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Experimental Infection of *Aedes sollicitans* and *Aedes taeniorhynchus* with Two Chimeric Sindbis/Eastern Equine Encephalitis Virus Vaccine Candidates

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Abstract

Two chimeric vaccine candidates for Eastern equine encephalitis virus (EEEV) were developed by inserting the structural protein genes of either a North American (NA) or South American (SA) EEEV into a Sindbis virus (SINV) backbone. To assess the effect of chimerization on mosquito infectivity, experimental infections of two potential North American bridge vectors of EEEV, *Aedes sollicitans* and *Ae. taeniorhynchus*, were attempted. Both species were susceptible to oral infection with all viruses after ingestion of high titer blood meals of ca. 7.0 log₁₀ plaque-forming units/mL. Dissemination rates for SIN/NAEEEV (0 of 56) and SIN/SAEEEV (1 of 54) were low in *Ae. taeniorhynchus* and no evidence of transmission potential was observed. In contrast, the chimeras disseminated more efficiently in *Ae. sollicitans* (19 of 68 and 13 of 57, respectively) and were occasionally detected in the saliva of this species. These results indicate that chimerization of the vaccine candidates reduces infectivity. However, its impact on dissemination and potential transmission is mosquito species-specific.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) (*Togaviridae: Alphavirus*) is an important mosquito-borne pathogen that can cause severe encephalitis and case fatality rates between 30% and 80% in humans of North America, and up to 95% in equines throughout the Americas.^{1,2} In contrast to the high morbidity and mortality associated with North American (NA) EEEV, South American (SA) EEEV strains appear to be less virulent and/or infectious for humans.³ Despite the severity of disease associated with symptomatic NAEVV infection and its potential to become aerosolized and used as a biologic weapon, there are currently no licensed human vaccines for this virus. Although the natural attack rate in North America is relatively low,⁴ a safe and effective vaccine is needed to routinely vaccinate laboratory personnel and first-line epidemic responders in the event of a biologic attack. Formalin-inactivated vaccines are available for veterinary use⁵; however, these are poorly immunogenic, require repeat doses, and have the potential to contain virulent, wild-type EEEV.⁶ As an alternative approach to designing a safer and more effective vaccine, we developed two recombinant Sindbis (SINV)/EEEV chimeric viruses by inserting the structural protein genes of either NAEVV (strain FL93-939) or SAEEEV (strain BeAr436087) into a backbone containing SINV (AR339) non-structural protein genes and cis-acting RNA genome elements, as previously described.⁷ These

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chimeric viruses replicate efficiently in both African green monkey (Vero) and *Aedes albopictus* mosquito (C7/10) cells. In addition, they are highly attenuated in mice, which develop high levels of neutralizing antibodies without detectable disease or viremia and are protected against challenge with a lethal dose of NAEDEV.⁷

To our knowledge, the ability of interspecific chimeric alphaviruses to infect mosquito vectors has not been studied. Both EEEV and SINV, the parents of our vaccine candidates, are arthropod-borne viruses and their transmission cycles include mosquito vectors and avian amplifying hosts. Although the chimeric vaccine candidates do not produce detectable viremia in mice, evaluating their abilities to infect and potentially be transmitted by mosquitoes of epidemiologic importance is essential in determining their safety for veterinary or human use. In North America, EEEV is sustained in an enzootic transmission cycle by its ornithophilic mosquito vector, *Culiseta melanura*, and passerine birds in freshwater swamp habitats.¹ However, under favorable amplification conditions, sporadic epizootic and epidemic transmission occurs via bridge vectors to dead-end hosts, such as humans, horses, and other domestic animals.⁴ These bridge vectors have more catholic feeding preferences and overlap with *Cs. melanura* in habitat and geographic distribution. Because there have been numerous isolates of NAEDEV from *Ae. (Ochlerotatus) sollicitans* and *Ae. (Ochlerotatus) taeniorhynchus* during epidemics,⁸⁻¹⁰ and experimental infections have demonstrated their susceptibility to NAEDEV,^{11,12} these species are considered potential bridge vectors and ideal candidates to assess the likelihood of secondary transmission of these SIN/EEEV chimeric vaccine candidates. Because *Cs. melanura* is ornithophilic and almost exclusively feeds on avian species, its likelihood to take a blood meal from a vaccinated person or domestic animal is extremely low and this species was therefore not included in this study.

