Pathogenesis of Japanese Encephalitis Virus Infection in a Golden Hamster Model and Evaluation of Flavivirus Cross-Protective Immunity

Angela Bosco-Lauth, Gary Mason, and Richard Bowen*

Department of Microbiology, Pathology, and Immunology and Department of Biomedical Sciences,
Colorado State University, Fort Collins, Colorado

Abstract. Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus endemic to Southeast Asia and surrounding Pacific Islands, and it has most recently emerged in northern Australia. JEV is closely related to West Nile virus (WNV) and St. Louis encephalitis virus (SLEV), both endemic to the United States. In the event that JEV is introduced into the Americas, it will be important to determine whether immunity to WNV or SLEV might afford protection from infection and development of viremia in susceptible hosts. We investigated a hamster model of JEV infection and showed that a large fraction of animals infected with either a genotype I or III isolate of virus developed viremia and encephalitic lesions without clinical signs of disease. Using this model, we showed that prior infection with WNV or SLEV, vaccination using a chimeric WNV vaccine, and passive immunization with anti-JEV immune sera prevented viremia in hamsters challenged with JEV.

INTRODUCTION

Within the family *Flaviviridae*, several vector-transmitted pathogens exist that cause significant disease in both humans and animals on every continent except Antarctica.¹ Because of their 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 after established, they are virtually impossible to eradicate. Japanese encephalitis virus (JEV) is one such pathogen. It is the leading cause of viral encephalitis in humans worldwide, and it is responsible for more than 30,000 cases of human disease each year and up to 25% mortality, many of those in children under 12 years old.^{2–4} JEV is also a significant pathogen of horses, causing death from encephalitis in as many as 40% of clinical cases.² 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 consequences.⁵ Although JEV has historically been found only in Southeast Asia, Japan, India, and China, in the early 1990s, 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.^{6,7} There exist five genetically distinct genotypes of JEV, three of which are associated with the endemic cycle (II, IV, and V), whereas the other two (I and III) are culpable in epidemics.^{8,9} Significant differences in virulence or pathogenicity have not been detected between the two epidemic genotypes, but there has been a recent trend to more outbreaks of genotype I versus the traditionally isolated genotype III.^{10–12}

The recent emergence and establishment in North America of a related pathogen, West Nile virus (WNV), has led scientists and the public alike to consider the probability of other exotic arboviruses like JEV being introduced into this country and the extent that prior infection with endemic flaviviruses like WNV and St. Louis encephalitis virus (SLEV) might alter the course of infection by JEV in susceptible hosts.^{13–15} JEV, WNV, and SLEV are all in the JEV serocomplex of flaviviruses, which potentially allows for some cross-reactivity in host

immune response.^{16,17} Indeed, previous studies have shown that immunity to JEV can protect against fatal WNV infection in mice, hamsters, pigs, and bonnet macaques.^{18–22} These viruses also have in common the use of multiple bird species as reservoir hosts, and WNV, like JEV, is a serious pathogen of horses.^{2,23}

The hypothesis investigated in the experiments reported here is that immunity against heterologous flaviviruses would provide protection against JEV in mammalian hosts. A hamster model was used to evaluate several flaviviruses and flaviviral vaccines for their ability to induce protective immunity against infection by JEV. We also examined the ability of passively transferred anti-JEV or anti-WNV antiserum to protect against JEV infection. Cross-protective immunity could be a significant factor in altering the course of an outbreak of JEV in the United States. Although the level of immunity in populations with high turnover rates and annual births, such as occurs in birds, is likely to be too low to have a significant effect on virus circulation, in hosts such as horses 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, the response necessary in the event of an outbreak could be significantly diminished because of established herd immunity.

