TRANSMISSION OF EASTERN EQUINE ENCEPHALOMYELITIS VIRUS IN CENTRAL ALABAMA

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Abstract. A site near Tuskegee, Alabama was examined for vector activity of eastern equine encephalomyelitis (EEE) virus in 2001. More than 23,000 mosquitoes representing 8 genera and 34 species were collected during a 21-week period, and five species, Culiseta melanura, Aedes vexans, Coquillettidia perturbans, Culex erraticus, and Uranotaenia sapphirina, were examined for the presence of virus using a nested reverse transcriptase–polymerase chain reaction for EEE virus. Each species was infected at various times of the mosquito season (May–September) with different minimum infection rates (MIRs). Culiseta melanura had the highest MIR (20.2) and positive pools were detected from late May to mid-September. Aedes vexans had an MIR of 2.2 and was infected early in the season (June), while Cq. perturbans exhibited a much higher field infection rate (9.9) with all positive pools collected in August. Culiseta melanura is a likely endemic vector in central Alabama, while Ae. vexans and Cq. perturbans probably function as bridge vectors. Culex erraticus, the most common mosquito in the habitat (54% of total collections), had an MIR of 3.2, and was persistently infected from mid-June to mid-September. This is the first report of high rates of EEE virus infection in this species, a member of the tropical subgenus Melanocion. Uranotaenia sapphirina, considered to feed on amphibians and possibly reptiles, had an MIR of 5.6, with positive pools spanning a four-month period. This suggests that species other than birds may serve as a reservoir for EEE in hardwood swamps in the Southeastern United States and elsewhere. The lengthy period of mosquito infection with EEE virus, coupled with the diverse habits of the vectors and their proximity to a population center, indicate the importance of monitoring EEE virus activity in the Mid-South.

INTRODUCTION

Eastern equine encephalomyelitis (EEE) virus is focally endemic primarily throughout eastern Canada, the eastern United States, and southward into the Caribbean and tropical/subtropical Latin America. Circulation of the virus occurs in a mosquito-avian cycle usually within well-defined habitats, and endemic maintenance of the virus in North America is correlated with the presence of Culiseta melanura, a highly ornithophilic mosquito species. Periodic EEE epizootics and epiornitics may occur in horses, resident birds, and exotic avian species such as emus. These outbreaks are associated with the activity of bridge vectors such as Coquillettidia perturbans, Ochlerotatus sollicitans, and Aedes vexans that are catholic in host selection and therefore more likely to move the virus to the periphery or beyond the endemic swamp/hardwood forest habitat. Although human cases occur sporadically, infection with EEE is particularly virulent with about 70% of symptomatic cases in epidemics being fatal. Most survivors have residual neurologic sequelae and health care during acute phase illness and afterwards may result in the expenditure of several million dollars per person.

With the exception of Florida, the ecology of EEE in the Southeastern United States is much less understood than in other endemic locations in the eastern United States. This is problematic because habitat fragmentation is occurring rapidly throughout the region due to increased population movement and expansive peri-urban development in the South Atlantic and Southeastern states. Consequently, susceptible human populations are migrating into or near sylvatic areas, wetlands, and permanent standing water habitats in which the virus may be endemic.

Eastern equine encephalomyelitis virus is endemic in Alabama, with occasional epidemic outbreaks that vary from year to year. The summer of 2001 was particularly active for this virus, with one human and more than 30 equine infections reported in the southern and central portions of the state. To provide an understanding of EEE ecology in a hardwood swamp habitat in the Mid-South near a population center, we describe the species composition and dynamics of mosquito populations in a focus in east central Alabama, provide data on onset and rate of EEE virus infection in several vector species, compare these findings with those reported from other locations in the United States and tropical South America, and discuss their importance with regard to the possible emergence of EEE virus in the southern United States.

MATERIALS AND METHODS

Study site. The study site was located in Tuskegee National Forest in Macon County, Alabama (32°00'26” W, 85°44'38” N). This general area is within the narrow Fall Line transition zone from upland Piedmont to Black Belt, a physiographic region that is characterized by heavy calcareous soils of Cretaceous origin. Within the site, there has been extensive reencroachment of forest over depleted farmland that was abandoned in the early 1900s. Water tupelo (Nyssa aquatica), eastern red cedar (Juniperus virginiana), sweetgum (Liquidambar styraciflua), red maple (Acer rubrum), and laurel oak (Quercus hemisphaerica) occur as mixed stands with loblolly pine (Pinus taeda) as the dominant canopy species. Five beaver ponds, which are interconnected and fluctuate in size and depth, provide standing water for much of the year. Plants such as duckweed (Lemna sp.), arrow root (Maranta arundinacea), softstem bulrush (Scirpus tabernaemontani), and switchcane (Arundinaria gigantea) are common in and around the ponds. Hazel alder (Alnus serrulata) is prevalent in thickets along the wet margins of the ponds and the edges of temporary pools in the forest islands. This plant and red maple has open, aboveground root mats that serve as excellent resting places for adult mosquitoes. The town square of Tuskegee, Alabama is located approximately 3.0 kilometers from the site.

