UNIVERSITI PUTRA MALAYSIA

SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS (JEV) IN BIRDS IN MALAYSIA

ANISAH BINTI ABDUL RASID

FPV 2016 5

SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS (JEV) IN BIRDS IN MALAYSIA

ANISAH BINTI ABDUL RASID

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
43400, Serdang,
Selangor Darul Ehsan.

MARCH 2016

It is hereby certified that we have read this project paper entitled “Seroprevalence of Japanese Encephalitis Virus (JEV) in Birds in Malaysia”, by Anisah Binti Abdul Rasid 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 – Final Year Project.

ASSOCIATE PROFESSOR DR SITI SURI BINTI ARSHAD
DVM (UPM), MSc (UPM), PhD (London)
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia
(Supervisor)

ASSOCIATE PROFESSOR DR JALILA BINTI ABU
DVM (UPM), MSc (UPM), PhD (Minnesota)
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia
(Co-Supervisor)

DR NOR YASMIN BINTI ABD. RAHAMAN
DVM (UPM), MSc (UPM), PhD (UPM)
Senior Lecturer
Department of Veterinary Laboratory Diagnostic,
Universiti Putra Malaysia
(Co-Supervisor)

DEDICATION

"Animals are such agreeable friends.

They ask no questions, they pass no criticism"

-George Eliot-

To my lovely parents,

Abdul Rasid Bin Bakar & Rasiahwati Binti Sanudin

for always believe in me and for the endless motivation and support.

To my family,

for the love and care.

To my dearest cats,

especially Kiki and Lala.

To all dearest birds,

for allowing me to complete my project.

Lastly, to all avian enthusiasts.

ACKNOWLEDGEMENTS

Alhamdulillah, I would like to express my deepest gratitude to Assoc. Prof. Dr Siti Suri Arshad, my supervisor for her patient guidance, enthusiastic encouragement and useful critics of my project. Assoc. Prof. Dr Jalila Abu and Dr Nor Yasmin Bt Abdul Rahaman my co-supervisors for their willingness to give their time and constructive suggestions to help me in this project. Not to forget Dr Shaik Mohd Amin Babjee, Dr Gayathri Thevi Selvarajah and Dr Ooi Peck Toung for their advice and assistance.

I would like to give my appreciation to my family, especially my mom and dad for the endless support and encouragement throught my study. I would like to thank Jamil, for being so understanding and helpful throughout my study. My grateful thanks are also extended to, En Din, En Rusdam, Pn Siti,En Azri, Mira and all Virology and Bacteriology laboratory technician for guiding me with my laboratory work. I would like to extend my thanks to postgraduate student, for useful advice and guidance in my project. I wish to thanks my friends, especially Norsuzana, Heshini and Chee Yien as my project mate with all the support and advices. To be included also Ainul, Husna Atikah, Nabila, Nik Nur Fatin Amira and all my bestfriends for their encouragement and support. Finally, special thanks to my classmates DVM Class 2016 and everyone that help me directly or indirectly throughout this study.

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

JEV	Japanese Encephalitis Virus
JE	Japanese Encephalitis
RNA	Ribonucleic Acid
WHO	World Health Organization
ELISA	Enzyme-linked Immunosorbent Assay
IACUC	Institutional Animal Care and Use Committee
G	Gauge
OD	Optical Density
Ag	Antigen
Ab	Antibody
HRP	Horseradish Peroxidase
°C	Degree Celcius
nm	Nanometer
-ve	Negative
+ve	Positive
%	Percentage
n	Total
C.I	Confidence Interval
g	Gravity
mL	Millilitre

ABSTRACT

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek Ilmiah Tahun Akhir

PREVALEN ANTIBODI VIRUS JAPANESE ENCEPHALITIS PADA BURUNG DI MALAYSIA

Oleh

ANISAH BINTI ABDUL RASID

2016

Penyelia: Professor Madya Dr Siti Suri Arshad

Penyelia Bersama: Professor Madya Dr Jalila Binti Abu

Dr Nor Yasmin Binti Rahaman

Japanese Encephalitis (JE) merupakan salah satu daripada penyakit zoonotik yang disebabkan oleh virus Japanese Encephalitis yang berasal daripada famili *Flaviviridae* dan genus *Flavivirus.* Virus ini disebar melalui nyamuk *Culex*

terutamanya nyamuk *Culex tritaeniorhynchus* dengan burung ardeid sebagai perumah takungan. Babi dan burung memainkan peranan yang penting sebagai perumah pemangkin dengan manusia dan kuda sebagai perumah terakhir. Empat puluh lima burung terdiri daripada ayam kampung, ayam hutan kacukan dan burung air di sampel di Tanjung Piandang, Perak, Jenderam Hulu, Sepang, Selangor dan di Taman Wetland Putrajaya. Sampel darah diproses dan serum diuji dengan menggunakan ELISA sandwic antigen-berganda untuk mengenalpasti antibodi terhadap virus JE (MyBiosource®). Daripada empat puluh lima sampel, 28.89% (13/45) didapati positif terhadap antibodi virus JE. Berdasarkan lokasi, Jenderam Hulu mempunyai prevalen antibodi tertinggi (50%) diikuti oleh Tanjung Piandang (21.74%) dan akhir sekali Taman Wetland Putrajaya (20%). Berdasarkan faktor umur, burung muda menunjukkan prevalen antibodi tertinggi (35.71%) berbanding burung dewasa (25.81%). Berdasarkan kepada spesis, kedua- dua spesis ayam hutan kacukan dan flamingo America mempunyai prevalen antibodi (50%) diikuti dengan ayam kampung (21.74%). Burung jantan menunjukkan prevalen antibodi tertinngi dengan (50%) diikuti burung betina (28.13%). Analisis chi square menunjukkan tiada hubung kait antara faktor risiko dan prevalen antibodi terhadap virus JE. Secara kesimpulannya, terdapat antibodi yang dapat dikesan terhadap virus JE dalam burung di Malaysia dan kesemua burung mempunyai risiko yang sama kepada jangkitan virus JE dalam kondisi umur, spesis dan lokasi. Kajian selanjutnya perlu dilakukan untuk mengenalpasti genotip virus yang berada di dalam populasi burung melalui kajian molekul.

Kata kunci: Burung, Japanese Encephalitis, Malaysia, prevalen, antibodi, faktor risiko, ELISA sandwic antigen-berganda

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine as a partial requirement in the course VPD 4999- Final Year Project.

