Natural Enzootic Vectors of Venezuelan equine encephalitis virus, Magdalena Valley, Colombia

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To characterize the transmission cycle of enzootic Venezuelan equine encephalitis virus (VEEV) strains believed to represent an epizootic progenitor, we identified natural vectors in a sylvatic focus in the middle Magdalena Valley of Colombia. Hamster-baited traps were placed into an active forest focus, and mosquitoes collected from each trap in which a hamster became infected were sorted by species and assayed for virus. In 18 cases, a single, initial, high-titered mosquito pool representing the vector species was identified. These vectors included Culex (Melanoconion) vomerifer (11 transmission events), Cx. (Mel.) pedroi (5 transmissions) and Cx. (Mel.) adamesi (2 transmissions). These results extend the number of proven enzootic VEEV vectors to 7, all of which are members of the Spissipes section of the subgenus Melanoconion. Our findings contrast with previous studies, which have indicated that a single species usually serves as the principal enzootic VEEV vector at a given location.

Venezuelan equine encephalitis (VEE) is an emerging zoonotic arboviral disease that affects equines and humans in the Americas (1). Venezuelan equine encephalitis virus (VEEV) has caused sporadic outbreaks since the early part of the 20th century, with some epidemics affecting 100,000 persons. For many years, the source of the epizootic/epidemic VEEV strains belonging to subtypes IAB and IC viruses remained unknown. After antigenically related but distinct, equine-avirulent, enzootic strains of VEEV were isolated in the 1960s, researchers hypothesized that epizootic/epidemic strains evolve from enzootic VEEV progenitors (2). The first genetic evidence supporting this hypothesis came from RNA fingerprinting studies that indicated a close relationship between subtype ID–enzootic VEEV strains from Colombia and epizootic/epidemic isolates belonging to subtype IC (3). Later, sequencing (4) and phylogenetic (5,6) studies also supported the evolution of the epizootic/epidemic serotype IAB and IC strains from enzootic ID VEEV progenitors. Recently, comprehensive phylogenetic analyses have indicated that the epizootic/epidemic strains evolved independently on at least three occasions from a single lineage of ID VEEV that circulates in eastern and central Colombia, western Venezuela, and northern Peru (7–10). Other ID-like VEEV lineages that occur in Panama, Amazonian Peru, southwestern Colombia, coastal Ecuador, north-central Venezuela, and Florida have not generated any of the epizootic/epidemic strains sequenced (10–12).

Enzootic VEEV (subtypes ID–IF, II–VI) circulate nearly continuously in sylvatic or swamp habitats in various tropical and subtropical locations in the New World (1,13). These viruses generally use small mammals as their reservoir hosts and are transmitted by mosquitoes. Enzootic mosquito vectors have been identified for four VEEV variants: 1) Culex (Melanoconion) portesi transmits Mucambo virus (VEE complex subtype IIIA) in Trinidad (14), 2) Cx. (Mel.) cedecci transmits Everglades virus (VEE complex subtype II) in southern Florida (15), 3) Cx. (Mel.) aikenii sensu latu (ocossa and pano-cossa) transmits subtype ID VEEV in Panama (16,17), and 4) Cx. (Mel.) taeniopus (formerly opisthopus) is the primary enzootic vector of subtype IE VEEV in Guatemala (18). More than 70% of enzootic field isolations have come from the subgenus Melanoconion, suggesting that these mosquitoes are the principal vectors of most or all enzootic VEE complex strains (17).

The infrequency of VEE emergence is probably determined by the infrequent, simultaneous occurrence in time and space of viral mutations that mediate host range changes, combined with ecologic and epidemiologic conditions that permit efficient amplification (1). To understand the mechanisms of VEE emergence from enzootic progenitors in Colombia and Venezuela, we are studying the hosts in which epizootic mutations may occur and in which the selection of epizootic strains may follow. However, the vector and reservoir hosts of the particular subtype ID VEEV lineage implicated in epizootic emergence have not been identified. Using an efficient system of vector identification employing hamster baited traps, we identified Cx. (Mel.) vomerifer, Cx. (Mel.) pedroi, and Cx. (Mel.) adamesi as natural enzootic vectors in an active focus of subtype ID VEEV in the middle Magdalena Valley of Colombia.

