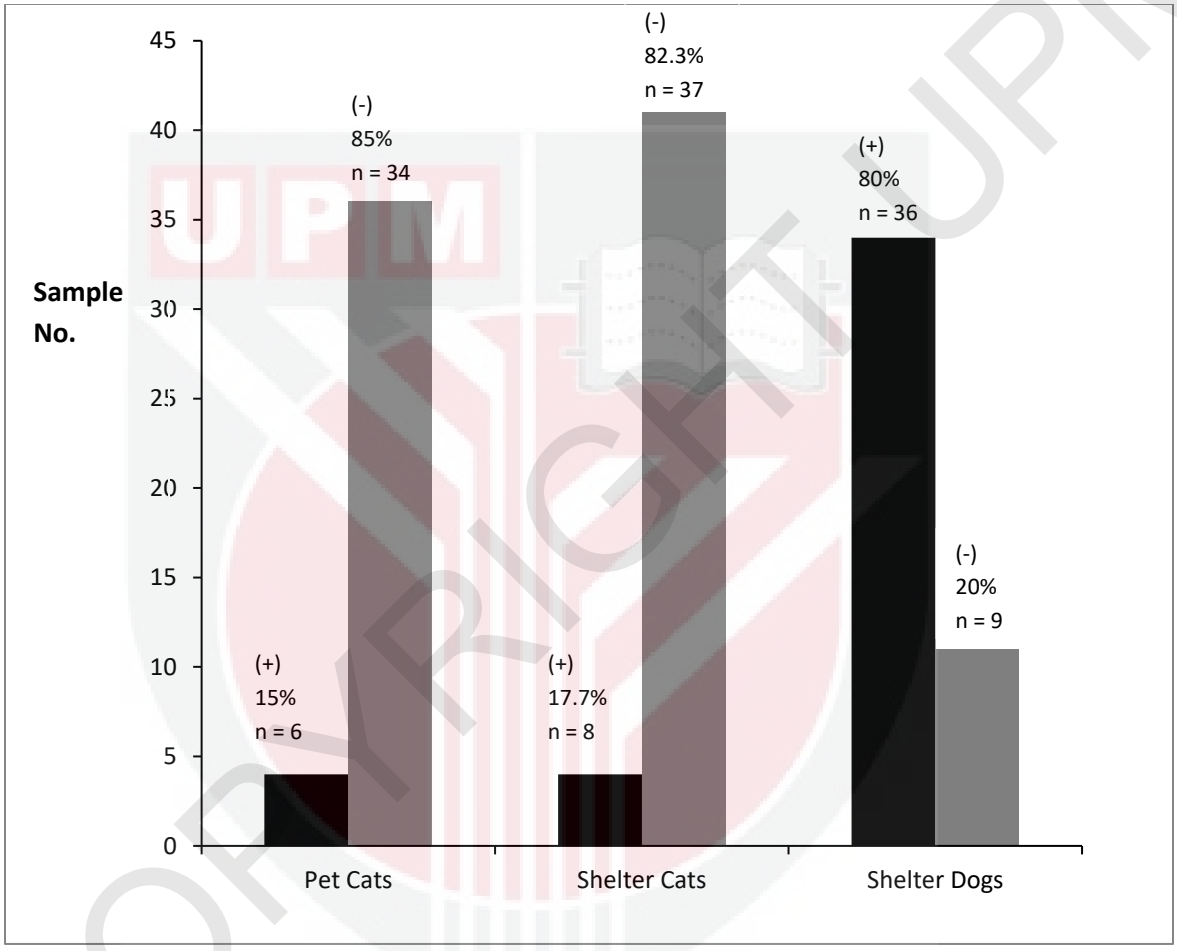

Fig. 4.1: Out of 40 pet cats, 6 were seropositive for JE IgG antibodies (15%). Out of 45 shelter cats, 8 proved to be seropositive for JE IgG antibodies (17.7%) and out of 45 shelter dogs, 36 were seropositive (80%).

4.1 Sample Demographics

Table 4.2: Sample demographics of pets cats, shelter cats and shelter dogs.

	Pet Cats	**Shelter Cats**	**Shelter Dogs**
Samples collected	40	45	45
Age	<1 year: 25% >1 year: 75%	<1 year: 20% >1 year: 80%	<1 year: 13.3% >1 year: 86.7%
Sex	Male: 65% Female: 35%	Male: 28.9% Female: 71.1%	Male: 33.3% Female: 66.7%
Breed	DSH: 75% DLH: 10% Mainecoon: 5% Persian: 5% BSH: 2.5% Somali: 2.5%	DSH: 100%	Local: 100%
Location	Selangor: 70% Kuala Lumpur: 12.5% Putra Jaya: 10% Negeri Sembilan: 2.5% Pahang: 2.5% Perlis: 2.5%	Ipoh SPCA: 40% PPK Putrajaya: 60%	Ipoh SPCA: 48.9% PAWS Subang: 51.1%

4.2 Serological Test Results Against JEV

Table 4.3: Serological test results per each plate.