SEROPREVALENCE OF JAPANESE ENCEPHALITIS VIRUS (JEV) IN BIRDS IN MALAYSIA

By

ANISAH BINTI ABDUL RASID

2016

Supervisor: Associate Professor Dr Siti Suri Arshad

Co-Supervisor: Associate Professor Dr Jalila Binti Abu

Dr Nor Yasmin Binti Rahaman

Japanese Encephalitis is one of the most important zoonotic diseases caused by Japanese Encephalitis virus from family *Flaviviridae* and genus *Flavivirus*. The virus is transmitted through *Culex* mosquito primarily by *Culex tritaeniorhynchus* with ardeid birds as reservoir. Pigs and birds play an important role as the main vertebrate amplifier with human and horse as the dead-end host. Forty-five birds consisted of village chicken, jungle fowl cross and waterbirds were sampled in Tanjung Piandang, Perak, Jenderam Hulu, Sepang, Selangor and Putrajaya Wetland, respectively. Blood samples were processed and the serum were subjected to double-antigen sandwich ELISA for detection of antibody against Japanese Encephalitis virus (MyBiosource®). Out of forty-five sample, 28.89% (13/45) were positive for JEV antibodies. Based on location, Jenderam Hulu has the highest seroconversion (50%) followed by Tanjung Piandang (21.74%) and Putrajaya Wetland (20%). Based on age, young birds showed higher seroconversion (35.71%) than adult (25.81%). According to species, both jungle fowl cross and American flamingo has 50% seroconversion followed by village chicken with 21.74%. Male showed highest seroconversion (50%) followed by female (28.13%). Chi square test analysis revealed that there were no association between the risk factors and seroprevalence of JEV. In conclusion, there was presence of seroconversion against JEV in birds in Malaysia and all birds have similar risk to JEV infection in terms of age, sex, species and location. Further work should examine the genotype of the virus circulating in the birds' population by molecular studies.

Key words: Birds, Japanese Encephalitis, Malaysia, seroconversion, antibody, risk factors, double-antigen sandwich ELISA

1.0 INTRODUCTION

Japanese Encephalitis virus (JEV) is a mosquito-borne *Flavivirus* in the family of *Flaviviridae.* It is the most important cause of viral encephalitis in both humans and animals in Asia (Chen *et al*., 1990). JEV also has been deemed the most important cause of epidemic encephalitis worldwide and is the leading recognized cause of childhood encephalitis in Asia (Nemeth *et al.*, 2010). *Flavivirus* is a spherical, enveloped, single stranded linear RNA virus measuring about 40-50μm in diameter. The capsid of the virus has an icosahedral symmetry and the lipid bilayer of the *Flavivirus* is featuring as a glycoprotein spikes. Three structural protein present in a *Flavivirus* is nucleocapsid protein, a membrane like protein and an envelope glycoprotein (Himani *et al*., 2014).

Transmission of JEV involves *Culex tritaeniorhynchus* mosquito and its similar species that lay eggs in rice paddies and other open water source, with pigs and aquatic birds as the major vertebrate amplifying host (Halsted *et al*., 2008). According to Tsuchie *et al*. (1994) pigs and some avian species are important amplifier because of its significant viraemia following infection, large number of population, high turnover rate and preferential feeding by vectors. Two JEV transmission has been observed and in general, Japanese encephalitis is epidemic in temperate regions of Asia and endemic in tropical regions. The reasons for this two pattern is unknown but the best explanation is because there is differences in virulence among JEV strain (Burke and Leake, 1988).

Most of the monsoon areas in Asian countries which includes Malaysia have climate condition of sufficiently high temperature during summer and precipitation during rainy season, allowing for rice cultivation in watered paddy fields (Tsuchie *et al*., 1994) which eventually will become a place for the mosquito to lay eggs. Chicken rearing is quite common in Malaysia especially in village where it is nearby the paddy fields, swamp area or river. Both of this condition help in maintaining the JEV in nature. Other than that, according to Chen *et al*. (1990) JEV might reintroduced annually by migrating birds from tropical region of Asia. Chickens and ducklings have been considered to pay the best minor role in outbreaks because of their low or absent viremia. In endemic region which includes the tropical region, large number of free-ranging chickens and ducks lived and breed nearby humans, providing an abundant pool of potential amplifying hosts of JE virus. Domestic poultry also produce high enough viraemia for an extended period of time to serve as a possible alternative source of JEV infection and transmission in humans (Cleton *et al*., 2014). As a viral reservoir or amplifying hosts, birds do not develop clinical symptoms (Yang *et al*., 2011).

In Malaysia, the first JE case was reported in 1952 (Erlanger *et al*., 2009) and several outbreaks had occur years after that. In 1974, outbreaks had occur in

Langkawi with 10 cases causing 2 deaths (Fang *et al*., 1980), 1988 in Penang with 9 cases and 4 deaths (Cardosa *et al*., 1995) and in 1992 in Serian district of Sarawak with 9 cases and 4 deaths (WHO, March 1999). In Malaysia, there are about 9 to 91 cases of JE were reported each year (WHO, March 1999) and recently until June 2014, there are 16 reported cases of JE that causing 4 deaths. This disease occurs in almost every state in Malaysia, with greater number of cases in Penang, Perak, Selangor and Johor in West Malaysia and Sarawak in East Malaysia (Tsuchie *et al*., 1994).

JE is an important and endemic zoonotic disease in many country including Malaysia, Thailand, Philippines and other developing countries. However, currently there is no report on the JE in birds in Malaysia and the status of this species involvement in maintaining the virus in the environment is not known despite them act as a potential amplifier (Tsuchie *et al*., 1994; Halsted *et al.*, 2008; Cleton *et al.,* 2014). Therefore, this study will give some a clear view on the serological prevalence of JE in birds in Malaysia. This study will hope to give path and served as a foundation for future epidemiological research on JE virus in Malaysia. In this preliminary study, the aim is to document the serological prevalence of JEV in birds as it is the important potential amplifying host for JEV based on previous study. The antibodies will be detected using MyBiosource® Chicken Japanese Encephalitis IgG Antibody enzyme-linked immunosorbent assay (ELISA) kit.

Thus, the objective of this study includes:

1. To determine the serological prevalence of JEV in birds in Malaysia.

2. To determine the association between seropositivity against JEV and potential risk factors.

The hypothesis for this study is:

1. There is presence of seroconversion against JEV in birds in Malaysia

2.0 LITERATURE REVIEW

2.1 Japanese Encephalitis (JE)

Japanese encephalitis (JE) is one of the common mosquito borne flaviviral encephalitis. Worldwide, JE is the leading forms of viral encephalitis and mostly prevalent in south east Asia (Tiwari *et al.*, 2012) such as Malaysia, Indonesia, Thailand and Philippines. Due to almost half of the world population is estimated to live in JE endemic countries (Ghosh and Basu, 2009), JE has become the public health concern worldwide. Annual incidence of JE is about 30,000-50,000 in the world (Solomon, 2006).

2.2 Japanese Encephalitis Virus (JEV)

JEV is a 40-50 nm enveloped, positive sense single-stranded RNA virus, with an isometric 30 nm nucleocapsid core. The envelope is spiked with a mature membrane (M) protein and a glycosylated envelope (E) protein, which comprises of 3 domains (I, II and III) that are involved in antigenic properties, cell receptor binding and penetration of the virion into the host-cell. The 10,976 bases long single-stranded viral RNA encodes an uninterrupted open reading frame that is translated into a polyprotein precursor and eventually processed into capsid (C), membrane (M/prM) and envelope (E) structural proteins and into seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Sumiyoshi *et al*., 1987; Solomon *et al*., 2003; Weaver and Barrett, 2004).

