DISSERTATION

JAPANESE ENCEPHALITIS VIRUS: PATHOGENESIS AND IMMUNITY

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANGELA M. BOSCO-LAUTH, ENTITLED JAPANESE ENCEPHALITIS VIRUS: PATHOGENESIS AND IMMUNITY, BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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Japanese encephalitis virus (JEV) is a vector-borne disease of Asian origin that has the potential to spread into temperate regions across the globe. The recent incursion of the virus into Northern Australia illustrates its ability to replicate in different vectors and has led to an increase in the need for disease surveillance in the U.S. and other locations. The focus of this dissertation was to investigate the potential of some common North American mosquitoes to transmit JEV and to study the pathogenesis and immunity in animal models, namely horses and hamsters.

Four mosquitoes were tested for JEV vector competence: *Culex tarsalis, Culex pipiens, Aedes aegypti*, and *Aedes albopictus*. Both *Culex* species and *Aedes aegypti* can become infected with the virus through an artificial blood meal and will replicate and disseminate the virus over several weeks time. Mosquitoes with a disseminated infection can then transmit the virus back into blood meal during feeding, thus demonstrating the capacity to infect a host while feeding.

Horses, like humans, are incidental hosts for JEV and can develop severe neurological deficits and in some cases die from an infection. Pathogenesis of the virus in horses as well as their potential role in the virus replicative cycle has not been clarified experimentally. The experiments in this thesis revealed that *Aedes aegypti* mosquitoes

can transmit JEV to horses and that horse infection results in low level viremia. Mosquitoes allowed to feed on viremic horses failed to become infected, thus confirming the likely role of horses as dead-end hosts. Three of six horses developed viremia, two with mild pyrexia, but no clinical or post-mortem pathological findings suggested the development of encephalitis.

Pre-existing immunity to related flaviviruses such as West Nile virus (WNV) is thought to produce some protective effect against JEV infections. Prior immunization or infection with live flaviviruses within the JEV serocomplex of viruses, including WNV and St. Louis encephalitis virus (SLEV), protected Golden Syrian hamsters from JEV infection. Immunization with recombinant or DNA vaccines for WNV has a reduced protective effect, suggesting that humoral immunity is best derived from active viral infections.

The results of these studies provide some insight into potential vectors of JEV in North America and elucidate some of the mechanisms of viral pathogenesis and immunity in mammals. The overall purpose is to demonstrate a need for emerging disease surveillance for JEV in the U.S. and to try to predict what amount of spread could be reduced by prior immunity to WNV or SLEV.

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CHAPTER 1

LITERATURE REVIEW

Japanese encephalitis virus (JEV) is the causative agent of Japanese encephalitis, a disease that has plagued Southeast Asia and surrounding areas for the past two centuries. The etiologic agent is a small, single-stranded RNA virus that can replicate in a variety of vertebrate hosts and is spread via the bite of infected mosquitoes. Typically, wildlife reservoir hosts are unaffected by the virus, but humans and horses can develop symptomatic infections ranging from mild febrile illness to severe meningoencephalitis and sometimes death. Human survivors are often left with permanent neurological disorders, making the impact of the disease all the more devastating. The increase in spread and reemergence of many vector-borne diseases with changes in climate, human movements, and other natural factors has led to an increase in the research involving some of the more clinically important diseases, which include JEV.

History of Japanese Encephalitis Virus

Japanese encephalitis virus is a relatively new virus thought to have emerged from ancestral viruses in the Malay Archipelago as recently as the late 19th century (Solomon et al., 2003). Human outbreaks of illness thought now to be Japanese encephalitis date back to 1871. The first large epidemic occurred in Japan in 1924 and the first virus isolation was made in 1933 when inoculation of brain emulsion from an encephalitic

patient into monkeys led to neurological disease and death similar to what occurs in humans (Hayashi, referenced by Halstead and Jacobson, 2003; Erlanger et al., 2009). In the summer of 1935 another Japanese outbreak of more than 5000 cases was documented with a mortality rate of approximately 33%. It was during this epidemic that scientists began to study the virus in vivo and define it as the transmissible agent responsible for summer encephalitis (Kasahara et al., 1936). Several groups of scientists inoculated homogenates of brain from clinically diagnosed, fatal cases of summer encephalitis into monkeys, rabbits, guinea pigs and mice. While rabbits and guinea pigs were found to be refractory to infection, monkeys and mice inoculated intracerebrally were found to be susceptible based on mortality and clinical symptoms; subsequent passage of brain matter from these animals into additional generations yielded similar results, with more lethality in the mouse passaged virus compared to the monkey. (Kasahara et al.,1936; Kawamura et al., 1936; Taniguchi et al., 1936). Through these studies investigators were also able to verify that virus circulated in the blood and could be isolated from a variety of organs. In 1938 the virus was isolated from *Culex tritaeniorhynchus* mosquitoes, thus elucidating the mechanism of hematogenous spread of the virus from infected mosquitoes to susceptible hosts (Mitamura et al., 1938).

During the 1950's a group of researchers led by Buescher and Scherer from the U.S. Army Medical General Laboratory in Japan elucidated the complex vector-host relationship of the virus in a series of elegant studies spanning the years between 1952 and 1958 in the Kanto plain of Japan (Buescher et al., 1959b). The two major study sites were the Shinhama heronry and the Sagiyama heronry along with the surrounding areas. Within the heronries the major bird species were ardeids such as black-crowned night

herons, plumed egrets, little egrets, cattle egrets and great egrets. The surrounding areas had high human densities (10,000 people per square mile) and pig populations of 500-1000 pigs per 3 mile radius. Mosquito traps were set up in the heronries and surrounding areas and the primary species collected and tested were *Cx. tritaeniorhynchus.* It was later revealed that both virus and antibody were present in ardeid birds with an incidence of viremia as high as 47% during the peak summer months of July to September (Scherer et al., 1959a). Additionally, experimental tests on *Cx. tritaeniorhynchus* revealed that mosquitoes could acquire virus from infected birds and also pass it on to naïve birds (Gresser et al., 1958). From these studies came the first understanding of how the virus cycled and was maintained in nature.

The role of pigs as natural amplifying hosts was also elucidated during the studies conducted by the U.S. Army Medical General Laboratory in Japan. Swine are present in high numbers in the Kanto plain around Tokyo and the high annual population turnover creates large numbers of susceptible hosts. These observations were coupled with a higher than expected rate of fetal death and abortions in shoats and the detection of virus and antibody from multiple serum samples to conclude that pigs are indeed a potential source for virus propagation (Gresser et al., 1958; Scherer et al., 1959d). From here, the link to the incidental hosts, humans and horses, was discovered. During years of peak virus activity, such as extended rainy seasons followed by warmer weather, where mosquito populations were at their peak, the natural hosts, birds and pigs, amplify the virus to transmissible levels in their blood. Due the close proximity of humans and other livestock such as horses to these reservoir hosts, especially pigs, infected mosquitoes will feed on all available hosts and thus cause outbreaks of disease. Humans and horses were

deemed incidental hosts by those same scientists, because infection is not as frequent and isn't seen until after the appearance of the virus in birds, swine and mosquitoes (Scherer et al., 1959c). Later studies confirm these observations, but from these classical investigations came most of the initial understanding of JEV enzootic and epidemic cycles.

Geographic Features of Japanese Encephalitis Virus

Japanese encephalitis virus is found throughout Asia and is spreading into the Pacific islands and Australia. The first clinical case was recorded in Japan in 1924 and since then it has become endemic in nearly every mainland country in Asia including Bangladesh, Cambodia, China, India, Indonesia, Japan, North Korea, South Korea, Laos, Malaysia, Myanmar, Nepal, Papau New Guinea, Pakistan, the Philippines, Singapore, Sri Lanka, Thailand, Vietnam, and most recently Australia. (Erlanger et al., 2009). The first isolations of the virus in Papua New Guinea and the Torres Strait of Northern Australia took place in 1995 (Hanna et al., 1996); mainland Australia, however, has had only sporadic cases and does not appear to harbor the virus in a sylvatic cycle. (Mackenzie et al., 2002a). Isolated incidences of Japanese encephalitis have been reported in Russia, and according to the World Health Organization, countries such as Afghanistan, Bhutan, Brunei Darussalam, and the Maldives are at risk but no cases have been reported in the last 30 years. Nonetheless, this trend towards emergence in new locations is concerning and has been partially attributed to alterations in vector habitats such as deforestation and irrigation, increased pig breeding throughout Asia, and increased travel and commerce.

Additionally, natural factors such as windblown mosquitoes and climate change could facilitate geographic spread of the virus.

The normal transmission cycle is at its peak during the late summer months, but in more tropical climates disease can be acquired as early as April and as late as October. Maintenance of JEV in nature during the non-vector season is made possible by overwintering in mosquitoes and subsequent vertical transmission to offspring (Rosen, 1986). From there, infected mosquitoes feeding on ardeid birds and swine are able to perpetuate the cycle by passing the virus on to new vectors and hosts (Endy and Nisalak, 2002; Ellis et al., 2000).

Molecular Characterization of Japanese Encephalitis Virus

Japanese encephalitis virus is a positive sense, single-stranded RNA virus belonging to the *Flaviviridae* family, and is in the JEV serocomplex of flaviviruses (Mackenzie et al., 2002b). The genome is approximately 11kb in length and has a poly(A) tail and a 5' cap. There are 3 major structural proteins: the nucleocapsid (C) protein, the membrane protein (M) and the envelope glycoprotein (E) (Sumiyoshi et al., 1987). The E protein is the major inducer of neutralizing antibodies and also functions as a hemagglutinin, facilitating binding of virus to host cell receptors (Chen et al., 2004). There are seven nonstructural (NS) proteins encoded in the viral genome, some of which may play a role in host antibody response as well, although these proteins are poorly understood. When sequencing JEV, the premembrane and envelope proteins are commonly used to determine genotype and strain as these regions in the genome have the most nucleotide variability (Mangada et al., 1999). Recent research implicates the NS1

protein in viral neuroinvasiveness (Melian et al 2010), and this protein may also play a role in host immune modulation by activating the complement pathway (Pierson et al 2009).

There are 5 known genotypes of JEV. Genotypes I and III are predominantly associated with epidemic encephalitis whereas genotypes II, IV, and V are thought of as sylvatic strains. The virus itself is a recently diverged member of the flaviviruses, with its first isolate thought to be only approximately 130 years old (Solomon et al., 2003). The fifth genotype was most recently discovered in Muar, India only a few years ago and is most likely a divergent from the sylvatic genotype IV (Uchil et al., 2001). All five genotypes can be found in Malaysia and Indonesia, suggesting that this is where the most recent divergence from ancestral flaviviruses to modern JEV originated (Solomon et al., 2003). Presently, all genotypes are still endemic in Malaysia and Indonesia but more temperate regions favor the circulation of the more recent genotypes, which are I, II, and III (Pond et al., 1954; Ompsunggu et al., 2009).

Prior to the mid 1970's, most JEV isolates from humans and mosquitoes during epidemics belonged to the genotype III group. Recently, however, there has been a dramatic shift from the predominant genotype III to genotype I during epidemics. In China, most of the isolates obtained prior to 1971 belonged to genotype III but after 1974 virus isolations have been dominated by genotype I strains (Wang et al., 2007). In the 1980s the same switch was observed in Thailand, thus, most isolates obtained from patients and mosquitoes during the 1990's and later have been characterized as genotype I (Ali et al., 1995; Nitapattana et al., 2008.) Japan experienced a similar shift in the 1990's (Saito et al., 2007; Yoshida et al., 2005). In Vietnam the genotype shift occurred

in the 1990's as well and now, most isolates from humans in that region belong to genotype I (Nga et al., 2004). The same genetic shift has been observed in Korean isolates from pigs in the last decade (Yang et al., 2004). In spite of the seeming shift in epidemic genotypes, genotype III viruses are still circulating throughout Asia. A recent outbreak in Yuncheng, China in 2006 involving 66 human patients revealed both genotypes I and III were responsible for causing disease, which is the first account of an outbreak involving two genotypes (Doi et al., 2007). It is not known at this time whether or not current human vaccines are equally effective in protecting against infection with all genotypes, although major deficits in this regard appear unlikely. Although pathogenicity doesn't seem to change, it is thought that receptor binding or affinity is enhanced by amino acid changes in the genotype I viruses (Ali et al., 1995; Wang et al 2007), which may be a factor driving recent shifts in genotype prevalence.

JEV is closely related to 10 other flaviviruses within the same serocomplex, including West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV), Kunjin virus, Usutu virus and a few other less medically relevant tick-borne subtypes (Endy and Nisalak, 2002). Historically, viruses within the JE serocomplex have held geographically distinct niches: JEV in Southeast Asia, WNV in Western Asia and Northern Africa, MVEV in Australia, and SLEV primarily in the U.S. (Gaunt et al., 2001). However, recent emergences of JEV farther South into Australia and west past India, as well as WNV expanding into Europe, North and South America has led researchers to conclude that there are no real ecological boundaries among these arboviruses. Cross-protective immunity, however, may prevent further spread, but could also complicate diagnostic virus identification.

Mosquito Vectors of Japanese Encephalitis Virus

In 1936 JEV was isolated from a *Culex tritaeniorhynchus* mosquito, a species of Culicine mosquitoes that feeds predominantly at night on birds and mammals (Mitamura et al., 1938). In 1946, laboratory transmission of JEV to mice was demonstrated in three species of mosquito native to Guam: *Cx. quinquefasciatus, Ae. vexans,* and *Cx. jepsoni* (Hodes, 1946). In 1949 laboratory transmission of JEV was demonstrated in *Cx. tritaeiorhynchus* and *Cx. pipiens* var. *pallens,* two species native to Japan (Hammon et al., 1949). Since then, JEV has been isolated from a large number of other field caught species, including but not limited to *Cx. vishnui, Cx. gelidus, Cx. p. pallens, Cx. p. pipiens, Cx. quinquefasciatus, Cx. annulirostris, Cx. sitiens, Cx. rubithoracis, Ar. subalbatus, Ae. albopictus, Ae. subpictus, Ae. vexans nocturnus, Anopheles hyrcanus,* and *Mansonia uniformis* (Van Den Hurk et al., 2003b; Weng et al., 1999, Weng et al., 2005). Laboratory transmission of JEV has been demonstrated in a large number of mosquito species as well, summarized in Table 1. Typically, *Culex spp* are the most efficient vectors, although lower levels of infection and transmission have been observed in *Aedes, Anopheles, Culiseta, Ocleratatus,* and *Mansonia* species. (Turell et al., 2004; Turell et al., 2006; Van den Hurk et al., 2003b; Weng et al., 1997; Weng et al., 2000; Thenmozhi et al., 2006; Hemmerter et al., 2007; Leake et al., 1987; Reid et al., 2006). The best vectors, such as *Cx. tritaeniorhynchus,* can become infected by feeding on blood with virus titers as low as 1log10 pfu/ml (Soman et al 1977, Takahashi et al., 1976). What makes a vector highly efficient, however, is not just laboratory transmission and infection rates. Other factors, such as vectorial capacity and host preference are extremely important as well. As a point in fact, *Cx. annulorostis* moquitoes, which are most

commonly implicated in Australian arboviral diseases, are not considered exceptional vectors of JEV because they typically feed on marsupials, and much more rarely on birds and swine (Van Den Hurk et al., 2003a). In addition, biodiversity within a mosquito species can influence viral infectivity. *Cx. annulorostris* and *Cx. palpalis* exhibit genetic changes in different geographic locations that alter the ability of these mosquitoes to disseminate JEV (Hemmerter et al., 2007). It is possible that mosquito biodiversity and host preference contribute to the lack of an endemic JEV cycle on mainland Australia.

Feeding patterns and climate greatly influence the transmission cycle of JEV. Because *Cx. tritaeniorhynchus* feed primarily at night and often feed on the two major reservoirs, swine and birds, these mosquitoes are ideal vectors of the virus. Humans in close proximity to water birds and pigs and who live in the warmer and more humid areas are at the greatest risk of infection. In Asia, rice paddies and other long-standing water sources provide the best environments for mosquitoes and make vector control an unlikely possibility for decreasing JEV presence in nature (Endy and Nisalak, 2002; Rosen, 1986).

Mosquitoes can obtain virus via feeding on an infected host, vertical transmission, infection at oviposition and sexual transmission. Overwintering has been observed in *Cx. tritaeniorhynchus* and *Cx. pipiens. Cx. tritaeniorhynchus, Cx. bitaeniorhynchus,* and *Anopheles subpictus* are all capable of vertical transmission as evidenced by viral antigen in ovarian cells and infected male progeny (Thenmozhi et al., 2006; Soman et al., 1977; Weng et al., 1999). Both vertical transmission and overwintering are thought to maintain the virus during the winter months. These findings imply that the mosquito vector is the most probable source of viral maintenance. Migratory birds could also play a role in

moving or re-introducing JEV, but at this point there is no evidence to support this possibility.

Perhaps the first study demonstrating vector competence in North American species was carried out between the years 1943-1945 in the United States (Reeves, Hammon, 1946). These scientists tested ten native North American mosquitoes native to either California or Washington for JEV infection and transmission in the laboratory. Cotton pledgets soaked in blood mixed with virus (Nakayama strain) were placed on top of screened mosquito cartons and mosquitoes were allowed to feed for approximately 30 minutes. Infection was confirmed by inoculating a suspension of ground mosquitoes into suckling mice and if the mice showed signs of encephalitis and died, mosquitoes were considered positive. Transmission was demonstrated by feeding infected mosquitoes on mice and observing mice for symptoms of encephalitis. If symptoms were detected, the mouse was euthanized and brain suspensions were inoculated into three suckling mice to check for encephalitis and death. In all, seven of the ten species transmitted virus and nine of ten species had individuals that tested positive even in the absence of transmission. Most transmission events occurred 3-16 days post infectious blood meal. The following species transmitted JEV in these investigations: *Cx. pipiens* Linn, *Cx. quinquefasciatus, Cx. tarsalis, Ae. dorsalis, Ae. nigromaculis, Culiseta incidens,* and *Culiseta inornata.* In addition to these species, *Ae varipalpus* [now sierrensis] and *Anopheles freeborni* both tested positive, but did not transmit the virus. *Ae. vexans* did not become infected with JEV in the laboratory. In 1948 another species was added to the list of potential North American vectors when laboratory transmission of JEV to mice was demonstrated in *Cx. quinquefasciatus* from the east coast (Hurlbut et al., 1948).

Although these reports did not provide details regarding infectious dose and dissemination rates, they brought attention to the potential for emerging vector-borne diseases in the U.S.

