

VECTOR COMPETENCE OF PERUVIAN MOSQUITOES (DIPTERA: CULICIDAE) FOR A SUBTYPE IIIC VIRUS IN THE VENEZUELAN EQUINE ENCEPHALOMYELITIS COMPLEX ISOLATED FROM MOSQUITOES CAPTURED IN PERU

M. J. TURELL,¹ D. J. DOHM,¹ R. FERNANDEZ,² C. CALAMPA,³ AND M. L. O'GUINN¹

ABSTRACT. We evaluated mosquitoes collected in the Amazon Basin, near Iquitos, Peru, for their susceptibility to a subtype IIIC strain of the Venezuelan equine encephalomyelitis complex. This virus had been previously isolated from a pool of mixed *Culex vomerifer* and *Cx. gnomatos* captured near Iquitos, Peru, in 1997. After feeding on hamsters with viremias of about 10⁸ plaque-forming units of virus per ml, *Cx. gnomatos* was the most efficient vector. Other species, such as *Ochlerotatus fulvus* and *Psorophora cingulata*, although highly susceptible to infection, were not efficient laboratory vectors of this virus due to a significant salivary gland barrier. The *Cx. (Culex)* species, consisting mostly of *Cx. (Cux.) coronator*, were nearly refractory to subtype IIIC virus and exhibited both midgut infection as well as salivary gland barriers. Additional studies on biting behavior, mosquito population densities, and vertebrate reservoir hosts of subtype IIIC virus are needed to determine the role that these species play in the maintenance and spread of this virus in the Amazon Basin region.

KEY WORDS Peru, Venezuelan equine encephalomyelitis virus, Mucambo virus, transmission, mosquitoes, vector competence

INTRODUCTION

Members of the Venezuelan equine encephalomyelitis (VEE) complex are responsible for sporadic epizootics of severe disease in Central and South America (Walton and Grayson 1989), extending from central South America to as far north as Texas. Disease caused by infection with VEE virus (VEEV) continues to be a problem in Central and South America as indicated by the 1995 epidemic in Colombia and Venezuela (Weaver et al. 1996, Rivas et al. 1997) that resulted in 75,000 to 100,000 human cases and at least 300 fatalities. Identification of human illness associated with VEEV infections near Iquitos, Peru (Watts et al. 1998), led to the initiation of field and laboratory studies to evaluate Peruvian mosquitoes for their ability to transmit this virus.

As part of a field ecology study of mosquitoes in the Amazon Basin region of Peru (Jones et al. 2004), over 160 virus isolations were made from mosquitoes (Turell et al. 2005). These included 24 isolations of VEE complex viruses, including 2 isolations of a subtype ID enzootic strain of VEEV and 21 isolations of a subtype IIIC virus, currently considered to be in the species Mucambo virus. However, Aguilar et al. (2004) recently demonstrated that the subtype IIIC virus from the Iquitos area of Peru was serologically distinct from both Mucambo (subtype IIIA) and Tonate (subtype IIIB) viruses. For simplicity, the subtype IIIC virus in the

VEE complex used in this study will be referred to as subtype IIIC virus throughout the rest of this article. Because various studies (Kramer and Scherer 1976; Scherer et al. 1982, 1986; Weaver et al. 1984; Turell et al. 1999, 2000, 2003) indicate that certain mosquito species may be significantly more susceptible to one subtype in the VEE complex than to another, it is necessary to determine the ability of a particular species to be able to transmit a particular subtype. Therefore, we evaluated the vector competence of several mosquito species, captured in an area where subtype IIIC virus strains were actively being transmitted, for a strain of subtype IIIC virus isolated from mosquitoes captured in January 1997 near Iquitos, Peru.