Viremia has not been detected in rodents after vaccination with either SIN/EEEV vaccine candidate. However, to determine the environmental safety of these new chimeric alphavirus vaccine candidates in the event that an immunocompromised, vaccinated human or equid became viremic, we exposed orally *Ae. taeniorhynchus* and *Ae. sollicitans* to high titer artificial blood meals and assessed infection and transmission potential.

MATERIALS AND METHODS

Viruses

The chimeric vaccine strains contained nonstructural protein genes from SINV strain AR339, as well as cis-acting RNA sequence elements. The structural protein genes were derived either from North American lineage I EEEV strain FL93-939 (SIN/NADEV) or South American lineage IV EEEV strain BeAr436087 (SIN/SADEV).⁷ The chimeric and parent viruses were all rescued from cDNA clones, as described previously, by transfection of transcribed RNA into baby hamster kidney cells using electroporation.⁷ Approximately 24-hours later, the viruses were harvested, their titers were determined by plaque assay on Vero cells, and the harvested medium was aliquoted to produce frozen virus stocks for use in these experiments.

Mosquitoes

Adult female *Ae. sollicitans* and *Ae. taeniorhynchus* mosquitoes were collected in Galveston, Texas (29°13.13'N, 94°56.06'W), using CDC light traps and mechanical aspiration and maintained in an insectary at 27°C and a relative humidity of 70–75% with a 16:8 light:dark photoperiod. Feral adult mosquitoes were presented blood meals for egg development. The F₁ eggs were hatched in distilled water, the larvae reared on a diet of TetraMin fish flakes (Doctors Foster and Smith, Thineland, WI) and crushed Prolab 2500 rodent diet (PMI Nutrition International, Brentwood, MO) in a 1:1 mixture, and the F₁ adults were maintained

on a diet of 10% (w/v) sucrose/water solution *ad libitum*. The F₁ adults from field-collected mosquitoes were used in all experiments.

Mosquito infections

Aedes sollicitans and *Ae. taeniorhynchus* were allowed to ingest artificial blood meals containing each of the chimeras (SIN/NAEEEV and SIN/SAEEEV), as well as the parent viruses (SINV, NAEEEV, and SAEEEV). Cohorts of 50–100 female adult mosquitoes (7–10 days post-emergence) were placed in 0.9-liter cartons and sucrose-starved for several hours before allowing them to feed on artificial blood meals. The artificial blood meals contained 35% (v/v) packed defibrinated sheep erythrocytes (Colorado Serum Company, Denver, CO), 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Omega Scientific, Inc., Tarzana, CA), and 0.25 μ M adenosine triphosphate and 0.03 μ M sucrose as phagostimulants. The remaining volume was virus suspension in minimum essential medium (MEM). The blood meal was encased in either an artificial membrane or sausage skin, warmed to 37°C in a Hemotek feeding apparatus (Discovery Workshops, Accrington, United Kingdom), and placed on the nylon mesh cloth that covered the top of the carton containing the mosquitoes. After one hour, fully engorged mosquitoes were removed from the carton and incubated under the same rearing conditions for 10–14 days, which is greater than the observed extrinsic incubation period for most alphaviruses including EEEV.^{13,14} A sample of each mosquito species was given an uninfected blood meal and monitored under the same conditions to serve as negative controls.

Mosquito processing

Mosquitoes were cold-anesthetized and the legs and wings were removed. The proboscis of each mosquito was then inserted into the end of a glass 10- μ L capillary tube containing immersion oil (Cargille Laboratories, Cedar Grove, NJ) and allowed to salivate for approximately one hour. Each saliva sample was transferred separately to an Eppendorf (Hamburg, Germany) tube with 100 μ L of 10% FBS/MEM. The bodies and legs/wings were transferred separately to individual round bottom Eppendorf safe-lock tubes containing 350 μ L of 10% FBS/MEM and a stainless steel bead for trituration. All samples were stored at –80°C.