MATERIALS AND METHODS

Viruses. Two strains of JEV were used. The first, strain 826309 (hereafter, JE:8J), is an isolate from a human brain in India, and it was passaged two times in suckling mice and two times in Vero cells. The second virus was isolated from a *Culex tritaeniorhynchus* mosquito in Vietnam, and it was passaged one time in suckling mice and one time in Vero cells (JE:VN) before use. Molecular sequencing of the premembrane (prM) region of the genomes of the two JE viruses revealed that JE:8J is a genotype III strain and JE:VN is a genotype I strain (Bosco-Lauth A, unpublished data). WNV strain NY99-4132 was isolated from an American crow and passaged one time in Vero cells, one time in C6/36 mosquito cells, and one time in baby hamster kidney-21 cells. The other viruses used were SLEV strain TBH28, yellow fever virus (YFV) strain 17D, and

*Address correspondence to Richard Bowen, College of Veterinary Medicine and Biomedical Sciences, 3701 Rampart Road, Fort Collins, CO. E-mail: richard.bowen@colostate.edu

Sindbis virus strain AR339. YFV is a flavivirus in the Yellow fever virus serogroup and is less closely related to those viruses in the JEV serocomplex. Sindbis virus is an alphavirus belonging to the *Togaviridae* family, and it was included as a control for non-specific antiviral immunity.

Animals. Golden hamsters 8–10 weeks of age were purchased from Charles River Laboratories. The animals were housed in cages of no more than four, were fed a diet of commercial rodent chow, and had free access to water. All animal care was in compliance with National Institutes of Health guidelines for the Humane Care Use of Laboratory Animals, Wilmington, MA.

Vaccines. Two WNV vaccines were used to immunize groups of hamsters: Recombitek WNV vaccine (lot number 54027; Merial Ltd., Deluth, GA) and Prevenile WNV vaccine (lot number 07966003; Intervet, Inc., Summit, NJ). The Recombitek vaccine (referred to hereafter as canarypox WNV) is a recombinant live virus vaccine in which the prM and E genes from WNV are inserted into the canarypox genome. The Prevenile vaccine (referred to hereafter as chimeric WNV) is a chimeric vaccine based on a 17D YFV backbone with the prM and E genes replaced by those from WNV. Pooled normal sera and immune sera from hamsters infected previously with WNV or JEV were used for passive immunization experiments. The two immune sera had 90% neutralizing titers of 640 against homologous virus, whereas the control antisera had a 50% neutralization titer of < 10 against JEV.

Virus titration and serology. Titers of virus in sera and tissue homogenates were determined by plaque assay on Vero cells, as previously described.²⁴ Neutralizing antibody titers were determined using plaque reduction neutralization assays (PRNT) as previously described.²⁵

Statistical analysis. Differences in titer of JEV viremia among groups of hamsters with different vaccinations and pre-JEV inoculation infections were compared using χ^2 values from $2 \times n$ contingency tables with StatCrunch software (www.statcrunch.com). Peak viremias among 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 did not meet the normality assumption required for parametric tests. Differences were considered statistically significant at $\alpha \leq 0.05$ with 95% confidence intervals.

Experimental design: hamster model development and pathogenesis. Twelve male and twelve female hamsters were inoculated subcutaneously (SC) with 10^4 plaque-forming units (pfu) of JEV in 0.1 mL; one-half of the animals of each gender received JE:8J virus, and the other one-half received JE:VN virus. Blood samples (100 μ L) were collected by saphenous venipuncture one time daily for 5 days post-infection (DPI) and mixed with 0.45 mL BA-1 (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 μ g/mL streptomycin, 2.5 μ g/mL amphotericin B in 0.05 M Tris, pH 7.6) supplemented with 10% fetal bovine serum to yield a solution of approximately 10% serum. Blood samples were maintained at room temperature for 15–30 minutes and then centrifuged at $12,000 \times g$ for 2.5 minutes, and the supernates were recovered and frozen to -80°C until assay. On days 2, 4, 6, 8, 12, and 16 post-inoculation, one male and one female hamster inoculated with each JEV strain were euthanized and necropsied, and samples of brain, heart, lungs, liver, spleen, kidney, muscle, gonads, and intestine were collected. One-half of each organ was fixed in neutral buffered

formalin and subsequently embedded and sectioned to prepare hematoxylin & eosin-stained sections for histopathology. The other one-half of each organ was frozen to -80°C and later homogenized in BA-1 to prepare 10% suspensions, which were assayed for virus concentration by plaque assay on Vero cells. Sera collected on or after day 8 post-infection were assayed for neutralizing antibody.