MATERIALS AND METHODS

Mosquitoes: Adult female mosquitoes were collected in dry ice-baited Centers for Disease Control miniature light traps in a forested area in the Amazon Basin near Iquitos, Peru (3°07'S, 73°3'W), from February 1998 through August 1999. Mosquitoes were transported to a Biological Safety Level 3 (with HEPA-filtered exhaust air, treated sewage, exit shower, and a 100% clothing-change policy) laboratory at the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD), provided apple slices as a carbohydrate source, and held at 26°C for 1–3 days until exposed to subtype IIIC virus. In addition to wild-caught mosquitoes, F₁ progeny of some species also were evaluated. Species studied included *Ochlerotatus (Ochlerotatus) fulvus* (Wiedemann), *Oc. (Och.) ser-ratus* (Theobald), *Culex (Melanoconion) vomerifer* Komp, *Cx. (Mel.) gnomatos* Sallum, Huchings, and Ferreira, *Cx. (Mel.) pedroi* Sirivanakarn and Belkin, *Cx. (Mel.) portesi* Senevet and Abonnenc, *Cx.*

¹ Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011.

² Department of Entomology, U.S. Naval Medical Research Center Detachment, Lima, Peru.

³ Ministry of Health, Loreto Department, Iquitos, Peru.

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(*Mel.*) *spissipes* (Theobald), *Cx. (Mel.) theobaldi* (Lutz), *Cx. (Culex) coronator* Dyar and Knab, *Pso-rophora (Janthinosa) albigena* (Peryassu), *Ps. (Grabhamia) cingulata* (Fabricius), and *Ps. (Jan.) ferox* (Von Humboldt).

Voucher specimens were placed in the collection at the National Museum of Natural History, Smithsonian Institution, Washington, DC.

Virus and virus assay: We exposed mosquitoes to an enzootic (subtype IIIC) strain of VEE complex virus, PE-4.0904, which had been isolated from a pool of mixed *Cx. vomerifer* and *Cx. gnomatos* captured near Puerto Almendras, Peru, on January 27, 1997. This virus had undergone 2 passages in Vero (African green monkey kidney cells) before its use in the present study.

Serial 10-fold dilutions of specimens were tested for infectious virus by plaque assay on Vero cell monolayers as described by Gargan et al. (1983), except that the neutral red stain was added 2, rather than 4, days after applying the initial agar overlay.

Determination of vector competence: An anesthetized, adult female Syrian hamster, inoculated intraperitoneally 1 or 2 days earlier with 0.2 ml of a suspension containing $\sim 10^4$ plaque-forming units (PFU) of subtype IIIC virus, was placed on top of a cage containing 50–250 field-collected or F_1 progeny mosquitoes. There were a total of 17 mosquito-feeding trials. Immediately after mosquito feeding, a 0.2-ml sample of blood was obtained from the hamster by cardiac puncture and added to 1.8 ml of diluent (10% heat-inactivated fetal bovine serum in medium 199 with Earle's salts and antibiotics). The blood suspensions were frozen at -70°C until assayed on Vero cell monolayers to determine the viremia levels at the time of mosquito feeding. After exposure to the viremic hamsters, engorged mosquitoes were transferred to 3.8-liter cardboard cages and the nonengorged mosquitoes were destroyed, or in some cases, inoculated intrathoracically (Rosen and Gubler 1974) with virus to determine transmission rates for individuals with a disseminated viral infection. An apple slice or a 10% sucrose solution was provided as a carbohydrate source, and mosquitoes were held at 26°C at a 16:8 h light:dark schedule for 12–16 days. To determine if the mosquitoes could transmit virus by bite, mosquitoes were allowed to feed on susceptible hamsters either individually or in small groups of 2–5 mosquitoes. Immediately after each

transmission trial, mosquitoes were killed by freezing at -20°C for 2–5 min, identified to species, and their legs and bodies triturated separately in 1 ml of diluent. These suspensions then were frozen at -70°C until tested for virus. If more than 1 mosquito in a pool had a disseminated infection and fed, then transmission data from that pool were not used to determine a transmission rate.

Infection was determined by recovering virus from the mosquito body-tissue suspension. If virus was recovered from its body, but not its legs, the mosquito was considered to have a nondisseminated infection limited to its midgut. Alternatively, if virus was recovered both from body and leg suspensions, the mosquito was considered to have a disseminated infection (Turell et al. 1984). Because infection with PE-4.0904 is consistently fatal to hamsters (Turell, unpublished data), death of these animals was used to indicate viral transmission. Transmission was verified by isolating virus from brain tissue. Any hamster that survived 21 days after being fed on by a mosquito with a disseminated infection was challenged with 10^4 PFU of the PE-4.0904 strain of subtype IIIC virus to determine its immune status.