	Pet cat ELISA	**Shelter cat ELISA**	**Shelter dog ELISA**
Critical value	0.1977	0.0193	0.1796
Positive result (%)	15	17.7	80
Negative result (%)	85	82.2	20
Individual shelter results (n)	N/A	PPK Putrajaya: 3/25 Ipoh SPCA: 5/20	PAWS Subang: 16/23 SPCA Ipoh: 20/22

4.3 Association of Host Factors and Environment with JEV Seropositivity

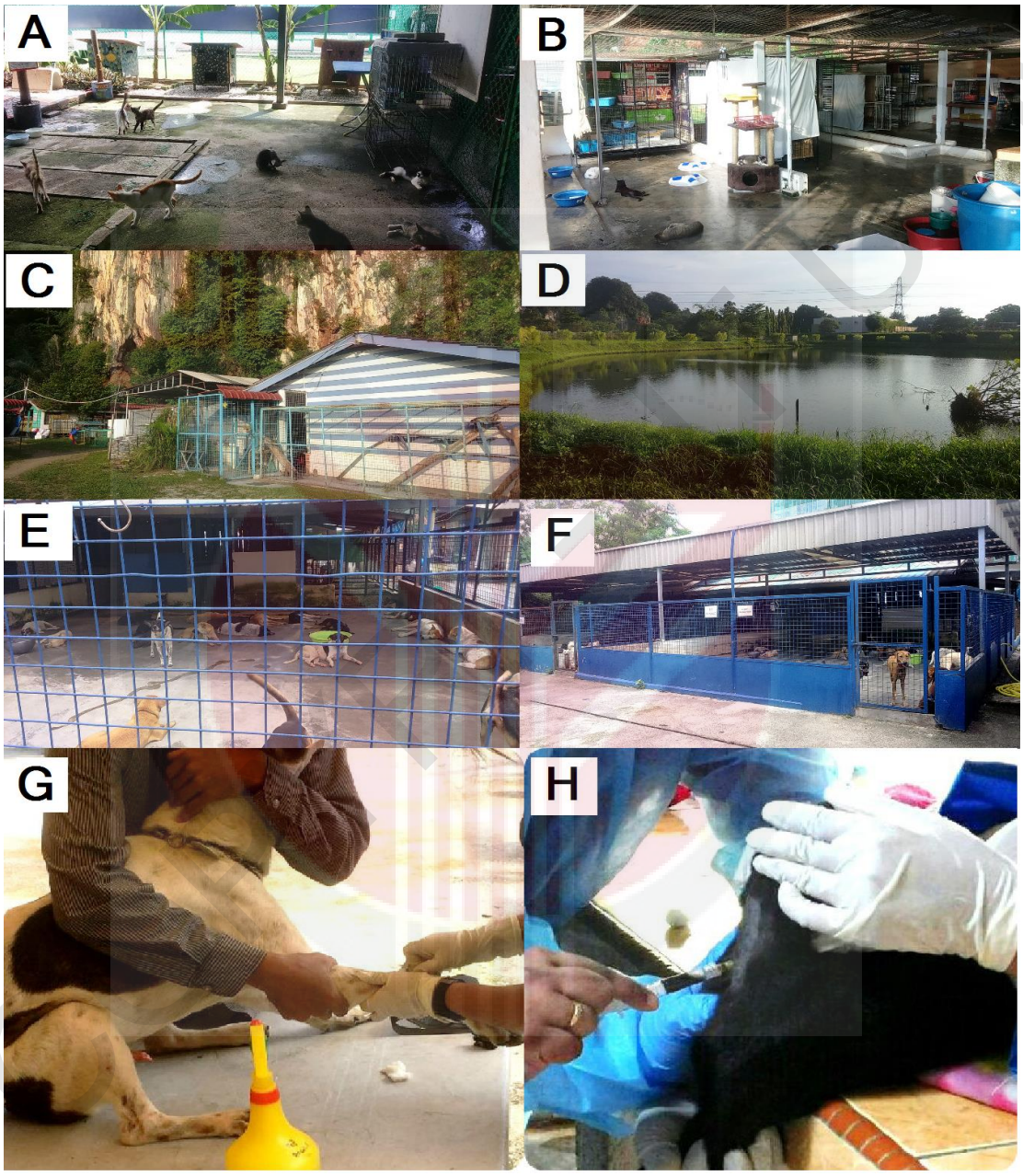

Fig. 4.4: (A) Environment within Putrajaya cat shelter, (B) Environment within Ipoh cat shelter, (C) Environment of Ipoh dog and cat shelter, with mountain in the background, (D) Lake nearby Ipoh shelter, (E, F) Environment of Selangor dog shelter, (G, H) Withdrawal of blood from shelter animals.

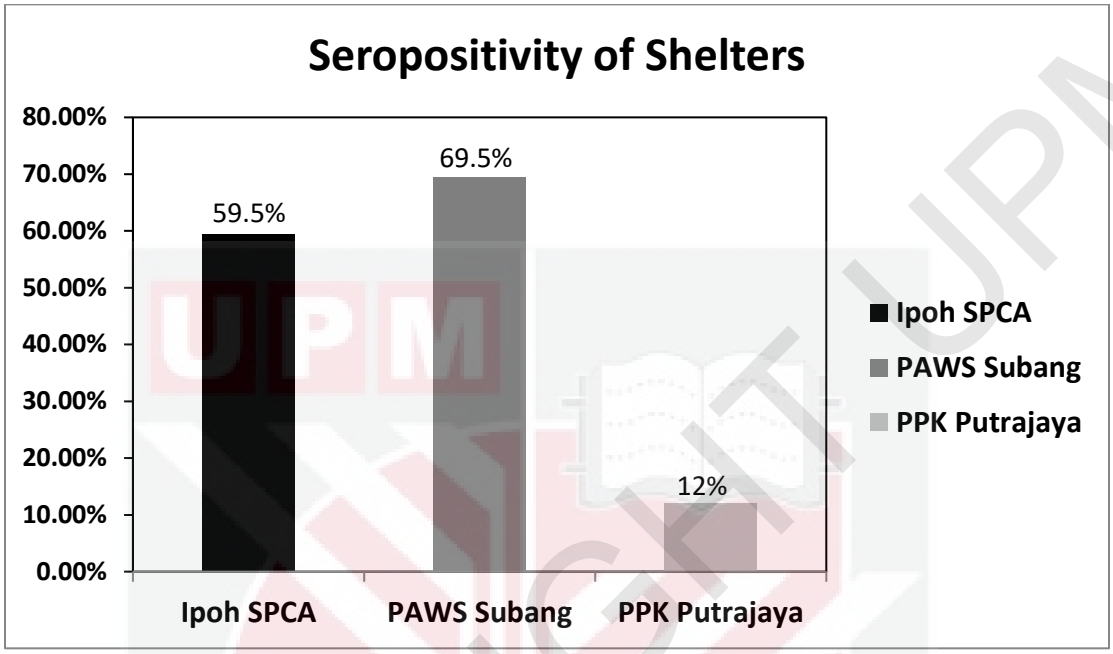

Fig. 4.5: Seropositivity of each shelter from which cats and dogs were sampled.