Experimental design: cross-protective immunity to JEV. Antiviral immunity was induced by virus infection or administration of vaccines. Groups of hamsters were inoculated SC with 0.1 mL WNV (10^5 pfu), SLEV (10^5 pfu), YFV (5×10^4 pfu), or Sindbis virus (2×10^4 pfu). Additional groups of hamsters were vaccinated intramuscularly (IM) with canarypox WNV vaccine (full dose as recommended for horses but reconstituted into 0.3 mL diluent) or chimeric WNV vaccine (one-half dose reconstituted into 0.3 mL diluent). Eight hamsters from each of the groups that received a vaccine virus were given a booster dose 2 weeks after the initial dose. One final group (naïve hamsters) consisted of animals that were neither vaccinated nor infected before JEV challenge. Twenty-eight days after the initial vaccination or infection, hamsters were bled, and serum used to determine antibody levels; they were then infected SC with 10^4 pfu of JEV in 0.1 mL. Blood (100 μ L) was collected by saphenous puncture into 0.45 mL BA-1 one time daily for 5 DPI to provide roughly 10% serum. Hamsters were euthanized, and serum was collected 14 DPI.

Experimental design: protection by passive transfer. Antiviral immunity was induced by passive transfer of immune sera against either JEV or WNV. Groups of hamsters were injected intraperitoneally with 0.2 mL immune or control serum. One day later, blood was collected to determine antibody concentration. Using techniques described above, the animals were then inoculated with JEV and bled daily for 5 days to characterize viremia; they were then euthanized.

RESULTS

Pathogenesis of JEV infection in hamsters. None of the hamsters inoculated with either strain of JEV developed detectable clinical disease. Of 24 inoculated hamsters, 23 developed viremia by 2 DPI, with peak titers on day 3 (Table 1). Viremia was not detected after day 5 in any animal. Although virus was isolated from a variety of organs within the first 6 days after inoculation, histopathological lesions attributable to JEV infection were limited to the brain. The most prevalent histopathological finding was moderate to severe non-suppurative meningoencephalitis, with diffuse inflammation of the brain and leptomeninges extending into the subarachnoid space (Figure 1). This lesion was observed in tissues from one hamster euthanized 6 DPI and was found in the brains of all but two hamsters euthanized 8 DPI or later. There was no apparent difference in lesion occurrence or severity between animals infected with the two strains of JEV. Four hamsters had mild splenic follicular expansion at days 12 or 16 post-inoculation, likely a hyperplastic response to virus infection, and one hamster had mild hepatitis but no other pathology; these lesions were unlikely associated with JEV infection.

Cross-protective immunity. Before JEV challenge, adverse effects of virus infection or immunization were not observed except in hamsters infected with WNV, where a total of 4 of 15 animals were euthanized or died, which is similar to what we

TABLE 1
Viremia and encephalitis in naïve hamsters infected with JEV

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

*Viremia was not detected after 5 DPI. NA = not applicable.

have observed in other experiments with WNV and hamsters (unpublished data). Thus, only 11 hamsters previously infected with WNV were included in the JEV challenge.

All of the hamsters infected with WNV or SLEV were viremic 3 days post-inoculation, and none of these animals developed viremia in response to JEV challenge. Similarly, hamsters immunized with either one or two doses of chimeric WNV vaccine also failed to become viremic after exposure to JEV. The canarypox WNV vaccine was only partially protective against

JEV; 15 of 20 immunized hamsters developed JEV viremia, and there was not a significant difference among the groups that received one versus two doses of vaccine ($P > 0.1$). As anticipated, immunization with the yellow fever 17D vaccine virus also failed to protect, with 12 of 14 hamsters developing JEV viremia, and there was no difference between groups receiving one and two doses. Sindbis virus was used to rule out the possibility of a generic antiviral response as the primary cause of immunity, and 4 of 5 animals infected with Sindbis

FIGURE 1. Histopathologic lesions in the brains of four infected hamsters. (A) Mesencephalon of hamster 17. (B) Cerebral cortex of hamster 10. (C) Thalamus of hamster 11. (D) Thalamus of hamster 24.

became viremic after JEV challenge. A summary of results is presented in Table 2.