Because few mosquitoes took 2 blood meals (e.g., an infectious meal on day 0 and a transmission feed 12–16 days later), it was difficult to calculate a transmission rate for many of the species. However, by including the data from the inoculated mosquitoes and the orally exposed mosquitoes with a disseminated infection, it was possible to calculate a transmission rate for mosquitoes with a disseminated infection. To calculate an estimated transmission rate for mosquitoes orally exposed to subtype IIIC virus, we multiplied the percentage of mosquitoes that developed a disseminated infection after oral exposure times the transmission rate for mosquitoes with a disseminated infection. If we did not observe transmission for a particular species, then the estimated transmission rate was defined as less than the rate we would have observed if the next mosquito tested had been a transmitter.

RESULTS

Hamster viremias during the 17 infectious feedings by mosquitoes ranged from $10^{6.6}$ to $10^{9.1}$, with a mean \pm standard error of $10^{8.2\pm 0.2}$ PFU/ml. Within this range of viremias, for each of the species tested, infection and dissemination rates were similar, regardless of the dose of virus ingested. Therefore, results were combined for further analysis. Because the *Cx. vomerifer/gnomatos* contained individuals of both species, they were not considered in this evaluation, but they had results intermediate between those for *Cx. gnomatos* and *Cx. vomerifer*.

Susceptibility to infection

Nearly all of the species were susceptible to infection; however, the *Cx. (Cux.)* spp. (consisting

⁴ The experiments reported herein were conducted in compliance with the Animal Welfare Act and according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. This work was supported by Work Unit Number 62787A 870 U 8517 of the U.S. Navy.

Table 1. Susceptibility of Peruvian mosquitoes to a subtype I11C strain of a Venezuelan equine encephalitis complex virus after feeding on viremic hamsters with a mean viremia of $10^{8.2}$ PFU/ml.

Species	Number tested	Infection rate ¹	Dissem. rate ²	Estimated trans. rate ³
<i>Culex (Mel.) gnomatos</i>	34	94 (83–99) d	88 (75–96) f	59
<i>Ochlerotatus (Och.) fulvus</i>	28	89 (75–97) d	50 (33–67) e	<5
<i>Cx. (Mel.) gnomatos/vomerifer</i>	42	88 (77–95) d	79 (66–88) f	63
<i>Psorophora (Gra.) cingulata</i>	5	80 (34–99) cd	60 (20–92) de	8 ⁴
<i>Cx. (Mel.) spissipes</i>	4	75 (24–99) cd	50 (10–90) cde	36 ⁴
<i>Ps. (Jan.) albigenu</i>	31	58 (42–73) c	26 (14–42) cde	4
<i>Cx. (Mel.) vomerifer</i>	21	29 (13–49) bc	19 (7–38) bcde	14 ⁴
<i>Cx. (Mel.) pedroi</i>	61	16 (9–26) b	7 (2–14) ab	7
<i>Cx. (Mel.) theobaldi</i>	25	12 (3–28) b	8 (1–23) abc	≤4
<i>Oc. (Och.) serratus</i>	42	12 (4–23) b	0 (0–7) a	<1
<i>Cx. (Mel.) portesi</i>	22	5 (<1–20) ab	5 (<1–20) abc	3 ⁴
<i>Cx. (Cx.) spp.</i>	104	2 (<1–6) a	1 (<1–4) a	<1
<i>Ps. (Jan.) ferox</i>	12	0 (0–22) ab	0 (0–22) abc	<1

¹ Percentage of mosquitoes containing virus in their bodies. Infection rates followed by the same letter are not significantly different at $\alpha = 0.05$.

² Dissemination rate, percentage of mosquitoes containing virus in their legs. Dissemination rates followed by the same letter are not significantly different at $\alpha = 0.05$.

³ Estimated transmission rate, the percentage of mosquitoes with a disseminated infection 12–15 days after ingesting a subtype I11C strain of VEE complex virus multiplied by the transmission rate for those individuals with a disseminated infection (see Table 2).