Since the initial investigative studies in the 1940's, there was a relative dearth in vector competence studies for flaviviruses until the late 1990's. Two important events occurred that sparked a renewed interest in researching JEV and related WNV. First, JEV was isolated as far south as the Torres Strait of Australia (Hanna et al., 1996), and second; WNV was identified in New York and swept across the U.S. over a period of just 4 years. The flurry of research that followed has expanded the list of potential vectors for both viruses and provided valuable information about infectious dose as well as infection and dissemination rates. Table 1 summarizes some of the most explicit and comprehensive studies involving vector competence for both JEV and WNV, with primary focus on mosquitoes native to Australia and the U.S.

Table 1.1: Vector Competence of JEV and WNV

Author	Species	Infection rate (%)	Dissemination rate (%)	Transmission rate (%)	# tested	Method	Virus	Titer (log units)
Turell, 2006a	*Cx. pipiens pallens*	64-70	7 – 57	100 (6 tested)	134	Live chicks	WNV	6.8/ml
	Cx. tritaeniorhynchus	100	100	100 (1 tested)	13	Live chicks	WNV	6.8/ml
	Cx. pipiens pallens	0	0	NT*	40	Live chicks	JEV	4.3/ml
	Cx. pipiens pallens	6	0	NT	32	Live chicks	JEV	5.2/ml
	Cx. tritaeniorhynchus	100	80	67 (6 tested)	10	Live chicks	JEV	4.3/ml
	Cx. tritaeniorhynchus	100	93	NT	14	Live chicks	JEV	5.2/ml
Turell, 2006b	*Cx. pipiens pipiens*	47-56	25-26	8 (37 tested)	142	Live chicks	JEV	4.5-5.4/ml
	Cx. pipiens pipiens	89-100	76-87	47 (15 tested)	140	Live chicks	WNV	6.3-7/ml
Van Den Hurk, 2003	*Cx. annulirostris*	78-100	6 – 78	24-81	90	Glass membrane	JEV	4.5/ml
	Cx. sitiens	83-92	6 – 33	7 - 67	90	Glass membrane	JEV	4.5/ml
	Cx.quinquefasciatus	98	28	50	51	Glass membrane	JEV	4.5/ml
	Ochlerotatus vigilax	19-39	18-39	0	75	Glass membrane	JEV	4.5/ml
	Oc. notoscriptus	27	8	27	48	Glass membrane	JEV	4.5/ml
	Ae. aegypti	27	17	NT	60	Glass membrane	JEV	4.5/ml
	Oc.notoscriptus	20	20	NT	5	Glass membrane	JEV	4.5/ml
	Oc. normanensis	0	0	NT	1	Glass membrane	JEV	4.5/ml
	Oc. purpureus	100	0	NT	2	Glass membrane	JEV	4.5/ml
	Ma. septempunctata	67	54	NT	24	Glass membrane	JEV	4.5/ml
	Ma. uniformis	100	100	NT	1	Glass membrane	JEV	4.5/ml
	Ve. carmenti	0	0	NT	2	Glass membrane	JEV	4.5/ml

*NT= not tested

Table 1.1: Vector Competence of JEV and WNV (continued)

Author	Species	Infection rate (%)	Dissemination rate (%)	Transmission rate (%)	# tested	Method	Virus	Titer (log units)
Weng, 1997	*Ae. albopictus*	2.03-4.98 MID50**	NT	NT	?	Hanging drop	JEV	5/mosq
	Cx. tritaeniorhynchus	1.02 MID50	NT	NT	?	Hanging drop	JEV	5/mosq
Weng, 2000	*Cx. tritaeniorhynchus*	1.02 MID50	NT	NT	?	Hanging drop	JEV	5.5/mosq
	Cx. pipiens molestus	2.83 MID50	NT	NT	?	Hanging drop	JEV	5.5/mosq
Vanlandingham, 2008	*Cx. quinquefasciatus*	59-100	86-100	NT	162	Hemotek	WNV	5.22-8.22/5ul
	Cx. quinquefasciatus	0-90	86-100	NT	327	Hemotek	WNV	0.22-4.65/5ul
Turell, 2005	*Cs. melanura*	0-26	0-11	0	21	Chicks	WNV	6.3-7.1/ml
	Oc.canadensis	13-50	0-13	0-100 (of 1)	32	Chicks	WNV	6.3-7.1/ml
	Oc. cantator	22	18	0	51	Chicks	WNV	6.3-7.1/ml
	Ps. ferox	29-33	0-12	0	41	Chicks	WNV	6.3-7.1/ml
	Ae. vexans	44	19	11	73	Chicks	WNV	6.3-7.1/ml
	Oc. triseriatus	31	17	12	29	Chicks	WNV	6.3-7.1/ml
Sardelis, 2001	*Cx. nigripalpus*	29-84	0-12	0-10	266	Chicks	WNV	4.6-6.8/ml
	Cx. quinquefasciatus	50-94	0-22	0-20	124	Chicks	WNV	5-7/ml
	Cx. restuans	100	55	55	11	Chicks	WNV	6.6/ml
	Cx. salinarius	95	60	34	20	Chicks	WNV	6.6/ml
	Cq. perturbans	18	9	2	11	Chicks	WNV	6.6/ml

**MID50= minimum infectious dose for 50% of the tested mosquitoes

Table 1.1: Vector Competence of JEV and WNV (continued)

Author	Species	Infection rate (%)	Dissemination rate (%)	Transmission rate	# tested	Method	Virus	Titer (log units)
Vanlanding-ham, 2007	*Ae. albopictus*	13-69	66-100	NT	50	Blood meal	WNV	5.72 $TCID_{50}$/5ul
	Ae. aegypti	73	86	NT	30	Blood meal	WNV	6.43 $TCID_{50}$/5ul
	Cx.quinquefasciatus	100	100	NT	30	Blood meal	WNV	5.22 $TCID_{50}$/5ul
Goddard, 2002	*Cx. tarsalis*	74-100	NT	10-100	185	Hanging drop	WNV	7/ml
	Cx. quinquefasciatus	8-86	NT	0-52	373	Hanging drop	WNV	7/ml
	Cx. pipiens	100	NT	0-71	48	Hanging drop	WNV	7/ml
	Cx. stigmatasoma	67-77	NT	0-19	63	Hanging drop	WNV	7/ml
	Cx. erythrothorax	100	NT	33-64	40	Hanging drop	WNV	7/ml
	Oc. dorsalis	41-50	NT	13-34	59	Hanging drop	WNV	7/ml
	Oc. melanimon	46-48	NT	10-20	110	Hanging drop	WNV	7/ml
	Oc. sierrensis	5 -14	NT	3-6	90	Hanging drop	WNV	7/ml
	Ae. vexans	32	NT	23	22	Hanging drop	WNV	7/ml
	Culiseta inornata	75	NT	21	28	Hanging drop	WNV	7/ml
	Cx. tarsalis	0-36	NT	0-82	141	Hanging drop	WNV	4.9/ml
	Cx. quinquefasciatus	0-58	NT	0	205	Hanging drop	WNV	4.9/ml
	Cx. pipiens	23-36	NT	0-60	60	Hanging drop	WNV	4.9/ml
	Cx. stigmatasoma	69	NT	34	29	Hanging drop	WNV	4.9/ml
	Cx. erythrothorax	15-67	NT	0-30	79	Hanging drop	WNV	4.9/ml
	Oc. dorsalis	3 – 4	NT	0-4	54	Hanging drop	WNV	4.9/ml
	Oc. melanimon	0-3	NT	0-2	110	Hanging drop	WNV	4.9/ml
	Oc. sierrensis	0-4	NT	0	55	Hanging drop	WNV	4.9/ml

Avian Infections with Japanese Encephalitis Virus

One of the first experiments on the susceptibility of wild birds to JEV took place in 1951 by Hammon and colleagues (Hammon et al., 1951). These scientists found that tri-colored blackbirds and house finches develop significant virus titers following infection while English sparrows and chickens were less competent hosts, although still capable of infection. Over the next decade, and in large part thanks to the work of Buescher (1959a) and Scherer (1959a), it was determined that the most commonly infected bird species in Asia are the night herons, plumed egrets, lesser egrets, pond herons and cattle egrets. In endemic areas these birds had a high seroprevalence and were shown to replicate virus to a high enough titer in their blood that mosquitoes can acquire it from blood feeding, which for competent vectors can be as low as 1 log pfu/ml of virus (Takahashi et al., 1976; Soman et al., 1977; Rodrigues et al., 1981). *Cx. tritaeniorhynchus* mosquitoes can become infected by feeding on viremic Cattle egrets and Pond herons and can then transmit the virus to naïve birds (Soman et al., 1977). Domestic ducks and chickens often have high seroprevalence in areas of JEV transmission and both ducks and chickens have been shown to amplify virus to a transmissible level experimentally (Dhanda et al., 1977). Domestic chickens are also susceptible to JEV infection, although their age may play a significant role in whether or not they develop viremia; particularly, young chicks are much more likely to develop disseminated infection than mature adult chickens when fed upon by infected mosquitoes *(Cx. bitaeniorhynchus)* (Banerjee et al., 1987). Maternal antibody transfer has been demonstrated in wild caught plumed egrets, lesser egrets, and Bblck-crowned night heron nestlings, suggesting that fledglings may have some protection against JEV (Scherer et

al., 1959a). Interestingly, birds in general don't appear to have any adverse clinical effects following infection, which makes them ideal reservoir hosts for disease.

While seroprevalence is high in many wild caught bird species, little is known about the role of non-ardeid bird species in regards to JEV competence. Passerines such as tri-colored and red-winged blackbirds, European sparrows, and house finches can all be infected experimentally with JEV (Hammon et al., 1951; Nemeth et al., 2009). Passerines seem to be primary source of virus for several other viruses in the JEV serocomplex, including WNV and SLEV, and typically, infection does not lead to clinical symptoms (Stamm et al., 1966). The major exception to this observation is the role corvids play in the WNV cycle in North America where the virus amplifies to incredibly high titers and often kills the birds within a matter of days (Brault et al., 2004). While this is certainly not a desirable effect ecologically, it is a good predictor of WNV presence within an area prior to human and horse infections. In the case of JEV and SLEV, the lack of clinical signs in the reservoir bird populations implies that incidental hosts such as humans are often the only indicators of outbreaks. Therefore, serosurveillance of known competent avian species may aid in the prevention of outbreaks in human populations.

Japanese Encephalitis Virus Infection in Swine

The role of pigs in JEV infection was initially defined as sentinel animals in which the presence of JEV antibody indicated virus activity in the area (Scherer et al., 1959b). Scherer and colleagues were the first to seriously delve into the more important role of pigs as reservoir hosts. Starting in Japan in 1956, this group took serum samples

from commercially farmed pigs as well as sentinel pigs housed in mosquito traps and tested the sera for antibodies and presence of virus from April through September. They were able to determine that maternal antibody in young pigs was typically high during the early months of the summer season but waned into August and September; at least two-thirds of swine were susceptible to infection by early August. Because of the high turnover of pig populations and annual births, swine are unable to maintain a high enough level of herd immunity (approx. 80% of the population) to prevent infection and are thus good reservoirs of disease. These studies also found that viremia occurs in pigs 1-2 days following infection and virus multiplies to high enough titers and persists long enough to infect biting mosquitoes. (Scherer et al., 1959b; Sazawa et al., 1968). In 1958, it was demonstrated that infected pigs could be fed upon by *Cx. tritaeniorhynchus* mosquitoes and that these mosquitoes could then transmit the virus to susceptible pigs (Gresser et al., 1958). Vaccination using a modified vaccinia virus encoding the prM and E genes of JEV as well as DNA vaccines can produce sufficient neutralizing antibody in pigs, but the vaccines require boosters and are not agriculturally cost effective as the virus does not cause enough disease in pigs to warrant immunizations (Nam et al., 2002). Serological surveillance has consistently demonstrated that pigs are frequently exposed to JEV. In some locations, such as Hokkaido, Japan, seroprevalence can reach 100%, which far surpasses the serological responses of other domestic animals such as goats, horses, cattle, rabbits, dogs, and chickens (Sazawa et al 1968). Surveillance in Nepal revealed a similar pattern; pigs are almost universally antibody positive, ducks and horses between 5 and 50% (Pant et al., 2006).

Clinically, infected mature pigs are asymptomatic, but pregnant sows can deliver still-born or developmentally damaged offspring at rates reaching 60% during peak JE transmission seasons (Sazawa et al., 1968). Except for horses, other domestic animals do not seem to be as affected, although there could be a link between the virus and fetal malformations in cattle. In spite of these problems, the high turnover of swine and the cost of vaccination is reason enough for most Asian pig farmers to forego vaccination. This could perpetuate the pig-mosquito-human transmission cycle, which is the most likely scenario for human and horse infection, but there is little evidence to support the idea that pig immunity would quell JEV outbreaks. Indeed, on Badu Island, Australia, pigs were removed to see if the lack of a mammalian host would stop the infection of mosquitoes, but this wasn't the case. Infection rates among various *Culex* mosquitoes did decrease, but the drop was not statistically significant and there were still more than enough mosquitoes carrying the virus to infect incidental hosts (Van Den Hurk et al., 2008). It is likely that the mosquitoes were becoming infected by feeding on nearby herons and egrets, the most common natural source of virus. Therefore, while keeping large populations of pigs farther from domestic dwellings is likely to decrease the chance of transmission to humans, vector control is probably still a better prevention method.

Japanese Encephalitis Virus Infection in Horses

The first experimental infection of horses with JEV was reported in 1964 (Gould et al., 1964), and with the purpose of better understanding their role in disease transmission. *Cx. tritaeniorhynchus* mosquitoes that had fed on JEV-infected chicks were used to transmit JEV to horses. Two horses were fed upon by infected mosquitoes

and both were found to have become viremic via intracerebral inoculation of their sera into suckling mice. Mosquitoes were then fed on one of the viremic horses and after incubation, were allowed to feed on chicks to determine infectivity. One out of 62 attempts was successful. Horse-to-horse transmission via mosquito was also attempted and found to be successful. Of the three horses used in this study, only one developed overt signs of encephalitis but recovered after a week of illness. Since this experiment, little has been done in horses to confirm or refute the claim that they can in fact be reservoirs of disease and while experimental horse-to-horse transmission may be possible, horses are still largely considered incidental hosts due to the infrequency of infection compared to pigs and birds during non-epidemic years and also because horse infection typically doesn't occur until the end of summer at the end of the natural transmission cycle (Scherer et al., 1959c).

Inapparent JEV infections in horses are far more prevalent than clinical presentation of disease, but in those horses that are symptomatic, infection can be quite severe. There are three primary types of disease presentation in horses: transient, lethargic, and hyperexcitable (Ellis et al., 2000). Transient disease is characterized by fever and occasional anorexia with sluggish movement, but horses generally recover from this type. Lethargic type refers to fever, anorexia, nasal discharge, staggering, petechial hemorrhaging on mucous membranes, and sometimes radial paralysis. Horses usually recover within 5 days. Hyperexcitable type is the least common, but is also the most severe. In this stage, horses often have high fevers, are more prone to shying and startling, profuse sweating, muscle twitching, teeth grinding, blindness, and violent

behavior. Some horses recover, but many collapse and die within 24 to 48 hours of presenting with severe symptoms.

Since 1948, horses in Japan have been regularly vaccinated against JEV and therefore the incidence in horses in developed countries is rare (Goto et al., 1976). A formalin inactivated vaccine has been available for decades (Nakamura, 1972), and more recently attenuated vaccines have been marketed as well (Monath et al 2002). These vaccines are highly effective and have few if any side effects in horses. Despite the decreases in horse and human cases in more developed areas, those animals not vaccinated are still very much at risk of acquiring infection. In 2000, two unvaccinated horses in Taiwan were diagnosed with JEV after developing neurological symptoms and dying of encephalitis. Both were determined to have been infected with genotype III viruses (Lian et al., 2002). In Japan in 2003 an unvaccinated horse died of a neurological disorder that was later confirmed as the first genotype I virus implicated in the death of a horse (Yamanaka et al., 2005). More recently, a previously vaccinated horse in Hong Kong died of JEV infection and the isolate was thought to be a hybrid genotype I/genotype II strain (Lam et al., 2005). Since then, veterinary authorities in Hong Kong have implemented a six month booster vaccine regimen for racehorses. These isolated cases coupled with evidence of natural infection of horses occurring as detected by antibodies specific for NS1 protein, which is not present in the vaccine, clearly show that the virus is present at any given point in time during the mosquito season in endemic areas (Konishi et al., 2006). For horses, like people, the best method of prevention is vaccination and vector control.

Japanese Encephalitis Virus Infection in Other Vertebrates

Clearly, birds and swine are the premier reservoir hosts for JEV and the most likely sources for perpetuating the human transmission cycle, yet there are other likely hosts in the sylvatic cycle that could contribute to viral maintenance as well. Rodents typically have low seroprevalence rates and are not thought to play much of a role in transmission cycles (Scherer et al., 1959d, Endy and Nisalak, 2002). Reptiles and amphibians can be experimentally infected and virus can overwinter in these animals and in some insectivorous bats (Oh et al., 1974). Between 1989 and 1997, genotype III viruses were isolated in China from *Rousettus leschenaultia,* a fruit bat, and *Murina aurata,* an insectivore (Wang et al., 2009), and serological reports indicate that multiple bat species and other mammals such as raccoons, raccoon dogs, mongoose, and wild boars are constantly exposed and develop neutralizing antibodies. However, the role of these animals in the transmission cycle, if any, is unknown (Ciu et al., 2008; Ohno et al., 2009; Saito et al., 2008). In Australia, flying foxes may play a role in the sylvatic transmission cycle, as these animals can be used to infect mosquitoes under experimental conditions (Van Den Hurk et al., 2009). Other domestic animals such as cattle, goats, sheep, dogs, bullocks, and buffalo are often seropositive, but virus has not been isolated from these species. Clearly, much work remains to be done to examine what role, if any, these other vertebrates may play in epidemic JEV (Carey et al., 1968; Sazawa et al., 1968).