Methods

Study Area

The study was carried out from 1999 to 2000 in the Monte San Miguel Forest in the middle Magdalena Valley of Colombia (6° 23’ 30”N; 74° 21’ 41” W; 50 m elevation). This is a lowland tropical rainforest surrounded by cattle ranches cr-
ated by deforestation. Mean minimum and maximum daily
temperatures are 23°C and 33°C (overall mean of 29°C),
respectively, and annual rainfall averages 2,700 mm. Mean rel-
ative humidity is 80%. Generally, the peaks of the rainy sea-
sons occur in April–May and October–November. Numerous
previous isolations of subtype ID VEEV from sentinel ham-
sters (9) indicate that this forest site is a stable enzootic focus.

Mosquito Traps
Hamster-baited traps were used for detection of natural
VEEV vectors. These traps were a version of the Trinidad No.
10 trap (19) with the following modifications: 1) the metal can
comprising the trap opening was replaced by a polyvinyl chlo-
ride pipe, 10 cm in diameter; 2) the cylindrical animal cage
was enlarged to 11 cm in diameter and 12 cm in height; 3) the
roof was constructed from plexiglass; and 4) the opening for
mosquito aspiration was a simple buttonhole sewn into the
polyester collection net (Figure). The traps were baited with
adult golden Syrian hamsters obtained from a colony main-
tained at the Instituto Nacional de Salud in Bogota. Baited
traps were suspended approximately 1.5 m above the ground
and placed in transects at 10-m intervals. Carrots and rat chow
were provided for food and water. The traps were checked
each morning between 0600 and 0800 h, and some were also
checked in the evening between 1700 and 1900 h. Mosquitoes
were removed from the traps by using an aspirator, and the
daily or semi-daily collections from each trap were frozen as a
single pool in a plastic bottle immersed in liquid nitrogen
vapor. When hamsters within the traps became moribund or
died, serum samples were obtained by cardiac puncture or
their hearts were dissected aseptically and frozen for virus iso-
lation.

Detection of Natural Transmission to Hamsters
To confirm VEEV infection in dead or moribund hamsters,
virus was isolated from a 10% heart tissue suspension in
Eagle’s minimal essential medium (MEM), supplemented with
20% fetal bovine serum (FBS) and antibiotics. The suspension
was prepared in a Ten Broeck tissue grinder and centrifuged at
15,000 x g for 5 min; 200 µL of the supernatant was added to a
25-cm² flask containing a monolayer of Vero cells and
adsorbed for 1 h at 37°C; 6 mL of additional MEM containing
2% FBS was then added. Cultures were incubated at 37°C for
5 days or until cytopathic effects were evident.

Mosquito pools from traps in which hamster infection with
VEEV was confirmed were assayed for infectious virus. Pools
containing 1–40 individuals of each mosquito species were
triturated with a Minibeadbeater (BioSpec Products, Inc., Bar-
tlesville, OK) or a Ten Broeck tissue grinder containing 1.0
mL of MEM supplemented with 20% FBS, penicillin, strepto-
ymycin, and amphotericin B. The triturated pool was centri-
fuged for 5 min at 15,000 x g, and 200 µL of the supernatant
was added to a 10-mL plastic tube or a 25-cm² cell culture dish
containing a monolayer of Vero cells and 2–5 mL of MEM.
Cultures were monitored for cytopathic effects for 5 days.

Genetic and Antigenic Characterization of VEEV Isolates
Viruses isolated from hamster heart tissue suspensions and
mosquito pools were characterized antigenically by using
immunofluorescence of infected cells and a panel of mono-
clonal antibodies described previously (20). Subtype ID
VEEV isolates were further characterized genetically by
reverse transcription–polymerase chain reaction (PCR) ampli-
fication of an 856-nucleotide portion of the PE2 (sometimes
called p62) envelope glycoprotein precursor gene as described
previously (8), followed by single-stranded conformation
polymorphism (SSCP) or sequence analysis (9). For SSCP
analysis, PCR products were purified on agarose gels by using
the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). A
2-µL volume of the PCR amplicon DNA suspension was mixed
with 8 µL of SSCP loading buffer (95% formamide, 0.05% bro-
mophenol blue, and 0.05% xylene cyanol). The DNA was
heated to 95°C for 5 minutes, rapidly cooled on ice, loaded
onto an 8% polyacrylamide gel, and underwent electrophoresis
in 1X Tris-borate EDTA buffer at room temperature for 20 h at
8 mA. Single-stranded DNA products were visualized by
using silver staining (21). SSCP patterns were compared by
measuring the migration of single-stranded DNA of the vari-
ous isolates in comparison to one another and to a standard
DNA ladder.

Results
For vector identification studies, 87 hamsters were
exposed in traps within the Monte San Miguel Forest for 5–7
days. Of these, 38 became moribund or died and were pro-
cessed for virus isolation. VEEV was isolated from 37 ham-
sters, and the mosquito collections from the corresponding
traps were assayed for virus.

