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ISOLATION OF POTOSI VIRUS FROM *AEDES ALBOPICTUS* IN NORTH CAROLINA

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ABSTRACT. A total of 4,169 adult mosquitoes were aspirated from 3 tire disposal sites in North Carolina for virus assays. *Aedes albopictus* was the dominant species, with a relative abundance of approximately 99%. Potosi virus was isolated from one pool of 68 female *Ae. albopictus*. Priorities for future Potosi virus research and the implications of the North Carolina isolate are discussed.

INTRODUCTION

During 1993, a survey for *Aedes albopictus* (Skuse) was conducted in tire disposal sites across North Carolina (Apperson, unpublished data). This survey revealed several disposal sites that contained larvae of both *Ae. albopictus* and *Culiseta melanura* (Coquillett). *Aedes albopictus* has been found naturally infected with eastern equine encephalitis (EEE) virus in Florida (Mitchell et al. 1992) and has been infected easily with this virus in *per oral* studies using bird reservoirs (Mitchell et al. 1993). Also, *Cs. melanura* is generally recognized as the primary enzootic vector of this virus in North American wild birds. The fact that both species occurred in the same tire disposal site suggests the possibility of enzootic and epizootic transmission of EEE virus near the site.

On March 28, 1994, a horse died in extreme eastern Union County, NC, and EEE virus was isolated and confirmed (No. 94-20034) by the U.S. Department of Agriculture's National Veterinary Services Laboratory, Ames, IA. The occurrence in North Carolina of an EEE case this early in the year is extremely unusual; however, there was a warm period in mid-March 1994. Furthermore, the month of March precedes the normal emergence of the enzootic vector, *Cs. melanura*, and the epizootic vector(s) such as *Coquillettidia perturbans* (Walker). Although Crans et al. (1994) have documented EEE virus survival in overwintering native North American birds, this extremely early case suggests that the virus was transmitted by an infected overwintering female mosquito such as *Anopheles punctipennis* (Say) or *Culex salinarius* Coq., which were plentiful one month later (unpublished data) at the horse case site, or through a nonmosquito vector. For more than 3 years before its death the horse continuously resided on a small farm located only 2 km from a tire site (state designation 124) that was found to be heavily infested with *Ae. albopictus* during the 1993 survey. Furthermore, in adjacent Anson County another 2 *Ae. albopictus*-infested tire sites (state designations 143, 144) were only 35 km (by air) from the horse farm in Union County. One of these sites (144) also contained *Cs. melanura* immatures. The proximity of the EEE virus activity and the horse case to the 3 *Ae. albopictus*-infested tire sites prompted us to initiate adult mosquito collections from the sites to attempt virus isolations for EEE, La Crosse (LAC), or previously undetected viruses in North Carolina, such as Potosi virus (POT).

MATERIALS AND METHODS

On July 27, 1994, mosquitoes were collected from the tire disposal site in Union County (124) and the 2 sites (143, 144) in Anson County (Fig. 1). Another trip was made to site 144 on August 10 to collect additional *Ae. albopictus* specimens for virus isolation attempts. Site 124 contains an estimated 8,000 tires and is located in Union County east of Marshville on the north side of route U.S. 74 just west of the Anson County line. Site 143 contains an estimated 5,000 tires and is located in southeastern Anson County, just northeast of Morven, which is on route U.S. 52. Site 144 contains an estimated 4,000 tires and is located in southeastern Anson County about 1 km northwest of Morven.

Mosquitoes were collected using 2 battery-operated backpack aspirators (Meyer et al. 1983) that were constructed at North Carolina State University. Mosquitoes were aspirated from the tires, while flying from kicked tires, and from around the collector and other persons working at the sites. Specimens were collected by the aspirator into pint-sized ice cream containers with a fine mesh screen on the bottom. Once several hundred specimens were seen in a container it

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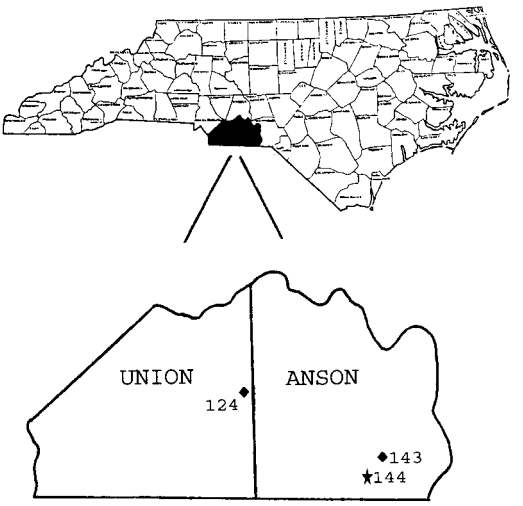

Fig. 1. Three North Carolina tire disposal sites where mosquitoes were collected for virus assay. A ★ indicates the site of the Potosi virus isolate.

Table 1. Relative abundance of mosquito species aspirated for virus assays from 3 North Carolina tire disposal sites in 1994.

