EXPERIMENTAL INFECTION OF *CULEX ANNULIROSTRIS*, *CULEX GELIDUS*, AND *Aedes vigilax* WITH A YELLOW FEVER/JAPANESE ENCEPHALITIS VIRUS VACCINE CHIMERA (CHIMERIVAX™-JE)

MARK REID,* DONNA MACKENZIE, ANDREW BARON, NATALIE LEHMANN, KYM LOWRY, JOHN AASKOV, FARSHAD GUIRAKHOO, AND THOMAS P. MONATH
Australian Army Malaria Institute, Brisbane, Queensland, Australia; School of Life Sciences, Queensland University of Technology, Brisbane, Queensland, Australia; Acambis Inc., Cambridge, Massachusetts

Abstract. Australian mosquitoes from which Japanese encephalitis virus (JEV) has been recovered (*Culex annulirostris*, *Culex gelidus*, and *Aedes vigilax*) were assessed for their ability to be infected with the ChimeriVax™-JE vaccine, with yellow fever vaccine virus 17D (YF 17D) from which the backbone of ChimeriVax™-JE vaccine is derived and with JEV-Nakayama. None of the mosquitoes became infected after being fed orally with 6.1 log10 plaque-forming units (PFU)/mL of ChimeriVax™-JE vaccine, which is greater than the peak viremia in vaccinees (mean peak viremia = 4.8 PFU/mL, range = 0–30 PFU/mL of 0.9 days mean duration, range = 0–11 days). Some members of all three species of mosquito became infected when fed on JEV-Nakayama, but only *Ae. vigilax* was infected when fed on YF 17D. The results suggest that none of these three species of mosquito are likely to set up secondary cycles of transmission of ChimeriVax™-JE in Australia after feeding on a viremic vaccinee.

INTRODUCTION

Japanese encephalitis virus (JEV) is a member of the family *Flaviviridae* and is a leading cause of viral encephalitis in Asia. Case fatality rates of 29% have been reported with an additional 33–50% of survivors developing long-term neurologic sequelae.1 ChimeriVax™-JE is a live, attenuated, vaccine for JEV in which the premembrane (prM) and envelope (E) proteins genes of the yellow fever virus strain 17D (YF 17D) have been replaced with the vaccine strain JEV SA14-14-2 prM-E sequence.2 Persons inoculated subcutaneously with ChimeriVax™-JE develop a self-limiting, very low–titer viremia that poses a theoretical risk of secondary transmission by a mosquito vector. In a recent study (Acambis Inc., unpublished data), the viremia in humans after immunization with 3.0–5.0 log10 plaque-forming units (PFU) of ChimeriVax™-JE developed a mean peak viremia of 4.8 PFU/mL (range = 0–30 PFU/mL of 0.9 days mean duration, range = 0–11 days). The level of viremia was lower than reported for yellow fever 17D vaccine (YF-VAX®) with a mean peak viremia of 21.8 PFU/mL (range = 0–80 PFU/mL of mean duration 1.2 days, range 0–3 days).3,4 The low levels of viremia in humans after vaccination with ChimeriVax™-JE and YF 17D vaccines is a barrier to oral infection of hematophagous vectors. In addition, it has been known for many years that YF 17D vaccine virus has lost its ability to be transmitted by the yellow fever vector *Aedes aegypti*.5 In previous vector competence studies of ChimeriVax™-JE, *Ae. aegypti*, *Ae. albopictus*, and *Culex tritaeniorhynchus* mosquitoes did not become infected after oral feeding on blood-virus suspensions containing 6.9 log10 PFU/mL.6

The objective of the present study was to assess the potential of mosquito species *Cx. annulirostris*, *Cx. gelidus*, and *Ae. vigilax* to become infected with ChimeriVax™-JE after ingesting a virus laden blood meal or after intrathoracic (IT) inoculation. Japanese encephalitis virus genotypes I or II have been isolated from these three mosquito species in Australia,7–9 and all three species of mosquito have been infected experimentally by membrane feeding with JEV isolates obtained from the Torres Strait of Australia.10