Ipoh SPCA and PAWS Subang housed both cats and dogs within houses that were open to the environment through open walls (Refer to Fig. 4.2, (A, B, C, E & F)). However, while both cats and dogs were sampled from Ipoh SPCA, only dogs were sampled from PAWS Subang. PPK Putrajaya sheltered cats, also open to the environment, with certain areas lacking cover from above.

Prior to further analysis, data from the sampled animals were collected and the environment was observed. The data collected was grouped according to age, sex, management, health status and location.

Age was grouped as such; animals less than one year old were grouped as "Young" and animals more than one year old were grouped as "Adult". Sex groups were "Male" and "Female", Health status was "Sick" and "Healthy". Management was "Indoors" and "Outdoors".

Of the total cat samples, 16.5% of cats were positive for JEV antibodies, however, statistical analysis revealed no significant difference in results when associated to the health ($p = 1.000$), age ($p = 0.370$), sex ($p = 1.000$), location ($p = 0.621$) of all cats, both pet and shelter together.

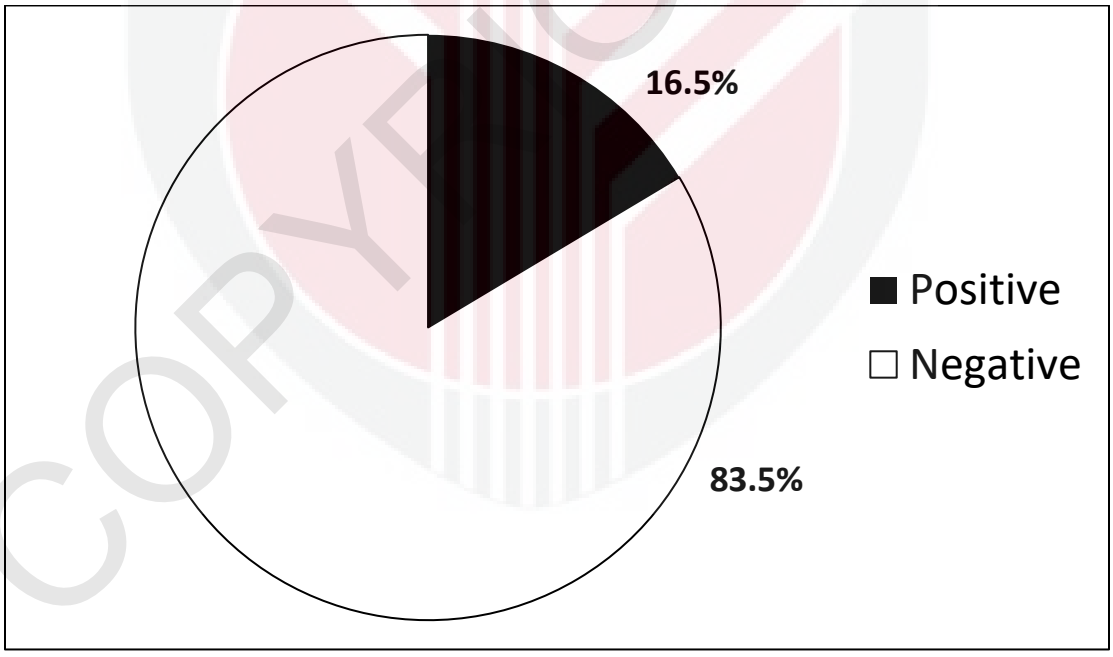

Fig. 4.6: Percentage of cats positive for JEV IgG antibodies from a total of 85 samples.

Analysing the pet cat samples, revealed that 15% (n = 6) were positive for JEV antibodies.