Species	Number	Relative abundance (%)
Aedes albopictus	3,965	95.1
Aedes (*Stegomyia*) spp.[1]	165	4.0
Aedes canadensis	2	0.0
Aedes triseriatus	35	0.8
Aedes sp.	1	0.0
Psorophora sp.	1	0.0

[1] Poor or rubbed specimens—the possibility of *Aedes aegypti* (Linn.) could not be ruled out.

was covered with a lid and removed from the aspirator. A label noting the collection site and date was placed on the lid and the container was placed in an ice chest containing dry ice. Specimens from the 3 disposal sites were kept separate and transported to North Carolina State University in Raleigh, where they were placed on a chill table, sorted from other insects and arthropods, and separated from damaged or engorged specimens. The remaining specimens were placed in shell vials with appropriate labels on the outside and shipped on dry ice by overnight express to the Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, CO.

After arriving at CDC, the mosquitoes were sorted by species and sex and placed in pools of up to 100 mosquitoes. Mosquito pools were triturated in 2 ml of BA-1 diluent (0.2 M Tris, pH 8.0, 0.15 M NaCl, 1% BSA, 10 mg/liter phenol red, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml Fungizone) using cold mortars and pestles. Suspensions were centrifuged in Eppendorf tubes at 14,000 rpm for 2 min. Supernatants to be tested for virus by plaque assay in Vero cell culture were poured into 1-dram screw-cap vials and stored at −70°C until tested as previously described (Mitchell et al. 1987). Thirty-eight pools from the July 27 collections were placed in Vero cell culture on August 2, and an additional 13 pools from the August 10 collections were placed in Vero cell culture on August 16. Wells containing potential virus isolates were given to the Arbovirus Diseases Branch, CDC, for identification.

Harvested isolate plaques were inoculated into a 25-cm² flask of Vero cell cultures and observed daily until very early cytopathic effects (CPE) were noted. Infected cells were scraped from the surface of the flask and resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 5% fetal bovine serum. Twelve-well spot slides were prepared, dried, and fixed in cold acetone. The spot slides were tested in an indirect fluorescent antibody (IFA) assay (Wulff and Lange 1975) against a variety of NIH hyperimmune ascitic grouping fluids to antigenically group the viral isolate. In most instances, viral type-specific monoclonal antibodies also were used in the test in an attempt to definitively identify the isolate at that step. Viral isolates that had been antigenically grouped then were tested in a neutralization assay versus reference polyclonal immune reagents prepared against known individual virus members of the antigenic group in question. Homologous neutralization titers were predetermined for those reference reagents used in the identifying neutralization test.

RESULTS

A total of 4,169 adult mosquitoes were collected from the tire sites and identified at CDC as *Ae. albopictus, Aedes* (*Stegomyia*) spp., *Aedes canadensis* (Theobald), *Aedes triseriatus* (Say), *Aedes* sp., and *Psorophora* species. Table 1 shows the relative abundance of these species and the dominance of *Ae. albopictus* at the 3 tire sites. In fact, most of the specimens in the *Ae.* (*Stegomyia*) spp. category were probably *Ae. albopictus*. Adding these to the identified *Ae. albopictus* means that the Asian tiger mosquito probably comprised nearly 99% of the collected specimens.

The 4,169 adults were sorted into 51 pools and assayed for virus (Table 2). One pool (No. NC94-658) produced 35 and 42 plaques, respectively,

Table 2. Adult mosquitoes from 3 North Carolina tire disposal sites that were tested for virus in 1994.

Site	Date	Species	Sex	Specimens	Pools	Test results +	Test results −
124	Jul. 27	*Aedes albopictus*	♀	800	8		8
		Aedes albopictus	♂	937	10		10
		Aedes triseriatus	♀	26	1		1
143	Jul. 27	*Ae. albopictus*	♀	811	8		8
		Ae. albopictus	♂	165	2		2
		Aedes (*Stegomyia*) spp.	♀	114	1		1
		Aedes (*Stegomyia*) spp.	♂	51	1		1
		Ae. triseriatus	♀	4	1		1
144	Jul. 27	*Ae. albopictus*	♀	262	3	1[1]	2
		Ae. albopictus	♂	252	3		3
	Aug. 10	*Ae. albopictus*	♀	325	4		4
		Ae. albopictus	♂	368	4		4
		Ae. albopictus	mixed	45	1		1
		Aedes canadensis	♀	2	1		1
		Ae. triseriatus	♀	5	1		1
		Aedes sp.	♀	1	1		1
		Psorophora sp.	♀	1	1		1
				4,169	51	1[1]	50

[1] Potosi virus isolated from one pool (No. NC94-658) containing 68 females.

in the 2 wells by day 3 postinoculation. It was subsequently identified as Potosi (POT) virus, a Bunyawera serogroup virus in the genus *Bunyavirus* (family Bunyaviridae). The POT-positive pool consisted of 68 unfed females of *Ae. albopictus* collected on July 27, 1994, at site 144. The isolate from this pool has been preserved at CDC, Fort Collins, CO. The remaining 50 pools were negative for virus.

