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ISOLATIONS OF POTOSI VIRUS FROM MOSQUITOES COLLECTED IN THE UNITED STATES, 1989–94

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ABSTRACT. Potosi (POT) virus, a recently characterized Bunyamwera serogroup virus, was discovered when it was isolated from *Aedes albopictus* collected at a waste-tire site in Potosi, Washington County, Missouri, during 1989. During the following year, POT virus was not isolated from 39,048 mosquitoes, including 17,519 *Ae. albopictus,* collected in Washington County. In 1991, mosquito collections from South Carolina, Ohio, and Michigan yielded 8 strains of POT virus: 6 from *Coquillettidia perturbans* and one each from *Culex restuans* and *Psorophora columbiae.* Additional collections of *Ae. albopictus* from several states during 1990–93 failed to yield further isolates of POT virus. In 1994, POT virus was isolated from *Ae. albopictus* and *Anopheles punctipennis* in North Carolina and from *Ae. albopictus* in Illinois. These represent the first virus isolations of any type from *Ae. albopictus* in those states. Thus far, POT virus has been isolated from 5 mosquito species in different genera in 6 states. The known geographic range of POT virus, based on virus isolations, has been extended from Missouri to the upper Midwest and the Atlantic seaboard. Potential vector relationships and possible transmission cycles of POT virus are discussed.

INTRODUCTION

Potosi (POT) virus was first isolated from *Aedes albopictus* (Skuse) collected in Potosi, Washington County, Missouri, in August and September 1989 (Francy et al. 1990, Mitchell et al. 1990). This previously uncharacterized Bunyamwera (BUN) serogroup virus has since been shown to have a wider distribution. Our laboratory has isolated POT virus from *Ae. albopictus* collected in North Carolina (Harrison et al. 1995) and Illinois (Centers for Disease Control and Prevention [CDC], unpublished data) and from other mosquito species collected in Ohio (Nasci et al. 1993), North Carolina, South Carolina, and Michigan (CDC, unpublished data). Also, McLean et al. (1995) found neutralizing antibody against POT virus in deer sera from Missouri, Iowa, Arkansas, and Colorado. Our previously unpublished findings concerning virus isolations from mosquitoes, plus the results of intensive follow-up entomologic studies in Washington County, Missouri, during the summer of 1990, are reported here to add to the limited body of knowledge concerning the ecology and distribution of POT virus.

MATERIALS AND METHODS

Mosquitoes were collected in Washington County, Missouri, during 3 periods in 1990, the year following that in which the original isolations were made. Collections were made during June 11–16, July 27–August 1, and September 7–13, 1990, at 22 sites, although collections were concentrated in and around the waste-tire site that had previously yielded POT-infected *Ae. albopictus.* Mosquitoes were collected by CDC light traps baited with dry ice, by duplex cone traps (Freier and Francy 1991) baited with dry ice, and by using small aerial sweep nets and mechanical aspirators to collect specimens attracted to the investigators. To optimize collection of *Ae. albopictus,* the CDC light traps were baited and set in the afternoon 3–4 h before sunset and were retrieved the following morning 1–2 h after sunrise. Duplex cone traps, designed specifically for collecting *Ae. albopictus,* were used only at the waste-tire site. These were baited early in the morning and operated throughout the daylight hours.

Some mosquito collections, or supernatants from triturated mosquito pools, were submitted to our laboratory for testing by vector control personnel conducting arbovirus surveillance, sometimes in the course of outbreak investigations. Other collections were submitted by collaborators as part of our ongoing effort to test *Ae. albopictus* from different areas of the United States for field-acquired arbovirus infections (Mitchell et al. 1992). Collection methods for the mosquitoes from Ohio (Nasci et al. 1993) and North Carolina (Harrison et al. 1995) have been described. Mosquitoes from South Carolina and Michigan were collected by CDC light traps baited with dry ice. The mosquitoes from Illinois were collected by CDC light traps baited with dry ice and by gravid traps (Haramis et al., unpublished data).

Specimens from Michigan, collected in the course of eastern equine encephalitis (EEE) surveillance activities, were identified and sent to us as supernatants from triturated mosquito pools. In all other cases, whole mosquitoes were placed in stoppered shell vials, transported on dry ice to the laboratory in Fort Collins, and stored in mechanical freezers at −70°C for varying lengths of time before further processing and testing. Mosquitoes were thawed and identified with the aid of a dissecting microscope placed on a chill table at about 4°C. Mosquitoes were pooled by species in groups of up to 100 specimens. Only female mosquitoes were routinely tested except in the case of *Ae. albopictus,* where both sexes were tested. Mosquito pools were triturated in 2 ml of BA-1 diluent (1 × M199 containing Hanks' balanced salt solution, 0.05 M Tris (pH 7.6), 1% bovine serum albumin, 0.35 g/liter sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml Fungizone, and 10 mg/liter phenol red) by using cold mortars and pestles. Suspensions were centrifuged in Eppendorf tubes at 14,000 rpm for 2 min. Supernatants were poured into 1-dram screw-cap vials and stored at −70°C until tested.