Determination of infection, dissemination, and transmission

Suspensions of the body and legs/wings of each mosquito were individually assayed to determine overall body infection and hemocoel dissemination rates, respectively, and saliva samples were tested to determine potential transmission rates. The bodies and legs/wings were triturated for 4 minutes at 26,000 motions per minute using a Mixer Mill 300 (Retsch, Newton, PA) and then centrifuged at 10,000 rpm and 4°C for 5 minutes, and the saliva samples were clarified by centrifugation. One hundred microliters of each body sample were inoculated onto confluent Vero cell monolayers in 24-well plates in duplicate. The cultures were incubated for one hour at 37°C, after which 1 mL of 2% FBS/MEM was added to each well. The plates were maintained at 37°C and microscopically monitored daily for cytopathic effect (CPE). If virus was detected in the body samples, the corresponding legs/wings were assayed in the same format, and if positive, the corresponding saliva samples were assayed by inoculating 50 μ L onto confluent baby hamster kidney (BHK) cell mono-layers as described for the body samples. The BHK cells were shown to be approximately 10 times more sensitive to various EEEV strains than Vero cells (Arrigo NC, unpublished data) and were thus used for the detection of virus in the saliva. Plaque assays on Vero cells were conducted using randomly selected CPE-positive samples to establish that viral infection was responsible for the observed CPE, rather than toxicity. The infection, dissemination, and potential transmission rates were expressed as percentages derived from the number of virus positive samples divided by the total number of respective sample-types generated during the study period.

Statistical analysis

Overall body infection, hemocoel dissemination, and saliva infection (potential transmission) rates were compared among virus groups for each mosquito species using Fisher's exact test in Prism 4.0c for Macintosh (Graph-Pad Software, San Diego, CA). A P value less than 0.05 was considered statistically significant. Replicates of each experiment that did not differ statistically significantly from one another were combined for analysis and the concatenated values were compared between virus groups. Alternatively, replicates that differed significantly were compared individually.

RESULTS

Aedes taeniorhynchus

Overall body infection by all parent strains (NAEEEV strain FL93-939, SINV strain TR339, and SAEDEV strain BeAr436087) exceeded 50% of exposed mosquitoes in each experimental replicate (Table 1). However, the dissemination and saliva infection rates were considerably lower for all virus groups. Oral ingestion of the SIN/NAEEEV chimeric vaccine strain resulted in significantly lower overall infection ($P < 0.001$) and dissemination ($P < 0.01$) rates compared with both parent strains (SINV strain TR339 and NAEEEV strain FL93-939) (Figure 1A). Disseminated infections by SINV (7 of 51) and NAEEEV (8 of 49) also led to virus in the saliva (3 of 51 and 4 of 49, respectively). However, SIN/NAEEEV did not disseminate (0 of 56) or infect the saliva of any mosquito tested (0 of 56), indicating that transmission of this chimeric strain by *Ae. taeniorhynchus* would be highly unlikely (Table 1).

Although the overall infection rates of the SIN/SADEV chimera varied between mosquito cohorts, most comparisons were significantly lower ($P < 0.05$) than the parent strains SINV TR339 and SA DEV BeAr436087 (Figure 1A). Both parent viruses disseminated to the hemocoel and infected the saliva of *Ae. taeniorhynchus* (Table 1), but infection with the SIN/SADEV chimera resulted in only a single disseminated infection (1 of 54). In addition, SIN/SADEV was not present in the saliva of any mosquitoes and is unlikely to be transmitted by this species.

Aedes sollicitans

Aedes sollicitans was highly susceptible to body infection, hemocoel dissemination, and saliva infection for all parent viruses and chimeric vaccine strains tested. Overall infection rates generally exceeded those observed in *Ae. taeniorhynchus*, and all strains disseminated significantly more efficiently in *Ae. sollicitans* (Figure 1B). Sindbis virus was also extremely efficient in its overall infection (35 of 37), dissemination (35 of 37), and saliva infection (24 of 37) of this species (Table 2).

Collectively, the overall infection and dissemination rates of SIN/NAEEEV in *Ae. sollicitans* did not differ significantly from its NAEEEV FL93-939 parent, and its dissemination rates were significantly lower ($P < 0.001$) than its SINV TR339 parent for all experimental replicates. Virus was also detected in the saliva of SIN/NAEEEV-infected (4 of 68) mosquitoes, but at a significantly lower rate than either of its parent strains ($P < 0.01$). The infection trends of the SIN/SADEV chimera were similar to SIN/NAEEEV (Figure 1B), but dissemination and saliva infection rates were significantly lower ($P < 0.01$) than both of its parent strains for most experimental replicates. In contrast to *Ae. taeniorhynchus*, both chimeric vaccine strains were capable of disseminating and infecting the saliva of *Ae. sollicitans*, suggesting the transmission potential of this species.