The viral E protein is a major antigen responsible for eliciting neutralizing antibody response and protective immunity in host (Konishi *et al*., 1991; McMinn, 1997). The non-structural proteins play important role in viral genome replication and expression. The NS1 protein (48 kDa) is a secreted glycoprotein and contains 2 or 3 conserved N-linked carbohydrates (Chambers *et al*., 1990) and six conserved disulphide bridges (Wallis *et al.,* 2004). The NS2B and NS3 proteins together work as a virus specific protease and cleave polyprotein to generate 3 structural and 7 non-structural proteins (Chambers *et al*., 1990; Yusof *et al*., 2000). The NS3 also has helicase activity (Matusan *et al*., 2001). Both NS4A and NS4B have a role in viral replication. The NS5 protein is the viral RNA dependent RNA polymerase.

2.3 Genotype of JEV

All known JEV isolates comprise a single serotype and this fact is supported by phylogenetic analysis performed for different genome regions of Japanese encephalitis virus (Tsarev *et al*., 2000). Major alterations in the genome of resulting viral variants frequently occur in the envelope (E) protein (Deubel *et al*., 1993). Until now, five genotypes of JEV have been described based on phylogenetic analysis of the viral E gene (Williams *et al*., 2000; Uchil and Satchidanandam, 2001; Solomon *et al*., 2003). Most virus strains of genotype I were isolated from northern Thailand, Cambodia and Korea; those from genotype II were isolated from southern Thailand, Malaysia, Indonesia and Australia; those from genotype III were isolated from areas of Asia that are largely temperate, such as Japan, Korea, China, Taiwan, Philippines, India and Sri Lanka; and those from genotype IV have only been isolated from Indonesia (Chen *et al*., 1992).

The Muar strain, isolated from Malaysia in 1952 is the only known representative of the proposed fifth genotype (Uchil and Satchidanandam, 2001). The genotype III is the most widely distributed genotype with Nakayama strain being the prototype (Mackenzie *et al.*, 2004). It is widely distributed in Asian countries, including India. However, during the past decade, genotype I has been introduced into South Korea, Thailand and China and has replaced the genotype III strains that had been circulating in Japan and Vietnam during the mid-1990s (Nga *et al.*, 2004).

2.4 Transmission of JEV

The infection is transmitted among birds, pigs, humans and other vertebrate host through the bite of an infected mosquito (Himani *et al*., 2014) particularly *Culex tritaeniorhynchus*. Human and horse are dead end host meaning that they do not participate in the spread of JE because of low level and also short-lived viraemia (Rosen, 1986; Solomon and Vaughn, 2002; Weaver and Barrett, 2004). Therefore, JEV naturally cycles between mosquitoes and birds or mosquitoes and pigs. Domestic pigs are the major virus-amplifying host for virus transmission to humans, not only because they develop high titres and long-lasting viraemia after natural infection but also because they live on farms in close proximity to human habitats (Yun and Lee, 2006). Other important amplifying hosts are herons, egrets and other ardeid birds that also act as maintenance hosts and may contribute to the long-distance dissemination of JEV into new geographic locations, since the virus does not cause any clinical signs in these natural hosts (Solomon *et al.*, 2003; Nga *et al.*, 2004). Several researchers have demonstrated vertical transmission of JE virus in mosquito vectors (Rosen, 1986). JE virus has also been isolated from the mosquito larvae, supporting vertical transmission and overwintering in mosquito eggs (Rosen *et al.*, 1989). Therefore, vertical transmission in mosquitoes might be an additional mechanism by which virus maintains itself in nature.

2.5 Birds as Reservoir and Amplifying Host

JEV transmission cycle had involved *Culex tritaenirhynchus* mosquitoes and its similar species that lay egg in rice paddies and other open water source, with pigs and aquatic birds as principal vertebrae amplifying hosts (Halsted *et al.,* 2008). Domestic and migrating birds has been deemed as an effective host for JEV transmission. Avian species are the most important amplifier because of their significant viraemia following JEV infection, large numbers of population, high turnover rate and preferential feeding by vectors (Tsuchie *et* *al*., 1994). It has been proven that 86.7% of serum samples of wild birds catched in Korea were positive for JEV (Yang *et al*., 2011). Migratory birds such as egrets and herons may serve as viral reservoirs or amplifying hosts but do not develop clinical signs. 85.9% wild ducks captured in Hokkaide, Japan have an antibody against JEV (Saito *et al*., 2009). Other than that, ducklings and chickens also have been considered to play at best minor role in outbreaks because of their low or absent viraemia (Cleton *et al*., 2014). In India, 35.6% of wildbirds have specific antibody against JEV (Rodrigues *et al*., 1981)

2.6 Enzyme-linked Immunorsorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is an analytical methods that show antigen-antibody reactions through the colour change obtained by using an enzyme-linked conjugate and enzyme substrate that will serve to identify the presence and concentration of molecules in biologic fluids (Hornbeck, 2001). This method can be used to measure even substances in very low concentrations with hardly any risk of interference since it is almost impossible for an antibody or antigen to be bound to a molecule other than its own antigen or antibody. With ELISA, the type and amount of antibody able to be identified and not to forget the specific antigen and the amount of the antigen. This methods are generally referred to as enzyme immunotest (Engvall, 2010; Hornbeck, 2001). This study uses a 96-well pre-coated with antigen against JE IgG which will provides a qualitative assessment through a double-antigen sandwich ELISA concept. In double-antigen sandwich ELISA, the target antibody that is presence in the serum sample will be sandwiched in between two antigen. The presence of antibody in the serum sample then will be detected by coloration showed in each well due to reactions between the enzyme and the substrate. Coloration shows a positive result, while lack of coloration indicates lack of enzymes, or a negative results (Aydin, 2015).

3.0 MATERIALS AND METHODS

3.1 Animals

A total of forty-five birds were sampled from three different locations in Malaysia. The locations were Tanjung Piandang, Perak, Putrajaya Wetlands and Jenderam Hulu, Sepang, Selangor in January 2016. Out of forty-five birds, twenty-three samples are village chicken (*Gallus gallus domesticus*) from Tanjung Piandang, Perak, twelve samples are jungle fowl cross (*Gallus gallus*) from Jenderam Hulu, Sepang, Selangor and ten samples are waterbirds from Putrajaya Wetlands. Species chosen from waterbirds group are white swan and black swan (*Cygnus atratus*), egyptian goose (*Alopochen aegyptiaca*), great white pelican (*Pelecanus onocrotalus*), pink-backed pelican (*Pelecanus rufescens*) and American flamingo (*Phoenicopterus ruber*). The birds were selected through random sampling. All the birds were healthy and the area never had a history of Japanese Encephalitis outbreak. Sampling of the animals was approved by IACUC dated 21st December 2015 (AUP No: FYP.2015/FPV.038).