Specimens were tested for virus in Vero cell culture grown in 6-well plates. Specimens were inoculated in 0.1-ml quantities in 2 wells each and adsorbed for 1 h at 37°C. Then the cells were overlaid with 1% Noble agar in M199 + 2% fetal bovine serum, 2.0 g $NaHCO_3$, 150 g/liter of DEAE-dextran, and 1:40,000 neutral red plus 100 units/ml penicillin, 100 µg/ml streptomycin, 250 µg gentamicin/ml, and 4.5 µg Fungizone/ml. Cell cultures were incubated at 37°C and examined for 10 days for plaques.

Virus-positive cell cultures were harvested in 2 ml of BA-1 and frozen at −70°C until passed into fluid cultures of Vero cells in 25-cm^2 flasks. When early cytopathic effects were noted, infected cells were scraped from the surface of the flask and resuspended in phosphate-buffered saline (pH 7.4) containing 5% fetal bovine serum. Twelve-well spot slides were prepared, air-dried, and fixed in cold acetone. These were tested in an indirect fluorescent antibody assay (Wulff and Lange 1975) against a battery of National Institutes of Health- and CDC-hyperimmune ascitic grouping fluids. Usually, viral type-specific monoclonal antibodies against common or suspected viruses also were included in the test to definitively identify isolates at this stage. Otherwise, antigenically grouped viral isolates were tested by neutralization (N) assay in Vero cell culture against reference polyclonal immune reagents prepared against specific members of the antigenic group. Homologous N titers were predetermined for reference reagents used in the identifying N tests.

Additionally, hyperimmune (4 injections) antisera were prepared for 2 representative Cache Valley (CV) and one representative POT virus isolates from Michigan and one POT virus isolate from South Carolina. All of these isolates, and antibodies to them, were cross-tested in N tests using prototype POT and CV viruses and their respective antibodies. This was done after using antibodies, with predetermined homologous N titers, to the 6 North American BUN serogroup viruses for preliminary identification.

RESULTS

Missouri: During follow-up studies in Washington County, Missouri, in 1990, we collected and tested 27,948 mosquitoes in 1,301 pools (Table 1). Potosi virus was not isolated from these mosquitoes; however, 3 strains of other viruses were recovered. Flanders (FLA) virus (strain MO90-1409) was isolated from *Culex restuans* Theobald collected on July 28, 1990. Two virus strains (MO90-2219 and MO90-2320) isolated from *Culex erraticus* (Dyar and Knab) collected on September 10 and 11, 1990, reacted with a rhabdovirus grouping fluid in the IFA assay; however, these strains have not been definitively identified. Our 1990 Washington County collections also contained 124 *Culicoides* spp. and 12 Simuliidae. These were tested in 15 and 4 pools, respectively, with negative results.

Included in the above collections were 9,301 *Ae. albopictus* consisting of 9,109 females and 192 males. Temporally, 63.4% of these were collected during September 7–13, 1990, approximately the same period during which POT- virus-infected specimens had been collected the previous year at the waste-tire site. During September 1990, 91% of our *Ae. albopictus* collection came from the same waste-tire site. We also tested mosquitoes collected by M. L. Niebylski at the waste-tire site during 1990. These consisted of 11,100 specimens, including 4,515 *Ae. albopictus* females and 3,703 males (Niebylski 1992[1]). All 11,100 specimens were tested in 229 pools in Vero cell culture with negative results.

South Carolina: During the summer of 1991, the U.S. Navy, Disease Vector Ecology and Control Center (DVECC), Jacksonville, Florida, collected mosquitoes in Florida and South Carolina for arboviral encephalitis surveillance (Cope et al., unpublished data). We assisted their efforts by testing 2,738 mosquitoes from Florida

[1] Niebylski, M. L. 1992. Bionomics of *Aedes albopictus* (Skuse) in Potosi, Missouri. Ph.D. dissertation. University of Notre Dame, Notre Dame, IN.

Table 1. Mosquitoes collected in Washington County, Missouri, by CDC personnel during 1990 and tested for virus.

