Characterization and Mosquito Infection of the Tengah Isolate of Japanese Encephalitis Virus

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by

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Abstract

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Japanese encephalitis (JE) is a brain infection caused by JE virus (JEV). It has an estimated worldwide incidence of 68,000 cases and 10,000 to 15,000 deaths annually. Despite large effective immunization campaigns, Japanese encephalitis remains a disease of global health concern, because the virus is spreading. There are five genotypes of JEV (genotype I – V), each associated with different geographical areas and associated epidemiology. The Muar strain of JEV, the fifth genotype, is believed to represent the oldest lineage from which genotype I - IV evolved. Muar was isolated in Singapore in 1952. At the same time as Muar was isolated; the Tengah strain of JEV was also isolated from a nearby location. However, Tengah and the characteristics of Tengah have largely remained unknown. Muar was considered the only known representative of genotype-V prior to 2009.

Vector competence studies have examined genotype-I, II and III of JEV. However, genotype-IV and genotype-V have never been investigated in vector competence studies. Therefore, the infectivity of these viruses to mosquitoes is unknown. The competence of non-Asian mosquitoes to JEV has been demonstrated suggesting potential for emergence in some other regions. In Great Britain, JEV is considered a potential threat to animals and public health. However, the level of competence of British mosquitoes to any arbovirus is not known.

The overall objective of this thesis was to characterise Tengah, investigate molecular and mosquito factors that might relate to the lack of circulation of genotype-V isolates and assess the potential of arbovirus (JEV) emergence in Great Britain.

Molecular characterization of Tengah strain showed that it is another isolate of genotype-V, with 99% sequence similarity to Muar. Evolutionary analysis performed using the Bayesian Evolutionary analysis of Sampling Trees (BEAST) program estimated that JEV is evolving at a rate

of 3.53×10^4 nucleotide substitution per site per year. Vector competence studies demonstrated *Culex quinquefasciatus* mosquitoes are able to transmit Muar with transmission rates of 23% at 21-days post infection. Comparison of transmission between Muar (genotype V) and Nakayama (genotype III) found no significant difference between the two genotypes. *Ochlerotatus detritus*, a British mosquito, was susceptible to JEV at both 23°C and 28°C as determined by the detection of virus in the saliva 7 days post infection. The overall transmission rate was 13% at 23°C and 25% at 28°C. There was no significant difference between the two temperatures. Infection rates for *Ochlerotatus detritus* and *Culex quinquefaciatus* were similar.

This thesis has shown that on account of its similarity to the Muar isolate, Tengah represents a variant of Muar and is another genotype-V isolate. Evolutionary analysis showed that JEV originated from its ancestral virus in the year 1120 while the Time to Most Recent Common Ancestor for genotype V was in the year 1840. Muar has the ability to infect, replicate and be transmitted in *Culex quinquefasciatus*, suggesting that the limited distribution, isolation and circulation of genotype-V is probably not explained solely by mosquito factors. *Ochlerotatus detritus* is competent to transmit JEV and would therefore pose a threat should this virus occur in Great Britain.

Declaration

I, Lucy Mwende Mackenzie-Impoinvil declare that this thesis and the work presented in it are the result of my own work. Except for the assistance as outlined in the acknowledgements, the work described is my own work and has not been submitted either wholly or in part for a degree or other qualification to this or any other university. The research work was carried out at the department of Vector Biology, at the Liverpool School of Tropical Medicine and at the Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool.

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List of abbreviations

µg	Microgram
µl	Microliter
ACDP Ar CL3	Advisory Committee on Dangerous Pathogens Arthropod containment level 3
ACDP CL-3	Advisory Committee on Dangerous Pathogens Containment level 3
ATP	Adenosine 5'-triphosphate
BATV	Batai virus
BEAST	Bayesian evolutionary analysis of sampling trees
BFV	Barmah forest virus
BSA	Bovine serum albumin
C	Capsid gene
CHIKV	Chikungunya virus
CI	Confidence intervals
CNS	Central nervous system
CO_2	Carbon dioxide
CPE	Cytopathic effect
df	degree of freedom
DMEM	Dulbecco Modified Eagle's Minimal Essential Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPI	Days post infection
E	Envelope gene
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EEEV	Eastern equine encephalitis virus
EIP	Extrinsic incubation period

ER	endoplasmic reticulum
ESS	Effective sample size
FBS	Fetal bovine serum
FCS	Fetal calf serum
GPS	Global position systems
GTR	General time reversible
GTR+G+I	General time reversible (GTR) model for substitution with combination of gamma distribution and proportion of invariant sites
HPD	High posterior density
IFN	Interferon
INKV	Inkoo virus
JAK-STAT	Janus kinase-Signal transducer and activator of transcription
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
Kb	Kilo base
KUNV	Kunjin virus
LB	Luria Broth
LEDV	Lednice virus
LSTM	Liverpool School of Tropical Medicine
MCC	maximum clade credibility
MCMC	Markov chain Monte Carlo
MEM	Minimum Essential Media
mg	Milligram
ml	Millilitre
mM	milimolar
MVEV	Murray valley encephalitis virus
MYXV	Myxoma virus
$NaHCO_3$	Sodium hydrogen carbonate
NCBI	National center for biotechnology information

nt	Not tested
NS1	Non-structural protein- 1
NS2A	Non-structural protein- 2A
NS2B	Non-structural protein- 2B
NS3	Non-structural protein- 3
NS4A	Non-structural protein- 4A
NS4B	Non-structural protein- 4B
NS5	Non-structural protein- 5
ORF	Open reading frame
PBS	Phosphate buffered Solution
PCR	Polymerase chain reaction
pfu	Plaque forming unit
prM	Pre-Membrane
RACE	Rapid Amplification of cDNA Ends
rER	rough endoplasmic reticulum
RNA	Ribonucleic acid
rpm	Rotations per minute
RRV	Rose river virus
RT-PCR	Reverse transcriptase polymerase chain reaction
RVFV	Rift valley fever virus
S.O.C	Super otimal broth
SINV	Sindbis
SISA	Simple Interactive Statistical Analysis
SLEV	St. Louis encephalitis virus
TAE	Tris-acetate-EDTA
TAHV	Tahyna
TBEV	Tick-borne encephalitis virus
TEA	Triethylamine
TGN	Trans-Golgi network

TMRCA	Time to most recent common ancestor
USUV	Usutu virus
UTR	Un-translated region
UUKV	Uukuniemi virus
UV	Ultraviolet
VEEV	Venezuela equine encephalitis virus
WEEV	Western equine encephalitis virus
WHO	World Health Organization
WNF	West Nile fever
WNV	West Nile
YF	Yellow fever
YFV	Yellow fever virus

CHAPTER 1: Overview, literature review and aims

1.1 Overview

Japanese encephalitis virus (JEV) is a major cause of viral encephalitis worldwide with an estimated annual incidence of 68,000 cases occurring annually and 10,000 to 15,000 deaths (Solomon, 2006; Campbell *et al.*, 2011). This mosquito-borne virus poses a major threat to public health (Mackenzie *et al.*, 2004). Approximately 75% of these cases occur in children aged 0–14 years (Ghosh & Basu, 2009). Approximately 25% of encephalitic patients die while about 50% of the survivors develop permanent neurologic sequelae including memory loss, impaired cognition, behavioural disturbances convulsions, motor weakness or paralysis and abnormalities of tone and coordination (Solomon *et al.*, 1998; Unni *et al.*, 2011).

Currently JEV is found throughout most countries in Asia, extending north into maritime Siberia, and it has shown the propensity to expand. In recent years the virus' geographical reach has expanded south into Australia in 1995 (Hanna *et al.*, 1996), west into Pakistan in 1992 (Igarashi *et al.*, 1994) and east into Saipan in 1990 (Paul *et al.*, 1993). There has also been some evidence of viral activity in Italy where JEV RNA was isolated from *Culex pipiens* mosquitoes collected in Italy in 2010 (Ravanini *et al.*, 2012). It is likely JEV will appear in places it has never been reported before. JEV is composed of five genotypes. These genotypes are spread throughout different geographic regions except for genotype IV which is confined in the Indo-Malaysia region. It was believed that different genotypes occupied different geographic regions (Chen *et al.*, 1990; Chen *et al.*, 1992; Williams *et al.*, 2000). However, current studies show the genotypes are shifting in their distribution. For example genotype III was the predominant genotype throughout Asia; however, over the past two decades, it has been displaced by genotype I viruses in a number of Asian countries including China (Wang *et al.*, 2007), Thailand (Nitatpattana *et al.*, 2008), South Korea (Nam *et al.*, 1996), Japan (Ma *et al.*, 2003), Malaysia (Tsuchie *et al.*, 1997), Vietnam (Nga *et al.*, 2004), India (Fulmali *et al.*, 2011) and Taiwan (Chen

et al., 2011). It is still not clear what determines which genotype will appear where or what drives these genotypes to spread.

One of the most curious genotypes of JEV is genotype V. Up until 2010, there had only been one isolate of genotype V the Muar strain. Muar was isolated in Singapore from the brain of a patient in 1952. This genotype is believed to be the most ancient JEV genotype since it is more divergent from the other genotypes with approximately 20% sequence divergence while the divergence between other genotypes is approximately 10 – 17% (Mohammed *et al.*, 2011). At the same time and the same geographical location of Muar isolation, another JEV isolate Tengah strain, was also isolated. This strain which has not been fully characterized is also thought to be a genotype V. Prior to 2010 genotype V had only been found in the Indo-Malaysia region which is the only region that all the JEV genotypes have been isolated. Hence, it is believed that this is where they emerged (Solomon *et al.*, 2003). After nearly 60-years, a JEV isolate from *Culex tritaeniorhynchus* mosquitoes in China in 2009 was found to belong to genotype V (Li, Fu*, et al.*, 2011). Another isolate was reported from *Culex bitaeniorhynchus* Korea in 2010 (Takhampunya *et al.*, 2011). The implications of these further findings are unclear.

Evolutionary studies can provide an insight into how viruses spread by presenting information such as evolutionary rate between and within genotypes. This information can be used to infer whether some genotypes evolve faster than others. However for this to be possible it is essential to have a good amount of sequence information representative of each genotype in order to make a reliable conclusion. To date, there are several complete genome sequences available for genotype I and genotype III, while there are only four for genotype II, only one complete genome sequence of genotype IV and only two complete genome sequences of genotype V. There is therefore a need to determine more complete genome sequences of JEV viruses especially for genotype II, genotype IV and genotype V.

Across Asia JEV appears to use a wide range of mosquito vectors (Burke & Leake, 2000). Another approach to understanding how the virus has expanded its geographic range is to

compare the fitness of the different genotypes in mosquito vectors. It could be possible that some genotypes are transmitted more efficiently than others hence allowing the virus to establish in new areas, or displacing currently circulating genotypes. Such questions have never been experimentally addressed for JEV and would provide valuable information.

For mosquito-borne viruses the term extrinsic incubation period (EIP) describes the duration between the acquisition of an infectious bloodmeal by the mosquito to the time the mosquito is able to transmit the virus. A study using West Nile virus (WNV) genotypes, reported that WN02 strain had an extrinsic incubation period (EIP) that was 4 days shorter than that of NY99 strain. This difference in the EIP of WN02 and NY99 provided a possible explanation of the displacement of NY99 by WN02 which was transmitted much earlier hence leading to more infection rates (Moudy *et al.*, 2007). In the case of JEV genotype-V one of the reasons for the long hiatus in its rediscovery may have been that the original genotype V had low infectivity rates compared to other genotypes. Therefore it may have become relatively displaced. For this reason it is important to compare the infectivity of the original genotype V to other JEV genotypes such as the Nakayama strain which is not only the JEV prototype but also the belongs to the most isolated genotype, genotype III. The recent isolations of genotype V may indicate that it has now evolved and possibly is able to infect a variety of host and vectors that it did not initially. In fact, the isolate from Korea was from *Culex bitaeniorhynchus* mosquitoes which are not usually the main vector for JEV. Furthermore the 86% nucleotide sequence homology between Muar and the Chinese isolate (Li, Fu*, et al.*, 2011) does indeed support the suggestion that evolution may have aided in the emergence in China and Korea.

Some of the factors associated with the spread of viruses include climatic factors such as wind. It is believed that JEV may have spread into Australia through wind-borne mosquitoes (Ritchie & Rochester, 2001). Warmer temperatures can increase the distribution and density of vectors and also enhance transmission potential in temperate climates by elongating transmission seasons. It can also lead to shorter extrinsic incubation times of the viruses in their vector (Weaver & Reisen, 2010). Another factor to consider is migratory birds. For instance viraemic

birds may have been responsible for the spread of JEV into India (Fulmali *et al.*, 2011), Taiwan (Huang *et al.*, 2010) or Papua New Guinea (Johansen *et al.*, 2000).

Man-made factors such as importation of infected animals or air transportation of disease carrying vectors, changes in agricultural practises and land use may also be responsible for introduction and spread of viruses in places they were not found before. Once these viruses are introduced into an area, they are more likely to spread and become established if the climate is conducive to allow breeding of the vector, availability and abundance of susceptible hosts and the presence of competent vectors to transmit the viruses (Pfeffer & Dobler, 2010). This spread and emergence diseases is of most importance to countries where they have never been reported before because this countries provide a large number of naive hosts that would lead to major epidemics. Several vector competence studies have been conducted in different countries to evaluate the level of competence of local disease vectors that have been implicated for transmission elsewhere (Romi *et al.*, 2004; Moutailler *et al.*, 2008; Nett *et al.*, 2009; Abdel-Hamid *et al.*, 2011; Kramer *et al.*, 2011; Vega-Rua *et al.*, 2013).

Currently there is no circulation of any mosquito-borne viruses in Great Britain, however with JEV viruses emerging in other temperate regions of Europe it is likely that they will in due time appear in Great Britain. There is also the abundance of mosquitoes in Great Britain with 34 species recorded to date. Some of these species have been implicated in the transmission of other viruses elsewhere and most importantly there are 13 species that are capable of acting as bridge vectors (Medlock *et al.*, 2005). Bridge vectors are mosquitoes that due to their ability to feed on both humans and birds can transmit viruses circulating in birds to humans. One such species in Great Britain is the *Ochlerotatus detritus* mosquito. This mosquito has been implicated for the transmission of WNV in Italy (Romi *et al.*, 2004), Egypt (Abdel-Hamid *et al.*, 2011) and Portugal (Osorio *et al.*, 2012). Other than acting as a bridge vector this mosquito also causes the greatest human biting nuisance of any British mosquito (Snow, 1990; Clarkson & Setzkorn, 2011) especially in residential areas near its breeding site i.e. the marshes. Should a mosquito-borne virus appear in the Great Britain, this mosquito would be a suitable candidate

to transmit that virus to humans. However it is not known if this mosquito is competent to transmit any viruses and hence evaluating its competence is of ultimate significance.

1.2 Literature review

1.2.1 History of JEV

Japanese encephalitis virus (JEV) is an arthropod-borne virus belonging to the genus Flavivirus in the family *Flaviviridae*. The genus Flavivirus is comprised of more than sixty-six virus species, many of which are arthropod-borne human pathogens (mainly mosquitoes and ticks) and are highly pathogenic for both humans and animals. The diseases caused by flaviviruses range from fevers and encephalitides to hemorrhagic fever. Yellow fever virus (YF) is the prototype member of the Flavivirus genus.

The Flavivirus genus is divided into serological complexes that are related serologically, genetically, and etiologically (Table 1). JEV belongs to the JE serological complex (Table 2) which includes members from around the world. (Thiel H-J *et al.*, 2005). JEV is the most important and widespread member of this sero-complex.

Table 1: List of virus groups in the Flavivirus genus

Flavivirus virus groups	Dengue virus group
	Yellow fever virus group
	Japanese encephalitis virus group
	Kokobera virus group
	Ntaya virus group
	Kedougou virus group
	Edge Hill virus group
	Mammalian tick-borne virus group
	Seabird tick-borne virus group
	Kadam virus group
	Aroa virus group

Table 2: List of viruses in the Japanese encephalitis serological complex

Japanese encephalitis serological complex	Japanese encephalitis virus
	St. Louis encephalitis virus
	West Nile virus
	Murray Valley encephalitis virus
	Alfuy virus
	Koutango virus
	Cacipacore virus
	Usutu virus
	Kunjin virus
	Yaounde virus

JEV causes a disease called Japanese encephalitis (JE). JE was recognized in horses and humans as early as 1871. JE gained its recognition in 1924 when a great epidemic resulted in approximately 4000 deaths and 6000 cases reported in Japan (Rappleye, 1939). A filterable agent was extracted from human brain and passed to rabbits, although the agent could not be characterized. Every 10 years, major epidemics were reported in Japan affecting over 6000 patients (Miyake, 1964). In 1934, Hyashi reproduced the disease in monkey by intra-cerebral inoculation. It was later isolated for the first time in 1935 from the brain tissue of a fatal encephalitis case in Tokyo, Japan (Mitamura *et al.*, 1936; Erlanger *et al.*, 2009; McArthur & Holbrook, 2011) and its virological and serological prototype, Nakayama strain, was established. JEV was also isolated from the brain of a sick horse in 1937. The seasonal occurrence of the disease in Japan suggested a vector relationship and in 1938 the virus was first isolated from *Culex tritaeniorhynchus* (Mitamura, Kitaoka, Mori*, et al.*, 1938). The role of pigs and birds as reservoir in the transmission of JEV was established in 1959 (Buescher & Schere, 1959). The term Japanese B encephalitis was used to distinguish it from summer epidemics of Von Economo's encephalitis lethargica which is also known as type A encephalitis. Later the term type B was dropped and now it is known as Japanese encephalitis.

JEV causes encephalitis an inflammatory disease of the brain. JEV is the major cause of viral encephalitis with an estimated annual incidence of 68,000 cases and 10,000 to 15,000 deaths (Solomon, 2006; Campbell *et al.*, 2011). This may be an underestimate, due to inadequate surveillance and reporting which may be a result of lack of funds or financial constraints or complex logistics. About 19% of these cases occur in areas with little or no JE vaccination implementation, while 81% of these cases occur in areas with well-established or developing JE vaccination programmes. The high number of cases reported in areas with established or developing vaccinations programmes most likely reflects the areas with the highest risk of JEV transmission hence better reporting and surveillance for such areas. Approximately 75% of these cases occur in children aged 0–14 years and in countries like India it has been referred to as the "Kid killer" (Ghosh & Basu, 2009). Most adults in endemic countries have natural immunity acquired from childhood infection (Campbell *et al.*, 2011). However there is an increase in number of cases in the elderly, as protective immunity decreases.

Approximately 25% of encephalitic patients die while about 50% of the survivors develop permanent neurologic sequelae including memory loss, impaired cognition, behavioural disturbances, convulsions, motor weakness or paralysis and abnormalities of tone and coordination (Solomon *et al.*, 1998; Unni *et al.*, 2011).

1.2.1.1 Clinical manifestation in Humans

JEV infection and disease are usually the result of the bite of an infected mosquito. The initial infection event is thought to be uptake of virus into the dendritic cells in the skin. The antigen presenting cells carry the virus to peripheral lymph nodes and viral replication occurs within the macrophages and other cells of the peripheral lymphatic system (Sapkal *et al.*, 2007). This is followed by a short lived viremia usually less than a week, which precedes the entry of virus in to the central nervous system. In most patients the infection resolves at this stage. Nervous system involvement sufficient to produce clinical disease occurs in only 1:200 to 1:1000 cases and this is usually via penetration of the blood brain barrier through the vascular endothelium.

The JEV encephalitic illness classically is preceded by fever, headache and gastrointestinal symptoms, followed by deteriorating consciousness. JE has an incubation period of usually less than 1 week, but may be up to 16 days. Neck stiffness is present in about half of the cases. Sudden onset with fever and convulsions may occur in children and occasionally adults and is generally a bad prognostic feature (Mackenzie *et al.*, 2007)

1.2.1.2 Humoral immunity

Humoral immunity plays an important role in JEV infection. Cell free virus is usually cleared by antibodies through neutralization of the virus and infectivity and phagocytic clearance of the virions (Griffin, 1995). For this reason, JEV is rarely isolated from peripheral blood in humans. Other reasons include low viremia and clinical symptoms being seen after the virus has invaded the central nervous system which is usually after the viremia has finished. Virus can be isolated from CSF early in the course of acute encephalitis, but this is consistent with a poor prognosis. Most viruses have been isolated from the brains of patients at autopsy. After infection most patients produce immunoglobulin M (IgM), both in serum and cerebrospinal fluid (CSF). IgM in CSF is detected as early as day 1 after the onset of the symptoms, and by seven days all patients have raised IgM titers (Burke, Nisalak*, et al.*, 1985), while it is not detected in the serum until 9 – 10 days. The presence of JEV specific IgM antibodies in the serum or CSF is necessary for laboratory confirmation of JEV infection (Solomon *et al.*, 2008). Failure to mount IgM response is associated with viral isolation and fatal outcome (Leake *et al.*, 1986). JEV specific IgM has been used for clinical diagnosis of JEV infected patients through IgM capture enzyme linked immunosorbent assay (IgM capture ELISA). In surviving patients immunoglobulin class switching occurs where IgM declines and immunoglobulin M (IgG) starts rising and by 30 days most patients have serum IgG against JEV (Burke, Nisalak*, et al.*, 1985). However if a person has been infected with Dengue virus prior to JEV infection, high titres of IgGs have been reported (Innis *et al.*, 1989) due to serological cross-reactions with other flaviviruses. Many other flaviviruses overlap with JE virus including Dengue and West Nile viruses and this can result in misinterpretation of test results. Therefore it is necessary to show a fourfold increase in

antibody titres to JE virus between paired serum samples collected 14 days apart to have a presumptive diagnosis of JE (Burke, Nisalak*, et al.*, 1985)

1.2.2 Transmission cycle

JEV is transmitted through a zoonotic (enzootic) cycle between mosquito vectors and vertebrate hosts, particularly pigs and birds; humans get accidentally infected when bitten by an infected mosquito and are a dead end host meaning they do not participate in the spread of JE because of low level and short-lived viraemia (Rosen, 1986; Solomon & Vaughn, 2002; Weaver & Barrett, 2004). Therefore, JEV naturally cycles between mosquitoes and birds or mosquitoes and pigs (Figure 1.) (Lindenbach *et al.*, 2007; van den Hurk *et al.*, 2009). Although many vertebrate animals can be infected with JEV, 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 & 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). Of other vertebrate species, horses can develop central nervous system (CNS) infections but are a dead-end host; other domestic animals become infected, but show no evidence of viraemia; rodents are refractory to infection; and amphibians, reptiles and bats can become infected experimentally and virus can persist, but their role in overwintering and maintenance of the virus in the environment is not known (Mackenzie *et al.*, 2004). A variety of mosquito species may act as vectors in the enzootic cycle, but Culicine mosquitoes primarily *Culex tritaeniorhynchus* are the principal vector for human infection (Gubler *et al.*, 2007). In addition to its mosquito-specific horizontal transmission, JEV is also vertically transmitted to the progeny of infected mosquitoes through eggs (Dhanda *et al.*, 1989; Rosen *et al.*, 1989). Therefore Japanese encephalitis is largely a rural disease, with *Culex tritaeniorhynchus* mosquitoes breeding in rice paddies and pigs providing the main source of blood meals, with the consequence of transmission cycles in close proximity to human habitation. There are two epidemiological patterns of transmission: an endemic

pattern in tropical areas with virus circulation in most months of the year, but with a broad seasonal peak probably resulting from irrigation practices; and an epidemic pattern in more temperate areas with clear summer seasonality (Vaughn & Hoke, 1992a).

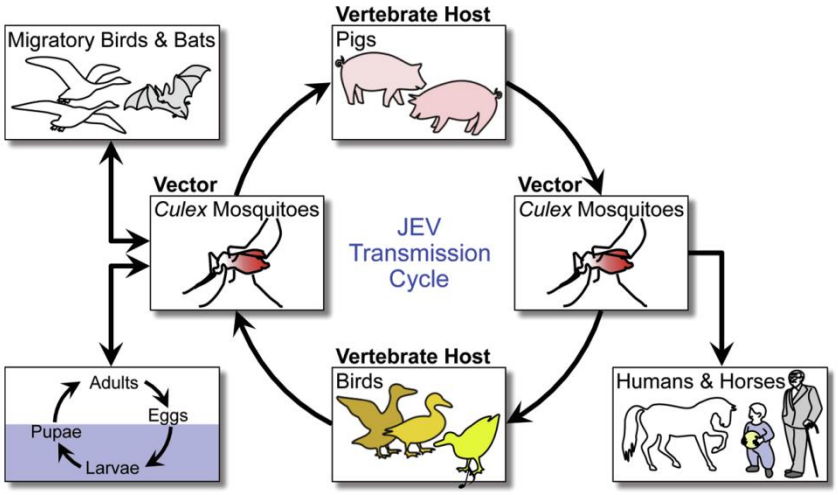


Figure 1: Japanese encephalitis virus transmission cycle (Yun & Lee, 2013)

JEV is amplified in an enzootic cycle that involves mosquito vectors (mainly Culex species) and vertebrate hosts (primarily pigs and birds). Incidentally, JEV is also transmitted to dead-end hosts, such as humans and horses.

1.2.3 Vectors of JEV

Although JEV has been isolated from over 30 mosquito species, paddy-breeding mosquitoes of the *Culex vishnui* subgroup, particularly *Culex tritaeniorhynchus*, are the major vectors of the virus. A number of other species, such as C*ulex gelidus*, *Culex fuscocephala* and *Culex annulirostris*, *Culex annulus, Culex sitiens* have yielded numerous isolates, implicating them as important secondary or regional vectors (Peiris *et al.*, 1992; Vythilingam *et al.*, 1994; Ritchie *et al.*, 1997). Mosquitoes belonging to the genus *Anopheles* such as: *Anopheles peditaeniatus*, *Anopheles barbirostris* and *Anopheles subpictus* have also been reported from India as harbouring JEV (Thenmozhi *et al.*, 2006). Distinctive seasonal patterns of virus activity in mosquitoes occur, with increases in infection rates linked to the warmer summer months in

temperate areas (Buescher & Schere, 1959) and the onset of the monsoon season in tropical areas (Gajanana *et al.*, 1997). Table 3 below gives a list of mosquitoes from which JEV has been isolated and also shows their level of efficiency in transmitting JEV.

Table 3: List of mosquito species from which Japanese encephalitis virus has been isolated.

***Culex* species (*Cx.*)**	***Anopheles* species (*An.*)**	***Aedes* species (*Ae.*)**	***Mansonia* species (*M.*)**
Cx. annulius	*An. barbirostris*	*Ae. albopictus*	*M. annulifera*
Cx. bitaeniorhychus	*An. hyrcanus*	*Ae. curtipes*	*M. bonneae/dives*
Cx. epidesmus	*An. sinesis*	*Ae. togot*	*M. uniformis*
Cx. fuscocephalas	*An. subpictus*	*Ae. vexans*	
Cx. gelidus	*An. tessalatus*		
Cx. Pipiens fatigans			
Cx. p. pallens			
Cx. p. quinquefasciatus			
Cx. pseudovishnui			
Cx. tritaeniorhynchus			
Cx. vishnui			
Cx. whitmorei			
High competence	Moderate competence	Low competence	

Other mosquito species from which JE virus has been isolated (Burke & Leake, 2000). The coloured mosquitoes are those that have been also been tested in the laboratory for competence of JEV

1.2.4 Vertebrate hosts

1.2.4.1 Avian vertebrates

Birds are an important component in the transmission cycle of JE virus and they are thought to be the "basic" vertebrate hosts (Hammon, Sather*, et al.*, 1958; Buescher, Scherer, Mc*, et al.*, 1959). The virus has been isolated in nature from a variety of wild species, and both wild and

domestic species have been shown to develop viraemia high enough to infect mosquitoes (Hasegawa *et al.*, 1975; Dhanda *et al.*, 1977; Soman *et al.*, 1977). Some of these birds are night herons, plumed egrets, lesser egrets, pond herons and cattle egrets (Scherer, Buescher*, et al.*, 1959). In general, younger birds exhibit higher viraemia than older individuals of the same species (Boyle *et al.*, 1983). Ducks and chickens have been shown to amplify virus to a transmissible level experimentally (Dhanda *et al.*, 1977). The migratory patterns of herons coincide with the seasonal transmission of JE in Japan with herons migrating from China, Taiwan, the Philippines, and Java to Japan at the beginning of the summer and peak JE transmission season (Ogata *et al.*, 1970). *Culex tritaeniorhynchus* can be successfully infected by feeding on viraemic birds and can in turn infect susceptible birds. It is suspected that they are relatively important as a source of human infection in India, where JE infection rates in swine are not as high as they are in many other endemic areas (Banerjee, 1975).

1.2.4.2 Domestic pigs

Pigs play an important role in the epidemiology of JE since they provide a significant source of infection for those mosquito species that transmit JE virus to humans and also due to their susceptibility for JE infection. For that reason they are used to monitor the annual seasonal appearance of JE virus in Japan (Konno *et al.*, 1966). Field studies have demonstrated that when JE naive swine are placed into a JE endemic area, they develop infection within one week of placement and develop a viraemia lasting for 4 or more days (Maeda *et al.*, 1978). Given the rapid population turnover of pigs, this domestic animal is clearly a significant source of mosquito infection.

1.2.4.2.1 Clinical manifestation in Pigs

The most common symptom of Japanese encephalitis in pigs is the birth of stillborn or mummified foetuses, usually at term. Piglets born alive often have tremors and convulsions and die soon after birth. The foetuses from infected pigs are mummified and dark. Hydrocephalus, cerebellar hypoplasia, and spinal hypomyelinogenesis (defective formation of myelin in the spinal cord, brain, or peripheral nerves) may be seen. The mortality rate is high in piglets born

to infected sows, but close to zero in adult pigs (Cirad, 2007). Pregnant sows may also abort. Non- pregnant animals are usually asymptomatic or experience a transient febrile illness, but symptoms of encephalitis are occasionally seen in pigs up to six months of age (Spickler *et al.*, 2010). In addition, disturbances of spermatogenesis can cause infertility in boars; although this is usually temporary, it can be permanent in severely affected animals (Habu *et al.*, 1977).

1.2.4.2.2 Economic impact on Pigs

Piglet acquires passively maternal antibodies which could be detected by neutralization test and hemagglutination inhibition (HI) assay. The neutralizing antibody against the JEV remains detectable in the majority of pigs until the age of 3–6 months and after this period, pigs become susceptible to virus (Geevarghese *et al.*, 1987). Almost 2/3 (60.7%) of pigs are slaughtered at the age greater than or equal to 6 months. In pigs over 6 months, which is the reproduction age, the HI test was positive in 95.2%, highlighting the potential economic impact of JEV infection in swine particularly on those who basically rely upon pig rearing (Duong *et al.*, 2011) . These results also reinforce the important role of over 6 month-old pigs in the maintenance of virus in the nature as they become probably rapidly infected and repeatedly re-exposed to the virus. Vaccines are available for swine in Japan and Taiwan and are expected to provide good immunity.

It is not known how JEV changes during swine infection and within the enzootic cycle of JEV between birds and swine and its transmission to humans. The effects of interspecies transmission of JEV from an avian host to swine and back again on mutation rates and recombination events are also not known. Considering viruses such as the influenza virus that have an avian-swine transmission, swine is known to play an important role in the recombination events of this virus and its ability to infect humans (Stech *et al.*, 1999). Swine are susceptible to infection with avian influenza due to the presence of a receptor similar to that found in birds. Upon infection, they serve as a mixing vessel that allows recombination events to occur which then lead to emergence of a dominant strain that has acquired the ability to infect humans (Webby *et al.*, 2000). It is not known if similar mechanisms apply to JEV.

1.2.4.3 Horses

Equines can develop encephalitis following JE virus infection (Burns *et al.*, 1949), and the clinical picture of JE disease is characterized by fever, anorexia, weakness, congested or jaundiced mucous membranes and neurologic signs varying from a mild lethargy to hyperirritability, ataxia and paralysis similar to human disease (Paterson *et al.*, 1952). In the severe form of the disease, symptoms include, high fever, hyper-excitability, aimless wandering, violent and demented behaviour, occasional blindness, profuse sweating, and muscle tremors. While some horses will usually recover without complications, those that go on to develop encephalitis will usually die in 1–2 days from onset (Spickler *et al.*, 2010). Viraemia in horses develops from 1 to 4 days after infection and lasts 2 to 6 days. Horses represent a dead-end host for JE transmission, although experimental transmission of JE from birds to horses, from horse to horse, and horse back to birds by *Culex tritaeniorhynchus* has been demonstrated (Gould *et al.*, 1964).

1.2.4.4 Other vertebrate hosts

Cattle and water buffalo are hosts to the same mosquito species that feed on swine and can therefore be infected by JE virus with the development of JE antibody (Ilkal *et al.*, 1988). Significant antibody titres to JE virus have been found in cattle and goats and have been correlated to the occurrence of human diseases (Peiris *et al.*, 1993). JE antibody sero-prevalence in domestic livestock was studied in Malaysia. The highest sero-prevalence was observed in swine (88.1 %), followed by buffalo (45%), cattle (42%), sheep (17.9%), and goats (13.8) (Oda *et al.*, 1996). Despite the fact that these domestic livestock appear to have the ability to be infected by JE, they do not contribute to the overall cycle of JE transmission due to low level of viraemia and are therefore considered dead-end hosts. Other possible vertebrate-hosts for JE virus that have been examined include reptiles and amphibians which can be infected experimentally with JE virus and overwintering of the virus can occur in snakes, frogs, and bats (Oh *et al.*, 1974). However since they are not the main source of blood meals for the principal JE vector, *Culex tritaeniorhynchus* there is inadequate contact for successful field

transmission. Rodents are refractory to JE virus infection and in ecologic studies have had no or very little JE antibody present (Williams & Imlarp, 1972). Several species of bats are susceptible to JE with sufficient viraemia to infect mosquitoes that last for 6 days. Bats have also demonstrated persistence of viral infection under low environmental temperatures suggesting an ability to overwinter the virus (Sulkin *et al.*, 1970). A study in China reported that two JE viruses isolated from bats showed a close relationship to JE viruses isolated from mosquitoes and humans in the same region over two decades supporting the fact that bats may play an important role in human JE outbreaks in that region (Wang, Pan, Zhang, Fu, Wang*, et al.*, 2009).

1.2.5 Persistence of JEV in nature

1.2.5.1 Virus survival and re-introduction

A variety of mechanisms may explain the ability of JEV to survive in areas such as Japan, China, and Korea where there are very cold winters. Possible mechanisms include persistence in enzootic foci within vertebrate hosts and/or mosquitoes and reintroduction of the virus by migratory birds and/or mosquitoes (van den Hurk *et al.*, 2009). Given the regularity of appearance of JE virus each year in so many different types of habitat, it seems likely JEV survives adverse conditions in the vector either by overwintering in the adult or by being transovarially passed on to the next generation (Burke & Leake, 2000). It is also suggested that infected mosquitoes from areas where transmission occurs throughout the year are blown north by the wind or the virus is carried north by viraemic or latently infected migrating birds or bats (Solomon *et al.*, 2003; Nga *et al.*, 2004). A study in China reported subsequent long-distance southern migration of *Culex tritaeniorhynchus* before winter in northern latitudes during autumn, with a potential dispersal of 200 km per night (Ming *et al.*, 1993), And in another study *Culex tritaeniorhynchus* mosquitoes were collected up to 500 km offshore in the Pacific Ocean (Asahina, 1970). JE could have been introduced into Australia by wind-blown mosquitoes. This was indicated by backtrack simulations that indicated winds sufficient to transport mosquitoes from New Guinea to Badu Island occurred frequently during the large incursions of virus in 1995 and 1998 (Ritchie & Rochester, 2001).

1.2.6 Overwintering mechanisms

The duration of viraemia of JEV in birds and pigs is too short for these animals to effectively maintain the virus during adverse conditions. On the other hand, bats that have been experimentally inoculated have been shown to sustain low levels of virus in the blood during simulated hibernation at low temperatures and later exhibited recurrent viraemia high enough to infect mosquitoes when they are removed from simulated hibernation (La Motte, 1958).

Experimentally infected lizards, snakes, and frogs also develop a viraemia under simulated hibernation (Lee, 1971; Doi *et al.*, 1983; Oya *et al.*, 1983). Since the major vector of JE virus to humans and domestic animals, *Culex tritaeniorhynchus*, survives the winter by hibernation of inseminated adult females, JE virus could be carried over the winter by mosquitoes that had acquired infection by feeding on a viraemic host before entering hibernation. In fact, experimentally infected *Culex tritaeniorhynchus* and *Culex quinquefasciatus* were shown to transmit the virus to susceptible hosts following overwintering (Hurlbut, 1950; Mifune, 1965). However, there is limited evidence for maintenance of JEV in overwintering mosquitoes. JEV has only been isolated once from field-collected overwintering *Culex tritaeniorhynchus* in Japan, although only low numbers have ever been processed during the winter months (Hayashi *et al.*, 1975). In Korea, JEV has been isolated twice during winter from *Culex pipiens* (Lee, 1971). Importantly, the female *Culex tritaeniorhynchus* rarely takes a blood meal prior to hibernation, thus reducing its exposure to viraemic animals (Oda *et al.*, 1981). Vertical transmission (transovarial) which occurs when an infected female mosquito passes the virus to its progeny can facilitate overwintering of JEV (Rosen, 1987). Laboratory transmission studies have demonstrated that vertical transmission occurs through the F1 generation of larvae and adults of numerous species, including *Culex tritaeniorhynchus, Culex pipiens pallens, Culex pipiens molestus, Culex quinquefasciatus, Culex vishnui, Aedes albopictus, Aedes alcasidi, Aedes japonicus, Aedes togoi, Aedes vexans, and Armigeres flavus* (Rosen *et al.*, 1989; Takashima & Rosen, 1989). However, these results are difficult to interpret in terms of natural transmission cycles, as parenteral inoculation was used as the mode of infection in many instances and JEV is rarely isolated from field collected immatures or adult male mosquitoes. Indeed, over a 3.5-

year period in Taiwan, only one isolate of JEV was obtained from almost 400,000 *Culex tritaeniorhynchus* larvae, compared with 164 isolates obtained from about 142,000 adult females (Rosen, 1987)

1.2.7 Molecular epidemiology of JEV

1.2.7.1 JEV genome

JEV is a spherical, enveloped virus about 50 nm in diameter with a single stranded, positive sense, RNA genome of ˜11 kb in length. The genome is organized into a capsid formed by multiple copies of capsid (C) protein; which is covered by a host derived lipid bilayer. The surface proteins are arranged in an icosahedral-like symmetry.