Small Animal Models for Japanese Encephalitis Virus Infection

Domestic farm animals, such as pigs and horses, and wild birds are all affected by JEV. However, in order to study the virus in vivo, it is convenient to use small animal disease models. Historically, mice have been widely used for laboratory experimentation with flaviviruses. Mice are susceptible to many of these viruses, including JEV and WNV, and often develop frank clinical symptoms of neuropathies similar to what is exhibited by horses and humans (Charlier et al., 2006). However, infected mice often die of disease following infection with either JEV or WNV, which makes long term or immunity studies difficult to carry out. Golden Syrian hamsters, while susceptible to both viruses, have a better survival rate and are therefore a better small animal model than mice for longer duration experiments involving either JEV or WNV (Tesh et al., 2002; Takehara et al., 1975a). Compared to mice, hamsters are more susceptible to peripheral inoculation with JEV, meaning that viremia is evident more rapidly after infection and reaches higher titers. Hamsters typically develop a subclinical infection marked by viremia and antibody production whereas mice require a higher dose for infection but also have a much higher mortality rate (Takehara et al., 1975a). In hamsters, fatalities due to JEV are rare but stillbirth and abortions are quite common, making them an excellent model for JEV in swine. No birth abnormalities were observed in pregnant infected mice (Takehara, 1975b).

Clinical Aspects of Infection with Japanese Encephalitis Virus and Vaccination

While most human JEV infections are asymptomatic, there are 30,000-50,000 cases of clinical disease due to JEV infection reported each year in endemic countries,

and it is likely that these numbers are quite low due to underreporting and poor surveillance (World Health Report, 1996-2008). In reported cases, the case-fatality rate ranges from 5% to 50% with the average somewhere around 25-30%, and the majority of fatal cases are in children under the age of ten (Halstead and Jacobson, 2003). One of the biggest problems with JEV infection regarding the effect on the daily adjusted life years (DALYs) is that >50% of survivors will have permanent neurological sequelae (Ding et al., 2007). Disease occurs within 5-15 days following infectious mosquito bite and symptoms can vary from febrile illness to severe meningoencephalitis, aseptic meningitis, or flaccid paralysis (Solomon et al 1998, 2002). Recent analysis of key cytokines produced in mice during JEV infection indicate that the expression of proinflammatory cytokines, particularly IFN-gamma, are responsible for the clinical progression of disease such that the inflammatory response to the virus in the CNS causes encephalitis and leads to symptomatic disease (Biswas et al., 2009, 2010). The majority of severe cases in endemic areas are found in children or non-natives, as most adults are immune either through natural infection or vaccination (Schneider et al., 1974). However, when the virus spreads to new locations, the susceptible population becomes any exposed individual and disease prevalence is indiscriminate across age groups and gender. Survivors of both asymptomatic and clinical infection are thought to have immunity against future infections, but the duration of immunity of is not clearly defined (Kurane et al., 2002).

Diagnosis of JEV is based upon the observation of clinical symptoms of encephalitis, virus isolation and serological response (Solomon and Vaughn, 2002b). The classic presentation of JE is observation of muscle tremors, hypertonia, rigidity of limbs

and torso, and dull, flat mask-like facial appearance with wide, unblinking eyes (Halstead and Jacobson, 2003). Virus isolation is achieved by inoculating cell culture with potentially infectious samples and evaluating cytopathic effect on the cells. Mosquito cell lines such as C6/36 cells derived from *Aedes albopictus* mosquitoes and mammalian cell lines including Vero African green monkey kidney cells are often useful for in vitro virus growth (Leake et al., 1986). Serological response can be determined either by ELISA or by plaque reduction neutralization assays, both of which test for the presence of antibody against JEV in serum (Burke et al., 1985; Solomon and Vaughn, 2002b). However, closely related flaviviruses can cross-react in neutralization tests and if the antibodies used in ELISAs are not specific enough, it is possible to misdiagnose which flavivirus is responsible for infection. This is particularly problematic in areas where multiple flaviviruses circulate, such as the U.S. were both WNV and SLEV are endemic as well as Australia with JEV, MVEV, and Kunjin virus are all likely culprits in human infection. Newer technology using epitope-blocking and IgM capture ELISAs allow for differentiation between the related viruses based on more specific antibodies that recognize antigens on individual viruses and focus on those proteins that would be present only if the virus had been allowed to replicate, thus discerning between vaccinated patients and those with active infections (Ravi et al., 2006; Kitai et al., 2007). These modern diagnostic methods coupled with the more traditional techniques of neutralization assays and virus isolation could be key in determining which virus is present in an infection, particularly in areas where emergence is a concern.

Fortunately, there are several effective vaccines available for human use and in developed countries such as Japan, these vaccines are responsible for an almost complete

elimination of outbreaks. The most commonly used vaccine is an inactivated mouse brain homogenate, and while it is manufactured and distributed in multiple countries, it has several drawbacks including cost, adverse reactions, variable protection and a requirement for multiple boosters (Monath, 2002). There are a variety of new vaccines in the developmental stage today, including inactivated Vero-cell culture derived vaccines, a chimeric 17D yellow fever virus backbone vaccine, recombinant modified vaccinia vaccine and DNA vaccines (Nam et al., 2002). Especially promising is that vaccination using DNA plasmids encoding the prM and E regions of the JEV genome protected 100% of mice from a lethal infectious dose of virus (Konishi et al., 1998). In spite of the efficacy of current vaccines and the promise of new ones, the burden of disease still rests in underdeveloped areas where vaccination is cost and availability prohibitive. Currently, India is one of the countries with the highest reported incidence of JEV annually (Yadav et al., 2006). Vaccination and vector control are likely the best methods of controlling disease in areas most affected.

Immunity to Japanese Encephalitis Virus

JEV, like many flaviviruses, is thought to confer life-long immunity following natural infection, but the mechanisms of immunity are not well understood. Children receiving two doses of inactivated vaccine have titers between 1:10 and 1:60 after 1 year, and the protection rate of this vaccine is 91% (Hoke et al., 1988). In experimental animal infections using rodents, pigs, and horses, neutralizing antibody shows up as early as 8 days post-infection and is almost always detectable by 14 days post infection, with titers anywhere from 1:10 to 1:640 (Beasley et al., 2004; Lobigs et al., 2003). Duration of

immunity is not known, but based on clinical vaccine trials it is thought that a 1:10 neutralizing antibody titer is sufficient for preventing JEV infection (Monath, 2002).

Humoral immunity to flaviviruses is thought to be the most important mechanism by which hosts combat disease. The E (envelope) protein is the primary target for neutralizing antibody, followed by the prM (premembrane) protein. Additionally, antibodies specific for non-structural proteins (NS) have been found in infected hosts; these antibodies are not thought to neutralize virus directly, but may still play a role in directing the immune response, perhaps through complement activation. The strength of the bond between antibody and antigen is the best indicator of neutralization; high affinity antibodies tend to be able to neutralize virus at relatively low concentrations compared to antibodies that don't bind as strongly (Pierson et al., 2009). Neutralization can happen via several mechanisms such as blocking viral attachment to cellular receptors, inhibition of viral entry into the cell, and triggering complement activation. The lower affinity antibodies may play more of a role in activating Fc-dependent effector functions than direct neutralization (Pierson et al., 2009). Passive transfer of immune serum from mice 2 weeks after primary infection protected against JEV challenge, but if serum was first treated with 2-ME to remove IgM antibodies, the protective response was eliminated. Transfer of immune mouse spleen cells also protected against JEV infection, suggesting that T-lymphocyte production may also have some inhibitory effects on JEV (Mathur et al., 1983). It is possible that there are different regions on the E protein gene that elicit different monoclonal antibody responses, and some of these antibodies may exhibit more neutralizing capabilities than others (Kimura-Kurodan et al., 1988).

Among the JEV serocomplex viruses, there is quite a bit of genetic similarity and this potentiates some cross-protective immunity between these viruses. One of the first observations of this phenomenon was made in the early 1950s in California involving three children and 13 horses with natural St. Louis encephalitis virus infections followed by JEV vaccination. In both species, administration of a single dose of a killed-virus JE vaccine showed an increase in the levels of both complement fixing and neutralizing antibody titers for both SLEV and JEV. Three horses with no prior SLEV immunity were also given the JE vaccine and no neutralizing antibodies could be detected following the vaccine. These results indicate that while the vaccine by itself is not especially effective at inducing an immune response, there is a clear secondary immune reaction that is likely responding to a common antigen present in both viruses (Hammon et al., 1956). In 1992, a series of cross-protection experiments involving WNV and JEV in bonnet macaques showed similar results. Monkeys immunized against JEV all survived intranasal WNV challenge with no clinical symptoms and only 1 of 3 developing viremia. WNV immune monkeys all survived intranasal JEV challenge, but 2/5 showed clinical signs of encephalitis and JE virus was isolated from one brain. Three of four control monkeys infected with either virus with no prior immunity died from viral infection. This appears to confirm previous predictions that secondary infection with a related flavivirus induces a neutralizing antibody response that helps protect the host, although the duration of this immunity is unknown (Goverdhan et al., 1992).

Swine play a very important role in propagating JEV in Asia but are not thought to be involved in any other flavivirus cycles. However, exposure to related viruses and a subsequent immune response could dampen the ability of JEV to spread into a pig

population in areas where other flaviviruses are already endemic. Experimental infection of pigs with JEV followed by WNV and vice versa show that pigs are poor hosts of WNV but that WNV antibodies could lessen the titer of secondary JEV infection (results not significant). In both instances, secondary viral infection with either WNV or JEV boosted the antibody response to both viruses (Ilkal et al., 1994). A similar study investigated the role of cross-protective immunity in pigs using two Australian flaviviruses, Murray Valley encephalitis virus (MVEV) and Kunjin virus (KUNV) in addition to JEV. Primary JEV infection resulted in viremia in pigs, but primary infection with MVEV and KUNV virus primary infections did not. However, secondary JEV infection following either of the other viruses did not produce any viremia, and boosted antibody levels were observed after any secondary flaviviral infection. KUNV and MVEV are endemic in Australia and it is likely that pigs are frequently exposed to these viruses and thus already have some inherent immunity against JEV, which could explain the lack of endemic JEV in the country (Williams et al., 2001).

The incursion of JEV into Northern Australia and WNV into the U.S. has sparked a renewed interest in the idea of cross-protection. Golden Syrian hamsters were immunized with JE SA14-2-8 vaccine, Yellow fever 17D vaccine, or wild-type SLEV and then challenged with WNV. Hamsters are typically susceptible to WNV infection with a mortality rate up to 47%. JEV and SLEV immune hamsters all survived WNV challenge and had much lower viremia. Yellow fever virus is a non-JEV serocomplex flavivirus so there is much less genetic similarity between this virus and WNV. The 17D vaccine provided a little bit of protection against WNV by lessening the overall titers in the blood and decreasing the mortality rate to about 4%. Thus, the more closely related

flaviviruses provide a more significant level of protection against heterologous secondary infection (Tesh et al., 2002). Monoclonal antibodies specific for the E protein gene region of JEV administered to mice either just prior to or shortly after infection with JEV, WNV, and dengue-2 virus elicited a partial protective effect against WNV and dengue virus, while increasing the survival time of lethal JEV intracerebral challenge (Gupta et al., 2008). This concept of passive transfer of immunity has implications for rapid treatment in the face of an epidemic.

A similar study using mice showed that JE vaccination using mouse-brain derived vaccine reduced the mortality rate of intraperitoneal WNV infection from 90% (9/10) to 20% (2/10), which reinforces the idea that JEV immunity can lessen the severity of WNV infection (Takasaki et al., 2003). In another study, immunity derived from a "naked" DNA vaccine made up of a eukaryotic expression vector encoding the prM and E genes of MVEV protected against lethal JEV infection in mice (Lobigs et al., 2003). Vaccination using a killed JE vaccine afforded some immunity to MVEV in mice, but using a live chimeric vaccine with a yellow fever backbone and the prM and E genes of JEV gave substantially more cross-protective immunity against MVEV and WNV, which seems to support the idea that live attenuated vaccines induce a more prolific and general anti-flaviviral immunity than killed or subunit vaccines (Lobigs et al., 2009).

Wildlife reservoirs can greatly impact the spread and emergence of zoonotic diseases. In the U.S, naturally acquired WNV immunity in birds has the potential to influence the outcome of a JEV introduction. Red-winged blackbirds are susceptible to peripheral JEV infection but naturally acquired or experimentally induced WNV immunity protects against the development of secondary JEV infection in 93% of birds

(Nemeth et al., 2009.) High WNV seroprevalence in birds has the potential to lessen the ability of JEV to infect birds and could alter the role of avian hosts in an endemic cycle. If the same reaction is observed in pigs and horses, then there is reason to believe that JEV may be less likely to establish a continuous sylvatic or epidemic cycle in areas where WNV is already endemic.

Animal models provide some important insight for researchers regarding infection and immune response, but often human reactions to disease are much different. Obviously, ethics prevent the use of humans as model for experimental viral infections, but using human serum with naturally or artificially acquired antibodies can bypass the ethical issues and help illuminate at least in part the humoral immune response to JEV and cross-protective immunity with other flaviviruses. In one study, a group of volunteers vaccinated either with an inactivated JEV vaccine or an attenuated dengue vaccine failed to produce neutralizing antibodies against WNV (Kanesa-Thasan et al., 2002). Another study examined patients who had either been infected with JEV or vaccinated with a live attenuated JEV vaccine for IgG and neutralizing antibodies against WNV and this group found that only 2 of 82 JEV infected patients developed neutralizing WNV antibodies (Tang et al., 2008). However, a similar study tested cross-neutralizing WNV response in volunteers vaccinated with either YF 17D or inactivated JEV vaccine found that prior vaccination with heterologous flaviviruses produced significant levels of neutralizing antibodies against WNV (Yamshchikov et al., 2005). These contradictory results indicate that there is much need for more elucidation regarding the molecular mechanisms of cross-protective flaviviral immunity before any solid conclusions can be reached, especially regarding human response to infection.

Although rare, there is some evidence of antibody-dependent immune enhancement in flaviviral infections. This occurs when a secondary infection with an antigenically related virus results in an increase in the severity of secondary infection. This is thought to happen quite frequently with dengue virus, a flavivirus in the dengue virus group (Lei et al., 2001). There are 4 serotypes of dengue and it appears as though secondary infection with a different serotype from the primary infection within a short time interval causes the symptoms of dengue fever to increase in severity to dengue hemorrhagic fever. It is even possible that some antibody response to related flaviviruses can induce this effect when followed by dengue infections (Kimuro-Kuroda et al., 1988). Although this effect has never been observed naturally with JEV infections, in experimentation, mice immunized with a killed MVEV vaccine had a 75% mortality rate following JEV infection compared to 0% mortality in non-immunized mice infected with JEV alone (Lobigs et al., 2003). Antibody-dependent immune enhancement is a hotly debated subject among flavivirologists, and much more research needs to be done to better understand this phenomenon.

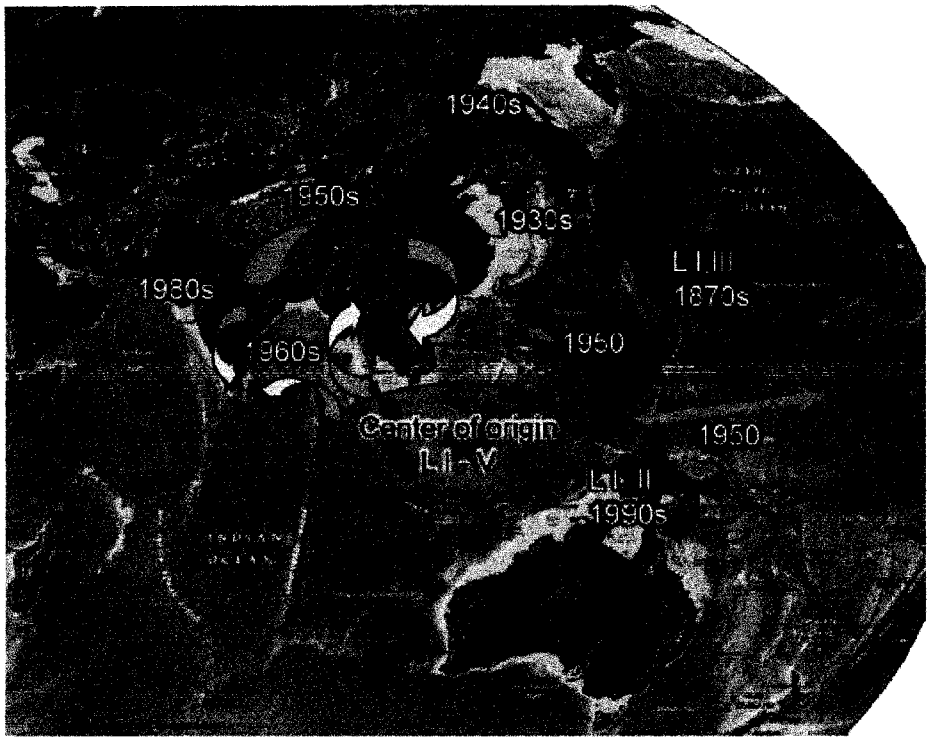
Japanese Encephalitis Virus as an Emerging Infectious Disease

Arboviral diseases have some of the most complex transmission cycles of any diseases, and are frequently emerging and re-emerging into new areas with little or no definitive explanation. The most likely mechanisms of spread for JEV include wind-blown mosquitoes, bird migration, and movement or transport of infected people, birds or mosquitoes via aircraft or container ship. Other factors could include changing agricultural usage to include more areas of standing water, thus increasing vector habits,

and natural climate change that could increase the vector season (Mackenzie et al., 2004). Recent studies show that arboviruses can frequently cocirculate in the same environmental habitats as long as there are sufficient competent vectors and hosts (Reimann et al., 2008). In the case of JEV, understanding which mosquito vectors in the U.S. are most likely to transmit the virus could lead to some knowledge about how the virus could become established. Reservoirs such as pigs and ardeid birds are abundant throughout the U.S, which means that proper vectors are the only major question mark in the emergence equation.

The map shown in Figure 1 (courtesy of Weaver and Reisen, 2010) depicts the mobile trend of JEV dispersion. The origin in the Malaysian archipelago is where all five genotypes circulate, but the outward spread is linked to the primary epidemic genotypes I and III. It appears that Japan was the first country to be hit with epidemic JEV in the 19th century, but since then the virus quickly worked its way North into China, West into India, and eventually South into Australia. While the exact reasons for the spread of JEV are unknown, it seems likely that increased international human movements have played an important part.