In 18 of the traps yielding infected hamsters, a vector spe-
cies was identified by using the following criteria: 1) the ham-
ster-baited traps used to identify vectors
of Venezuelan equine encephalitis virus. Arrow shows entry route of
mosquitoes.
ster died at least 24 h after the collection of the presumed vector, consistent with the incubation time of VEEV in hamsters (22); 2) during the first day in which infected mosquitoes were collected from the trap, only one species pool had a high titer (>5.0 log10 PFU/pool) consistent with an infectious mosquito, as determined by previous experimental studies of enzootic VEEV vectors (18,23–26); 3) the remaining pools, from the first day in which infected mosquitoes were collected, were uninfected, or had low titers (<5.0 log) shown previously to be inconsistent with an infectious mosquito (18,23–26); 4) the mosquito collections on the days subsequent to that of the vector collection were mostly infected, reflecting hamster viremia and the ingestion of infectious blood by mosquitoes biting ≥12 h after the transmission event; and 5) virus isolates from the hamster and corresponding vector were indistinguishable antigenically and genetically with SSCP analysis, sequencing, or both. In 18/37 infected hamster events studied, these criteria were fulfilled, vector was identified unambiguously. Typical data for one of these transmission events (hamster 164) is shown in Table 1. In this example, transmission by Cx. vomerifer occurred ≤24 h after exposure of the trap, and the vector pool had a titer of 5.8 log10 PFU/pool. The other two infected pools from day 2, Cx. pedroi and Aedes serratus, had log titers ≤3.3, indicating that they were not capable of transmission. These pools presumably contained one or more mosquitoes that engorged on the hamster after viremia began, probably just before the daily trap collection. On the next day, all mosquito pools contained infectious virus in their midguts, representing viremic hamster blood ingested by mosquitoes within the trap.

A total of 18 transmission events were characterized as described above. The most common interval of collection of the identified vector was 24–48 h after exposure, reflecting a very high level of enzootic VEEV transmission in the Monte San Miguel Forest. Cx. vomerifer was implicated in 11 of these events, Cx. pedroi in 5, and Cx. adamesi in 2 transmissions (Table 2). The minimum infection/transmission rate for the mosquitoes we collected could not be determined directly because we did not identify the mosquito collections for traps where transmission to the hamster did not occur. However, rates on the order of 1/200–1/1000 can be estimated for these three vector species if the species composition is assumed to be similar in traps where transmission did not occur. Even if this assumption is incorrect, the error in this estimate should not be more than twofold because VEEV transmission occurred in most traps.

Discussion

Use of Hamster-Baited Traps for Arbovirus Vector Identification

Traditional criteria for arthropod vector identification include the following: 1) demonstration of feeding or other effective contact with pathogen’s host; 2) association in time and space of the vector and pathogen; 3) repeated demonstration of natural infection of the vector, and 4) experimental transmission of the pathogen by the vector (27). Infection rates for arbovirus vectors tend to be relatively low, usually <1%. Therefore, fulfillment of these criteria for arbovirus vectors usually relies on the capture of large numbers of arthropods for virus isolation, followed by experimental laboratory transmission studies to ensure that species found infected in nature are competent vectors. Although this strategy is the most comprehensive and unbiased, it is extremely costly and time consuming, accounting for the relative paucity of information on natural vectors of many arboviruses. Some studies of VEEV vectors have also relied on oral infection from experimentally infected hamsters with viremia levels of very high titer, on the order of 8 log10 PFU/mL (28,29), a titer at least 100–1,000 times greater than that generated by experimentally infected rodent reservoir hosts (30,31), equines (13,30,32), or naturally infected humans (8,33) (Some studies of equine viremia have