DISCUSSION

This study evaluated the environmental safety of two chimeric alphavirus vaccine candidates by attempting to infect experimentally two potential mosquito bridge vectors for NAEDEV. The chimeras were constructed using the non-structural protein genes of SINV (AR339) and the structural genes of either NAEDEV (FL93-939) or SADEV (BeAr436087). The latter DEV strain is naturally attenuated in mice, hamsters, and marmosets (Weaver SC, unpublished data) adding to the safety of the corresponding vaccine candidate. Although the attenuated nature of both of these chimeras has been demonstrated in a murine model,⁷ the effect of their chimeric nature on vector competence was unknown. Mosquitoes were orally presented high viral titer blood meals of approximately $7.0 \log_{10}$ plaque-forming units (PFU)/mL to mimic a worst-case scenario whereby an immunosuppressed, or otherwise compromised, vaccinated horse or human developed viremia and was exposed to a potential bridge vector.

To our knowledge, this is the first description of a chimeric alphavirus infection in any mosquito species, as well as the first published results of experimental infections of *Ae. taeniorhynchus* and *Ae. sollicitans* with the SINV and SADEV parent viruses. Experimental mosquito infection studies have been conducted with recently developed chimeric flavivirus vaccine candidates. Oral infectious doses of $6.1\text{--}6.9 \log_{10}$ PFU/mL of the ChimeriVax™-JE vaccine for Japanese encephalitis virus, an infectious dose much greater than its mean peak viremia in humans (0–30 PFU/mL), were unable to infect a wide variety of mosquito species.^{15,16} The ChimeriVax™-DEN1–4 vaccine candidates for dengue virus and the licensed YF-VAX® (17D) vaccine for yellow fever virus (YFV) were also presented orally to their most significant epidemic vectors, *Ae. aegypti* and *Ae. albopictus*. *Aedes albopictus* was susceptible to infection and dissemination with ChimeriVax™-DEN1, 3, and 4 and 17D, although at lower levels than the respective wild-type strains. In contrast, only the ChimeriVax™-DEN4 was orally infectious for a single *Ae. aegypti* mosquito, while all others were unable to infect this species.¹⁷ These results demonstrate the variable degrees of attenuation observed with chimeras of different recombinant construction and underscore the need to characterize their infection in multiple mosquito species to assess environmental safety.

Aedes taeniorhynchus and *Ae. sollicitans* were chosen for experimental infection with the SIN/DEV vaccine candidates on the basis of previous implications as potential bridge vectors for DEV,^{8–10} their aggressive and opportunistic feeding preferences, and geographic distributions that coincide with enzootic DEV foci, as well as human and equine cases of DEV. *Aedes sollicitans* was an efficient vector for all parent alphaviruses, especially SINV strain TR339, which infected and disseminated in nearly all exposed mosquitoes. In addition, although slightly less efficient in infection and dissemination than the parent strains, both chimeras were capable of infecting the saliva of *Ae. sollicitans*. As the non-structural gene component of the chimeric vaccine strains, the efficiency of SINV in this vector may have influenced the ability of these chimeras to disseminate and infect the saliva of *Ae. sollicitans*.

In contrast to *Ae. sollicitans*, both SIN/DEV chimeric vaccine candidates were poorly infectious for *Ae. taeniorhynchus*. The overall infection rates of the North American chimera (SIN/NADEV) were considerably lower than those of the South American chimera (SIN/SADEV), indicating that the SADEV structural gene component of the latter recombinant may play an important role in infection of this species. Despite this difference, the chimeric nature of both SIN/NADEV and SIN/SADEV resulted in extremely low dissemination rates (0 of 56 and 1 of 54, respectively). Because of these low dissemination rates, transmission rates would also be extremely low and none of the orally exposed *Ae. taeniorhynchus* demonstrated a potential to transmit either chimera. This infection pattern is similar to the widely accepted YFV 17D vaccine strain, which has historically demonstrated its ability to infect, but not disseminate in, *Ae. aegypti*.^{18,19}