Figure 1: JEV distribution



The geographic location of Hawaii makes it an ideal location for a primary introduction of JEV into the United States. The possibilities exist that air or boat travel could introduce infected mosquitoes, or that infected birds being imported (legally or otherwise) could expose the islands to JEV (Quisenberry and Wallace, 1959). In 1964, a serological survey of 749 birds and over 21,000 mosquitoes failed to provide any evidence of arboviral antibodies or virus isolation (Wallace et al., 1964). Mosquitoes were not found in Hawaii prior to the early 19th century, but human travel has likely been the cause of the introduction of several species that now thrive in the tropical climate. There are several mosquito species in Hawaii that may serve as potential vectors, including *Aedes albopictus, Aedes aegypti* and *Culex quinquefasciatus* (Joyce et al., 1961). Additionally, there have been numerous instances of mosquitoes intercepted on aircraft arriving in Honolulu, including some major JEV vectors such as *Cx.*

tritaeniorhynchus, Cx. annulorostris, and *Cx. pipiens* (Joyce et al., 1961). Thus far, no instances of JEV have been reported in Hawaii, and a survey of 1,835 serum samples taken from 11 species of native and migratory birds on the islands between 2004 and 2005 revealed only three birds with some JEV neutralization via plaque reduction neutralization assay (PRNT). These three birds could have been exposed to a related flavivirus, but it is unlikely that their serology is indicative of JEV arriving in Hawaii (Nemeth et al., 2010).

California is the next most likely place for a JEV incursion into the U.S. There are several mosquito species commonly found in California that have been deemed competent laboratory vectors of JEV, including *Cx. tarsalis, Cx. quinquefasciatus* and *Cx. pipiens* (Reeves and Hammon, 1946). It is also quite possible that many of the mosquitoes that have tested positive for WNV infection such as *Culiseta inornata, Ochlerotatus dorsalis,* and *Ochlerotatus nigromaculis* could also transmit JEV, although this theory has not yet been tested. Potential vertebrate hosts in California include pigs, both domestic and feral, birds, and even lizards. As of 2002, there are 1,521 pig farms in California which house over 163,000 pigs, but the real problem could lie in the expanding feral swine population, which at present is more of an issue in the Southeast but could easily allow for a more widespread JEV reservoir host population (Nett et al., 2009). The role of feral pigs in JEV transmission is largely unknown and is should be considered an important research priority if we are to fully understand the epidemiology of a JEV invasion.

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CHAPTER TWO

VECTOR COMPETENCE FOR JAPANESE ENCEPHALITIS VIRUS

Abstract

Japanese encephalitis virus (JEV) is a vector-borne disease that is transmitted primarily through culicine mosquitoes in Asia. The primary vector is *Culex tritaeniorhynchus*, but the virus has been isolated from a large variety of other mosquito species both in the wild and in the laboratory. Although *Cx. tritaeniorhynchus* mosquitoes are not found in North America, it is quite possible that there are mosquitoes native to the U.S. that can vector the virus as well. In this study, we investigated the vector competence for JEV in four North American mosquitoes already known to transmit West Nile virus (WNV): *Culex tarsalis, Culex pipiens, Aedes aegypti,* and *Aedes albopictus* (Goddard et al., 2002; Vanlandingham et al., 2007). Using a membrane feeder system for blood-feeding we were able to demonstrate that *Cx. tarsalis, Cx. pipiens,* and *Ae. aegypti* are all weakly competent laboratory vectors. Infection rates were low, between 2 and 20 percent, but dissemination of virus in *Cx. tarsalis* and *Ae. aegypti* was 100%, indicating that infected mosquitoes will likely be able to transmit the virus to a host through salivary secretions when blood-feeding. These results imply that an introduction of JEV into North America could lead to an established natural cycle in local mosquito populations.

Introduction

Many viruses in the family *Flaviviridae* are arboviruses, viruses that are transmitted by arthropods such as ticks and mosquitoes, and many of these viruses utilize one or more vertebrate hosts to maintain their replicative cycle. Among the most medically important flaviviruses, there are four antigenically related viruses that are known for causing human encephalitis: West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV) and Japanese encephalitis virus (JEV) (Mackenzie et al., 2002). MVEV is endemic in Australia and SLEV is found in North America; WNV and JEV, however, are more diverse geographically. West Nile virus was first isolated in Africa, but since the late 1990's it has spread rapidly into new areas and is now found in Europe, North and South America, including the U.S (Weaver and Reisen, 2010). Japanese encephalitis virus is thought to have originated in the Malaysian region and is typically found throughout Southeast Asia, but the virus has been isolated from mosquitoes and patients in Western Asia and Northern Australia beginning in the 1990's (Solomon et al 2003, Hanna et al 1995). Both WNV and JEV can cause neurologic disease in humans and horses and these viruses utilize wild birds as reservoir vertebrate hosts and can replicate in a variety of mosquitoes, primarily *Culicine* species (Endy et al., 2002; Halstead and Jacobson, 2003). The spread of WNV into the U.S. has created an increased awareness for the ease with which arboviruses can reach novel geographic locations and become endemic over a very short period of time. It is thought that increases in human travel and changing agricultural practices in addition to natural causes such as bird migration and climate change are likely mechanisms for introducing viruses into foreign areas (Mackenzie et

al., 2004; Erlanger et al., 2009). But beyond the initial introduction, the remarkable ability of arboviruses to adapt to alternate vertebrate hosts and spread through novel vectors makes them challenging pathogens to study and understand. Knowing which mosquito species can vector various viral diseases provides a key emerging disease surveillance tool, and early detection can lead to prevention of establishment and epidemics. This is the rationale for investigating several common North American mosquitoes for their ability to vector JEV. In this study, we assessed the ability of four common North American mosquitoes for their capacity to become infected by JEV and then transmit the virus using an artificial blood-feeding system.

Materials and Methods

Mosquitoes

Cx. tarsalis mosquitoes from a colony established in 1951 in Bakersfield, California were received by Colorado State University (CSU) in 2004 and raised in the Arthropod-borne Infectious Disease Laboratory (AIDL). Larvae were hatched and then brought into the BSL-3 facility where they were divided among 6"x12" Tupperware pans with tap water and fed a mixture of Tetramin fish food and crushed rodent feed twice a week. Pupae were picked daily as needed and kept in cardboard cartons with approximately 100 mosquitoes per carton and maintained on sugar cubes and water. Adults were kept in an incubator with an 8:16 hours dark:light cycle at 75% relative humidity and 25° C.

Cx. pipiens pipiens from Iowa were raised in the AIDL and were received into the BSL-3 facility as adults, females only. Mosquitoes were maintained in cardboard cartons

on sugar cubes and water with a 8:16 hour light:dark cycle at 25 ° C and 75% relative humidity.

Ae. aegypti Rex strain from Puerto Rico were raised in the AIDL and were received into the BSL-3 facility as adult females. Mosquitoes were maintained in cardboard cartons on sugar cubes and water with a 12:12 hour light:dark cycle at 78% relative humidity and 27° C.

Ae. albopictus from Florida were raised in the AIDL and were received into the BSL-3 as adult females. Mosquitoes were maintained on sugar cubes and water with a 12:12 hours light:dark cycle at 78% relative humidity and 27 ° C.

Viruses

Two strains of JEV were used for this study. The first, strain 826309 (hereafter JE:8J), is an isolate from a human brain in India and passaged twice in suckling mice and twice in Vero cells. The other virus is an isolate from a *Cx. tritaeniorhynchus* mosquito in Vietnam passaged once in suckling mice and once in Vero cells (JE:VN). Molecular sequencing of the two JE viruses using the prM region of the genome revealed that JE:8J is a genotype III strain and JE:VN is a genotype I strain (Bosco-Lauth, unpublished data). WNV strain NY99-4132 was isolated from an American crow and passaged once in Vero cells, once in C6/36 mosquito cells, and once in baby hamster kidney-21 cells.

Mosquito infection

Sugar cubes were removed from mosquito cartons 24 hours prior to blood feeding. Defibrinated sheep blood with 5% sucrose added was used for the blood meals.

Virus was added to blood immediately prior to feeding. A Hemotek™ Membrane (Discovery Workshops, UK) was used to deliver blood meals. Briefly, 3 ml of virus-blood mixture was added to the metal feeding pools covered by a cellulose membrane. Feeders are attached to the Hemotek™ machine which heats the blood meal to 35 ° C and maintains constant temperature while feeding. Feeding pools were placed with the membrane side down on top of the screened cardboard carton. Mosquitoes were allowed to feed for 1-1.5 hours on artificial feeders. Immediately following feeding, blood from the feeder was collected and frozen at -80 ° C. Mosquitoes were chilled briefly in -20 ° C freezer and were then sorted to save the fully engorged and discard the rest via autoclave. Engorged mosquitoes were then placed in 1 pint cartons and maintained at the previously mentioned incubator settings for 12-14 days. After the intrinsic incubation period, mosquitoes were frozen for approximately 30 minutes and were then dissected. Legs were separated from bodies and both were frozen at -80 ° C.

Virus isolation

Frozen mosquitoes were thawed briefly before adding 0.5 ml bovine albumin media (BA-l: M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B in 0.05 M Tris, pH 7.6) along with a single copper BB in a conical cryovial. Samples were then triturated at 25 cycles/second for 3 minutes at room temperature using a mixer mill. Ground samples were then centrifuged for 1.5 minutes at 1127 x *g* (1200 rpm). After centrifugation, 150 µl of each sample were added into individual wells of a 96 well plate. Serial 10-fold dilutions were prepared in BA-1 and 100 µl samples from each

dilution were immediately plated onto 6 well plates with a monolayer of Vero cells that were 90-100% confluent, as previously described (Westaway et al 1966). Cell culture was as follows: Vero cells were grown in 6 well plates in Dulbecco's Modified Eagle media (DMEM) with 7.5% calf serum and antibiotics until a monolayer of cells formed. A 0.1ml sample was added to the monolayers and incubated at 37 ^{o} C for one hour before an overlay of 2 ml minimum essential media (MEM) and agarose supplemented with 2% fetal bovine serum, sodium bicarbonate and antibiotics per well. Plates were then incubated for 48 hours at 37 ^{o} C at which time a second overlay was added, identical to the first, but containing 0.004% neutral red dye. Plaques were counted one and two days following addition of the second overlay. Virus was back-titrated from blood meals using the same methods.

Statistical Analysis

Infection rates among mosquito species and virus strain variation were analyzed by Chi-square values from 2x2 contingency tables using StatCrunch, on online statistical analysis program. Differences were considered statistically significant at alpha $\geq$0.05.

Experimental Design

Cx. tarsalis and *Cx. pipiens* were tested first, feeding 1 carton per species containing approximately 100 mosquitoes each week. The first feedings used JE virus at titers greater than 10^5 pfu/ml. If any mosquitoes tested positive, later trials with virus titers less than 10^5 pfu/ml were attempted. JE:VN and JE:8J were both tested to check for variations in infection rates between genotypes. Mosquitoes were also experimentally

infected with WNV to compare infection rates with those for JEV. This was done as a control to make sure that these known WNV vectors would test positive, which verifies that the infection method is acceptable. *Ae. aegypti* and *Ae. albopictus* were then tested in the same manner. If mosquitoes of a certain species failed to become infected at the higher titer, then the lower titer was not attempted. Species that tested positive for JEV infection were then tested for ability to transmit virus during blood-feeding.

Transmission

To test for transmission while taking a blood meal, intrathoracic inoculation was performed (Rosen and Gubler, 1974) and after an intrinsic incubation period mosquitoes were allowed to feed on a blood meal using the Hemotek™ Membrane feeder system as described above. Briefly, 15-20 adult female mosquitoes were chilled in a refrigerator until immobile and then were transferred to a glass Petri dish on ice. Virus was injected into the thoracic cavity using a micro-needle made by heating a 100 µl glass tube until it could be pulled apart at the center, making two very small needles. Approximately 10^5 plaque forming units (pfu) of JE:VN virus were injected into each mosquito (1-2 µl). Following inoculation, mosquitoes were transferred to cardboard cartons and incubated at appropriate temperatures for 14 days. On day 13, sugar cubes were removed from cartons and on day 14 mosquitoes were offered a non-infectious blood meal using the Hemotek™ Membrane Feeder as described above for 1-1.5 hours. Immediately following feeding, engorged mosquitoes were chilled and then dissected for assay. The blood from the Hemotek™ feeder was then serially diluted and assayed after the

mosquitoes were allowed to feed in order to determine presence and titer of virus transmitted into the blood meal during feeding.

Results

Experimental infection of Culex spp

Table 2.1 summarizes the infection and dissemination rates of all the mosquitoes tested during the course of this study. A total of 419 individual *Culex spp* mosquitoes survived the intrinsic incubation period and were tested for either JEV or WNV infection. *Cx. tarsalis* mosquitoes that fed on infected blood meals were able to ingest and disseminate JEV and WNV using an artificial blood feeding system, although the infection rates were quite low. Over the course of numerous feedings, only 10 out of 189 *Cx. tarsalis* mosquitoes that survived the 12-14 day incubation period were shown to be infected with JE virus, and all 10 had disseminated the virus as evidenced by virus isolation from the legs. Ten out of 86 WNV infected mosquitoes developed disseminated infection, nearly double the rate for JEV but much lower than expected. JEV infection was evaluated to determine the effect of genotype and titer variations. There was no detectable difference between the two genotypes, but the titer of virus could be an important determining factor in vector infection. Only 2.4% of mosquitoes became infected when fed on a blood meal containing less than 10^5 pfu/ml of virus; infection rate doubled with titers above 10^5 pfu/ml.

Cx. pipiens were found to be extremely weakly competent vectors for JEV, with only two out of 95 mosquitoes over multiple feedings developing infection. No conclusions could be made about genotype or titer; as both genotypes are represented and

both low and high titers resulted in infection. WNV infection was more successful, with a 20.4% infection rate and 80% dissemination rate. Of note, *Cx. pipiens* often wouldn't feed when a blood meal was offered, and when they did they had a low survival rate following feeding (41.7%), making them a rather difficult species to work with.

Experimental infection of Aedes spp.

A total of 384 *Aedes spp* mosquitoes survived the intrinsic incubation period and were tested for infection with either JEV or WNV, as shown in Table 2.1. *Ae. aegypti* Rex were found to be the most competent JEV vectors of the species tested in this study. The overall JEV infection rate was 12.7%, however, there was a large discrepancy between infectious dose and infection rate; none of the 38 mosquitoes that were offered a blood meal with less than 10^5 pfu/ml became infected, while 16 of 88 mosquitoes offered a higher titer developed infection, and all 16 disseminated. Both genotypes were represented in the infection; while more of the JE:8J mosquitoes became infected, it is unlikely that this variation is reflective of genotype alone, especially considering that the results represent multiple trials. Ten of 71 mosquitoes became infected with and disseminated WNV, which is rather close to the overall infection rate of JEV (11.6% compared to 12.7% respectively) and could indicate that these vectors are likely to transmit JEV in a similar manner as WNV.

The *Ae. albopictus* mosquitoes used in this experiment were not found to be competent vectors for JEV, although they become infected with WNV. Previous studies have shown that *Ae. albopictus* from other locations are JEV competent, so these results were not expected.

Vector competence comparison between mosquito species and JEV genotype

Infection rates for all four species were evaluated for species variation and were found to be statistically different when all four species were examined at once ($p<0.0001$). Thus, vector competence between these species varies significantly. Additionally, *Cx. tarsalis, Cx. pipiens,* and *Ae. aegypti* were analyzed for variations in the infection rates between the two JEV genotypes, but none were found to be statistically significant ($p>0.05$).

Transmission

Cx. tarsalis, Cx. pipiens, and *Ae. aegypti* were all found to be weakly competent laboratory vectors of JEV, so members of these three species were examined for the potential to transmit the virus during blood-feeding. Table 2.2 summarizes the results of the transmission attempts. All three species transmitted virus to the blood meal as evidenced by the recovery of live virus from the blood meal immediately following feeding. *Ae. aegypti* transmitted the most virus (10^5 pfu/ml blood), followed by *Cx. tarsalis* at 10^4 pfu/ml and *Cx. pipiens* (10^3 pfu/ml). While these results reflect the infectious capacities of pools of 15-20 mosquitoes feeding at the same time, it is likely that individual mosquitoes could still release enough virus particles during feeding to infect a susceptible host.

Discussion

In Asia, the primary mosquito vector that transmits JEV is *Cx. tritaeniorhynchus,* a species known to feed in the evenings on both birds and mammals (Hammon et al.,

1949; Mitamura et al., 1938; Leake et al., 1987). JEV, like WNV, utilizes birds as a vertebrate reservoir, but JEV is unique in that the virus also has a mammalian host: domestic swine (Gresser et al., 1958). Indeed, it is likely that most epidemics in humans and horses occur when infected pigs are fed upon by mosquitoes which then bite the humans and horses in close proximity (Buescher et al., 1959). In pigs, infection is mostly asymptomatic, although occasional stillbirths and abortions have been observed in infected shoats (Gresser et al., 1958). Because JEV has both mammalian and avian reservoirs, it is important to investigate vectors that are non-specific feeders, as these mosquitoes are just as likely to bite birds as mammals and would pose a significant threat to humans. Additionally, species that are ornithophilic (those that feed primarily feed on birds) are important in maintaining the sylvatic cycle.

Previous studies have shown that some strains of *Cx. pipiens, Cx. annulorostris, Cx. taeniorhynchus, Cx. sitiens, Cx quinquefasciatus, Ae. vexans, Ae. albopictus* and *Cx. nigripalpus* are just some of the competent vectors that can transmit JEV in the laboratory (Turell et al., 2006a; Turell et al., 2006b; Van Den Hurk et al., 2003; Weng et al., 1997; Weng et al., 2000). In the U.S, mosquito species that are highly associated with WNV transmission such as *Cx. tarsalis* and *Cx. pipiens* are thought to be potential JEV vectors due to the close relation between the viruses (Goddard et al., 2002). In addition to the *Culex* species, other important North and Central American arbovirus vectors include members of the *Aedes spp,* such as *Ae. aegypti* and *Ae. albopictus,* as well as *Ocleratatus spp* and *Culiseta spp* (Turell et al., 2005; Nett et al., 2009, Sardelis et al., 2001). For this study, four important arbovirus vectors in the U.S. and Central America were tested for their ability to disseminate and transmit JEV. *Cx. tarsalis* and *Cx. pipiens* are both bird-

feeders that are major WNV vectors across much of the U.S. *Ae. aegypti* and *Ae. albopictus* are mammalophilic mosquitoes that are known to transmit a variety of arboviruses, including yellow fever virus and western equine encephalitis virus (Mackenzie et al 2004, Weaver and Reisen, 2010). All four species have been previously implicated as competent WNV vectors, and so they were all evaluated for their ability to disseminate and transmit JEV and the laboratory infection rates were compared to those of WNV. Overall, there was a statistically significant difference between the infection rates of the four species for JEV ($p<0.0001$) but not for WNV ($p=0.423$). We found that both *Culex* species are weakly competent laboratory vectors for these viruses, although WNV infection rates were higher than those of JEV. These results indicate that both species could play a role in maintaining the sylvatic cycle of JEV in birds if the virus reaches the U.S. *Ae. aegypti,* a subtropical mosquito found in Africa, South and Central America as well as much of the southern U.S, was found to be the most competent JEV vector, with approximately equivalent WNV infection rates. Previous studies have also determined that *Ae. aegypti* can become infected with JEV, and with similar infection rates as those observed in this study through artificial blood feeding (Van Den Hurk et al., 2003). Although *Aedes spp* are not typically implicated as major vectors for WNV or JEV, these findings are of some importance because of the mammalophilic feeding habits of these mosquitoes and their vast distribution. Clearly, *Ae. aegypti* mosquitoes should be monitored carefully in countries such as the U.S and much of Central America where JEV could emerge. The *Ae. albopictus* mosquitoes tested in this study could not be infected in the laboratory, but that doesn't mean that as a species *Ae. albopictus* should be discounted as potential vectors, especially considering the other studies that have found

them to be competent for JEV (Weng et al., 1997). There are several explanations for finding discrepancies within a laboratory vector study, including differences in experimental design, such as infection methods and virus strains, as well as biodiversity among mosquitoes within a species. *Cx. annulorostris* mosquitoes in Australia vary in their genetic makeup between those that live on the mainland and the island mosquitoes (Hemmerter et al., 2007). Incidentally, the island *Cx. annulorostris* are better vectors of JEV than the mainland mosquitoes, which could help to explain why the virus hasn't become established on the Australian continent but remains active on the nearby islands. Before ruling out a particular species from being a potential vector, testing representative individuals from different geographic locations is a necessary step to ensure a comprehensive evaluation has been made.