Species	No. specimens	No. pools	No. pools positive
Aedes albopictus	9,301	248	
Ae. canadensis	66	9	
Ae. epactius	1,463	64	
Ae. vexans	2,418	107	
Ae. triseriatus-hendersoni	5,343	177	
Ae. spp.	353	32	
Anopheles barberi	2	2	
An. crucians	80	19	
An. punctipennis	512	70	
An. quadrimaculatus	689	73	
An. spp.	15	3	
Coquillettidia perturbans	476	57	
Culex erraticus	4,335	104	2[1]
Cx. quinquefasciatus	277	35	
Cx. restuans	250	25	1[2]
Cx. salinarius	422	51	
Cx. territans	21	13	
Cx. (Mel.) spp.	527	45	
Cx. (Cux.) spp.	1,064	94	
Cx. spp.	10	3	
Culiseta inornata	3	1	
Cs. melanura	36	14	
Orthopodomyia signifera	2	1	
Or. spp.	3	1	
Psorophora ciliata	5	3	
Ps. columbiae	15	3	
Ps. cyanescens	23	8	
Ps. ferox	208	29	
Ps. howardii	15	5	
Ps. spp.	1	1	
Uranotaenia sapphirina	13	4	
Total	27,948	1,301	

[1] Probable *Rhabdovirus*, identification pending.
[2] Flanders virus.

and 8,531 from South Carolina by plaque assay in Vero cell culture. Coincidental to the arboviral encephalitis surveillance objectives, we isolated a strain of POT virus (SC91-2202) from a pool of *Psorophora columbiae* (Dyar and Knab) collected on July 8, 1991, at the Naval Weapons Station in Charleston County (Table 2).

Ohio: An epizootic of EEE occurred in Wayne and Holmes counties, Ohio, during August and September, 1991 (Nasci et al. 1993). Five strains of a BUN serogroup virus were isolated from pools of *Coquillettidia perturbans* (Walker) collected on July 23, 1991, from 3 CDC light traps baited with dry ice. Trap locations were in Wayne County within 2 km of the Killbuck marsh basin. The 5 viral strains (OH91-3832, -4134, -4138, -4240, and -4241) were subsequently identified by N test as POT virus (Table 2).

Michigan: During the summer of 1991, mosquitoes collected in southern Michigan as part of an EEE investigation were sent to us for viral assay (Walker et al., unpublished data). During the following fall and winter, we tested 4,372 mosquitoes in an attempt to determine which species were involved in transmitting EEE virus during the epizootic. Seven virus strains were isolated and identified. Two of these, MI92-62 from *Cq. perturbans* and MI92-68 from *Cx. restuans,* were POT virus isolated from pools of mosquitoes collected on August 26, 1991, in Jackson County, Michigan (Table 2).

Prompted by the finding of POT virus in *Cq. perturbans* from both Michigan and Ohio, we

Table 2. Potosi virus strains isolated from mosquitoes collected in 6 states during 1989–94.

State	County	Species	Date collected
Missouri	Washington	*Aedes albopictus*	Aug.–Sep. 1989
	Washington	*Ae. albopictus*	Aug. 1989
South Carolina	Charleston	*Psorophora columbiae*	Jul. 1991
Ohio	Wayne	*Coquillettidia perturbans*	Jul. 1991
Michigan	Jackson	*Cq. perturbans*	Aug. 1991
	Jackson	*Culex restuans*	Aug. 1991
North Carolina	Anson	*Ae. albopictus*	Jul. 1994
	Transylvania	*Anopheles punctipennis*	Jul. 1994
Illinois	Jasper	*Ae. albopictus*	Sep. 1994

[1] Minimum infection rate/1,000 tested for months indicated.
[2] Referred to as BUN serogroup virus by Nasci et al. (1993).

reidentified 18 strains of BUN serogroup virus isolated from this species in Michigan in 1981 and identified as CV virus by Calisher et al. (1986) prior to the discovery and characterization of POT virus. Identification of the virus strains was confirmed as CV virus in all cases and no further strain of POT virus was found.

North Carolina: We tested 4,169 mosquitoes, mainly *Ae. albopictus,* collected from waste-tire sites in Union and Anson counties, North Carolina, during 1994. One pool of *Ae. albopictus* collected in Anson County on July 27, 1994, yielded a strain of POT virus (NC94-658) (Harrison et al. 1995) (Table 2). Also, during a survey for California encephalitis vectors, a strain of POT virus (NC95-227) was isolated from a pool of 48 *Anopheles punctipennis* (Say) collected in Transylvania County on July 20, 1994 (CDC and North Carolina Department of Environmental Health and Natural Resources [NCDEHNR], unpublished data). A total of 446 specimens of *An. punctipennis* was collected and tested.

Illinois: During 1994, the Division of Environmental Health, Illinois Department of Public Health, submitted mosquitoes collected at waste-tire sites to us for viral assay. Collections from Jasper County, Illinois, contained 2,275 *Ae. albopictus,* 500 *Aedes triseriatus* (Say), 440 *Aedes atropalpus* (Coquillett), and 28 additional specimens in 7 other taxa (CDC, unpublished data). Two strains of POT virus (IL94-1899 and IL94-1921) were isolated from pools of *Ae. albopictus* collected on September 22, 1994, at the waste-tire site in Jasper County (Table 2).