3.2 Risk Factors

Tanjung Piandang, Perak is a foreland or promontory which was very close to paddy field, swamp area and river which eventually will become a place for a mosquito to lay eggs. As stated from previous study, *Culex tritaeniorhynchus* which is main vector for JEV was the most predominant mosquitoes in ricefield ecosystem (Macdonald *et al*., 1965, 1967; Hill *et al*., 1969; Vythilingam *et al*., 1993). Paddy field area was also a habitat for migrating birds such as egret which is the reservoir for JEV (Figure 1). The second location at Putrajaya Wetland had about similar risk factors as the first location, which is an area that were saturated with water and can become a possible place for mosquito to lay eggs. Birds at this location also had indirectly contact with other stray birds such as painted stork and pigeon which can act as possible amplifying vector for JEV. The third location was at Jenderam Hulu, Sepang, Selangor which was a possible place to have high population of mosquito since the farm was surrounded with bushes and vegetation areas. *Aedes albopictus* which is one of the possible vector carrying JEV is more commonly found in open spaces with shaded vegetation and in water containers (Saleeza *et al*., 2011). Information on each bird and history of the farm was retrieved from the owner and all the information were tabulated (Appendix 8.1). The risk factors investigated in this study include 1) species [village chicken, waterbirds or jungle fowl cross]; 2) age [categorized into young or adult birds according to species]; 3) sex [male, female or undetermined for waterbirds]; and 4) location.

3.3 Sample Collection

The birds were physically restrained, and blood sample was collected using 23G or 25G needles (B.Braun®, Germany) and a 3mL syringe (TERUMO®

syringe, Philipines). For each bird, approximately 1-3mL (depending on the bird's size) of blood was withdrawn via the wing vein as it is easily accessible because the vein crosses the elbow medially and medial metatarsal vein for waterbirds only. Blood collected was then transferred into 3mL Plain vacutainer tubes (BD Vacutainer®, United States) and gently inverted to ensure thorough mixing of the blood with the clot activator and to prevent haemolysis. All tubes were labeled clearly with number according to the order in the information table (Appendix 8.1) prior to blood collection. The tubes were then transported back to Virology laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM).

3.4 Sample Processing

All tubes with blood sample was left standing for one to three hours in room temperature before centrifuged at 2000 x *g* for 10 minutes (Hettich Universal 32R, United Kingdom). Serum was extracted using a micropipette and placed into 1.5mL Eppendorf® tubes. Eppendorf® tubes were labelled accordingly and stored in -20°C (Sanyo, Japan) until it further analysis.

3.5 Serological Test

All the sera collected were assayed using MyBiosource® Chicken Japanese Encephalitis IgG Antibody enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction. The kit uses a 96 strip wells, pre-coated with Chicken JE IgG antigen, and provides qualitative determination of activated

Japanese Encephalitis IgG antibody (JE-IgG) by using a double-antigen sandwich ELISA concept (Figure 2). When pipetting the serum sample into the ELISA kit wells, each sample was repeated once. The blank sample was placed in well A1, B1 followed by negative control in well C1, D1 and positive control in well E1 and F1. Serum samples from village chicken (*Gallus gallus domesticus*) were placed first starting from well G1 followed by waterbirds from well E7 and jungle fowl cross (*Gallus gallus*) from well A10. Repeated sample was placed one after another to ensure least possibility of cross contamination between wells (Figure 3).

After the addition of Stop solution in the final step of the assay procedure, the optical density (O.D) of the plate was read at 450nm using a microplate reader (TECAN Infinite M200, Switzerland) within 15 minutes, as indicated in the manual. The O.D measurement was repeated three times without removing the plate from the ELISA reader. This was done to reduce inconsistency of readings due to machine error.

The test is validated if the mean value of Negative Control O.D ($O.D_{NC}$) is less or equal to 0.15 and the mean value of Positive Control O.D ($O.D_{PC}$) is more or equal to 1.00. For result interpretation, the cut off value was calculated using the following formula

$$\text{CUT OFF} = O.D_{NC} + (0.15 \times O.D_{PC})$$

The sample is considered positive if the mean value of sample O.D ($O.D_{SAMPLE}$) is more or equal to the cut off value and considered negative if less than the cut off value.

3.6 Statistical Analysis

Categorical data were assessed using GraphPad Prism 5 software. The chi-square test was used to study the association of the risk factor and presence of antibodies against JEV. If the calculated P value is less than 0.05, it is considered statistically significant. Odds ratio was also calculated to study the potential risk factor.

Figure 1: Migrating birds spotted on paddy field area at Tanjung Piandang, Perak

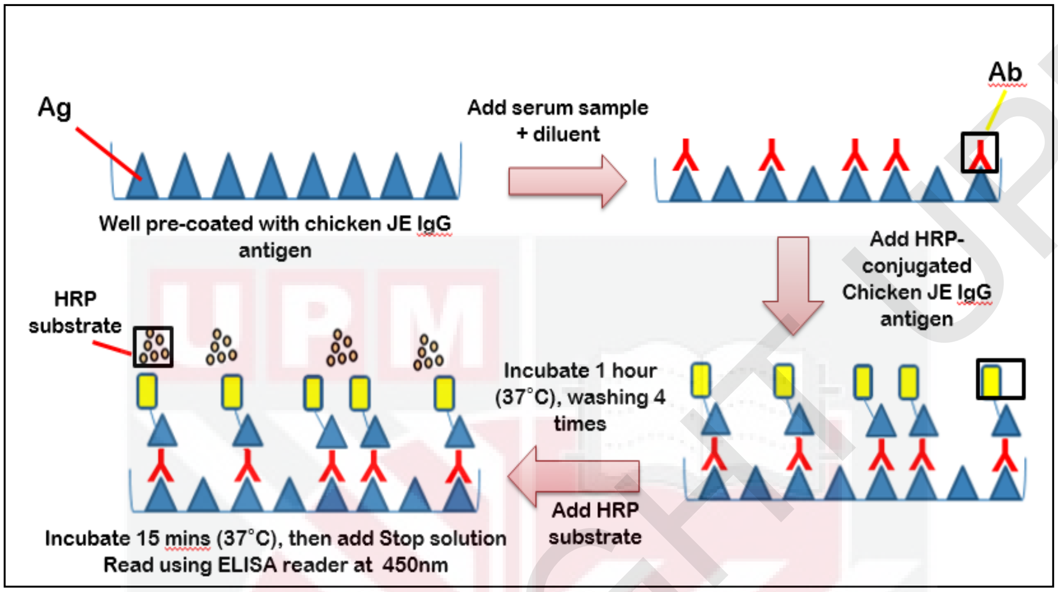

Figure 2: Double-antigen sandwich ELISA concept

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	Blank											
C	-ve											
D	-ve											
E	+ve											
F	+ve											
G												
H												

Figure 3: Illustration of sample arrangement on ELISA plate

Legend:

Blank	Blank sample which did not contain any serum sample or HRP-conjugate reagent
-ve	Negative control sample, using the negative control solution provided in the kit
+ve	Positive control sample, using the positive control solution provided in the kit
	Serum sample from village chicken (*Gallus gallus domesticus*)
	Serum sample from waterbirds
	Serum sample from Jungle fowl cross (*Gallus gallus*)

RESULTS

A total of 45 sera samples of birds were collected from three different location at Peninsular Malaysia and had been analyzed for presence of IgG antibodies against JEV using double-antigen sandwich ELISA kit. In general, IgG antibodies against JEV has been detected in 28.89% (13/45) sample which had an optical density value of more than 0.1582.