Table 1. Mosquito collections from hamster-baited trap no. 164

| Species                  | Fraction of pools positive | Pool titers | Species                  | Fraction of pools positive | Pool titers | Species                  | Fraction of pools positive | Pool titers |
|--------------------------|----------------------------|-------------|--------------------------|----------------------------|-------------|--------------------------|----------------------------|-------------|
| Culex (Melanoconion) pedroi | 0/1                        | NT*         | Culex (Mel.) pedroi      | 1/1                        | 3.3         | Culex (Mel.) pedroi      | 1/1                        | 5.9         |
| Cx. (Mel.) spissipes     | 0/1                        | NT          | Cx. (Mel.) spissipes     | 0/1                        | NT          | Cx. (Mel.) spissipes     | 1/1                        | 4.7         |
| Cx. (Mel.) vomerifer     | 0/1                        | NT          | Cx. (Mel.) crybda        | 0/1                        | NT          | Cx. (Mel.) ferreri       | 1/1                        | 4.8         |
| Cx. (Mel.) adamesi       | 0/1                        | NT          | Cx. (Mel.) vomeriferb    | 1/1                        | 5.8         | Cx. (Mel.) vomerifer     | 1/1                        | 5.5         |
| Aedes serratus           | 0/1                        | NT          | Cx. (Mel.) adamesi       | 0/1                        | NT          | Cx. (Mel.) adamesi       | 1/1                        | 5.1         |
| Cx. (Cx.) nigricalpus    | 0/1                        | NT          | Ae. serratus             | 1/1                        | <2          | Ae. serratus             | 1/1                        | 5.2         |
| Cx. (Ae.) amazonensis    | 0/1                        | NT          | Cx. (Cx.) nigricalpus    | 0/1                        | NT          | Cx. (Cx.) nigricalpus    | 1/1                        | 5.2         |
| Coquilletidia venezuelensis | 0/1                        | NT          | Cx (Ae.) amazonensis     | 0/1                        | NT          | Cx (Ae.) accelerans      | 1/1                        | 4.5         |
| Ae. fulvus               | 0/1                        | NT          | Ae. fulvus               | 0/1                        | NT          | Mansonia titillans       | 1/1                        | 5.2         |

*NT, not tested.

bIncriminated vector pool.
yielded titers of $>10^8$ suckling mouse intracerebral 50% lethal doses, but this method for quantifying VEEV titers is 100- to 1000-fold more sensitive than PFU [30,34]). Results from these studies are therefore inconclusive regarding natural transmission potential.

Other investigators have streamlined the vector identification process by collecting suspected vectors and sorting them according to species, then exposing single-species pools to naive animals in a field or laboratory setting to detect transmission (16,18). We have taken this approach one step further by combining collection and transmission detection using hamster-baited traps. This method simplifies the vector identification process in several ways: 1) Hamster-baited traps attract and capture only arthropod species that are attracted to small mammals, the natural reservoir hosts of the enzootic VEEV (Proechimys spp. spiny rats in the case of subtype ID VEEV circulating in this focus [35]), minimizing collection and mosquito processing efforts. 2) Arthropod collections from traps where no transmission occurs do not need to be sorted, greatly reducing a laborious step in the vector identification process. 3) Only a small number of arthropod pools must be tested for virus, eliminating much of the cost, labor, and biosafety hazard associated with traditional vector identification approaches. In addition, the hamster-baited traps can serve as sentinels for detection of active virus circulation in a forest and reveal the presence of other viruses in a focus. However, unlike other sentinel enclosures that allow arthropods to escape after biting a viremic bait animal and thereby initiate artificial amplification, the hamster-baited traps capture most of the arthropods that bite the viremic host and prevent most or all artificial amplification. A similar strategy for detecting transmission of western equine encephalitis and St. Louis encephalitis viruses to chickens in baited traps was described by Reeves et al. (36).

Using these hamster-baited traps alone, we were not able to measure directly the capture efficiency of our traps. However, in the case of five infected hamsters, the lack of any collections with a single or few high titer mosquito species pools on the day preceding total infection of collected mosquitoes indicates that the arthropod responsible for transmission may have escaped. In other cases, two or more mosquito pools collected on the first day virus was detected had titers consistent with infectious vectors, precluding vector identification. We are currently experimenting with funnel-shaped openings to reduce the frequency of vector escape from this trap design. As with any passive trap design, a compromise between ease of vector entry and frequency of escape must be sought to maximize collections.

### Enzootic Vectors of Venezuelan Equine Encephalitis Complex Viruses

Previous studies of VEE complex enzootic transmission have each identified a single, principal mosquito species in a given geographic region. All of these species, including Cx. portesi (14), Cx. cedecei (15), Cx. aikenii sensu lato (ocossa and panocossa) (16,17), and Cx. taeniopus (18) are members