⁴ For those species for which data for transmission rates for individuals with a disseminated infection were not available, the average for that genus [i.e., 0.73 and 0.14 for *Cx. (Mel.)* spp. and *Psorophora* spp., respectively] was used to calculate the estimated transmission rate.

primarily of *Cx. (Cux.) coronator* and the *Ps. (Jan.) ferox* were essentially refractory, with infection rates of 2% and 0%, respectively (Table 1). Four species, *Cx. gnomatos*, *Oc. fulvus*, *Cx. spissipes*, and *Ps. cingulata*, were highly susceptible, with infection rates $\geq 75\%$. However, sample sizes for the latter 2 species were small, so results for these may not be meaningful. Infection rates were not significantly different among these 4 species. The other *Culex (Mel.)* species tested, *Cx. vomerifer*, *Cx. pedroi*, *Cx. portesi*, and *Cx. theobaldi*, were all relatively inefficient vectors of subtype I11C virus (Table 1).

Dissemination to the hemocoel

Patterns of viral dissemination were similar to those for infection rates, with the same 4 species, *Cx. gnomatos*, *Oc. fulvus*, *Cx. spissipes*, and *Ps. cingulata*, having the highest dissemination rates. The highest dissemination rate (88%) occurred in *Cx. gnomatos*, and this rate was significantly greater than that observed for each of the other species (except *Cx. spissipes* and *Ps. cingulata*) ($\chi^2 \geq 9.9$, $df = 1$, $P \leq 0.002$). However, if only the infected mosquitoes were evaluated, viral dissemination occurred in about half of the individuals in the other species, regardless of species. This indicated that only a moderate midgut escape barrier was present in these species.

Transmission studies

There was little evidence of a salivary gland barrier in the *Cx. (Mel.)* species tested, with 73% (8

of 11) of the mosquitoes with a disseminated infection successfully transmitting virus by bite (Table 2). In contrast, only 14% (3 of 21) of the *Psorophora* species with a disseminated infection transmitted virus by bite, indicating a significant salivary gland barrier. Although *Oc. fulvus* were highly susceptible to infection and most of the infected individuals developed a disseminated infection, none of 22 individuals (10 with a disseminated infection) transmitted virus when refeeding (Table 2). This indicated a significant salivary gland barrier in this species.

DISCUSSION

Culex gnomatos was the most efficient vector in our study, with an estimated transmission rate about 10-fold higher than any of the other species tested. This is consistent with virus isolation studies conducted in the same region as where the mosquitoes used in the present study were captured (Turell et al. 2005). In that study, 14 of the 19 subtype I11C virus isolations that could be attributed to a specific species were from pools of *Cx. gnomatos*. Four of the remaining 5 isolates were from *Cx. pedroi*, the species with the second highest estimated transmission rate in our current study. Therefore, *Cx. gnomatos* appears to be the principal vector of this virus in the Amazon Basin region of Peru. This is supported by the recent demonstration that a female *Cx. gnomatos* transmitted subtype I11C virus to a sentinel hamster in this region (Yanoviak et al. 2005).

Previous studies indicate that the enzootic cycle for members of the VEE complex probably in-

Table 2. Potential for Peruvian mosquitoes to transmit a subtype IIIC strain of a Venezuelan equine encephalitis complex virus.

Species	Route of virus exposure			
	Oral			
	Total ¹	Dissem. ²	Inoculated	Trans. ³ (D)
<i>Culex (Cux.)</i> spp.	0 (22) ⁴	n.t. ³	0 (2)	0 (2)
<i>Cx. (Mel.) gnomatos</i>	40 (5)	67 (3)	n.t.	67 (3)
<i>Cx. (Mel.) pedroi</i>	0 (3)	n.t.	100 (2)	100 (2)
<i>Cx. (Mel.) portesi</i>	0 (12)	n.t.	n.t.	unk. ³
<i>Cx. (Mel.) theobaldi</i>	0 (6)	0 (1)	n.t.	0 (1)
<i>Cx. (Mel.) vomerifer</i>	0 (4)	n.t.	n.t.	unk.
<i>Cx. (Mel.) gnomatos/vomerifer</i>	40 (10)	80 (5)	n.t.	80 (5)
<i>Ochlerotatus (Och.) fulvus</i>	0 (22)	0 (10)	n.t.	0 (10)
<i>Oc. (Och.) serratus</i>	0 (15)	n.t.	0 (1)	0 (1)
<i>Psorophora (Jan.) albigena</i>	0 (22)	0 (7)	28 (7)	14 (14)
<i>Ps. (Gra.) cingulata</i>	0 (4)	n.t.	n.t.	unk.
<i>Ps. (Jan.) ferox</i>	0 (8)	n.t.	14 (7)	14 (7)

¹ Data for all orally exposed mosquitoes that took a second blood meal.