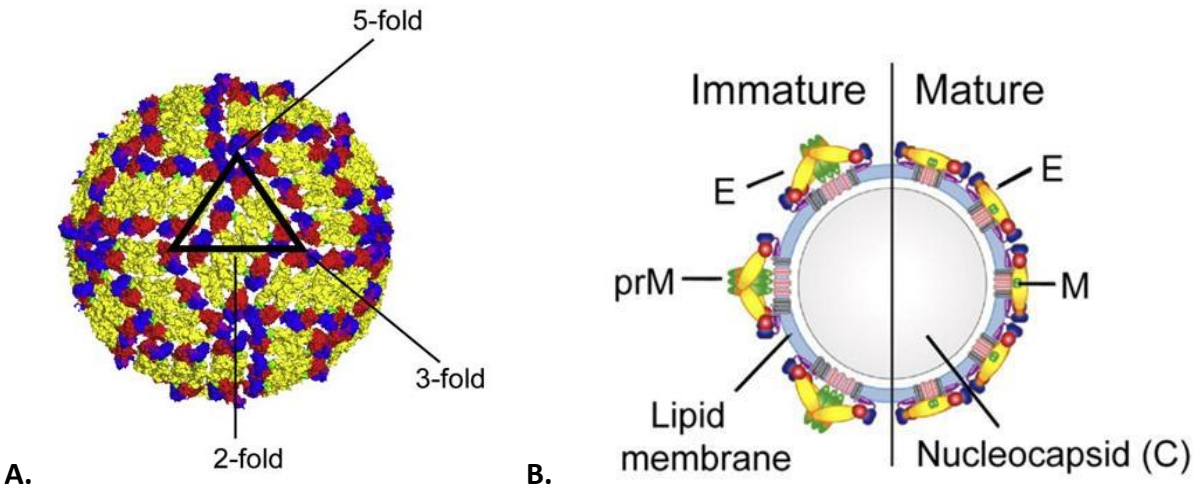


Figure 2: Schematic diagram of a flavivirus particle.

A) Enveloped, spherical, about 50 nm in diameter. Herringbone-like arrangement of 90 E protein dimers at the virion surface as determined by cryo-electron microscopy. The surface proteins are arranged in an icosahedral-like symmetry (Heinz & Stiasny, 2012). B) In its immature (prM-containing) and mature form after proteolytic cleavage of prM (Fritz *et al.*, 2008).

The genome has one open reading frame (ORF) encoding for a single polyprotein of 3432 amino acids, which is cleaved by viral proteases into 3 structural proteins, the capsid (C), precursor to

membrane (prM), envelope (E) and 7 non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5,. The ORF is flanked by 5' and 3' non-coding regions (NCRs), which are crucial cis-acting elements for replication, transcription and translation. The genomic RNA has a type I cap at its 5' end (m7GpppAmp) (a distinguishing feature of the Flavivirus genus since it is not found in other genera) and lacks a 3'-terminal poly (A) tract (Unni *et al.*, 2011). See figure 3.

Figure 3: Japanese encephalitis genome organization.

The complete genome consists of 3 structural proteins capsid (C), precursor to membrane (prM), envelope (E) and 7 non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5,. The ORF is flanked on the sides by 5' and 3' non-coding regions (NCRs), (Saxena *et al.*, 2013)

The C protein has ≈ 120 amino acids and forms homodimers. It is involved in packaging of the viral genome and formation of the nucleocapsid (Mukhopadhyay *et al.*, 2005).The prM protein (≈165 amino acids is closely associated with the E protein and forms a heterodimer. It is thought that the prM acts as a chaperone for folding, assembly and impairing the function of the E protein until the virus is released. Just before the virion is released the prM gets cleaved by cellular furin-like protease to form M protein (≈75 amino acids) its mature protein form during the maturation of the flaviviruses in the Golgi complex (see figure 2). This allows the formation of E protein homodimers which are thus activated (Stadler *et al.*, 1997).

E protein is a large structural protein consisting of ≈495 amino acids, with two potential glycosylation sites. It contains cellular receptor-binding sites(s) and a fusion peptide and is important for the entry of the virus into the host cell. The E protein is the main target of neutralizing antibodies (Ding *et al.*, 2003). Ninety homodimers of E protein present in the host derived lipid bilayer form the major mature virion component (Mukhopadhyay *et al.*, 2005). The E gene sequences of flaviviruses have been considered responsible for virulence in experimental models. The E protein has a major role in determining the virulence phenotype

and a single amino acid substitution may result in loss of virulence or neuroinvasiveness (Ni & Barrett, 1996). JEV E protein possesses the three domains characteristic of flavivirus E with symmetry operators that allow for generation of the canonical E dimer (Luca *et al.*, 2012).

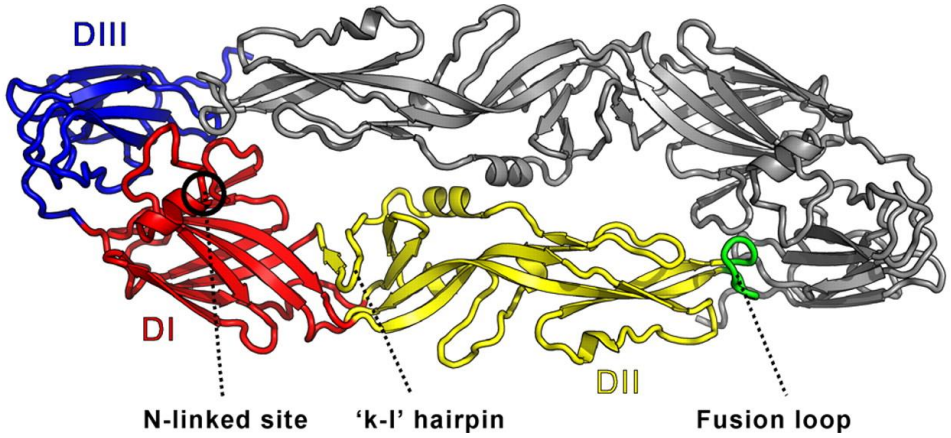


Figure 4: Crystal structure of JEV E ectodomain.

A JEV envelope diagram representation crystal structure. Domain I highlighted in red, domain II highlighted in yellow and domain III highlighted in blue. The fusion loop is shown in green, and the "k-l" loop and glycosylation site are indicated (Luca *et al.*, 2012).

NS1 is required for viral replication (Lindenbach & Rice, 1997), and high levels are produced during flavivirus infection, resulting in the production of specific antibodies (Konishi *et al.*, 1991; Libraty *et al.*, 2002). It has also recently been shown to have a potential role in immunomodulation hence a potential candidate for the development of vaccines and diagnostic reagents. NS1 is known to be more specific than the E protein in serological testing of flavivirus infections (Hua *et al.*, 2013). Novel vaccines containing only virus envelope proteins may raise fears over antibody mediated enhancement (ADE) of disease. However, NS1 is able to elicit protective immunity without the risk of antibody-dependent enhancement hence making it an attractive alternative immunogen. As such, much research is currently being devoted to NS1-based vaccine development. In one study a plasmid containing the coding sequence of NS1 was shown to be a successful genetic vaccination against tick borne

encephalitis in an experimental animal (Timofeev *et al.*, 2004). NS2A is the first of four relatively small hydrophobic membrane associated proteins (NS2A, NS2B NS4A and NS4B) that are conserved in position and not in sequence. NS2A acts in a *cis* fashion to cleave the NS1-NS2A junction after translation and plays functional role in viral replication, viral assembly and secretion. It also modulates the antiviral response of the host by inhibiting the interferon (IFN) signaling pathway (Leung *et al.*, 2008). NS2B remains as a heterodimer along with NS3 and helps in stabilization, substrate recognition and anchoring of this heterodimeric complex to the endoplasmic reticulum (ER) membrane. It acts as a cofactor for the NS2B-NS3 serine protease, which cleaves the viral polyprotein at the NS2A/ NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions (Bera *et al.*, 2007). The non-structural protein 3 (NS3) of JEV has been proposed to originate from rough endoplasmic reticulum (rER), Golgi apparatus or the trans-Golgi network (TGN), and serves as a reservoir for viral proteins during virus assembly (Sahoo *et al.*, 2008). It also participates in viral replication and viral assembly by virtue of its RNA helicase and NTPase activity (Utama *et al.*, 2000). High hydrophobicity of the NS4 protein supports the fact that this protein plays a role as a membrane component and the poorly conserved nucleotide sequence among JEV strains suggested that this region might be important to adapt each virus to different viral growth environments (Kim *et al.*, 2007). This protein is also acts as an IFN antagonist (Lin *et al.*, 2008). NS5 is the largest among all the proteins of JEV and the most highly conserved. It is the key component of the viral RNA replicase complex that presumably includes other viral nonstructural and cellular proteins, and carries both methyltransferase and RNA-dependent RNA polymerase (RdRp) domains (Chambers *et al.*, 1990). It also acts as an IFN antagonist by blocking IFN induced JAK-STAT signalling cascade (Lin *et al.*, 2006).

1.2.8 Viral replication

JEV enters the host cell by receptor mediated endocytosis. Host cells that are targeted include monocytes, macrophages and dendritic cells (Mason, 1989). Subsequent fusion of the lipid membrane of the virus with the endosome membrane caused by low pH allows viral RNA to penetrate into the cytoplasm of the infected cell (Chambers et al., 1990). Viral RNA replication occurs in the endoplasmic reticulum and the Golgi-derived membranes called vesicle packets

(Salonen *et al.*, 2005). The newly synthesized viral RNA is either packaged within progeny virions or used to translate additional viral proteins. Flaviviruses assemble within the ER to form immature particles that display the prM protein. Following transport through the trans-Golgi network, furin-mediated cleavage of prM to M generates mature, infectious virions that are released by exocytosis (Mukhopadhyay *et al.*, 2005)

1.2.9 Phylogenetic variation

All JEV viruses fall under one serotype as reported by Tsarev and others in 2000 after performing phylogenetic analysis involving 92 sequences of JEV using the complete envelope region. The sequences were obtained from samples collected in different geographical regions. In this study the occurrence of a different serotype i.e. the minimum amino acid difference threshold that must be crossed to lose cross-protection, was 18% as reported for Poliovirus type 1-3 or 22% as reported for dengue serotypes 1-4. In this case the maximum observed differences for JEV was 12% which was less than the estimated serotype threshold hence consistent with the proposition that JEV isolates belong to one serotype (Tsarev *et al.*, 2000). This is unlike viruses such as dengue which now has five serotypes (Normile, 2013).

Several techniques have been used to examine strain variation among JEV isolates from various regions. Using complement fixation, haemagglutination-inhibition and antibody-absorption, two immunotypes were differentiated (Okuno *et al.*, 1968). While using polyclonal antiserum, JE isolates in the northern Thailand were grouped into four subtypes (Ali & Igarashi, 1997). Another technique that was used was oligonucleotide fingerprints of viral RNA. This methodology suggested that geographic boundaries limited migration of the JE virus and that viruses isolated at approximately the same time were quite similar (Hori, 1986). However a later study using the same method did not find any relationship between geographic boundaries and migration (Banerjee & Ranadive, 1989).

The availability of the entire nucleotide sequence of the genome of JEV strain JaOArS982 in 1987 demonstrated the feasibility of undertaking detailed nucleotide sequence studies on JE viruses (Sumiyoshi *et al.*, 1987). Phylogenetic analysis performed for JEV using complete

genome, envelope, NS-1, NS-3, NS-5, pre-M, M region showed similar topologies; however, the number of sequences available for each region varied greatly. In GenBank, the most sequences available were for the envelope (E) protein gene (Tsarev *et al.*, 2000). The E protein of the flaviviruses is the major antigen for the viruses and is also believed to be associated with virus binding and entry to host cells. Envelope (E) gene analysis was shown to be a good representative of the phylogenetic analysis of JEV. The choice of the E protein for analysis also provides the best chance of finding variability related to serotypic groups of the virus. Variability in this region should therefore be directly correlated to changes in viral surface epitopes (Monath & Heinz, 1996).

To date, five genotypes of JEV (genotype I, II, III, IV and V) have been described based on phylogenetic analysis of the viral envelope gene (Ni & Barrett, 1995; Williams *et al.*, 2000; Uchil & Satchidanandam, 2001; Solomon, 2003; Nitatpattana *et al.*, 2008). The maximum difference between genotypes was found to be 12% amino acids and the maximum within genotypes was 6% amino acids

Several studies have reported that JEV originated in the Indonesia-Malaysia region from an ancestral virus common to JEV and MVEV (Solomon *et al.*, 2003; Mohammed *et al.*, 2011; Schuh *et al.*, 2013). From this ancestral virus JEV genotypes IV and V diverged, followed by the more recent genotypes I, II, and III. This is supported by the fact that all five genotypes are found in this region and no large epidemics have been reported in these areas (Solomon *et al.*, 2003). These studies also showed that genotype V formed the oldest genotype. A study by Bakonyi and others suggested that Asian JEV and Australian Murray Valley encephalitis virus may have evolved from a virus related to the African Usutu virus in the Southeast Asia-Australasia region based on the results of an amino acid signature analysis (Bakonyi *et al.*, 2004).

There are no reports of difference of virulence of different genotypes. This was tested in a study by Solomon and others in 2003, where mice were inoculated intracerebrally using representative strains of JEV. The results showed no significant differences between genotypes in mouse neurovirulence (Solomon *et al.*, 2003).

1.2.10 Geographic distribution

JEV is found throughout most of Asia, extending north into maritime Siberia. In recent years the geographical distribution of JEV has expanded, reaching south into Australia in 1995 (Hanna *et al.*, 1996), west into Pakistan in 1992 (Igarashi *et al.*, 1994) and east into Saipan in 1990 (Paul *et al.*, 1993). Transmission of JEV in temperate zones is epidemic with the majority of cases occurring in summer months, while transmission in tropical zones is endemic and occurs year-round at lower rates (Innis, 1995).

The molecular epidemiology of JEV has changed and the geographical distribution of JEV has expanded in recent years. Previously, studies, suggested that genotypes I and III occurred principally in temperate, epidemic areas, and genotypes II and IV occurred principally in tropical, endemic regions based on their geographic distribution (Chen *et al.*, 1990; Chen *et al.*, 1992; Williams *et al.*, 2000), conversely, further analysis found several anomalies, especially with respect to movement from epidemic to endemic areas. For example, isolates of epidemic genotype III were found in various endemic areas, such as Indonesia, southern Vietnam (Williams *et al.*, 2000) and Malaysia (Tsuchie *et al.*, 1997). Epidemic genotype I isolates were found in Malaysia (Tsuchie *et al.*, 1997), and the same genotype has also recently become established in the Torres Strait of northern Australia (Pyke *et al.*, 2001). In addition to these anomalies, from the isolation of the prototype Nakayama strain of JEV in 1935, GIII was the predominant genotype throughout Asia; however over the past two decades, it has been supplanted by genotype I viruses in a number of Asian countries including China (Wang *et al.*, 2007), Thailand (Nitatpattana *et al.*, 2008), South Korea (Nam *et al.*, 1996), Japan (Ma *et al.*, 2003), Malaysia (Tsuchie *et al.*, 1997), Vietnam (Nga *et al.*, 2004), India (Fulmali *et al.*, 2011) and Taiwan (Chen *et al.*, 2011). Further, following the isolation of the GV Muar isolate (Uchil & Satchidanandam, 2001; Mohammed *et al.*, 2011), in 1952 from an encephalitic patient originating in Malaysia, the genotype remained undetected for almost 60 years until a pool of *Culex tritaeniorhynchus* collected in the Tibetan Province of China in 2009 yielded the GV XZ0934 isolate (Li, Fu*, et al.*, 2011) and a pool of *Culex bitaeniorhynchus* collected in South Korea in 2010 yielded the GV 10-1827 isolate (Takhampunya *et al.*, 2011).

To address all these anomalies, a study was conducted in 2013 using Bayesian phylogeographic, categorical data analysis and phylogeny-trait association test techniques to determine whether the viruses circulating in the temperate and tropical geographical zones were genetically distinct (Schuh *et al.*, 2013). This study utilized the envelope (E) gene of 487 isolates collected from 12 countries over 75 years which is the largest JEV dataset compiled to date. This study also represents the most recent description of the geographical distribution of JEV as detailed below.

1.2.10.1 Genotype I

Genotype I is currently divided into two groups genotype I-a and genotype I-b. Both groups emerged in tropical Asia around the mid-20th century. Genotype I-a includes samples from tropical regions which are Cambodia, Thailand and northern Australia between 1967 and 2005, and genotype I-b includes samples from temperate regions i.e., Japan, S. Korea, China and Taiwan between 1979 and 2009. Genotype I-b, a temperate genotype, has recently displaced genotype III as the dominant viral genotype of JEV throughout Asia like genotype III, genotype I-b may be maintained in temperate Asia throughout the winter months in hibernating mosquitoes, vertical transmission in mosquitoes, poikilothermic vertebrates, and/or bats. This suggests that the spread and establishment of genotype I-b throughout Asia may have been due to its ability to efficiently overwinter in temperate Asia

1.2.10.2 Genotype II

Genotype II is geographically distributed in tropical climates such as N. Australia, Indonesia, and Malaysia between 1951 and 1999. Like genotype I, it also evolved in tropical Asia around the early 20th century. The Bennett isolate, isolated in Korea around 1951, is the only example of a genotype II virus collected outside of tropical Asia (Schuh *et al.*, 2010). It is suggested that the isolation of this single strain may represent a single imported case from nearby Japan or that genotype II became endemic in Korea for a period of time and subsequently disappeared (Schuh *et al.*, 2010).

1.2.10.3 Genotype III

Genotype III includes samples mainly from temperate climates such as China, N. India, Japan, S. Korea, Sri Lanka, Taiwan and Vietnam between 1935 and 2009.This genotype evolved in temperate Asia (Japan) around the late 19th century. Due to the relatedness of genotype III viruses sampled years apart, it is suggested that GIII is most likely maintained year-to-year by hibernating mosquitoes, vertical transmission in mosquitoes, poikilothermic vertebrates and/or bats.

1.2.10.4 Genotype IV

Genotype IV includes seven isolates collected from mosquitoes only between 1980 and 1981 and is geographically confined to Indonesia. This genotype is estimated to have existed in the late 20th century. It is not known why this genotype has not spread to other regions. However some of the reasons suggested are that the vector competence of *Culex tritaeniorhynchus* for genotype IV may be low, the replicative ability of genotype IV in birds may be low, there could be a narrow host/vector range for genotype IV, or the genotype IV transmission cycle may involve a non-migratory amplifying host (Schuh *et al.*, 2010).

1.2.10.5 Genotype V

Genotype V includes three isolates sampled from temperate and tropical locations which are China, South Korea and Malaysia between 1952 and 2010. It is estimated that genotype V evolved in Malaysia in the early 20th century. JEV was first described in the 1940s in Malaysia when an outbreak occurred during the Second World War among British prisoners of war (Cruickshank, 1951). It is thought that genotype V may have circulated undetected in tropical Asia for much longer, causing only sporadic cases of encephalitis that may have been mistaken for cerebral malaria or other encephalitic diseases. Prior to 2009, only one isolate of genotype V had been described that was isolated in 1952 in Malaysia. So it was quite surprising when after almost 60 years of undetected virus circulation, a pool of *Culex tritaeniorhynchus* collected in the Tibetan Province of China in 2009 yielded the genotype V XZ0934 isolate (Li, Fu*, et al.*, 2011)

and a pool of *Culex bitaeniorhynchus* collected in South Korea in 2010 yielded the genotype V 10-1827 isolate (Takhampunya *et al.*, 2011). Interestingly, despite surveillance that was established in 1951 (Wang, Li*, et al.*, 2009) neither JEV nor *Culex tritaeniorhynchus* had been detected in Tibet prior to 2009 (Li, Li*, et al.*, 2011). Tibet, had been internationally recognized as a Japanese encephalitis (JE)–non-endemic area because the average altitude (above 4,500 meters) was thought to be too high to facilitate the cycle of Japanese encephalitis virus (JEV) between mosquitoes and vertebrates (Brunette *et al.*, 2010). Therefore genotype V of JEV may have entered Tibet shortly before it was initially isolated in 2009. It is possible that GV arrived in Tibet via JEV-infected migratory birds or perhaps by wind-blown mosquitoes (Schuh *et al.*, 2013).

A map is provided showing the currently known distribution of JEV genotypes in figure 5.

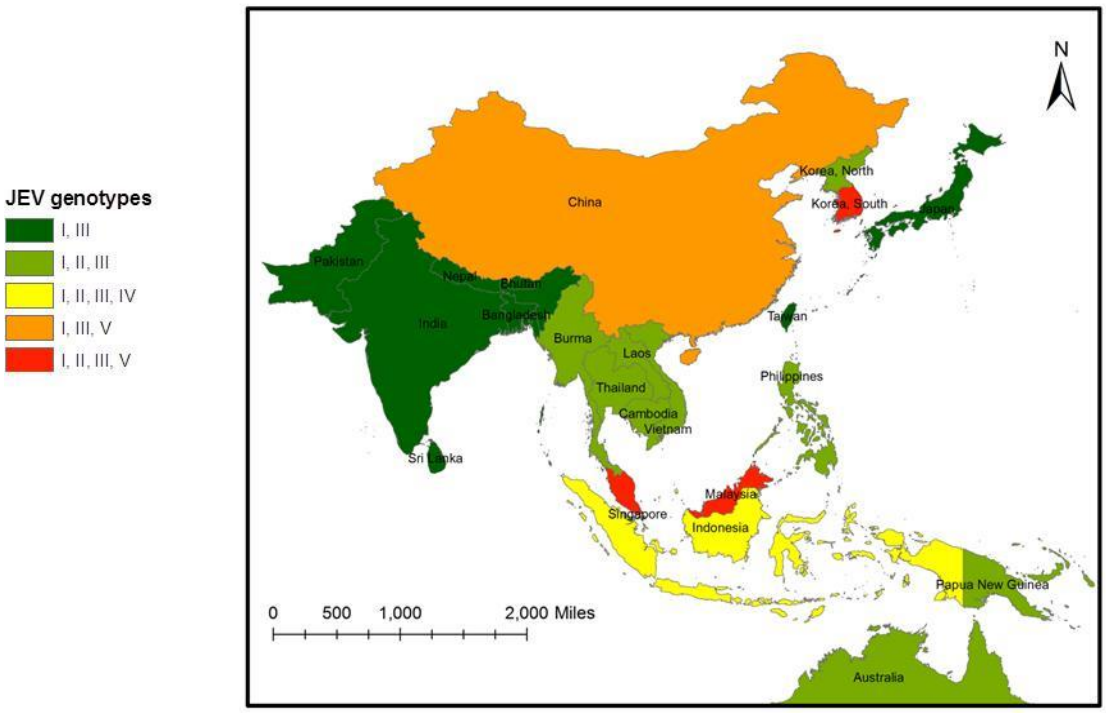


Figure 5: Distribution of Japanese encephalitis virus

The map shows the different countries JEV has been reported. The genotypes that circulate in these countries are shown beside them

1.2.11 The emergence and spread of JEV

JEV is a prominent emerging neurotropic disease. The expansion of JEV over the past decades has seen the progression of genotypes I and II to the east into New Guinea, across the Torres Strait into northern Australia (Hanna *et al.*, 1999; Mackenzie *et al.*, 2002b). There has also been the replacement of genotype III by genotype I in Asia (Pan *et al.*, 2011b) and further the isolation of two genotype V isolates from China (Li, Fu*, et al.*, 2011) and Korea (Takhampunya *et al.*, 2011). The emergence of genotype V in this two areas is thought to be due to JEV gaining fitness to a new competent vector *Culex bitaeniorhynchus* in Korea or greater host availability resulting from the increase in pig farming in Tibet (Li, Li*, et al.*, 2011) It is possible that genotype V arrived in Tibet via JEV-infected migratory birds or perhaps by wind-blown mosquitoes which is similar to the introduction of JEV into Australia (Ritchie & Rochester, 2001). In regards to genotype I, its rapid and widespread expansion was associated closely with increases in human populations, in acreage of irrigated rice, and in pig farming (Erlanger *et al.*, 2009; van den Hurk *et al.*, 2009). Other studies also suggested that following changes in agricultural practices in the 19th century, there was a dramatic expansion of the Asiatic cattle egret across Asia which coincided with the expansion of genotype I (Hancock & Kushlan, 1984).

1.2.12 Factors associated with emergence

1.2.12.1 Environmental factors

1.2.12.1.1 Land-cover and land-use

Changing agricultural practices, can lead to the spread of JEV such as increasing irrigation which provides mosquito breeding sites and animal husbandry which provides host animals (Tsai, 1997). In Asia paddy field surfaces have constantly extended since the early 1960s (http://faostat3.fao.org/) hence driving the JE risk and incidence. Given that paddy fields provide long-term Culex sp. breeding sites and attract many wading birds for foraging and resting, they enhance the circulation and expansion of mosquito and wading bird populations (Elphick *et al.*, 2010). Likewise, the increase in amount of pigs since the 1960s (http://faostat3.fao.org/), has provided a continuous potential source of blood meals for

mosquitoes. In Nepal, the percentage of irrigated land was significantly associated with confirmed JE cases. This was possibly because irrigated land provided a habitat for mosquito development and water bird foraging in Nepal (Impoinvil *et al.*, 2011).

1.2.12.1.2 Climatic variables (rainfall, temperature and wind)

The major climatic variables capable of influencing the level of JEV transmission are rainfall and temperature. They have been shown to strongly affect vector density (McMichael *et al.*, 2006). Recently, Miller *et al.,* identified an optimal range of temperature of 22.8 to 34.5 during the wet season that is favourable to the *Culex tritaeniorhynchus* biology and also found that most of JE cases were located in areas of high probability of vectors. A study in China observed that districts with high temperature in the preceding months of the JEV transmission season had higher JE incidence (Mogi, 1983). It was suggested that the high temperature decreased development time for larval and pupal mosquito stages and also increases the rate of virus replication and dissemination. However, in a study to identify potential environmental drivers of Japanese encephalitis virus (JE) transmission in Nepal, it was reported that districts with low precipitation in the preceding months of the JEV transmission season also had higher JE incidence (Impoinvil *et al.*, 2011). In this case, it was thought that drought increased the association between mosquito vectors and birds by reducing the number of water sites available, hence concentrating birds and mosquitoes in one area (Landesman *et al.*, 2007). Another climatic variable is wind. It is believed that JEV may have spread into Australia through wind-borne mosquitoes (Ritchie & Rochester, 2001). JE vectors have been found at altitudes over 380 m above ground (Ming *et al.*, 1993) and collected 500 km offshore in the Pacific Ocean (Asahina, 1970). It would seem likely for wind to be involved in transport of mosquitoes to such extreme altitudes and distance from land.

1.2.12.2 Non-environmental factors

1.2.12.2.1 Trade

In south-eastern Asia, trade of live animals occurs between farms, local markets, and more importantly within the Indo-chinese peninsula and China, and also to Hong Kong and Singapore (Di Nardo *et al.*, 2011). These movements of either naive or infected animals through trade and transportation can influence human exposure to JEV.

1.2.12.2.2 Bird migration

Migrating birds, have a complex migration system over a large geographical area,. Considering hosts of JEV in Asia particularly the black-crowned night heron (*Nycticorax nycticorax*) and the Asiatic cattle egret (*Bubulcus ibis coromandus*), are thought to be important in the virus' dispersal to new geographic areas (Innis, 1995). Viraemic birds, for instance, may have been responsible for JEV spread and introductions into India (Fulmali *et al.*, 2011), Taiwan (Huang *et al.*, 2010) or Papua New Guinea (Johansen *et al.*, 2000). However, little is known on the large-scale movement patterns of the main wading birds species implicated in JEV transmission (Le Flohic *et al.*, 2013).

1.2.12.2.3 Vaccination

Immunization of humans with JE is the only reliable and effective method to control the disease with efficacies of up to 98% reported (Hennessy *et al.*, 1996). Vaccination is mainly focused on children 1-15 years old who are at most risk of developing JE disease. However human immunization does not contribute to the interruption of virus transmission in animal reservoir cycles. Vaccinating pigs is thought to decrease the amplification of the virus, and help protect horses and humans (Rosen, 1986). However, pig vaccination is not practical and sustainable because of the rapid turnover in pigs, the relative cost of vaccines, and not necessarily effective in piglets (they must be immunized after the disappearance of maternal antibodies) (Igarashi, 2002). In addition, pigs represent a relevant sentinel model used to predict potential JE

outbreak in a human population nearby hence immunizing sentinel pigs would also impede the detection of such a threat (Nitatpattana *et al.*, 2011).

1.2.13 JEV vaccines

Despite the use of effective vaccines including both inactivated whole virus and live attenuated vaccines, JEV remains as an important cause of arthropod-transmitted viral encephalitis. The first JE vaccines available were inactivated vaccines prepared using the prototype Nakayama strain, in mouse brains or primary hamster kidney cells with protection efficacy of 76% to 95% (Halstead & Thomas, 2010) and was manufactured and exported by the Biken Institute in Japan (Shlim & Solomon, 2002). Use of this vaccine together with vector control and alternative agricultural practices almost eliminated the incidence of disease in Japan (Igarashi, 2002), Korea (Sohn, 2000), and Taiwan (Wu *et al.*, 1999). Inactivated cell culture vaccines prepared in primary hamster kidney (PHK) or African green monkey kidney (Vero) cells, and a live attenuated SA14-14-2 vaccine have been used in China (Liu *et al.*, 2006). The SA14-14-2 vaccine has also been used successfully in Nepal (Tandan *et al.*, 2007)and in India (Beasley *et al.*, 2008). Recently, a new purified inactivated JE vaccine derived from Vero cell-adapted SA14-14-2 strain (IXIARO, Intercell AG, Vienna, Austria) has been licensed in the US, Europe, Canada, and Australia (Tauber *et al.*, 2007). In addition, a live chimeric vaccine containing prM and E proteins of JEV in a backbone of attenuated YFV 17D strain has been developed by Sanofi Pasteur (Chimerivax/IMOJEV, Lyon, France)(Guy *et al.*, 2010). The Chimerivax/IMOJEV showed outstanding immunogenicity without adverse effects, thus, it was recently licensed in Australia and is currently under review in Thailand (Halstead & Thomas, 2011).

1.2.14 Potential future trends

Air transport of mosquitoes was the probable cause of JEV outbreaks on isolated Pacific Islands such as Guam (Hammon, Tigertt*, et al.*, 1958) and Saipan (Mitchell *et al.*, 1993), so the possibility of long-distance spread cannot be discounted. Countries with a significant pig population would perhaps be at most risk of human disease (Mackenzie *et al.*, 2002b). This also demonstrates the potential of this virus to invade new areas such as the west coast of the USA.

Here the particular risk for the introduction of JEV is due to the fact that California is a large state functioning as a hub for international travel and commerce with Asia and potentially allowing the introduction of mosquitoes infected with JEV. Of importance is the availability of a significant number of susceptible mosquito vectors and vertebrate hosts (Nett *et al.*, 2009). Other areas of the Pacific could be at risk of JE in the future as indicated by the spread of JE to India and Pakistan in the west and Australasia in the east. Certainly, many islands in the Pacific have potential vectors and vertebrate hosts for JE virus transmission (Mackenzie *et al.*, 2002b). With the spread of JEV into much of the Indian subcontinent, other destinations served by frequent routes of commerce or passenger air travel, such as Africa and Europe, also could be at risk (Weaver & Reisen, 2010).

1.2.15 Vector competence of Japanese encephalitis virus

1.2.15.1 General vector competence concepts

Vector competence is the intrinsic ability of a vector to biologically transmit a pathogen (Higgs & Beaty, 2005). The biological transmission of a pathogen occurs when the pathogen actively reproduces or develops in the vector prior to being transmitted to the next host. In the case of arboviruses, they usually undergo propagative development, which simply means that pathogen propagates in the vector remaining in its same developmental form. However, more units of the virus are usually transmitted than the number of virus that was actually able to infect the vector. A diagram of the cycling of virus through the mosquito is shown (Figure 6). In the biological transmission of arboviruses, the virus is ingested when the mosquito feeds on an infected vertebrate host. The virus usually undergoes an eclipse phase where levels of virus are untraceable because the virus undergoes inactivation in the midgut or infecting virions are entering into the mosquito cells and starting to undergo replication. After the virus replicates, it disseminates to other mosquito tissues and organs, and once it reaches the salivary glands, the mosquito can go on to transmit the virus to another vertebrate host by bite.

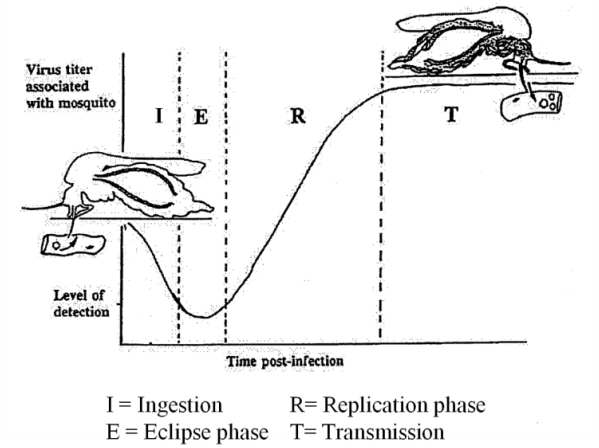


Figure 6: Virus cycle in mosquito (modified from (Higgs & Beaty, 2005).

Virus is ingested (**I**) when the mosquito bites an infected vertebrate host. The virus usually undergoes an eclipse phase (**E**) where levels of virus detection are untraceable because the virus is being inactivated in the midgut. After replication (**R**) in mosquito cells, the mosquito will go on to transmit (**T**) the virus to another vertebrate host.

The concept of vector competence includes several factors such as the extrinsic incubation period (EIP), infection susceptibility, pathogen reproduction and development, and transmission efficiency. Therefore, vector competence studies directly describe the pathogen-vector interaction.

The first key concept in the biological transmission of pathogen in a vector is EIP. EIP is the period from ingestion of the pathogen to the point where onward transmission is possible. EIP is important as it determines at what point the vector will be infectious and able to transmit infectious virus. In poikilothermic vectors, the duration of EIP varies with temperature, with the general trend of higher temperature leading to faster pathogen replication and dissemination and hence shorter EIP duration and lower temperature leading to slower pathogen replication and dissemination, and hence longer EIP longer duration. However, the intrinsic nature of the vector and /or the virus can also influence EIP. For example, it is typical to observe alphaviruses with very short EIP (Dubrulle *et al.*, 2009) duration relative to flaviviruses (Takahashi, 1976) or bunyaviruses (Higgs & Beaty, 2005). Mosquito species have also been shown to have different EIP duration at the same extrinsic incubation temperature (Turell *et al.*, 1984; Turell *et al.*, 1985). The significance of EIP is that if a vector does not survive longer than the EIP, then it will not be able to transmit the pathogen.

Other key concepts in the biological transmission of a pathogen in a vector are infection susceptibility, pathogen reproduction and development, and transmission efficiency. These factors are related to EIP since the pathogen must infect, replicate and be transmitted by the host. For example, in a mosquito-arbovirus system once virus is ingested in a blood meal the virus must infect and replicate in the midgut epithelia then spread to secondary tissues and the mosquito hemolymph (blood system) for further infection and replication. The desired destination for the virus is the salivary gland, because once virions are shed into the salivary ducts of the mosquito the virus can now be transmitted by bite. However, the virus may encounter barriers which prevent it from being transmitted. Two main mosquito barriers to arboviruses are the midgut and the salivary gland. However, 4 scenarios exist in the ability of the virus to cross the barrier(s) at the cellular level (see figure 7). The permissive infection

represents the ability to cross into subsequent tissues and replicate within those cells. The infection and escape barriers represent the inability to transverse into subsequent tissues with or without replication. The leaky gut phenomenon is a scenario where promptly after exposure to a pathogen, the pathogen can then be found in the hemocoel before the pathogen has replicated. It is thought that damage to the midgut causes this phenomenon. It is important to note that these barriers are not absolute and other factor such as viral dose-response and environmental factors such as temperature may influence competence.

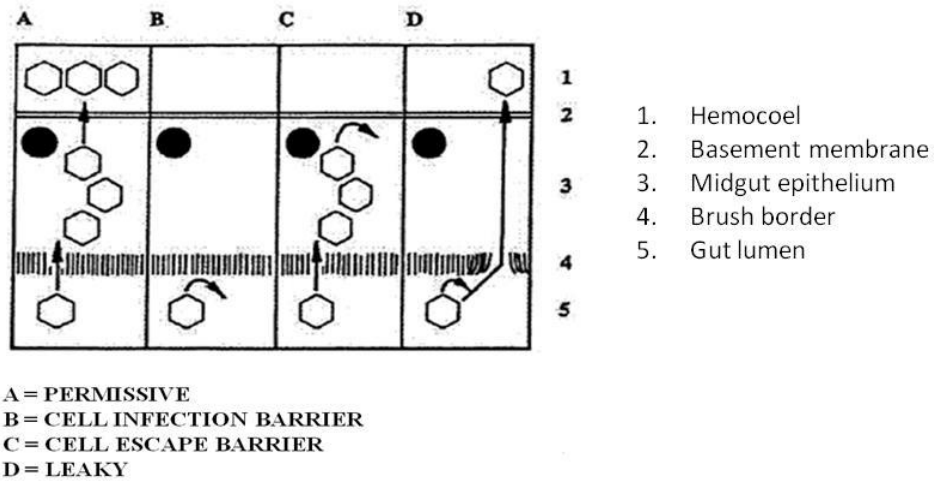


Figure 7: Mosquito barriers to arbovirus transmission modified from (Higgs & Beaty, 2005).

1-Hemocoel; **2**- basement membrane; **3**-Midgut epithelium; **4**- Brush border; **5**- Gut lumen.
A=Permissive-Virus is able to cross into subsequent tissues and replicate within those cells and infects tissues in hemocoel including salivary glands.
B=cell infection barrier-lack of specific viral receptors on the brush border may prevent access to mosquito cell.
C=Cell escape barrier or Midgut escape barrier –prevents viruses that have penetrated into and replicated within the cells of the midgut epithelium from escaping from them into the hemocoel.
D=Leaky -promptly after exposure to virus, the virus by passes gut cells and can then be found in the hemocoel before the replication in the midgut.

When measuring vector competence, three common parameters that are measured are infection rate, dissemination rate and transmission rate. These are usually associated with the barriers to virus infection mentioned above. Infection rate is defined as the number of

mosquitoes that are infected over the total number of mosquitoes that are tested. The dissemination rate is number of mosquitoes that have a disseminated infection over the total number of mosquitoes tested. Dissemination refers to when the infection in the mosquito is no longer confined to the midgut but has indeed infected other secondary organs or tissues of the mosquitoes. This is usually measured by testing mosquito legs (Anderson *et al.*, 2010) or other mosquito organs. The transmission rate is the number of mosquitoes that are actually able to transmit virus over the total number of mosquitoes tested. These are mosquitoes in which the virus has penetrated the salivary glands and are able to transmit the virus by bite. This measure is perhaps the most relevant measure in vector competence studies as it indicates the proportion of mosquitoes that are able to transmit virus. Methods used to quantify transmission rates include forced salivation of mosquitoes into capillary tubes and feeding infected mosquitoes on naïve animals.

In regards to mosquitoes, intra- and inter-specific variation in competence exist which is likely related to co-evolution between the virus and the mosquito. For example, in the case of inter-specific variation some viruses have become more associated with a particular vector and its particular behaviour and physiology. While it may be able to infect other vectors outside its typical range the efficiency is usually lower. In the case of intra-specific variation in vector competence, geography and genetics most likely are the major determinants where again the variability in behaviour and physiology of conspecific (same species) mosquitoes influences competence.