An overview of the distribution of all 45 sera samples based on location had been shown in Figure 4. The highest prevalence was at Jenderam Hulu,Sepang, Selangor (50%) followed by Tanjung Piandang, Perak (21.74%) and lastly Putrajaya Wetland (20%). Statistically, there was no association between location and seroprevalence of JEV since the P value was more than 0.05 (P value = 0.1049). However, Jenderam Hulu, Selangor has 4 times greater risk of having seroconversion against JEV as compared to Putrajaya Wetland while Tanjung Piandang have about the same risk with Putrajaya Wetland. For seroprevalence of JEV according to age, the highest prevalence was in young birds (35.71%). Statistically, there was no association between age and seroprevalence of JEV since the P value was more than 0.05 (P value = 0.5024) (Figure 6). Having said that, young birds have 1.6 times greater risk of having seroconversion against JEV compared to adult birds. For the seroprevalence of JEV based on species of birds, it shows that both jungle fowl cross (*Gallus gallus*) and American flamingo

(*Phoenicopterus ruber*) had the same prevalence (50%) followed by village chicken (*Gallus gallus domesticus*) (21.74%) (Figure 5). Statistically, there was no association between species of birds and seroprevalence of JEV (P value= 0.9848). Seroprevalence of white swan and black swan (*Cygnus atratus*), egyptian goose (*Alopochen aegyptiaca*), great white pelican (*Pelecanus onocrotalus*) and pink-backed pelican (*Pelecanus rufescens*) were unable to be calculated since the sample size was too small which is less or equal to two samples. For seroprevalence of JEV based on sex, it shows that the highest prevalence was in male (50%) (Figure 7). Statistically, there was no association between sex and seroprevalence of JEV since the P value was more than 0.05 (P value= 0.1610). However, male birds has 2.6 times greater risk of having seroconversion against JEV compared to female birds. The undetermined age was focusing solely on waterbirds since some of the waterbirds age are unable to be identified.

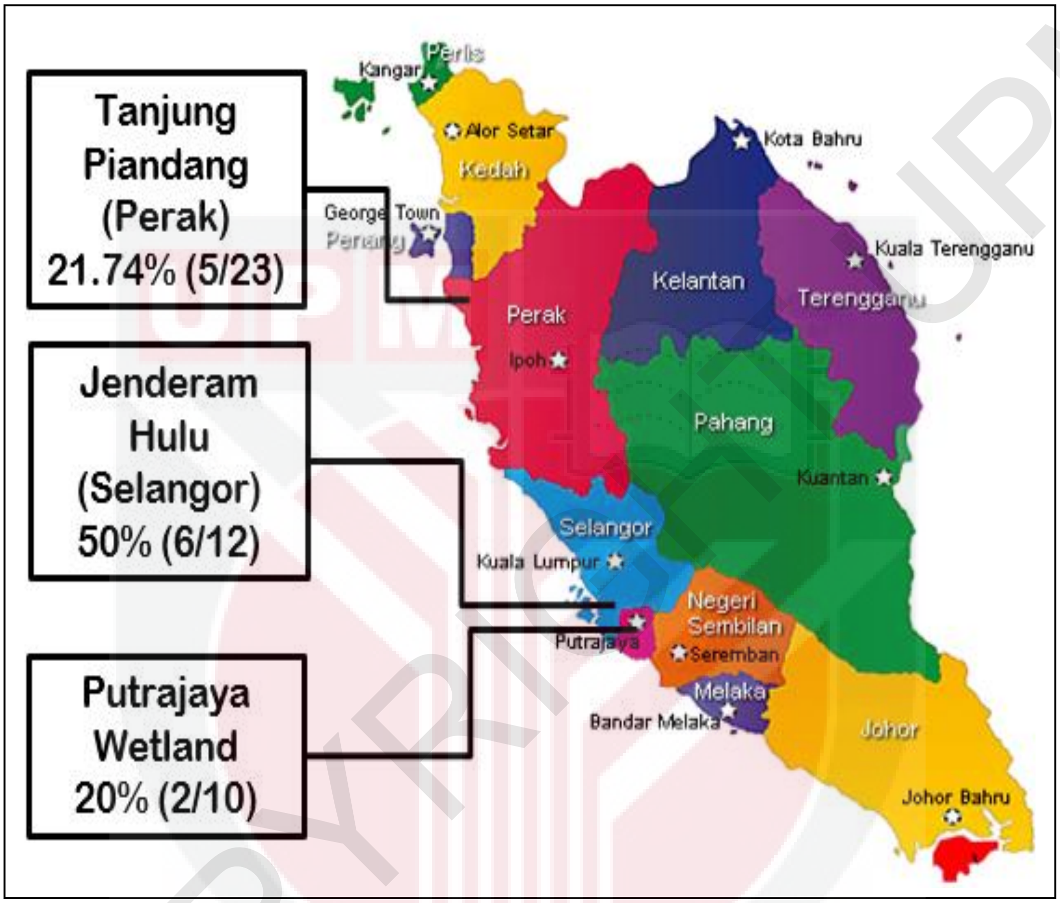

Figure 4: Seroprevalence of JEV in West Coast of Malaysia. Highest JEV prevalence in birds are detected in Jenderam Hulu, Sepang, Selangor followed by Tanjung Piandang, Perak and Putrajaya Wetland.

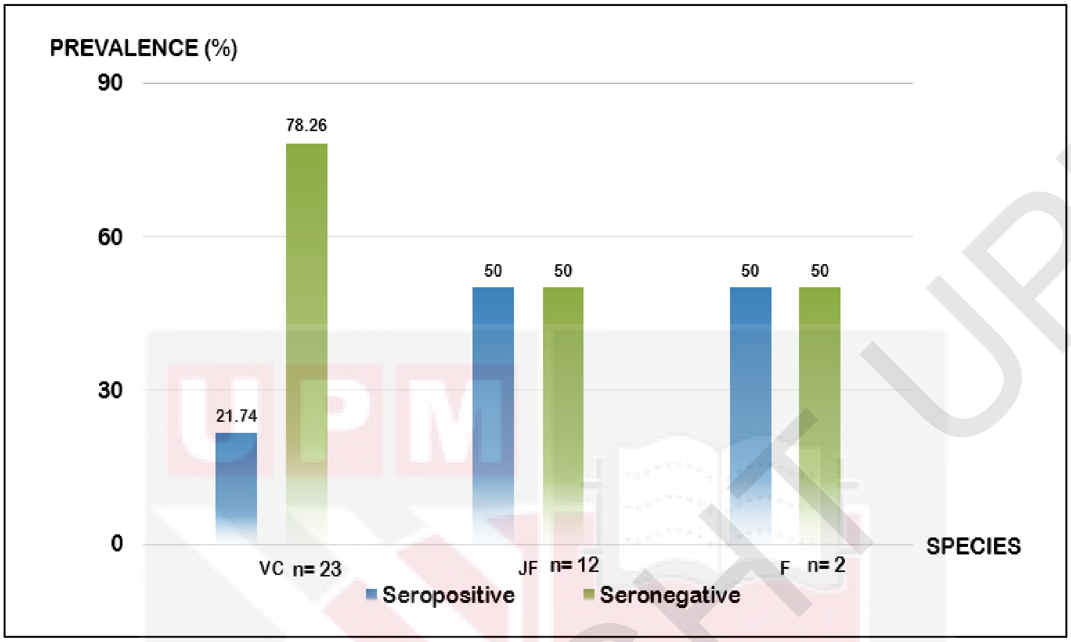

Figure 5: Seroprevalence of JEV based on birds species. Jungle fowl (JF) and American flamingo (F) have 50% prevalence against JEV antibodies whereas village chicken (VC) only showed 21.74%.

