UNIVERSITI PUTRA MALAYSIA

SERO-EPIDEMIOLOGY OF JAPANESE ENCEPHALITIS VIRUS AMONG LIVESTOCK, AVIAN AND COMPANION ANIMALS IN SELECTED STATES OF MALAYSIA

KIVEN KUMAR A/L KARUNANITHI

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By

KIVEN KUMAR A/L KARUNANITHI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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November 2017

Chairman : Professor Siti Suri Arshad, PhD
Faculty : Veterinary Medicine

Japanese encephalitis (JE) is vector-borne disease causes encephalitis in human and horse as well as reproductive failure in sows. Pigs, bats, wild boar, and *ardeidae* birds play an important role as a main vertebrate amplifier of JEV. In Malaysia the disease is endemic in east Malaysia, Sarawak and epidemic in Peninsular Malaysia. The encephalitis cases were observed among human and animals reported in earlier 1950s. However, they are no data regarding seroprevalence pattern among livestock (buffaloes, pigs, cattle), avian (captivity water birds, poultry and migratory birds) and companion animals (cats, dogs). At the same time, risk factors associated with JEV seroprevalence in high risk areas in Malaysia are not documented. In this study, the objectives are to determine JEV seroprevalence rate, risk factors associated with JEV seroprevalence and determination of JEV genotype circulating among livestock (buffaloes, pigs, cattle), avian (water captivity birds, poultry and migratory birds) and companion animals (cats, dogs) were documented. Total of 461 serum samples were collected from dogs, cats, captivity water birds, village chickens, domestic pigs, buffaloes and cattle in selective states of Perak, Selangor and Sabah and screened for evidence of JEV antibodies by using a double sandwich IgG ELISA kits (DAS-ELISA). The risk factors associated with JEV seroprevalence of the animals were analysed by using IBM SPSS 22 which included *Chi*-square (χ^2 test is not appropriate if one of expected cell value is less than 5, Fisher's exact test was used at $\alpha = 0.05$) and multiple logistic regression. A total of 13 survey criteria which include gender (male vs female), health status (healthy vs sick), age (young vs adult), breeding types (local, vs import), ownership of animal (own, shelter or none) and source of animals (same area, same district, same state vs epidemic areas) were collected. At the same time, environmental factors of sampling areas including presence of stagnant water (yes or no), paddy cultivation (yes or no), presence of ardeid birds (yes or no), locality (urban vs rural) and presence of mosquitos (yes or no). In the molecular assay, total of

791 samples of plasma, serum, and buffy coat from livestock (buffaloes, pigs, cattle), companion animals (cats, dogs) and avian (water captivity birds, poultry and migratory birds) were subjected to one step RT-PCR to detect the JEV by targeting the conserved region of NS3 of the JEV. In seroprevalence study among the animals studied, dogs show the highest seropositive rate of 80% (36/45; 95%CI: ±11.69) followed by pigs with 44.4% (40/90; 95% CI: ±1.715), buffaloes with 33.3% (15/45; 95% CI: ± 6.661), cattle with 32.2% (29/90; 95% CI: ±1.058), avian 28.9% (13/45; 95% CI: ±5.757), and cats with 14.4% (13/90; 95% CI: ±7.38). This study reveals that the risk factors associated with JEV seroprevalence are varied among different species of animals. The risk factors involved in different species of animals can be either singly or more than one. In additional to that, geographical factors may influence on the seropositivity of animals to JEV. In dogs, significant risk factor associated with JEV seroprevalence is the source of dog. In cats, significant risk factors associated with JEV seroprevalence are the age, locality, breeding types, and source of cats. In cattle, significant risk factors associated with JEV seroprevalence are gender, healthy status, breeding types and source of cattle. In pigs, significant risk factors associated with JEV seroprevalence are age and ownership of pigs. In buffaloes, significant risk factors associated with JEV seroprevalence are the source of buffalo and breeding types. In avian, significant risk factors associated with JEV seroprevalence are age and the breeding types. In this study, the high JEV seroprevalence in dogs observed is believed due to the preference and selective feeding pattern of *Culex* spp. in dogs compared to cats or human, thus making the dogs more prone to infection. JEV seroprevalence in pigs in this study are lower than reported previously probably due to the location of the subject matter in a non-paddy cultivation areas. This study also observed that JEV seroprevalence rate in buffaloes and cattle are low and support previous finding of this species play minimum role in JEV transmission cycles. The low viremia following JEV infection is too low of viral load to infect vector. Low JEV seroprevalence observed in chickens in this study is rather unexpected as avian was sampled in high risks areas with paddy plantation, water bodies and mosquitoes breeding sites. This finding may be further support by the lack of pig farming in this area, as the residents are engaged in fishing activities besides paddy cultivation. In this study, JEV antigen was unable to be detected in all the samples collected from various species of animals. Preliminary findings show that, all the animals were negative for JEV antigen. This could be due to the presence of antibodies of JEV naturalised the antigen. Apart from that, the sero-complex characteristics of flaviviruses make the antigen-antibodies cross-react with each other members. This makes the antigen being neutralised and unable to be detected in blood of the host. Samples collected during acute stage of JEV infection in animals have high chances of virus being isolated. In addition to that, the oronasal fluid is preeminent for isolation and detection of JEV antigen compared to serum, cerebrum spinal fluid, plasma and buffy coat.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