After JEV challenge, one naïve hamster and one hamster previously infected with Sindbis virus died 10 DPI. In the naïve group, two hamsters failed to develop viremia or serologic response to JEV infection; every other hamster infected with JEV had neutralizing antibody titers greater than 10 by 14 DPI. It is noteworthy that the anti-WNV antibody response to the initial viral infection/vaccination did not necessarily predict whether subsequent JEV infection would occur (Table 2). Throughout this study, no clinical differences between JEV genotypes were observed, which is consistent with observations during human and horse outbreaks.^{26,27}

Peak viremia among infected/vaccinated animals was compared to evaluate whether there were significant differences in levels of JEV viremia because of pre-exposure immune status (Table 3). 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 equivalent JEV viremia ($P = 0.54$); all other vaccinated groups that developed infection had significantly lower viremia compared with the naïve controls ($P < 0.05$). Those hamsters infected or vaccinated with Sindbis virus, YFV, or canarypox WNV all developed similar peak JEV titers ($P > 0.05$), indicating a lack of statistical difference among these groups. The chimeric WNV vaccinates and the hamsters infected with virulent WNV or SLEV all failed to develop JEV viremia.

Passive transfer. Zero of six hamsters injected with serum containing anti-JEV antibodies became viremic. However, the anti-WNV serum protected only three of six hamsters from JEV viremia, and five of six hamsters from the control group developed viremia in response to JEV infection. Signs of illness or adverse reactions were not observed in any of the hamsters, and there were no differences in virulence or magnitude of viremia between the two strains of JEV. These results are consistent with previous research, indicating that passive transfer of neutralizing antibodies is more protective against homologous versus heterologous viruses and can be useful in prophylactic treatment of JEV infection if administered rapidly after exposure.^{28–30}

DISCUSSION

The most frequently used animal models for JEV infection are mice and primates; however, adult mice are often resistant to parenteral infection, and primates are expensive and quite difficult to work with.³¹ One purpose of this study was

to investigate the potential of adult Golden hamsters as a useful model for JEV infection, including vaccine efficacy trials. The results presented here indicate that a high proportion of mature hamsters infected by SC inoculation with either epidemic genotype of JEV become viremic and developed encephalitic lesions but failed to display overt clinical signs of disease. A large majority of humans infected with JEV also fail to develop significant clinical disease.³¹ Peak viremia was not different between the two genotypes. Considering their outbred nature and ease of handling, hamsters may well be a good substitute for mice in a variety of JEV-related studies.

To further characterize the hamster model of JEV infection, we addressed the question of how well immunity to WNV or other flaviviruses would protect against JEV. As expected, recent infection of hamsters with either WNV or SLEV totally prevented development of viremia after JEV challenge, whereas similar infection with YFV or Sindbis virus failed to prevent JEV viremia. Furthermore, vaccination using a commercially available chimeric WNV vaccine completely protected hamsters from infection with JEV. The other commercial WNV vaccine tested, canarypox WNV, was much less efficacious in preventing JEV viremia, but the reasons for this divergence remain unclear. As with the pathogenesis experiment, no differences were observed between the two genotypes of JEV. Pathogenesis of JEV infection in hamsters may not perfectly model the 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 similar to what is observed in other flaviviral infections in natural hosts. In humans, as many as 50% of survivors of clinical JE have permanent neurological damage, 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.^{27,32–34} We have found it difficult to reliably evaluate neurologic disease in hamsters.

A final demonstration of cross-protective flaviviral immunity using this hamster model showed that passive transfer of JEV antibodies 24 hours before JEV infection protects completely against JEV infection, whereas administration of antiserum to WNV was only partially protective against JEV infection. The homologous neutralizing titers of both antisera were the same, confirming assumptions that heterologous immunity is less effective than homologous.