There are five distinct genotypes of JEV. Genotypes I and III are found throughout Southeast Asia, including the more temperate countries, and are typically associated with epidemic JE. Genotypes II, IV and V are mostly found in the Malaysian and Indonesian islands and are considered sylvatic strains of the virus (Solomon et al., 2003; Uchil et al., 2001). Prior to the late twentieth century, genotype III viruses were the most frequently isolated in human cases, but recently there has been an increase in the prevalence of genotype I viruses circulating and causing outbreaks in locations such as Thailand, China, Japan, Vietnam, and Korea (Ali et al., 1995, Doi et al., 2007, Saito et al., 2007, Yang et al., 2004, Yoshida et al., 2005). There do not appear to be any virulence differences between genotypes I and III in susceptible hosts, and while both are isolated from human cases and from mosquitoes each year, the recent upsurge of genotype I virus isolates could indicate increased infectivity or some other beneficial

adaptation (Ali et al., 1995, Wang et al., 2007). For this experiment we used two strains of JEV to represent the two epidemic genotypes. Both genotypes were represented in the oral infection rates, and there were no statistically significant differences in these rates between the two strains, so it is unlikely that genotypic variation at least between I and III has any effect on vector competence for JEV in the mosquitoes tested here. It is possible that the genetic variation of these viruses has a larger impact on vertebrate hosts than on the vectors, but more research needs to be done in this area before any conclusions can be made.

Laboratory infection and transmission in mosquitoes is unlikely to be absolutely reflective of what occurs in nature because of the highly controlled and often unnatural mechanisms by which these studies are carried out. Studies with dengue virus, a more distantly related flavivirus, have shown that using frozen virus stocks for an artificial bloodmeal can lead to reduced infection rates in *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes (Richards et al., 2007). Additionally, laboratory raised mosquitoes can have different characteristics than their wild relatives, which could complicate the nature of their competence as mosquito vectors. Even the blood meal itself can affect virus transmission; in a viremic host, virus is circulating throughout the blood and is kept at a constant temperature, whereas an artificial blood meal is static and may regulate temperature inappropriately, which could decrease the amount of virus that the vector is actually exposed to. Therefore, in the laboratory setting, it is difficult to eliminate a species of mosquito as a vector; rather, these studies should be interpreted in terms of which mosquitoes can be artificially infected, as this is good evidence that a natural infection can also take place.

From these experiments we can determine that mosquitoes representative of three species indigenous to North and Central American can ingest virus through a blood meal, replicate it, and pass it on through the saliva. Infection rates in the lab may be very different in nature, but even very low infection rates could lead to outbreaks or epidemics, especially when climatic conditions allow for higher than average numbers of potential vectors. Thus, monitoring laboratory competent vectors such as *Cx. tarsalis, Cx. pipiens,* and *Ae. aegypti* could help forewarn of an impending outbreak. An increasingly international climate with constant movement of people and animals makes preventing the spread of disease extremely challenging, but preparation in the face of an outbreak is key in stopping foreign diseases like JEV from causing severe epidemics. When WNV reached the U.S, we as a nation were largely unprepared for the amount of damage the disease would cause in humans, horses and wildlife, and had very little idea which mosquitoes were spreading the virus. Regular surveillance of suspected JEV vectors is necessary to prevent a repeat of the WNV epidemic that took this country by storm. There are several effective JEV vaccines on the market that could be rapidly distributed if need be to both humans and horses given enough advance notice and assuming that there are sufficient amounts of vaccines stockpiled (Goto 1976, Monath 2002). An advantage that is provided by mosquito monitoring is the ability to pinpoint which areas are more likely to have high numbers of vectors and then target these locations for rapid vaccinations and mosquito control, with the hope that reaching these "hot-spots" first would slow the spread of the virus and possibly even prevent disease from occurring in susceptible hosts.

Table 2.1: Infection and dissemination rates of *Culex spp* and *Aedes spp* mosquitoes fed on artificial blood meals containing JEV and WNV

Species	Virus	Strain	# tested	# positive	# disseminated	Dose (pfu/ml)	Infection rate	Dissemination rate
Cx. tarsalis	JEV	826309	41	1	1	$<10^5$	2.40%	100%
Cx. tarsalis	JEV	826309	55	3	3	$>10^5$	5.50%	100%
Cx. tarsalis	JEV	VN	93	6	6	$>10^5$	6.50%	100%
Cx. tarsalis	WNV	NY99	86	10	10	$>10^6$	11.60%	100%
Cx. pipens	JEV	826309	58	1	NT	$<10^5$	1.70%	NT
Cx. pipens	JEV	826309	1	0	0	$>10^5$	0%	0%
Cx. pipens	JEV	VN	14	0	0	$<10^5$	0%	0%
Cx. pipens	JEV	VN	22	1	0	$>10^5$	4.5%	0%
Cx. pipens	WNV	NY99	49	10	8	$>10^6$	20.40%	80%
Ae. aegypti	JEV	826309	38	0	0	$<10^5$	0%	0%
Ae. aegypti	JEV	VN	42	4	4	$>10^5$	9.50%	100%
Ae. aegypti	JEV	826309	46	12	12	$>10^5$	26%	100%
Ae. aegypti	WNV	NY99	71	10	10	$>10^6$	14.10%	100%
Ae. albopictus	JEV	VN	82	0	0	$>10^5$	0%	0%
Ae. albopictus	JEV	826309	62	0	0	$>10^5$	0%	0%
Ae. albopictus	WNV	NY99	43	5	5	$>10^5$	11.60%	100%

Table 2.2: Transmission of JEV by intrathoracically inoculated mosquitoes during blood feeding

Species	Overall JEV Oral Infection Rate*	Intrathoracic Inoculation JE:VN Titer	Intrathoracic Dissemination Yes/No	Transmission Yes/No	Virus titer in Hemotek™ blood meal (pfu/ml)
Cx. tarsalis	5.3%	1.1^5pfu/mosquito	Yes	Yes	10^4
Cx. pipiens	2.1%	1.1^5pfu/mosquito	Yes	Yes	10^3
Ae. aegypti	12.7%	1.1^5pfu/mosquito	Yes	Yes	10^5

*Overall infection rate is the percent of infected mosquitoes for each virus without differentiating between strain or virus titer at the time of infection

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CHAPTER THREE

EXPERIMENTAL INFECTION OF HORSES WITH JAPANESE ENCEPHALITIS VIRUS

Abstract

Japanese encephalitis virus (JEV) is a vector-borne neurotropic virus that can cause severe encephalitic disease in horses. The virus is endemic in Southeast Asia and surrounding areas, but the threat of spread is increasing with changes in international human activities and natural climatic factors (Mackenzie et al., 2004; Weaver and Reisen, 2010). The focus of this study is to elucidate the role of horses in the transmission cycle and investigate the potential of *Aedes aegypti,* a mosquito found all over the world, to transmit the virus. Six horses were infected with three strains of JEV by mosquito bite and were studied for two to three weeks for clinical and serological signs of disease. On the third day post inoculation, uninfected mosquitoes were allowed to feed on horses to attempt horse-to-mosquito transmission. Three of the six horses developed low level viremias that persisted for five days. One horse had a low grade fever lasting two days; no other clinical signs of illness were observed and all horses survived until the end of the study. Tissue pathology of the brains of the viremic horses did not reveal any neurological changes. Still, the ability of *Ae. aegypti* to transmit JEV to horses indicates

that this mosquito could be a potential vector of the virus. Horses have long been considered dead-end hosts for JEV and in this study, none of the mosquitoes that fed on viremic horses developed infections, thus confirming that their role in disease transmission is likely incidental and probably does not play a part in perpetuating the natural virus cycle.

Introduction

Japanese encephalitis virus (JEV) is an arthropod-borne virus in the family *Flaviviridae,* genus *Flavivirus.* It is a positive-sense single-stranded RNA virus closely related to West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) (Mackenzie et al., 2002). JEV is endemic throughout much of Southeast Asia and the surrounding Pacific Islands and has extended its reach into the northern Torres Strait of Australia as recently as the early 1990's (Hanna et al 1995, Rosen 1986). The virus is primarily transmitted by *Culex tritaeniorhynchus* mosquitoes in Asia, but can be vectored by many different *Culex* and *Aedes* species in nature and in the laboratory (Turell et al., 2006a; Turell et al., 2006b; Van Den Hurk et al., 2003; Weng et al 1997, Weng et al., 2000). Serologic evidence suggests that JEV continually circulates at low levels in the primary reservoirs, ardeid birds and domestic swine, but during epidemic years when rainfall is above average and mosquito populations are at their peak, transmission can lead to outbreaks in human and horses (Scherer et al 1959a; Solomon et al., 2002). There are approximately 35,000 annual human cases reported worldwide, which is likely far under-representative of the actual number of infections that occur (World Health Report, 1996-2008). Horse outbreaks have been greatly reduced since the introduction of vaccination

in the 1940's, but during outbreaks the case fatality rate can reach levels as high as 42% (Ellis et al., 2000). Horses and humans are considered dead-end hosts but very little is known about JEV pathogenesis in either species.

In 1964, one study demonstrated that horses experimentally infected with JEV by the bite of *Culex tritaeniorhychus* mosquitoes were subsequently infectious to naïve mosquitoes, but this event has never been observed in nature and these results have not been replicated (Gould et al., 1964). Additionally, it is not known if any species of mosquito found in North America can vector the disease to horses. Lastly, although two of the five distinct genotypes of JEV have been isolated from infected horses, there is no evidence to date that there is any difference in virulence between strains (Lian et al., 2002; Yamanaka et al., 2005).

The purpose of this experiment was three fold. First, to evaluate the course of infection of JEV in horses infected by mosquito bite using several different virus strains representative of the two epidemic genotypes, I and III. Second, attempt transmission of JEV to horses using *Aedes aegypti,* a mosquito found in the southern states of North American and throughout Central America and well known for its invasive nature into many regions of the world. Third, attempt horse-to-mosquito infection in order to either determine if horses are indeed a dead-end host or if they can be competent reservoirs for the virus. In all, this study was designed to elucidate the role of horses in the JEV cycle and also to give researchers a better understanding of the virus-vector-host interaction with the hope that this understanding can lead to preparedness in the event of an introduction of JEV to North America.

Materials and Methods

Horses

Yearling horses were obtained in Montana and transported to a BSL-3 facility at Colorado State University in Fort Collins, CO. Horses were pre-screened for anti-WNV antibodies prior to the study using plaque reduction neutralization as described later. They were kept in 12' by 18' rooms with cedar shavings for bedding, and were provided clean water and fed alfalfa pellets twice daily. Rooms were cleaned daily and maintained at approximately 18 ° C (65° F) with 20% humidity. Natural lighting was provided via skylights and plastic jugs were supplied for enrichment.

Mosquitoes

Ae. aegypti Rex strain from Puerto Rico were raised in the Arthropod-borne Infectious Disease Laboratory at Colorado State University and were received into the BSL-3 facility as adult. Mosquitoes were maintained in an ambient light incubator at 80% humidity and 28° C and were provided sugar cubes and water.

Viruses

JEV strain 826309 (hereafter JE:8J) is an isolate from a human brain in India and passaged twice in suckling mice and twice in Vero cells. JEV strain Miller Vietnam (hereafter JE:VN) is an isolate from a *Culex tritaeniorhynchus* mosquito in Vietnam passaged once in suckling mice and once in Vero cells. Molecular sequencing of the two JE viruses using the prM region of the genome revealed that JE:8J is a genotype III strain and JE:VN is a genotype I strain (Bosco-Lauth, unpublished data). The JE:8J strain has been previously catalogued in Genbank. The JE:P3 strain was obtained from the

University of Texas Medical Branch, Galveston, TX and is a Genotype III virus isolated from a mosquito in Beijing.

Experimental Design

Intrathoracic infection of mosquitoes

Infection of mosquitoes was performed intrathoracically as previously described (Rosen and Gubler, 1974). Briefly, 100 µl capillary tubes were heated and stretched until pulled in half in order to make ultra-fine inoculation needles. These were attached to 2 mm plastic tubing with a syringe at the other end to aspirate virus. Mosquitoes were chilled briefly at -20° C and then were placed in glass Petri dishes on ice to keep them immobilized. For inoculation, virus was used from stock samples without dilution to allow for maximum titers and to minimize contamination. The ultra-fine needles were then gently inserted into the thorax and virus was injected into the thoracic cavity of the mosquito. Following infection, mosquitoes were kept in 1 pint cartons with water and sugar cubes for seven days under the same environmental conditions as previously described. Twenty-four hours prior to attempted feeding on horses, sugar was removed from mosquito cartons to help increase blood-feeding appetite.

Mosquito-to-Horse Transmission

Transmission from horse to mosquito was attempted using the four horses infected with JE:VN and JE:8J. Just prior to mosquito feeding, sera were collected and rectal temperatures recorded for all four horses. Then, cartons with approximately 25 infected mosquitoes were held to a shaved area over the shoulder of each horse so that the

screened lids were pressed against the skin. Mosquitoes were allowed to feed for 10 minutes per horse. Immediately following feeding, mosquitoes were chilled and engorged individuals were saved for testing. Legs were pulled from engorged mosquitoes and tested separately for virus isolation to verify disseminated infection.

Sampling Scheme:

Serum collection: Blood was taken from the jugular vein and collected in serum separator tubes every twelve hours for the first seven days post-inoculation (DPI) and then once daily for 8,9,10, and 14 DPI.

Temperatures: Glass thermometers were used to take temperatures rectally twice daily for the duration of the study (temperature were measured in degrees Fahrenheit).

Tissues: Horses were euthanized either on 14 or 20 DPI and the following tissues were collected into 10% neutral-buffered formalin: heart, lung, spleen, liver, kidney, adrenal gland, brain, and spinal cord. Tissues were transferred into 70% ethanol solution after fixing in formalin for 48 hours.

Horse-to-Mosquito Transmission

Cartons of approximately 25 mosquitoes that had been starved of sugar for 24 hours were placed on the shoulders of each horse as described above three days after the initial mosquito infection and were allowed to feed for 10 minutes. This time point was chosen based on the assumption that peak viremia occurs 3 DPI, which is typical for WNV infection in horses (Bowen, unpublished data). Following feeding, mosquitoes were maintained in the incubator for 14 days after which time they were killed, legs were separated and tested to check for viral dissemination, and frozen at -80° C for later assay.

Virus isolation

Plaque assays were performed for determination of viremia using mammalian cells in culture as previously described (Westaway et al., 1966). Briefly, Vero cells were grown in 6 well plates in Dulbecco's Modified Eagle Medium (DMEM) with 7.5% bovine calf serum and antibiotics until a monolayer of cells formed. Horse sera (0.1ml) were added to the monolayers and incubated at 37° C for one hour before an overlay of 2 ml Minimum Essential Media (MEM) and agarose supplemented with 2% fetal bovine serum, sodium bicarbonate and antibiotics per well. Plates were then incubated for 48 hours at 37° C at which time a second overlay was added, this time with 0.004% neutral red dye. Plates were counted for plaques 24 and 48 hours following the second overlay.

Mosquitoes were placed in 1.5 ml centrifuge tubes and 0.5 ml BA-1 (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B in 0.05 M Tris, pH 7.6). was added to the tubes along with 1 copper BB. Tubes were placed in a tissue homogenizer and triturated at 25 cycles/second for three minutes. Samples were then centrifuged at 1127 x *g* (1200 rpm) for 2 minutes and immediately added to wells of 96 well plates. Samples were serially diluted (10-fold) and plated on six well plates as described above.

Serology

Antibody determination was performed using plaque reduction neutralization assays (PRNT) as previously described (Lindsey et al., 1976). Serum samples were

diluted out to 1:320 in 96 well plates in BA-1. JE strain VN virus was added to the wells containing samples at appropriate dilutions to yield approximately 100 plaques/well for the control titration and 100 µl virus was added to 100 µl sample in the 96 well plate and incubated at 37° C for one hour or refrigerated overnight. Samples were then inoculated onto Vero cells in 6 well plates and allowed to incubate for one hour at 37° C. Overlay was added as for plaque assay and plates were counted 24 hours following the second overlay. Titers are measured as percent neutralization for serial dilutions from 1:10 to 1:320.

Serum cross-neutralization

Sera from four of the JEV challenged horses was tested for cross-neutralizing antibodies against WNV. Additionally, sera from five control horses previously used in a WNV vaccine efficacy trial (challenge E-07-07) was tested for cross-neutralizing JEV antibodies. The WNV infected horses were challenged by intrathecal inoculation of 10^6 pfu/ml of virus and the serum used for this study was collected on 14 DPI (Bowen et al., unpublished data). PRNT assays as described in the previous paragraph were used to calculate neutralizing antibody titers. Samples were serially diluted out to 1:2560 before virus was added. JE VN strain was used for JEV neutralization and WNV NY99 for WNV neutralization. Titers were measured as serum dilution with neutralizing capabilities of 80% or greater.