Collections. Mosquitoes were collected using portable Centers for Disease Control (CDC) light traps baited with CO₂
and by vacuum collection. Light traps ran from dusk to dawn and were positioned approximately two meters above ground. Sampling (twice a week) began during the first week of May and was concluded during the first week of October 2001. Vacuum collections were made twice a week from resting boxes\(^2\) and natural resting sites during this same time period. These collections complemented those from light traps and allowed sampling of mosquitoes in different physiologic/behavioral conditions, i.e., nulliparous/parous host-seeking mosquitoes in light traps versus blood-engorged or gravid ones in resting boxes, or allowed collection of species not attracted to light. Live material was returned to the laboratory, sorted, identified using a chill table and binocular microscope, and frozen at \(-7^\circ C\). Mosquitoes containing blood were excluded and specimens were transported periodically to the University of Alabama at Birmingham on dry ice for virus detection.

**Virus identification.** Pools of mosquitoes containing up to 50 individuals were homogenized in 1.5 mL of BA-1 tissue culture medium and subjected to centrifugation at 13,000 × \(g\) for five minutes at room temperature. A total of 140 \(\mu\)L of the resulting supernatant was removed and RNA was purified from the aliquot using the QiaAMP viral RNA extraction kit (Qiagen, Valencia, CA). The RNA was prepared following the manufacturer’s instructions, with the exception that the number of washes with buffers AW1 and AW2 were increased from one to two.

Viral RNA was detected in the RNA prepared from pools of mosquitoes using a nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay. This assay was a modification of a previously published protocol\(^1\) that included a nested amplification step to increase the limit of detection of the assay. Briefly, 4 \(\mu\)L of RNA prepared as described earlier was used in a 50-\(\mu\)L total volume one-step RT-PCR amplification reaction using reagents provided by Qiagen (One step RT-PCR) and the EEE virus-specific primers EEE c7601 (5\'-TACCCCTACAACATTAAYCCGC-3\') where \(Y = C\) or \(T\) and EEE nc7873 (5\'-TGTCGTTTGC-CTGGTTAAGT-3\'). The amplification reactions contained 1× Qiagen OneStep RT-PCR buffer, 400 \(M\) each of dATP, dGTP, dCTP, and dTTP, 0.6 \(M\) each of primer, and 2 \(\mu\)L of Qiagen OneStep RT-PCR enzyme mixture. Reaction conditions consisted of 50°C for 30 minutes and 95°C for 15 minutes, followed by 40 cycles each consisting of 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for two minutes. Reactions were completed with a final extension at 72°C for 10 minutes. Nested PCRs were carried out in a total volume of 50 \(\mu\)L using 0.5 \(\mu\)L of the first-step PCR product as a template. The nested PCRs contained 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl\(_2\), 50 mM KCl, 200 \(\mu\)M each of dATP, dGTP, dCTP, and dTTP, 0.5 \(M\) each of primer, and 2.5 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). The primers used in the nested PCRs were EEE c7643 (5\'-ATGGCCTACCCGGATCCTAATC-3\'), where \(Y = C\) or \(T\) and EEE nc7848 5\'-ACGTTTTGTTCCTGTGCGATT-3\'). Cycling conditions consisted of 40 cycles of 95°C for 45 seconds, 58°C for one minute, and 72°C for 30 seconds, followed by a final extension at 72°C for seven minutes. Products were visualized by electrophoresis on a 1.5% agarose gel, followed by staining with 1 \(\mu g/\text{ml}\) of ethidium bromide. Each experiment was conducted with a series of positive and negative controls. The positive control for each experiment consisted of RNA extracted from inactivated EEE virus culture supernatants kindly provided by the Centers for Disease Control and Prevention (Fort Collins, CO). Negative controls consisted of sham extractions done with each set of 24 samples at the time of sample RNA preparation, and RT-PCR negative samples set up on each plate, which contained water instead of RNA. Samples producing an amplicon of the expected size (228 basepairs) were retested with a second independent RT-PCR. Samples giving amplicons of the predicted size in both independent reactions were scored as putative positive, and the identity of the amplicons in the putative positive samples were then confirmed by DNA sequencing.