DISCUSSION

The tire site (144) producing the POT virus isolate was located just northwest of Morven, a small rural community in southeastern Anson County, approximately 6 km north of the South Carolina line. This site contained an estimated 4,000 tires (primarily automobile) that were piled in the open and under *Quercus* spp. (oak), *Pinus taeda* (loblolly pine), *Acer rubrum* (red maple), and *Liriodendron tulipifera* (yellow poplar) trees. The age of the trees was estimated to be between 15 and 25 years; however, the tires were a more recent addition. Many of the tires were in the shade for most of the daylight period. The area surrounding the immediate vicinity of site 144 was wooded except for a road and 3 residences, and conducive for wild birds and populations of small to large wild mammals, including white-tailed deer. Anson County has a large deer population (Kibler 1994). One residence in the area was a trailer housing 4–6 people that is immediately adjacent (15–20 m) to the tire site. Two other occupied houses were within 100 m of the tire site. Residents openly complained of the mosquito problem in the area.

During 1993, *Cs. melanura* immatures were collected in tires from this site. However, no adults of this species, or of *Culex* spp., were collected using the backpack aspirators. Also, *Ae. albopictus* was not nearly as abundant at this site as it was at sites 124 and 143 (Table 2). Although nearby residents complained about mosquitoes, there was no evidence that site 144 had been sprayed.

Since Potosi virus was first isolated and identified from 16 *Ae. albopictus* pools collected in 1989 from a tire disposal site in Potosi, MO (Francy et al. 1990, Mitchell et al. 1990), considerable additional work has been conducted in Washington County, MO (CDC, unpublished data). In follow-up studies to the 1989 work, intensive field investigations were conducted by CDC personnel in and around the Potosi, MO, area during the spring and summer of 1990. These resulted in the collection of large numbers of mosquitoes and vertebrates. However, there were no additional isolations of POT virus. The animal reservoir(s) for POT virus have not been defined, although the suspected role of deer in this virus cycle is currently under investigation at CDC, Ft. Collins (Robert McLean, CDC, un-

published data). Furthermore, only 2 strains of *Ae. albopictus* have been found competent as laboratory vectors of this virus (Lexington, KY, strain—Mitchell et al. 1990; Potosi, MO, strain—Heard et al. 1991). Although North Carolina strains of *Ae. albopictus* have not been tested for laboratory transmission of this virus, our data show that North Carolina *Ae. albopictus* can be infected with POT virus. Attempts to identify vertical transmission of this virus through the eggs of *Ae. albopictus* have been negative, which suggests that POT virus may be a previously undetected endemic virus in the United States and not a virus introduced with *Ae. albopictus* (Mitchell et al. 1990, Heard et al. 1991).

There is no evidence that Potosi virus causes subclinical or clinical illness in humans. In fact, Mitchell (1991) referenced a serosurvey of people in the Potosi, MO, area that showed no evidence of human exposure to this virus. During the present study a serosurvey of the residents adjacent to site 144 was not conducted. However, we believe that the rapidly expanding populations of *Ae. albopictus* in many states, the ability of this species to be infected by this virus, and its close proximity to and ease of feeding on humans suggests that humans are probably being exposed to POT virus.

A number of aspects of the POT virus cycle need to be investigated. Studies are needed to determine antibody response and the effects of POT virus in humans and in domestic pets, livestock, and poultry. Research is needed on potential reservoirs, including rodents, small mammals, birds, and domestic pets. More virus isolation studies are needed to expand knowledge of the distribution of this virus, and to implicate the native North American mosquito species that are involved in the maintenance of the POT cycle. At least one other species, besides *Ae. albopictus,* has been found positive for POT virus. Mosquito collections in South Carolina in 1991 resulted in the isolation of POT virus from a pool of *Psorophora columbiae* (Dyar and Knab) (CDC and Stan Cope, unpublished data). If POT virus is like other Bunyamwera serogroup viruses in the United States (Tensaw, Cache Valley), a number of mosquito species in several genera will be involved in the maintenance of its natural cycle (Berge 1975).

The isolation of Potosi virus from *Ae. albopictus* in North Carolina has at least 2 significant implications: 1) POT virus has a much wider distribution in the United States than previously indicated in publications, and 2) *Ae. albopictus* may be much more involved in the enzootic transmission cycle of POT virus than previously suspected. *Aedes albopictus* may become a maintenance vector of this virus in certain areas, and additional isolations of POT virus from *Ae. albopictus* may become more frequent.

This virus isolation was a major factor in gaining the immediate attention of local public health officials. Before the virus isolation, the 3 tire disposal sites had low priority ratings for cleanup. When the North Carolina Public Health Pest Management Section was notified of a virus isolate (then unidentified) on August 5, the Anson County Environmental Health personnel were notified. They immediately visited site 144 and made plans for its removal. The afternoon of August 10, after we made our last mosquito collections, Anson County personnel sprayed site 144 with a pesticide. One week later the tires at this site were loaded onto trucks and shipped to a buyer that sold chipped rubber. Shortly thereafter, the tires at site 143 were also removed.

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