| Hamster no. | Vector species   | Titer of vector poola | Collection interval of vector pool (h) | Titer of other mosquito pools in the same collection as the vectora | Fraction of mosquito species infected on the subsequent day’s collectionb |
|-------------|------------------|------------------------|----------------------------------------|-------------------------------------------------------------------|---------------------------------------------------------------------------|
| 65          | Culex pedroi     | 5.2                    | 0–24                                   | ≤2                                                                | 6/10                                                                      |
| 66          | Cx. vomerifer    | 5.0                    | 48–72                                  | ≤2                                                                | 18/18                                                                     |
| 144         | Cx. vomerifer    | 5.4                    | 24–48                                  | ≤2                                                                | NA                                                                        |
| 150         | Cx. adamesi      | 5.5                    | 24–48                                  | ≤2                                                                | 9/9                                                                        |
| 164         | Cx. vomerifer    | 5.8                    | 0–24                                   | ≤3.3                                                               | 10/10                                                                     |
| 172b        | Cx. vomerifer    | 5.4                    | 24–48                                  | ≤3.8                                                               | 9/9                                                                        |
| 184         | Cx. pedroi       | 5.1                    | 0–24                                   | ≤2.3                                                               | 6/6                                                                        |
| 186         | Cx. vomerifer    | 6.1                    | 0–24                                   | ≤2                                                                | 9/9                                                                        |
| 264         | Cx. vomerifer    | 5.4                    | 0–24                                   | ≥3.9                                                               | 12/16                                                                     |
| 272         | Cx. pedroi       | 5.1                    | 0–24                                   | ≤2                                                                | 11/11                                                                     |
| 277         | Cx. adamesi      | 5.3                    | 0–24                                   | ≤2                                                                | 18/18                                                                     |
| 279         | Cx. vomerifer    | 6.1                    | 24–48                                  | ≤2                                                                | 14/16                                                                     |
| 286         | Cx. vomerifer    | 5.5                    | 120–144                                | ≤2                                                                | 14/15                                                                     |
| 287         | Cx. pedroi       | 5.4                    | 0–24                                   | ≤2                                                                | 15/15                                                                     |
| 296         | Cx. vomerifer    | 5.7                    | 0–24                                   | ≥4.9                                                               | 10/10                                                                     |
| 290         | Cx. pedroi       | 5.4                    | 0–24                                   | ≤2.8                                                               | 13/15                                                                     |
| 304         | Cx. vomerifer    | 5.3                    | 48–72                                  | ≤2                                                                | 8/8                                                                        |
| 305         | Cx. vomerifer    | 5.7                    | 2                                     | ≤2.1                                                               | 8/8                                                                        |

aLog10 Vero PFU per pool
bA second infected pool of C. vomerifer with a log titer of 3.8 was collected on day 1, but was presumed not to have been transmitted to the hamster due to its low titer.
of the Spissipes section of the subgenus *Melanoconion* within the genus *Culex* (37). Previous studies of enzootic VEEV transmission in the Catatumbo region of northeastern Colombia also suggested that *Cx. pedroi* might be the principal vector, based on abundance in active foci (38). *Cx. vomerifer* from Iquitos, Peru, also has been shown to be susceptible to infection by several strains of VEEV (28), but was only tested after mosquitoes ingested 8 log_{10} PFU/mL from viremic hamsters, a viremia titer at least 100 times greater than that generated by infection in the Magdalena Valley.

Our findings of at least three enzootic vectors of subtype ID VEEV in Colombia contrast with the findings of all previous studies of enzootic VEEV vectors, which suggested that enzootic VEEV strains are each adapted to a single, principal vector species (13,18,39–41). In Colombia, subtype ID VEEV appears to utilize efficiently both *Cx. vomerifer* and *Cx. pedroi* in the Magdalena Valley. *Cx. adamesi*, which is usually less abundant in the Monte San Miguel Forest, appears to serve as a secondary vector.

All three of the mosquito species that we identified as VEE vectors are members of the Spissipes section of the subgenus *Culex* (*Melanoconion*), bringing the total to seven confirmed vectors within this section of closely related mosquitoes. The genetic or ecologic basis for the exclusive use of these mosquitoes by enzootic VEE complex viruses deserves further study. Hypotheses to explain this phenomenon include possible shared, derived characteristics of the Spissipes section, such as particularly high susceptibility to infection by enzootic VEE complex viruses, a particularly high degree of association with the *Proechimys* spp. (35) and other small mammalian reservoir hosts (13), or both. Mosquito longevity and population sizes in habitats that support large populations of reservoir hosts may also favor transmission by members of the Spissipes section (35).

### Role of Enzootic Vectors in VEEV

#### Emergence and Disappearance

Identification of the principal enzootic vectors (*Cx. vomerifer* and *Cx. pedroi*) of subtype ID VEEV strains believed to be closely related to epizootic progenitors will allow us to assess the role of these mosquitoes in the generation of mutations that mediate VEEV emergence by enhancing equine viremia and infection of epizootic mosquito vectors such as *Ochlerotatus taeniorynchus*. The hypothesis that epizootic VEEV is not recovered from sylvatic foci because these strains lose their fitness for the enzootic vectors (25) can also be tested in the two principal vectors that we identified.

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