1.2.15.2 Vector competence studies of JEV: Mosquito determinants

Over the years, there have been several vector competence studies using JEV and a wide array of mosquitoes. These studies have been reviewed to some extent (Burke & Leake, 1988; Vaughn & Hoke, 1992b; Endy & Nisalak, 2002; Bosco-Lauth, 2010). One conclusion that can be collectively drawn from these studies is that JEV does not appear limited to a specific range of vectors. Rather, given its ability to replicate in several mosquito species and other insects (Wang *et al.*, 2007; Pan *et al.*, 2011a), it can be concluded that JEV has the potential to be

transmitted by a wide array of vectors. However, the mosquito that has been studied in greatest detail is *Culex tritaeniorhynchus*. Detailed studies of this vector showed that it could be infected with relatively low viraemia (Takahashi, 1976) and transmit virus to a wide array of vertebrate hosts including pigs, birds and horses (Gresser, Hardy, Hu*, et al.*, 1958). Generally, *Culex tritaeniorhynchus* is considered to have high infection and transmission efficiency based on laboratory experiments; though, variation in competence has been demonstrated (Takahashi, 1980a). While, *Culex tritaeniorhynchus* is considered the primary vector of JEV globally, other species have been shown to be important vectors, regionally. For example, in areas such as India *Culex vishnui*, *Culex pseudovishnui*, and *Culex tritaeniorhynchus* are usually taken as a complex (i.e. *Culex vishnui* complex) (Hati & Bhattacharya, 1987; Toma *et al.*, 2000). The reason for this is that these mosquitoes are very difficult to distinguish microscopically, share similar behaviour and are similar in their competence to JEV. Therefore, this trio of mosquitoes likely works together to vector JEV transmission in areas where the 3 mosquitoes coexist. With the expansion of JEV into Papua New Guinea, the Torres Strait region of Australia and the tip of northern mainland Australia, *Culex annulirostris* has been identified as the mosquito likely to vector JEV transmission in Australia and its northern territory (Hanna *et al.*, 1996; Mackenzie *et al.*, 2002a; van den Hurk *et al.*, 2003); this is due to the abundance and distribution of this mosquito in the region, and the lack of *Culex tritaeniorhynchus* in Australia (van den Hurk *et al.*, 2009).

While there have been several studies done with mosquitoes within the transmission range of JEV, several vector competence studies have been done on mosquitoes outside of the known JEV transmission range. The countries where these studies have been done include the US (Reeves & Hammon, 1946; Bosco-Lauth, 2010), mainland Australia (van den Hurk *et al.*, 2003), New Zealand (Kramer *et al.*, 2011) and Uzbekistan (Turell, Mores, Dohm, Komilov*, et al.*, 2006). The early vector competence studies in the US were likely due to recognition of the potential for arboviruses to expand beyond their existing range. Later studies in other countries are likely the result of the emergence of JEV in Australia and emergence of West Nile virus in the US.

Table 4 below summarises studies of vector competence of different vectors for JEV. This table also highlights the differences in vector competence methods used by different authors. For the infection rate some studies used MID50 (midgut 50%infective dose) which is the infectious virus titre at which 50% of the mosquitoes get infected midguts. Most of the authors did not determine the transmission rate and reported mainly the infection rate only. The number of mosquitoes tested varied from as little as one mosquito to 800 mosquitoes. The method used to infect mosquitoes also varied some used live chicks while others used a glass membrane feeder. The glass feeder consists of an outer chamber for circulating water which is heated to 37°C and an inner chamber into which the infectious blood is added. A membrane is stretched over the lower portion of the inner chamber from which mosquitoes feed on. The hanging drop methods allow mosquitoes to feed on hanging blood drops. Studies also used either field collected or colony mosquitoes for their vector competence experiments.

Table 4: Review of vector competence studies of Japanese encephalitis virus

Author	Species	Infection Rate (%)	Dissemination Rate (%)	Transmission Rate (%)	# tested	Method	Infecting titre (pfu/mL)	Genotype	Temp (°C)	Colony or Field collected
(Turell, Mores, Dohm, Lee*, et al.*, 2006)	*Culex pipiens pallens*	0	0	NOT TESTED	40	Live chicks	4.3	?	26	FIELD
	Culex pipiens pallens	6	0	NOT TESTED	32	Live chicks	5.2	?	26	FIELD
	Culex tritaeniorhynchus	100	80	67 (6 tested)	10	Live chicks	4.3	?	26	FIELD
	Culex tritaeniorhynchus	100	93	NOT TESTED	14	Live chicks	5.2	?	26	FIELD
(Turell, Mores, Dohm, Komilov*, et al.*, 2006)	*Culex pipiens pipiens (f. molestus)*	47-56	25-26	8 (37 tested)	142	Live chicks	4.5 - 5.4	?	26	FIELD
(van den Hurk *et al.*, 2003)	*Culex annulirostris*	78-100	6-78	24-81	90	Glass membrane	4.5	2	28	FIELD
	Culex sitiens	83-92	6-33	7-67	90	Glass membrane	4.5	2	28	FIELD
	Culex quinquefasciatus	98	28	50	51	Glass membrane	4.5	2	28	FIELD
	Ochlerotatus vigilax	19-39	18-39	0	75	Glass membrane	4.5	2	28	FIELD
	Ochlerotatus notoscriptus	27	8	27	48	Glass membrane	4.5	2	28	FIELD
	Aedes aegypti	27	17	NOT TESTED	60	Glass membrane	4.5	2	28	FIELD
	Ochlerotatus notoscriptus	20	20	NOT TESTED	5	Glass membrane	4.5	2	28	FIELD
	Ochlerotatus normanensis	0	0	NOT TESTED	1	Glass membrane	4.5	2	28	FIELD

Author	Species	Infection Rate (%)	Dissemination Rate (%)	Transmission Rate (%)	# tested	Method	Infecting titre (pfu/mL)	Genotype	Temp (°C)	Colony or Field collected
	Ochlerotatus purpureus	100	0	NOT TESTED	2	Glass membrane	4.5	2	28	FIELD
	Mansonia septempunctata	67	54	NOT TESTED	24	Glass membrane	4.5	2	28	FIELD
	Mansonia uniformis	100	100	NOT TESTED	1	Glass membrane	4.5	2	28	FIELD
	Verrallina funerea	0	0	NOT TESTED	2	Glass membrane	4.5	2	28	FIELD
(Weng *et al.*, 1997)	*Aedes albopictus*	2.03-4.98 MID50	NOT TESTED	NOT TESTED	?	Hanging drop	5	1	28-32	COLONY
	Culex tritaeniorhynchus	1.02 MID50	NOT TESTED	NOT TESTED	?	Hanging drop	5	1	28-32	COLONY
(Weng *et al.*, 2000)	*Culex tritaeniorhynchus*	1.02 MID50	NOT TESTED	NOT TESTED	?	Hanging drop	5.5	1	28-32	COLONY
	Culex pipiens molestus	2.83 MID50	NOT TESTED	NOT TESTED	?	Hanging drop	5.5	1	28-33	COLONY
(Kramer *et al.*, 2011)	*Opifex fuscus*	74	70	0	50	Pledgets	8.1	3	24	FIELD
	Aedes notoscriptus	0	NOT TESTED	NOT TESTED	39	Pledgets	8.1	3	24	FIELD
	Culex quinquefasciatus	17	0	NOT TESTED	36	Pledgets	8.1	3	24	FIELD
	Culex quinquefasciatus	86	0	0	50	Pledgets	8.1	3	24	COLONY
(Vythilingam *et al.*, 2002)	*Culex sitiens*	67	NOT TESTED	NOT TESTED	?	Glass membrane	?	3	?	COLONY
	Aedes togoi	80	NOT TESTED	NOT TESTED	?	Glass membrane	?	3	?	COLONY

Author	Species	Infection Rate (%)	Dissemination Rate (%)	Transmission Rate (%)	# tested	Method	Infecting titre (pfu/mL)	Genotype	Temp (°C)	Colony or Field collected
(Samuel *et al.*, 2010)	*Culex tritaeniorhynchus*	2-17	NOT TESTED	NOT TESTED	800	Pledgets	6	3	29	FIELD
(Bosco-Lauth, 2010)	*Culex tarsalis*	2	100	NOT TESTED	41	Hemotek	<5.0	3	25	COLONY
	Culex tarsalis	5	100	NOT TESTED	55	Hemotek	>5.0	3	25	COLONY
	Culex tarsalis	6	100	NOT TESTED	93	Hemotek	>5.0	1	25	COLONY
	Culex pipiens pipiens	2	NOT TESTED	NOT TESTED	58	Hemotek	<5.0	3	25	COLONY
	Culex pipiens pipiens	0	0	NOT TESTED	1	Hemotek	>5.0	3	25	COLONY
	Culex pipiens pipiens	0	0	NOT TESTED	14	Hemotek	<5.0	1	25	COLONY
	Culex pipiens pipiens	4	0	NOT TESTED	22	Hemotek	>5.0	1	25	COLONY
	Aedes aegypti	0	0	NOT TESTED	38	Hemotek	<5.0	3	27	COLONY
	Aedes aegypti	9	100	NOT TESTED	42	Hemotek	>5.0	1	27	COLONY
	Aedes aegypti	26	100	NOT TESTED	46	Hemotek	>5.0	3	27	COLONY
	Aedes albopictus	0	0	NOT TESTED	82	Hemotek	>5.0	1	27	COLONY
	Aedes albopictus	0	0	NOT TESTED	62	Hemotek	>5.0	3	27	COLONY
(Chen *et al.*, 2000)	*Armigeres subalbatus*	Not reported	Not reported	79	14	Hanging drop	7.0	3	28	COLONY

1.2.15.3 Vector competence studies of JEV: Virus determinants

Few studies have evaluated differences between virus strains or genotypes. A recent study evaluated the difference in infection rates between genotype 1 and genotype 3 in four mosquitoes: *Culex tarsalis, Culex pipiens pipiens, Aedes aegypti* and *Aedes albopictus.* No difference was found in the infection within each species (Bosco-Lauth, 2010). A study using an attenuated strain of JEV (JEV strain 2-8) and its parent (JEV strain SA 14) found that when colony of *Culex tritaeniorhynchus* was infected intrathoracically with the attenuated strain of JEV, only 3% went on to transmit the virus while 100% of mosquitoes transmitted virus when infected with the parent (non attenuated) JEV strain. In oral infection studies 11% of mosquitoes became infected when feeding on attenuated virus but none of them actually transmitted the virus (Chen & Beaty, 1982). Conversely, 100% of mosquitoes became infected when feeding on parent virus while 75 – 78% of the mosquitoes transmitted the virus. This study shows that differences in infectivity occurring at the virus level may impact transmission of the virus by the mosquito. However, it is important to note that the extent to which mosquitoes in the field actually encounter attenuated viruses is not known.

1.2.16 Mosquito-borne viruses in Europe

In Europe eleven mosquito-borne viruses have been reported into date: six of them are autochthonous and cause human infections (Sindbis, West Nile, Ťahyňa, snowshoe hare, Inkoo, and Batai viruses); three of the viruses are exotic, being occasionally imported to Europe (Chikungunya, dengue and yellow fever); and two of the viruses are associated with birds and are not pathogenic to humans (Lednice, Usutu) (Hubalek, 2008). Figure 8 below shows the known distribution of mosquito-borne viruses across Europe. Mosquito-borne virus outbreaks are strictly determined by the presence and/or import of particular competent vectors of the disease. Ecological variables affect mosquito-borne viruses considerably, the main factors are: presence of appropriate habitats for mosquitoes, abundance of mosquito vectors and their vertebrate hosts and climatic factors. Some of the important flaviviruses in Europe are discussed below.

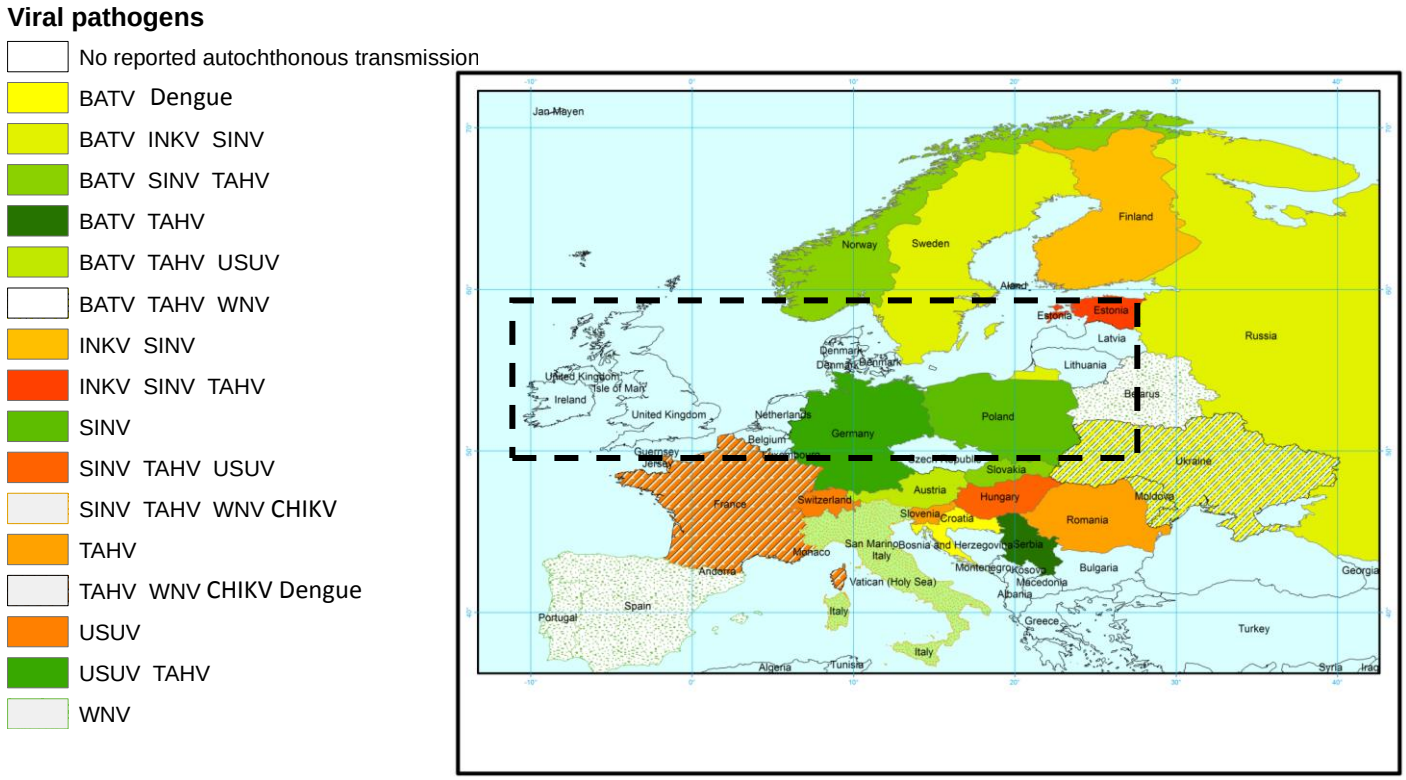


Figure 8: Distribution of mosquito-borne pathogens in Europe modified from (Hubalek, 2008). (The dotted line indicates countries that occur at the same latitude as Great Britain). BATV=Batai virus, INKV=Inkoo virus, SINV=Sindbis virus, TAHV=Tahyna virus, USUV=Usutu virus, WNV=West Nile virus.

1.2.16.1 West Nile Virus

West Nile virus (WNV) is a mosquito-borne flavivirus in the Japanese encephalitis antigenic group. WNV is classified within the Japanese Encephalitis serological complex on the basis of cross-neutralization (Calisher *et al.*, 1989) and molecular genetic studies (Kuno *et al.*, 1998). WNV has a natural transmission cycle in *Culex spp*. mosquitoes and wild and captive birds. In contrast, humans and horses are incidental dead-end hosts (Kramer *et al.*, 2007). WNV was first isolated in 1937 from a febrile woman in the West Nile district of Uganda (Hubalek & J., 1999) and subsequently was associated with sporadic cases of disease as well as major outbreaks in Africa, Eurasia, Australia, and the Middle East.

Epidemics that occurred prior to 1996 generally involved hundreds to thousands of cases in mostly rural populations, with few cases of severe neurological disease (Hayes, 2001). However, beginning in the 1990s, outbreaks began to occur more frequently, especially in the Mediterranean Basin, and were associated with increased numbers of cases with severe disease including viral encephalitis and neurological symptoms (Marfin & Gubler, 2001). The largest human outbreaks occurred in Bucharest in 1996 (393 hospitalized cases; 17 deaths) and Russia in 1999 (318 human cases; 40 deaths) (Platonov *et al.*, 2001; Zeller & Schuffenecker, 2004). This was the largest outbreaks of arboviral illness in Europe since Sindbis virus (*Alphavirus*: Togaviridae) caused an epidemic in northern Europe in the 1980s and also the first epidemics reported in large urban populations (Kramer *et al.*, 2008).

This disease was previously unrecognized in the Western Hemisphere and occurred for the first time in North America in 1999. Its current epizootic/epidemic in North America appeared to have been the result of a single point introduction into the New York City area followed by a dramatic range expansion that now encompasses the United states, Canada, Mexico, Central America and the Caribbean and South America (Hayes *et al.*, 2005; Komar & Clark, 2006; Bosch *et al.*, 2007). Since 1999 to 2013, WNV has caused 39 557 cases of West Nile disease and 1668 deaths in the United States (CDC, 2014). In 1999 recognition of human cases was foreshadowed by weeks by reports of dead exotic and domestic birds in the New York City area (Steele *et al.*, 2000).

In North America, the New York 1999 (NY99) strain of WNV was first isolated from a dead American Crow (Lanciotti *et al.*, 1999) and subsequently from carcasses of 22 other bird species collected between August and November 1999 (Anderson *et al.*, 1999; Steele *et al.*, 2000) Steele et al., 2000). After the initial outbreak in 1999, WNV overwintered in New York, with mid-winter infections discovered in hibernating mosquitoes (Nasci *et al.*, 2001). WNV continued to cause sporadic equine and human disease in the United States (Marfin *et al.*, 2001) reaching Canada in 2001. In 2002, the largest outbreak of WNV encephalitis ever recorded occurred in

the United States, with numerous epicenters spread across the nation's mid-section, and virus activity occurring coast-to-coast, breaching both the Canadian (Pepperell *et al.*, 2003)and Mexican borders (Blitvich *et al.*, 2003).

1.2.16.1.1 Mosquitoes and Birds

Throughout its worldwide distribution, WNV is maintained in nature in an enzootic cycle between ornithophilic mosquitoes, predominantly Culex (*Culex*) species, and birds. Approximately 59 species of mosquitoes and 284 species of birds (48) have been found infected in North America (Hayes *et al.*, 2005). Essentially, all vertebrate hosts that were exposed, whether by inoculation or by infectious mosquito bite, developed viremia and/or raised antibodies. However, birds stand out from other vertebrates as being important WNV amplification hosts due to the development of viremias of sufficient duration and magnitude to infect vector mosquitoes.

Culex spp. are important in their potential role for overwintering WNV in temperate climates, where they hibernate as adult mosquitoes. Field evidence of this phenomenon was observed in the cold months of early 2000 when three WNV-infected hibernating adult *Culex. pipiens* mosquitoes were collected in Queens, New York City, near the epicenter of the 1999 outbreak. In the fall, *Culex pipiens* mosquitoes destined for hibernation undergo a developmental arrest (diapause) determined by the effect on the pupal stages of shortening day-length. The mosquitoes entering diapause feed only on plant sugars and do not blood-feed, so presumably the overwintering mosquitoes acquired their infection by vertical transmission (Nasci *et al.*, 2001).

The emergence of West Nile virus (WNV) in eastern North America in 1999 was a major event in modern arbovirology, not because of its disease impact or the potential threat it represented, but because it alerted the world that pathogens may turn up anywhere at any time. However, New York City, the epicenter of the 1999 outbreak, had no capacity for surveillance and control of arboviral diseases.

1.2.16.1.2 Control of WNV

WNV infection can be prevented by avoiding exposure to infected mosquitoes. Coordinated mosquito control programs that eliminate mosquito breeding sites, apply larvicides to breeding areas, and spray pesticides targeted at adult mosquitoes can reduce their abundance, but the impact of such programs on human disease depends on multiple ecologic determinants of mosquito abundance and human exposure to mosquitoes (Gubler *et al.*, 2000). To reduce their exposure to mosquito bites, people should wear insect repellent on skin and clothes and avoid being outdoors during hours of peak feeding by WNV mosquito vectors, usually from dusk to dawn. Repellents containing N,N-diethyl-m-toluamide (DEET) have excellent safety records and are effective (Fradin & Day, 2002). Oil of lemon eucalyptus, soybean oil, and picaridin also appear to provide effective protection (Barnard & Xue, 2004). Blood donations in WNV endemic areas should be screened for evidence of WNV infection to prevent transmission of WNV through blood transfusions (Custer *et al.*, 2004). Because WNV is known to cause viremia in humans, blood transfusion was considered a potential risk factor for WNV infection after the 1999 epidemic in New York City.

Research toward an effective vaccine to prevent WNV disease in humans is rapidly expanding. Both an inactivated WNV vaccine and a recombinant vaccine based on canarypox expression of WNV antigens are currently licensed for use in horses (Minke *et al.*, 2004). Vaccine candidates for use in humans include an inactivated WNV vaccine, an attenuated WNV vaccine, chimeric live virus vaccines that incorporate WNV E and preM genetic sequences into a 17-D yellow fever vaccine or serotype-4 dengue virus backbone, DNA vaccines that elicit WNV antigen or attenuated Kunjin virus antigen expression, and a recombinant vaccine that uses measles vaccine as a vector for WNV antigens (Tesh *et al.*, 2002; Hall & Khromykh, 2004). Thus far, only the chimeric vaccine using a yellow fever 17-D vaccine backbone has been tested in clinical trials in humans (Hall & Khromykh, 2004).

1.2.16.2 Dengue

Dengue is a mosquito-borne infection that has re-emerged as a major international public health concern over the last four decades. It is currently regarded as the most important

arboviral disease internationally as over 50% of the world's population live in areas where they are at risk of the disease, and approximately 50% live in dengue endemic countries (Gubler, 2011; WHO, 2012) (WHO, 2014). Dengue fever (DF) is caused by any of four closely related yet antigenically distinct single-stranded RNA viruses (genus Flavivirus, family Flaviridae) or serotypes: The serotypes are termed DENV-1, DENV-2, DENV-3, and DENV-4. Infection with one serotype does not protect against the others but results in lifelong immunity to that specific serotype (Halstead, 1974; Wilder-Smith *et al.*, 2010) and sequential infections put people at greater risk for dengue hemorraghic fever (DHF) and dengue shock syndrome (DSS). Each of the four serotypes has been individually found to be responsible for dengue epidemics and associated with more severe dengue (Gibbons & Vaughn, 2002; Asia., 2011) unlike all other flaviviruses, such as JEV and WNV, DENVs that cause most human disease are not zoonoses, but exclusively utilize humans as reservoir and amplification hosts (Weaver & Reisen, 2010).

Epidemic DHF/DSS emerged 50 years ago in Southeast Asia (Hammon *et al.*, 1960) and did not become hyperendemic until the 1980s and later. It was first seen in the Americas only in 1981 (Kouri *et al.*, 1989) and in South Asia in 1989 (Messer *et al.*, 2002b). Since the 1950s, the incidence of DHF/DSS has increased over 500-fold, with more than 100 countries affected by outbreaks of dengue (WHO, 2000). Increases in human population, uncontrolled urbanization, and the increase in human air travel and perhaps commerce have undoubtedly facilitated the spread of DENV strains and enhanced hyperendemicity. In addition, the lack of sustained mosquito control programs, the increase in use of disposable containers and tyres have enhanced conditions for *Aedes aegypti* and for efficient interhuman transmission and can explain much of the spread and persistence of dengue (Rosen, 1977; Gubler, 1997; Weaver & Reisen, 2010).

The arthropod vector for the dengue virus is the *Aedes spp.* mosquito. Two species of the Aedes mosquito are known to transmit the dengue virus to humans. These are *Aedes aegypti* and *Aedes albopictus*. (Pourrut *et al.*, 2011). Humans become infected with the dengue virus when a female Aedes spp. mosquito takes a blood meal from an infected human host, and then

bites another uninfected human, thereby transmitting the virus during the second feeding event. Thus, humans are in fact a natural reservoir for the dengue virus and play a critical role in the spread of dengue virus to new geographic regions (Bain, 2011). Interhuman DENV transmission is highly efficient due to the relatively high viremia titers found in many infected persons, and the susceptibility, but more importantly, the behavior and ecology of *Aedes aegypti*. This mosquito prefers artificial water containers as its larval habitat, human habitations as a resting and host-seeking habitat as adults, and human blood as both a protein source for oogenesis (egg development) and energy for flight. In addition, adult females often feed on multiple human hosts during a single gonotrophic cycle (Harrington *et al.*, 2001).

1.2.16.2.1 Mosquitoes

The principal vector of DENV is the *Aedes aegypti* mosquito, an anthropophilic (one that prefers to feed on humans) species that has adapted extremely well to the urban environment, which is found both indoors and outdoors in close proximity to human dwellings (Rodhain & Rosen, 1997). *Aedes aegypti* is believed to have originated in the jungles of Africa and was most likely spread throughout the rest of the world via slave and trading ships during the seventeenth to nineteenth centuries (Romi, 1995). It was noted some time ago that epidemics of dengue seemed to correlate with the spread of *Aedes aegypti* in South and Southeast Asia, appearing first in port towns and moving inland over time along waterways (Romi, 1995). Now a fully domesticated mosquito, *Aedes aegypti* is an efficient vector of DENV because of its preference for laying its eggs in artificial containers, biting humans, and remaining indoors, where it has access to its favourite host (Rodhain & Rosen, 1997).

Aedes albopictus is a secondary vector of DENV in Southeast Asia, the Western Pacific, and increasingly in Central and South America (Gratz, 2004), but it has also been documented as the sole vector during certain dengue epidemics (Ali *et al.*, 2003). Prior to 1979, this species was found only in Asia and in the Western Pacific, but it has spread to much of the rest of the world in recent decades (Gratz, 2004). The invasion of North America by *Aedes albopictus* was first confirmed with its discovery in Houston, Texas, in 1985 (CDC, 1986) probably arriving in shipments of used tyres from Japan (Hawley *et al.*, 1987). The range of *Aedes albopictus* stretches farther north than that of *Aedes aegypti*, and its eggs are somewhat resistant to

subfreezing temperatures (Hawley *et al.*, 1987) raising the possibility that *Aedes albopictus* could mediate a re-emergence of dengue in the United States or Europe. For example, *Aedes albopictus* can survive the winters in northern Italy (Romi, 1995) and was recently implicated in an outbreak of Chikungunya virus in Italy (Rezza *et al.*, 2007).

Infection with JEV confers lifelong immunity to the virus. However, patients who fail to produce antibody are more likely to have virus isolated from their CSF and are more likely to die (Burke, Lorsomrudee*, et al.*, 1985). While with Dengue infection, epidemiological studies have demonstrated that secondary infection with a heterologous serotype or primary infection of infants born to dengue immune mothers significantly increases the risk of developing severe disease. These clinical observations have led to the widely accepted hypothesis of antibody-dependent enhancement (ADE) of disease (Halstead & Simasthien, 1970; Halstead & O'Rourke, 1977).

Antibody-dependent enhancement (ADE) of infection occurs when pre-existing antibodies present in the body from a primary (first) dengue virus (DENV) infection bind to an infecting DENV particle during a subsequent infection with a different dengue serotype. The antibodies from the primary infection cannot neutralize the virus. Instead, the Antibody-virus complex attaches to receptors called Fcγ receptors (FcγR) on circulating monocytes. The antibodies help the virus infect monocytes more efficiently. The outcome is an increase in the overall replication of the virus and a higher risk of severe dengue (Whitehead *et al.*, 2007).

Differences in severity associated with individual Dengue serotypes or particular sequences of serotypes in sequential infection have been observed. For example, DENV2 viruses have most commonly been associated with DHF/DSS (Thein *et al.*, 1997; Guzman *et al.*, 2002; Balmaseda *et al.*, 2006) along with DENV1 and DENV3 viruses (Harris *et al.*, 2000; Messer *et al.*, 2002a); While DENV4 appears to be the most clinically mild, although it too can cause severe disease (Nisalak *et al.*, 2003). DENV2 and DENV4 have been associated with increased disease severity as a secondary infection, whereas DENV1 and DENV3 seem to cause more severe disease in

primary infection than do the other two serotypes (Vaughn *et al.*, 2000; Balmaseda *et al.*, 2006). None the less, secondary infection by any of the four DENV serotypes remains the greatest risk factor for severe disease (Halstead, 2007). There are no reports of difference of virulence of the different JEV genotypes (Solomon *et al.*, 2003).

In mosquitoes, the Asian DENV2 strains also disseminated in a larger percentage of field caught *Aedes aegypti* mosquitoes compared with American DENV2 strains (Armstrong & Rico-Hesse, 2003), and when the mosquitoes were co-inoculated with equal titres of Asian and American strains, the Asian strains were consistently recovered from a larger percentage of mosquitoes than were the American strains (Cologna *et al.*, 2005). Studies have also shown that the Thai DENV strains (Asian genotype) replicated to higher titers than American genotype DENV2 strains in human monocyte-derived macrophages and dendritic cells (Cologna & Rico-Hesse, 2003). Thus, it is possible that the success of the Southeast Asian DENV2 strains is due in part to more efficient replication in human target cells as well increased transmission by vector mosquitoes.

1.2.16.2.2 Control of Dengue

Mosquito control measures are important and play a central role worldwide in the control of Dengue due to the current lack of dengue-specific vaccines or therapeutics. Since *Aedes aegypti* facilitated the emergence of epidemic dengue in urban centres around the world and is still the primary vector of dengue today, most control efforts have focused on this species (Keller *et al.*, 2006; Hombach, 2007; Whitehead *et al.*, 2007).

A fundamental distinction in the design of a vector control program is whether it takes a government-led, vertical (top-down) approach or a community-led, horizontal (bottom-up) approach (Gubler, 1989). Two examples of successful vertical control programs were undertaken by the governments of Singapore and Cuba. DHF was first reported in Singapore in 1960 (Chew *et al.*, 1961), and beginning in 1968, the Vector Control Unit of the Ministry of Health established a program of entomologic surveillance, larval source reduction, public

education, and law enforcement targeted to control both *Aedes aegypti* and *Aedes albopictus* (Ooi *et al.*, 2006). This program succeeded in bringing the house index (HI) down from almost 50% to approximately 2% by 1973, where it has remained until the present time. In the case of Cuba, a devastating epidemic of DHF/DSS in 1981, the first in the Americas, resulted in over 10,000 cases of severe illness and 158 deaths. The Cuban government initiated a vertical, systematic campaign aimed at eradicating the *Aedes aegypti* vector from the island, and *Aedes aegypti* was eliminated from 13 of Cuba's 14 provinces (Kouri *et al.*, 1989). Some 10,000 health workers remained committed to the control program, and for 15 years no dengue cases were reported in Cuba (Kouri *et al.*, 1989; Kouri *et al.*, 1998). With their past successes, Singapore and Cuba had long been considered to have model dengue control programs, owing in part to their unique political and geographical situations. These two countries implemented consistent programs and policies that made possible the long-term control of dengue, rather than relying only on emergency responses to manage epidemics. However, both locales have faced reintroductions of dengue in spite of low reported vector indices, likely due in large part to the continued influx of people from endemic regions either as tourists, migrant workers, or recipients of cultural exchange, combined with a highly susceptible native population that, ironically, resulted from the success of vector control programs in these countries (Kyle & Harris, 2008).

1.2.17 Mosquito-borne viruses in Great Britain

Mosquito-borne viruses belong to an ecological group of viruses characterized by their specific biological transmission via competent mosquitoes to vertebrates. Competent vectors are those that are able imbibe the virus in the course of blood-feeding on an infected donor host, to support the replication of the virus in their body and to biologically transmit the virus to the recipient host (Weaver & Reisen, 2010). Mosquito-borne viruses are important causes of human disease worldwide. They circulate among wild animals, and many cause disease after spill-over transmission to humans and agriculturally important domestic animals that are incidental or dead-end hosts. In the past three decades, many mosquito-borne viruses have

emerged, creating new challenges for public health. Some are exotic pathogens that have been introduced into new regions, and others are endemic species that have greatly increased in incidence or have started to infect local human populations for the first time.

One such example is the current spread of Chikungunya virus in the Caribbean. Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus belonging to the *Togaviridae* family, first isolated in Tanzania in 1952. The main vectors are mosquitoes from the *Aedes* genus (Chretien *et al.*, 2007). Chikungunya virus causes Chikungunya fever an acute febrile illness associated with severe, often debilitating polyarthralgias and is transmitted to humans primarily via the bite of an infected mosquito (Caglioti *et al.*, 2013). CHIKV transmission is reported to have begun with just 10 confirmed cases of the Chikungunya virus on the French side of St. Martin in December 2013, has quickly spiralled into a much larger outbreak with nearly 300 confirmed cases in two months spanning the Caribbean from Martinique to the British Virgin Islands (Promed_mail, 2013). This is the first report for the local transmission of this virus in the New world reminding us the very real risk of introduction of exotic pathogens in new regions. This is not the first time CHIKV emergence has surprised everyone. Between 2005 and 2007 CHIKV epidemic in the Indian Ocean area caused millions of cases and significant morbidity (Kumar *et al.*, 2011). The virus was imported into the first European country in 2007 and caused an autochthonous-transmitted CHIKV outbreak in Italy (Rezza *et al.*, 2007).

Another example is the West Nile Virus (WNV) which gained notoriety during the 1999 – 2004 US epidemic which resulted in >16,600 human cases with >650 deaths (CDC, 2008). WNV is a member of the genus Flavivirus in the family *Flaviviridae*. The virus is closely related to other flaviviruses in the Japanese encephalitis virus serological complex. WNV has a natural transmission cycle in *Culex* spp. mosquitoes and wild and captive birds. Most humans infected with WNV remain asymptomatic. Approximately 20–40% of infected humans develop symptoms, the vast majority of which range from a mild flu-like syndrome, West Nile fever (WNF), to severe West Nile encephalitic disease (WNED) (Rossi *et al.*, 2010). WNV has a wide geographic distribution, but is commonly found in Africa, Asia, and the Middle East. During the past 40 years, WNV outbreaks have occurred in many European countries including the Czech

Republic, France, Italy, Poland, Portugal, Romania, Russia and Spain (Murgue *et al.*, 2002; Higgs *et al.*, 2004).

The two examples given above (i.e. CHIKV and WNV) are among the best understood mosquito-borne viruses to have emerged in the last two decades indicating just how explosive epidemics can be in new regions. In addition, autochthonous occurrence of dengue fever occurring in Madeira in 2012 were reported to start with two confirmed cases in October of 2012 which eventually turned to approximately 2144 probable cases in just three months (Tomasello & Schlagenhauf, 2013).

1.2.18History of mosquito-borne pathogens in the Great Britain

1.2.18.1 Yellow fever

Great Britain has not completely been free of mosquito-borne disease. As early as the 16th century, there have been sporadic events of mosquito-borne pathogen circulation.

In 1865, Yellow fever (YF) was introduced into Swansea when the 'Hecla' a wooden sailing vessel, docked with from Cuba with *Aedes aegypti* mosquitoes carrying the YF virus on board. It coincided with a spell of exceptionally hot weather therefore leading to an epidemic of Yellow fever in the town. Twenty five days after its introduction, at least 27 inhabitants were infected and 15 of them died. (Meers, 1986). Yellow fever, the original viral haemorrhagic fever, was one of the most feared lethal diseases before the development of an effective vaccine. Today, the disease still affects as many as 200,000 persons annually in tropical regions of Africa and South America (Monath, 2001).

1.2.18.2 Myxomatosis

Myxomatosis which is caused by the myxoma virus, (MYXV) a type of poxvirus that only affects rabbits appeared in Britain in 1953. The first case in England was confirmed at Bough Beech, near Edenbridge, Kent in October 1953. It is not clear how myxomatosis entered England. It may have been brought by rabbit fleas on birds, through wind carriage of infected insects or by

the deliberate introduction of diseased rabbits (Andrewes, 1954). It was first discovered in 1896 in Uruguay and was imported to Australia in 1951 to control its large rabbit populations - initially having the desired devastating effect (Kerr, 2012). The disease was illegally introduced to France in 1952 and it quickly spread to both wild and domestic rabbit populations and within a few years it had spread throughout Europe (Sellers, 1987). A study by Sellers showed that the first outbreaks of myxomatosis in S.E. England in 1953 could have resulted from wind carriage of insects infected with myxoma virus from northern France. The most likely insect was the mosquito *Anopheles atroparvus* which breeds along the coastal marshes of England and northern France and which has been shown experimentally and in the field to transmit myxoma virus mechanically (Andrewes *et al.*, 1956). In England it was caught in 1954 biting rabbits in a built-up area in Newhaven, East Sussex, indicating that flight had occurred away from its normal coastal marshes habitat (Muirhead-Thomson, 1956).

1.2.18.3 Malaria

Malaria a mosquito-borne infectious disease of humans and other animals was common in marsh communities in southern England between the 16th and 19th centuries (Dobson, 1997) where it was referred to the “ague” meaning acute and is usually used to describe fever. Malaria is caused by parasitic protozoa of the genus *Plasmodium*. The disease is transmitted via a bite from an infected female *Anopheles* mosquito. Malaria causes symptoms that typically include fever and headache, which in severe cases can progress to coma or death. The disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia, and the Americas (WHO, 2013). Its introduction into England coincided with the arrival of many Dutch refugees who came to England's marshes to escape the Catholic persecution in Holland (Cracknell, 1959). Malaria was endemic in Holland (Shute, 1944) and it is likely that many Dutch refugees would have brought *Plasmodium vivax* with them. More indigenous malaria cases occurred at the beginning of the 20th Century. The *Plasmodium vivax* parasite was being transmitted by local *Anopheles atroparvus* whose normal habitat is brackish water (Newman, 1919). In 1917 and 1918 there were around 330 cases of locally-transmitted vivax malaria when infected servicemen returning from overseas were

billeted near salt marshes on the Thames Estuary (James, 1920). Those areas most badly affected included the Fens, Thames Estuary, South-East Kent, the Somerset levels, the Severn Estuary and the Holderness of Yorkshire (Shute & Maryon, 1974). From 1840 to 1910, a total of 8,209 deaths were reported. The highest rates were reported from Kent, Essex, and Cambridgeshire consistent with historical observations of high ague mortality in coastal and marshy areas (Whitley, 1863) where the principal mosquito vector, *Anopheles atroparvus,* is still present (Kuhn *et al.*, 2002). These deaths declined steadily over time due to a number of factors. Marsh drainage could have eliminated many breeding sites of the main local vector, *Anopheles atroparvus*, in the brackish waters of coastal marshes, river deltas, and fens (MacArthur, 1951). *Anopheles atroparvus* feeds mainly on livestock but will take human blood when available. Hence, increasing livestock densities may have diverted biting from humans toward cattle, pigs, or horses. Improved housing, better access to health care and medication, and improved nutrition, sanitation, and hygiene all may have reduced transmission and/or mortality rates. At present, more than one thousand imported cases of falciparum malaria are reported in Great Britain each year, mostly from West Africa. Many of these people live in London and other urban areas in the south of England, in areas where *Anopheles* mosquitoes occur (Williams *et al.*, 2002).