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

TABLE 2
Responses to JEV challenge in hamsters after previous flaviviral infection or immunization

Pre-challenge treatment	Geometric mean anti-WNV PRNT pre-infection	No. viremic/total	Mean peak viremia (log ₁₀ pfu/mL) and range*
None	< 5	17/20	4.1 (2.0–6.5)
WNV infection	300 (160–320)	0/11	NA
SLEV infection	< 5	0/6	NA
Sindbis virus infection	< 5	4/5	4.4 (2.0–5.3)
Yellow fever virus vaccine (one dose)	< 5	5/6	2.7 (2.0–3.4)
Yellow fever virus vaccine (two doses)	< 5	7/8	2.4 (2–3)
Chimeric WNV vaccine (one dose)	48 (10–320)	0/12	NA
Chimeric WNV vaccine (two doses)	34 (20–320)	0/8	NA
Canarypox WNV vaccine (one dose)	8 (< 5–20)	9/12	2.9 (2–4.2)
Canarypox WNV vaccine (two doses)	7 (< 5–20)	6/8	2.8 (2–3.4)

* Detection threshold was 2 log₁₀ pfu/mL serum. Only viremic hamsters were included in data.

TABLE 3
Peak viremia for each treatment group and comparison of peak titer with other treatment groups

Treatment group	Control	Sindbis virus	YFV	Canarypox WNV	DNA WNV	Chimeric WNV	WNV	SLEV
Mean peak titer	6.5 (a)	5.3 (a and 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. Groups with the same parenthetical letters showed no statistically significant differences in peak viremia. Different letters indicate that a significant difference was detected.

emergence of JEV into Australia and WNV into North and South America has increased the concern that these niches are not necessarily separate.^{16,35} Vector distribution and competence are likely contributors to the regional differences between these viruses, but their ability to induce cross-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; however, Murray Valley encephalitis virus, another in the JEV serocomplex, is a constant threat there.^{13,36} There is every reason to believe that JEV will, at some point, be introduced into North America, where WNV and SLEV are endemic. The question is to what extent immunity to these closely related flaviviruses would mitigate an incursion of JEV.

Neutralizing antibodies to WNV in birds, horses, and rodents have been detected after both symptomatic and unapparent infections,^{22,37,38} and the experiments in this study indicated that the antibodies produced against WNV and SLEV protected against JEV infection. It is, thus, likely that heterologous immunity in humans and horses would similarly protect against JEV infection. Considering that a very large fraction of horses in the United States are vaccinated annually against WNV, the results of this study suggest that they would be at least partially protected against JEV, although further testing needs to be done to confirm such a hypothesis.

Protection from secondary infection by heterologous flaviviruses has been shown many times for WNV, SLEV, MVEV, and JEV in a variety of species, including bonnet macaques, pigs, and laboratory rodents.^{18,19,21,22,39} Although laboratory 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 shown that a variety of bird species in the United States could serve as reservoirs for JEV, including red-winged blackbirds, tri-colored blackbirds, European sparrows, and house finches.^{40,41} Additional research has shown that WNV immunity in red-winged blackbirds prevents JEV infection,⁴¹ which further supports the potential importance of cross-protective immunity in natural hosts.

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.¹⁴ 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 United States.

Received January 6, 2011. Accepted for publication February 11, 2011.

Acknowledgments: We are grateful to Drs. Barbara Johnson and Barry Miller for providing JEV strains. This work was funded by contract N01-AI25489 from National Institute of Allergy and Infectious Disease (NIAID).

Authors' addresses: Angela Bosco-Lauth, Gary Mason, and Richard Bowen, College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO, E-mails: angela.bosco-lauth@colostate.edu, gary.l.mason@colostate.edu, and richard.bowen@colostate.edu.