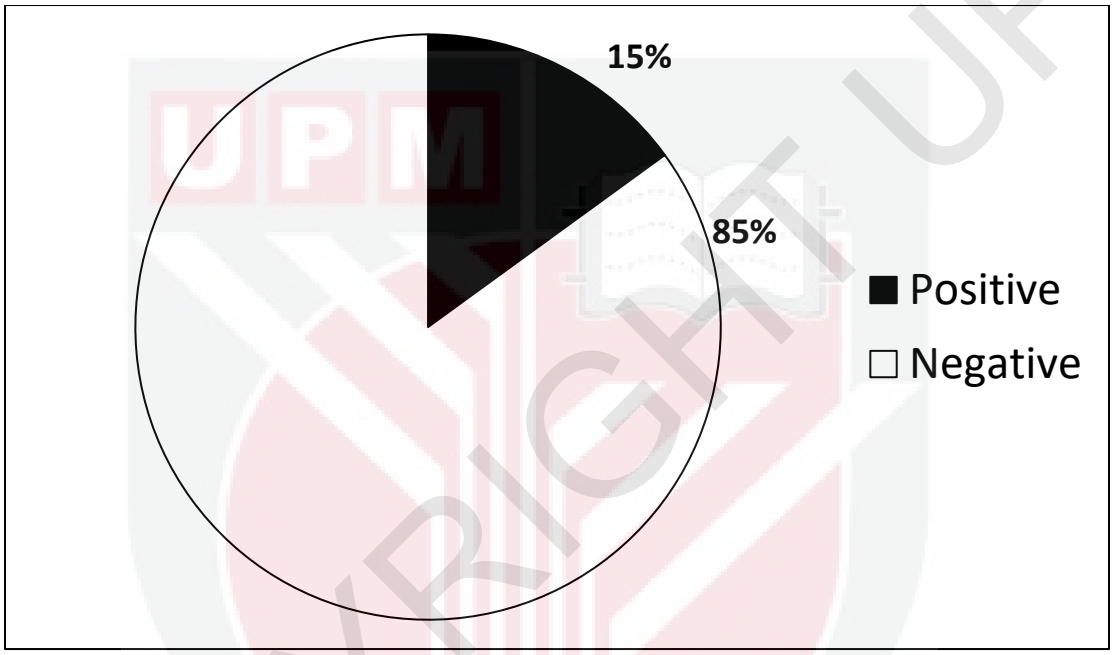

Fig. 4.7: Percentage of pet cats positive for JEV IgG antibodies from a total of 40 samples.

Analysing the shelter cat samples, revealed that 17.7% (n = 8) were positive for JEV antibodies.

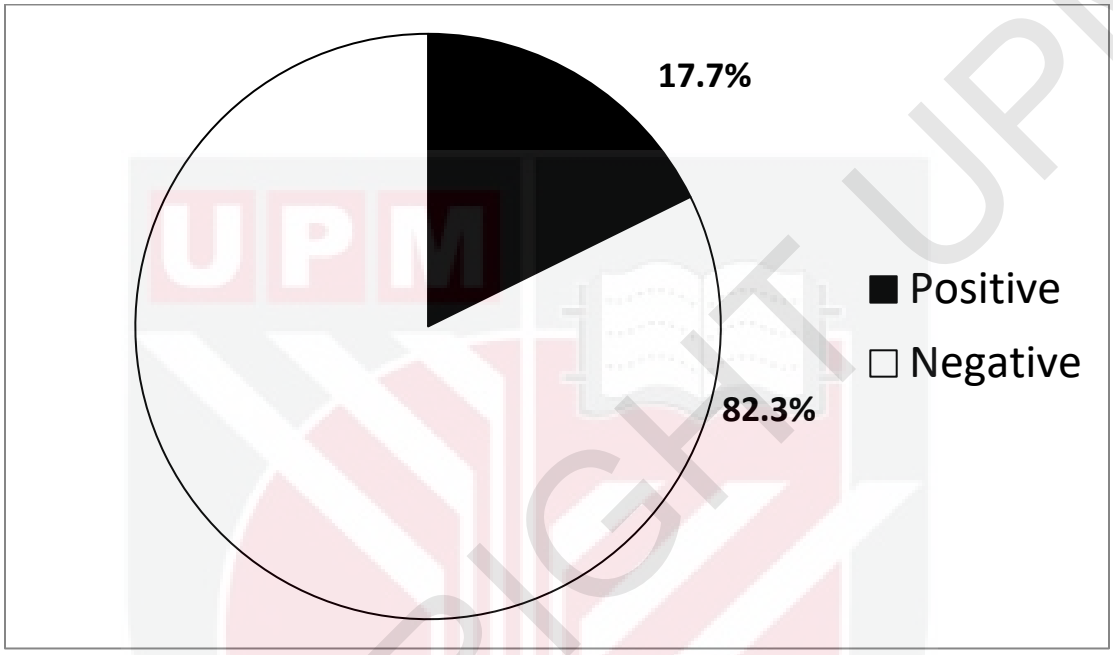

Fig. 4.8: Percentage of shelter cats positive for JEV IgG antibodies from a total of 45 samples.

All cats, regardless of age, sex, location or health, appeared to have equal chance of infection, and no studies currently exist, that prove otherwise. This result may be attributed to the endemicity of the vector within Malaysia, allowing non-selective infection among cats.

Of the total shelter animal samples, 48.9% of animals were positive for JEV antibodies.

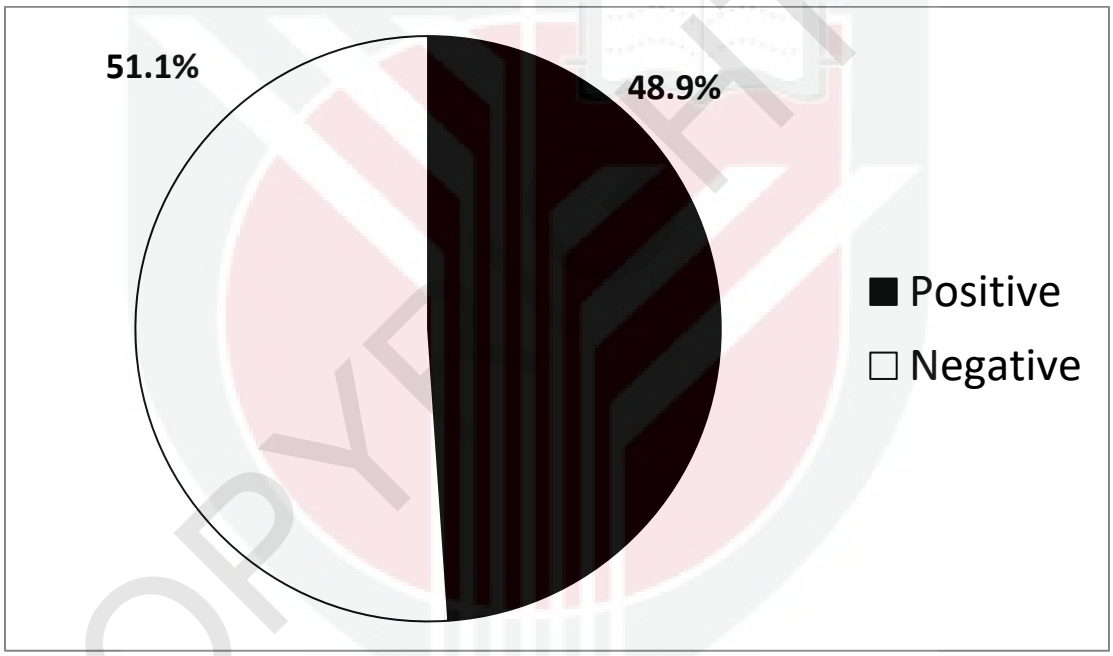

Fig. 4.9: Percentage of shelter dogs and cats positive for JEV IgG antibodies from a total of 90 samples.