1.2.19 British Mosquitoes

At present, there are thirty-four species of mosquitoes recorded in the British Isles comprising six species of Anophelinae (genus *Anopheles*) and 28 species of Culicinae in seven genera: *Aedes* (3), *Coquillettidia* (1), *Culex* (4), *Culiseta* (7), *Dahliana* (1), *Ochlerotatus* (11) and *Orthopodomyia* (1) (Medlock & Vaux, 2009; Golding *et al.*, 2012). These mosquitoes occupy different habitats and differ in their feeding habits. Some develop in permanent water bodies such as ditches and ponds (e.g. *Anopheles claviger*-human-biting, *Coquillettidia richiardii* bird-biting and human biting), while others occupy temporary freshwater pools in woodlands (e.g. *Ochlerotatus cantans*-bird-biting and human biting), *Ochlerotatus rustius*-human biting) and flooded meadows (e.g. *Aedes cinereus* bird-biting and human biting) or saline pools in salt marshes (*Ochlerotatus detritus* bird-biting and human biting) and grazing marsh (*Culex*

modestus bird-biting and human biting). A few species occupy tree-holes (e.g. *Anopheles plumbeus* bird-biting and human biting, *Dahliana geniculata* human biting); while in urban areas others can use containers such as rainwater butts (e.g. *Culex pipiens pipiens* biotype human biting, *Culiseta annulata* bird-biting and human biting). One species also favours underground water in flooded basements, the foundations of dwellings, drains and underground railway tunnels (*Culex pipiens molestus* biotype human biting) (Service, 1969; Service, 1971; Snow *et al.*, 1998; Medlock *et al.*, 2005). Mosquitoes that feed on both birds and mammals can spread viruses from birds to humans such as WNV and JEV which are maintained in birds and are infectious to humans. These mosquitoes are called bridge vectors as they bridge the gap between birds and mammals. These bridge vectors are of great public health importance. Table 5 gives a list of British mosquitoes that are considered bridge vectors due to their feeding habits. It also shows their distribution, habitat and mosquito-borne viruses they have been implicated to transmit elsewhere.

Table 5: Important British mosquitoes considered bridge vectors

Mosquito species	Distribution	Habitat	Implicated for mosquito-borne virus transmission elsewhere†
Aedes cinereus	Widespread ,patchy	Flooded habitat	WNV, SINV, TAHV
Anopheles plumbeus	widespread	Tree holes	
Coquillettidia richiardii	widespread	Permanent waters	WNV, TAHV, BATV
Culex modestus*	Widespread	Fresh and brackish waters	WNV, TAHV, UUKV
Culex pipiens sensu lato	Widespread abundant	Permanent waters	WNV, SINV, TAHV, USUV RNA, LEDV
Culex pipiens* biotype *molestus	Locally sporadic	Underground	WNV
Culex europaeus*	Widespread, few records	Small permanent collections of ground water	WNV
Culiseta annulata	widespread	Permanent waters	TAHV, USUV RNA
Culiseta litorea	Widespread in south	Coastal waters	
Culiseta morsitans	Wide spread	Permanent waters	WNV, SINV, TAHV
Ochlerotatus cantans	widespread	Woods and scrublands	WNV, SINV
Ochlerotatus detritus	Widespread, patchy	Coastal waters	WNV, TAHV
Ochlerotatus punctor	Widespread	Woodland pools	BATV, UUKV

*****These mosquitoes are very rare in Great Britain hence do not pose threat to public health.
†Based on virus isolation or autochthonous disease.
WNV=West Nile virus, SINV=Sindbis virus, TAHV=Tahyna virus, USUV=Usutu virus, LEDV=Lednice virus, BATV=Batai virus, UUKV=Uukuniemi (Romi *et al.*, 2004; Medlock *et al.*, 2005, 2007; Li *et al.*, 2010)

1.2.20 Invasive mosquitoes

In addition to the 34 species of mosquitoes, there is threat from invasion of new mosquito species. *Culex modestus*, a mosquito vector suspected of WNV transmission to animals and humans in France and other European countries, was identified in Kent, Essex and Cambridgeshire (Golding *et al.*, 2012). Six exotic species (*Ochlerotatus atropalpus, Aedes aegypti*, *Ochlerotatus japonicas, Aedes albopictus, Aedes triseriatus*, and *Aedes koreicus* and have also recently been found in Europe hence raising the prospect that they might also be

introduced to the British Isles. Two main mosquitoes that have a great impact on public health, *Aedes aegypti* and *Aedes albopictus* are discussed in detail below.

1.2.20.1 Aedes albopictus

This mosquito is considered the most invasive mosquito in the world and presents a major threat to public health (Paupy *et al.*, 2009). The eggs of *Aedes albopictus* have successfully been transported globally via the used tyre trade and the importation of lucky bamboo and its success in colonizing new geographic locations is due to its ability to adapt to different climates through the production of cold-resistant eggs (Scholte *et al.*, 2007). *Aedes albopictus* is an important known vector of Chikungunya virus (CHIKV). It was the primary vector involved in outbreaks of CHIKV on La Reunion Island (Pialoux *et al.*, 2007), Italy (Rezza *et al.*, 2007), and France (Grandadam *et al.*, 2011). It has also been implicated as a vector for dengue virus causing outbreaks in Hawaii (Effler *et al.*, 2005), Le Reunion Island (Pierre *et al.*, 2005) Mauritius (Ramchurn *et al.*, 2009), Croatia (Gjenero-Margan *et al.*, 2011) and France (La Ruche *et al.*, 2010). So far *Aedes albopictus* has been detected in 20 European countries including Netherlands, Albania, Belgium, Bosnia, Germany, Greece, Italy and France with Italy being the most infested (Medlock, Hansford, Schaffner*, et al.*, 2012).

1.2.20.2 Aedes aegypti

This species was previously established in Brest and Odessa in Europe up to the beginning of the 20th century (Reiter, 2010) and has recently re-established in Europe in Madeira (Almeida *et al.*, 2007) and around the Black Sea in southern Russia, Abkhazia, and Georgia (Yunicheva *et al.*, 2008). It was reported for the first time in the Netherlands in 2010, associated with imported used tyres (Scholte *et al.*, 2010). *Aedes aegypti* can utilize sheltered sites in a domestic setting, which provides protection against environmental conditions and numerous aquatic habitats suitable for oviposition (Reiter, 2010). It is also a highly effective vector of YFV, a disease found in west, central, and east Africa and in South America. The historical, YF outbreak that occurred in Swansea in the 19th century was caused by importation of *Aedes*

aegypti (Buchanan, 1865). This mosquito has a major impact on public health as it causes dengue fever epidemics in the Americas, Southeast Asia, and the western Pacific, with an estimated 50 million infections every year (Wilder-Smith *et al.*, 2010).

1.2.21 Risk of arbovirus introduction into the Great Britain

There is currently no transmission of mosquito-borne arboviruses to humans in Great Britain, However some evidence pointed to the serological detection of antibodies to West Nile virus (WNV), Usutu virus and Sindbis virus in both migrant and non-migrant wild bird species (Buckley *et al.*, 2003) and to WNV in sentinel chickens raised on a farm (Buckley *et al.*, 2006). Prior to this, the only previous record of a mosquito-borne arbovirus in Great Britain was serological evidence of Tahyna virus in small mammals in Devon (Chastel, 1985).

With a number of mosquito-borne arboviruses being endemic in other parts of Europe where they cause human disease and the continuing invasion of exotic mosquito species, it is likely that some of these diseases and mosquitoes could appear in Great Britain. This may occur through movement of infected humans, animals and insects leading to subsequent transmission. For example Chikungunya virus was introduced into Italy by a viraemic traveller (Rezza *et al.*, 2007) and so was the dengue outbreak in France (La Ruche *et al.*, 2010). The growth in air travel not only enables global transit of pathogens but also accelerates their introduction by allowing infectious host to reach other continents in a few days. Transportation also could aid in the introduction of exotic mosquito species that could in turn lead to epidemics of disease as was the case with CHIKV in France (Grandadam *et al.*, 2011). This could be through importation in used tyres. Climatic factor could also influence the introduction of arboviruses.

Arthropods are an important part of the transmission cycle and are dependent on specific climatic conditions for their development and maintenance. Warmer summers and milder winters could favour the abundance of disease causing mosquitoes. Global warming also has the potential to increase the distribution of vectors and to enhance transmission potential in temperate climates by elongating transmission seasons, increasing host-vector contact and

shortening extrinsic incubation times (Weaver & Reisen, 2010). Wind has also been shown to play a part for example several epidemics of bluetongue are thought to have been as a result of windborne infected *Culicoides* from affected areas across seas (Gibbs & Greiner, 1988). Another potential way that arboviruses could be introduced into Great Britain is through migratory birds. Several studies have implicated migratory birds in the spread of arboviruses (Johnston & Conly, 2000; Farfan-Ale *et al.*, 2004; Lvov *et al.*, 2004). There is one study that has shown migratory birds testing positive for WNV and Usutu antibodies in Great Britain (Buckley *et al.*, 2003). However, follow up studies have not shown evidence (Phipps *et al.*, 2008). Nonetheless, bird migrations likely only offer a partial explanation to the emergence of a pathogen in an area.

Great Britain had recently seen the emergence of Culicoides biting midge-borne viruses, bluetongue (2007) (Baylis, 2002; Landeg, 2007) and Schmallenberg viruses (2012) (Beer *et al.*, 2013). Both viruses affect ruminants e.g. sheep and cattle. While these are not mosquito-borne viruses and do not cause disease in humans it still suggest that there is potential for transmission of arboviruses in the UK.

One of the major mosquito-borne viruses considered a threat to Europe is Japanese encephalitis virus (JEV). Unlike WNV which causes relatively mild disease in humans, JEV is known to cause severe illness in humans mainly children between the ages of 1 through 15 years (Ghosh & Basu, 2009). JEV has also been reported to have a high potential to expand to new areas and indeed one possible introduction was reported in Italy in 2010 when JEV RNA was detected in a pool of *Culex pipiens* mosquitoes collected in north-eastern Italy (Ravanini *et al.*, 2012). It is thought that this virus may have been introduced into Italy through migratory waterfowl or wild water-birds (Ravanini *et al.*, 2012). However, it is important to note that autochthonous cases of Japanese encephalitis (JE) have never been reported in Europe (Erlanger *et al.*, 2009).

It is thought that significant levels of immunity in avian species, and the low temperatures that in turn lead to low densities of mosquitoes in Great Britain may provide a barrier for the

introduction of arboviruses (Gould & Higgs, 2009). However there has been transmission of mosquito-borne viruses in countries occurring at the same latitude as Great Britain or even higher as indicated by the map in figure 6 above.

With all the factors of introduction of mosquito-borne pathogens considered one very crucial aspect is the availability of competent vectors to actually sustain the circulation of these pathogens. Some of the mosquito species in Great Britain have been implicated for transmission of mosquito-borne viruses elsewhere (see Table 3). However one important factor lacking is the evaluation of the fitness of these local species to transmit viruses.

1.3 Aims and hypothesis:

1.3.1 **Aim 1**:

Sequence the previously uncharacterised Tengah isolate of JEV, and determine the evolutionary rate of JEV, determined using complete genomes, including three complete sequences of genotype V that have not been used in evolutionary studies before.

1.3.1.1 Hypothesis 1

A. Evolutionary trees that use all the available information on complete genome sequences of JEV will reveal different evolutionary rates than those based on partial genome only.

B. The rate of evolution for genotype V will be slower than that of the other genotypes

1.3.2 **Aim 2**:

Assess the infectivity of Muar (genotype V) stain of JEV in *Culex quinquefaciatus* mosquitoes and compare its infection, dissemination and transmission rates to Nakayama (genotype III).

1.3.2.1 Hypothesis 2

A. Muar will infect and will be transmissible in *Culex quinquefaciatus* mosquitoes

B. The infectivity efficiency of Muar GV will be lower when compared to that of Nakayama genotype III.

1.3.3 **Aim 3**:

Investigate for the first time, the vector competence of a British mosquito species *Ochlerotatus detritus* for JEV at different temperature and compare the vector competence to *Culex quinquefasciatus*.

1.3.3.1 Hypothesis 3

A. *Ochlerotatus detritus* will be susceptible to infection with JEV

B. If *Ochlerotatus detritus* is capable of transmitting JEV, it will be more competent at the higher temperature of 28°C than at the lower temperature of 23°C

C. The transmission efficiency of *Ochlerotatus detritus* will be lower than that of the known JEV *vector Culex quinquefaciatus.*

1.4 Thesis outline

The results chapters of this thesis are organized as follows: Chapter 3 characterizes the full genome sequence of the Tengah isolate of JEV using molecular biology methods of RNA extractions, polymerase chain reaction, sequencing analysis and phylogenetic methods. The Tengah sequence provides additional sequence information which is then included in evolutionary studies using all published full genome sequences of JEV. Of the published sequences, three from genotype II and one from genotype V were recently published hence they have not being used in evolutionary studies before. The evolutionary analysis aims to provide a more comprehensive evolutionary rate of JEV together with the evolutionary rate of genotype V which has not been provided before due to lack of adequate sequence information.

Chapter 4 continues to explore genotype V isolate Tengah (now referred to as Muar due to the fact that its sequence is virtually identical to Muar as revealed in chapter 3) to understand the factors that may have led its limited distribution and isolation. Isolates in genotype V have never been used in vector competence studies before hence the rate of infectivity is not known. The infectivity of Muar in *Culex quinquefaciatus* mosquitoes (a known vector for JEV) is assessed for the first time. The ability of Muar to infect *Culex quinquefaciatus* mosquitoes is also compared to that of Nakayama, an isolate belonging to genotype III which is the most frequently isolated genotype of JEV.

With the possibility of JEV emerging in places it has never been seen before, there is a concern of such an arbovirus emerging in Great Britain which is has yet to see any autochthonous transmission of mosquito-borne viruses. Increased global trade and transportation together with climatic factors may enable the emergence of arboviruses in Great Britain. However it is not known whether the local British mosquitoes can maintain and support the circulation of these viruses. Hence in chapter 5 I determine the competence of a local British mosquito *Ochlerotatus detritus* to JEV at two different temperatures. The work in this chapter has been accepted for publication in the Medical and Veterinary Entomology journal (manuscript attached).

2 Chapter 2: General materials and methods

2.1 Cell culture

2.1.1 Cells

The cell line used in this study was the Vero cell line. Vero cells are a mammalian continuous cell line derived from the Kidney epithelial cells of an African green monkey in the 1960s (Simizu & Terasima, 1988). African green kidney epithelial cells (Vero) were maintained in Dulbecco Modified Eagle's Minimal Essential Medium (DMEM) (Sigma-Aldrich, Dorset, UK) media containing 10% heat-inactivated Fetal bovine serum (FBS), 2 mM L-glutamine and 50 µg/ml Penicillin/Streptomycin in 75 cm^2 flasks with vented cap (Corning® New York, USA). The cells were incubated in a humidified 37°C incubator in the presence of 5% CO_2. The cells were sub-cultured once they reached 80-90% confluence. This was done by pipetting out medium and washing the monolayer. The media was aspirated and the monolayer was washed with 10 ml of 1 × Potassium phosphate buffer (PBS) twice. The cells were then incubated with 1 × trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin, 0.53 mM EDTA, 1 ml/25 cm^2) for 5 minutes at 37°C until cells start to streak as they detach from the flask. To help detach the cells, the flask was tapped gently from side to side, then 10 mls of fresh DMEM with 10% FBS was added to inactivate trypsin. The cells were aspirated several times to ensure single cell suspension and dispensed into new flasks. The cells were diluted 1:20 and passed every 7 days until virus inoculation.

2.1.2 Long-term storage of cells

Cells were grown until they attained 80-90% confluence. Growth medium was removed and the monolayer washed with 10 ml of 1× Phosphate buffered Solution (PBS) twice. The cells were then incubated with 1 × trypsin-EDTA for 5 minutes at 37°C. Five mls of fresh DMEM with 20% FBS was added to inactivate trypsin. The cell suspension was transferred to a sterile 15 ml conical tube and centrifuged for 10 minutes at 1000 × g to pellet the cells. The supernatant was removed and replaced with cell freezing medium containing 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Dorset, UK) and DMEM with heat-inactivated 20% FBS. The DMSO is added to

prevent the formation of ice crystals during the freezing process and hence to preserve the cells during the freezing and thawing process respectively. Re-suspended cells are added to cryo vials 1 ml each and slowly frozen to -80°C in a Cryo 1°C freezing container (Mr. Frosty, Nalgene, UK) which provides the recommended -1°C/minute cooling for successful cell cryopreservation for 24 hours, then transferred to liquid nitrogen.

2.1.3 Cell counting

To assess cell viability and to determine cell density, cell culture suspensions were analysed using a hemacytometer Improved Neubauer (Hausser Scientific USA). Cell viability was determined using trypan blue exclusion. Only dead cells were stained with the trypan blue dye while the viable cells remained clear. Cultured cells were pelleted by centrifugation and re-suspended in 2-5ml of growth medium. Equal amounts of cell suspension and 0.4% v/v trypan blue solution (Gibco) were mixed and allowed to stand for 5-10 minutes and loaded on to an assembled hemacytometer. The number of clear (viable) cells and the number of blue (non-viable) cells were counted within 4 random 1 $mm^2$1mm^2 squares. The percentage of viable cell density (cells/ml) was calculated as below and only cell suspensions containing >90% viable cells were used.

Cell count (cells/mL) = Average cell count per 1mm^2square × dilution factor 10^4

Cell viability (%) = Cell count (viable)/Total cell count × 100

2.2 Virus

Two strains of Japanese encephalitis virus (JEV) were used in this study Tengah and Nakayama.

2.2.1 Tengah

Tengah strain of JEV was originally obtained from the brain of a nine year old Malay girl in Singapore in 1952 (Hale *et al.*, 1952; Okuno *et al.*, 1968) and was kindly donated to us by Professor Ichiro Kurane from the Department of Virology, National Institute of Infectious

Diseases, Tokyo, Japan. The virus has been passaged 5 times in Vero cells before use in this study. This strain has been fully sequenced and characterized as a JEV genotype V strain in Chapter 3 of this thesis.

2.2.2 Nakayama

Nakayama is a genotype III strain of JEV and was originally isolated in Japan in 1935 from human cerebellum (Lewis, Taylor, & et al., 1947). It was kindly donated by Dr. David Beasley and Dr. Alan Barrett from University of Texas Medical Branch, Texas, USA.

2.2.3 Virus culture and harvest

All procedures using 'live' virus were carried out in Advisory Committee on Dangerous Pathogens containment level 3 facilities (ACPD CL3) at the University of Liverpool. All viruses were propagated in Vero cells. Vero cells were grown till 60-80% confluence was reached. Growth media was removed and 0.5- 2ml of virus growth media (DMEM supplemented with 5% FBS and penicillin/Streptomycin) was added to the flask (in T25 tissue culture flask). 100 µl of seed virus was added into the flask and rocked gently to ensure that the monolayer was evenly covered. The flask was incubated at room temperature for 30 -60 minutes with gentle rocking every 5-10 minutes. After incubation, 7-8 ml of virus growth medium was added to the flask and incubated in a humidified incubator at 37°C with 5% CO_2.

Cells were monitored daily for development of cytopathic-effect (CPE) and the virus was harvested when 50-70% CPE was observed. Supernatant from the infected cells was transferred to a 15 ml centrifuge tube and centrifuged at 2000 rpm for 15 minutes in sealed centrifuge buckets to remove cellular debris. The clarified medium was transferred to a sterile 15 ml tube then aliquoted in screw-top cryovials (Nalgene®, UK) and stored at -80°C freezer.

2.2.4 Virus titration

Plaque assays were performed to determine virus titre (plaque forming units/ml) in Vero cells. Cells were grown until they were 100% confluent, and then counted using the method stated

above. The cells were diluted to a 7.5 x 10^5 cells/ml density with growth medium. One milliliter of the cells was added to each well in a 6 well tissue culture plate then topped with 2-3 ml of growth medium and incubated overnight in a 37°C incubator with 5% CO_2. A 2% agarose overlay was prepared by dissolving 2 g of SeaPlaque® agarose-(Lonza, Basel, Switzerland) in sterile distilled water and then autoclaved. This medium was allowed to cool in a 56°C water bath before 2 x nutrient medium was added. The composition of 2 x nutrient medium is 5 x Minimum Essential Media (MEM) (Sigma-Aldrich Dorset, UK) 4% FBS, Gentamicin (50 mg/ml 10000x) (Thermo Fisher Scientific, UK), Amphotericin B (Fungizone) (250ug/ml, 1000x) (Thermo Fisher Scientific, UK), 7.5% Sodium hydro carbonate $NaHCO_3$ and sterile distilled water. Equal volumes of 2% agarose and 2x nutrient medium were mixed to obtain a 1% agarose and 1x nutrient medium. The overlay medium was held at 42°C until required. Virus was diluted ten-fold from 10^{-1} to 10^{-6}, medium was removed from all wells on the plate and 500 µl of virus dilution was inoculated into each well on the plate (one dilution per plate). The plates were then incubated at 37°C for 1 hour with rocking every 10 minutes; after incubation 4ml of agarose overlay were added to each well. Once the overlay had set the plates were incubated at 37°C in the presence of 5% CO_2 for 5- 7 days.

2.2.5 Assay development

After incubation 2 ml of 10% neutral buffered formalin solution was added to each well and incubated at room temperature for 3 hrs or overnight. The formalin solution inactivates any virus present and fixes the cells to the plate. The fixative and agarose plugs were removed from the wells and 0.5 ml of crystal violet solution was added to each well and left to stain for 5 minutes. The stain was washed off using water and the plates were dried on tissue paper. The plates were placed on a light box and plaques were counted in each well.

To calculate the plaque forming units per ml:

Plaque forming units per ml (pfu/ml) = Number of plaques/dilution factor × volume of diluted virus added to the well.

2.3 Molecular biology

2.3.1 Viral RNA extraction

RNA extraction was undertaken using the QIAamp Viral RNA Mini Kit (Qiagen®, Valencia, CA, USA.) according to manufacturer's instructions. Briefly, 140 µl of cell culture supernatant was added to 560 µl of AVL buffer containing carrier RNA and vortexed for 15 seconds. The sample was incubated for 10 minutes to inactivate the virus at room temperature. After a brief centrifugation, 560 µl of 100% ethanol was added and mixed by vortexing for 15 seconds. The solution was then applied to a QIAamp mini spin column and centrifuged at 8000 rpm for 1 minute and the filtrate discarded. AW1 buffer (500 µl) was then added to the spin column and centrifuged for 1 minute at 8000 rpm. After discarding the filtrate, 500 µl of AW2 buffer was added and centrifuged for another 3 minutes at 14,000 rpm. To make sure that there is no residue of the AW2 buffer the spin column was then transferred to a clean collection tube and centrifuged again for 1 minute at 14000 rpm. To elute viral RNA the spin column was transferred to a clean collection tube and 60 µl of AVE buffer (RNase-free water with 0.04% Sodium azide) was added. The sample was incubated at room temperature for 3 minutes then centrifuged at 8,000 rpm for 1 minute. The obtained RNA was then stored at -70°C for further analysis. The extracted RNA was used as a template for the amplification of cDNA by reverse transcriptase-PCR (RT-PCR).

2.3.2 Agarose gel electrophoresis

A 1% agarose gel was prepared in 1 × Tris-Acetate (TAE) buffer (40 mM Tris-Acetate, 1mM EDTA, pH 8.3, Fisher products Loughborough, UK). The suspension was heated in a convectional microwave oven until the agarose had completely dissolved. A gel-casting try was prepared by sealing both ends with tape and inserting small combs to produce wells. After the agarose had cooled to 50-60°C, ethidium bromide was added to a final concentration of 0.5 µg/ml and the solution was transferred to a casting tray. Once set, the tape and combs were removed and the tray transferred to an electrophoresis tank containing TAE buffer. The samples were mixed with 10 × loading buffer (50% v/v glycerol, 10 mM Tris-HCL pH 7.5, 1 mM EDTA, 0.2% (w/v)

Bromothymol blue to a final concentration of 2 × loading buffer and loaded on to the gel. A 1 kilo base DNA ladder (New England Biolabs Ontario, Canada) was included in each gel (0.5 µg/well) to allow approximation of band size and concentration during visualization. The DNA fragments were separated by electrophoresis using 100-120 volts for the appropriate amount of time. PCR products were analyzed visually by electrophoresis through ethidium bromide-stained 1% agarose gels under UV light. Bands were visualized and photographed using a Syngene gel documentation system. Products were purified by using a QIAquick Spin PCR Purification kit (Qiagen®). Amplicons were sequenced on both strands via an automated ABI 3730XL sequencer by the Eurofins MWG Operon® Company Ebersberg, Germany.

2.3.3 Cloning of PCR products

Sections of the Tengah sequence which had double peaks were later cloned to verify the nucleotides in these positions. These were 5500 to 6000, 7000 to 7500 and 8900 to 9100 regions. Cloning was done using the TA Cloning® Kit with pCR™2.1 Vector and One Shot® TOP10 chemically competent E. coli (Invitrogen Corporation Carlsbad, California) following the manufacturer's instructions. Briefly, PCR products were obtained using primers specific for these regions (A table of primers is provided in chapter 3). These products were ligated into the cloning vector, pCR®2.1 by pipetting 2 µl of the PCR product, 1 µl of the ligation buffer, 2 µl of the vector, 1 µl of T4 DNA ligase and 4 µl of water to a final volume of 10 µl. The reaction was incubated at 14°C overnight. After ligating the insert into the pCR®2.1 vector, the construct was transformed into One shot® *E. coli* competent cells (TOP10). The vials containing the ligation mix were centrifuged briefly and placed on ice. 2 µl of the reaction was added to a vial with 50µl of thawed competent cells and incubated on ice for 30 minutes. The cells were then heat shocked without shaking for 30 seconds in a 42°C water bath, then immediately transferred to ice. After 30 seconds, 250 µl of S.O.C (Super Optimal Broth) medium equilibrated to room temperature was added to the vial. The vials were incubated in a shaking incubator at 225 rpm for 1 hour at 37°C. The transformation was spread on Luria broth (LB) agar plates containing 50 µg/ml Kanamycin after they had been equilibrated at 37°C for 30 minutes. The LB plates were then incubated overnight at 37°C. Screening of colonies: Ten colonies for each reaction were

picked and screened by PCR using the Qiagen HotStarTaq Master Mix kit, according to the manufacturer's instructions. Primers specific for these regions were used and the following PCR conditions: An initial activation of 95°C for 15 minutes followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The positive colonies were then incubated overnight in LB medium containing 50 µg/ml of Kanamycin for plasmid extraction. Incubation was at 37°C with vigorous shaking at 250 rpm.

2.3.4 Plasmid extraction

Plasmids were extracted using the QIAprep Spin Miniprep kit as described in the protocol. Briefly, bacterial cells were centrifuged at 8000 rpm in a conventional tabletop centrifuge for 3 minutes at room temperature. The supernatant was carefully removed and the pelleted bacterial cells were re-suspended in 250 µl of buffer P1 and transferred to a 1.5 ml micro-centrifuge tube. A volume of 250 µl of buffer P2 was added and mixed thoroughly by inverting the tube 4-5 times. This was done to lyse the cells. Buffer N3 was added at a volume of 350 µl and tubes mixed immediately. The tubes were then centrifuged at 13,000 rpm for 10 minutes and the supernatant obtained was pipetted on to a QIAprep spin column. After another centrifugation for 1 minute at 13,000 rpm, the spin column was washed by adding 500 µl of buffer PB and centrifuged for 1 minute at 13,000 rpm. An additional wash was followed using 750 µl of buffer PE and centrifuged at 13,000 rpm for 1 minute. The flow through was discarded and the tubes centrifuged again for 1 minute at 13,000 rpm to ensure complete removal of buffer PE. To elute DNA, 50 µl of buffer EB was added to the center of the QIAprep spin column, incubated for 2 minutes, then centrifuged for 1 minute at 13,000 rpm. The obtained plasmid DNA was sequenced on both strands using an automated ABI 3730XL sequencer by the Eurofins MWG Operon ® Company Ebersberg, Germany.

2.3.5 Sequence analysis

Obtained sequences were compiled together to form the complete genome using the Contig assembly application of Vector NTI Advance ™11. The complete sequence was compared to

sequences in the public database using NCBI-Blast program. ClustalW2 Cambridge, UK (Chenna *et al.*, 2003) was also used to align the Tengah genome with other complete genome sequences of different JEV genotypes.

2.4 Vector competence methods

2.4.1 Mosquito acquisition and maintenance

2.4.1.1 Mosquitoes

Mosquitoes used in this study were derived from wild-caught larvae of *Ochlerotatus detritus* sourced locally and *Culex quinquefasciatus*, Say (Recife strain), a colonized mosquito from Brazil. *Culex quinquefasciatus* was used for validation since JEV has been isolated from this mosquito previously (Weng, Lien, Wang, Lin, Lin, & C., 1999; Halstead & Tsai, 2004; Nitatpattana *et al.*, 2005; Changbunjong *et al.*, 2013) and it has also been shown to be competent for JEV in infection studies (Mourya *et al.*, 2002; van den Hurk *et al.*, 2003; Liu *et al.*, 2012). All mosquitoes were reared and maintained at the Liverpool School of Tropical Medicine (LSTM) insectaries.

2.4.1.2 Ochlerotatus detritus

Ochlerotatus detritus immatures (larvae and pupae) were collected from pools on a saline marsh in northwest England (GPS coordinates 53.277073N, -3.067728W) (Figure 9A). This was undertaken using the standard dipping technique (Service, 1993) or the net method (Robert *et al.*, 2002) using a fish net (Figure 9B). The collected samples were labelled with the date and site of collection and kept in separate containers for identification. They were transported to the LSTM insectary where they were reared in 15 × 30 × 5 cm trays, in the same saline water they were collected from at the marsh, and fed on brewer's yeast tablets (Holland & Barrett, Nuneaton, Warwickshire, UK) as needed. Identification was carried out using the fourth instar larvae following the identification keys for British mosquitoes (Cranston *et al.*, 1987). Pupae were collected daily once the field collected larvae started pupating and transferred to a

BugDorm cages® (BioQuip, Rancho Dominguez, CA) (30 × 30 × 30 cm) where they would emerge as adults. A colony was not established as eggs laid in the laboratory failed to hatch.

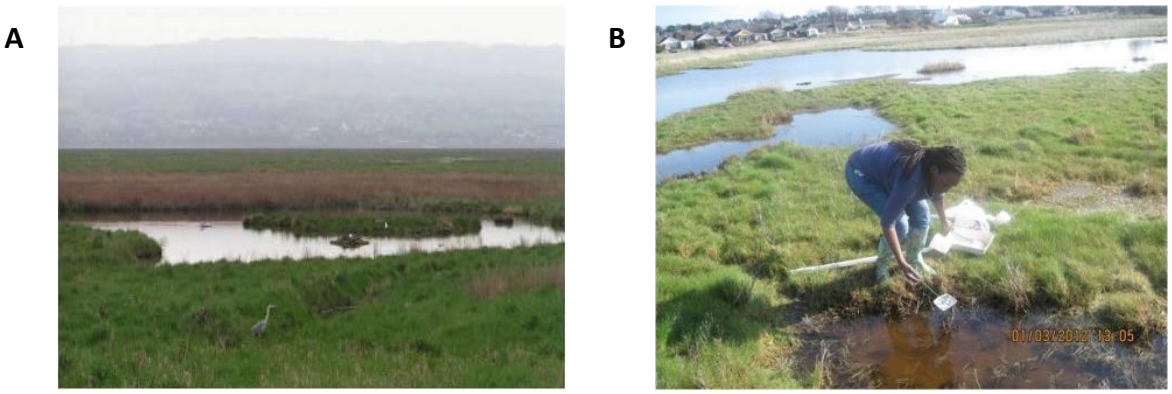


Figure 9: (A) Mosquito sampling site at the saline Marsh in Cheshire – GPS coordinates: 53.277073N –3.067728W and (B) mosquito sampling method using a fish sweep net.

2.4.1.3 Culex quinquefaciatus

The *Culex quinquefaciatus* (Recife strain) mosquitoes were obtained from a colony maintained in the LSTM insectary that was originally established in Brazil. Adult female mosquitoes were provided with a bloodmeal using a Hemotek feeding system (Discovery Workshops, UK). Two days later they were provide with cups filled with water to lay their eggs. Larvae hatched a day after and were divided into 15 × 30 × 5 cm trays (Figure 10A) with approximately 1 litre of de-chlorinated water and fed on brewer's yeast tablets (Holland & Barrett, Nuneaton, Warwickshire, UK) as needed. The amount of food provided was variable depending on the average size of the larvae.

2.4.2 Maintenance

Once the larvae started pupating, the pupae for both species of mosquito were collected daily and transferred to separate 30 × 30 × 30cm BugDorm® cages (BioQuip, Rancho Dominguez, CA) (Figure 10B) where they would emerge as adults. The larval stages pupated after 7 days and the adults emerged after 2 to 3 days at an optimal temperature of 27°C. All mosquitoes were

reared under standard insectary conditions with a relative humidity of 70-90% and 12: 12 light: dark cycle at 27°C. The adults were maintained with cotton wool soaked in 10% sucrose solution placed on top of the cage as a carbohydrate source, and water *ad libitum.* The sugar was then removed twenty four hours before the infection experiments.

A **B**

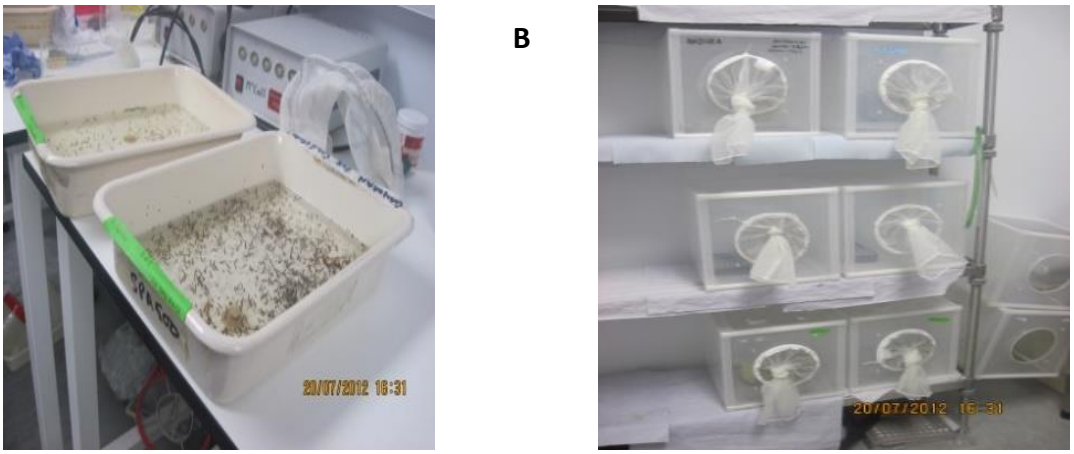


Figure 10: Larval trays (**A**) and cages used to rear mosquitoes (**B**)

2.4.3 Vector competence studies

2.4.3.1 Infection of mosquitoes by artificial bloodmeal

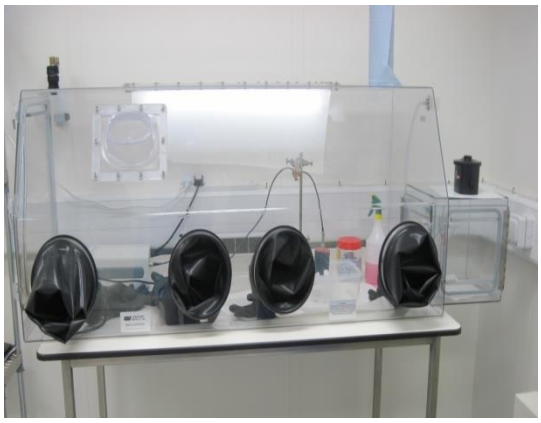
Five to seven day old adult female mosquitoes were mechanically aspirated from their cages into small plastic cups. They were deprived of sugar and maintained on water for 24 hours before they were used for vector competence studies. About 100 mosquitoes were allowed to feed to try and achieve a minimum of 50 mosquitoes for each experiment.

All work with infectious blood meals was undertaken in an Advisory Committee on Dangerous Pathogens Arthropod containment level 3 (ACDP ArCl3) facilities at the Liverpool School of Tropical Medicine (LSTM), Liverpool, UK. Infectious blood meal containing virus from frozen stock was prepared by combining 1 ml of defibrinated horse blood (Thermo Oxoid Remel) with the appropriate amount of virus stock and 100 µl of adenosine 5'-triphosphate (ATP 0.02 µm) as a phagostimulant to a final concentration of 6 logs pfu/ml.

Peroral infection was achieved by exposing mosquitoes to the infectious bloodmeal artificially using a Hemotek feeding system (Discovery Workshops, UK) (Figure 11B) enclosed in a glove box (Figure 11A) for 1-hour in the dark, at room temperature. This is done by using an artificial membrane (Parafilm®). The membrane is stretched on to the Hemotek reservoir connected to the feeding apparatus which warms the blood to 37°C. The reservoir is then placed on the top of the cage with the membrane towards the mosquitoes and mosquitoes allowed to engorge. In all cases a 0.5 ml aliquot of the infectious blood meal was taken both before and after the mosquitoes were fed, and was stored at -80° C for subsequent plaque assay analysis.

Mosquitoes were chilled and sorted on ice and placed in 0.5 litre plastic cups with lids. Only the fully engorged mosquitoes were used for the study. Fed females were maintained with 10% sucrose, under a 70-90% relative humidity and a photoperiod of 12: 12 light: dark cycle. Experiments for the different temperatures were set on different days and so mosquitoes were either held at 23° C or 28° C.

A



B

Figure 11: Glove Box (A) Oral infection of mosquitoes on JEV infectious bloodmeal (B) Hemotek feeding system containing infectious bloodmeal with Parafilm as membrane is placed on top of container holding female mosquitoes to allow mosquitoes to feed. Feeding was carried out in a glove box in the ACDP ArCL3 facility.

2.4.3.2 Determination of infection, dissemination and transmission

Mosquitoes were sampled at 0, 1, 3, 7, 14, 21 and 28 days post infection (dpi) at both 23°C and 28°C. All mosquitoes collected at 0, 1, and 3 dpi were frozen individually in 1.5ml tubes containing virus diluents media. All mosquitoes collected at 7, 14, 21 and 28 dpi had their saliva collected before they were then separated into bodies and legs. This was achieved by anesthetizing using Triethylamine (TEA) (Sigma, St. Louis, MO). Studies have shown that low-level exposures to TEA anesthetize mosquitoes for several hours and do not affect heart physiology or reduce mosquito survival thus making it suitable for mosquito anaesthesia and ideal for experiments where mosquitoes must be restrained for prolonged periods of time (O'Guinn & Turell, 2002).

The corner of a 10 × 10 cm cotton gauze pad was dipped into TEA and placed on the screen of a 0.5 litre cardboard container and the container was placed in a plastic bag for 4-5 minutes. Mosquitoes remained alive but were incapacitated for several hours.

Salivary secretions were collected using a modified *in vitro* capillary transmission assay (Aitken, 1977). Mosquito mouth parts were inserted into a capillary tube containing approximately 10 µl of a mixture of virus diluent (Minimum essential medium (MEM), containing 1% Bovine serum albumin (BSA), 50 µg/ml penicillin/streptomycin, 0.3% Sodium hydrogen carbonate and 2.5 µg/ml Fungizone), sucrose and adenosine 5'-triphosphate (ATP, 0.02 µM) for 45 minutes (Figure 12). One µL of 1% pilocarpine, an analogue of the acetylcholine, prepared in phosphate buffered solution (PBS) and 0.1% Tween 80, was applied on the thorax to stimulate salivation. Active movements of the maxillary palpi and the stylets observed under a stereoscopic microscope were interpreted as a sign of salivation. After 45 minutes, medium containing the saliva was expelled under pressure into 1.5 ml tubes containing 0.5 ml of virus diluent and frozen at -80° C for subsequent determination of transmission rates.