MATERIALS AND METHODS

Mosquitoes. *Culex gelidus* larvae were collected from Tannent Creek (Northern Territory, Australia) by Peter Whelan (Northern Territory Health Services). *Culex annulirostris* eggs were provided by Stephen Doggett (University of Sydney and Westmead Hospital). Colonies of each species were established at the Army Malaria Institute in an insectary maintained at 24–28°C and a relative humidity of 40–60%, with a 12:12 light:dark photoperiod. To maintain the colony, the larvae of each species were reared in dam water on a diet of commercial fish food (Kyorin, Himiji, Japan). For egg development, adults were offered a blood meal 3–5 times a week. All adult mosquitoes were maintained on a 20% (v/v) sucrose/water solution containing multivitamins (Myadech multivitamins and minerals; Nelson Laboratories, Warriendale, New South Wales, Australia) ad libitum. Mosquito colonies were established from larvae but to limit the possibility of adventitious infection of the colonies, 60 adults from each of the three colonies were screened for the presence of arboviruses by adding mosquito homogenate to 2-cm2 cultures of baby hamster kidney 21 (BHK-21) clone c15 cells11 and observing daily for 7–10 days for cytopathic effects (CPEs).

Viruses. Yellow fever 17D vaccine (lot no. W6440-1 Stama®; Sanofi-Pasteur, Lyon, France) was passaged once in Vero (African green monkey) cells12 (European Type Culture Collection lot no. CB2617). Supernatant fluid was harvested when cell monolayers showed ≥ 75% CPE and stored at ≤ −60°C in 20% (v/v) heat-inactivated (56°C for 30 minutes) fetal bovine serum (FBS, Gibco-Invitrogen, Carlsbad, CA). Japanese encephalitis virus (Nakayama strain) was obtained from Queensland Health Scientific and Pathology Services (Townsville, Queensland, Australia) and was passaged in Vero cells in the same manner as YF 17D. ChimeriVax™-JE was supplied lyophilized (Acambis Inc., Cambridge MA) and was reconstituted in sterile 0.9% saline (AstraZeneca, Australia) ad libitum.

* Address correspondence to Mark Reid, Australian Army Malaria Institute, Weary Dunlop Drive, Gallipoli Barracks, Enoggera, Queensland 4051, Australia. E-mail: markreid2@optusnet.com.au
Alderley Park, United Kingdom) before use. Titers of virus stocks were as follows: YF 17D = 7.3 log$_{10}$ PFU/mL (in porcine stable-equine kidney cells [PS-EK]),$^{25}$ JEV-Nakayama = 6.7 log$_{10}$ PFU/mL (in BHK-21 c15 cells), and ChimeriVax™-JE = 6.1 log$_{10}$ PFU/mL (in Vero cells).

**Virus titrations.** Mosquitoes were killed with CO$_2$ gas and triturated using plastic pestles in sterile micro-centrifuge tubes in 1 mL volumes of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 0.3 g/L of sodium bicarbonate and supplemented with 20% (v/v) heat-inactivated FBS (HI-FBS), 100 µg/mL of streptomycin, 100 U/mL of penicillin, 2 mM L-glutamine (SPG) (Sigma-Aldrich), and 2.5 µg/mL of amphotericin B (Gibco-Invitrogen). The homogenate was clarified by centrifugation (10,377 × g for 2 minutes) and the supernatant was stored at −60°C until assayed. Virus titers were assayed using a modified plaque titration method.$^{25}$ Brieﬂy, supernatants from the mosquito homogenates were diluted 10-fold in duplicate 2% (v/v) HI-FBS RPMI 1640 medium/SPG and 100 µL was added to 2-cm$^2$ cell monolayers. Japanese encephalitis virus (Nakayama) and ChimeriVax™-JE were titrated on BHK-21 c15 cells and YF 17D on PS-EK cells (because YF 17D did not plaque consistently on BHK-21 c15 cells). After 1 hour ± 10 minutes, 1.5% (w/v) carboxymethylcellulose (Sigma-Aldrich) with a ﬁnal concentration of 2% (v/v) HI-FBS RPMI 1640 medium/SPG was added to each culture and incubated at 37 ± 1°C for 3–5 days in an atmosphere of 5% (v/v) CO$_2$ in air. Monolayers were ﬁxed and stained with 0.5% (w/v) CI basic violet in 5% (v/v) formalin:phosphate buffered saline (Sigma-Aldrich) before plaques were counted. The limit of sensitivity of the assay was ≥1.7 log$_{10}$ PFU/mosquito. To enable log transformations of average plaque counts, cell monolayers with no detectable plaques were given the nominal value of half the limit of detection (0.85 log$_{10}$ PFU).