*Culiceta melanura*, *Ae. vexans*, and *Cq. perturbans* were chosen for analysis based on their historical involvement in EEE virus transmission. *Culex erraticus* was included because of previous EEE virus isolations in Florida and South Carolina and its relatively high abundance at the site. *Uranotaenia sapphirina* was also analyzed because of the large reptile/amphibian population at the site and previous, though infrequent, reports of EEE virus isolations from this species. All pools of these species collected during 2001 were examined for EEE virus as follows: *Ae. vexans*, May 24–September 28; *Cs. melanura*, May 16–October 3; *Cx. erraticus*, May 16–October 3; *Cq. perturbans*, May 1–September 26; and *Ur. sapphirina*, May 24–October 3. Minimum infection rates (MIRs) were calculated for each species during the collection periods reported using a standard formula.\(^1\)

**RESULTS**

More than 23,000 mosquitoes representing 8 genera and 34 species were collected during a 21-week period (Table 1). Mosquito activity peaked in July and decreased gradually until the first week of October, at which time collections were terminated (Figure 1). A total of 20,448 mosquitoes were collected during 163 trap nights (average of 125 mosquitoes per trap night). *Culex erraticus* was the most abundant species taken by this method (11,507) and was present throughout the entire collection period. On average, 70 *Cx. erraticus* females were collected per trap night. Other mosquitoes routinely collected in light traps included *Anopheles crucians* (2,171), *Ae. vexans* (1,611), *Oc. infrirmatus* (1,051), and *Ur. sapphirina* (1,007) (Table 2).

A total of 2,669 female mosquitoes were taken in 541 vacuum collections from resting boxes and natural sites such as root mats and animal burrows, with an average of 4.9 mosquitoes per collection. During the 21-week period, 1,041 *Cx. erraticus* (109), and *Ae. vexans* (82) (Table 2). A total of 2,669 female mosquitoes were taken in 541 vacuum collections from resting boxes and natural sites such as root mats and animal burrows, with an average of 4.9 mosquitoes per collection. During the 21-week period, 1,041 *Cx. erraticus* (109), and *An. crucians* (82) (Table 2). Analysis for EEE virus was conducted from collections made from the second week of May through the first week of October (Figure 2). From a total of 477 pools screened for EEE virus using the nested RT-PCR, 54 were positive (11.3%), with 37 positive pools occurring in pools of *Cx. erraticus* (7.75%). *Culiceta melanura*, although the least abundant among the five species tested (Figure 3), had the highest MIR (20.2). The first EEE PCR-positive pool of that species was detected on May 30 and the last was detected on September 20. Two suspected bridge vectors (*Ae. vexans* and *Cq.*...
Mosquitoes collected in Tuskegee National Forest in central Alabama, May–October 2001

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of adults collected</th>
<th>% of total collected</th>
<th>No. of pools tested/No. of pools positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx. erraticus†</td>
<td>12,548</td>
<td>54</td>
<td>268/11,369</td>
</tr>
<tr>
<td>An. crucians</td>
<td>2,753</td>
<td>10</td>
<td>22/1,235</td>
</tr>
<tr>
<td>Ae. vexans‡</td>
<td>1,629</td>
<td>7</td>
<td>43/1,357</td>
</tr>
<tr>
<td>Ur. sapphirina‡</td>
<td>1,116</td>
<td>5</td>
<td>70/1,079</td>
</tr>
<tr>
<td>Oc. infirmatus</td>
<td>1,061</td>
<td>5</td>
<td>10/1,007</td>
</tr>
<tr>
<td>Cs. melanura</td>
<td>818</td>
<td>4</td>
<td>5/394</td>
</tr>
<tr>
<td>Ps. ferox</td>
<td>586</td>
<td>3</td>
<td>10/390</td>
</tr>
<tr>
<td>Cs. peccator</td>
<td>386</td>
<td>2</td>
<td>1/191</td>
</tr>
<tr>
<td>Cx. erraticus</td>
<td>320</td>
<td>1</td>
<td>33/303</td>
</tr>
<tr>
<td>Oc. atlanticus</td>
<td>298</td>
<td>1</td>
<td>7/291</td>
</tr>
<tr>
<td>An. quadrimaculatus</td>
<td>296</td>
<td>1</td>
<td>5/291</td>
</tr>
<tr>
<td>Cs. melanura†</td>
<td>273</td>
<td>1</td>
<td>63/248</td>
</tr>
<tr>
<td>Cx. salinarius</td>
<td>174</td>
<td>1</td>
<td>2/172</td>
</tr>
<tr>
<td>Cs. spp.</td>
<td>149</td>
<td>1</td>
<td>1/148</td>
</tr>
<tr>
<td>Oc. f. pallens</td>
<td>89</td>
<td>L‡</td>
<td>1/89</td>
</tr>
<tr>
<td>Oc. dupreii</td>
<td>48</td>
<td>L‡</td>
<td>1/48</td>
</tr>
<tr>
<td>Cx. nigricalpus</td>
<td>32</td>
<td>L‡</td>
<td>1/32</td>
</tr>
<tr>
<td>An. spp.</td>
<td>29</td>
<td>L‡</td>
<td>1/29</td>
</tr>
<tr>
<td>All others§</td>
<td>115</td>
<td>L‡</td>
<td>1/115</td>
</tr>
<tr>
<td>Total</td>
<td>23,046</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* Cx. = Culex; An. = Anopheles; Ae. = Aedes; Oc. = Ochlerotatus; Ur. = Uranotaenia; Ps. = Psorophora; Cs. = Culex; Coquillettidia; Cx. = Culiseta.
† Examined for eastern equine encephalitis virus.
‡ Less than 1% of the total collection.
§ All others in descending order of abundance: Oc. canadensis, Cs. restuans, Ps. howardi, Ps. spp., Ae. trietus, Oc. tibubiti, Ps. columbium, Oc. sticticus, Orthopodomyia signifera, An. perplexus, Ae. albopictus, Ps. ciliata, Ps. cyanescens, Ae. cinereus, Oc. grossbecki, An. barberi, Orthopodomyia spp., Ps. horsida, Ps. varipes.