As the first description of a chimeric alphavirus vaccine candidate in mosquitoes, this study demonstrates that the chimeric nature of the SIN/EEEV virus strains did confer some loss of infectivity for the mosquito vector *Ae. taeniorhynchus* but not for *Ae. sollicitans*. Because the chimeras do not produce detectable pathology or viremia in mice,⁷ equines (Bowen RA, unpublished data), or Chinese painted quail (Arrigo NC, unpublished data), the likelihood of further transmission by either mosquito species is low. However, the attenuation of the chimeric vaccine strains that occasionally appear in the saliva of *Ae. sollicitans* should be assessed, which will require large numbers of mosquito infections.

The observed variations in susceptibility between mosquito species could help identify genomic markers of vector infectivity and additional alterations in their genetic design to further limit replication within mosquitoes. Overall, our results are encouraging for the environmental safety of these vaccine candidates and the use of chimerization as an alternate strategy for the development of future alphavirus vaccines.

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Figure 1.

Overall body, hemocoel (dissemination), and saliva infection rates of chimeric Sindbis/Eastern equine encephalitis virus vaccine candidates and parental virus strains. **A**, *Aedes taeniorhynchus*. **B**, *Ae. sollicitans*. The mean is plotted and the standard error bars represent the variation between experimental replicates. The lower portions of the error bars have been removed for clarity.

Overall alphaviral body, hemocoel (dissemination), and saliva infection rates for *Aedes taeniorhynchus* mosquitoes exposed orally to chimeric alphavirus vaccine candidates and parent viruses*

Virus strain	Blood meal titer (log ₁₀ PFU/mL)	Total no. engorged	% Infected [†] (no. infected)		
			Body	Hemocoel (legs, wings)	Saliva
SIN/NAEEEV	7.2 7.4	17 39	0 (0) 15 (6)	0 (0) 0 (0)	0 (0) 0 (0)
NAEEEV (FL93-939)	4.9 7.3	16 33	69 (11) 52 (17)	31 (5) 9 (3)	13 (2) 6 (2)
SINV (TR339)	7.0 7.4	19 32	74 (14) 56 (18)	26 (5) 6 (2)	5 (1) 6 (2)
SIN/SAEEEV	7.2 7.4	24 30	21 (5) 57 (17)	0 (0) 3 (1)	0 (0) 0 (0)
SAEEEV (BeAr 436087)	6.9 7.0	27 42	52 (14) 81 (34)	7 (2) 14 (6)	4 (1) 5 (2)

* PFU = plaque-forming units; SINV = Sindbis virus; NAEEEV = North American Eastern equine encephalitis virus; SAEEEV = South American EEEV.
[†] Percentages are no. infected/total no. engorged.

Overall alphaviral body, hemocoel (dissemination), and saliva infection rates for *Aedes sollicitans* mosquitoes exposed orally to chimeric alphavirus vaccine candidates and parent viruses*

Table 2

Virus strain	Blood meal titer (log ₁₀ PFU/mL)	Total no. engorged	% Infected† (no. infected)		
			Body	Hemocoel (legs, wings)	Saliva
SIN/NAEEEV	6.6 7.7	22 13	86 (19) 70 (9)	55 (12) 23 (3)	14 (3) 0 (0)
NAEEEV (FL93-939)	7.2 4.9	33 05	55 (18) 100 (5)	12 (4) 80 (4)	3 (1) 40 (2)
SINV (TR339)	8.0 7.5	20 23	45 (9) 91 (21)	30 (6) 91 (21)	25 (5) 70 (16)
SIN/SAEEEV	7.6 7.4	14 36	100 (14) 53 (19)	100 (14) 38 (8)	57 (8) 24 (5)
SAEEEV (BeAr 436087)	7.2 7.2	16 20	56 (9) 65 (13)	14 (5) 55 (11)	3 (1) 44 (7)

* PFU = plaque-forming units; SINV = Sindbis virus; NA EEEV = North American Eastern equine encephalitis virus; SAEEEV = South American EEEV.
† Percentages are no. infected/total no. engorged.