**Inthoracic inoculation of mosquitoes.** Freshly thawed stocks of virus were maintained on ice for 2 hours ± 20 minutes. Female mosquitoes of the three species 1–4 days post-emergence, were immobilized with CO$_2$ gas and manipulated in a laboratory cold plate (2 ± 1°C) (Thermolene Scientiﬁc, Sydney, New South Wales, Australia). Mosquitoes were inoculated IT with 0.15 ± 0.08 µL of virus stock using heat-drawn, 1-mm capillary tubes (Harvard Apparatus, Edenbridge, United Kingdom) as previously described (Narishige, Tokyo, Japan).$^{14}$ After inoculation, mosquitoes were transferred in batches of 20 to primary mesh-covered plastic cups and maintained at 27 ± 1°C, at a relative humidity of 80 ± 5%, with a 12:12 light:dark photo phase for up to 18 days. Mosquitoes were sampled every 24 hours for 8 days and again at days 10, 12, 15, and 18.

**Oral infection of mosquitoes.** Deﬁbrinated sheep’s blood (Institute of Medical and Veterinary Science, Adelaide, Queensland, Australia) was washed twice in serum free RPMI 1640 medium and the erythrocyte pellet was reconstituted to its original volume using freshly thawed virus stocks. Batches of 50, 1–4-day-old female mosquitoes of each species werestarved for 24 ± 6 hours prior to feeding on the virus/erythrocyte suspension under a commercial sausage skin membrane warmed to 37 ± 1°C in a water-jacketed membrane feeder (University of Queensland, Brisbane, Queensland, Australia). *Culex gelidus* would not feed from membrane feeders and therefore were offered cotton pledges soaked in the virus/erythrocyte suspensions warmed to 37 ± 1°C immediately prior to feeding. All mosquitoes were fed over a time interval of 2 hours ± 20 minutes and fully engorged mosquitoes maintained for up to 18 days as described for mosquitoes infected IT. The average blood meal for each species was estimated by weighing a sample of 20 mosquitoes pre-feeding and post-feeding (Cx. annulirostris = 1.3 ± 0.9 µL, Cx. gelidus = 1.9 ± 0.6 µL, and Ae. vigilax = 1.7 ± 1.5 µL).

**Stability of virus stocks.** Experiments were conducted to quantify the reduction in virus titer for the virus/erythrocyte suspensions and IT inocula for each virus at 37 ± 1°C or 0°C after a 2 hour ± 20 minute time interval. Five replicates were assayed without freeze-thawing before and after the time interval and the average titer was compared by a paired, one-tailed Student’s t-test (α = 0.05) to determine whether signiﬁcant decreases in virus titer had occurred.

**Nucleotide sequencing.** *Culex annulirostris* mosquito homogenates 876, 877, and 878 obtained 12 days after IT inoculation with ChimeriVax™-JE were passaged once in Vero cells and titrated in BHK-21 c15 cells as previously described. Viral RNA was extracted from the supernate of infected cultures using the High Pure Viral RNA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Viral RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using random hexamers (Roche Diagnostics). The region of the genome coding for capsid (C) and prM proteins was ampliﬁed by polymerase chain reaction (PCR) using *Taq* polymerase (Roche diagnostics) and primers previously described,$^{7}$ as well as with JEV E protein–speciﬁc primers (JEV-19F: 5'-GGCAATCGTGACTTCATAGAAG-3' and JEV-591R: 5'-TCCACTCCCTTGCTCACAGTC-3') on a thermocycler (Eppendorf, Hamburg, Germany). Cycling conditions were 94°C for 2 minutes followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. The PCR products were stained with ethidium bromide after electrophoresis on a 2% (w/v) agarose Tris-borate-EDTA gel (Sigma-Aldrich) and visualized with ultraviolet light. The 380-base pair C/prM and 572-base pair envelope gene bands were sequenced in both directions by dideoxynucleotide dye termination (BigDye terminator cycle sequencing kits; Applied Biosystems, Foster City, CA) using the oligonucleotide primers previously mentioned in this report and an automated sequencer (Applied Biosystems).