Legs were removed and placed in 1.5 ml tubes containing 0.5 ml of virus diluent and frozen at -80° C for subsequent determination of dissemination rates. Bodies were placed in separate tubes containing 0.5 ml virus diluent and frozen at -80° C for subsequent determination of infection rates.

Infection was determined by recovery of virus from the mosquito tissue suspension by plaque assay. If virus was recovered from its body and but not its legs, the mosquito was considered to have a non-disseminated infection. If virus was recovered from both the legs and the body suspension the mosquito was considered to have a disseminated infection (Turell *et al.*, 1984) and if virus was recovered from its saliva the mosquito was considered to have a transmissible infection. Infection, dissemination and transmission rates were defined as the percentage of mosquitoes tested that contained virus in their bodies, legs and saliva respectively. In all cases 0.5 ml aliquots of the infectious blood meal were collected both before and after the mosquitoes were fed and stored at -80°C for subsequent virus isolation. This was done to confirm that the virus was viable before and after the blood feed.

Figure 12: Mosquito saliva collection
Mosquito proboscis was inserted into a capillary tube containing medium. Mosquitoes were anesthetized using Triethylamine and 1 µl of pilocarpine in PBS was applied on the thorax to stimulate salivation. Saliva collection was carried out under a dissecting microscope in a glove box in the ArCL3 facility.

2.4.3.3 Mosquito sample plaque assay

Body and leg samples were prepared for virus titration by homogenizing using a Disruptor Genie® for 5 minutes. Plaque assays were performed by inoculating 100 µl of the salivary secretions or the supernatant of the homogenized bodies or legs onto a confluent monolayer of Vero cells on a 6-well plate (Costar®, Corning Life Sciences). The plates were then incubated at 37°C at an atmosphere of 5% CO_2 for 30 -60 minutes with rocking every 10 minutes to allow the virus to enter the cells. A 4 ml overlay of Minimum essential medium (MEM), 4% Fetal bovine serum (FBS), 50 µg/ml gentamycine, 0.5% Sodium hydrogen carbonate and 2.5 µg/ml Fungizone (amphotecerine B) to limit contamination and 1% SeaPlaque® low melting point agarose was then added to the wells and the plates were incubated at 37°C in 5% CO_2. After 5 days of incubation, 2 ml of 10% neutral buffered formalin solution was added to each well and the plates left for at least 3 hours with the fixative to ensure complete inactivation of the virus. In order to visualize the plaques the wells were stained with 0.5 ml of crystal violet solution. Samples were scored as virus-positive or virus-negative based on the presence or absence of plaques. Infection, dissemination and transmission rates were determined by Fisher exact test at the 95% confidence level.

2.4.4 Statistical analysis

Fisher Exact Test was used to determine if there were significant differences ($p < 0.05$) in rates of infection, dissemination and transmission between temperatures and species. SISA, an open access online statistics calculator (http://www.quantitativeskills.com/sisa/) was used to conduct Fisher Exact Test (sum of small p's). Confidence intervals of proportions were calculated using VassarStats (http://vassarstats.net/prop1.html). VassarStats uses the Wilson Score Interval method which is more robust when dealing with small number of trials and/or an extreme probability (Newcombe, 1998). Sample size in each group was dictated by the feeding success and the survival rates through the days post infection (dpi).

3 Chapter 3: Molecular characterization of Tengah isolate of Japanese encephalitis virus

3.1 Abstract

Molecular studies have suggested that flaviviruses are rapidly evolving and may have originated from a common ancestor about 10,000 to 20,000 years ago. There are five genotypes of JEV (genotype I, II, III, IV and V), each associated with different geographical distributions and epidemiology. The Muar strain of JEV, the fifth genotype, is believed to represent the oldest lineage from which genotype I – IV evolved. This single characterized isolate (Muar) was last seen in 1952 and considered the only representative of genotype V. However, two recent isolates belonging to genotype V were reported in 2009 and 2010 in China and Korea, respectively. At the same time as Muar was isolated (1952) another virus (Tengah strain) was also isolated in Singapore from a nine year old child but this strain has never been fully characterized. In this chapter I characterise the full genome of Tengah and utilize the Bayesian evolutionary analysis of sampling trees (BEAST) program to examine the evolutionary rate of JEV using published complete genome sequences. Molecular characterization of Tengah strain showed that it is another isolate of genotype V, with 99% sequence similarity to Muar. Evolutionary analysis performed using BEAST program estimated that JEV is evolving at a rate of 3.53×10^{4} nucleotide substitution per site per year. Tengah represents a variant of the Muar strain due to its close similarity. Evolutionary analysis revealed the time to the most recent common ancestor of genotype II strains and genotype V strains. Genotype II isolates appear to have evolved from a common ancestor around 1910 while genotype V evolved from a common ancestor in 1814.

3.2 Introduction

Japanese encephalitis virus is divided into five genotypes. Genotype I, II, III, IV and V. These genotypes are distributed throughout Southeast Asia and the Indian subcontinent, through the Indonesian archipelago, and into the Australasian geographical region (van den Hurk *et al.*, 2009). Genotype V which is believed to be the oldest had initially only been isolated in one instance in Singapore at the Singapore general hospital from a patient in 1952 (Hasegawa *et al.*, 1994; Mohammed *et al.*, 2011). In the same hospital in the same year, three other virus isolations from three patients were also reported to be Japanese encephalitis virus (Hale *et al.*, 1952). Tengah, one of the three isolates was thought to be genotype V but has never been fully characterised (Okuno *et al.*, 1968). More recently, this genotype has been isolated from *Culex tritaeniorhynchus* mosquitoes collected in China, after 57 years (Li, Fu*, et al.*, 2011). Another isolation of Genotype V has also been reported from Republic of Korea (Takhampunya *et al.*, 2011).This shows that this virus is not limited to Southeast Asia as previously thought.

Japanese encephalitis (JE) virus belongs to the family F*laviviridae* and is endemic in several regions of Asia and the pacific (Lindenbach *et al.*, 2007). JE virus (JEV) shares many virological, epidemiological and clinical features with other encephalitis-causing viruses such as the flaviviruses, St. Louis encephalitis virus (SLEV) in North America, West Nile virus (WNV) in Africa and the Middle East, Murray Valley encephalitis virus (MVEV) in Australia, Rocio virus in South America and the Tick-borne encephalitis virus (TBEV) in Russia (Solomon, 2004). In addition there are similarities to mosquito-borne alphaviruses, such as Eastern equine encephalitis virus (EEEV).

Molecular studies have suggested that flaviviruses are rapidly evolving and may have originated from a common ancestor about 10,000 to 20,000 years ago (Solomon *et al.*, 2003). JEV has a genome comprising a positive sense single-stranded RNA molecule of approximately 11 kilo bases (Kb) in length, comprising a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR). The ORF encodes a large polyprotein which is post-translationally processed into three structural proteins (capsid (C), the precursor of the membrane (prM), and envelope (E) and seven non-structural proteins (NS1 NS2A, NS2B, NS3, NS4A NS4B and NS5)

(Chambers *et al.*, 1990; Lindenbach *et al.*, 2007). They have been isolated from many vertebrate and invertebrate vectors and hosts (Johansen *et al.*, 2000), such as bats (Wang, Pan, Zhang, Fu, & Wang, 2009), a variety of mosquito species (van den Hurk *et al.*, 2009) humans (Wang *et al.*, 2010; Zhang *et al.*, 2011), horses (Gulati *et al.*, 2012), pigs (van-den-Hurk *et al.*, 2008) and birds (Yang *et al.*, 2011). A genetic distance of 12% at the amino acid level is used to classify different genotypes while the maximum genetic distance within a genotype is 6% at the amino acid level (Chen *et al.*, 1990; Chen *et al.*, 1992; Tsarev *et al.*, 2000).

Genotype I is distributed widely in Asia, including Japan, Korea, China, India, Vietnam, Cambodia, Malaysia, Thailand, Taiwan, northern Australia and Philippines. Genotype II includes isolates from southern Thailand, Malaysia, Indonesia and northern Australia and Papua New Guinea. Genotype III includes isolates from Southeast Asia, China, Japan, Korea, Taiwan and Central Asia sub-continent. Genotype IV has been isolated only in Indonesian archipelago (Solomon *et al.*, 2003).

In order to understand how these viruses spread to new areas, it is important to sequence their full genomes to assess their genetic diversity which in turn could be used to evaluate how they evolve. However, with JEV some genotypes lack enough sequence information to make this possible. To date, there are several complete genome sequences available for genotype I and genotype III, while there are only four for genotype II, only one complete genome sequence of genotype IV and only two complete genome sequences of genotype V. There is therefore a need to determine more complete genome sequences of JEV viruses in order to provide more accurate evolutionary rate for JEV.

3.2.1 Evolutionary analysis

JEV is a single stranded RNA virus whose transmission cycle involves, mosquito vectors, vertebrate host (birds and pigs) and humans as incidental dead-end host. RNA viruses are thought to evolve faster than DNA viruses because of a combination of highly error-prone replication with RNA polymerase or reverse transcriptase, large population sizes, and rapid replication rates (Domingo & Holland, 1997; Moya *et al.*, 2004). These high mutation rates

facilitate their ability to replicate alternately in disparate vertebrate and invertebrate hosts(Holland & Domingo, 1998). The study of rates of nucleotide substitution in RNA viruses is central to our understanding of their evolution which can provide information for development of an evolutionary model of viral emergence (Holmes & Drummond, 2007). In a study that utilized 50 RNA viruses to determine the rates of evolution in RNA viruses a significant relationship between genetic divergence and isolation time was reported for majority of the viruses indicating a molecular clock (Jenkins *et al.*, 2002). The molecular clock hypothesis states that DNA and protein sequences evolve at a rate that is relatively constant over time and among different organisms (Ho, 2008). However in RNA viruses, a higher rate variation exists among lineages than would be expected under the constraints of a molecular clock. This high variation may be as a result of mutation rates, replication rates, or undefined selective constraints, which might be expected if RNA viruses infect a variety of host species. The general lack of a molecular clock or clock-likeness clearly reduces the power of gene sequences to estimate divergence times for RNA viruses accurately. The study by Jenkins and others showed that fluctuations in clock-like behavior increased the error in rate estimates. They however concluded that, since the error is random, multiple random deviations from rate constancy would be expected to have no net effect on the overall rate estimate. Therefore, substitution rates estimated from large data sets should still be reliable indicators of the average speed of evolution, even if rate heterogeneity is present (Jenkins *et al.*, 2002).

3.2.1.1 Evolutionary analysis using Bayesian Evolutionary Analysis Sampling Trees (BEAST)

BEAST is a program for evolutionary inference of molecular sequences orientated toward rooted, time-measured phylogenies inferred using molecular clock models. Molecular clock is a technique in molecular evolution that uses fossil constraints and rates of molecular change to deduce the time in geologic history when two species diverged. It is used to estimate the time of occurrence of events where strict molecular clock means that the rate of evolutionary change of any specified protein is approximately constant over time and over different lineages

and relaxed molecular clock means that the rate of evolution can vary over time and over different lineages.

BEAST analysis generates a set of trees which are often condensed into a single tree representing the whole set. BEAST uses Bayesian MCMC analysis to average over tree space, so that each tree is weighted proportional to its posterior probability (Drummond *et al.*, 2002). In BEAST, divergence time estimation has also been extended to include relaxed phylogenetics models, in which the rate of evolution is allowed to vary among the branches of the tree. (Drummond *et al.*, 2006). Dates of sequence isolations can be incorporated into the model providing a source of information about the overall rate of evolutionary change, (Rambaut, 2000) hence estimating the time to the most recent common ancestor (TMRCA) (Drummond & Rambaut, 2007).

Some of the advantages of Bayesian analysis are it provides a natural and principled way of combining prior information with data and it also provides interpretable answers, such as "the true parameter has a probability of 0.95 of falling in a 95% credible interval"(Berger, 1985). However with Bayesian analysis, there is no correct way to choose a prior. Bayesian inferences require skills to translate subjective prior beliefs into a mathematically formulated prior. If priors are not chosen with caution, they can generate misleading results. In addition, the computational cost can be high especially in models with a large number of parameters and simulations provide slightly different answers unless the same random seed is used (Wasserman, 2004).

3.2.2 Sequence analysis

Sequence analysis uses sequence alignment methods to compare a new sequence to those with known functions as a way of understanding the biology of an organism from which the new sequence comes from. This information can be used to assign function to genes and proteins by the study of the similarities between the compared sequences (Durbin *et al.*, 1998). Results can reveal sequences that are 100% similar or show variations in the sequences referred to mutations. Some mutations can either be silent mutations or missense mutations. In silent

mutations the changed nucleotide does not result in a change in the amino acid sequence and are also known as synonymous mutations. Missense mutations also known as non-synonymous mutations the changed nucleotide results in a different amino acid which can code for a different protein (Loewe, 2008). Sequence analysis can also reveal quasispecies.

3.2.2.1 Quasispecies

The term 'quasispecies' was first introduced in 1977 by Eigen and Schuster. They used this term to describe the cluster of closely related molecular species produced by errors in the self replication of nucleic acids (Eigen & Schuster, 1977). Hence selection and mutation form a distribution of mutants that are called quasispecies. The target selection is not an individual mutant but the whole quasispecies. Therefore fitness is a property of the quasispecies and not of individual mutants (Nowak, 1992).Quasispecies are a cluster of variant viruses that arise from mutations over time within a viral isolate. They arise following error-prone replication associated with the viral RNA polymerase, which is less accurate at copying template molecules than those of DNA viruses. The quasispecies generated by mutation are acted on by complex and powerful selective pressures in the host, with some viruses having a survival or fitness advantage over others (Lauring & Andino, 2010).

Four viral encephalitis cases were reported in Malaya (n= 1) and Singapore (n= 3) in the summer of 1952. All patients exhibited high fever, vomiting, headache, disturbance of consciousness, stiff neck and deep coma with rapid progression to death by respiratory failure (Hale *et al.*, 1952). Four virus isolates were isolated from brain tissue specimens and identified as JEV by neutralization tests using the Japanese Nakayama strain of JEV (Okuno *et al.*, 1968). Of these, the Muar strain, isolated from a 19-year-old male patient in Singapore in 1952, was classified as a genotype V (Okuno *et al.*, 1968; Hasegawa *et al.*, 1994; Uchil & Satchidanandam, 2001; Mohammed *et al.*, 2011). The Tengah isolate of JEV is one of the three viral isolates originally isolated in Singapore in 1952 from a nine year old Malaysian patient (Hale *et al.*, 1952). Using an antibody-absorption test, it was characterised as JaGAr01 immunotype. However slight quantitative differences between Tengah and JaGAr01 were noted. They also

reported very close similarity between the Tengah and the Muar strain based on antibody absorption test (Okuno *et al.*, 1968). To this date, this strain remains to be fully characterised. This study was undertaken in order to:

- Determine the complete nucleotide and predicted amino acid sequence of the Tengah strain of JEV.
- Compare the nucleotide and predicted amino acid sequence of Tengah to other published JEV strains.
- Determine a more comprehensive rate of evolution of JEV by including three complete sequences of genotype V which have not been used in evolutionary analysis before.

3.3 Materials and methods

Refer to chapter 2 (general materials and methods) for detailed source of the virus, viral RNA extraction, reverse transcription and genome sequencing analysis. Chapter specific protocols and alterations are detailed below.

3.3.1 Virus propagation and amplification

The Tengah strain of JEV was originally obtained from a nine year old patient in Singapore in 1952 and was kindly donated by Prof. Ichiro Kurane from the Department of Virology, National Institute of Infectious Diseases, Tokyo, Japan. All procedures using live virus were carried out in a containment level-3 facility. The virus was propagated in Vero cells and cells were monitored daily for development of cytopathic-effect (CPE). The virus was harvested when 50-70% CPE was observed. RNA extraction was done using the QIAamp Viral RNA Mini Kit (Qiagen®, Valencia, CA, USA.) according to manufacturer's instructions. Reverse transcription and PCR amplification were achieved in one reaction using the Titan-One tube reaction kit (Roche Applied Science, Indianapolis, IN, USA) according to manufacturer's instructions. Primers used were synthesized based on the published sequence of a JEV strain, JKT6468 (Solomon *et al.*, 2003) and later on from the Tengah sequence already derived. The amplified products were then run on a 1% agarose gel for visualization to allow approximation of band size and concentration by comparing them to a 1 kb DNA ladder (New England Biolabs) with fragments of similar size and known concentration. Products were then purified using a QIAquick Spin PCR Purification kit (Qiagen® Valancia, CA, USA.) before sequencing to get rid of residual PCR primers and unincorporated nucleotides. If multiple products were amplified, the correct band based on the size of the amplicon was excised and gel eluted using QIAquick® Gel Extraction Kit (Qiagen® Valencia, CA, USA). Amplicons were sequenced on both strands using the same primers that were used for amplification via an automated ABI 3730XL sequencer by the Eurofins MWG Operon® Company. Table 6 below shows a list of all primers used to amplify the open reading frame (ORF) of the Tengah complete genome.

Table 6: Primers used to amplify and sequence the ORF of the Tengah JEV strain.

No.	Primer Name	Sequence (5'-3')
1	4 S	AGTTTATCTGTGTGAACTTCTTGG
	1265A	AARCCTTGYTTGCACACRTA
2	1202S	CCACGACTGGAGAAGCYCACAA
	1720A	GTGGCGTGCGCCTCTTCAAA
3	1202S	CCACGACTGGAGAAGCYCACAA
	2518A	CCACACCTCATCTCTTTTCTTG
4	1202S	CCACGACTGGAGAAGCYCACAA
	2598A	TCTGGGCGTYTCTGGCARRT
5	2534 S	CTTCGTACACAACGATGTGGAAGCTTGGG
	3200A	AAGATCACTTTCCTCAACGCCATCTCCC
6	3172S	GGGGAGATGGCGTTGAGGAAAGTGATC
	3866A	TGCCCCTAGGACCAAAACCATGTTTTCT
7	3172S	GGGGAGATGGCGTTGAGGAAAGTGATC
	5122A	TCTTGACGGTCACCTTGCACAATAGCG
8	5005S	GGAACATCCGGCTCACCCAT
	6378A	CTTYCTCTCACCCATYCGGG
9	5500S	GCTGCAAGAGGATACATATCTACC
	6000A	GGGCTAGGTTGGTGTCATCC
10	6331S	GARGAYAACACYGAGGTRGA
	7653A	GTTCTTRATGAGAGTCCAGG
11	6652S	TGACAGGAGGATTCTTCCTGCTCATGATG
	7376A	AACCATTCCGTCTACGACGGCATTCTT
12	7000S	GGAAGACATAAGGAGCATCCTTGG
	7500A	CCTCTCGTACTGTTGTGACATTAG
13	7601S	TGCGAGGYAGCTACCTRGCT
	8842A	GTGACAAGTGGGCCCACAGC
14	7601S	TGCGAGGYAGCTACCTRGCT
	9011A	AGGTGGTTTTCCCTCTCCAC
15	8600S	GGACATACCATGGAAGTTACGAAG
	9100A	GCTCCAAGCCACATGAACCAGAT
16	10131S	GAACAGRGTVTGGATTGAAG
	10965A	AGATCCTGTGTTCTTCCTCA

3.3.2 Sequencing of the 5' UTR and 3' UTR

Sequencing of the 5' and 3' UTR were determined using Invitrogen's Rapid Amplification of cDNA Ends (RACE) system – 5' RACE and 3' RACE. 5' RACE was performed according to standard protocols (Invitrogen 5'RACE kit). 3' RACE was performed by first adding a poly A tail polymerase (New England Biolabs) to the RNA template, then conducting RT-PCR with gene specific primers and an oligo-dT-adapter primer (details in Chapter 2). A list of the primers used to amplify the 5' and 3' region are listed below in Table 7.

Table 7: Primers used for the amplification and sequencing of the 5, and 3, untranslated regions (UTR)

Application	Name	Oligonucleotide sequence(5'-3')
5' RACE	Abridged anchor primer	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
5'RACE	Abridged Universal Amplification Primer (AUAP)	GGCCACGCGTCGACTAGTAC
5'RACE	Universal Amplification Primer (UAP)	CUACUACUACUAGGCCACGCGTCGACTAGTAC
PCR GSP1/5'RACE	1720aJE	GTGGCGTGCGCCTCTTCAAA
PCR GSP2/5'RACE	1265aJE	AARCCTTGYTTGCACACRTA
3'RACE	Adapter Primer (AP)	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
PCR GSP1/3'RACE	10131S	GAACAGRGTVTGGATTGAAG
PCR GSP2/3'RACE	10944a	CCACCAGCTACATGTTTCGGCGCTC

3.3.2.1 Cloning of PCR products

To investigate quasispecies of the Tengah strain of JEV, sections of the Tengah consensus sequence which had double peaks were reamplified, cloned, and multiple clones sequenced to verify the nucleotides in these positions. These were regions 5500 to 6000, 7000 to 7500 and 8900 to 9100. Cloning was done using the TA Cloning® Kit with pCR™2.1 Vector and One Shot® TOP10 chemically competent E. coli (Invitrogen Corporation) following the manufacturer's instructions. Colonies for each reaction were picked and screened by PCR using primers specific for these regions. PCR was done using the Qiagen HotStarTaq Master Mix kit, according to the manufacturer's instructions. (Detailed protocol in Chapter 2)

3.3.2.2 Plasmid extraction

Plasmids were extracted using the QIAprep Spin Miniprep kit as described in the protocol. The obtained plasmid DNA was sequenced on both strands using the same primers used for amplification as listed in Table 4 above using an automated ABI 3730XL sequencer by the Eurofins MWG Operon ® Company (detailed protocol in Chapter 2).

3.3.3 Sequence analysis

Consensus sequences were compiled together to form the complete genome using the Contig assembly application of Vector NTI Advance ™11 (Invitrogen). The complete sequence was compared to sequences in the public database using NCBI-Blast program. ClustalW2 (Chenna *et al.*, 2003) was also used to align Tengah with other complete genome sequences of different JEV genotypes.

3.3.4 Phylogenetic analysis

The genetic relationship to other JEV genotypes was determined by phylogenetic analysis using the MEGA 5.1 (Tamura *et al.*, 2011). Analysis was performed using published sequences of various JEV strains obtained from the Genbank database. A list of these sequences, their year of isolation, geographic origin, accession numbers and genotype are listed in Table 8 below. Sequences were aligned and phylogenetic trees were generated by Neighbour joining and confidence levels for internal nodes estimated by 1000 bootstrap replicates.

3.3.5 Evolutionary analysis

Bayesian analysis was performed using the BEAST (Bayesian evolutionary analysis of sampling trees) software package v1.7.4 (Drummond & Rambaut, 2007). The maximum clade credibility (MCC) phylogenetic tree was inferred using the Bayesian Markov Chain Monte Carlo (MCMC) method to estimate the rate of nucleotide substitution and the Time to the Most Common

Recent Ancestor (TMRCA). This was done by incorporating the date of sample collection as the age of the virus. Path-O-Gen Version 1.2 software (by A. Rambaut; http://tree.bio.ed.ac.uk/software/pathogen) was used to investigate the temporal signal and 'clocklikeness' of the sequence data. In BEAUti v1.7.4, the analysis utilised the general time reversible (GTR) model for substitution with combination of gamma distribution and proportion of invariant sites (GTR + G + I) to describe rate heterogeneity among sites. This model was selected after the sequence datasets were subjected to TOPALi v2 (Milne. I *et al.*, 2004) to determine the most suitable nucleotide substitution model. Uncorrelated lognormal relaxed clock model was chosen in order to accommodate for variation in substitution rate among branches (Drummond *et al.*, 2006). MCMC chains were 100 000 000 generations with 10% burn-in to make an effective sample size (ESS) for parameter estimates >200. BEAST output was viewed with TRACER v1.5 for convergence. Trees from multiple runs were combined using the LogCombiner v1.7.4 program and the evolutionary tree was generated in the FigTree program v1.3.1. To reveal uncertainty in the estimations 95% high probability density (HPD) intervals in each case was also determined. Posterior probability values were provided as an assessment of the degree of support for each node on the tree. Sequences used for the evolutionary analysis are listed in table 6 below.

Since the XZ0938 isolate in genotype V was isolated 57 years after Muar and Tengah, BEAST analysis were performed with and without this isolate in order to examine the effect this isolate would have on the estimation of JEV evolutionary rate.

Table 8: Details of the Japanese encephalitis virus isolates sequences retrieved from GenBank for use in this study.

No.	Genotype	Strain	Year	Origin	Host	GenBank accession no.
1	I	HEN0701	2007	China	Pig	FJ495189
2	I	Ishikawa	1998	Japan	Mosquito	AB051292
3	I	JEV/sw/Mie/40/2004	2004	Japan	Pig	AB241118
4	I	JEV/sw/Mie/41/2002	2002	Japan	Pig	AB241119
5	I	JX61	2008	China	Pig	GU556217
6	I	K94P05	1994	Korea	Mosquito	AF045551
7	I	SC04-17	2009	China	Mosquito	GU187972
8	I	SH17M-07	2007	China	Mosquito	EU429297
9	I	XJ69	2007	China	Mosquito	EU880214
10	I	XJP613	2007	China	Mosquito	EU693899
11	II	Bennett	1951	Korea	Human	FJ515927/872376
12	II	FU	1994	Australia	Human	AF217620
13	II	JKT654	1978	Indonesia	Mosquito	HQ223287
14	II	WTP-70-22	1970	Malaysia	Mosquito	HQ223286
15	III	14178	2001	India	Human	EF623987
16	III	57434	2005	India	Human	EF623988
17	III	04940-4	2002	India	Mosquito	EF623989
18	III	B58	1986	China	Bat	FJ185036
19	III	Beijing-1	1949	China	Human	L48961
20	III	CH1392	1990	Taiwan	Mosquito	AF254452
21	III	CH2195LA	1994	Taiwan	Mosquito	AF221499
22	III	GB30	1997	China	Bat	FJ185037
23	III	GP78	1978	India	Human	AF075723
24	III	HVI	1967	Taiwan	Human	AF098735
25	III	JaGAr01	1959	Japan	Mosquito	AF069076
26	III	JaOArS982	1982	Japan	Mosquito	M18370
27	III	JaOH0566	1966	Japan	Human	AY508813
28	III	K87P39	1987	Korea	Mosquito	AY585242
29	III	Ling	1965	Taiwan	Human	L78128
30	III	Nakayama	1935	Japan	Human	EF571853
31	III	NJ2008	2008	IU	IU	GQ918133
32	III	P3	1950	China	Human	U47032
33	III	RP-9	1985	Taiwan	Mosquito	AF014161
34	III	SA14	1954	China	Mosquito	U14163
35	III	T1P1	1997	Taiwan	Mosquito	AF254453
36	III	Vellore-P20778	1958	India	Human	AF080251
37	IV	JKT6468	1981	Indonesia	Mosquito	AY184212
38	**V**	**Tengah**	**1952**	**Singapore**	**Human**	**This study**
39	V	Muar	1952	Malaysia	Human	HM596272

40	V	XZ0938	2009	China	Mosquito	HQ652538

3.4 Results

3.4.1 Sequencing of complete genome of Tengah

The full-length Tengah strain genome was compiled, edited and analysed using the Vector NTI software (Invitrogen). The complete genome was 10,988 nucleotides long, and encoded a predicted polyprotein of 3433 amino acids. Nucleotide and amino acid sequence for the complete Tengah sequence can be found in appendix I and II. Nucleotide sequence homology to sequences in the public database using NCBI-Blast program revealed 99% nucleotide and amino acid sequence similarity to JEV isolate Muar (Mohammed *et al.*, 2011) which belongs to genotype V (Table 9).

Table 9: Percentage nucleotide and amino acid sequence similarity with other JEV genotypes.

Strain	Genotype	K94P05	FU	JaOAr982	JKT6468	Muar	Tengah
		I	**II**	**III**	**IV**	**V**	
K94P05	I	100	**96**	**97**	**93**	**90**	**90**
FU	II	89	100	**97**	**94**	**91**	**91**
JaOAr982	III	89	89	100	**95**	**91**	**91**
JKT6468	IV	83	83	84	100	**90**	**90**
Muar	V	78	78	79	78	100	**99**
Tengah		78	78	79	78	99	100

Nucleotide similarity in lightface type; amino acid in bold-face type

3.4.2 Quasispecies

ClustalW2 (Chenna *et al.*, 2003) was used to align Tengah with other complete genome sequences of JEV and revealed three nucleotide differences when compared to Muar sequence. Two were synonymous substitutions while one was non-synonymous. The non-synonymous mutation occurred in position 7173 of the nucleotide sequence and 2391 of the predicted amino acid sequence, and resulted in a cytosine (C)-guanine (G) substitution, in the NS4B region.

3.4.3 Phylogenetic analysis

The results of the nucleotide and amino acid sequence similarity in Table 9 above which shows a 99% sequence similarity between Muar and Tengah isolates of JEV are further supported by the phylogenetic tree which shows that Tengah clusters in the same branch as Muar which belongs to the fifth genotype of JEV. Both Muar and Tengah are significantly divergent from the other four JEV genotypes as shown on the phylogenetic tree in Figure 13. This suggests that Tengah is a fourth isolate belonging to the fifth genotype along with the JEV isolate Muar, XZ0934 and 10-8027 (Hasegawa *et al.*, 1994; Li, Fu*, et al.*, 2011; Mohammed *et al.*, 2011; Takhampunya *et al.*, 2011).

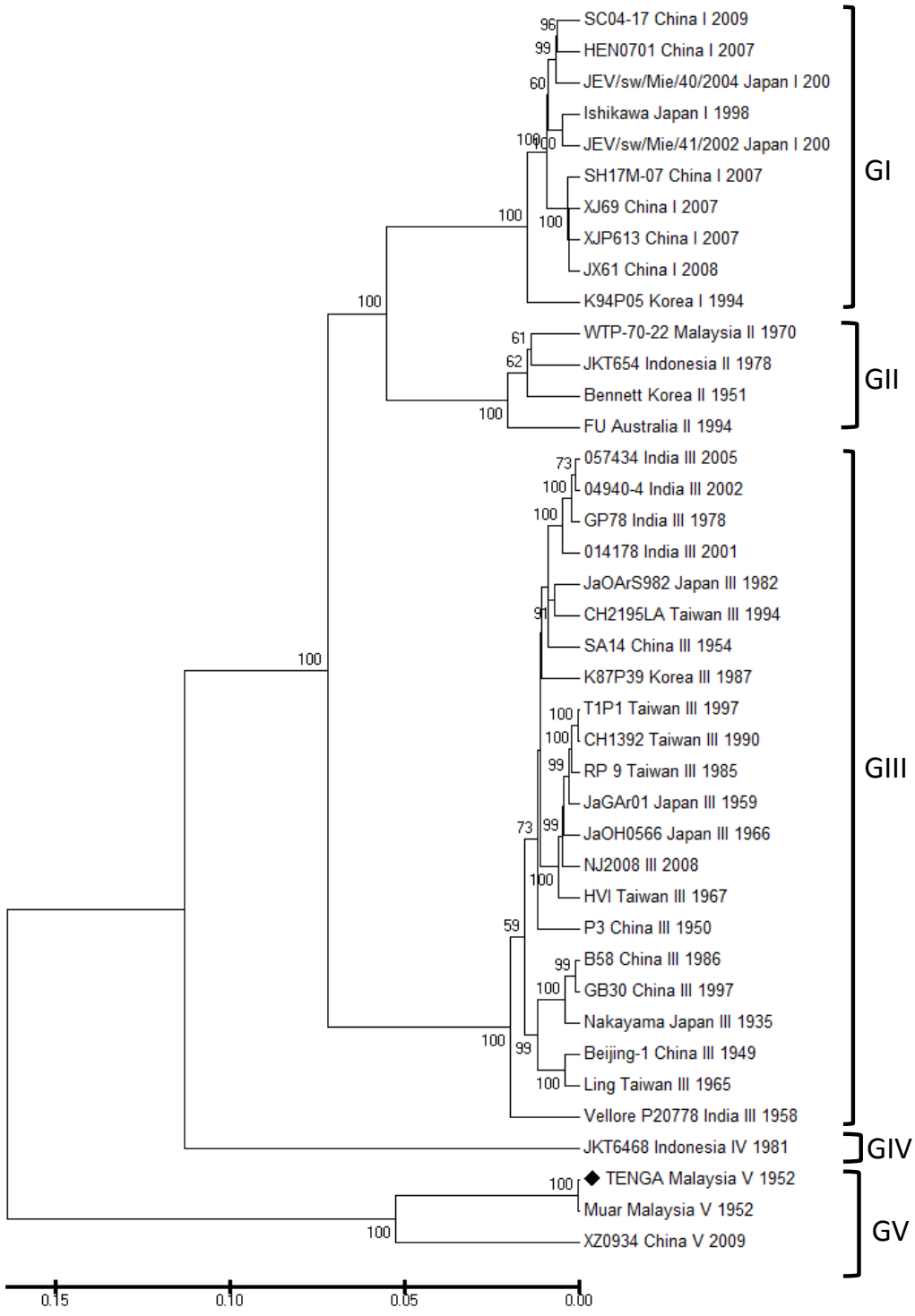


Figure 12: Maximum likelihood tree based on complete nucleotide genome sequences for 40 Japanese encephalitis virus isolates.

Phylogenetic analysis was performed using MEGA 5 based on the Tamura-Nei model. Genotype (GI-GV) is represented to the right of the tree. Bootstrap percentages are based on 1000

replicates and are shown next to the branches. Scale bar represents the number of nucleotide substitutions per site per year.

3.4.4 Evolutionary analysis

3.4.4.1 Path-O-Gen

3.4.4.1.1 Evaluation of the temporal signal and 'clock-likeness' of the data

A strict molecular clock states that the rate of evolutionary change of any specified protein is approximately constant over time and over different lineages. Hence any mutations will accumulate in a clock-like rate (Zuckerkandl & Pauling, 1962). Therefore, the lack of clock-likeness indicates that the evolutionary rate of one sequence cannot predict the evolutionary rate of another.

Regression of root-to tip distances (the root being the oldest sequence and the tip being the youngest sequence) against date of sampling of 40 sequences to investigate the 'clock-likeness' of its molecular phylogeny using Path-O-Gen revealed an R squared value of 0.3 as shown in figure 14 below. Based on these results there is some but limited clocklike data.

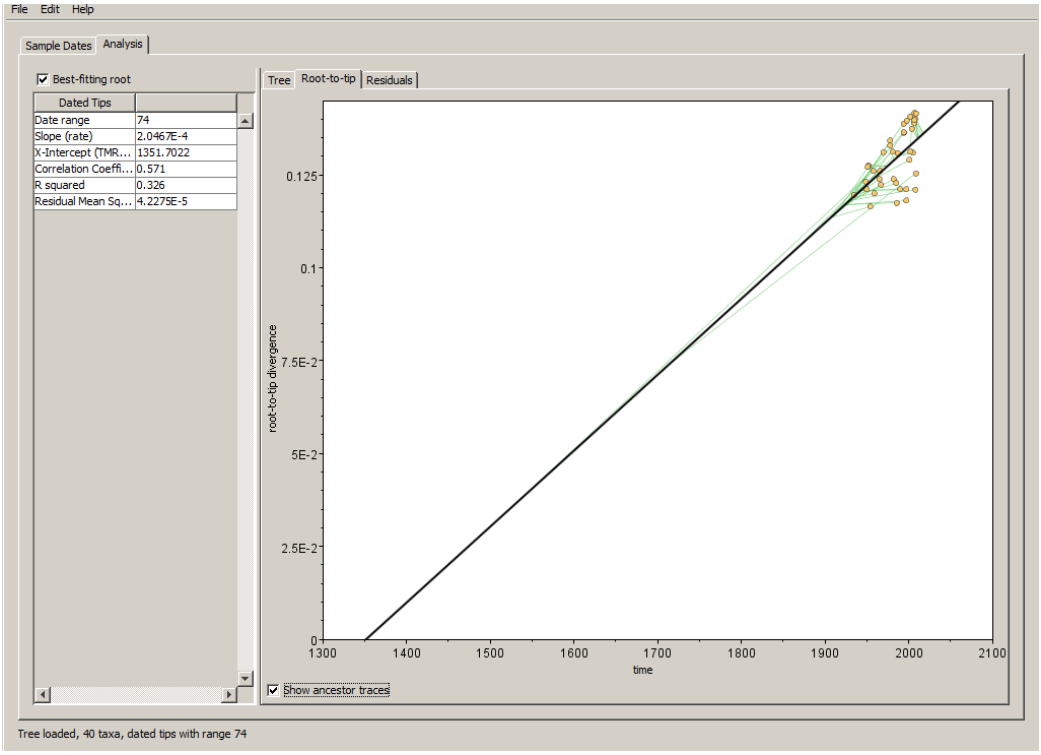


Figure 13: Extract of the output from the Path-O-Gen programme, where 40 JEV sequences (listed in Table 6) have been analysed.

Parameter values were set as: date range = 74 years which is the range from the youngest to the oldest sequence. The line of best fit through the points is shown. The line explains about 30% of the variance in the data. The y-axis represents the amount of sequence variation from oldest sequence (root) to the youngest sequence (tip).

3.4.4.2 Bayesian evolutionary analysis of sampling trees

The maximum clade credibility (MCC) tree for 39 whole genomes of JEV in Figure 15 contains five distinct clades corresponding to genotypes V, IV, III, II, and I. The most common recent ancestor for all genotypes is estimated to have occurred 778 years ago (95% highest posterior density -212 to -1602 years). The branching of the genotypes was predicted to have occurred in the following order: Genotype III at -142 years, (95% HPD -93 to -206 years), Genotype II at -98 years (95% HPD -60 to -151 years), and Genotype I at -64 years (95% HPD -26 to -114 years). Genotype IV and genotype V in figure 15 are ignored since genotype IV has only one sequence and the two sequences for genotype V Muar and Tengah are very similar hence the results are not reliable

Based on the Bayesian MCMC approach assuming a relaxed uncorrelated log-normal molecular clock, the mean nucleotide substitution rate for the entire 39 sequence set was estimated at 4.39×10^{-4} substitutions per site per year (95% HPD, 1.86×10^{-4} to 7.19×10^{-4}). Genotype I and II had a high evolutionary rate of 6.11×10^{-4} substitutions per site per year (95% HPD, 1.43×10^{-4} to 1.64×10^{-3}) for genotype I and 6.14×10^{-4} substitutions per site per year (95%HPD, 9.34×10^{-5} to 2.45×10^{-3}) for genotype II. Genotype III had the slowest rate with 3.29×10^{-4} substitutions per site per year (95%HPD, 3.98×10^{-5} to 1.12×10^{-3}) (Table 10).