Pathology

Horse brains and spinal cord sections were collected immediately following euthanasia and placed into 10% neutral-buffered formalin. Tissues were then transferred into 70% ethanol solution after 48 hours and brains were sectioned by cerebrum, cerebellum, thalamus/hypothalamus, pons, rostral medulla and spinal cord before being sent to Colorado Histoprep for Hemotoxylin & Eosin staining. The following grading system was used to identify and distinguish lesions:

Grade 0 = No significant lesions

Grade 0.5 = Rare, small, multifocal glial nodules scattered throughout the parenchyma and/or minimal lymphoplasmacytic leptomeningeal infiltrates.

Grade 1 = Mild nonsuppurative encephalitis. This is characterized by mild multifocal perivascular cuffs with lymphocytes, plasma cells, and rare neutrophil and scattered multifocal glial nodules composed of glial cells with a few mononuclear inflammatory cells. Occasionally within this grade, there may be minimal perivascular cuffing and more moderate scattered glial nodules as well asmild lymphoplasmacytic infiltration of the leptomeninges.

Grade 2 = Moderate nonsuppurative encephalitis characterized by moderate lymphoplasmacytic perivascular cuffs around many vessels, multifocal accumulations of glial nodules scattered throughout the parenchyma, and mild to moderate expansion of the leptomeninges by mononuclear inflammatory infiltrates.

Grade 3 = Severe nonsuppurative encephalitis characterized by severe and thick lymphoplasmacytic perivascular cuffing, multiple scattered glial nodules throughout the

parenchyma, and expansion of the leptomeninges by mononuclear inflammatory infiltrates.

Results

Horse infection

Mosquito feeding was successful in all horses with at least nine of 25 (~36%) mosquitoes per horse becoming fully engorged. Engorged mosquitoes were dissected and pooled; legs and bodies were assayed separately to verify disseminated infection. All mosquitoes had virus present in the legs. JEV titers in pooled mosquitoes immediately following feeding are recorded in Table 3.1.

Three of the six horses developed detectable JE viremia following mosquito transmission. Both horses infected with the VN strain and one of the 8J-infected horses were viremic; neither of the P3 horses had virus in the blood up to 14 DPI. Of the viremic animals, virus was detected in the blood as early as 24 hours PI and persisted through the morning of the sixth day PI. The highest JE viremia titer was $10^{2.7}$ pfu/ml on 4 DPI. Horse 1 had mild pyrexia for days 1-4 PI with temperatures at or above 102° F (39° C). Horse 2 also had a fever for days 1 and 2 PI with temperatures at or above 102° F (39° C). None of the other horses had temperatures that exceeded this temperature for more than one measurement. It is likely that the three non-viremic horses that exhibited high temperatures for only one reading could have been excitable due to handling and were not actually febrile. Throughout the study, none of the horses exhibited any of the potential neurological signs associated with viral encephalitis, such as ataxia, hyperesthesia, head tilt, loss of appetite, muscle tremors or circling. Pathologic

observations indicated that five of six horses were graded as 0.5 in portions of the brain, and one of the three non-viremic horses was scored as 1 in the cerebrum and thalamus. A score of 0.5 or 1 indicates mild lymphocytic infiltration in non-specific regions of the brain, but these results do not clearly indicate any clinical manifestations or marked pathology associated with JEV infection, rather, only mild abnormal change in the cellular makeup of the nervous system tissues.

Mosquito infection

Attempted horse-to-mosquito transmission was unsuccessful. Twenty-five mosquitoes fed on both horses 1 and 3 and became fully engorged after feeding. Fourteen days post-feeding, mosquitoes were dissected and legs and bodies were immediately assayed for virus isolation. Even though both horses were viremic at the time of feeding, none of the mosquitoes had detectable virus in bodies or legs.

Serum cross-neutralization

All six horses were screened for WNV antibodies prior to JEV infection using PRNT and all were found to be negative for antibody (less than 50% neutralization at 1:10). Sera collected on 14 DPI was tested for anti-JEV antibodies and all six horses had at least 80% neutralization at serum dilution 1:10 with titers ranging from 1:10 to 1:320 (Table 3.1).

Table 3.2 summarizes the results of the serum cross-neutralization study. The four JEV infected horses had anti-JEV titers between 1:40 and 1:320 and the corresponding WNV neutralizing titers were between 1:10 and 1:20. The five WNV

infected horses had anti-WNV titers between 1:40 and 1:640 and the corresponding JEV titers were between 1:10 and 1:20. These results indicate a reduced antibody response to the secondary virus, but a neutralizing response to the infecting virus none-the-less. All four JEV infected horses were screened for anti-WNV antibodies prior to JEV challenge and all were negative (<50% neutralization at 1:10 serum dilution), thus, these results clearly indicate that JEV neutralizing antibodies do indeed afford some anti-WNV immunity. While the WNV infected horses were not screened for JEV antibodies prior to their infection, it is highly unlikely that they would have had any antibodies given that JEV has never been detected in the U.S.

Discussion

The potential for emerging vector-borne diseases to spread globally and cause epidemics is seemingly increasing, as evidenced by the intercontinental spread of viruses including WNV, Chikungunya virus, Rift Valley fever virus, and bluetongue virus (Weaver and Reisen, 2010). As a close relative of WNV, JEV could have an equally significant impact were it to spread to new locations. Both of these viruses are known to cause severe disease in humans and horses and WNV has been wreaking havoc across the U.S. since its arrival in 1999. While both viruses have a relatively low incidence rate in humans and horses, in countries like the U.S. where there is no established vaccination regimen or acquired community immunity, outbreaks can have devastating results. This was experienced for WNV between 1999 and 2003, during which time several thousand people were diagnosed with WNV infection and at least as many horses, many of which experienced clinical disease and death in some cases (Gubler et al., 2007). Since this

outbreak, the fear of other arboviruses emerging in the U.S. and other countries has been more pronounced (Reimann et al., 2008; Nett et al., 2009). JEV, as the leading cause of encephalitis in Asia and worldwide in children, is one of the most important emerging infectious diseases with potential to reach the Western Hemisphere (World Health Report 1996-2008). The ability of these viruses to infect and disseminate within a variety of mosquitoes, including *Ae. aegypti, Cx. tarsalis,* and *Cx. pipiens,* all of which are found in various locations across the U.S. and much of Central and South America, is just one reason to maintain an active mosquito surveillance program. The results of this study show that horses can easily be infected with JEV via the bite of infected *Ae. aegypti* mosquitoes; however, infection does not necessarily lead to overt disease. Among the three viremic horses in the present study, the only clinical sign observed was transient low grade fever, which would likely go unnoticed in a natural setting. These results are not surprising; like WNV, less than 30% of JEV-infected horses show obvious clinical signs and approximately 70% of those animals will recover (Ellis et al., 2000). This still leaves a large population of at-risk animals, however, and humans could fare worse. In Asia, human JEV cases are recorded every year, and often are most devastating in children or adults with no prior immunity (Solomon et al., 2002). More alarming, though, is that nearly 50% of patients who recover from acute Japanese encephalitis will have permanent neurological sequelae (Ding et al., 2007). An entire population without immunity, as in the U.S, could be fertile grounds for JEV to take hold and wreak havoc.

There are five distinct genotypes of JEV, of which only I and III are implicated in human and horse outbreaks (Lian et al., 2002; Yamanaka et al., 2005). The other genotypes, II, IV, and V appear to exist primarily in the natural enzootic cycle between

mosquitoes and birds and may be attenuated in virulence (Uchil et al., 2001; Solomon et al., 2003). Little is known about the differences, if any, between genotypes I and III, so viruses from each genotype were used for this study. Both genotypes were represented in the horse infection, although the P3 strain (a genotype III virus) did not infect either of the two horses attempted. Interestingly, genotype I viruses have recently been implicated in more human cases than genotype III viruses, indicating a shift in the predominant epidemic genotype (Nga et al., 2004; Nitatpattana et al., 2008). It is thought that perhaps minor genetic shifts could lead to a higher receptor binding affinity in genotype I strains, making them more infectious, if not more virulent (Ali et al., 1995; Wang et al., 2007). The sample size in this study was too small to make any statistically significant conclusions, but 100% (2/2) of genotype I infected horses developed viremia compared to only 25% (1/4) of genotype III infected horses. Because no significant clinical symptoms were observed, we can not confidently say that there are any variations in virulence between the genotypes represented in this study, but the idea of some enhanced infectiousness may have merit.

This study illustrates the ability of *Ae. aegypti* mosquitoes to infect horses with JEV. However, the viremic horses in this experiment did not develop sufficient JEV titers to infect mosquitoes, indicating that horses are likely dead-end hosts. Indeed, this has long been assumed, since horse outbreaks are typically only seen during epidemic years and horse infection does not seem to play any role in the natural virus cycle (Scherer et al., 1959b; Rosen et al., 1986). The most logical explanation for this phenomenon is that horses do not develop sufficiently high viremias to infect these mosquitoes, however, the most highly competent vectors have been known to become

infected by feeding on blood meals with JEV virus titers as low as 10log pfu/ml (Soman et al., 1977). *Aedes aegypti* are therefore not likely to be categorized as highly competent vectors, but rather as mildly competent. It is quite possible that a better vector could still obtain a blood meal from an infected horse and then replicate the virus, thereby including the horse in the infectious cycle. It has been previously reported that horse-to-mosquito transmission is possible experimentally using *Cx. tritaeniorhynchus,* the mosquito that is largely considered the most effective JEV vector (Gould et al., 1964). This study does not refute previous conclusions regarding horses as potential reservoirs, but rather supports the idea that some mosquitoes may be more efficient at vectoring JEV than others. In general, *Aedes spp.* are not considered the primary vectors for flaviviruses within the JEV serocomplex, so there exists the possibility that *Culex* mosquitoes here in the U.S. would be more efficient vectors. The reasons for using *Aedes aegypti* to transmit JEV to horses in this investigation were based upon the infection rates from previous mosquito work, which showed that *Ae. aegypti* were slightly more competent than *Cx. tarsalis* or *Cx. pipiens* (infection rates in order: 13%, 5%, 2%), but more importantly, neither of the *Culex* mosquitoes would feed on horses in preliminary attempts. As a point in fact, transmission using *Cx. tarsalis* intrathoracically inoculated with JEV was attempted on horses prior to using *Ae. aegypti,* but the mosquitoes did not even probe on horses after 15 minutes, and so they were removed from the study. This does not mean that in the wild, infected *Culex* mosquitoes won't feed on horses, but laboratory strains of mosquitoes can sometimes lose the preference for feeding on live hosts if they are used to artificial blood sources. Also, host preference could be a factor, and while *Culex tarsalis*

have been implicated in horse WNV infection, they typically feed on birds as opposed to mammals.

There are multiple North American bird species that can become infected with JEV and develop higher viremias than horses, including red-winged blackbirds, tri-colored blackbirds, English sparrows, and house finches (Hammon et al., 1951; Nemeth et al., 2009). Preliminary results from our work with wild birds implicate the following additional species: ducks, seagulls, grackles, European starlings, herons and egrets. Some of these birds, such as gulls and finches, can develop titers as high as 10^5 pfu/ml, which is probably a sufficiently high titer to infect even moderately competent vectors such as *Culex pipiens*. Additionally, domestic swine play a large role in perpetuating JEV transmission cycles in Asia, and are a pivotal source of virus that can be then transferred to humans by mosquitoes that feed on both species in close proximity with one another (Scherer et al., 1959a; Gresser et al., 1958). Serosurveillance of birds and pigs is a good way to predict and potentially prevent JEV from reaching human populations, but in terms of emerging zoonotic infections, once free-ranging birds and mammals become infected with JEV in a novel area, it may be difficult to contain viral transmission and spread. In the U.S, mosquito surveillance and control is critical to preventing outbreaks. Although effective vaccines are commercially available for both horses and humans, the amount of time it would take to induce immunity through vaccination would be too long to prevent an outbreak if JEV behaves anything like WNV in a new location. Mammalophilic mosquitoes, such as *Ae. aegypti, Ae. vexans,* or *Culiseta ionornata* for example, are a probable source of infection; monitoring these and other known vector

species could be the key to minimizing the impacts of JEV circulation should it arrive in the U.S.

Cross-protective antibody response between JEV and WNV has been studied extensively in laboratory animals such as bonnet macaques, hamsters and mice as well as domestic swine (Tesh et al., 2002; Ilkal et al., 1994, Goverdhan et al., 1992, Williams et al., 2001, Lobigs et al., 2003; Lobigs et al., 2009). In addition, red-winged blackbirds with naturally acquired WNV immunity fail to develop JEV infection after experimental inoculation, thus extending the idea of cross-neutralization into potential wildlife disease reservoirs (Nemeth et al., 2009). It is not known exactly how high a neutralizing titer needs to be before this phenomenon is seen, but in the case of the blackbirds and some raptors, antibody titers as low as 1:20 against WNV protect against JEV (Nemeth et al., 2008). The results of the serum cross-neutralization of the horses in this study provide some evidence that horses with moderate to high anti-WNV titers (1:40-1:640) may be afforded some protection against JEV. Although there are effective vaccines available for horses, they are not currently distributed in the U.S (Goto et al., 1976). Here in the U.S, many horse owners vaccinate annually against WNV, which could afford protection against JEV in the event of an outbreak.

The infectious cycle of JEV is complex and multidimensional, involving vectors, reservoir hosts and incidental hosts. As such, preventing the emergence of JEV into North America or other new areas requires investigating potential vectors and vertebrate hosts. In addition, many flaviviruses, including WNV, can produce cross-protective antibodies against JEV, which could mitigate the impact of an incursion. Here we have demonstrated that *Ae. aegypti,* a common mosquito found in warm temperate regions

from the southern U.S. southward to Central and South America, can transmit JEV to horses experimentally. However, naturally induced immunity to WNV coupled with the current vaccination regimen against WNV could prevent JEV infection in horses and provides some insight into methods of preventing JEV infection in other vertebrate hosts as well.

Table 3.1: Horse experimental infection results: mosquito infectious titers, viremias, temperatures, and serology

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6
Sex	Female	Male	Female	Female	Female	Female
# engorged mosquitoes	15/18	9/21	18/26	15/15	9/12	25/26
JE strain	VN	VN	8J	8J	P3	P3
JE titer in mosquito	$5.4x10^7$	$4.5x10^7$	$4.3x10^7$	$4.8 x10^7$	$1.9 x10^7$	$2.2 x10^7$
Horse viremias (log pfu/ml)*	0.7-2.6	0.7-2.7	0.7-2.1	<0.7	<0.7	<0.7
Duration of viremia (peak day)	Days 1-6 (4)	Days 1-6 (4)	Days 1-6 (4)	N/A	N/A	N/A
Temperature range (degrees F)	99.6-104	99.4-103	99.6-101.9	99.1-100.9	99.4-102.8	99.4-101.2
JEV PRNT80*	320	160	320	40	10	20

*PRNT80 is the serum dilution titer at which 80% or more virus was neutralized.
Viremia detection threshold was 0.7 log pfu/ml

Table 3.2: Horse serum cross-neutralization antibody titers following primary infection with either JEV or WNV

		WNV screen		
Horse	**Challenge virus**	**Pre-challenge[1]**	**JEV PRNT80***	**WNV PRNT80***
1	JE:VN	<50%	320	10
2	JE:VN	<50%	160	20
3	JE:8J	<50%	320	10
4	JE:8J	<50%	40	10
203	WNV NY99	<50%	10	320
221	WNV NY99	<50%	10	80
590	WNV NY99	<50%	20	320
593	WNV NY99	<50%	10	40
608	WNV NY99	<50%	10	640

*PRNT80 is the serum dilution titer at which 80% or more virus was neutralized.
[1]Pre-challenge WNV screen is the percent neutralization of virus due to suspected antibody in sera.

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CHAPTER FOUR

PRE-EXISTING IMMUNITY TO RELATED FLAVIVIRUSES PROTECTS AGAINST INFECTION BY JAPANESE ENCEPHALITIS VIRUS IN HAMSTERS

Abstract

Japanese encephalitis virus (JEV) is a mosquito-borne virus in the genus *Flavivirus,* family *Flaviviridae.* JEV is endemic in Southeast Asia and the surrounding Pacific Islands and most recently emerged in northern Australia (Hanna et al., 1995; Mackenzie et al., 2002a). The primary reservoirs of JEV are ardeid birds and domestic pigs, but spillover into humans and horses can cause severe encephalitic disease. JEV is closely related to West Nile virus (WNV) and St. Louis encephalitis virus (SLEV), two flaviviruses that are endemic to the U.S. (Reimann et al., 2008). If JEV were to reach the U.S. it is important to determine whether or not prior infection with WNV or SLEV can confer any immunity. To investigate this we used a small animal model and tested a variety of flaviviruses and flaviviral vaccines for their ability to protect against subsequent JEV infection. Our findings indicate that prior infection with closely related viruses like SLEV and WNV are protective against JEV infection. Additionally, vaccination using a chimeric WNV vaccine provides immunity from JEV as does passive

transfer of JEV antiserum one day prior to infection. These results suggest that in the face of an introduction of JEV into the U.S, prior immunity to heterologous flaviviruses through either vaccination or infection may alter the course of the disease.

Introduction

Within the family *Flaviviridae* a variety of vector-transmitted pathogens exist that can cause significant disease of both humans and animals on every continent except Antarctica (Rosen, 1986). Because of their incredibly complex and diverse lifecycles, often involving multiple species of reservoir hosts and mosquito vectors, these viruses tend to be very adaptable to nearly any climate and once established are virtually impossible to eradicate. Japanese encephalitis virus is one such pathogen. It is the leading cause of viral encephalitis in humans worldwide and is responsible for more than 30,000 cases each year and 10,000-15,000 deaths, many of those in children under 12 years old (Ellis et al., 2000; Endy et al., 2002; World Health Report 1996-2008). JEV is also a significant pathogen of horses, causing death from encephalitis in as many as 42% of cases (Ellis et al., 2000). The endemic cycle of JEV allows it to circulate between ardeid birds, culicine mosquitoes and domestic pigs, but during epidemic years the spillover into humans and horses can have disastrous consequence (Scherer et al., 1959a). While historically JEV has only been found in Southeast Asia, Japan, and China, in the early 1990's it was isolated for the first time in the Torres Strait and mainland Australia, putting it on the global radar as a potential emerging infectious disease (Hanna et al., 1995; Erlanger et al., 2009). There exist five genetically distinct genotypes of JEV, three of which are associated with the endemic cycle (II, IV, and V) while the other two (I and

III) are culpable in epidemics (Uchil et al., 2001; Solomon et al., 2003). No significant differences in virulence or pathogenicity have been detected in either epidemic genotype, but a recent trend towards more outbreaks of genotype I versus the traditionally isolated genotype III are cause for question (Nga et al., 2004; Nitatpattana et al., 2008; Saito et al., 2008).