Five most abundant mosquitoes collected by light-trap and vacuum collection in Tuskegee National Forest in central Alabama, 2001*

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of pools positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx. erraticus</td>
<td>11,507</td>
</tr>
<tr>
<td>Ae. vexans</td>
<td>2,171</td>
</tr>
<tr>
<td>Oc. infirmatus</td>
<td>1,051</td>
</tr>
<tr>
<td>Ur. sapphirina</td>
<td>1,007</td>
</tr>
<tr>
<td>An. crucians</td>
<td>82</td>
</tr>
</tbody>
</table>

* Cx. = Culex; An. = Anopheles; Ae. = Aedes; Oc. = Ochlerotatus; Ur. = Uranotaenia.

Eastern equine encephalomyelitis virus was transmitted in central Alabama during the late spring and summer of 2001. All five mosquito species analyzed by a nested RT-PCR yielded positive pools, indicating that each came in contact with vertebrate hosts infected with EEE virus. The nested RT-PCR assay used in this study is extremely sensitive, with a detection level that is roughly two logs lower than that for the single-step RT-PCR. Because it was demonstrated in a previous study that the single-step PCR is capable of detecting 3–4 logs of the 50% tissue culture infectious dose (TCID50).16 This suggests that the nested RT-PCR used here is capable of detecting 1–2 logs of TCID50 viral particles, a level of sensitivity that is comparable to that seen in similar nested assays developed for flaviviruses.17 In addition, alphaviruses produce a greater proportion of field-collected mosquito pools with high titers than do flaviviruses.15 This information, coupled with the extremely high sensitivity for EEE virus detection by the nested PCR, suggests it was possible that some of the virus-positive pools may have contained mosquitoes with developing infections that had not yet disseminated from the gut to the salivary glands and other parts of the body for further replication. Thus, mosquitoes with low viral titers that would have been missed using serologic and culture-based tests, which are generally 2 logs less sensitive than the test used here, may have been detected and included in the calculation of MIRs. Similarly, it is possible that the assay might have detected low titers of virus in mosquito species that are poor vectors. Despite the inability of the nested PCR to distinguish infected from infective mosquitoes, the data presented here may be used as a measure of the degree of contact of various mosquito species with EEE virus-infected vertebrate hosts, a variable that is of importance in determining the vectorial capacity of a given mosquito species.

Four of the five species tested have been shown in previous laboratory studies to be competent vectors for EEE virus.1,18 Culex erraticus was the most abundant mosquito collected during this study and yielded the highest number of virus-infected vertebrate hosts.
infected pools. Reports of EEE virus isolations from this species are relatively rare, with one and two positive pools, respectively, noted in two studies in Florida\textsuperscript{10,11} and a single positive pool in South Carolina.\textsuperscript{19} Culex\textit{erraticus} is a member of the subgenus \textit{Melanoconion}, a largely tropical group of mosquitoes.\textsuperscript{20} However, this species is the most common member of that subgenus in the United States and is distributed throughout the eastern United States, the upper Midwest, and westward to California.\textsuperscript{23} It is a competent vector of EEE virus\textsuperscript{18} and the habitat in which it occurs in the Tuskegee National Forest is generally similar to that described for Cx. (\textit{Mel.}) \textit{taeniopus} and Cx. (\textit{Mel.}) \textit{ferreri}, enzootic vectors of EEE virus in humid forests in Brazil, Trinidad, Panama,\textsuperscript{22–24} and Venezuela.\textsuperscript{25} 