Legend:

JF: Jungle fowl cross

F: American Flamingo

VC: Village chicken

Risk factor	Seropositive	Seronegative	Seroprevalence (%)	95% C.I
Young	5	9	35.71	0.41 to 6.2
Adult	8	23	25.81	

Figure 6: Seroprevalence of JEV based on birds age. In young birds, 35.71% (5/14) sample have an antibodies against JEV while in adult birds, only 25.81% (8/31) sample have an antibodies against JEV.

Risk factor	Seropositive	Seronegative	Seroprevalence (%)	95% C.I
Male	3	3	50	0.43 to 15
Female	9	23	28.13	

Figure 7: Seroprevalence of JEV based on birds sex. Male birds, 50% (3/6) sample showed seroprevalence against JEV while in female birds, only 28.13% (9/32) sample showed seroprevalence against JEV.

DISCUSSION

In this study, the seroprevalence of JEV in west coast of Malaysia was found to be 28.89%. The number can be considered low as compared to study done by Yang *et al*. (2011) with 86.7% and Saito *et al*. (2009) with 85.9%. The reasons behind this could be due to location of the samples where it is focusing more on the presence of water body which at the paddy field area, wetlands and plantation area without pig rearing area nearby the samples location. According to study by Erlanger *et al*. (2009), the impact on JEV transmission is stronger in areas where rice production and pig rearing overlap than in areas where both activities are physically separated.

All the risk factors investigated shows no association with the seroprevalence of JEV since the chi-square value is more than 0.05. Study based on locations showed that the highest prevalence is in Jenderam Hulu, Sepang, Selangor with 50% as compared to Tanjung Piandang with 21.74% and Putrajaya Wetland with 20%. Higher rate in Jenderam Hulu, Sepang, Selangor could be supported by Vythilingam *et al.* (1997) report which stated that *Culex tritaeniorhynchus* and *Culex gelidus* were the most abundant species in Sepang district and JEV were successfully isolated from *Culex tritaeniorhynchus*, *Culex gelidus* and Culex *quinquefasciatus. Culex tritaeniorhynchus* had been known as the major vector carrying and transmitting JEV to vertebrate host. However for

Culex gelidus and *Culex quinquefasciatus*, both species are considered as important secondary or regional vectors (Peiris *et al.*, 1992; Vythilingam *et al.*, 1994; Ritchie *et al.*, 1997) to transmit the JEV infection. Based on Burke and Leake (2000), other than *Culex tritaeniorhynchus*, *Culex gelidus* is also considered to have high competence to transmit JEV infection. On the other hand, *Culex quinquefasciatus* is considered to have moderate competence to transmit JEV infection. High number of these three mosquitoes species might increase the risk of birds in Jenderam Hulu, Sepang, Selangor to have seroconversion against JEV.

According to age, young birds have higher prevalence with 35.71% as compared to adult birds and also have greater risk of having seroconversion against JEV. The reasons for this might be because younger birds tend to develop significantly higher viraemia level than older birds (Cleton *et al.*, 2014) and according to study by Soman *et al*. (1977), antibodies against JEV have been detected in chickens and ducklings at a young age in JEV-endemic region. For the seroprevalence according to age of the birds, this study showed that male birds have higher seroprevalence as compared to female birds. Results in this study shows that there is no association between species of birds and seroprevalence of JEV but it showed that jungle fowl cross and flamingo having higher seroprevalence with 50% as compared to village chicken. However, there was still no explanation regarding this.

There are few factors that might influence endemic of JEV in a location or country. As according to study done by Vaughn and Hoke (1992), JE cases occur more sporadically and peaks usually observed during rainy season in tropical areas such as Malaysia, Indonesia, southern Vietnam, southern Thailand, southern India and Philippines. Raining season or so called wet monsoon in Southwest Malaysia is usually between May and October and this might contribute to the transmission of JEV since there will be a lot of water body which act as favorable breeding sites for mosquitoes. Extreme rainfall after a period of drought can trigger outbreaks in situations in which vector populations which is the mosquitoes rapidly proliferate and blood feeding is spilling over to human (Erlanger *et al*., 2009). Since this study was conducted during the dry spell of January, the seroprevalence of JEV in birds might be reduced. Other than that, as described by Tsai (1998), changing in agricultural practices, can lead to the spread of JEV such as increasing irrigation for example in rice agriculture which provides mosquito breeding sites and animal husbandry which provides host animals. It also had been proven by Impoinvil *et al*. (2011) in his study in Asia that the extended of paddy field areas and increased in number of pigs since the early 1960s had drove the JE risk and incidence and also in Nepal which the percentage of irrigated land was significantly associated with confirmed JE cases. Both of the study showed that changing of land use for agricultural practices actually does influence transmission of JEV infection especially when there is increase of irrigation area and animal rearing area.

CONCLUSIONS AND RECOMMENDATIONS

This study had revealed that there was presence of seroconversion against JEV in birds in Malaysia with the seroprevalence of 28.89%. The highest seroprevalence had been recorded in birds at Jenderam Hulu, Sepang, Selangor where it has been suggested for having high number of mosquitoes which helps in transmitting JEV infection to the birds. In addition, young birds had showed higher seroprevalence as compared adult birds. Statistically, all birds have similar risk of having seroconversion against JEV in terms of age, sex, species and location.

The presence of IgG antibodies in birds indicate previous exposure to JEV. This study provides the first database on antibodies status against JEV of birds in Malaysia. Transmission of JEV would be very challenging to prevent and control due to the habitat of the vectors in agricultural areas. Further studies should be focusing on determination of JEV genotype circulating in bird population in Malaysia by molecular studies. Although vaccines are available for humans and animals, but these vaccines are not included as mandatory vaccines in the health programme in Malaysia.

REFERENCES

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, *72*, 4-15. http://dx.doi.org/10.1016/j.peptides.2015.04.012.

Burke, D. S. and Leake, C. J. (1988). Japanese encephalitis. In *The Arboviruses: Epidemiology and Ecology* (ed. by T. P. Monath), Vol. 3. Boca Raton: CRC Press Inc., Pp. 63-92.

Burke, D. S. and Leake, C. J. (2000). Japanese encephalitis. In *The Arboviruses: Epidemiology and Ecology* (ed. by T. P. Monath), pp. 64-84. CRC Press Inc., Boca Raton, Florida.

Cardosa, M. J., Tio, P. H. and Kaur, P. (1995*). Japanese encephalitis virus is an important cause of encephalitis among children in Penang*. Southeast Asian Journal of Tropical Medicine and Public Health 26, 272–275.

Chambers, T. J., Hahn, C. S, Galler, R. and Rice, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annual Review of Microbiology*, 44: 649-688.