Here in the United States, the recent emergence and establishment of a related pathogen, West Nile virus (WNV) has led scientists and the public alike to consider the possibility of other serious diseases like JEV being introduced into this country (Mackenzie et al., 2002; Nett et al., 2009; Weaver et al., 2010). One question that should be addressed is whether or not prior infection with endemic flaviviruses like WNV and St. Louis encephalitis virus (SLEV) has the ability to alter the course of infection by JEV in susceptible hosts. JEV, WNV, and SLEV are all in the JEV serocomplex of flaviviruses, which potentially allows for some cross-reactivity in host immune response (Mackenzie et al., 2002). Indeed, previous studies have shown that JEV immunity can protect against fatal WNV infection in mice, hamsters, pigs, and bonnet macaques (Ilkal et al., 1994; Goverdhan et al., 1992; Lobigs et al., 2003; Takasaki et al., 2003; Tesh et al., 2002). These viruses also have in common the use of multiple bird species as reservoir hosts and WNV, like JEV is serious pathogen of horses (Ellis et al., 2000; Scherer et al., 1959b).

The hypothesis investigated in the following experiments is that antibody-mediated immunity against heterologous flaviviruses can provide some protection against JEV in susceptible hosts. To investigate this idea, a hamster model was used to examine a variety of flaviviruses and flaviviral vaccines for their ability to induce immunity

against infection by JEV. Also examined was the ability of passively transferred anti-JEV or anti-WNV antiserum to provide some protection against JEV infection. Cross-protective immunity could be a significant factor in altering the course of an outbreak of JEV in the U.S. The level of immunity in a natural population with high turnover rates and annual births, such as occurs in birds, is likely to be too low to reach a majority of the susceptible population. However, in hosts such as horses or humans for whom annual vaccinations are prescribed, the majority could have some level of acquired immunity. If there is clear indication that prior immunity to similar viruses has the ability to prevent JEV infection and disease in susceptible hosts such as horses and humans, then the response necessary in the event of an outbreak could be significantly diminished due to established herd immunity.

Materials and Methods

Viruses

Two strains of JEV were used throughout this study. The first, strain 826309 (hereafter JE:8J), is an isolate from a human brain in India and passaged twice in suckling mice and twice in Vero cells. The other virus is an isolate from a *Culex tritaeniorhynchus* mosquito in Vietnam passaged once in suckling mice and once in Vero cells (JE:VN). Molecular sequencing of the two JE viruses using the prM region of the genome revealed that JE:8J is a genotype III strain and JE:VN is a genotype I strain (Bosco-Lauth, unpublished data). WNV strain NY99-4132 was isolated from an American crow and passaged once in Vero cells, once in C6/36 mosquito cells, and once in baby hamster kidney-21 cells. The other JEV serocomplex flavivirus used was SLEV

strain TBH28. Yellow fever virus strain 17D, and Sindbis virus strain AR339 were used as well. Yellow fever virus (YFV) is a flavivirus in the Yellow fever virus group and is less closely related to those viruses in the JEV serocomplex. Sindbis virus is an alphavirus belonging to the *Togaviridae* family and was included as a control to verify that any induced immunity was specific for flaviviruses and not a generic humorally mediated anti-viral response.

Animals

Juvenile male Golden Syrian Hamsters age 8-10 weeks were held in cages of no more than four and fed a diet of rodent feed and cared for in compliance with NIH guidelines for the care and use of lab animals.

Vaccines

The following vaccines were used: West Nile-Innovator® DNA by Fort Dodge, Recombitek™ WNV vaccine by Merial, Inc lot number 54027 and Prevenile™ WNV vaccine by Intervet, Inc lot number 07966003. The Recombitek™ vaccine (hereafter referred to as Canarypox WNV) is a recombinant virus vaccine that uses a canarypox virus backbone with the prM and E regions of WNV inserted into the genome. The Prevenile™ vaccine (hereafter referred to as Chimera WNV) is a chimeric vaccine that uses another flavivirus, Yellow Fever virus, as the backbone with the YF prM and E genes replaced by WNV prM and E genes. West Nile-Innovator® DNA (hereafter called WNV DNA vaccine) is a purified DNA plasmid that encodes WNV proteins. For passive immunity determination, pooled hamster WNV antiserum titered at 1:640 and pooled

hamster JEV antiserum titered at 1:640 were used along with control hamster serum that had <50% neutralization for either virus at a 1:10 dilution.

Statistical Analysis

Differences in JEV infection parameters between groups of hamsters with different vaccinations and pre-JEV inoculation infections were compared using Chi-square values from 2x2 contingency tables in StatCrunch, an online statistical analysis program. Peak viremias between groups were then compared overall and individually using a pair-wise approach with Kruskal-Wallis non-parametric tests on StatCrunch. This non-parametric approach was used because the data do not meet the normality assumption required for parametric tests. Differences were considered statistically significant at alpha ≤ 0.05 with 95% confidence intervals.

Experimental Design

Hamster model development and pathogenesis

Twelve male and twelve female hamsters were used in this portion of the study. Hamsters were inoculated subcutaneously (s.c.) with JEV (10^4 pfu/0.1ml) on Day 0, half the males and females with JE:8J and the other half with JE:VN. 100 µl of blood was collected into 0.45mls BA-1 (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B in 0.05 M Tris, pH 7.6) supplemented with 10% fetal bovine serum once daily for 5 days post-infection (DPI) with JEV via saphenous puncture. Following sera collection, samples were centrifuged at 1127 x *g* (1200 rpm) for 2.5 minutes. On days 2,

4, 6, 8, 12, and 16 post inoculation four hamsters, one male and one female for each JEV strain, were euthanized and necropsied. The following organs were collected: brain, heart, lungs, liver, spleen, kidney, muscle, gonads, and intestine. Half of each organ was fixed in neutral buffered formalin, embedded and sectioned to prepare H&E stained sections for histopathology. The other half was homogenized in BA-1 to prepare 10% suspensions which were assayed for virus isolation on Vero cells. Starting on day 8 post infection, serum collected from remaining hamsters was assayed for antibody detection on Vero cells.

Cross-protection

This part of the study involved inducing immunity either through viral infection or vaccination. Hamsters were divided into groups and treated as shown in Table 4.1. The WNV and SLEV infected groups were inoculated s.c. with 0.1 ml virus at a titer of $1x10^5$ pfu/0.1ml. The YFV hamsters were inoculated with 0.1 ml s.c. at $5x10^4$ pfu/0.1ml and the Sindbis virus group 0.1 ml s.c. with $2x10^4$ pfu/0.1ml. The Canarypox WNV group was vaccinated intramuscularly (i.m.) with a full dose (as recommended for horses by the manufacturer) reconstituted into 0.3 ml diluent and the Chimeric WNV group was vaccinated i.m. with a half dose reconstituted into 0.3 ml diluent. The DNA vaccinates were given a roughly half-dose of 150 µl i.m into the shoulder. Eight hamsters from each of the following vaccine groups were given a booster dose two weeks following the initial dose: YF 17D, WNV DNA vaccine, Canarypox WNV vaccine, and Chimeric WNV vaccine. Twenty eight days following the initial treatment, hamsters were first bled to determine antibody levels and then infected s.c with 0.1 ml of JEV at $1x10^4$

pfu/0.1ml. 100 µl of blood was collected into 0.45 ml BA-1 once daily for 5 DPI with JEV via saphenous puncture. Hamsters were euthanized and serum collected 14 DPI.

Passive transfer

This part of the experiment investigated the passive transfer of immunity using serum containing antibodies against either JEV or WNV and went as described in Table 4.2. Hamsters were injected with 0.2 ml serum intraperitoneally (i.p.) 24 hours prior to JEV inoculation. At this time, blood was collected for antibody profile and then JEV infection was performed s.c. as described above. Blood was again collected for 5 days post-inoculation and hamsters were euthanized on day 5.

Virus isolation

Plaque assays were performed for determination of viremia (viral load in the blood) using a mammalian cell line previously described (Westaway et al., 1966). Briefly, Vero cells were grown in 6 well plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 7.5% bovine calf serum and antibiotics until a monolayer of cells formed. One hundred microliters of hamster sera was added to the monolayers and incubated at 37° C for one hour before an overlay of Minimum Essential Medium (MEM) and agarose supplemented with 2% fetal bovine serum, sodium bicarbonate and antibiotics was added, 2 ml per well of the six-well plate. Plates were then incubated for 48 hours at 37° C at which time a second overlay was added, this time with 0.004% neutral red dye. Plates were counted for plaques 24 and 48 hours following the second overlay.

Serology

Antibody determination was performed using plaque reduction neutralization assays (PRNT) as previously described (Lindsey et al., 1976). Briefly, monolayers of Vero cells were grown as described above. Serum samples were diluted out to 1:320 in 96 well plates in BA-1 with antibiotics. Virus samples were mixed at appropriate dilutions to yield approximately 100 plaques/well for the control titration and 100 µl virus was added to 100 µl sample in the 96 well plate and incubated at 37° C for one hour or refrigerated overnight. Samples were then inoculated onto 6 well Vero plates and allowed to incubate for one hour at 37° C. First and second overlays were added as described above and plates were counted 24 and 48 hours following the second overlay.

Results

Pathogenesis

Of the 16 infected hamsters, 15 developed detectable viremia by 2 DPI with peak titers on day 3 as shown in Table 4.3. While virus was isolated from a variety of organs in the infected hamsters within the first 6 days post-infection, histopathological findings showed no significant lesions or abnormalities associated with JEV infection in any organ other than the brain. The most prevalent histopathological finding was the appearance of moderate to severe non-suppurative meningoencephalitis in the brains of 12/24 hamsters (Figure 4.1). Four of the hamsters had mild splenic follicular expansion at days 12 or 16 PI which could possibly be associated with increased lymphocytic production due to disease. Three hamsters had some renal abnormalities and one hamster had mild hepatitis

but no other pathology; these pathologies are unlikely associated with JEV infection. No other histologic lesions were detected in any other organs. Meningoencephalitis showed up at the earliest in one hamster 6 DPI and was detected in the brains of all but 2 hamsters euthanized 8 DPI or later. There was no apparent difference in the encephalitic disease between the two genotypes. In spite of the appearance of encephalitis in the brains of many of these hamsters, no clinical symptoms could be detected and no hamster died of disease.

Meningoencephalitis is characterized by diffuse inflammation of the brain and inflammation of the leptomeninges, or subarachnoid space. The images in Figure 4.1 illustrate the increase in inflammatory cells in locations throughout the brains of certain hamsters, with noticeable gathering of these cells near vessels, as shown in Hamsters 17 and 11, as well as around the cortical lining in Hamster 10 and clustered near glial cells in Hamster 24. The evidence of inflammation of the tissue throughout various parts of the brain dictates that the infection caused by JEV affects the entire brain and is not localized in specific areas.

Cross protection

Table 4.4 shows the results of the plaque assay for detection of viremia. Overall, there was a significant difference between JEV infections between all eight treatment groups, including the controls ($p<0.0001$). Those animals that received a booster dose or respective vaccines, however, did not have any significant variations in infection rates compared to the non-booster groups ($p=0.21$). Hamsters infected with wild-type WNV and wild-type SLEV were completely protected against infection by JEV as determined

by inability to isolate JEV from sera. The Chimeric WNV vaccine was also completely protective. The Canarypox WNV vaccine was only partially protective; 9/12 hamsters over the course of two trials developed viremia. The yellow fever 17D vaccine strain virus failed to protect as well; 5/6 hamsters developed viremia. The same was seen with Sindbis virus (4/5 viremic). Sindbis virus was used to rule out the possibility of a generic antiviral response as the primary cause of immunity; these results indicate that a specific immune response is responsible for cross protection. Over the course of two trials, 4/15 WNV infected hamsters died prior to JEV challenge (previous work in our lab has shown that approximately 25% of hamsters die following WNV infection, therefore we started our study using 15 hamsters for the WNV challenge and thus the results only include data from the 11 survivors.) No adverse effects were seen in any of the other pre-JEV challenge groups. Infection was confirmed in each of the live-virus infected groups by isolating virus 3 days post-inoculation using plaque assays. Following JEV infection, one control hamster and one Sindbis hamster died 10 DPI. In the control group, two hamsters failed to develop viremic or serologic response to infection; every other hamster infected with JEV had detectable neutralizing antibodies against JEV at a 1:10 serum dilution at the end of the 14 day post infection with JEV. Interestingly, JEV infection enhanced the titer of anti-WNV antibodies (all >1:320), but seemed to have no enhancing effect on any other group, including SLEV. Figure 4.2 shows the antibody response to the initial infection/vaccination and the whether or not the hamsters developed JEV infection. It is noteworthy that the antibody response to the initial viral infection/vaccination did not necessarily predict whether or not subsequent JEV infection would occur (see Figure 4.2). Throughout this study, no significant clinical differences between JEV genotypes

were observed, which is consistent with observations during human and horse outbreaks (Lian et al., 2002, Solomon et al., 2002).

Peak viremias between vaccinated animals were compared to evaluate whether or not there were any significant differences in levels of JEV viremia due to pre-exposure immune status (Table 4.6). Overall, there was a significant difference in viremic response when all groups were compared together ($p<0.0001$). The control hamsters and those infected with Sindbis virus had approximately equivalent levels of viremia ($p=0.54$), all other vaccinated groups that developed infection had significantly lower viremias compared to the controls ($p<0.05$). Those hamsters vaccinated with either Sindbis virus, YF, DNA WNV or Canarypox WNV all developed similar peak JEV titers ($p>0.05$) to each other, indicating no strong statistical differences in viremic levels between these vaccinated groups. The Chimeric WNV vaccinates and the hamsters infected with virulent WNV or SLEV all failed to develop any level of viremic infection, thus, no comparison for peak viremia is necessary.

Passive transfer

Hamsters injected with serum containing anti-JEV antibodies showed complete protection from JEV infection 24 hours later, as shown in Table 4.5. However, the anti-WNV serum protected only 3/6 hamsters from JEV infection while 5/6 hamsters from the control group developed viremia in response to JEV infection. These results show that administration of JEV antisera prior to JEV infection is quite different compared to the other two groups ($p=0.014$). No signs of illness or adverse reactions were seen in any hamsters and as before, there did not appear to be any differences in virulence or viremic

profiles between the two strains of JEV. Each hamster was bled just prior to JEV challenge to determine if there were any detectable antibodies to either JEV or WNV; none of the hamsters had neutralizing antibodies at a 1:10 dilution. These results are not surprising, however, as previous studies have shown that passive serum transfer can elicit a primed T cell response and still prevent infection even in the absence of neutralizing antibodies (Gupta et al., 2008; Konishi et al., 1998; Kimura-Kurodan et al., 1988).

Discussion

Flaviviruses are a large and diverse group of viruses, many of which can cause significant disease in humans and animals. Historically, the JEV serocomplex viruses have clustered in geographically distinct locations, but the recent spread and emergence of JEV into Australia and WNV into North and South America has increased the concern that these niches aren't necessarily separate (Gaunt et al., 2001; Mackenzie et al., 2002a). Vector competence is a likely contributor to the regional differences between these viruses, but their ability to induce protective immunity against each other may also impact their propensity to spread to novel locations. Australia has seen several instances of JEV infection, but it does not seem to have become well established on the mainland, while Murray Valley encephalitis virus, another in the JEV serocomplex, is a constant threat there (Hemmerter et al., 2007; Mackenzie et al., 2002b). Both SLEV and WNV are endemic throughout most of the U.S, but the most severe neuroinvasive disease cases of SLEV are limited and are mostly found in Texas, Louisianna and along the Missouri river while WNV has a stronger clinical impact near the Rockies (Reimann et al., 2008). WNV neutralizing antibodies in birds, horses, and rodents have been detected following

both symptomatic and inapparent infections (Nemeth et al., 2008; Sanchez et al., 2007; Tesh et al., 2002), and the experiments in this study indicate that the antibodies produced against WNV may also be neutralizing against JEV. Therefore, it is possible that the impact of an introduction of JEV into the U.S. may be mitigated due to herd immunity against the flaviviruses already circulating here both in humans and animals. In Asia, vaccination against JEV in both humans and horses has greatly reduced the disease burden on susceptible populations (Monath, 2002; Goto et al., 1976). The results of this study also indicate that the current vaccination regimen for horses against WNV could be protective against JEV, although further testing needs to be done to confirm such a claim. It appears that using a chimeric WNV vaccine induces more immunity against heterologous viruses than do the recombinant WNV vaccine or the DNA WNV vaccine; whether or not the inactivated JEV vaccine currently used for humans has the same effect is undetermined. The transfer of anti-JEV antibody-containing serum could have implications for rapid treatment, but more research needs to be done to determine whether or not serum given as a prophylactic or post exposure has any impact on the outcome of disease during an epidemic.

The idea of cross-reactivity among related flaviviruses is not a new one; indeed, multiple studies have demonstrated this phenomenon both as a cross-protective effect and also as an infection enhancing effect. Dengue virus, for example, has four serotypes and in multiple instances a second infection with a new genotype seems to actually worsen the virulence of disease in the host (Lei et al., 2001). In the case of JEV, mouse models have been used to show that a very low magnitude humoral immune response to primary infection fails to produce sufficient antibodies to protect against subsequent infection and

allows for a more rapidly deleterious effect upon re-infection (Lobigs et al., 2003). Conversely, other studies show that vaccination with sufficient antibody production or prior virulent infection greatly reduces the severity of secondary infection in mice (Konishi et al., 1998). Protection from secondary infection of heterologous flaviviruses has been shown many times over for WNV, SLEV, MVEV and JEV in a variety of species including bonnet macaques, pigs and lab rodents (Lobigs et al., 2009; Goverdhan et al., 1992; Ilkal et al., 1994, Takasaki et al., 2003; Tesh et al., 2002). While lab animals are useful models, it is important to examine parallel effects in the natural hosts of disease as well, which in the case of JEV includes birds. Prior research has already determined that there are a variety of bird species here in the U.S. that could be reservoirs for JEV, such as red-winged blackbirds, tri-colored blackbirds, European sparrows, and house finches (Hammon et al., 1951; Nemeth et al., 2009). Additional research has shown that WNV immunity in Red-Winged blackbirds prevents JEV infection (Nemeth et al., 2009), which lends support to our theory of cross-protective immunity in natural hosts as well as lab animal models.