\textit{Culex erraticus} is an opportunistic feeder\textsuperscript{26} and selects a wide variety of hosts, including cold-blooded vertebrates such as lizards.\textsuperscript{27} It can be an aggressive, persistent biter when humans enter forests\textsuperscript{28} or when abundant locally near inhabited areas.\textsuperscript{29} Females of this species are also capable of moving significant distances from larval locations. In a study conducted in central Florida, several marked Cx. \textit{erraticus} females were recaptured at distances of 1.4–2.2 kilometers from their release points.\textsuperscript{30} Therefore, it is possible that Cx. \textit{erraticus} may become important as a bridge vector of EEE virus in the southeastern United States as human populations continue to move closer to sylvatic sites, where populations of this mosquito have access to avian reservoirs. An earlier report\textsuperscript{26} indicated that 31% of the hosts selected for blood-feeding by Cx. \textit{erraticus} were from avian sources, thus confirming this species ability to become infected with EEE virus naturally, and suggesting that Cx. \textit{erraticus} might also serve as a secondary enzootic vector to Cs. \textit{melanura} in this region. Its relationship with other avian-associated encephalitic viruses such as Saint Louis encephalitis and West Nile virus is yet to be determined, although St. Louis encephalitis virus was isolated from Cx. \textit{erraticus} on several occasions during the 1976 epidemic in Memphis, Tennessee.\textsuperscript{31} 

Although Cs. \textit{melanura} was present in low numbers (Figure 1), it was infected with EEE virus throughout the summer. Thus, we assume that this mosquito is an important endemic vector in bottomland forests of central Alabama. The MIR of Cs. \textit{melanura} was also quite high when compared with other species in the Tuskegee focus and to previous reports for this species elsewhere. This can be correlated with collections made principally in the core area of the swamp and when populations were relatively low.\textsuperscript{32} In an earlier study in the Mobile Bay delta in southern Alabama, Stamm and others found that Cs. \textit{melanura} had a seasonal MIR of 2.8, but that as population size diminished in late summer, infection rates increased until approximately one of every 40 Cs. \textit{melanura} tested in October was infected.\textsuperscript{33} A later study near that same location found a similar pattern of high monthly infection ratios in late summer/fall, i.e., 1:75, 1:77, 1:98.\textsuperscript{34} Crans and Schulze\textsuperscript{7} also found an MIR of 12.93 in a Cs. \textit{melanura} population during a 1983 EEE equine epizootic in New Jersey and concluded that this mosquito functioned as an early endemic vector. 

Two other species, Ae. \textit{vexans} and Cq. \textit{perturbans}, were infected in early and late summer, respectively. The onset of EEE virus infection in the Cq. \textit{perturbans} populations in August (three positive pools, MIR = 9.9) was temporally associated with the first confirmed equine case in Macon County in mid-August. This species may have served as a bridge vec-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Reverse transcriptase–polymerase chain reaction (RT-PCR) pool screening of mosquitoes for eastern equine encephalomyelitis virus collected in Tuskegee National Forest in central Alabama. The lower half of the figure shows the number of pools screened from May to October 2001. The upper half shows the percentage of pools that were PCR+. Note the peaks seen during the second half of June and the first half of September.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Monthly distribution of five mosquito species at Tuskegee National Forest in central Alabama that were screened for eastern equine encephalomyelitis virus by reverse–transcriptase–polymerase chain reaction. Ae. = \textit{Aedes}; Cq. = \textit{Coquillettidia}; Cs. = \textit{Culiseta}; Cx. = \textit{Culex}; Ur. = \textit{Uranotaenia}.}
\end{figure}
tor because of its ability to fly relatively long distances and blood-feed on large mammals.  

Reports of *Ur. sapphirina* EEE virus-positive pools are very uncommon. The MIR for this species was the third highest of the five examined by the RT-PCR, with positive pools occurring from June to September. The blood-feeding behavior of *Ur. sapphirina* is largely unknown, but apparently does not include avian hosts. *Uranotaenia lowii*, a member of the same subgenus in the United States, has been observed feeding on amphibians. This observation suggests further investigation of *Ur. sapphirina* is needed to determine if amphibians are involved in the ecology of EEE virus in the central Alabama habitat and elsewhere in the region. Field and laboratory studies are currently underway to address this issue.

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