REFERENCES

- Hanna JN, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, Poidinger M, McCall BJ, Mills PJ, 1996. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. *Med J Aust* 165: 256–260.
- Mackenzie JS, Barrett ADT, Deubel V, 2002. The Japanese encephalitis serological group of flaviviruses: a brief introduction to the group. *Curr Top Microbiol Immunol* 267: 1–10.
- Reimann CA, Hayes EB, DiGuiseppi C, Hoffman R, Lehman JA, Lindsey NP, Campbell GL, Fischer M, 2008. Epidemiology of neuroinvasive arboviral disease in the United States, 1999–2007. *Am J Trop Med Hyg* 79: 974–979.
- Rosen L, 1986. The natural history of Japanese encephalitis virus. *Annu Rev Microbiol* 40: 395–414.
- Ellis PM, Daniels PW, Banks DJ, 2000. Japanese encephalitis. *Emerg Infect Dis* 6: 565–578.
- Endy TP, Nisalak A, 2002. Japanese encephalitis virus: ecology and epidemiology. *Curr Top Microbiol Immunol* 267: 11–48.
- World Health Organization, *World Health Report, 1996–2008*. 2010. Available at: <http://www.who.int/whr/en>. Accessed July 7, 2010.
- Scherer WF, Buescher EL, 1959. Ecologic studies of Japanese encephalitis virus in Japan. I. Introduction. *Am J Trop Med Hyg* 8: 644–650.
- Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K, 2009. Past, present, and future of Japanese encephalitis. *Emerg Infect Dis* 15: 1–7.
- Uchil PD, Satchidanandam V, 2001. Phylogenetic analysis of Japanese encephalitis virus: envelope gene based analysis reveals a fifth genotype, geographic clustering, and multiple introductions of the virus into the Indian subcontinent. *Am J Trop Med Hyg* 65: 242–251.
- Solomon T, Ni H, Beasley DWC, Ekkelenkamp M, Cardosa MJ, Barrett ADT, 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. *J Virol* 77: 3091–3098.
- Nga PT, Parquet Md C, Cuong VD, Ma SP, Hasebe F, Inoue S, Makino Y, Takagi M, Nam VS, Morita K, 2004. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from southeast Asia to East Asia. *J Gen Virol* 85: 1625–1631.
- Nitatpattana N, Dubot-Peres A, Gouilh MA, Souris M, Barbazan P, Yoksan S, de Lamballerie X, Gonzalez JP, 2008. Change in Japanese encephalitis virus distribution, Thailand. *Emerg Infect Dis* 14: 1762–1765.
- Saito M, Nakata K, Nishijima T, Yamashita K, Saito A, Ogura G, 2009. Proposal for Japanese encephalitis surveillance using captured invasive mongooses under an eradication project on Okinawa Island, Japan. *Vector Borne Zoonotic Dis* 9: 259–266.
- Mackenzie JS, Johansen CA, Ritchie SA, Van Den Hurk AF, Hall RA, 2002. Japanese encephalitis as an emerging virus: the emergence and spread of Japanese encephalitis virus in Australasia. *Curr Top Microbiol Immunol* 267: 49–74.
- Nett RJ, Campbell GL, Reisen WK, 2009. Potential for the emergence of Japanese encephalitis virus in California. *Vector Borne Zoonotic Dis* 9: 511–517.
- Weaver SC, Reisen WK, 2010. Present and future arboviral threats. *Antiviral Res* 85: 328–345.
- Ilkal MA, Prasanna Y, Jacob G, Geevarghese G, Banerjee K, 1994. Experimental studies on the susceptibility of domestic pigs to