Statistical analysis revealed there was significance in the difference of results between shelter cats and shelter dogs. Shelter dogs appeared to be four folds more likely to have acquired the JEV antibodies as compared to shelter cats (P = 0.001).

Previous studies also indicate that dogs tend to be more seropositive for JEV antibodies that cats. For example, a study in Japan showed that dogs were ten folds more seropositive than cats, with 17% of dogs being seropositive as compared to the 1% of seropositive cats. Also, it was reparted that there was no correlation between the sex of an animal and the seropositivity (Shimoda *et al.*, 2010), similar to the present study.

Studies involving other Flaviviruses also display similar results, with a USA study of West Nile Virus showing seropositivity in 26% of dogs and 9% of cats (Kile *et al.*, 2005).

The question as to why dogs were more seropositive, can be answered by observing the disease vector feeding patters. A study carried out in Taiwan, China proved that mosquitoes selectively preferred to feed on dogs as compared to cats (Mitchell *et al.*, 1973). Thusly, more dogs are bitten by mosquitoes carrying the virus, and as a result, more are infected and develop antibodies.

Of all the shelter dogs, 80% (n = 36) of 45 samples were positive for JEV antibodies. A study done in Bangkok, Thailand, revealed that 51% of dogs were seropositive, using an IgG ELISA (Shimoda *et al.*, 2013), showing results relatively high, similar to this study.

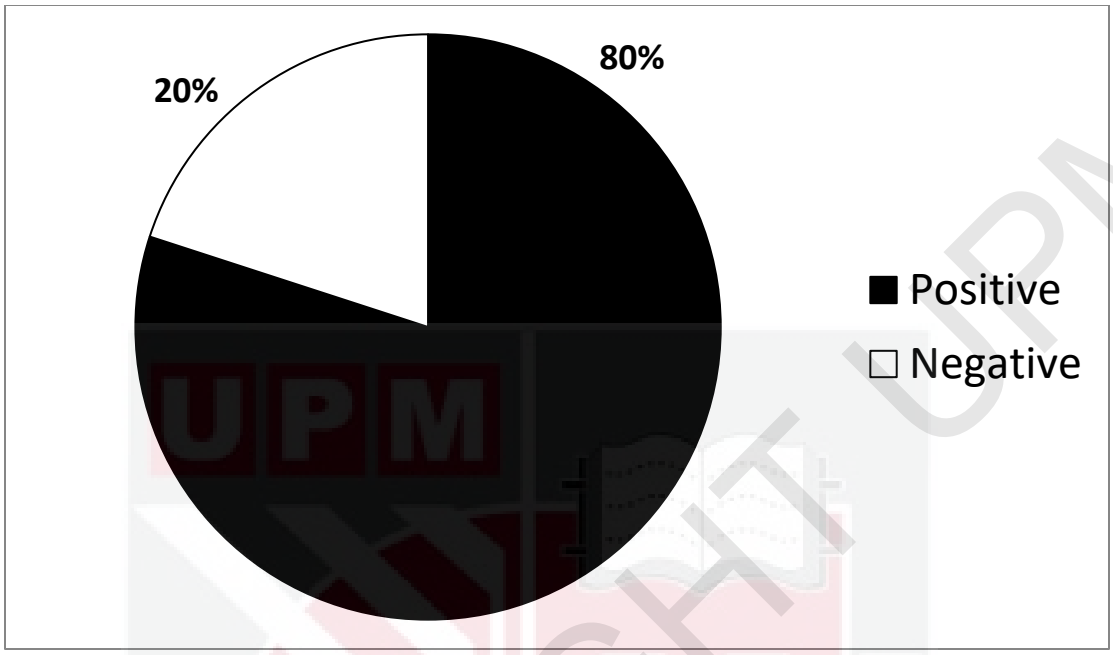

Fig. 5.0: Percentage of dogs positive for JEV antibodies from a total of 45 samples.

Statistical analysis revealed that there was no significant difference in results as compared to the location ($p = 0.071$) of the dogs as well as the age ($p = 0.084$), sex ($p = 0.704$) and health ($p = 1.000$) of the dogs.

The dogs were sampled from two locations, Ipoh and Selangor, with Ipoh proving to have a higher proportion of positive dogs , as calculating the odds ratio shows;

Table 5.1: Ipoh appears to have a higher risk for seropositivity (p = 0.229) when compared to Selangor.

	Odds Ratio	Lower (95% confidence interval)	Upper (95% confidence interval)
Location (Ipoh / Selangor)	**4.375**	**0.7962**	**24.04**

With these results, it cannot be denied that cats and dogs in Malaysia have definitely been exposed to the virus prior to testing.

5.0 DISCUSSION

Considering Malaysia's history with JEV and the endemicity of the disease, it is nothing short of surprising to see that this study was not carried out earlier, as exploring the possibility of dogs and cats as sentinals to warn of impending outbreak is one of the first steps that could ensure that outbreaks are kept at bay. In this study, only the states of Ipoh and Selangor were sampled due to their history with the disease and, as the objective of the study was only to identify whether dogs and cats were seropositive, random sampling throughout the country was not carried out. Instead, this study acts as a preliminary screening, to detect the presence of JEV in the environment that infected cats and dogs.