Chen, W. R., Rico-Hesse, R. and Tesh, R. B. (1992). A new genotype of Japanese encephalitis virus from Indonesia. *American Journal of Tropical Medicine and Hygiene*, 47: 61-69.

Chen, W., Tesh, R.., and Rico-Hesse, R. (1990). Genetic Variation of Japanese Encephalitis Virus in Nature. *Journal of General Virology*, *71*(12), 2915-2922.

Cleton, N., Bosco-Lauth, A., Page, M., and Bowen, R. (2014). Age-Related Susceptibility to Japanese Encephalitis Virus in Domestic Ducklings and Chicks. *American Journal of Tropical Medicine and Hygiene*, *90*(2), 242-246. http://dx.doi.org/10.4269/ajtmh.13-0161.

Deubel, V., Nogueira, M., Drouet, M. T., Zeller, H., Reynes, J. M. and Do, Q. H. (1993). Direct sequencing of genomic cDNA fragments amplified by the polymerase chain reaction for molecular epidemiology of dengue 2 viruses. *Archives of Virology*, 129: 197-210.

Engvall, E. (2010). The ELISA enzyme-linked immunosorbent assay. Clin Chem; 56:319–20.

Erlanger, T., Weiss, S., Keiser, J., Utzinger, J., and Wiedenmayer, K. (2009). Past, Present, and Future of Japanese Encephalitis. *Emerging. Infectious. Diseases,. 15*(1), 1-7. http://dx.doi.org/10.3201/eid1501.080311.

Fang, R., Hsu, D. R. and Lim, T.W. (1980). Investigation of a suspected outbreak of Japanese encephalitis in Pulau Langkawi. Malaysian Journal of Pathology 3, 23–30.

Ghosh, D. and Basu, A. (2009). Japanese Encephalitis – A Pathological and Clinical Perspective. *PLoS Neglected Tropical Diseases*, 3(9): 437-441.

Halsted, S.B. and Jacobson, J. (2008). Japanese encephalitis vaccines. In: Plotkin SA, orensten WA, oofit Pa, editors. *Vaccines*. 5th ed. Philadelphia: Elsevier;.pp. 311-52.

Hill, M. N., Varma, M. G. R., Mahadevan, S. and Meers, P. D. (1969). Arbovirus infections in Sarawak: observations on mosquitoes in the premonsoon period, September to December 1966. *Journal of Medical Entomology*. 6:398-406.

Himani, D., Kumar, H., Bhilegaonkar, K., and Kumar, A. (2014). Japanese Encephalitis: A Veterinary Perspective. *Journal of Foodbourne and Zoonotic Diseases*, *2*(4), 59-67.

Hornbeck, P. (2001). Enzyme-linked immunosorbent assays. Current Protocol Immunology, http://dx.doi.org/10.1002/0471142735.im0201s01 (Chapter 2: Unit 2.1).

Impoinvil, D., Solomon, T., Schluter, W., Rayamajhi, A., Bichha, R., and Shakya, G. *et al*. (2011). The Spatial Heterogeneity between Japanese Encephalitis Incidence Distribution and Environmental Variables in Nepal. *Plos ONE*, *6*(7), e22192. http://dx.doi.org/10.1371/journal.pone.0022192.

Konishi, E., Pincus, S., Fonseca, A. L., Shope, R. E., Paoletti, E. and Mason, P. W. (1991). Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology*, 185(1): 401-410.

Macdonald, W. W., Smith, C. E. G., andnWeb, H. E. (1965). Arbovirus infections in Sarawak: observations on the mosquitoes. *Journal of Medical Entomology* 1: 335-347.

Macdonald, W. W., Smith, C. E. G., Dawson P. S., GanapathipiUai, A. and Mahadevan, S. (1967). Arbovirus infections in Sarawak: further observations on mosquitoes. *Journal of Medical Entomology*. 4: 146-157.

Mackenzie, J. S., Guble, D. J. and Petersen, L. R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and Dengue viruses. *Nature Medicine*, 10(12): S98- S109.

Matusan, A., Pryor, M., Davidson, A. and Wright, P. (2001). Mutagenesis of the Dengue virus type 2 NS3 protein within and outside helicase motifs. *Journal of Virology*, 75: 9633-9643.

McMinn, P. C. (1997). The molecular basis of virulence of the encephalitogenic flaviviruses. *Journal of General Virology*, 78: 2711-2722.

Nemeth, N., Bosco-Lauth, A., Sciulli, R., Gose, R., Nagata, M., and Bowen, R. (2010). Serosurveillance for Japanese Encephalitis and West Nile Viruses in Resident Birds in Hawai‘i. *Journal of Wildlife Diseases*, *46*(2), 659-664.

Nga, P. T., Del Carmen Parquet, M., Cuong, V. D., Ma, S. P., Hasebe, F., Inoue, S., *et al.* (2004). Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. *Journal of General Virology*, 85, 1625-1631.

Peiris, J. S., Amerasinghe, F. P., Amerasinghe, P. H., Ratnayake, C. B., Karunaratne, S. H. & Tsai, T. F. (1992). Japanese encephalitis in Sri Lanka--the study of an epidemic: vector incrimination, porcine infection and human disease. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, 86, 307-313.

Ritchie, S. A., Phillips, D., Broom, A., Mackenzie, J., Poidinger, M. and van den Hurk, A. (1997). Isolation of Japanese encephalitis virus from Culex annulirostris in Australia. *American Journal of Tropical Medicine and Hygiene,* 56, 80-84.

Rodrigues, F., Guttikar, S., and Pinto, B. (1981). Prevalence of antibodies to Japanese encephalitis and West Nile viruses among wild birds in the Krishna-Godavari Delta, Andhra Pradesh, India. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *75*(2), 258-262. http://dx.doi.org/10.1016/0035-9203 (81)90330-8.

Rosen, L. (1986). The natural history of Japanese encephalitis virus. *Annual Reviews of Microbiology,* 40: 395-414.

Rosen, L. (1987). Overwintering mechanisms of mosquito-borne arboviruses in temperate climates. *American Journal of Tropical Medicine and Hygiene,* 37, 69S-76S.

Rosen, L., Lien, J. C., Shroyer, D. A., Baker, R. H. and Lu, L. C. (1989). Experimental vertical transmission of Japanese encephalitis virus by *Culex tritaeniorhynchus* and other mosquitoes. *American Journal of Tropical Medicine and Hygiene*, 40: 548- 556.

Saito, M., Osa, Y., Asakawa, M. (2009). Antibodies to flaviviruses in wild ducks captured in Hokkaido, Japan: risk assessment of invasive flaviviruses. Vector Borne Zoonotic Dis.; 9:253–258.

Saleeza, S., Norma-Rashid, Y., and Sofian-Azirun, M. (2011). Mosquitoes Larval Breeding Habitat in Urban and Suburban Areas, Peninsular Malaysia. *International Science Index, Bioengineering and Life Sciences*, *5*(10).

Solomon, T. (2006). Control of Japanese encephalitis–within our grasp? *New England Journal of Medicine*.; 355:869–71. DOI: 10.1056/NEJMp058263.