Table 10: Rates of evolution for GI to GV for the MCC tree with 39 whole genome sequences (Figure 15)

Genotype	TMRCA	year	95%HPD (height)	Rate-median	95% HPD (rate)
G I (n=10)	64	1944	26-114	6.11×10^{-4}	1.43×10^{-4} to 1.64×10^{-3}
G II (n=4)	98	1910	60-151	6.14×10^{-4}	9.34×10^{-5} to 2.45×10^{-3}
G III (n=22)	142	1866	93-206	3.29×10^{-4}	3.98×10^{-5} to 1.12×10^{-3}
G V (n=2)	57	1951	57-60	9.75×10^{-4}	2.20×10^{-6} to 3.35×10^{-3}
WHOLE TREE (n=39)	778	1230	212-1602	4.39×10^{-4}	1.86×10^{-4} to 7.19×10^{-4}

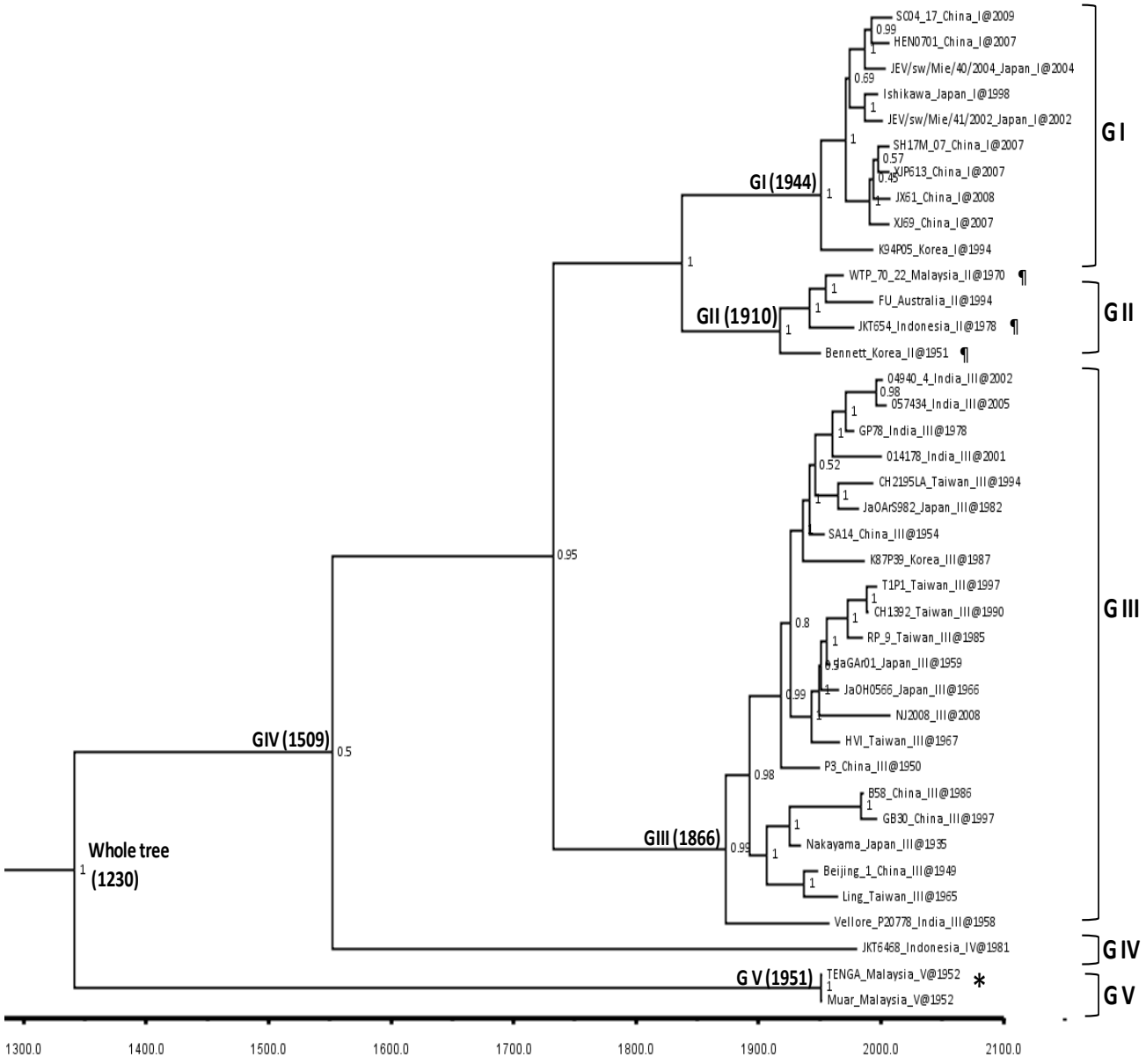


Figure 14: Maximum clade credibility (MCC) tree for 39 whole-genome sequences of JEV. The analysis was undertaken using GTR + Gamma + invariant sites substitution model, relaxed molecular clock. The high posterior probability values and the Time to the Most Recent Common Ancestor (TMRCA) of these lineages are shown beside the nodes. Overall, a rate of nucleotide substitution of 4.39 x10^{-4} (95% HDP: 1.86 -7.19 × 10^{-4}) per site per year was estimated.

¶=New sequences used in this study compared to previous studies (Mohammed *et al.*, 2011; Pan *et al.*, 2011b; Takhampunya *et al.*, 2011).*= Sample sequenced in this study.

Similarly the maximum clade credibility (MCC) tree for 40 whole genomes of JEV in Figure 16 contains five distinct clades corresponding to genotypes V, IV, III, II, and I. The most common recent ancestor for all genotypes is estimated to have occurred 888 years ago (95% highest posterior density -249 to -2065 years). The branching of the lineages occurred in the following order: Genotype V at -194 years (95% HPD, -66 to -466 years), Genotype III at -143 years, (95% HPD -95 to -224 years), Genotype II at 98 years (95% HPD -60 to -170 years), and Genotype I at 63 years (95% HPD -28 to -126 years).

The mean nucleotide substitution rate for the entire 40 sequence set was estimated at 3.53×10^{-4} substitutions per site per year (95% HPD, 1.40×10^{-4} to 5.80×10^{-4}). For genotype I, 5.17×10^{-4} substitutions per site per year (95% HPD, 1.26×10^{-4} to 1.25×10^{-3}), genotype II, 4.86×10^{-4} substitutions per site per year (95%HPD, 8.56×10^{-5} to 1.67×10^{-3}) genotype III, 2.65×10^{-4} substitutions per site per year (95%HPD, 4.56×10^{-5} to 8.99×10^{-4}) and genotype V, 5.60×10^{-4} substitutions per site per year (95% HPD, 9.00×10^{-6} to 2.67×10^{-3}) (Table 11).

Table 11: Rates of evolution for GI to GV for the MCC tree with 40 whole genome sequences (Figure 16)

Genotype	**TMRCA**	**year**	**95%HPD (height)**	**Rate-median**	**95% HPD (rate)**
G I (n=10)	63	1945	28-126	5.17×10^{-4}	1.26×10^{-4} to 1.25×10^{-3}
G II (n=4)	98	1910	60-170	4.86×10^{-4}	8.56×10^{-5} to 1.67×10^{-3}
G III (n=22	143	1865	95-224	2.65×10^{-4}	4.56×10^{-5} to 8.99×10^{-4}
G V (n=3)	194	1814	66-466	5.60×10^{-4}	9.00×10^{-6} to 2.67×10^{-3}
W Tree n=40	888	1120	249-2065	3.53×10^{-4}	1.40×10^{-4} to 5.80×10^{-4}

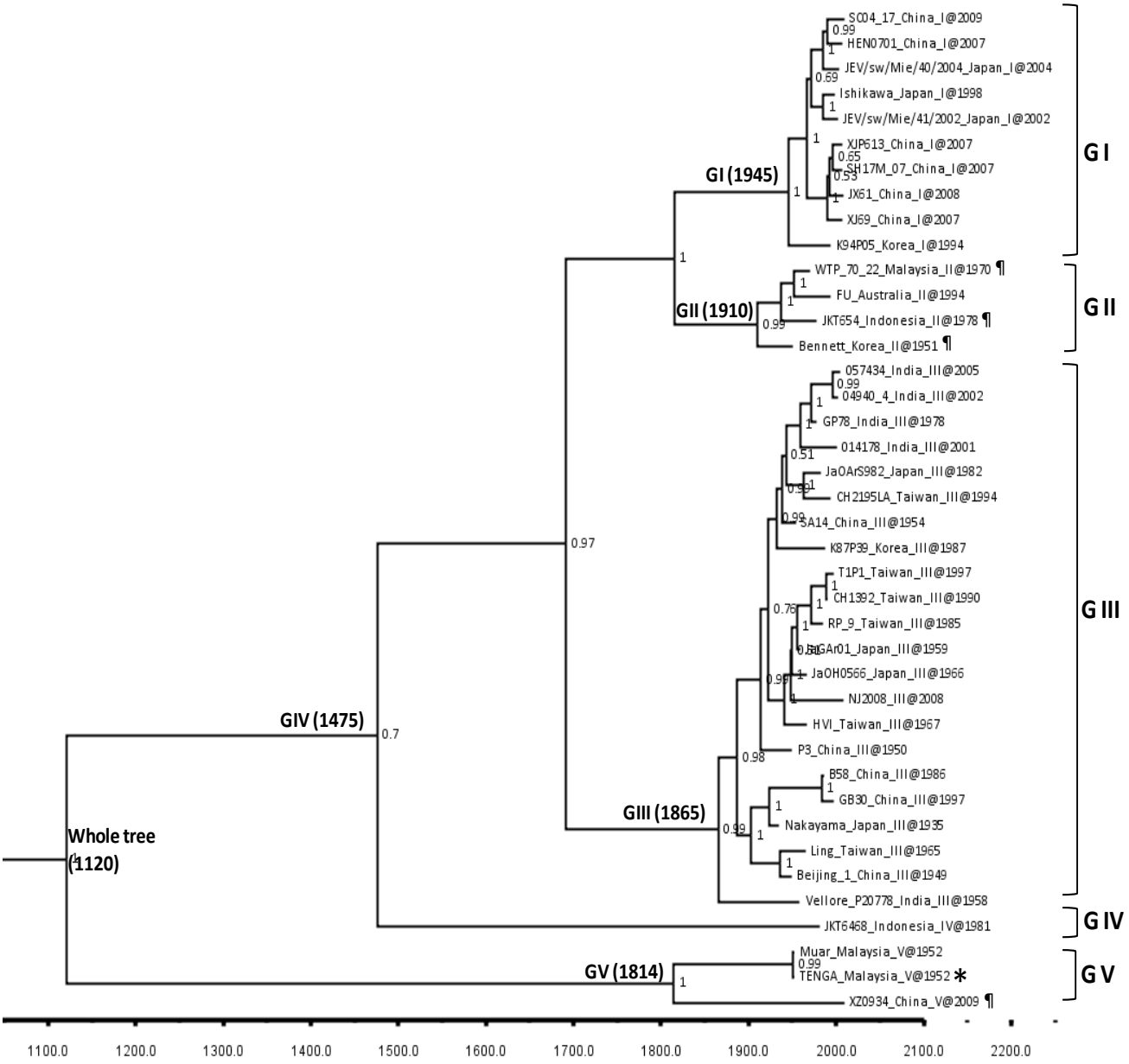


Figure 15: Maximum clade credibility tree (MCC) for 40 whole-genome sequences of JEV.

The analysis was undertaken using GTR + Gamma + invariant sites substitution model, relaxed molecular clock. The high posterior probability values and the TMRCA of these lineages are shown besides the nodes. Overall, a rate of nucleotide substitution of 3.53×10^{-4} (95% HDP: 1.40 -5.80 $\times 10^{-4}$) per site per year was estimated.¶=New sequences used in this study compared to previous studies (Mohammed *et al.*, 2011; Pan *et al.*, 2011b; Takhampunya *et al.*, 2011).*= Sample sequenced in this study.

The two MCC trees show a similar pattern for the age of the genotypes with genotype I being the youngest followed by genotype II then genotype III. Genotype V has the highest evolutionary rate in both MCC trees with 9.75×10^{-4} substitutions per site per year in figure 15 and 5.60×10^{-4} substitutions per site per year in figure 16, while genotype III had the lowest evolutionary rate with 3.29×10^{-4} substitutions per site per year in figure 15 and 2.65×10^{-4} substitutions per site per year in figure 16. The broad 95% HPD value especially for genotype II and genotype V in both trees is a result of insufficient sequence information with two to three complete sequences for genotype V available and only four for genotype II. This is further supported by the results on the temporal signal and 'clock-likeness' showing the sequences are not evolving at a constant rate.

3.5 Discussion

The complete sequence genome of Tengah contains 10,988 nucleotides and codes for 3433 amino acids. Phylogenetic analysis showed that the Tengah isolate belongs to genotype V of JEV. Sequence homology analysis revealed that Tengah had a 99% nucleotide sequence similarity and a 99% deduced amino acid sequence similarity to Muar isolate (GenBank accession number HM596272) isolated from a patient in Malaysia in 1952. The sequences of both isolates are almost identical, with a difference of only one nucleotide in the non-structural protein NS4B region. This suggests that Tengah is a fourth isolate belonging to the fifth genotype along with the JEV strains Muar, XZ0934 and 10-8027.

In order to understand how viruses evolve and spread to new areas, it is helpful to characterize their full genomes. To date, there are several complete genome sequences available for GI (n=10) and GIII (n=22). On the other hand, there are only four genotype II sequences, one genotype IV and three genotype V sequences. Therefore the objective of this study was to determine the complete nucleotide sequence and deduced amino acid sequence of JEV Tengah strain and to perform evolutionary analysis using BEAST for a more comprehensive evolutionary rate of JEV by including the Tengah strain, the new XZ0934 genotype V isolate and three new genotype II isolates (Bennett, JKY654 and WTP-70-22).

Based on the sequence comparison and phylogenetic results, there is only one non-synonymous nucleotide difference between Tengah and Muar. This non-synonymous mutation occurred in NS4B in position 7173 of the nucleotide sequence and position 2391 of the amino acid sequence and may be at the quasispecies level. Other genotype V isolates i.e. Muar, XZ0934, and 10-8027 have a cytosine (C) nucleotide in this position while the cloned isolates in this region in Tengah varied, with 50% showing C nucleotide and the other 50% showing a guanine (G) nucleotide. The nucleotide sequence in appendix 1 is reported using guanine (G) in this position. The NS4 protein has been shown to be hydrophobic hence supporting the fact that it plays a role as a membrane component. The poor nucleotide sequence conservation of this region among JEV strains suggests that it might be important to adapt each virus to different viral growth environments (Chambers *et al.*, 1990; Sahoo *et al.*, 2008).

In an effort to provide a more inclusive evolutionary rate of JEV, evolutionary analysis using BEAST was undertaken. Previous studies on the evolutionary rate of JEV using complete genomes have only used one sequence representing genotype V and two genotype II sequences (Solomon *et al.*, 2003; Mohammed *et al.*, 2011; Pan *et al.*, 2011b; Takhampunya *et al.*, 2011). This study utilized three GII sequences and two genotype V sequences that have not been used before. Since the gap between the isolation of the Indo-Malaysian strains of genotype V (Muar and Tengah) and that of the Chinese isolate (XZ0934) was 57 years, analysis was performed with and without this virus isolate. During the course of this study another genotype V strain 10-1827 was isolated from a pool of *Culex bitaeniorhynchus* collected in South Korea in 2010 (Takhampunya *et al.*, 2011); this strain was not included in this study since only the partial envelope gene sequence was available.

My results from both MCC trees (Figure 15 and 16) estimate the Time to the Most Recent Common Ancestor (TMRCA) for genotype I to have occurred around the mid-1900s which is similar to previous studies (Mohammed *et al.*, 2011; Schuh *et al.*, 2013). This genotype also shows a higher evolutionary rate than genotype III (Table 10 and 11) which is in agreement with a study by Pan *et al* 2011 that reports the rapid expansion of genotype I from the 1970s and a drop in the genetic diversity of genotype III hence making genotype I the dominant genotype in Asia (Pan *et al.*, 2011b).

From the BEAST analysis results we can now estimate for the first time the TMRCA for GII based on the full genome sequences which have not been reported before. Both MCC trees (Figure 15 and 16) predict the TMRCA for genotype II to have occurred approximately in the early 1900s These results are similar to those reported using the E gene sequence only by Schuh *et al* 2013 in which they utilized 28 isolates.

Our results predict genotype III to have the lowest evolutionary rate when compared to the other genotypes. This is in agreement with a study by Pan et al 2011 that reports a drop in the genetic diversity and also in the number of isolates collected from this group. Their study using skyline plot analysis also demonstrated that the genetic diversity of genotype III had already

reached a plateau by the time the first isolate (JEV prototype Nakayama) was collected in 1935. TMRCA of genotype III is predicted to have occurred in the 1860s in both trees. Results in figure 15 show genotype III to be the oldest when only two GV sequences are included. However, when an additional sequence of genotype V is included in the analysis (Figure 16), the results show genotype V to be the oldest lineage.

The TMRCA of genotype V is predicted to be in 1814 (figure 16 and table 11). This is the first time the TMRCA of genotype V is reported using complete genome sequences. This is however represented by a broad 95%HPD as a result of small number of sequences (n=3). The results utilizing three genotype V sequences (figure 16) are supported by a decrease in width of the 95% HPD (table 11) for rate of evolution making them more reliable for the prediction of evolutionary rate of JEV. They predict the overall TMRCA of JEV to have occurred in 1120 with a mean evolutionary rate of 3.53×10^{-4} and in agreement with other studies (Uchil & Satchidanandam, 2001; Solomon *et al.*, 2003; Mohammed *et al.*, 2011). These results are consistent with the hypothesis that genotype V to is the ancestral lineage of JEV. They also give an estimate that is consistent with that inferred for JEV when using the envelope gene which estimated the evolutionary rate of JEV to be 3.50×10^{-4} (Jenkins *et al.*, 2002).These findings support the need for isolation and characterization of more JEV isolates to aid in understanding the actual distribution of JEV and its significance in terms of risk of emergence in new areas and vaccine strategies.

Overall, the evolutionary rates for both MCC trees fall within the same range as that reported for other RNA viruses which is between 1×10^{-3} to 1×10^{-6} nucleotides substitutions per site per year (Steinhauer & Holland, 1987; Jenkins *et al.*, 2002). However we have to bear in mind that these results are based on sequences that show only moderate clocklikeness, therefore lowering the likely accuracy of these estimates.

Additionally, since genotypes I, II and III are more widely distributed while genotypes IV and V had only been reported in the Indonesian and Malaysian region prior to 1952 (Solomon et al 2003), further study of the Muar, Tengah and XZ0934 strains of genotype V at a molecular level

may help in identifying those properties of the virus that may have evolved and aided in the expansion of JEV distribution into new global habitats. However, the gap of 57 years between isolation of these viruses shows a vast amount of unsampled diversity missing for this genotype. Still, the three available complete sequences for genotype V provide data to investigate the phenotypic variations within genotype V and between genotype V and other genotypes. Nonetheless, in order for this to be more accurate the gap within genotype V itself would need to be filled by the isolation and characterization of more sequences.

Lack of diversity in genotype V challenges the fact that it is the ancestral lineage. For unknown reasons no viruses from this genotype were isolated for 57 years. However, it has now been isolated in China which is thousands of kilometres from the original Muar isolation location of Singapore. Some factors that may have aided in its spread would include wind-blown mosquitoes or migratory birds as suggested for the introduction of JEV into Australia (Hanna *et al.*, 1996) and also from mainland China into Taiwan and Japan (Nabeshima *et al.*, 2009; Huang *et al.*, 2010), change in agricultural practices that may provide new breeding sites and change in climatic factors that may now be favourable. Evolution of this genotype could have also played a major part. This is supported by the predicted high evolutionary rate within this genotype as shown on the MCC trees.

The re-emergence of genotype V after nearly 60 years raises questions on its once thought limited distribution, isolation and circulation. It is uncertain whether this is related to its virulence, host susceptibility, reduced transmission/ amplification or lack of surveillance. Hence future studies can now start to unravel the mysterious disappearance of this genotype by carrying out studies comparing the old and the new isolates in different mosquitoes and hosts and also under different environmental conditions.

My results based on sequence analysis and BEAST analysis showed that the Tengah strain of JEV belongs to genotype V. Muar and Tengah are very similar, suggesting Tengah represents a second isolate of Muar. This is further supported by the fact that both viruses were isolated in

the same region in the same year and for that reason Tengah is referred to Muar in the next chapters.

The lack of isolation or distribution of the Muar strain may be attributed to infectivity in mosquitoes hence studies to assess its infectivity in mosquitoes and also to compare its mosquito infection, dissemination and transmission rates with that of the most frequently isolated JEV genotype may provide valuable information. The next chapter addresses this question

4 Chapter 4: Comparative infectivity of the Nakayama (Genotype III) and Muar strains (Genotype V) of Japanese encephalitis virus in *Culex quinquefasciatus*

4.1 Abstract

In the previous chapter sequencing results showed that Tengah is a variant of the Muar strain of genotype V. Hence, in this chapter it is now referred to as Muar. Prior to 2009, Muar had been the only strain described belonging to genotype V. Nearly 60 years later, the report of two other genotype V isolates from China and Korea in 2009 and 2010 respectively, raised questions on the factors that may have led to the limited distribution of the original genotype V isolate Muar. In order to address this question, I undertook vector competence studies to determine the infectivity of Muar in *Culex quinquefasciatus* mosquitoes. Muar was then compared to Nakayama strain of genotype III which is the most frequently isolated genotype. Two hundred and fifty *Culex quinquefasciatus* mosquitoes were offered an infectious blood meal containing Muar and 130 *Culex quinquefasciatus* mosquitoes were offered an infectious blood meal containing Nakayama strain. All mosquitoes were incubated separately at 28°C for 21 days and the infection, dissemination and transmission rates were recorded at 7, 14 and 21 days post infection. Muar was able to infect *Culex quinquefasciatus* mosquitoes with a transmission rate of 23% at 21 days post infection. There was a significant difference in the infection and dissemination rates at 14 days post infection but no significant difference in infection and dissemination at 7 and 21 dpi. These findings argue against poor infectivity of mosquitoes being the key determinant which might explain why genotype V strains of JEV are apparently in limited circulation.

4.2 Introduction

Japanese encephalitis virus is divided into five genotypes (genotype I, II, III, IV and V) based on nucleotide sequencing and phylogenetic studies utilizing the capsid, pre-membrane and envelope genes (Chen *et al.*, 1990). These genotypes have spread widely in South East and South-eastern Asia and Australasia (Solomon *et al.*, 2003). Nakayama and Muar strains of JEV belong to Genotype III and Genotype V respectively.

Nakayama is the prototype strain of JEV and was isolated from the brain of a male that died of summer encephalitis in Tokyo, Japan in 1935 (Lewis, Taylor, Sorem*, et al.*, 1947). Genotype III has been the source of annually occurring epidemics of encephalitis and includes isolates collected in China, India, Indonesia, Japan, Korea, Malaysia, Myanmar, Nepal, Philippines, Sri Lanka, the former Soviet Union, Taiwan, Thailand and Vietnam between 1935 and present (Schuh *et al.*, 2013). The isolates have been derived from bats (Wang, Pan, Zhang, Fu, & Wang, 2009), birds (Hasegawa *et al.*, 1975; Yang *et al.*, 2011), horses (Singha *et al.*, 2013), humans (Xu *et al.*, 2013), mosquitoes (Van Den Hurk *et al.*, 2006) , and pigs (Deng *et al.*, 2011). This virus has been used extensively from vaccine development to vector competence studies.

Muar strain represents the first isolate of genotype V, having been isolated in 1952 from the brain of a fatal case in Singapore (Hale *et al.*, 1952) and had not been isolated since. However, after 57 years of undetected virus circulation, this genotype has recently been isolated from a pool of *Culex tritaeniorhynchus* in China in 2009 and in *Culex bitaeniorhynchus* in 2010 in the Republic of Korea (Li, Fu*, et al.*, 2011; Takhampunya *et al.*, 2011). Evolutionary studies have shown genotypes IV and V form the oldest JEV lineage that originated from an ancestral virus in the Indonesian-Malaysian region. Hence it is thought that JEV probably originally spread from this region (Solomon *et al.*, 2003; Mackenzie *et al.*, 2004). The underlying factors that have contributed to the once thought limited distribution of the genotype V of JEV (Solomon *et al.*, 2003) and the recent discovery in China and Korea are not clear. Several factors may have contributed to the re-emergence of this genotype ranging from climate and environmental changes, improved pathogen detection or mosquito-pathogen transmission factors.

JEV is maintained in ardeid wading birds, mosquito and pig cycle with humans and other non-avian vertebrates considered to be dead-end hosts. The principal vector for JEV is considered to be *Culex tritaeniorhynchus* mainly because the peak seasonal abundance of *Culex tritaeniorhynchus* coincided well with the seasonal occurrence of epidemic encephalitis, this lead to the subsequent recovery of the virus from this rice-paddy breeding mosquito in 1938 (Mitamura, Kitaoka, K*., et al.*, 1938). This mosquito is also distributed widely across the JEV-endemic regions (Impoinvil *et al.*, 2011; van den Hurk *et al.*, 2011).

Several other mosquitoes have been incriminated as JEV vectors and subsequently tested to assess their competence. These mosquitoes have been shown to vary in competence ranging from high level of efficiency to be infected with, disseminate and transmit the virus, to complete refractoriness to infection. The majority of these studies have used genotype III strains of JEV. These mosquitoes are listed in table 12 below.

Table 12: Comparison of different species of mosquitoes assessed for competence of JEV

Mosquito species	Level of Competence*	Country	JEV Genotype	Reference
Culex tritaeniorhynchus	High	Japan	Genotype III (JaGAr01)	(Takahashi, 1982a)
Culex tritaeniorhynchus	Moderate	Taiwan		
Culex tritaeniorhynchus	Low	Pakistan		
Opifex fuscus	Refractory	New Zealand	Genotype III (Nakayama)	(Kramer *et al.*, 2011)
Aedes notoscriptus	Refractory			
Culex quinquefasciatus	Refractory			
Culex quinquefasciatus	Refractory	USA		
Culex pipiens	Refractory			
Culex annulirostris	High	Australia (Southeast Queensland)	Genotype II (TS3306)	(van den Hurk *et al.*, 2003)
Culex sitiens	Moderate			
Culex quinquefasciatus	Moderate			
Culex gelidus	Moderate	Australia (North Queensland)		
Culex annulirostris	High			
Ochlerotatus vigilax	Low			
Culex quinquefasciatus	Refractory			
Aedes aegypti	Refractory			
Ochlerotatus kochi	Refractory			
Verrallina funerea	Refractory			
Aedes aegypti	Low	???	Likely Genotype III	
Aedes japonicas	Moderate	???	Likely Genotype III	(Takashima & Rosen, 1989)
Aedes notoscriptus,	Moderate	Taiwan	Genotype III (CH1392)	(Chen *et al.*, 2000)
Armigeres subalbatus	High			
Culex tritaeniorhynchus	Moderate			

*Refractory refers to lack of transmission or inability detected virus in the mosquito saliva

Vector competence refers to the ability of arthropods to acquire, maintain, and transmit microbial agents (Kramer & Ebel, 2003). It is usually a measure of the rate at which mosquito vectors are able to become infected, disseminate and transmit these agents. Vector competence looks at two aspects between the vector and pathogen: 1) the vector's innate ability to support transmission and 2) the pathogen's ability to infect the host.

Vector competence studies with JEV have been paramount in improving our understanding of transmission dynamics in mosquitoes. Many of the early vector competence transmission studies have used *Culex tritaeniorhynchus* (Hale *et al.*, 1957; Gresser, Hardy, Hu*, et al.*, 1958;

Gresser, Hardy, & Scherer, 1958; Buescher, Scherer, Rosenberg*, et al.*, 1959; Takahashi, 1976, 1980b). These studies revealed that 1) JEV could be transmitted as early as 5 days post infection depending on prevailing temperatures; 2) highly competent vectors, such as *Culex tritaeniorhynchus* are able to become infected with low doses of virus; ranging from $10^{1.0 - 3.5}$ suckling mouse intracerebral (SMIC) LD_{50} (lethal dose 50 %)/0.03 mL of blood; and 3) concentration of virus in the mosquito saliva can be as high as $10^{4.2}$ SMIC-LD_{50}/1 mL of saliva and virus diluents (Takahashi, 1976).

The brown medium-sized southern house mosquito, *Culex quinquefasciatus* is a tropical to sub-tropical mosquito species usually found within the latitudes 36° N and 36° S. This mosquito is a nocturnal, opportunistic blood feeder that is a vector of many of pathogens, several of which affect humans and both domestic and wild animals (i.e. birds or other mammals). Some of the pathogens well-established to be vectored by this mosquito include the filarial nematode, *Wuchereria bancrofti* (Nelson *et al.*, 1946; Janousek & Lowrie, 1989; Pothikasikorn *et al.*, 2008) and several arboviruses which include West Nile virus (Jansen *et al.*, 2008), St. Louis encephalitis virus (Meyer *et al.*, 1983). Western equine encephalitis virus (Wang *et al.*, 2012) and Rift Valley fever virus (RVF) (Turell *et al.*, 2007) (Table 13). These mosquitoes tend to have a ubiquitous distribution due to their ability to develop in diverse habitats ranging from nutrient-rich and sometimes organically polluted standing water such as stagnant drainage canals, water troughs and septic tanks (Weaver & Barrett, 2004), to relatively pristine water sources such as water tanks or rain-water filled tyres. *Culex quinquefasciatus* infected with JEV has been isolated in the field (Weng, Lien, Wang, Lin, Lin, & C., 1999; Nitatpattana *et al.*, 2005) suggesting a role in JEV transmission.

Table 13: Competence levels of *Culex quinquefaciatus* mosquitoes to various arboviruses isolated from different regions.

Source	virus	Level of competence*	Reference
Australian	WNV	High	(Jansen *et al.*, 2008)
Australian (southeast Queensland strains)	JEV	Moderate	(van den Hurk *et al.*, 2003)
Australian (north Queensland)	JEV	Refractory	
Australian	MVEV, KUNV, RRV	Low	(Kay *et al.*, 1982)
American	WNV	Moderate	(Sardelis *et al.*, 2001)
American	JEV	Refractory	(Kramer *et al.*, 2011)
Southern California	WNV	Low	(Goddard *et al.*, 2002)
California	SLEV	Moderate	(Meyer *et al.*, 1983)
New Zealand	BFV,	Low	(Kramer *et al.*, 2011)
	RRV	Low	
	SINV	Refractory	
	WNV	Moderate	
	MVEV	Low	
	JEV	Refractory	
Argentine	WNV	Moderate	(Micieli *et al.*, 2013)
China	WEEV	High	(Wang *et al.*, 2012)
North American	RVFV	Refractory	(Turell *et al.*, 2010)
China	WNV	Moderate	(Jiang *et al.*, 2010)
African	RVFV	Refractory	(Turell *et al.*, 2008)
Kenyan	RVFV	Low	(Turell *et al.*, 2007)
Mexican and Honduran	VEEV	Refractory	(Turell *et al.*, 2003)

WNV-West Nile Virus, **JEV**-Japanese encephalitis virus, **MVE**-Murray valley encephalitis virus, **BFV**-Barmah Forest virus, **RRV**-Rose River virus, **SLEV**-Saint Louis encephalitis virus, **KUNV**-Kunjin virus, **SINV**-Sindbis virus, **WEEV**-Western equine encephalitis virus, **VEEV**-Venezuela equine encephalitis virus.
*Refractory refers to lack of transmission or inability detected virus in the mosquito saliva

Susceptibility and competence has been shown to vary among and within mosquito species from different geographical regions. A study comparing the transmission efficiency among colonized strains of *Culex tritaeniorhynchus* showed that two strains from Japan had high transmission efficiency while three strains from Pakistan had low efficiency (Takahashi, 1982b) (see table 10). More important is the comparison of the vector competence and transmissibility of the different JEV genotypes by various mosquito species to determine whether there are any variations between the genotypes. These studies have not been done before using JEV and may

provide some answers as to why some genotypes emerge in places they were not found before. A route to disease emergence can be caused by the competitive displacement of a less virulent pathogen strain by a more virulent strain. One example is the displacement of the American genotype of dengue serotype 2 which causes dengue fever by the Southeast Asian genotype which causes a more severe dengue haemorrhagic fever that occurred in the Western Hemisphere and the South Pacific Islands (Rico-Hesse *et al.*, 1997). Previous studies have also shown variation in competence and susceptibility within mosquito species when infected with different strains of Chikungunya virus (CHIKV). An isolate of CHIKV with a mutation in the envelope protein gene, led to a significant increase in CHIKV infectivity in *Aedes albopictus* when compared to an isolate without the mutation (Tsetsarkin *et al.*, 2007). In another study the four dengue serotypes were shown to differ in the oral infection threshold with a significantly higher proportion of *Aedes aegypti* mosquitoes becoming infected with dengue 2 and 3 compared to the serotype 1 and 4 (Gubler *et al.*, 1979).

It is possible that the lack of detection or circulation of the Muar strain of JEV relates to the replication efficiency of the virus in mosquitoes. To date few studies have compared JEV genotypes to determine if there are differential infection rates in mosquitoes. In addition no studies have been done to determine the competence of Muar in mosquitoes. Therefore, the objective of this study is:

I. Assess the infectivity of Muar (genotype V) stain of JEV in *Culex quinquefasciatus* mosquitoes.
II. Compare infection, dissemination and transmission of the Nakayama (genotype III) and Muar (genotype V) strains of JEV in *Culex quinquefasciatus* mosquitoes.

4.3 Materials and methods

For detailed acquisition and maintenance of mosquitoes, cell and virus source and maintenance, vector competence studies and plaque assay methodology please refer to chapter 2 (general materials and methods) Chapter specific protocols and alterations are detailed below.

4.3.1 Mosquito acquisition and maintenance

Culex quinquefaciatus (Recife strain) mosquitoes were obtained from a colony maintained in the Liverpool School of Tropical Medicine insectary. The colony was originally established in Brazil. This mosquito was selected because JEV has been isolated from it previously (Nitatpattana *et al.*, 2005). Details of its maintenance can be found in chapter 2.

4.3.2 Cells and viruses

Vero cells were maintained in Dulbecco Modified Eagle's Minimal Essential Medium (DMEM) Sigma-Aldrich) media containing 10% heat-inactivated Fetal Calf Serum (FCS), 2 mM L-glutamine and 50 µg/ml Penicillin/Streptomycin. Viruses used for this study were Muar (genotype V) strain of JEV and Nakayama (genotype III) strain of JEV.

4.3.2.1 Muar

Muar was originally obtained from the brain of a patient in Singapore 1952(Hale *et al.*, 1952; Okuno *et al.*, 1968) and was kindly donated by Prof. Ichiro Kurane from the Department of Virology, National Institute of Infectious Diseases, Tokyo, Japan.

4.3.2.2 Nakayama

Nakayama strain was originally isolated in Japan in 1935 from human cerebellum (Lewis, Taylor, & et al., 1947) and was kindly donated by Dr. David Beasley and Dr. Alan Barrett from University

of Texas Medical branch. All procedures using virus were carried out in a containment level-3 facility.

4.3.3 Vector competence

Culex quinquefasciatus colony mosquitoes were tested for JEV vector competence using either Muar or Nakayama at 28°C which is the optimum temperature for maintenance of this tropical mosquito. Mosquitoes were sampled at 0, 1 3, 7, 14 and 21 days post infection (dpi; i.e. after offering an infectious blood-meal). Time point 0 represent mosquitoes collected 1-hour after offering an infectious blood meal.

4.3.3.1 Per oral infections

Details on per oral infections and plaque assay methodology can be found in Chapter 2 (Materials and Methods). Briefly, infectious blood meal containing virus from frozen stock was prepared by combining defibrinated horse blood (Thermo Oxoid Remel), with the appropriate volume of JEV Muar strain stock or Nakayama strain stock and 100 µl of adenosine 5'-triphospahte (ATP 0.02 µm) as a phagostimulant to a final concentration of 6 logs pfu/ml.

Seven day old females were selected and offered an infectious bloodmeal containing either Muar or Nakayama strain of JEV artificially using the Hemotek feeding system (Discovery workshops, UK) for 1 hour. A sample of 0.5 ml of the infectious bloodmeal was collected before and after the mosquitoes fed and stored at -80°C for subsequent plaque assay analysis. Engorged mosquitoes were selected and incubated at 28°C and maintained on 10% sucrose solution at 70-90% relative humidity and a photoperiod of 12: 12 light: dark cycle. Experiments for each virus strain were carried out on different days.

4.3.3.2 Determination of infection dissemination and transmission

Mosquitoes were sampled at 0, 1, 3, 7, 14 and 21 days post infection (dpi). All mosquitoes collected at 0, 1, and 3 dpi were frozen at -80°C individually in 1.5ml tubes containing virus

diluents media. All mosquitoes collected at 7, 14 and 21 dpi had their saliva collected before they were then separated into bodies and legs and frozen individually at -80°C.

Infection was determined by recovery of virus from the mosquito tissue suspension by plaque assay. If virus was recovered from its body and but not in its legs, the mosquito was considered to have a non-disseminated infection. If virus was recovered from both the legs and the body suspension the mosquito was considered to have a disseminated infection (Turell, Gargan et al. 1984) and if virus was recovered from its saliva the mosquito was considered to have a transmissible infection. Infection, dissemination and transmission rates were defined as the percentage of mosquitoes tested that contained virus in their bodies, legs and saliva respectively.

All samples were processed by plaque assay and scored as virus-positive or virus-negative based on the presence or absence of plaques.

4.3.4 Statistical analysis

Fisher Exact Test was used to determine if there were significant differences ($p < 0.05$) in rates of infection, dissemination and transmission between temperatures and species. SISA, an open access online statistics calculator (http://www.quantitativeskills.com/sisa/) was used to conduct Fisher Exact Test. Confidence intervals of proportions were calculated using VassarStats (http://vassarstats.net/prop1.html). VassarStats uses the Wilson Score Interval method which is more robust when dealing with a small number of trials and/or an extreme probability (Newcombe, 1998). The sample size in each group was dictated by the feeding success and the survival rates through the days post infection (dpi).

4.4 Results

One hundred and twenty eight mosquitoes offered an infectious bloodmeal containing Muar (genotype V) were sampled and 67 offered an infectious bloodmeal containing Nakayama (genotype III) were sampled making a total of 195 mosquitoes. Sixty five percent of 130 mosquitoes offered an infectious blood meal containing Nakayama successfully engorged, while 72% engorged when offered an infectious blood meal containing Muar strain. Of those that acquired the Nakayama strain infectious blood meal 21% of them died while 29% died out of those offered the Muar strain infectious blood meal (Table 14). The rates of engorgement and mortality in *Culex quinquefasciatus* mosquitoes that were fed either Muar or Nakayama strain infectious bloodmeal were very similar.

Table 14: Rate of engorgement and mortality of mosquitoes offered either Nakayama or Muar strain infectious bloodmeal

Virus strain	Initial no. of Mosquitoes	Bloodfed (%)[a]	Sampled (%)[b]	Dead (%)[c]
Nakayama	130	85 (65%)	67 (79%)	18 (21%)
Muar	250	181 (72%)	128 (71%)	53 (29%)

[a]Percentage Bloodfed = (no. of bloodfed mosquitoes/Initial no. offered bloodmeal) * 100
[b]Percentage sampled = (no. of mosquitoes sampled/total no. of bloodfed mosquitoes) * 100
[c]Percentage dead = (no. of mosquitoes that died /total no. of bloodfed mosquitoes) * 100

Early time points starting at day 0 to day 3 were sampled to ensure that virus detected in later time points was a result of new virus production rather than that of input virus. A typical eclipse phase was observed for Muar in which the virus titre decreased from 0 to 3 dpi followed by an increase after 7 dpi. This is attributable to the reduction in virus titre after day 0 as ingested virus either infects cells or gets digested; virus successful in infecting cells replicates to detectable levels several days later. Nakayama showed a similar pattern with a decrease in virus titre from 0 to 3 dpi and increase at 7 and 21 dpi. However no virus was detected at 14 dpi (figure 17).