Malaysia, ofcourse, is not the only country to have detected JEV antibodies in dogs and cats, with Japan showing 70% seropositivity in dogs and 1% seropositivity in cats (Shimoda *et al.*, 2010). Another study carried out in Thailand used an IgG ELISA kit to detect seropositivity in dogs, showing that 51% were seropositive for JEV (Shimoda *et al*., 2013). These findings are somewhat similar to the findings in this study, with the percentage of seropositive dogs being relatively high, especially compared to the seropositivity of cats.

Though there was no statistically significant correlation between age, sex and health factors to seropositivity, when it came to seropositivity of dogs, it appeared to be that Ipoh was four times higher at risk for seropositivity than Selangor. The reason as to why Ipoh has a higher proportion of positive dogs than Selangor may be due to

the environment in general. The area from which samples were collected in Ipoh was a lush environment with plenty of trees, a water body that could serve as a good breeding ground for vectors and was a mountaineous area (Refer to Figure 2, (C, D)), that could act as a home for bats, which were found to be seropositive for JEV antibodies in a study carried out in China (Jiang *et al.*, 2015). The Selangor shelter was situated in an urban area, with no visible water bodies and may not perform as well as the Ipoh shelter area in hosting vectors.

While this study used an IgG ELISA to detect presence of JEV antibodies, it is not strictly the only way to detect JEV. IgM ELISa has been used previously, to detect seropositivity in humans (Nyari *et al.*, 2015) and can detect the presence of JEV in a shorter time period due to it detecting IgM antibodies. Haemagglutination=inhibition (HI) can also be used to detect antibodies for JEV, as was used to detect seropositivity in wild birds in Korea (Yang *et al.*, 2011). However, though detection of antibodies can identify the general prescence of JEV, it cannot be used to identify whether JEV is present currently, at the point of sample collection. That is where, antigen detection methods come into play, such a Polymerase Chain Reaction (PCR), Virus Neutralisation Test (VNT) and Immunohistochemistry (IHC) (Iwasaki *et al.*, 1986). However, there are certain constraints to certain methods, such as VNT, wherein the virus must be grown and would require a Biosafety Level (BSL) of 3 or higher, which is hard to come across in Malaysia.

The findings of this study are indicative of the continuing presence of the Japanese Encephalitis Virus in Malaysia. In fear of a potential outbreak, certain measures have

been proven to work, reducing disease occurrence worldwide. JEV appears to have moved towards the south east, with sizeable epidemics in West Bengal, Korea, Nepal, Sri Lanka (Saxena *et al.*, 2008) and more, however, immunization programs that were introduced may be the key to control of the disease (Dong *et al.*, 2016; Vashishtha *et al.*, 2015; Fan *et al.*, 2015).

These vaccines, together with changing agricultural practices and vector control, have resulted in a reduction in disease occurrence (Igarashi, 2002).

One method of control of vectors that was adopted to an extent was ultra-low volume application of insecticide (Self *et al.*, 1973), however, it was considered to be costly and impractical considering the isolated areas, villages and vast paddy fields harboring the vectors, that are hard to reach as well as too large to employ chemical means of control during an outbreak. On top of that, the factors of resistance and toxicity to the humans and animals of the area have to be taken into consideration. (Lacey *et al.*, 1990). Another method involves using alternating wet and dry irrigation, together with larvivorous fish to reduce transmission of JEV by reducing the vector population (Keiser *et al.*, 2005).

Focusing on Malaysia itself, various disease control measures have been taken for prevention, vaccination being one of them. A study carried out in Sarawak demonstrates a 61% to 45% reduction in JE cases after the use of vaccines (Impoinvil *et al.*, 2013). Malaysia's climate, however, is a great contributing factor to the spread

of the disease, with vectors thriving off rainy seasons and humid climates. Therefore, the best form of control would be vector control.

In addition to controlling vectors, the results of this study revealed that Malaysian dogs and cats, especially dogs, can be used as sentinals to monitor the disease and whether there is an increase or decrease in seroprevalence. Thusly, they may play an integral part in the control of this disease.

6.0 CONCLUSION AND RECOMMENDATIONS

This study concludes that the Japanese Encephalitis Virus has infected 15% of pet cats, 17.7% of shelter cats and 80% of dogs in Malaysia, causing an immune response, resulting in seropositivity of the animals. However, the study does not inform us when the animal was infected and whether or not the animal is still infected. Upon infection by the JEV antigen, the first immune response would involve IgM antibodies that would last for a period of one to three months, and presence of these antibodies would be indicative of a recent infection. However, the ELISA kit used in this study is an IgG ELISA kit, detecting antibodies that are a second line of defence and last much longer than IgM. Therefore, the only conclusion that can be reached is that the animals have been infected at some point during their lifespan.

In order to truly identify the current infection status of the virus, further study would need to be carried out, using methods such as Virus Neutralisation Test (VNT) or Polymerase Chain Reaction (PCR) methods to isolate and identify the JEV antigen within the samples. However, as the purpose of this study was to identify the presence of JEV in dogs and cats, ELISA sufficed.

Furthermore, sampling of a wider area and more states at a higher risk, would produce a result that is closer to representing the population of dogs and cats in Malaysia.

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APPENDICES

APPENDIX A: Institutional Animal Care And Use Committee approval letter.