SERO-EPIDEMIOLOGI VIRUS ENSEFALITIS JEPUN DALAM HAIWAN TERNAKAN, BURUNG DAN HAIWAN KESAYANGAN DI NEGERI-NEGERI TERPILIH DI MALAYSIA

Oleh

KIVEN KUMAR A/L KARUNANITHI

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Ensefalitis Jepun (JE) adalah penyakit bawaan vektor yang menyebabkan ensefalitis pada manusia dan kuda serta kegagalan pembiakan dalam khinzir. Khinzir domestik, kelawar, khinzir liar, dan burung ardeidae memainkan peranan penting sebagai penguat vertebrata utama virus ensefalitis Jepun (JEV). Di Malaysia, penyakit ini adalah endemik di timur Malaysia iaitu Sarawak dan epidemik di Semenanjung Malaysia. Kes ensefalitis dilaporkan dalam kalangan manusia dan haiwan sebelum tahun 1950-an. Walaubagaimanapun, data mengenai corak seroprevalensi dalam kalangan haiwan ternakan (kerbau, khinzir, lembu), haiwan kesayangan (kucing, anjing) dan burung (burung air, unggas dan burung berhijrah) tidak didokumentasikan. Pada masa yang sama, faktor-faktor risiko yang dikaitkan dengan seroprevalensi JEV di kawasan berisiko tinggi di Malaysia juga tidak didokumentasikan. Dalam kajian ini, objektifnya adalah untuk menentukan kadar seroprevalensi JEV, faktor-faktor risiko yang dikaitkan dengan seroprevalensi JEV dan penentuan kitaran genotip JEV di kalangan haiwan ternakan (kerbau, khinzir, lembu), haiwan kesayangan (kucing, anjing) dan burung (burung air, unggas dan burung berhijrah) telah didokumentasikan. Empat ratus enam puluh satu sampel serum telah dikumpulkan daripada anjing, kucing, burung penangkapan air, ayam kampung, khinzir domestik, kerbau dan lembu daripada negeri-negeri terpilih iaitu Perak, Selangor dan Sabah dan diperiksa untuk megesan kehadiran antibodi JEV dengan menggunakan kit komersil berkonsep ‘sandwich berganda’ IgG ELISA (DAS -ELISA). Faktor-faktor risiko yang dikaitkan dengan seroprevalensi JEV haiwan dianalisis dengan menggunakan IBM SPSS 22 yang termasuk *Chi*-square (ujian $\chi 2$ tidak sesuai apabila jumlah sample kurang daripada 5, ujian ‘exact Fisher’ telah digunakan pada peringkat $\alpha = 0.05$) dan multivariasi regresi logistik. Sebanyak 13 kriteria telah dikaji termasuk jantina (jantan dengan betina), status kesihatan (sihat dengan sakit), umur (muda dengan dewasa), jenis baka (tempatan, dengan import), pemilikan haiwan (sendiri, tempat perlindungan