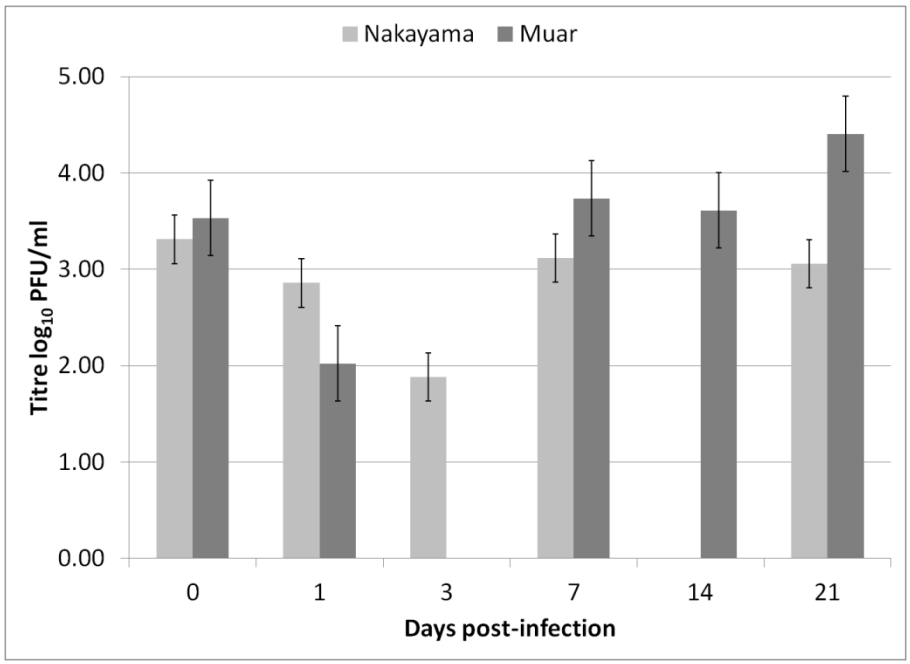


Figure 16: Graph showing the virus eclipse phase for Nakayama and Muar in Culex quinquefaciatus mosquitoes at 28°C

Muar strain of JEV replicated successfully in *Culex quinquefasciatus* as evident by presence of virus in the saliva at 14 and 21 dpi samples. The number of mosquitoes that tested positive for virus decreased from 0 to 1 dpi then increased from 7 to 21dpi. The infection rate for *Culex quinquefasciatus* when infected with Muar strain was 23% at 7dpi with no dissemination and transmission. Infection, dissemination and transmission rates were 41%, 30% and 7% at 14dpi respectively and 35%infection, 35% dissemination and 23% transmission rates at 21dpi (Table 15).

Table 15: Infection, dissemination and transmission rates of *Culex quinquefasciatus* mosquitoes exposed to 6 logs PFU of Muar strain of JEV.

Virus strain	DPI	No. tested	I	D	T	Infection rate[a] (95% CI)	Dissemination rate[b] (95% CI)	Transmission rate[c] (95% CI)
Nakayama (III)	0	11	11	nt	nt	100 (74-100)	nt	nt
	1	5	2	nt	nt	40 (11-77)	nt	nt
	3	6	3	nt	nt	50 (18-81)	nt	nt
	7	13	1	0	0	8(1.4-33)	0 (0-23)	0 (0-23)
	14	13	0	0	0	0	0	0
	21	19	8	8	5	42 (23-64)	42 (23-64)	26 (11-49)
Muar (V)	0	18	18	nt	nt	100 (82-100)	nt	nt
	1	15	11	nt	nt	73 (48-89)	nt	nt
	3	15	0	nt	nt	0	nt	nt
	7	22	5	0	0	23 (10-43)	0 (0-15)	0 (0-15)
	14	27	11	8	2	41 (25-59)	30 (16-48)	7 (2-23)
	21	31	11	11	7	35 (21-53)	35 (21-53)	23 (11-40)

DPI=Days post infection**; I**=number infected**; D**=number disseminated; **T**= number transmitting; nt=not tested

[a] Percentage of mosquitoes containing virus in their bodies out of no. tested (95% confidence interval).

[b] Percentage of mosquitoes containing virus in their legs out of no. tested (95% confidence interval).

[c] Percentage of mosquitoes containing virus in their saliva out of no. tested (95% confidence interval).

Nakayama strain of JEV replicated successfully in *Culex quinquefasciatus* as evident by presence of virus in the saliva at 21 dpi. The number of mosquitoes that tested positive for virus decreased from 0 to 3 dpi then increased by 21 dpi. The infection rate was 8% at 7 dpi and none at 14dpi. There was no dissemination and transmission at both 7 and 14 dpi. At 21 dpi, the infection and dissemination rate were at 42% and transmission rate was 26% (Table 15 above).

Table 16: Dissemination and transmission transition efficiency of *Culex quinquefasciatus* mosquitoes exposed to Nakayama and Muar strain of JEV

Virus strain	DPI	Dissemination/ % of no. infected (95% CI)	Transmission, % of no. disseminated (95% CI)	Transmission, % of no. infected (95% CI)
Nakayama (III)	7	0 (0-79)	0	0
	14	0	0	0
	21	100 (67-100)	63 (31-86)	63 (31-86)
Muar (V)	7	0	0	0
	14	72 (43-90)	25 (7-59)	18 (5-47)
	21	100 (74-100)	64 (35-84)	64 (35-84)

DPI= days post infection; **CI**= Confidence intervals

[a] Percentage of mosquitoes containing virus in their bodies out of no. infected

[b] Percentage of mosquitoes containing virus in their saliva out of no. disseminated

[c] Percentage of mosquitoes containing virus in their saliva out of no. infected

To describe the transition efficiency of the virus after overcoming the midgut barrier in the mosquito, the number of mosquitoes with a disseminated infection out of the number infected (dissemination efficiency) was examined (Table 16 above). The number of mosquitoes able to transmit out of those with disseminated infection (transmission efficiency) was also examined at 7, 14 and 21 dpi. Dissemination efficiency reached 100% at 21 dpi when the mosquitoes were infected with Nakayama strain and the transmission efficiency was 63%.

When infected with Muar strain, the dissemination efficiency was 72% at 14 dpi and the transmission efficiency was 25%. Out of the total numbers that were infected at 14 dpi, 18% of them developed a transmissible infection. There was 100% dissemination efficiency at 21 dpi and 64% transmission efficiency. Of the total number of mosquitoes that were infected at 21 dpi, 64% of them developed a transmissible infection (Table 16).

Table 17: Comparison of the rates of infection, dissemination and transmission at the different time points for the two strains of virus Muar and Nakayama.

dpi		Virus strain	N^{o} positive/ no tested[a]	Percent positive	df	Chi-square	*P values*
7	Infected	Muar Nakayama	5/22 1/13	23 8	1	0.780	0.377
	Disseminated	Muar Nakayama	0/22 0/13	0 0	1	0	1
	Transmission	Muar Nakayama	0/22 0/13	0 0	1	0	1
14	Infected	Muar Nakayama	11/27 0/13	41 0	1	7.273	**0.007**
	Disseminated	Muar Nakayama	8/27 0/13	30 0	1	4.350	**0.037**
	Transmission	Muar Nakayama	2/27 0/13	7 0	1	0	1
21	Infected	Muar Nakayama	11/31 8/19	35 42	1	0.089	0.766
	Disseminated	Muar Nakayama	11/31 8/19	35 42	1	0.089	0.766
	Transmission	Muar Nakayama	7/31 5/19	23 26	1	0	1

df= degrees of freedom

[a] Total number of mosquitoes positive for virus out of the total number tested at 7, 14 and 21 dpi.

When the infection, dissemination and transmission rates were compared for the two virus strains at 7, 14 and 21 dpi there was a significant difference in the infection and dissemination rate at 14 dpi (Table 17). There was no significant difference between the two viruses in their infection and dissemination rates at 7 or 21 dpi, nor in transmission rates at any of the dpi.

Table 18: Chi square values for rates of infection, dissemination and transmission when the two virus strains are compared from 7 to 21 dpi combined at α=0.05

	Species	N^{o} positive/ no tested[a]	Percent positive	df	Chi Square	*P values*
Infected	Muar	27/80	34	1	2.082	0.149
	Nakayama	9/45	20			
disseminated	Muar	19/80	24	1	0.451	0.502
	Nakayama	8/45	17			
Transmission	Muar	9/80	11	1	0	1
	Nakayama	5/45	11			

df= degrees of freedom

[a]Total number of mosquitoes positive out for virus out of the total number tested at 7, 14 and 21 dpi combined.

When 7 to 21 days post infections (dpi) are combined due to small sample sizes at individual time points, the overall infection rate when *Culex quinquefaciatus* mosquitoes are infected with the Muar strain was 34%, the dissemination rate was 24% and the transmission rate was 11%. The equivalent rates for Nakayama strain were 20%, 17% and 11% respectively. There was no significant difference in infection, dissemination and transmission rates between the two strains of virus in *Culex quinquefasciatus* mosquitoes (Table 18 above).

4.5 Discussion

One of the questions surrounding the lack of detection or circulation of the Muar strain of JEV is the replication efficiency of the virus in mosquitoes. Therefore, the objective of this study was to determine the rate of infection of the Muar strain of JEV (genotype-V) in *Culex quinquefasciatus* and compare these infection rates with Nakayama, the prototype genotype-3 strain. This is the first time a genotype-V virus has been used in mosquito infection studies. The results of this study have shown that Muar strain of JEV is indeed capable of infecting and disseminating in *Culex quinquefasciatus* mosquitoes, which can subsequently lead to transmission by bite. However the rates were low as compared to other studies with different mosquitoes and different virus strains. Of the mosquitoes tested at 14 dpi, 7% were able to transmit the Muar strain of JEV and by 21 days post infection, 23% of the mosquitoes had virus detected in their saliva (Table 15). The dissemination efficiency at 21 dpi was 100% (Table 16) indicating that in all mosquitoes the virus was not confined to the midgut but was able to infect various tissues throughout the mosquito body. Still, not all mosquitoes with a disseminated infection were shown to be able to transmit the virus. The transmission efficiency (i.e. the number of mosquitoes able to transmit after infection) was 64% (Table 16).

Another reason that may have contributed to the limited distribution of the Muar strain may have been competition from other genotypes. Hence the fitness of Muar infectivity was compared to a genotype III strain Nakayama under laboratory conditions at 28°C. This is also the first study to compare a JEV genotype V strain (Muar) to a genotype III strain (Nakayama). When *Culex quinquefasciatus* were infected with Nakayama (genotype III) strain, there was no dissemination and transmission at 7 dpi which was similar to the results obtained using the Muar strain. However, while Muar strain was detected at 14 dpi with a transmission rate of 7%, there was no infection, dissemination or transmission at this time point when the mosquitoes were infected with Nakayama strain. Infection, dissemination and transmission rates were 42%, 42% and 26%, respectively at 21 dpi for Nakayama (Table 15). These results did not differ from the rates at 21 days post infection when mosquitoes were infected with Muar (genotype V). The dissemination efficiency at 21 dpi was 100% for both Nakayama and Muar. The

transmission efficiency was 63% for Nakayama and 64% for Muar strain (Table 16). Therefore these two virus strains have similar transmission characteristics in *Culex quinquefasciatus*.

Culex quinquefasciatus was used mainly because it was readily available in the laboratory. It is important to note that *Culex quinquefasciatus* is not the main vector for JEV and its role as a secondary vector is likely to be variable in different locations. For example, vector competence of this vector to JEV has ranged from refractory to moderate (see Table 12). Vector competence studies using *Culex quinquefasciatus* and genotype II strains of JEV showed high infection rates of 98% at >17 days post infection, with 28% dissemination and 50% transmission (van den Hurk, Nisbet et al. 2003). However, a similar study using a genotype III strain of JEV reported no transmission at >14 dpi (Kramer, Chin et al. 2011). This suggests that is significant variation in the competence of *Culex quinquefasciatus* to JEV. In this study I would rank competence of this mosquito strain as low. Nonetheless, the results reveal that Muar is capable of infecting a mosquito and this did not vary greatly from Nakayama.

The results obtained here do not explain the underlying factors that have contributed to the once thought limited distribution, isolation and circulation of the Muar strain of JEV (genotype 5) (Solomon *et al.*, 2003) and the more recent the discovery of the genotype in China and Korea after a 57 year hiatus (Li, Fu*, et al.*, 2011; Takhampunya *et al.*, 2011) are not clear.

Several factors may have contributed to the manifestation of this genotype ranging from climate and environmental changes, improved pathogen detection or mosquito-pathogen transmission factors. This virus has been isolated from *Culex tritaeniorhynchus* and *Culex bitaeniorhynchus* hence it shows it has the ability to infect mosquitoes. Future studies should compare the different genotypes in *Culex tritaeniorhynchus*, the main vector for JEV or other mosquito species that are highly susceptible to JEV such as *Culex vishnui* or *Culex annulirostris*, to determine growth characteristics of Muar/genotype V relative to the other genotypes in permissive mosquitoes. Furthermore, studies using the different genotypes at different environmental conditions would also provide valuable information since studies have shown genotype distribution are associated with climatic conditions (Schuh *et al.*, 2013).

The results provided here show that the infection rate of Muar (genotype V) in *Culex quinquefaciatus* did not differ from that of Nakayama (genotype III). This observation, together with the recent emergence of viruses belonging to the genotype V in China and Korea, provide evidence of the potential of JEV and its genotype to appear in places they were never found before .Consequently it is important to assess the vector competence of mosquitoes in such areas to determine which mosquito species are likely to support the maintenance and circulation JEV should it emerge. The next chapter thus goes on to evaluate the vector competence of a local British mosquito *Ochlerotatus detritus* to JEV.

5 Chapter 5: Evaluation of a temperate climate mosquito, *Ochlerotatus (Aedes) detritus*, as a potential vector for Japanese encephalitis virus

5.1 Abstract

Great Britain has not yet experienced a confirmed outbreak of mosquito-borne virus transmission to people or livestock despite numerous autochthonous epizootic and human outbreaks of mosquito-borne diseases in the European mainland. Indeed, it has not been established if British mosquitoes are competent to transmit arboviruses. Therefore, in this chapter I assessed the competence of a local (temperate) British mosquito species, *Ochlerotatus (Aedes) detritus,* for a member of the Flavivirus genus, Japanese encephalitis virus (JEV) as a model for mosquito-borne virus transmission. I also evaluated JEV competence in a laboratory strain of *Culex quinquefasciatus*, an incriminated JEV vector, as a positive control. *Ochlerotatus detritus* adults were reared from field-collected juvenile stages. In oral infection bioassays, adult females developed disseminated infections and were able to transmit virus as determined by isolation of virus in saliva secretions. When pooled from 7 to 21 days post infection, 13 and 25% of *Ochlerotatus detritus* were able to transmit JEV when held at 23 and 28°C, respectively. Similar results were obtained for *Culex quinquefasciatus*. To my knowledge, this study is the first to demonstrate that a British mosquito species, *Ochlerotatus detritus,* is a potential vector of an exotic flavivirus.

5.2 Introduction

The emergence of mosquito-borne viruses in subtropical and temperate regions of Europe (Phipps *et al.*, 2008) in recent years has raised concerns about the risk of an outbreak occurring in Great Britain. However, the risk to Great Britain from mosquito-borne arboviruses is unknown. A major knowledge gap is the vector competence of Great Britain's indigenous mosquitoes for arboviruses. While there have been no reports of outbreaks of disease caused by mosquito-borne viruses, studies in Great Britain have reported the serological detection of antibodies to West Nile virus (WNV), Usutu virus and Sindbis virus in both migrant and non-migrant wild bird species (Buckley *et al.*, 2003), and to WNV in sentinel chickens raised on a farm (Buckley *et al.*, 2006), suggesting that some transmission of arboviruses may occur.

Vector competence is a measure of the ability of a mosquito to become infected with, allow replication of, and transmit virus to a susceptible host (Kramer & Ebel, 2003). At present, there are thirty-four species of mosquitoes recorded in the British Isles comprising six species of Anophelinae (genus *Anopheles*) and 28 species of Culicinae in seven genera: *Aedes* (3), *Coquillettidia* (1), *Culex* (4), *Culiseta* (7), *Dahliana (1), Ochlerotatus* (11) and *Orthopodomyia* (1) (Medlock & Vaux, 2009). With the exception of the recently (re)discovered *Culex modestus* (Marshall, 1945; Golding *et al.*, 2012; Medlock & Vaux, 2012), all of these mosquitoes are thought to be native species. However, to my knowledge there is no information on the vector competence of these resident British populations to any arbovirus.

Ochlerotatus detritus Haliday 1833 (Diptera: Culicidea) was selected in this study as a model to determine the vector competence of a temperate mosquito originating from Great Britain. Because of its relative abundance in the sampling site (Cheshire county, Great Britain), accessibility and biting behaviour, it was found to be ideal for vector competence evaluation at the time this study was implemented. It is one out of thirteen British species of mosquito that can be considered a potential bridge vector should any mosquito-borne virus emerge in the UK (Medlock *et al.*, 2005). *Ochlerotatus detritus* has been shown to feed on both birds and humans (Service, 1971) and therefore can potentially transmit flaviviruses from their natural cycle in birds over to humans. It is a salt marsh mosquito found in the low-lying coastal and

some inland saline waters (Rees & Snow, 1996). Though *Ochlerotatus detritus* has a widespread but patchy distribution in Great Britain (Figure 18) (Snow *et al.*, 1998; Medlock *et al.*, 2005), in coastal areas where it is found, this mosquito causes the greatest human biting nuisance of any British mosquito (Clarkson & Setzkorn, 2011).

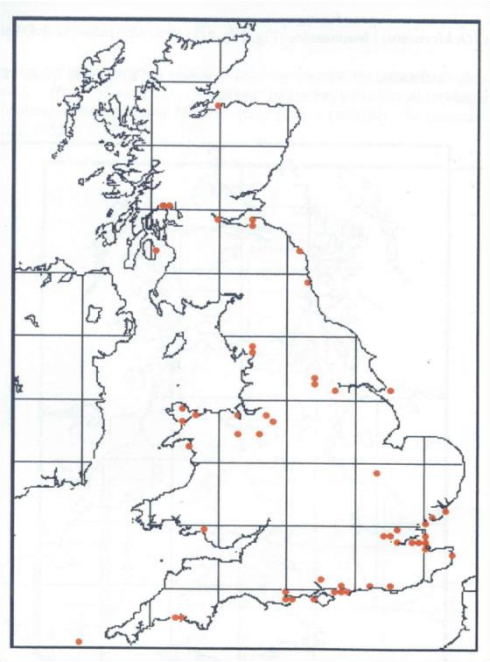


Figure 17: Distribution of *Ochlerotatus detritus* within Great Britain (Snow *et al.*, 1998)

Ochlerotatus detritus oviposits in salty ground prone to periodic flooding and usually a generation follows each immersion (Snow, 1990), hence it is multi-voltine. *Ochlerotatus detritus* bites humans persistently with adults appearing from March to November and overwinters as 4th instar larvae. Biting occurs mainly outdoors (Service, 1971). *Ochlerotatus detritus* is distributed throughout European coastal districts from the Baltic to the Aegean, Mediterranean and Red sea, and in inland saline areas in Europe and North Africa (Cranston *et al.*, 1987) Some of the countries where this mosquito have been identified include: China, Croatia, Cyprus, Egypt, France, Greece, Iran, Mongolia, Morocco, Poland, Portugal, Romania, Russia, Slovakia, Spain, Tajikistan, Turkey, Italy, Sweden and United Kingdom (Figure 19).

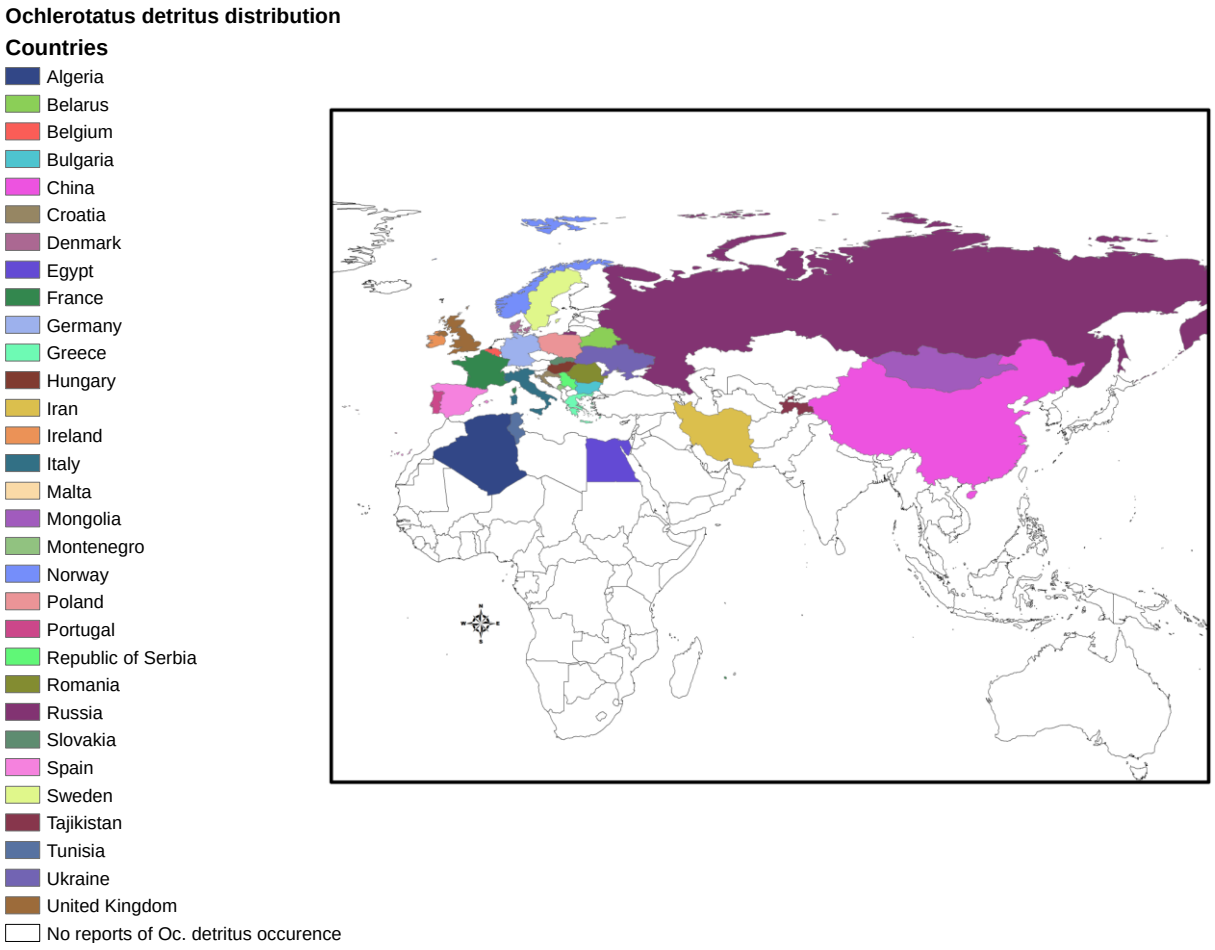


Figure 18: Worldwide distribution of *Ochlerotatus detritus.*

Japanese encephalitis (JE) virus was used as the model virus to evaluate vector competence in *Ochlerotatus detritus* because it was readily available and used in the laboratory. JE virus (JEV) is one of seven mosquito-borne zoonotic viruses considered a threat to Northern Europe (Johnson *et al.*, 2012); indeed, its genome was recently detected in *Culex pipiens* mosquitoes in Italy (Ravanini *et al.*, 2012). JEV is also the prototype of a sero-complex of closely related flaviviruses, which includes WNV and Usutu virus (USUV). JEV is an arbovirus that is maintained in a zoonotic cycle, which can be both enzootic and epizootic. This cycle involves pigs as the major reservoir/amplifying host, water birds as carriers and mosquitoes (in particular *Culex tritaeniorhynchus*) as vectors. Humans are considered dead-end hosts because they produce low viraemia levels over a limited time-frame that is insufficient to infect feeding mosquitoes (Scherer, Kitaoka*, et al.*, 1959; Chan & Loh, 1966; Impoinvil *et al.*, 2013). The disease caused by

JEV has an estimated worldwide annual incidence of 70,000 human cases with approximately three quarters occurring in children aged 0 to 14 years (Campbell *et al.*, 2011). Roughly, one quarter of encephalitis patients will die while about one half of the survivors will develop permanent neurologic and/or psychiatric impairment (Unni *et al.*, 2011). Although commercial inactivated vaccines are available against JEV, it still remains the most important member of the JEV sero—complex and the most widespread of a group of antigenically related mosquito-borne viruses that cause encephalitis in man.

Given the possible threat posed by mosquito-borne flaviviruses to Great Britain, I have investigated, for the first time, the vector competence of a resident British mosquito species for JEV. The aim of this study was to investigate JEV dissemination in a temperate mosquito species and its potential to transmit virus through detection of viable JEV in its saliva.

5.3 Materials and methods

5.3.1 Mosquitoes

Mosquitoes used in this study were derived from wild-caught larvae of *Ochlerotatus detritus* sourced locally and *Culex quinquefasciatus,* Say (Recife strain), a colonized mosquito from Brazil. *Culex quinquefasciatus* was used for validation since JEV has been isolated from this mosquito previously (Weng, Lien, Wang, Lin, Lin, & Chin, 1999; Halstead & Tsai, 2004; Nitatpattana *et al.*, 2005; Changbunjong *et al.*, 2013) and found to be competent for JEV in infection studies (Mourya *et al.*, 2002; van den Hurk *et al.*, 2003; Liu *et al.*, 2012).

Ochlerotatus detritus immatures (larvae and pupae) were collected from pools on Quayside saline marsh in northwest England (GPS coordinates: 53.277073N, -3.067728W) and transported to the Liverpool School of Tropical Medicine (LSTM) insectary. They were reared in trays (15 × 30 × 5 cm) in the same water from which they were collected. Identification of fourth instar larvae was carried out using the identification keys for British mosquitoes (Cranston *et al.*, 1987). A colony for this mosquito was not established because laid eggs failed to hatch; hence immatures were collected fresh for every experiment. *Culex quinquefaciatus* were obtained from a colony maintained in the LSTM insectary. Larvae were hatched, then divided among 15 × 30 × 5 cm trays with approximately one litre of de-chlorinated water and fed on brewer's yeast tablets (Holland & Barrett, Nuneaton, Warwickshire, UK) as needed.

Once the larvae started pupating, pupae for both mosquito species were harvested daily and transferred to separate BugDorm cages® (BioQuip, Rancho Dominguez, CA) (30 × 30 × 30 cm) where they would emerge as adults. All adults and larvae were maintained at 27°C with a relative humidity of 80% and 12:12 light: dark cycle. The adults were provided with 10% sucrose and water *ad libitum.*

5.3.2 Cells and viruses

The Muar strain of JEV was used in all infection experiments. Vero cells were maintained in Dulbecco Modified Eagle's Minimal Essential Medium (DMEM) Sigma-Aldrich) media containing 10% heat-inactivated Fetal Calf Serum (FCS), 2 mM L-glutamine and 50 µg/ml Penicillin/Streptomycin.

5.3.3 Vector competence

Field populations of *Ochlerotatus detritus* F_0 mosquitoes and *Culex quinquefasciatus* colony mosquitoes were tested for JEV vector competence at two temperatures (23°C or 28°C) and at time points 0, 1, 3, 7, 14, and 21 days post-infection (dpi; i.e. after offering an infectious blood-meal). Time point 0 represent mosquitoes collected 1-hour after offering an infectious blood meal. Two temperatures were used to provide preliminary evidence for any important effects of temperature on the level of vector competence of *Ochlerotatus detritus.*

5.3.4 Per oral infection and transmission assay

All work with infectious blood meals was undertaken in the ACDP Ar-CL3 facilities at LSTM. Viral stocks were diluted prior to infecting mosquitoes to ensure the final titre was correct. Infectious blood meal containing virus from frozen stock was prepared by combining defibrinated horse blood (Thermo Oxoid Remel), with the appropriate volume of virus stock and 100 µl of adenosine 5'-triphosphate (ATP 0.02 µm) as a phagostimulant to a final concentration of 6 logs pfu/ml.

Seven day-old adult female mosquitoes were aspirated from their cages into round 0.5 litre polypropylene plastic containers. Fine nylon netting was placed over the mouth of the container to provide ventilation and prevent the escape of the mosquitoes. The netting was secured by rubber bands and the hollowed-out lid of the container. A small slit was made in the net in order to fit the mouth aspirator. The slit was closed with cotton wool. The mosquitoes were deprived of sucrose solution and maintained on water soaked cotton balls for

24 hours prior to blood feeding. Approximately one hundred mosquitoes for each experiment were offered an infectious bloodmeal in order to achieve a minimum of 50 mosquitoes for assessment of infection.

Peroral infection was achieved by exposing mosquitoes to a suspension of defibrinated horse blood and the Muar strain of JEV, using a Hemotek membrane feeding system (Hemotek limited Accrington, Lancashire, UK) for 1 hr at ~23°C (50 – 70% humidity) in the dark. Parafilm® M was used as the membrane. In all cases 0.5 ml aliquots of the infectious blood meal were collected both before and after the mosquitoes were fed and stored at -80°C for subsequent virus isolation. This was done to confirm that the virus was viable before and after the blood feed, and determine if there was any change in the virus concentration.

Engorged mosquitoes were chilled and sorted on ice and placed in fresh round 0.5 litre polypropylene plastic containers with fine nylon netting. Fed females were maintained on cotton balls soaked with 10% sucrose solution. Excess sugar solution was squeezed out from the cotton ball to prevent it from dripping into the plastic cups. Cotton balls were changed daily. Mosquitoes were held at 23°C or 28°C in a Sanyo incubator model MIR-153 with a photoperiod of 12:12 light: dark cycle. A pan of water was kept in the incubator to maintain a relative humidity range of 70 – 90% relative humidity.

Mosquitoes were sampled at 0, 1, 3, 7, 14, and 21 dpi at both 23°C and 28°C. All mosquito samples were frozen individually at -80°C in 1.5 ml skirted conical microcentrifuge tubes with external thread O-ring screw-cap containing virus diluent media (Minimum essential medium (MEM), containing 1% Bovine serum albumin (BSA), 50 µg/ml penicillin/streptomycin, 0.3% Sodium hydrogen carbonate and 2.5 µg/ml Fungizone). For mosquitoes sampled at 0, 1 and 3 dpi, the whole mosquito body was placed in 1.5 ml tubes individually with virus diluent and then frozen. Early time points (0 to 3 dpi), representing the eclipse phase of virus production in a mosquito, were sampled to ensure that virus detection reported for later time points (7 to 21 dpi) was the result of new virus production rather than carry over from input virus. The eclipse phase is the period after the ingestion of an infectious bloodmeal by a mosquito where the

virus titre decreases to minimal or non-detectable levels which are reached at about 3 to 4 days depending on temperature, virus or vector. After multiplying in the midgut cells and spreading to other organs including the salivary glands, the virus can then be detected usually from about 7 days after feeding.

For mosquitoes sampled at 7, 14 and 21 dpi, saliva was collected before the mosquito legs were dissected from the remaining mosquito carcass. Each of these samples (saliva, dissected legs and the remaining mosquito carcass) were individually placed in a 1.5 ml tube with virus diluent and then frozen at -80°C. Manipulation of the mosquitoes was achieved by anesthetizing them using Triethylamine (TEA) FlyNap® (Blades Biological Limited, UK).

Salivary secretions were collected using a modified *in vitro* capillary transmission assay (Aitken, 1977). Mosquito mouth parts were inserted into a plastic Micro-Hematocrit capillary tube, (Drummond ®, Cole-Parmer, UK) containing approximately 10 µl of a mixture of virus diluent, 50% sucrose and adenosine 5'-triphosphate (ATP, 0.02 µM) for 30 to 45 minutes. One µl of 1% pilocarpine (Alfa Aesar, Ward Hill, MA, USA) solution in phosphate buffered saline (PBS) and 0.1% Tween 80 was applied to the thorax to stimulate salivation (Boorman, 1987; Dubrulle *et al.*, 2009). Active movement of the maxillary palpi and the stylets observed under a stereoscopic microscope, bubble formation in the media and engorgement of the mosquito were interpreted as a sign of salivation. The contents were then released under pressure into a tube containing 0.5 ml of virus diluent.

Infection was determined by recovery of virus from the mosquito tissue suspension. If virus was recovered from its body but not in its legs, the mosquito was considered to have a non-disseminated infection. If virus was recovered from both the legs and the body suspension the mosquito was considered to have a disseminated infection and if virus was recovered from its saliva the mosquito was considered to have a transmissible infection (Turell *et al.*, 1984).

The infection, dissemination and transmission rates percentage are defined as the number of mosquitoes testing positive for virus in their bodies, legs and saliva, respectively divided by the total number of mosquitoes tested, times 100. *Transition* efficiency – the proportion of

infected mosquitoes that have a disseminated or transmissible infection, or the proportion with disseminated infections that have a transmissible infection was also determined.

5.3.5 Plaque assay

Body and leg samples were prepared for virus titration by homogenizing using a Disruptor genie® cell disruptor (Scientific Industries, USA) for 5 minutes in a 1.5 ml tube containing 0.5 ml virus diluent and two 6mm glass beads (Merck KGaA, Germany). Plaque assays were performed by inoculating 100 µl of the salivary secretions or the supernatant of the homogenized bodies and legs onto a confluent monolayer of Vero cells on a 6-well plate (Costar®, Corning Life Sciences). The plates were then incubated at 37°C at an atmosphere of 5% CO_2 for 30 - 60 minutes with rocking every 10 minutes to allow the virus to enter the cells. A 4 ml overlay of MEM, 4% FBS, 50 µg/ml gentamycine, 0.5% Sodium hydrogen carbonate and 2.5 µg/ml Fungizone (amphotecerine B) to limit contamination and 1% SeaPlaque low melting point agarose was then added to the wells and the plates were incubated at 37°C in 5% CO_2. After 5 days of incubation, 2 ml of 10% neutral buffered formalin solution was added to each well and the plates left for at least 3 hours with the fixative to ensure complete inactivation of the virus. In order to visualize the plaques the wells were stained with 0.5 ml of crystal violet solution. Samples were scored as virus-positive or virus negative based on the presence or absence of plaques. Viraemia of mosquito carcases was determined for a small subset of mosquitoes (i.e. ~3 mosquitoes per each species and temperature for 0, 1, 3, 7, 14 and 21 days). Viraemia was also determined for a subset of saliva samples.

5.3.6 Statistical analysis

Fisher Exact Test was used to determine if there were significant differences ($p < 0.05$) in rates of infection, dissemination and transmission between temperatures. This was done both at each time point (dpi) and also in pooled analysis (7 to 21 dpi) to overcome issues of small sample size. SISA, an open access online statistics calculator (http://www.quantitativeskills.com/sisa/) was used to conduct Fisher Exact Test. Confidence intervals of proportions were calculated using VassarStats (http://vassarstats.net/prop1.html).

VassarStats uses the Wilson Score Interval method which is more robust when dealing with small number of trials and/or an extreme probability (Newcombe, 1998). Sample size in each group was dictated by the feeding success and the survival rates through the days post infection (dpi).

5.4 Results

There was a constant attrition of mosquitoes during the course of the study. A total of 873 field-collected *Ochlerotatus detritus* were offered an infectious bloodmeal and only 397 (45%) of them engorged, while 506 out of 695 (73%) of the colony mosquito *Culex quinquefasciatus* acquired a bloodmeal. Of those that acquired an infectious bloodmeal, more than half of the *Ochlerotatus detritus* died (224 of 397), while about a quarter (150 of 506) of the *Culex quinquefasciatus* died during the course of the experiment (Table 19). Mortality of *Ochlerotatus detritus* was especially high (65%) at 28°C. The rate of engorgement in *Culex quinquefasciatus* was higher than *Ochlerotatus detritus*.

Table 19: Rate of engorgement and mortality of *Ochlerotatus detritus* and *Culex quinquefasciatus* at 23 °C and 28 °C incubation temperatures.

Mosquito species	Temperature	Initial no. of mosquitoes	Bloodfed/ Initial (%)	Sampled/ Bloodfed (%)	Dead/ Bloodfed (%)
Ochlerotatus detritus	23 °C	430	198 (46)	103 (52)	95 (48)
	28 °C	443	199(45)	70 (35)	129 (65)
Culex quinquefasciatus	23 °C	385	277(72)	216(78)	61 (22)
	28 °C	310	229 (74)	140 (61)	89 (39)

Though freshly harvested viral stocks, prior to being frozen, were originally estimated to be ~6 logs PFU per ml, assessment of the infectious bloodmeal before and after being placed in the Hemotek artificial feeding system always yielded ~4 logs PFU/ml.

Both mosquito species displayed a typical eclipse phase following oral infection in which the virus titre and detection decreased from 0 to 3 dpi followed by an increase in virus titre and detection from 7 to 21 dpi (figure 20 and table 20). This is attributable to the reduction in virus titre after day 0 as ingested virus either infects cells or gets digested; virus successful in infecting cells replicates to detectable levels several days later. Viraemia of saliva samples ranged from ~1 log to ~ 3 logs PFU/ml for both mosquitoes at both temperatures.

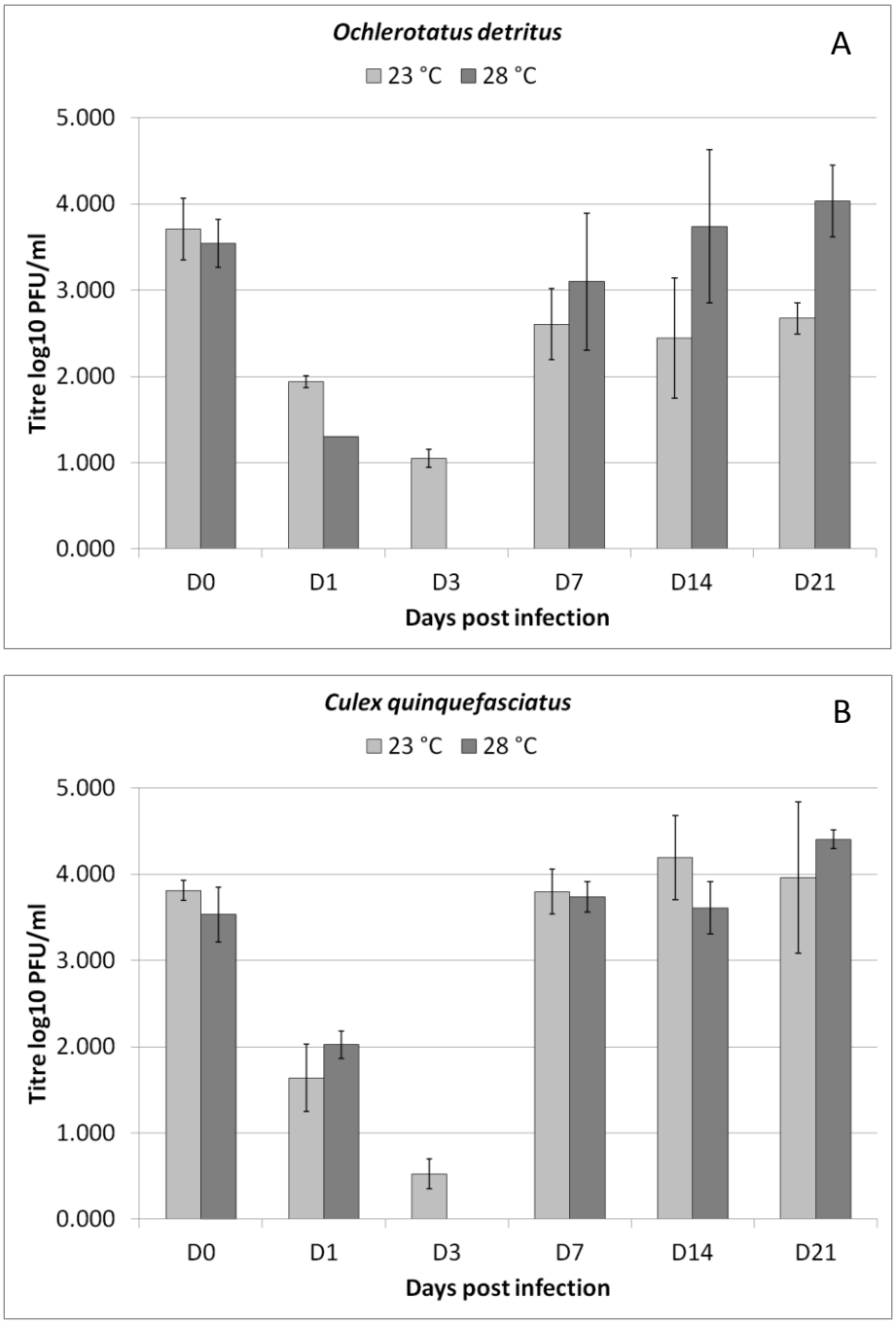


Figure 19: Graph showing the virus eclipse phase for JEV in both *Ochlerotatus detritus* (**A**) and *Culex quinquefasciatus* (**B**) at 23°C and 28°C.