UPM
UNIVERSITI PUTRA MALAYSIA
BENILMU BERBAKTI

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	11 August, 2015
Ref.:	UPM/IACUC/AUP- R008/2015
Project Title:	Japanese Encephalitis Virus detection and characterization from blood samples of domestic cats, dogs, birds and pigs in Peninsular Malaysia.
Principal Investigator:	Assoc. Prof. Dr Siti Suri Arshad
Associates:	Dr Gayathri Thevi Selvarajah, Dr Ooi Peck Tuong, Assoc. Prof. Dr Jalila Abu, Dr Reuben Sharma
Student:	Ms Norisal Nasai
Committee Decision:	The committee has reviewed and approved the proposed animal utilization protocol
AUP No.:	R008/2015
Project Classification:	Acute
Category of Invasiveness:	B
Source of Animals:	Cats & Dogs – SPCA & PAWS Animal Shelters, University Veterinary Hospital, UPM; Wild birds – Wetlands of Putrajaya, Penang and Perak; Pigs – Private owned farms.
Number of Animals Approved:	Dogs, cats, pigs and wild birds – 141 each.
Accommodation:	Dogs and cats – animal shelters and Veterinary hospital; pigs – selected farms; wild birds – wetland sanctuaries.
Duration:	20 August, 2015 – 19 August, 2016

17 AUG 2015

(Prof. Dr. Mohd Hair Bejo)
Chairman,
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Mal
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1293 603-8945 1646, Pejabat Pentadbiran TNCPI 603-8947 1608 603-8945 1
Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601 603-8945 1596, Pejabat Pengarah, Putra Science Park (PS
603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

APPENDIX B: Pet cats brought to University Veterinary Hospital, owned by public.

No	ID	Sex	Age group	Management	Location	Result
1	068564	Male	<1 year	Indoors	Selangor	Positive
2	067334	Male	>1 year	Indoors	Kuala Lumpur	Positive
3	041341	Male	>1 year	Indoors	Selangor	Positive
4	054393	Female	>1 year	Indoors	Selangor	Positive
5	068147	Female	>1 year	Unknown	Selangor	Positive
6	068543	Female	>1 year	Indoors	Putrajaya	Negative
7	068510	Female	>1 year	Outdoors	Selangor	Negative
8	066059	Female	<1 year	Indoors	Selangor	Negative
9	068078	Male	>1 year	Indoors	Selangor	Negative
10	066276	Female	>1 year	Indoors	Putrajaya	Negative
11	068895	Female	>1 year	Indoors	Kuala Lumpur	Negative
12	068922	Male	>1 year	Outdoors	Kuala Lumpur	Negative
13	068437	Male	>1 year	Outdoors	Kuala Lumpur	Negative
14	068825	Male	<1 year	Indoors	Kulala Lumpur	Negative
15	068558	Male	<1 year	Unknown	Selangor	Negative
16	068899	Male	<1 year	Indoors	Putrajaya	Negative
17	066301	Male	>1 year	Unknown	Selangor	Negative
18	039486	Male	>1 year	Indoors	Pahang	Negative
19	066286	Male	<1 year	Indoors	Negeri Sembilan	Negative
20	1396	Female	>1 year	Unknown	Selangor	Negative
21	068556	Male	>1 year	Indoors	Selangor	Negative
22	068818	Male	>1 year	Indoors	Selangor	Negative
23	067436	Male	>1 year	Indoors	Selangor	Negative
24	066071	Male	>1 year	Unknown	Selangor	Negative
25	068510	Male	<1 year	Unknown	Selangor	Negative
26	017088	Female	>1 year	Unknown	Selangor	Negative
27	068532	Female	>1 year	Unknown	Selangor	Negative
28	068513	Female	>1 year	Unknown	Selangor	Negative
29	068546	Male	>1 year	Indoors	Selangor	Negative
30	068530	Male	>1 year	Outdoors	Selangor	Negative
31	066083	Male	>1 year	Outdoors	Selangor	Negative
32	068270	Female	>1 year	Indoors	Selangor	Negative
33	066310	Female	>1 year	Indoors	Selangor	Negative
34	068095	Male	>1 year	Indoors	Perlis	Negative
35	068904	Male	>1 year	Unknown	Selangor	Negative
36	064831	Female	<1 year	Outdoors	Selangor	Negative
37	066239	Male	>1 year	Indoors	Kuala Lumpur	Negative
38	068290	Female	>1 year	Outdoors	Kuala Lumpur	Negative
39	030341	Male	>1 year	Indoors	Selangor	Negative
40	068916	Male	<1 year	Unknown	Putrajaya	Negative

APPENDIX C: Cats from animal shelters in Malaysia.