atau tiada) sumber haiwan (daripada kawasan yang sama; daerah yang sama; negeri yang sama dengan kawasan epidemik) telah dikumpulkan. Pada masa yang sama, faktor persekitaran persampelan termasuk kewujudan air bertakung (ya atau tidak), penanaman padi (ya atau tidak), kewujudan burung ardeid (ya atau tidak), persekitaran tempatan (bandar dengan luar bandar) dan kewujudan nyamuk (ya atau tidak). Dalam ujian molekular, sebanyak 791 sampel termasuk plasma, serum, dan 'buffy coat' daripada haiwan ternakan (kerbau, khinzir, lembu), haiwan kesayangan (kucing, anjing) dan burung (burung air, unggas dan burung berhijrah) telah dianalisis melalui 'reverse transcription polymerase chain reaction' (RT-PCR) untuk mengesan JEV antigen dengan berasaskan bahagian NS3 JEV. Dalam kajian ini, anjing menunjukkan kadar seropositif tertinggi sebanyak 80% (36/45; 95% CI: ± 11.69) diikuti oleh khinzir sebanyak 44.4% (40/90; 95% CI: ± 1.715), kerbau sebanyak 33.3% (15/45; 95 % CI: ± 6.661), lembu sebanyak 32.2% (29/90; 95% CI: ± 1.058), burung sebanyak 28.9% (13/45; 95% CI: ± 5.757), dan kucing sebanyak 15.6% (14/90; 95% CI: ± 7.38). Kajian ini mendedahkan bahawa faktor-faktor risiko yang dikaitkan dengan seroprevalensi JEV adalah berbeza di antara spesies haiwan yang berlainan. Faktor risiko yang terlibat dalam spesies haiwan yang berlainan boleh sama ada secara tunggal atau lebih daripada satu. Tambahan pula, faktor geografi boleh mempengaruhi seropositif JEV dalam haiwan. Faktor risiko yang signifikan dalam anjing yang dikaitkan dengan seroprevalensi JEV adalah sumber anjing. Dalam kucing pula, faktor risiko yang signifikan yang berkaitan dengan seroprevalensi JEV adalah umur, lokasi, baka, dan sumber kucing. Dalam lembu, faktor risiko yang signifikan yang dikaitkan dengan seroprevalensi JEV adalah jantina, status kesihatan, baka dan sumber lembu. Dalam khinzir, faktor risiko yang signifikan yang berkaitan dengan seroprevalensi JEV adalah umur dan pemilik khinzir. Dalam kerbau, faktor risiko yang signifikan berkaitan dengan seroprevalensi JEV adalah sumber kerbau dan baka. Dalam burung, faktor risiko yang signifikan yang berkaitan dengan seroprevalensi JEV adalah umur dan baka. Dalam kajian ini, seroprevalensi JEV yang tinggi dalam anjing dipercayai kerana pilihan dan pola pemakanan oleh *Culex* spp. pada anjing berbanding dengan kucing atau manusia, sehingga menjadikan anjing lebih terdedah terhadap jangkitan. Seroprevalensi JEV khinzir dalam kajian ini adalah lebih rendah daripada yang dilaporkan sebelum ini mungkin disebabkan tidak mempunyai lokasi penanaman padi di kawasan sampel yang diperolehi. Kajian ini juga mendapati bahawa kadar seroprevalensi dalam kerbau dan lembu adalah rendah dan menyokong penemuan sebelumnya kerana spesies ini memainkan peranan minimum dalam kitaran transmisi JEV. Viremia yang rendah berikutan jangkitan JEV dengan bebanan virus yang rendah didalam perumah tidak dapat menjangkiti vektor. Seroprevalensi JEV dalam ayam dalam kajian ini tidak dijangka kerana sampel ayam telah dikumpulkan diripada kawasan-kawasan yang berisiko tinggi dengan penanaman padi, air bertakung dan tapak pembiakan nyamuk. Penemuan ini disokong selanjutnya dengan kekurangan peternakan khinzir di kawasan ini, kerana penduduk terlibat dalam kegiatan penangkapan ikan selain daripada penanaman padi. Dalam kajian ini, antigen JEV tidak dapat dikesan dalam semua sampel yang dikumpulkan dari pelbagai spesies haiwan. Penemuan awal menunjukkan bahawa semua haiwan adalah negatif untuk antigen JEV. Ini disebabkan kewujudan antibodi JEV dalam haiwan mampu meneutralisasikan antigen JEV. Selain itu, ciri-ciri sero-kompleks flavivirus membuatkan antibodi antigen bertindak balas dengan virus yang lain. Ini menjadikan antigen itu dineutralkan dan tidak dapat mengesan di dalam darah perumah. Sampel