**Statistical analysis.** The Kruskal-Wallis test was used to compare the virus titer between the mosquito groups, viruses, and inoculation methods by ranking each data point and comparing mean ranks between the virus groups. Analysis was undertaken using SPSS version 7.0 software (SPSS Inc., Chicago, IL).

**RESULTS**

The titer of YF 17D virus in a suspension of erythrocytes decreased from 7.3 to 6.7 log$_{10}$ PFU/mL ($P = 0.023$) over 2 hours ± 20 minutes at 37 ± 1°C. The oral feeding dose for YF 17D was therefore adjusted accordingly. The titer of virus in other IT inocula or in virus/erythrocyte suspensions did not decrease signiﬁcantly.

ChimeriVax™-JE was not observed in *Cx. annulirostris* mosquitoes at detectable levels after oral or IT infection.
In contrast, wild-type JEV-Nakayama titers increased to approximately 6.0 log_{10} PFU/mosquito in Cx. annulirostris infected IT and several mosquitoes (7%, 4 of 60) infected orally developed titers of approximately 5.0 log_{10} PFU/mosquito (Figure 1B). No multiplication of YF 17D was detected in this species of mosquito after oral infection and titers in mosquitoes inoculated IT (22%, 13 of 60) did not increase above that of the estimated inoculum (3.5 log_{10} PFU/mosquito) (Figure 1C).

There was no evidence of ChimeriVax™-JE virus in Cx. gelidus after oral infection. Moreover, titers of ChimeriVax™-JE in this species inoculated IT (8%, 5 of 60) rarely increased above the titer of the estimated inoculum (2.3 log_{10} PFU/mosquito) (Figure 1D). In contrast, JEV-Nakayama virus was observed at high titer after IT inoculation in almost all (92%, 55 of 60) mosquitoes. Moreover, 18% (11 of 60) of Cx. gelidus mosquitoes infected orally contained JEV-Nakayama at an approximate titer of 5.5 log_{10} PFU/mosquito (Figure 1E). The titers of YF 17D in Cx. gelidus were similar to those of ChimeriVax™-JE with only 2% (1 of 60) of mosquitoes infected after feeding and 20% (12 of 60) after IT inoculation (Figure 1F).

ChimeriVax™-JE was not detected in Ae. vigilax infected orally, but titers were detected in 70% (42 of 60) of mosquitoes after IT inoculation but at a low mean titer of 2.4 log_{10} PFU/mosquito (estimated inoculum = 2.3 log_{10} PFU/mosquito) (Figure 1G). Japanese encephalitis virus (Nakayama) was detected in 88% (53 of 60) of Ae. vigilax inoculated IT reaching titers as high as 6.2 log_{10} PFU/mosquito. After oral infection, JEV-Nakayama was detected in 12% (7 of 60) of the Ae. vigilax assayed (Figure 1H). Yellow fever virus 17D was detected in 10% (6 of 60) of Ae. vigilax fed orally on this virus but titers were all less than the titers of the estimated inoculum (3.9 log_{10} PFU/mosquito). Yellow fever virus 17D was detected in 100% (60 of 60) mosquitoes inoculated IT but the mean titer (3.5 log_{10} PFU/mosquito) was also similar to that of the estimated inoculum (3.5 log_{10} PFU/mosquito) (Figure 1I).

Sequence analysis of the C, prM, and E protein genes of virus from mosquitoes 876, 877, and 878, the three Cx. annulirostris mosquitoes showing high virus titers after IT inoculation of ChimeriVax™-JE, indicated infection with JEV-Nakayama virus rather than ChimeriVax™-JE. A review of work logs suggests these three mosquitoes were misidentified during collection. The data points corresponding to these mosquitoes were removed from the Cx. annulirostris ChimeriVax™-JE IT analysis, giving a total of 57 mosquitoes for the analysis (Figure 1A).