No	ID	Sex	Age group	Management	Location	Result
1	PutraA	Male	<1 year	Outdoors	Putrajaya	Negative
2	PutraB	Male	<1 year	Outdoors	Putrajaya	Negative
3	PutraC	Male	<1 year	Outdoors	Putrajaya	Negative
4	PutraD	Male	<1 year	Outdoors	Putrajaya	Negative
5	PutraE	Female	<1 year	Outdoors	Putrajaya	Positive
6	PutraF	Female	<1 year	Outdoors	Putrajaya	Negative
7	PutraG	Male	<1 year	Outdoors	Putrajaya	Negative
8	PutraH	Female	<1 year	Outdoors	Putrajaya	Positive
9	PutraI	Male	<1 year	Outdoors	Putrajaya	Negative
10	Putra10	Male	>1 year	Outdoors	Putrajaya	Negative
11	Putra11	Female	>1 year	Outdoors	Putrajaya	Negative
12	Putra12	Female	>1 year	Outdoors	Putrajaya	Negative
13	Putra13	Female	>1 year	Outdoors	Putrajaya	Positive
14	Putra14	Female	>1 year	Outdoors	Putrajaya	Negative
15	Putra15	Female	>1 year	Outdoors	Putrajaya	Negative
16	Putra16	Female	>1 year	Outdoors	Putrajaya	Negative
17	Putra17	Male	>1 year	Outdoors	Putrajaya	Negative
18	Putra18	Female	>1 year	Outdoors	Putrajaya	Negative
19	Putra19	Female	>1 year	Outdoors	Putrajaya	Negative
20	Putra20	Female	>1 year	Outdoors	Putrajaya	Negative
21	Putra21	Female	>1 year	Outdoors	Putrajaya	Negative
22	Putra22	Female	>1 year	Outdoors	Putrajaya	Negative
23	Putra23	Female	>1 year	Outdoors	Putrajaya	Negative
24	Putra24	Female	>1 year	Outdoors	Putrajaya	Negative
25	Putra25	Female	>1 year	Outdoors	Putrajaya	Negative
26	PutraJ	Female	>1 year	Outdoors	Putrajaya	Negative
27	PutraK	Male	>1 year	Outdoors	Putrajaya	Negative
28	Ipoh1	Female	>1 year	Outdoors	Ipoh	Negative
29	Ipoh2	Male	>1 year	Outdoors	Ipoh	Negative
30	Ipoh3	Male	>1 year	Outdoors	Ipoh	Negative
31	Ipoh4	Male	>1 year	Outdoors	Ipoh	Negative
32	Ipoh5	Female	>1 year	Outdoors	Ipoh	Positive
33	Ipoh6	Female	>1 year	Outdoors	Ipoh	Negative
34	Ipoh7	Female	>1 year	Outdoors	Ipoh	Positive
35	Ipoh8	Female	>1 year	Outdoors	Ipoh	Positive
36	Ipoh9	Female	>1 year	Outdoors	Ipoh	Positive
37	Ipoh10	Female	>1 year	Outdoors	Ipoh	Negative
38	Ipoh11	Female	>1 year	Outdoors	Ipoh	Negative
39	Ipoh12	Female	>1 year	Outdoors	Ipoh	Negative
40	Ipoh13	Female	>1 year	Outdoors	Ipoh	Negative
41	Ipoh14	Female	>1 year	Outdoors	Ipoh	Negative
42	Ipoh15	Female	>1 year	Outdoors	Ipoh	Negative
43	Ipoh16	Male	>1 year	Outdoors	Ipoh	Negative
44	Ipoh17	Female	>1 year	Outdoors	Ipoh	Positive
45	Ipoh18	Female	>1 year	Outdoors	Ipoh	Negative

APPENDIX D: Dogs from animal shelters in Malaysia.

No	ID	Sex	Age group	Management	Location	Result
1	IpohD1	Female	>1 year	Outdoors	Ipoh	Positive
2	IpohD2	Female	>1 year	Outdoors	Ipoh	Positive
3	IpohD3	Male	>1 year	Outdoors	Ipoh	Positive
4	IpohD4	Female	>1 year	Outdoors	Ipoh	Negative
5	IpohD5	Male	>1 year	Outdoors	Ipoh	Positive
6	IpohD6	Female	>1 year	Outdoors	Ipoh	Positive
7	IpohD7	Female	>1 year	Outdoors	Ipoh	Positive
8	IpohD8	Female	>1 year	Outdoors	Ipoh	Positive
9	IpohD9	Female	>1 year	Outdoors	Ipoh	Positive
10	IpohD10	Male	>1 year	Outdoors	Ipoh	Positive
11	IpohD11	Female	>1 year	Outdoors	Ipoh	Positive
12	IpohD12	Female	>1 year	Outdoors	Ipoh	Positive
13	IpohD13	Female	>1 year	Outdoors	Ipoh	Positive
14	IpohD14	Female	>1 year	Outdoors	Ipoh	Positive
15	IpohD15	Female	>1 year	Outdoors	Ipoh	Positive
16	IpohD16	Female	>1 year	Outdoors	Ipoh	Positive
17	IpohD17	Male	>1 year	Outdoors	Ipoh	Positive
18	IpohD18	Male	>1 year	Outdoors	Ipoh	Positive
19	IpohD19	Female	>1 year	Outdoors	Ipoh	Positive
20	IpohD20	Male	>1 year	Outdoors	Ipoh	Positive
21	IpohD21	Female	>1 year	Outdoors	Ipoh	Negative
22	IpohD22	Male	>1 year	Outdoors	Ipoh	Positive
23	SelangorD1	Male	>1 year	Outdoors	Selangor	Positive
24	SelangorD2	Female	>1 year	Outdoors	Selangor	Positive
25	SelangorD3	Female	>1 year	Outdoors	Selangor	Positive
26	SelangorD4	Male	>1 year	Outdoors	Selangor	Positive
27	SelangorD5	Female	>1 year	Outdoors	Selangor	Negative
28	SelangorD6	Female	>1 year	Outdoors	Selangor	Positive
29	SelangorD7	Female	>1 year	Outdoors	Selangor	Positive
30	SelangorD8	Female	>1 year	Outdoors	Selangor	Positive
31	SelangorD9	Female	>1 year	Outdoors	Selangor	Positive
32	SelangorD10	Female	>1 year	Outdoors	Selangor	Negative
33	SelangorD11	Male	>1 year	Outdoors	Selangor	Negative
34	SelangorD12	Female	>1 year	Outdoors	Selangor	Positive
35	SelangorD13	Female	>1 year	Outdoors	Selangor	Positive
36	SelangorD14	Female	>1 year	Outdoors	Selangor	Positive
37	SelangorD15	Male	>1 year	Outdoors	Selangor	Negative
38	SelangorD16	Female	>1 year	Outdoors	Selangor	Negative
39	SelangorD17	Male	>1 year	Outdoors	Selangor	Negative
40	SelangorD18	Male	>1 year	Outdoors	Selangor	Positive
41	SelangorD19	Female	>1 year	Outdoors	Selangor	Positive
42	SelangorD20	Female	>1 year	Outdoors	Selangor	Positive
43	SelangorD21	Female	>1 year	Outdoors	Selangor	Positive
44	SelangorD22	Female	>1 year	Outdoors	Selangor	Negative
45	SelangorD23	Female	>1 year	Outdoors	Selangor	Positive