This is represented by the steady decline in the virus titre from day 0 then a steady increase from day 7 onwards.

Both mosquito species were susceptible to JEV infection with infection rates from 7 days post infection ranging from 32 to 100% for *Ochlerotatus detritus* and 25 to 100% for *Culex quinquefasciatus* (table 20 and figure 20). In general, higher infection, dissemination and transmission rates were reached at later time points although there was some variation. Dissemination rates of both species tended to be similar to the infection rates. Transmission rates tended to be lower than dissemination rates. Nevertheless, 33 – 67% of *Ochlerotatus detritus*, and 50 – 70% of *Culex quinquefasciatus*, had developed transmissible infections by 21 dpi (table 20).

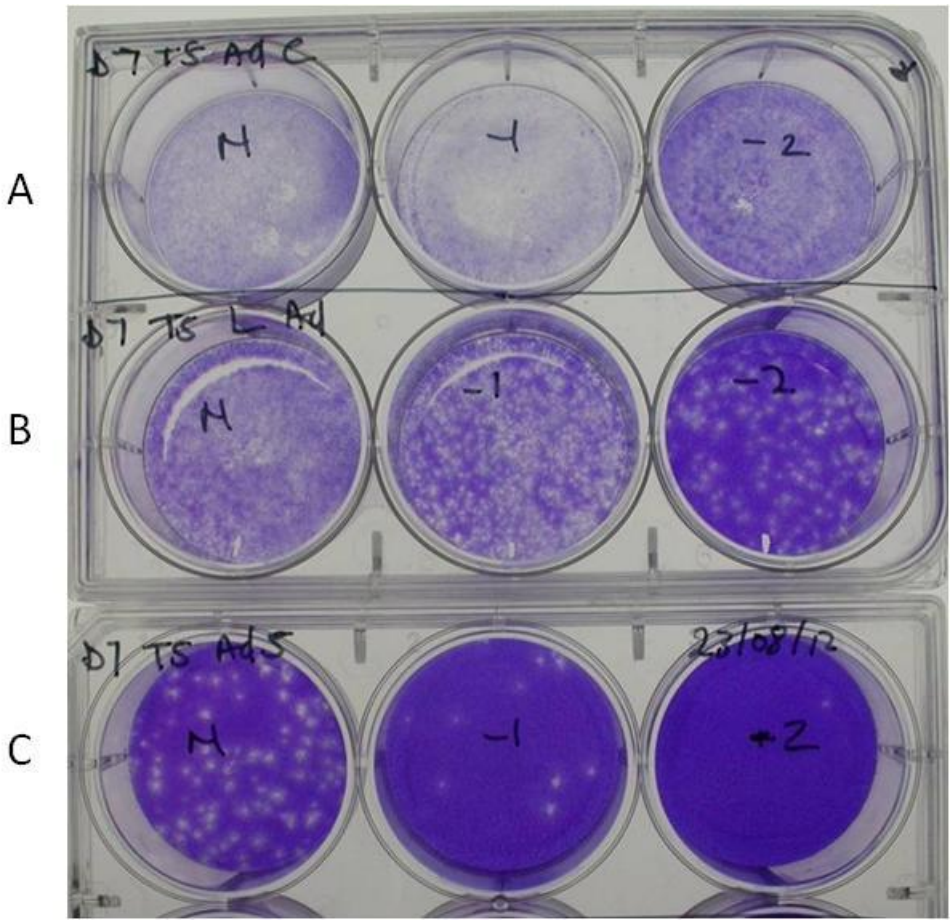


Figure 20: Sample plaque assay plate showing virus plaques obtained from *Ochlerotatus (Aedes) detritus* mosquito sampled at 7 days post infection serial diluted from neat (N) 10-1 and 10-2.

A) Plaques from whole body indicate that the mosquito is infected (carcass).
B) Plaques from legs indicate that the virus has disseminated to other organs in the body of the mosquito.
C) Plaques from saliva sample shows mosquito is able to transmit virus by bite.

Table 20: Infection, dissemination and transmission rates of mosquitoes exposed to 4 logs PFU/ml of the Muar strain of JEV**.**

Mosquito species	Temp	dpi * (days)	No. tested	I	D	T	Infection rate [a] (95% CI)	Dissemination rate [b] (95% CI)	Transmission rate [c] (95% CI)
O. d	23 °C	0	16	16	nt	nt	100 (81-100)	nt	nt
		1	11	4	nt	nt	36 (15-65)	nt	nt
		3	9	3	nt	nt	33 (12-65)	nt	nt
		7	25	8	5	3	32 (17-51)	20 (8-39)	12 (4-30)
		14	32	25	23	1	78 (61-89)	72 (54-84)	3 (0-15)
		21	6	6	6	4	100 (60-100)	100 (60-100)	67 (30-90)
		Total†	63	39	34	8	62 (50-73)	54 (42-66)	13 (7-23)
	28 °C	0	12	6	nt	nt	50 (25-75)	nt	nt
		1	7	1	nt	nt	14 (3-51)	nt	nt
		3	3	0	nt	nt	0	nt	nt
		7	15	9	9	4	60 (35-80)	60 (35-80)	27 (10-51)
		14	6	3	2	1	50 (18-81)	33 (9-70)	17 (3-56)
		21	3	3	3	1	100 (43-100)	100 (43-100)	33 (6-79)
		Total†	24	15	14	6	62 (42-79)	58 (39-76)	25 (12-45)
C. q	23 °C	0	17	17	nt	nt	100 (82-100)	nt	nt
		1	11	6	nt	nt	55 (28-55)	nt	nt
		3	11	3	nt	nt	27 (10-57)	nt	nt
		7	24	6	5	4	25 (12-44)	21 (10-40)	17 (7-36)
		14	32	20	11	1	62 (45-77)	34 (20-51)	3 (0-15)
		21	10	7	7	5	70 (39-89)	70 (39-89)	50 (24-76)
		Total†	66	33	23	10	50 (38-62)	35 (25-47)	15 (8-26)
	28°C	0	7	7	nt	nt	100 (65-100)	nt	nt
		1	10	3	nt	nt	30 (11-60)	nt	nt
		3	3	0	nt	nt	0	nt	nt
		7	9	4	0	0	44 (19-73)	0	0
		14	12	8	7	2	66 (39-86)	58 (31-80)	17 (4-45)
		21	10	7	7	7	70 (40-89)	70 (40-89)	70 (40-89)
		Total†	31	19	14	9	61 (44-76)	45 (29-62)	29 (16-47)

O. d= Ochlerotatus detritus ; ***C. q= Culex quinquefasciatus***; dpi=days post infection; I=number infected; D= number disseminated; T= number transmitting; nt = not tested; CI=confidence interval

* Days 0, 1, and 3 post infection represents input virus and is not true infection; † Totals include days 7 – 21 dpi only

[a] Percentage of mosquitoes containing virus in their bodies out of no. tested (95% confidence interval).

[b] Percentage of mosquitoes containing virus in their legs out of no. tested (95% confidence interval).

[c] Percentage of mosquitoes containing virus in their saliva out of no. tested (95% confidence interval).

Overall, when 7 to 21 dpi are combined, the field populations of *Ochlerotatus detritus* were competent for JEV with 62% infection, 54% dissemination and 13% transmission rate at 23°C and 62% infection, 58% dissemination and 25% transmission rate at 28°C. The rate of infection, dissemination and transmission in *Ochlerotatus detritus* did not differ significantly at the two temperatures in individual or pooled analysis. For *Culex quinquefasciatus*, when 7 to 21 dpi are combined, JEV competence rates were 50% infection, 35% dissemination and 15% transmission rate at 23°C and 61% infection, 45% dissemination and 29% transmission rate at 28°C. In addition, for *Culex quinquefasciatus*, when the analysis was done individually for each day post infection (7, 14, and 21dpi) or pooled, there was either marginal ($0.05 < p < 0.1$) or not a significant effect of temperature on the rate of infection, dissemination and transmission. However, sample sizes for individual time points are small. When the data for later time points (7 to 21 dpi) are pooled, the effect of temperature on the transmission rate was significant ($\chi^2 = 7.199$, df = 1, $p = 0.014$).

To describe the transition efficiency of the virus after overcoming the midgut barrier in the mosquito, the number of mosquitoes with a disseminated infection out of the number infected (dissemination efficiency) was examined and the number of mosquitoes able to transmit out of those disseminated (transmission efficiency) was also examined at day 7, 14, and 21 dpi (Table 21). Both mosquitoes attained 100% dissemination efficiency by 21 dpi.

Table 21: Dissemination and transmission transition efficiency of mosquitoes exposed to 4 logs PFU/ml of Muar strain of JEV.

Mosquito Species	Temp	dpi (days)	Dissemination, % of no. infected [a] (95% CI)	Transmission, % of no. disseminated [b] (95% CI)	Transmission, % of no. infected [c] (95% CI)
O. d	23 °C	7	62 (30-86)	60 (23-88)	37 (14-69)
		14	92 (75-98)	4 (0-21)	4 (0-19)
		21	100 (60-100)	67 (30-90)	67 (30-90)
	28 °C	7	100 (70-100)	44 (19-73)	44 (19-73)
		14	66 (20-94)	50 (9-90)	33 (6-79)
		21	100 (43-100)	33 (6-79)	33 (6-79)
C. q	23 °C	7	83 (44-97)	80 (38-96)	67 (30-90)
		14	55 (34-74)	9 (2-38)	5 (1-24)
		21	100 (65-100)	71 (36-92)	71 (36-92)
	28 °C	7	0	0	0
		14	87 (53-98)	28 (8-64)	25 (7-59)
		21	100 (65-100)	100 (65-100)	100 (65-100)

O. d = Ochlerotatus detritus ; ***C. q = Culex quinquefasciatus***; dpi= days post infection; CI=confidence interval

[a] Percentage of mosquitoes containing virus in their legs out of no. infected

[b] Percentage of mosquitoes containing virus in their saliva out of no. disseminated

[c] Percentage of mosquitoes containing virus in their saliva out of no. infected.

5.5 Discussion

This is the first study to investigate the biological competence of a mosquito of British origin (*Ochlerotatus detritus*) to an arthropod-borne virus. *Ochlerotatus detritus* was susceptible to laboratory infection with JEV at 23°C and 28°C, with virus detectable in the saliva of some individuals as early as 7 dpi, and it therefore appears to be a competent vector for this flavivirus.

Since the *Ochlerotatus detritus* mosquito population used in this study is a temperate variety, it showed poor survival when incubated at 28 °C, and there was high mortality during the experiments; hence, no mosquitoes survived greater than 21 dpi. This mosquito was also not adapted to acquiring a blood meal from an artificial feeder and that may have led to lower numbers of mosquitoes acquiring an infectious blood meal.

In this study, the transmission rate for *Ochlerotatus detritus* was only 19% when averaged for the two temperatures at the different days post infection. However, it is important to note that the medium used to collect the saliva can affect the amount of virus detected; because an aqueous solution was used in the capillary tube assay , this may have underestimated the amount of virus being secreted by the mosquito (Colton *et al.*, 2005; Turell, Mores, Dohm, Lee*, et al.*, 2006). While animal infections with the mosquito would have been a better model to confirm transmissibility, the facilities to do this were not available. In this study, salivary viraemia ranging from 1 log to 3 logs PFU/ml were produced. The viraemia produced in the saliva secretion of both mosquito species is likely to cause infections in susceptible birds, humans or other mammals.

The decrease in detectable titres of JEV in *Ochlerotatus detritus* and *Culex quinquefasciatus* during the first 3-days after an infectious blood meal indicated an eclipse phase in virus replication. At 23°C, for both species, the infection rates are very similar at 3 and 7 days (33 vs. 32%; 27 vs. 25%). The detection of viral dissemination and transmission by 7 dpi is likely an indication that viral replication has occurred by then and this is not “carry-over” input virus; the

increase in viral titre between 3 and 7 dpi suggests the same. Early in the eclipse phase, the rate of reduction in virus titre and detection appears to have been sharper at 28 compared to 23°C.

It remains to be seen whether *Ochlerotatus detritus* is competent at normal Great Britain temperatures. The extrinsic incubation period (EIP) is the duration required for a pathogen to complete its development within a vector from its initial acquisition via an infected blood meal to the point at which it can be transmitted to another host via another blood meal. EIP is heavily influenced by prevailing temperatures and is an essential piece of information when developing vectorial capacity models. There was no significant difference in the infection, dissemination and transmission rates in *Ochlerotatus detritus* at 23°C and 28°C. This was unexpected as studies have shown that increases in temperature often reduce the EIP, therefore increasing infection, dissemination and transmission rates (Davis, 1932; Takahashi, 1976; Kay *et al.*, 1989). In *Ochlerotatus detritus*, because a total of only 24 mosquitoes were assessed at 28°C while a total of 63 mosquitoes were assessed at 23°C, It is possible that my results may have been affected by small sample sizes, limiting the power of the study to detect a difference. It should also be noted that an increase in temperature could also reduce the adult lifespan of mosquitoes and this may interrupt transmission (See Table 19). In contrast, the pooled results for *Culex quinquefasciatus* at the two temperatures were significantly different.

JEV disseminated well in the bodies of both mosquito species, as demonstrated by high dissemination rates (i.e. virus found in the legs). However, transmission rates were considerably lower than dissemination rates. While these results may suggest the existence of barriers to the development of a transmissible infection in the mosquito, it is still premature to make this conclusion given the low sample size.

Some of the limitations of the study include the following: Firstly, relatively high temperatures (i.e. 23 and 28°C) where used which were beyond the average summer range temperature experienced in Cheshire where the *Ochlerotatus detritus* were sourced. For example, July is the

warmest month, with mean daily maximum temperatures approaching 21°C in Cheshire (http://www.metoffice.gov.uk/climate/uk/nw/print.html). The higher temperature certainly impacted mosquito survival; still it is not clear to what extent it played a role on the overall JEV susceptibility. There were no significant differences between *Ochlerotatus detritus* kept at 23 and 28°C but this may be due to sample size. Other studies have demonstrated transmission of JEV in mosquitoes held at 20°C (Takahashi, 1976). Secondly, though the initial number of mosquitoes used was relatively large, sample size was relatively low at the later time points (i.e. 14 and 21 dpi). The difficulty of consistently getting mosquitoes from the field and keeping them alive long enough in the laboratory for assessment was a challenge. Future study should focus on holding mosquitoes at more optimum survival conditions and doing more replicates to get larger sample sizes at later time points. Thirdly, freshly harvested virus was not used for infections. Rather frozen stocks were used out of convenience and convention. This may have affected the infection efficiency as suggested in other studies (Richards *et al.*, 2007). Nonetheless, the mosquitoes certainly received at least 4 logs pfu/ml of virus as determined by plaque assays conducted before and after offering mosquito an infectious blood meal. Fourthly, other physiological parameters such as mosquito size or daily mosquito survival were not recorded. While these parameters are important they were beyond the scope of the original aim of the study, which was to assess competence of *Ochlerotatus detritus*. Finally, only one mosquito species was used in this study, despite there being several potential arboviral vectors in Great Britain. Nevertheless, this study is one of the early contributions to the knowledgebase of vector competence of native British mosquitoes.

The result of 19% transmission rate by *Ochlerotatus detritus* must be gauged against the vectorial capacity indicators to determine the likelihood of sustained transmission of JEV for this vector. Early studies in Britain have estimated the feeding rate of *Ochlerotatus detritus* on birds to be 3.7% (3-bird blood positives of 81-decernible tests), while the feeding rate on humans was 33.3% (27 of 81) (Service, 1971). Feeding behaviour on other mammals are 1.2 % (1 of 81) for pigs and 49.4% (40 of 81) for bovids. Despite the low feeding rate on JEV amplifiers (i.e. birds and pigs), there is still a sizeable population of the *Ochlerotatus detritus* in Cheshire County (Clarkson & Setzkorn, 2011; Medlock, Hansford, Anderson*, et al.*, 2012), which may

make it possible for transmission to be sustained by this vector. Nonetheless, other factors to be considered are survival of mosquitoes at optimal conditions.

As mentioned earlier, *Ochlerotatus detritus* and JEV were selected primarily out of convenience. However, *Ochlerotatus detritus* is a relevant mosquito to study as it has high human biting rates (Clarkson & Setzkorn, 2011), and is considered a potential bridge vector for arboviruses such as WNV (Medlock *et al.*, 2005; Osorio *et al.*, 2012). JEV, recognised as a virus with the potential to expand in range (van den Hurk *et al.*, 2009), is also a relevant model to use; this is underscored by the recent detection of the JEV gene sequence in a pool of *Culex pipiens* in Italy. In 2010, the detection of the NS5 gene RNA sequence of the JEV was reported from one pool of *Culex pipiens* mosquitoes collected in north-eastern Italy (Ravanini *et al.*, 2012). This report suggested that the threat of the introduction of arboviral diseases of tropical origin to temperate regions is ever present and requires constant vigilance (Platonov *et al.*, 2012).

In the case of JEV, suitable vertebrate hosts for virus amplification are pigs and water-birds. The marsh where *Ochlerotatus detritus* was sourced is a protected conservation area that is frequented by several avian species including water birds such as the little egret and different varieties of ducks and geese and other aquatic avian. However, the susceptibility of British birds to JEV is not known.

Humans are considered dead-end hosts in the transmission of JEV and therefore its introduction in the UK would most likely be through transportation of infected mosquitoes on planes, ships or cars; trade in domestic animals and also infected migratory birds which may play a critical role in the long distance transportation of the virus (Platonov *et al.*, 2012). The demonstration of the presence of a competent local vector highlights the need for continued vigilance to prevent local transmission of arboviruses in the UK and suggests that mosquito control will form part of the intervention strategy in the event of disease emergence.

Future studies will determine the vector competence of this mosquito at lower temperatures and evaluate the possibility of vertical transmission since *Ochlerotatus detritus* mosquitoes are available all year round and hibernate as eggs and larvae. The data provided here will prove

useful for the development of Great Britain-specific models of the risk of mosquito-borne arbovirus outbreaks in Great Britain.

6 Overall discussion

The principal topic of this thesis is JEV transmission, replication in a competent host, spread and its possible introduction into new areas. JEV causes high mortality, morbidity and disability in Asian countries and is considered to have the potential to expand into new areas as has already been demonstrated in its introduction into northern Australia.

Several studies have been undertaken to understand the factors responsible for the different distribution patterns of JEV genotypes. These studies have utilised climate data (Schuh *et al.*, 2013), land use and land cover variables (Impoinvil *et al.*, 2011) agricultural practices (Lindahl *et al.*, 2012) and deduced different explanations. Some studies have also used sequence information to estimate the rate of evolution of JEV (Mohammed *et al.*, 2011; Chen, 2012). However one limitation is the lack of sequence information representative of each genotype. Nevertheless, these studies have provided information for the background of this thesis.

Rather than using evolutionary information only to explain the spread and expansion of JEV, this study has also addressed the potential difference that may occur in transmissibility of the different genotypes in mosquitoes. This study compares an old genotype V sequence, which had not been isolated for nearly 60 years; to a widespread genotype III. In addition, the possibility of emergence of JEV in Great Britain has been addressed in evaluating the competence of a local 'temperate' mosquito species *Ochlerotatus detritus.* Showing through experimental studies that temperate mosquito species are able to transmit exotic flaviviruses would be of public health importance.

Chapter 3 provides a complete sequence of a genotype V isolate that had been characterized using antibody absorption test only but no sequence information was available. There are so far three genotype V isolates (i.e. Muar, XZ0934 and 10-1827) but, until now, there were only two complete genome sequences available. Hence the availability of the complete genome sequence for Tengah brings that number to three complete sequences available for genotype V. Muar may have been the only strain circulating in Singapore at that time (1952) and hence the

only difference between Muar and Tengah was the isolation from different patients. The two names were derived from the places where patients came from before they were admitted to Singapore general hospital. Muar is a town in Malaysia and Tengah is a town in Singapore. The towns are just 200 km apart.

Previous studies on the evolutionary rate of JEV have only utilized one complete sequence for genotype V and genotype II. During the course of this study, the availability of three additional genotype II sequences and two genotype V sequences made it possible to evaluate the evolutionary rate of JEV in the hope of attaining a more comprehensive analysis for JEV genotypes. These results provide for the first time the time to the most recent common ancestor (TMRCA) for genotype II when using four complete sequences and for genotype V when using three complete genome sequences. The TMRCA for genotype II was estimated to have occurred in 1910 and that for genotype V in 1814. These results also show a higher evolutionary rate of GI than GIII. These data are in agreement with other studies that have reported the displacement of genotype III by genotype I (Chen *et al.*, 2011). The results of the analysis suggest that it has a higher evolutionary rate, however this might be due to the lack of sequence information between the initial genotype V isolates (isolated in 1952) and the recent XZ0934 isolate (isolated in 2009), or it might be true that viruses in genotype V are evolving at a high rate. For this reason studies to assess any differences in virulence and viral replication rates within genotype V and between genotype V and other four genotypes are required. This should also be followed by assessing the efficacy of existing vaccines against these strains. Information to date suggests the current vaccines (which are all based on genotype III isolates) are effective against genotypes I-IV (Beasley *et al.*, 2004), however genotype V is the most divergent, and there are few data available looking at potential efficacy of vaccines against the genotype. In addition, the two earlier isolations of genotype V (Muar and Tengah) were from humans who had fatal outcomes, which suggest that genotype V has virulent strains capable of causing death hence it is important to assess the efficacy of existing vaccines against these strains.

The recent isolates of genotype V differ in sequence similarities with Muar in the envelope gene with 93.2% amino acid similarity with the Chinese isolate XZ0934 and 98.8% amino acid similarity with the Korean isolate 10-1827 (Takhampunya *et al.*, 2011). This may indicate that this genotype is evolving and whether these differences are related to either higher virulence or lower virulence, or something else, is not known. Moreover, the isolation of the Korean isolate from *Culex bitaeniorhynchus,* a mosquito species previously unknown to carry JEV, may indicate that this genotype now has the ability to infect different mosquito species and possibly a variety of hosts too. Indeed, a mutation in the envelope gene of Chikungunya virus, which is primarily transmitted by *Aedes aegypti,* is reported to be directly responsible for the adaptation of this virus to *Aedes albopictus* mosquitoes which then lead to an epidemic in Reunion Island in 2005-2006, a region that lacks the typical vector. (Tsetsarkin *et al.*, 2007). The E gene sequence homology between the Muar and Tengah isolated in 1952 to that of XZ0934 isolated in 2010 is 89.4% for nucleotide sequence and 93.2% for amino acid sequence (Li, Fu*, et al.*, 2011). Classification using phylogenetic studies as described by Chen and others (Chen *et al.*, 1990) states that the maximum genetic distance within a genotype is 6% of amino acids. This shows that the genetic distance between the isolates in 1952 and that in 2010 of 6.8% is right at the border or has surpassed that percentage. Taking this into consideration may indicate that these viruses should be classified as different genotypes in JEV or the classification of JEV genotypes should be revisited.

Chapter 3 attempts to address the lack of circulation of genotype V by comparing its infectivity in *Culex quinquefaciatus* mosquitoes with the most isolated genotype III. There was no difference found between the two genotypes in their transmissibility in *Culex quinquefasciatus* mosquitoes. The fact that Muar was able to infect and be transmitted by *Culex quinquefasciatus* may indicate that the transmissibility by different species of mosquito may have not been the limiting factor in genotype distribution. The mosquito species used in this study does not have high efficiency for JEV compared to other mosquito species such as *Culex tritaeniorhynchus*. A highly susceptible vector may have yielded different results. Nevertheless, the same conditions were used for both genotypes. Therefore, any differences in infectivity should have been apparent in this study. Differences in transmission in mosquitoes by different genotypes have

been reported for viruses such as WNV where the WN02 genotype had an extrinsic incubation period (EIP) that was 4 days shorter than that of NY99 genotype. This difference in the EIP of WN02 and NY99 provided a possible explanation of the displacement of NY99 by WN02 which was transmitted much earlier hence leading to more infection rates (Moudy *et al.*, 2007). In another study, Dengue serotype 2 and 3 have been reported to infect a significantly high proportion of *Aedes aegypti* mosquitoes than dengue serotype 1 and 4 (Gubler *et al.*, 1979). However, the amino acid difference between dengue serotypes is between 30 to 40%, and they are effectively closely related but different viruses; in contrast while amino acid difference between JEV genotypes is 9 to 12% (Tsarev *et al.*, 2000) hence this may account for the differences in mosquito infectivity of dengue viruses. Some of the reasons that may have contributed to the lack of circulation of Muar may be that this virus has low infection and/or replication rates in pigs which are considered important amplifying host in the JEV cycle or low infection and replication rates in birds. It is possible that this virus circulates in non-migratory birds in the Indo-Malaysian region. Hence these factors should be investigated.

In addition to mosquito infectivity, virus spread and evolution is influenced by complex set of variables. There are several things to take into consideration, including climatic factors, specifically temperature, or replication in other hosts such as birds or pigs. A sequence homology of 86% between Muar and the recently isolated Chinese strain, XZ0934, shows evolution may have aided in its spread to China and Korea. Hence comparing these sequences at a molecular level could provide vital information.

With the concern of JEV expanding into different geographical regions, it is essential for European countries including Great Britain to investigate the potential of its resident 'temperate' vectors to transmit the virus. In Chapter 4, the competence of *Ochlerotatus detritus,* a local British mosquito, to transmit JEV at two different temperatures was evaluated. I hypothesized that British mosquitoes were refractory to arbovirus infection. However this study was conducted as a proof-of-principle investigation to demonstrate that the indigenous *Ochlerotatus detritus* was a competent vector and has the potential to transmit JEV. Hence, given the results of this study it is possible to conclude that the absence of arbovirus circulation

in Great Britain is likely due to other factors rather than lack of competent vectors. Only one mosquito was tested in this study hence other local mosquito species should be evaluated. There was no difference in susceptibility of *Ochlerotatus detritus* at the different temperatures. However this may have been due to high mortality at 28 °C. Therefore while the temperature was conducive for the virus it was not conducive for the vector. Competence of British mosquitoes using overall Great Britain summer temperatures (~ 15-18°C) may provide different results from my findings. But studies with JEV using temperatures ranging from 20°C to 22°C have shown this virus was able to infect the mosquito albeit at slower rates. The question remains whether mosquitoes survive long enough during British summer temperatures to infect a vertebrate host? However as indicated on the map in figure 8, mosquito-borne viruses have been reported in areas that share the same climatic conditions as Great Britain or are even cooler. Since *Ochlerotatus detritus* overwinters as fourth instar larvae, it may be important to evaluate vertical transmission of JEV and other mosquito-borne viruses in this mosquito. Vertical transmission means that an infected female mosquito is able to pass on the virus to its offspring. In this case, the virus would overwinter in the fourth instar larvae in the winter months when conditions are not favourable and would thereby resume transmission in the summer. Vertical transmission of WNV has been shown in *Culex* mosquitoes which are believed to serve as overwintering reservoir host (Nelms *et al.*, 2013). In the event of global-warming, the local mosquito population may actually decrease as shown by the high mortality of *Ochlerotatus detritus* at 28°C. It may take some time for the mosquitoes to adapt to warmer temperatures hence lowering chances of arboviral transmission. The serological detection of antibodies to WNV, Usutu virus and Sindbis virus in both migrant and non-migrant birds in Great Britain (Buckley *et al.*, 2003), the detection of JEV RNA in mosquitoes in Italy (Ravanini *et al.*, 2012), and the occasional importation of viruses such as dengue by returning travelers (Stephenson *et al.*, 2003) may indicate that arbovirus are actually entering Great Britain. The fact that there are also competent vectors as evidenced in this study but still no autochthonous cases of any arboviruses reported in Great Britain may indicate that other factors should also be considered.

Vector competence is a single piece in the jigsaw of arbovirus transmission risk. Other factors to take into consideration would include population dynamics, mosquito survival rates, biting behaviors and distribution of vector in relation to host. However vector competence studies are useful for prioritizing which mosquitoes would require more entomological surveillance in the event of arbovirus emergence.

6.1 Recommendations for future studies.

Given the results from this work the following suggestions are made for future studies.

1. Evolutionary studies performed in chapter 3 utilized only one sequence for genotype IV and only three for genotype V which are the only complete genome sequences available for these genotypes. Hence there is need for characterization of complete genome sequences for these genotypes. Evolutionary studies combined with more ecological/environmental type studies such as land-use, land-cover and animal populations may provide information on any relationship there may be between virus evolution and these factors.
2. Following chapter 3, it will be important to evaluate if the comparison of JEV genotypes transmissibility in the principle vector *Culex tritaeniorhynchus* would yield different results to those reported here. Comparison of genotypes should also be performed using different mosquito species. Growth curve studies can be done in vertebrate and mosquito cell lines, and live mosquitoes.
3. In chapter 4, evaluation of vector competence of other local British mosquitoes to different arboviruses beside JEV should be done. Vector competence should also be assessed at a wider range of temperatures. One limitation in my study was the small sample size of mosquitoes used. Hence future studies should consider using a higher number of mosquitoes.

7 Constraints

Some of the challenges faced during this project were

1. Short mosquito season in the UK

   The short mosquito season in the UK meant that mosquito collections could only be carried out during the summer. Hence when attempts to colonize failed I had to wait for the next mosquito season to obtain more mosquitoes. Similarly obtaining immatures from the field for infection experiments could only be done during the mosquito season. Hence not much work was possible in the winter.

2. Availability of containment laboratories.

   All experiments involving JEV virus were carried out in the ACDP containment laboratories level 3. With the move of our department to a new Ronald Ross building, the new ACPD CL3 laboratories had to undergo thorough inspections before issuing of a licence and so they were not available for use. The maintenance of these new facilities also meant that no infection experiments could be carried out awaiting inspection and validation that they were safe to use.

   Due to the strict regulations of the use of ACDP CL3 facilities, work could only be done 9.00am to 5.00pm and no weekend access. Hence time point experiments were difficult to set up.

3. Experimental troubleshooting

   As this was the first time for mosquito infection experiments to be carried out by our group, a lot of time was a spent troubleshooting experimental procedure. The main challenges were in keeping plaque assays plates free from fungus contamination and also collecting saliva samples from individual mosquitoes at the different time points. This was done in the glove box and proved to be very cumbersome. Only twenty samples could be carried out in one day. Fungus contamination was overcome by addition of Fungizone (amphotecerine B) in the virus diluent media used to freeze mosquito samples and also in the Minimum essential medium used to overlay the plaque assay plates. Preparation of both media can be found in chapter 2 of this thesis.

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Appendix I Attempt to establish laboratory colony of British mosquitoes

The need to colonize British mosquitoes is of extreme importance in order to establish working infection models. Attempts to colonize British mosquitoes have proved very challenging. However this is consistent with previous attempts to colonize these mosquitoes (Michael Service, Pers.Comm.) The largest barrier to colonizing British mosquitoes is overcoming their eurygamic behaviour i.e. preference for open spaces. In the quest to colonize British mosquitoes, several approaches have been used. This has included, increasing the light cycles (16 hours light: 8 hours dark), rotating mosquito cages, shifting mosquitoes into large cages, putting dark backgrounds under mosquito cages and using stroboscopic blue light to stimulate mating. In the UK, there is only one well-known stenogamous (i.e. mating in confined spaces) mosquito, *Culex molestus*. This mosquito species is the urban from of *Culex pipiens pipiens* and is a likely bridge vector for WNV since it feeds on both birds and humans. A source to provide this species has been identified but has not materialized yet. While, mosquito colonies have been established with eurygamous population in other parts of the world, this has usually been accomplished with great difficulty and painstaking effort. Further efforts are continued to establish British mosquitoes.

8.1.1 Establishment of laboratory colony of *Ochlerotatus cantans*

Ochlerotatus cantans mosquitoes were collected from the woods in Leahurst, Neston and reared as described in the materials and methods section above. Whatman filter paper was provided as the oviposition substrate. Four eggs were laid after two weeks. The eggs were collected and dried for three days and then immersed in water for hatching (Service, 1970; Andreadis, 1990). After 24hrs none of the eggs hatched. This was repeated three times. To determine the viability of the eggs, a 3% sodium hypochlorite solution was prepared and the eggs submerged for 45 minutes (Impoinvil *et al.*, 2007). They were observed under a microscope for presence of embryo. None of the eggs had any visible signs of segmentation, which would suggest embryo development. Eggs of *Aedes aegypti* were also tested for viability;

this was done as a positive control experiment. These eggs were found to be viable as indicated by the visibility of egg segmentation as shown in figure 22. These mosquitoes were kept for three months with blood meals provided every week. During the whole period only twelve eggs were collected and none of them hatched. Another attempt to colonize the mosquitoes was not possible since the availability of mosquitoes was low.

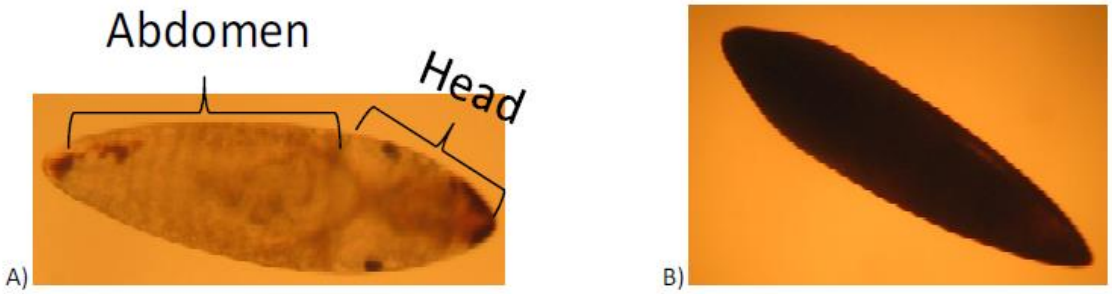

Figure 21: A) Segmentation of Head and abdomen in *Aedes aegypti* egg. B) No segmentation visible in *Ochlerotatus cantans* egg**.**

8.1.2 Establishment of laboratory colony of *Ochlerotatus detritus*

Ochlerotatus detritus mosquitoes were collected from the salt marsh water ponds in Parkgate, Cheshire and reared as described in the materials and methods section above. Whatman filter paper was provided as the oviposition substrate. Two eggs were laid after one week. Since the number of eggs was very low, the oviposition substrate (wet Whatman filter paper) was substituted with cotton wool soaked in water. With this, the number of eggs increased slightly. Eggs that were laid were collected and dried for three days and then immersed in water for hatching. After 24hrs none of the eggs hatched. The eggs were then dried again and immersed several times (Service, 1970; Andreadis, 1990). Hatching was not successful. To determine the viability of the eggs, a 3% sodium hypochlorite solution was prepared and the eggs submerged for 45 minutes. They were later observed under a microscope for presence of the embryo. None of the eggs had any visible sign of segmentation as shown in figure 22 above. Some of the

eggs did not bleach after 45 minutes or longer and so these were dissected under a microscope to observe for any cell differentiation. A positive control for egg viability was done using *Aedes aegypti* eggs. Mosquitoes were kept for seven weeks with blood meals provided every week. During the whole period about 30 eggs were collected in total and none of them hatched.

8.1.3 Establishment of Laboratory colony of *Culex pipiens pipiens*

Culex pipiens pipiens mosquitoes were collected from the cattle water troughs in Leahurst, Neston. Mosquitoes were reared as described in the materials and methods section above and maintained in a 30 × 30 × 30 cm cage to increase the density and encourage free mating. Cages were also rotated every evening. Water in small plastic cups, some with hay infusion and others with ordinary tap water, were provided for oviposition. After providing a blood meal two egg rafts were laid but none of the eggs hatched. Mosquitoes were then transferred to a 60 × 60 × 60 cm cage according to a study by Krishnan, 1964 and the same procedure followed to obtain eggs. Eggs were obtained but failed to hatch. Since *Cx pipiens pipiens* are eurygamous (need open spaces for mating), a bigger walk-in cage (100 × 50 × 150cm) was fabricated and all *Culex pipiens pipiens* mosquitoes transferred into it. A blue stroboscopic light was also provided to encourage the mosquitoes to copulate naturally under laboratory conditions according to a study by Lardeux and others, 2007. Several blood meals were provided and eggs laid but none of the eggs were viable hence none hatched.

Figure 22: A walk-in cage (100 × 50 × 150cm) fabricated in an attempt to establish a laboratory colony of *Culex pipiens pipiens*

Appendix II Tengah complete nucleotide sequence

```
LOCUS       Tengah                 10988 bp    RNA     linear       30-SEP-
2014
DEFINITION  Japanese encephalitis virus.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Japanese encephalitis virus
  ORGANISM  Japanese encephalitis virus
            Unclassified.
REFERENCE   1  (bases 1 to 10988)
  AUTHORS   Mackenzie-Impoinvil,L.M., Galbraith,S.E., Radford,A.D.,
            Impoinvil,D.E., Takasaki,T., Kurane,I., Fooks,A.R., Baylis,M.
and
            Solomon,T.
  TITLE     Molecular characterization of Tengah strain of Japanese
            encepahlitis virus and mosquito infection studies
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 10988)
  AUTHORS   Mackenzie-Impoinvil,L.M., Galbraith,S.E., Radford,A.D.,
            Impoinvil,D.E., Takasaki,T., Kurane,I., Fooks,A.R., Baylis,M.
and
            Solomon,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-SEP-2014) Clinical Infection Microbiology and
            Immunology, Brain Infections Group, Institute of Infection and
            Global Health, 8 West Derby Street, Liverpool, Merseyside L69
7BE,
            United Kingdom
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: Vector NTI v. 5.5
            Sequencing Technology :: 454
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organism="Japanese encephalitis virus"
                     /mol_type="genomic RNA"
                     /isolate="Tengah"
                     /host="human"
                     /country="Singapore"
                     /collection_date="1952"
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