Virus titers of ChimeriVax™-JE in all mosquito species were less than those for JEV-Nakayama inoculated IT or
DISCUSSION

Neither Cx. annulirostris, Cx. gelidus, nor Ae. vigilax mosquitoes became infected with ChimeriVax™-JE vaccine virus when fed with a blood meal containing doses of virus that exceeded the maximum viremia observed in humans inoculated with ChimeriVax™-JE vaccine. Furthermore, when the midgut infection barrier was circumvented by IT inoculation, titers of ChimeriVax™-JE were significantly less than those of either control viruses: JEV-Nakayama or YF 17D (P < 0.001).

This study has identified minor differences in the susceptibility of Culex and Aedes species to infection with ChimeriVax™-JE and YF 17D. Bhatt and others compared the differences in the susceptibility of Cx. tritaeniorhynchus, Ae. aegypti, and Ae. albopictus to infection with ChimeriVax™-JE, JEV SA14-14-2, JEV SA14, and YF 17D. In contrast, Cx. tritaeniorhynchus was a highly efficient vector for both the live, attenuated JEV SA14-14-2 vaccine (currently being used in China, South Korea, and Vietnam) and wild-type JEV (SA14). Culex tritaeniorhynchus, which was the only Culex species examined, did not become orally infected with either ChimeriVax™-JE or YF 17D viruses inoculated IT with approximately 5.5 log10 PFU/mosquito. In the present study, both Cx. gelidus and Cx. annulirostris demonstrated an ability to maintain low titer infections with ChimeriVax™-JE and YF 17D after IT inoculation. Bhatt and others demonstrated that neither Ae. aegypti nor Ae. albopictus species became infected after feeding with YF 17D. In contrast, YF 17D was detected in Ae. vigilax 12 and 18 days after feeding. Yellow fever virus 17D was also detected in one Cx. gelidus mosquito 24 hours after feeding. This may have been the inoculated virus that had survived digestion rather than indicating multiplication of the virus.

Although both JEV Nakayama and JEV SA-14-14-2 are JEV genotype III, this study would have benefited from the use of JEV SA-14-14-2 in place of JEV-Nakayama as the JEV control. At the time of this study, JEV SA-14-14-2 was not permitted to be used in our facilities. ChimeriVax™ vaccines for JE, dengue and West Nile have now been assessed in the arbovirus vectors Cx. tritaeniorhynchus, Cx. quinquefasciatus, Cx. nigripalpus, Ae. aegypti, and Ae. albopictus. These studies and the study presented here suggest that ChimeriVax™ vaccines are phenotypically similar to YF 17D for mosquito attenuation (first established in 1939), irrespective of the flavivirus prM or E gene presented in the chimeras. Yellow fever vaccines (and genetically modified derivatives) have been and continue to be manufactured from certified sub-strains of YF 17D under a seed lot system to maintain the attenuated vaccine phenotype. The inability of these vaccines to cause disseminated infection after oral ingestion of high viral doses, together with the very low viremia levels in humans after vaccination, suggest that mosquitoes pose limited risk for establishing secondary cycles of the ChimeriVax™-JE vaccine in Australia after feeding on a viremic vaccine.

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Authors’ addresses: Mark Reid, Donna MacKenzie, Andrew Baron, and Natalie Lehmann, Australian Army Malaria Institute, Weary Dunlop Drive, Gallipoli Barracks, Enoggera, Queensland, 4051 Australia. Kym Lowry and John Aaskov, Australian Army Malaria Institute, Weary Dunlop Drive, Gallipoli Barracks, Enoggera, Queensland, 4051 Australia and School of Life Sciences, Queensland University of Technology, GPO Box 2434, Brisbane, Queensland, 4001 Australia. Farshad Guirakhoo, Acambis Inc., 38 Sidney Street, Cambridge MA 02139, Thomas P. Monath, Kleiner Perkins Caufield & Byers, 21 Finn Rd., Harvard, MA 01451.

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