The results from these experiments indicate that genotypic variations don't seem to induce any symptomatic clinical differences in mammalian hosts and both genotypes appear to respond similarly to protective antibodies. In addition, infection rates in hamsters between the two genotypes were not found to be significantly different ($p=0.27$). It is possible that the recent shift in predominant genotype from III to I in Asia is associated with an increased receptor binding affinity, but from this study there is no evidence that virulence or infectivity in hamsters are enhanced (Ali et al., 1995; Wang et al., 2007.)

Pathogenesis of JEV in hamsters is not necessarily reflective of disease in humans, horses, or other susceptible hosts, but the presence of meningoencephalitis in the brains of hamsters that cleared infection and outwardly appeared normal is interesting. In humans, as many as 50% of survivors of JE infection have permanent neurological damage (Ding et al., 2007) and the range of abnormalities suggest that encephalitis is not focused in specific regions, but is diffuse and can lead to motor problems, speech impediments, and mental retardation (Solomon et al., 1998; Solomon et al., 2002; Schneider et al., 1974) In this particular study, no adverse effects were observed, but it is possible that the hamsters would eventually develop neurological problems or perhaps we simply have no way to determine the range of mental defect in hamsters. Either way, the presence of meningoencephalitis in an otherwise healthy host is pertinent to the overall response of the host to JEV disease.

The likelihood of JEV reaching this country remains high and it is generally acknowledged that the lack of theoretical transmission models could hamper the ability to react quickly to such an event (Nett et al., 2009). Being able to predict what will happen to animals in the event of a zoonotic disease outbreak like JEV can help us prepare for ways to mitigate the impact on humans and hopefully this information can be useful in preventing any future JEV epidemics in the U.S.

Table 4.1: Vaccine efficacy experimental design: initial treatment, number of animals used, and strain of JEV used for infection

Initial treatment	Number hamsters	JEV Strain
Infected with WNV	6	JE:8J
Infected with WNV	6	JE:VN
Infected with SLEV	3	JE:8J
Infected with SLEV	3	JE:VN
Infected with YF	7	JE:8J
Infected with YF	7	JE:VN
Infected with Sindbis virus	3	JE:8J
Infected with Sindbis virus	2	JE:VN
Vaccinated with Canarypox WNV	10	JE:8J
Vaccinated with Canarypox WNV	10	JE:VN
Vaccinated with Chimeric WNV	10	JE:8J
Vaccinated with Chimeric WNV	10	JE:VN
Vaccinated with DNA WNV	4	JE:8J
Vaccinated with DNA WNV	4	JE:VN
Untreated (control)	10	JE:8J
Untreated (control)	10	JE:VN

Table 4.2: Passive transfer of immunity experimental design: initial treatment (vaccination using antisera), number of animals used, and strain of JEV used for infection

Initial treatment	Number hamsters	JEV strain
Anti-WNV	3	JE:8J
Anti-WNV	3	JE:VN
Anti-JEV	3	JE:8J
Anti-JEV	3	JE:VN
Control antisera	3	JE:8J
Control antisera	3	JE:VN

Table 4.3: Results of pathogenesis experiment: JEV viremia and presence or absence of encephalitis in hamsters infected with one of two strains of JEV.

Hamster	Sex	JEV strain	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI*	Encephalitis +/-	Day Euthanized
1	M	VN	<2	3.6				-	2 DPI
2	M	VN	<2	<2	2.6	<2		-	4 DPI
3	M	VN	<2	2.6	3.8	2.6	<2	+	6 DPI
4	M	VN	<2	2.9	2.3	2.8	<2	+	8 DPI
5	M	VN	<2	<2	3.1	<2	<2	+	12 DPI
6	M	VN	<2	2.3	2.5	<2	<2	+	16 DPI
7	F	VN	<2	3.8				-	2 DPI
8	F	VN	<2	3.5	4.2	<2		-	4 DPI
9	F	VN	<2	<2	2.5	2.6	<2	-	6 DPI
10	F	VN	<2	2.5	3.7	3.4	<2	+	8 DPI
11	F	VN	<2	3.2	4.3	3.2	<2	+	12 DPI
12	F	VN	<2	3	<2	2	<2	+	16 DPI
13	M	8J	<2	2.5				-	2 DPI
14	M	8J	<2	3.2	3.6	<2		-	4 DPI
15	M	8J	2.3	<2	3.2	2.5	<2	-	6 DPI
16	M	8J	<2	2.3	3.3	2.8	2	+	8 DPI
17	M	8J	<2	3.2	4	2	<2	+	12 DPI
18	M	8J	<2	2.3	3.2	3	<2	+	16 DPI
19	F	8J	<2	2.7				-	2 DPI
20	F	8J	<2	2	3.3	<2		-	4 DPI
21	F	8J	<2	<2	<2	<2	<2	-	6 DPI
22	F	8J	<2	<2	2.8	2.5	<2	-	8 DPI
23	F	8J	<2	2.5	3.5	4	<2	+	12 DPI
24	F	8J	<2	2.9	3.2	<2	<2	+	16 DPI

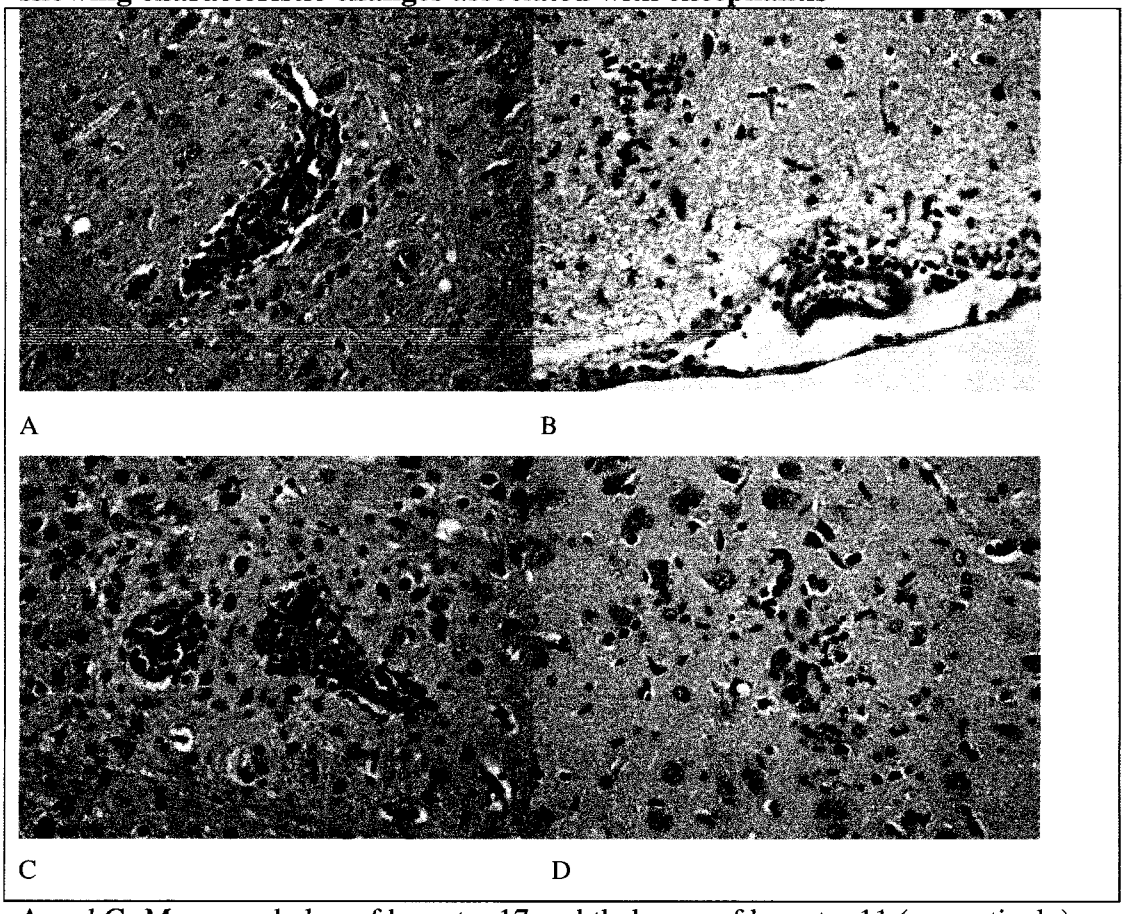
*No viremia was detected after day 5.

Hamsters were necropsied on the day of euthanasia; any encephalitic lesions detected are from that time point.

Values listed under DPI columns are viremia titers in log pfu/ml.

DPI= Days post-infection

Figure 4.1: Histopathologic legions in the brains of four of the infected hamsters showing characteristic changes associated with encephalitis



A and C- Mesencephalon of hamster 17 and thalamus of hamster 11 (respectively) showing infiltration of inflammatory cells surrounding small blood vessels.
B- Cerebral cortex of hamster 10 illustrating inflammation of the cortical lining
D- Thalamus of hamster 24 showing inflammatory cells clustered near glial cells

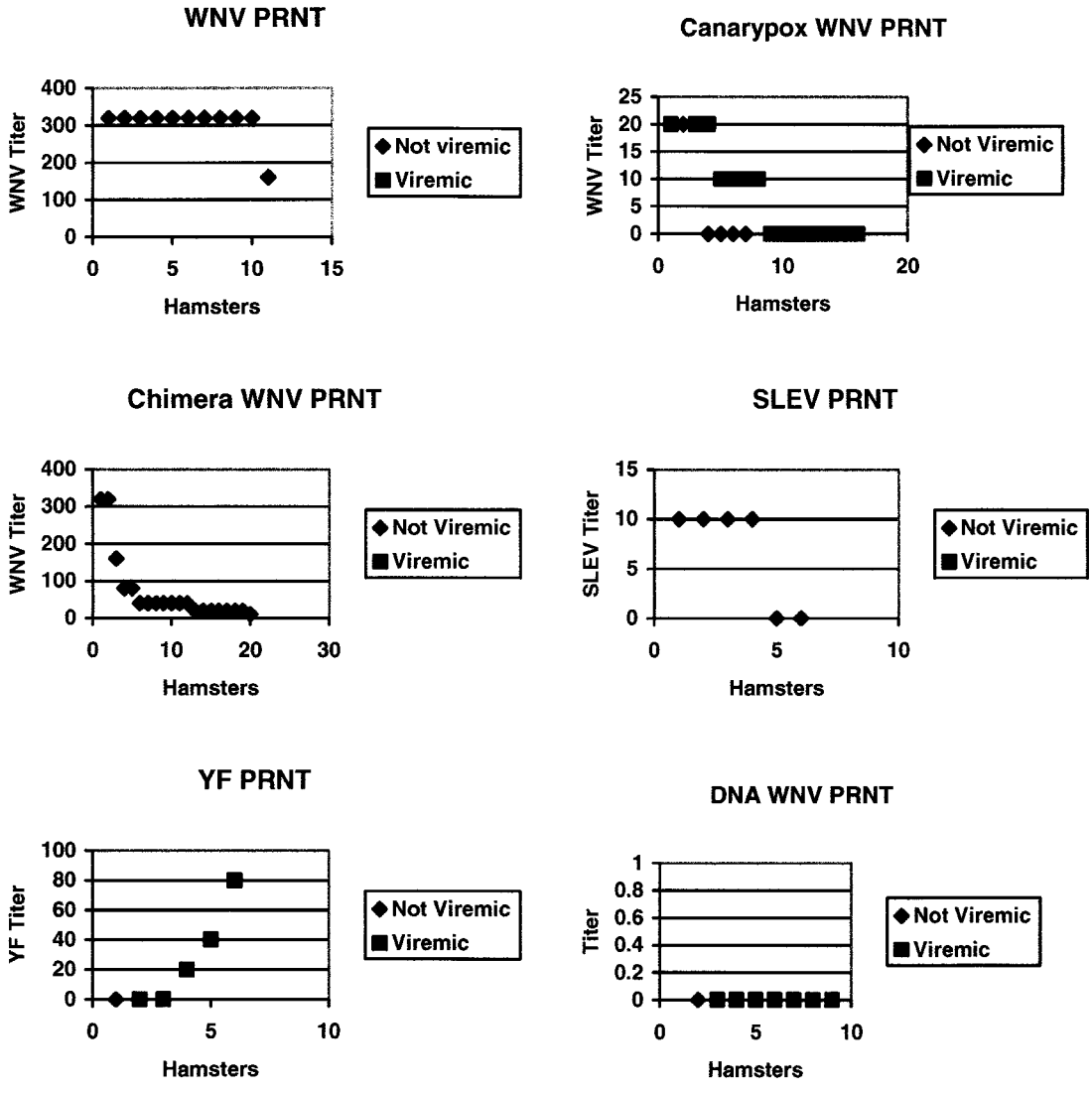
Table 4.4: Summary of results from cross-protection experiment: Initial treatment (vaccination or virus infection), viremic response to JEV infection and mean titer of viremia detected in hamsters

Initial treatment	No. Viremic/Total	Mean titer in log pfu/ml [range]**
No treatment (control)	17/20	4.1 [2-6.5]
WNV	0/11	N/A
SLEV	0/6	N/A
Yellow fever (YF)	12/14 (7/8)*	2.7 [2-3.7]
Chimeric WNV vaccine	0/20 (0/8)	N/A
Canarypox WNV vaccine	15/20 (6/8)	2.9 [2-4.2]
WNV DNA vaccine	(7/8)	2.6 [2-3.4]
Sindbis virus (non-flavivirus control)	4/5	4.4 [2-5.3]

*Red parentheses indicate viremias in hamsters given a booster vaccine.

** Detection threshold was 2 log pfu/ml serum. Only viremic hamsters were included in this column

Figure 4.2: Serologic response to initial infection or vaccination prior to JEV inoculation, followed by subsequent viremic response to JEV infection:
Hamster sera from YF, SLEV, or WNV infected/vaccinated hamsters was collected just prior to JEV infection and titrated out to determine antibody titer of the initial flavivirus exposure. Antibody titers were then correlated with a viremic or non-viremic response to JEV infection as indicated by the symbols above.



A titer of zero indicates >50% neutralization at 1:10.

Table 4.5: Summary for passive immunity experiment: initial treatment (sera vaccination), viremias and neutralizing antibody titers

Initial treatment	Viremic/Total	JEV PRNT80*	WNV PRNT80*
JEV antisera	0/6[1]	<10	<10
WNV antisera	3/6	<10	<10
Control sera	5/6	<10	<10

*PRNT80 is the serum dilution factor at which 80% or more virus was neutralized.
[1] Viremic response between JEV antisera group and the other groups was statistically significant, p=0.014.

Table 4.6: Peak viremia recorded for each treatment group and comparison of peak titer with other treatment groups. Groups with the same superscript letter showed no statistically significant differences between peak viremias; different letters indicate a significant difference was detected.

Treatment Group:	Control	Sindbis	YF	Canarypox WNV	DNA WNV	Chimera WNV	WNV	SLEV
Mean Peak Titer:	6.5^{a}	$5.3^{a,b}$	3.7^{b}	4.2^{b}	3.4^{b}	$<2^{c}$	$<2^{c}$	$<2^{c}$

Peak titer <2 indicates that no detectable viremias were observed in hamsters from these treatment groups.

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CONCLUSIONS

Japanese encephalitis virus (JEV) is a vector-borne pathogen of Asia and surrounding regions with demonstrated potential to spread into new locations across the globe. The experiments in this dissertation investigated vector competence for JEV using mosquitoes indigenous to the U.S., and examined pathogenesis and immunity of JEV infection in hamsters and horses. The conclusions from these investigations suggest that the U.S. is home to several species of mosquito vectors that could maintain JEV transmission and that host pathogenesis and immunity could be a discerning factor in epidemic JEV transmission.

Four mosquito species were evaluated for JEV competence; *Cx. tarsalis, Cx. pipiens, Ae. aegypti,* and *Ae. albopictus.* Three of these species, *Cx. tarsalis, Cx. pipiens,* and *Ae. aegypti,* were determined to be weakly competent laboratory vectors, and *Ae. aegypti* mosquitoes transmitted the virus to horses during blood-feeding. *Ae. aegypti* could not, however, become infected by feeding on viremic horses. These results confirm that there are at least three competent vectors for JEV native to the U.S.

Experimental infection of horses by mosquito bite resulted in asymptomatic viremic infection of three out of six horses. Histopathological lesions were not observed in the brains of horses 2-3 weeks following challenge, but all six horses developed a neutralizing antibody response by two weeks post infection. That JEV infection in horses does not necessarily lead to clinical disease or even viremia is not surprising considering

similar and much more extensive experience with the closely related West Nile virus (WNV). Nonetheless, it was clear that the horses became infected, as evidenced by an increased JEV neutralizing antibody titer following challenge. It is likely that most horses that are bitten by infected mosquitoes will exhibit a similar response, but severe disease will occur in some animals.

Cross-protective immunity to heterologous flaviviruses and flaviviral vaccines was examined using a hamster model. Prior infection with WNV or St. Louis encephalitis virus (SLEV), or vaccination using a live WNV/Yellow fever virus chimera completely protected hamsters against viremic or clinical infection by JEV. Conversely, infection with Yellow fever virus, Sindbis virus, or vaccination using a recombinant WNV vaccine or a subunit DNA WNV vaccine failed to protect the hamsters from developing a viremic infection with JEV. However, the peak JEV viremias were sufficiently reduced by vaccination or infection with Yellow fever virus, the recombinant vaccine or the DNA vaccine. In addition, vaccination using hamster anti-JEV immune sera 24 hours prior to JEV inoculation protected hamsters against infection. These results suggest that prior immunity to WNV or SLEV through natural infection or by a live chimera WNV/Yellow fever vaccine could protect the host from becoming infected by JEV. Thus, in animals such as horses that are commonly vaccinated against WNV in the U.S., there is the possibility that an emergence of JEV won't have much of an impact on the populations because of the cross-neutralizing properties of WNV vaccination. In wildlife, naturally acquired WNV or SLEV immunity could have benefits against JEV infection, but this effect would likely be lessened due to the high turnover in populations, which would reduce the number of animals with pre-acquired immunity.

In conclusion, the investigations of this dissertation are a starting point for looking at vector competence in North American species and also for evaluating the pathogen-vertebrate host interactions and infectious cycle of JEV. Future directions will include a more extensive investigation of vector competence and a better understanding of the immune response to JEV in horses vaccinated against WNV. Additionally, examination of potential wildlife hosts for JEV, both avians and mammals, is part of the plan to help predict and hopefully manage the impact of a likely future JEV emergence into the U.S.