Solomon, T. and Vaughn, D. W. (2002). Pathogenesis and clinical features of Japanese encephalitis and West Nile virus infections. *Current Topics in Microbiology and Immunology*, 267, 171-194.

Solomon, T., Ni, H., Beasley, D. W., Ekkelenkamp, M., Cardosa, M. J. and Barrett, A. D. (2003). Origin and evolution of Japanese encephalitis virus in Southeast Asia. *Journal of Virology,* 77: 3091-3098.

Soman, R. S., Rodrigues, F. M., Guttikar, S. N., Guru, P. Y., (1977). Experimental viraemia and transmission of Japanese encephalitis virus by mosquitoes in ardeid birds. *Indian Journa of Medical Research* 65: 709–718.

Sumiyoshi, H., Mori, C., Fuke, I., Morita, K., Kuhara, S., Kondou, J., Kikuchi, Y., Nagamatu, H. and Igarashi, A. (1987). Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology*, 161: 497-510.

Tiwari, S., Singh, R., Tiwari, R., & Dhole, T. (2012). Japanese encephalitis: a review of the Indian perspective. *The Brazilian Journal of Infectious Diseases*, *16*(6), 564-573. http://dx.doi.org/10.1016/j.bjid.2012.10.004.

Tsai, T. F., Popovici, F., Carnescu, C., Campbell, G. L. and Nedlelcu, N. I. (1998). West Nile encephalitis epidemic in Southeastern Romania. Lancet 352**:**767-771.

Tsarev, S. A., Sanders, M. L., Vaughn, D. W. and Innis, B. L. (2000). Phylogenetic analysis suggests only one serotype of Japanese encephalitis virus. *Vaccine,* 18: 36-43.

Tsuchie, H., Oda, K., Vythilingam, I., Thayan, R., Vijayamalar, B., and Sinniah, M. *et al*. (1994). Genetic study of Japanese encephalitis viruses isolated in Malaysia. *Japanese Journal of Medical Science and Biology*, *47*(2), 101-107.

Uchil, P. D. and Satchidanandam, V. (2001). Phylogenetic analysis of Japanese encephalitis virus: envelope gene based analysis reveals a fifth genotype, geographic clustering, and multiple introductions of the virus into the Indian subcontinent. *American Journal of Tropical Medicine and Hygiene*, 65: 242- 251.

Vaughn, D. W., Hoke, C. H. Jr. (1992). The epidemiology of Japanese encephalitis: prospects for prevention. *Epidemiologic Reviews*, 14:197–221.

Vythilingam, I., Inder Singh, K., Mahadevan, S., Zaridah, M. S., Ong, K. K., and Zainul Abidin, M. H. (1993). Studies on Japanese encephalitis vector mosquitoes in Selangor, Malaysia. *Journal of American. Mosquito Control Association*. 9: 467-469.

Vythilingam, I., Oda, K., Mahadevan, S., Abdullah, G., Thim, C., and Hong, C. *et al*. (1997). Abundance, Parity, and Japanese Encephalitis Virus Infection of Mosquitoes (Diptera: Culicidae) in Sepang District, Malaysia. *Journal of Medical Entomology*, *34*(3), 257-262. http://dx.doi.org/10.1093/jmedent/34.3.257.

Vythilingam, I., Oda, K., Tsuchie, H., Mahadevan, S. & Vijayamalar, B. (1994). Isolation of Japanese encephalitis virus from Culex sitiens mosquitoes in Selangor, Malaysia. *Journal of American Mosquito Control Association*, 10, 228-229.

Wallis, T. P., Huang, C. Y., Nimkar, S. B., Young, P. R. and Gorman, J. J. (2004). Determination of the disulfide bond arrangement of dengue virus NS1 protein. *Journal of Biological Chemistry*, 279: 20729-20741.

Weaver, S. C. and Barrett, A. D. T. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. *Nature Reviews Microbiology*, 2: 789-801.

Williams, D. T., Wang, L. F., Daniels, P. W. and Mackenzie, J. S. (2000). Molecular characterization of the first Australian isolate of Japanese encephalitis virus, the FU strain. *Journal of General Virology*, 81: 2471-2480.

World Health Organization, (1999). *1999-Epidemic Encephalitis in Malaysia*. Retrieved from http://www.who.int/csr/don/1999_03_26/en/.

Yang, D., Oh, Y., Kim, H., Lee, Y., Moon, O., and Yoon, H. *et al*. (2011). Serosurveillance for Japanese encephalitis virus in wild birds captured in Korea. *Journal of Veterinary Science, 12*(4), 373.

Yun, S. I. and Lee, Y. M. (2006) Japanese encephalitis virus: molecular biology and vaccine development. In *Molecular biology of the Flavivirus*, pp. 225-271. Horizon Scientific Press, Norwich, United Kingdom.

Yusof, R., Clum, S., Wetzel, M., Krishna Murthy, H. M. and Padmanabhan, R. (2000). Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. *Journal of Biological Chemistry,* 275(14): 9963-9969.

APPENDIX

List of Birds Sample and Information

No.	Sex	Age	Species	Health Status
1	Female	Young	VC	Healthy
2	Female	Young	VC	Healthy
3	Female	Young	VC	Healthy
4	Female	Young	VC	Healthy
5	Female	Young	VC	Healthy
6	Female	Young	VC	Healthy
7	Female	Young	VC	Healthy
8	Female	Young	VC	Healthy
9	Female	Young	VC	Healthy
10	Female	Young	VC	Healthy
11	Female	Young	VC	Healthy
12	Female	Young	VC	Healthy
13	Female	Young	VC	Healthy
14	Female	Young	VC	Healthy
15	Female	Adult	VC	Healthy
16	Female	Adult	VC	Healthy
17	Female	Adult	VC	Healthy
18	Female	Adult	VC	Healthy
19	Female	Adult	VC	Healthy
20	Female	Adult	VC	Healthy
21	Female	Adult	VC	Healthy
22	Female	Adult	VC	Healthy
23	Female	Adult	VC	Healthy
24	Undetermined	Young	BS	Healthy
25	Undetermined	Young	BS	Healthy
26	Female	Adult	EG	Healthy
27	Female	Adult	EG	Healthy
28	Undetermined	Adult	WP	Healthy
29	Undetermined	Adult	WP	Healthy
30	Undetermined	Adult	PBP	Healthy
31	Undetermined	Adult	WS	Healthy
32	Undetermined	Adult	F	Healthy
33	Undetermined	Adult	F	Healthy

34	Female	Adult	JF	Healthy
35	Female	Adult	JF	Healthy
36	Female	Adult	JF	Healthy
37	Female	Adult	JF	Healthy
38	Female	Adult	JF	Healthy
39	Female	Adult	JF	Healthy
40	Male	Adult	JF	Healthy
41	Male	Adult	JF	Healthy
42	Male	Adult	JF	Healthy
43	Male	Adult	JF	Healthy
44	Male	Adult	JF	Healthy
45	Male	Adult	JF	Healthy

Legend:

VC- Village chicken

JF- Jungle fowl cross

BS- Black sawn

EG- Egyptian goose

WP- White pelican

PBP-Pink-backed pelican

F- American flamingo

WS- White swan