yang dikumpulkan semasa peringkat akut jangkitan JEV pada dipercayai mampu untuk diasingkan virus. Selain itu, cecair oronasal adalah yang paling penting untuk pengasingan dan pengesanan antigen JEV berbanding dengan serum, cecair cerebrum tulang belakang, plasma dan 'buffy coat'.

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I certify that a Thesis Examination Committee has met on 16 November 2017 to conduct the final examination of Kiven Kumar a/l Karunanithi on his thesis entitled "Sero-Epidemiology of Japanese Encephalitis Virus among Livestock, Avian and Companion Animals in Selected States of Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	Alpha
Ab	Antibodies
amps	Ampere
BBB	Blood-Brain Barrier
bp	Base pair
CD	Cluster of Differentiation
CI	Confidence Intervals
C-Medium	Culture Medium
CNS	Central Nervous System
CSF	Cerebrospinal fluid
Cx.	*Culex*
˚C	Degree Celsius
DENV	Dengue virus
DI	Deionized water
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
ddPCR	Droplet Digital Polymerase Chain Reaction
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FVs	Flaviviruses
γ	Gamma
G	Gauge
g	Gravity

HRP	Horse Radish Peroxidase
IBM	International Business Machines Corporation
ID	Identity document
IgG	Immunoglobulin G
IgM	Immunoglobulin M
in	Inch
JE	Japanese encephalitis
JE-IgG	Japanese encephalitis Immunoglobulin G
JEV	Japanese encephalitis virus
kb	Kilo bases
kDa	Kilodalton
km	kilometer
LB agar	Luria broth agar
LIV	Louping ill virus
m	Meter
M	Marker
MHC	Major Histocompatibility Complex
µL	Microliter
mL	Milliliter
MVEV	Murray Valley encephalitis virus
n	Sub-total Population
N	Total number of Population
NA	Not Applicable
NAT	Nucleic acid test
NCBI	National Center for Biotechnology Information
NCR	Noncoding Region
NiV	Nipah virus

nm	Nanometer
NS	Non-structure
OD	Optical density
OD_{NC}	Optical density for Negative control
OD_{PC}	Optical density for Positive Control
OR	Odds Ratio
ORF	Open Reading Frame
%	Percentage
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
prM	Premembrane
qRT-PCR	Quantitative Polymerase Chain Reaction
Ref	Reference
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S E	Standard Error
SLEV	St. Louis encephalitis virus
Spp.	Species
SPSS	Statistical Package for the Social Sciences
ß	Beta
TAE	Tris-acetate Ethylenediaminetetraacetic acid
TCID	Tissue Culture Infective Dose
TNF	Tumor Necrosis Factor
U ml^{-1}	Unit Per Milliliter
UK	United Kingdom

UPM	Universiti Putra Malaysia
USA	United State of America
UV	Ultraviolet
UVH	University Veterinary Hospital
V	Volt
V	Vision
VN	Virus- neutralizing
VS	Versus
WNV	West Nile virus
WSLV	Wesselsbron virus
X	Times
X-gal	Indoxyl β-galactosidase
ZIKV	Zika virus

CHAPTER 1

INTRODUCTION

Japanese encephalitis (JE) is a vector-borne zoonotic disease spread by the bite of *Culex* spp. JE disease responsible for 30,000 to 70,000 cases every year and 10,000 deaths cases in the eastern Asia (Schuh *et al*., 2010; Campbell *et al*., 2011). Japanese encephalitis virus (JEV) causes central nervous system (CNS) disease in humans and animals. The virus induces abortion and weak piglets and causes fatal outcomes in humans and horses (Mackenzie *et al*., 2007). Wading birds, pigs and bats are considered as the amplifying agents, whereas humans and horses are the dead end host. Humans and other animals such as horses, cats and dogs are susceptible to JEV infection; however, they are not capable of developing enough viremia to turn mosquitoes into infected vectors. Some studies indicated that the high temperature during summer can lead to the high number of JE cases. The spread of JEV is correlated with rapid globalisation, population boom and global climate changes due to industrialisation and deforestation (Ghosh and Basu, 2009).