- West Nile virus followed by Japanese encephalitis virus infection and vice versa. *Acta Virol* 38: 157–161.
19. Goverdhan MK, Kulkarni AB, Gupta AK, Tupe CD, Rodrigues JJ, 1992. Two-way cross protection between West Nile and Japanese encephalitis viruses in bonnet macaques. *Acta Virol* 36: 277–283.
 20. Lobigs M, Pavy M, Hall R, 2003. Cross-protective and infection-enhancing immunity in mice vaccinated against flaviviruses belonging to the Japanese encephalitis virus serocomplex. *Vaccine* 21: 1572–1579.
 21. Takasaki T, Yabe S, Nerome R, Ito M, Yamada KI, Kurane I, 2003. Partial protective effect of inactivated Japanese encephalitis vaccine on lethal West Nile virus infection in mice. *Vaccine* 21: 4514–4518.
 22. Tesh RB, Travassos da Rosa APA, Guzman H, Araujo TP, Xiao SY, 2002. Immunization with heterologous flaviviruses protective against fatal West Nile encephalitis. *Emerg Infect Dis* 8: 245–252.
 23. Scherer WF, Buescher EL, McClure HE, 1959. Ecologic studies of Japanese encephalitis virus in Japan. V. Avian factors. *Am J Trop Med Hyg* 8: 689–697.
 24. Westaway EG, 1966. Assessment and application of a cell line from pig kidney for plaque assay and neutralization tests with twelve group B arboviruses. *Am J Epidemiol* 84: 439–456.
 25. Lindsey HS, Calisher CH, Mathews JH, 1976. Serum dilution neutralization test for California group virus identification and serology. *J Clin Microbiol* 4: 503–510.
 26. Lian WC, Liao MY, Mao CL, 2002. Diagnosis and genetic analysis of Japanese encephalitis virus infected in horses. *J Vet Med* 49: 361–365.
 27. Solomon T, Vaughn DW, 2002. Pathogenesis and clinical features of Japanese encephalitis and West Nile virus infections. *Curr Top Microbiol Immunol* 267: 171–194.
 28. Gupta AK, Lad VJ, Koshy AA, 2008. Survival of mice immunized with monoclonal antibodies against glycoprotein E of Japanese encephalitis virus before or after infection with Japanese encephalitis, West Nile, and Dengue viruses. *Acta Virol* 52: 219–224.
 29. Konishi E, Yamaoka M, Win KS, Kurane I, Mason PW, 1998. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. *J Virol* 72: 4925–4930.
 30. Kimura-Kurodan J, Yasui K, 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J Immunol* 141: 3606–3610.
 31. Monath TP, 2002. Japanese encephalitis vaccines: current vaccines and future prospects. *Curr Top Microbiol Immunol* 267: 105–138.
 32. Ding D, Hong Z, Zhao SJ, Clemens JD, Zhou B, Wang B, Huang MS, Zeng J, Guo QH, Liu W, Tao FB, Xu ZY, 2007. Long-term disability from acute childhood Japanese encephalitis in Shanghai, China. *Am J Trop Med Hyg* 77: 528–533.
 33. Solomon T, Kneen R, Dung NM, Khanh VC, Thuy TT, Ha DQ, Day NP, Nisalak A, Vaughn DW, White NJ, 1998. Poliomyelitis-like illness due to Japanese encephalitis virus. *Lancet* 351: 1094–1097.
 34. Schneider RJ, Firestone MH, Edelman R, Chieowanich P, Pronpibul R, 1974. Clinical sequelae after Japanese encephalitis: a one year follow-up study in Thailand. *Southeast Asian J Trop Med Public Health* 5: 560–568.
 35. Gaunt MW, Sall AA, de Lamballerie X, Falconar AKI, Dzhibanian TI, Gould EA, 2001. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. *J Gen Virol* 82: 1867–1876.
 36. Hemmerter S, Slapeta J, Van Den Hurk AF, Cooper RD, Whelan PI, Russell RC, Johansen CA, Beebe NW, 2007. A curious coincidence: mosquito biodiversity and the limits of the Japanese encephalitis virus in Australasia. *BMC Evol Biol* 7: 100–136.
 37. Nemeth NM, Kratz GE, Bates R, Scherpelz JA, Bowen RA, Komar N, 2008. Naturally induced humoral immunity to West Nile virus infection in raptors. *Ecohealth* 5: 298–304.
 38. Sanchez MD, Pierson TC, Degrace MM, Mattei LM, Hanna SL, Piero FD, Doms RW, 2007. The neutralizing antibody response against West Nile virus in naturally infected horses. *Virology* 359: 336–348.
 39. Lobigs M, Larena M, Alsharifi M, Lee E, Pavy M, 2009. Live chimeric and inactivated JEV vaccines differ in their cross-protective values against MVEV. *J Virol* 83: 2436–2445.
 40. Hammon WM, Reeves WC, Sather GE, 1951. Japanese B encephalitis virus in the blood of experimentally inoculated birds; epidemiological implications. *Am J Hyg* 53: 249–261.
 41. Nemeth NM, Bosco-Lauth AM, Bowen RA, 2009. Cross-protection between West Nile and Japanese encephalitis viruses in red-winged blackbirds (*Agelaius phoeniceus*). *Avian Dis* 53: 421–425.