² Data for orally exposed mosquitoes with a disseminated infection that took a second blood meal.

³ Trans., transmission; n.t., not tested; unk., unknown.

⁴ Percentage of mosquitoes that transmitted virus (number fed).

volves *Cx. (Mel.)* spp. and rodents (Barrera et al. 2002, Weaver et al. 2004). This is consistent with the study by Turell et al. (2005) in which all 25 isolations of VEE complex viruses were made from members of this subgenus. Also, in the present study, all 6 *Cx. (Mel.)* species tested developed disseminated infections after oral exposure to a subtype IIIC virus. Surprisingly, *Cx. vomerifer*, a species only recently separated from *Cx. gnomatos* (Sallum et al. 1997), was significantly less susceptible to infection with this virus than was *Cx. gnomatos*. In addition, when specimens of these 2 species collected in the same traps were tested separately for virus, all 14 isolations of subtype IIIC virus were from *Cx. gnomatos*, despite essentially equal numbers of the 2 species being captured and tested (Turell et al. 2005). As in several previous studies, members of the *Culex* subgenus *Culex* were essentially refractory to infection with VEE complex viruses (Schaffer and Scherer 1974; Kramer and Scherer 1976; Turell 1999; Turell et al. 2000, 2003). However, the present study extends the incompetence of members of this subgenus to subtype IIIC virus.

Despite relatively high infection and dissemination rates, *Oc. fulvus* did not appear to be an important vector. This species has a major salivary gland barrier, as indicated by the lack of transmission of subtype IIIC virus in the present study, even though 10 mosquitoes with a disseminated infection fed on susceptible hamsters. This is consistent with an earlier study (Turell et al. 2000) in which only 2 (4%) of 45 *Oc. fulvus* with a disseminated infection with subtype I strains of VEEV successfully transmitted virus by bite. The same is true for *Ps. cingulata*. In the earlier study (Turell et al. 2000), it also had a major salivary gland barrier as only 4

(11%) of 35 individuals with a disseminated infection successfully transmitted virus by bite. In contrast, the transmission rate for the combined *Cx. gnomatos* and *Cx. vomerifer* with a disseminated infection in that study was 13 (81%) of 16 specimens, a rate nearly identical to that observed in the present study, 6 (75%) of 8.

While we are not aware of any published data on viremia levels that occur in animals naturally infected with subtype IIIC virus, the viremias to which mosquitoes were exposed in our study, $\sim 10^8$ PFU/ml of blood, were consistent with those observed in burros (Gochenour et al. 1962) or horses (Kissling et al. 1956, Sudia et al. 1971) inoculated with an epizootic (IAB) strain or in bats inoculated with the enzootic IE strain of VEEV (Seymour et al. 1978). However, laboratory studies indicate that viremias with VEE complex viruses in spiny (*Proechimys* spp.) and cotton (*Sigmodon* spp.) rats (the likely vertebrate host of many of the VEE subtype viruses) tend to be lower, around 10^5 and 10^{5-7} PFU/ml, respectively (Young et al. 1969, Wang et al. 2001, Coffey et al. 2004), indicating that the viremias used in this study may be higher than those that occur in nature.

Although we did not conduct blood meal identification studies, *Cx. gnomatos* (identified only to *Cx. gnomatos/vomerifer*) readily fed on humans, with collection rates of 31 per day per person (Jones et al. 2004). Therefore, based on the numerous virus isolations from field-collected specimens, its vector competence in the laboratory, and the demonstration of its ability to transmit subtype IIIC virus to a hamster in the field, *Cx. gnomatos*, should be considered the principle vector of this virus in the Amazon Basin region of Peru.

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