JEV is a positive-sense single-stranded RNA virus with 50 nm in diameter. JEV RNA consists of 10 functional proteins, which made up three structural proteins (Capsid, Premembrance, and Envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). JEV strains can be classified into five genotypes based on gene E namely the JEV (GI), JEV (GII), JEV (GIII), JEV (GIV) and JEV (GV) (Schuh *et al*., 2013). JEV belongs to the *Flavivirus* genus under the family of *Flaviviridae*. Other members of Flaviviruses include West Nile virus (WNV), Zika virus (ZIKV), Tick-borne encephalitis virus (TBEV), Spondweni virus (SPOV), Yellow fever virus (YF), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), Dengue virus (DENV) and Usutu virus (USUV), which are the few examples of common viruses.

JE cases in human were first reported in Japan in 1924. Following that, more cases were reported in many parts of Asia especially in the South Asia countries namely Thailand, Japan, China, South Korea, Singapore, Taiwan, Vietnam, Malaysia, India, Burma, Cambodia, Laos, Philippines and Sri Lanka (Schuh *et al*., 2013). A prototype virus of Nakayama strain was isolated in Japan from the brain of encephalitis patient in 1935 and later in 1940, another strain known as Beijing JEV strain was isolated in China (Yin *et al*., 1997).

JEV has been shown to circulate in its enzootic stage, presenting a risk to unvaccinated or nonimmune humans and animals. JE cases usually occur in the absence of intensive pig farming and where pig density is relatively low to other livestocks in countries including Bangladesh and India (Lord *et al*., 2015). This indicates that other hosts can play a vital role in the transmission of JEV. Several factors contributing to JEV transmission include paddy cultivation, rubber plantation, climate change, bat population, semi-intensive pig farming and migratory bird. Most of the time,

mosquitoes become inactive during winter season; however, JE cases have been continuously detected during this season in countries like Japan, China, Korea and Russia (Mackenzie *et al*., 2007). The actual transmission cycle of JEV is yet to be revealed due to the variation of animals that can be infected by JEV.

In Malaysia, there are a higher number of high-risk zones for JEV especially in pig farmers, wetlands and paddy cultivation areas. JE cases in human are reported annually among children below 15 years old even though JE is not a serious infection disease in Malaysia. The seroprevalence rate of JEV was not reported since 20 years back among livestocks and companion animals in high-risk areas in Malaysia. Besides, the risk factors contributing to seroprevalence rate were not fully documented in Malaysia. The molecular characteristics of JEV from birds, buffalo, cats, cattle, dogs and pigs are still unclear. Moreover, the roles of these animals in disease transmission and as a sentinel to human JE in these high zones within Malaysia have not been fully discovered.

1.1 Hypothesis

In this study, the following alternative hypothesis was proposed:

Ho: JEV antibodies IgG and antigen are not detected in livestock (buffaloes, cattle and pigs), avian and companions animals (cats and dogs) at the high-risk areas in Malaysia.

Hi: JEV antibodies IgG and antigen are detected in livestock (buffaloes, cattle and pigs), avian and companions animals (cats and dogs) at the high-risk areas in Malaysia.

1.2 The objectives of this study are:

I. To determine the seroprevalence rate of JEV in livestock (buffaloes, cattle and pigs), avian and companions animals (cats and dogs) using JEV-IgG ELISA.
II. To identify the risk factors associated with seroprevalence of JEV in livestock (buffaloes, cattle and pigs), avian and companions animals (cats and dogs) using univariate and multivariate logistic regression analysis.
III. To detect JEV antigen in blood samples of livestock (buffaloes, cattle and pigs), avian and companion animals (cats and dogs) using one step RT-PCR.

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