Assuming that *Ae. albopictus* may be a vector

Table 3. *Aedes albopictus* tested for virus by CDC during 1987–94.

State	1987	1988	1989	1990	1991	1992
Alabama						
Arkansas					1,234	
Florida					9,904[1]	5,581
Illinois	351	4,372	361			1,811
Indiana	429	15	72			
Louisiana	953			1,693	78	
Mississippi						
Missouri			13,720[4]	17,519	750	
North Carolina						
Ohio		3				
South Carolina					3	69
Tennessee	2	1,769				
Totals	1,735	6,159	14,153	19,212	11,969	7,461

[1] Fourteen strains of EEE virus and one strain of KEY virus isolated (Mitchell et al 1992).
[2] One strain of KEY virus isolated (CDC, unpublished data).
[3] Two strains of POT virus isolated (CDC, unpublished data).
[4] Sixteen strains of POT virus isolated (Francy et al. 1990, Mitchell et al. 1990).
[5] One strain of POT virus isolated (Harrison et al. 1995).

Table 2. Extended.

M.I.R.[1]	No. strains isolated	Reference
0.4–8.9	10	Francy et al. (1990)
5.8	6	Mitchell et al. (1990)
0.7	1	CDC, unpublished data
0.5	5[2]	Nasci et al. (1993)
2.0	1	CDC, unpublished data
3.9	1	CDC, unpublished data
1.9	1	Harrison et al. (1995)
2.2	1	CDC and NCDEHNR, unpublished data
0.9	2	CDC, unpublished data

of POT virus in nature, we examined all of our data concerning the testing of field-collected specimens of this species from the United States during 1987–94. During this period we tested 77,223 specimens from 12 states (Table 3). As indicated above, POT virus was isolated from *Ae. albopictus* collected in Missouri in 1989 (Francy et al. 1990, Mitchell et al. 1990) and North Carolina (Harrison et al. 1995) and Illinois (CDC, unpublished data) in 1994. Other viruses have been isolated from *Ae. albopictus* as well. We isolated EEE and Keystone (KEY) viruses from *Ae. albopictus* collected in Florida in 1991, and the Texas State Department of Health isolated Tensaw virus from *Ae. albopictus* collected in Texas in 1991 (Mitchell et al. 1992). More recently, we isolated another strain of KEY virus (FL93-2972) from a pool of *Ae. albopictus* collected in Orange County, Florida, on August 4, 1993 (CDC, unpublished data).

Table 3. Extended.

1993	1994	Totals
	46	46
		1,234
3,377[2]		18,862
	2,833[3]	9,728
		516
	1,086	3,810
	128	128
3,124	684	35,797
	3,966[5]	3,966
979	311	1,293
		72
		1,771
7,480	9,054	77,223

DISCUSSION

Despite testing 39,048 mosquitoes, including 17,519 *Ae. albopictus,* from Washington County, Missouri, where POT virus had been discovered the previous year, that virus was not isolated from our 1990 collections. In 1991, mosquito collections from South Carolina, Ohio, and Michigan yielded 8 strains of POT virus, 6 from *Cq. perturbans* and one each from *Cx. restuans* and *Ps. columbiae.* Additional collections of *Ae. albopictus* from several states during 1990–93 failed to yield further isolates of POT virus. However, in 1994, POT virus was isolated from *Ae. albopictus* in North Carolina and Illinois, representing the first virus isolations of any type from this species in those states, and POT virus was isolated from *An. punctipennis* in North Carolina.

These disparate findings of POT virus in 5 mosquito species in 5 different genera, extension of the known geographic range of POT virus from Missouri to the upper Midwest and the Atlantic seaboard, and our failure to isolate POT virus from mosquitoes collected at the original collection site in the year following its discovery indicate that much remains to be learned about the transmission cycle and endemicity of this virus. Thus far, *Ae. albopictus* is the only arthropod species shown to be a competent experimental vector; no other species has been tested (Mitchell et al. 1990, Heard et al. 1991). These studies on vector competence include limited experimental data examining the question of whether vertical transmission of POT virus by *Ae. albopictus* occurs. The results indicate that vertical transmission of POT virus by *Ae. albopictus* is unlikely to occur with high frequency, if at all.

The finding of POT virus in *Ae. albopictus* in Missouri, North Carolina, and Illinois, coupled with the demonstrated competence of *Ae. albopictus* as an experimental vector, suggests that this mosquito is transmitting the virus in nature. Certainly, *Ae. albopictus* must be feeding frequently on the vertebrate host(s) that become viremic following infection with POT virus. Savage et al. (1993) and Niebylski et al. (1994) showed that populations of *Ae. albopictus* in the United States have catholic feeding habits. Collectively, these researchers found that 82% of 429 *Ae. albopictus* collected in Missouri, Florida, Indiana, Illinois, and Louisiana had fed on mammals. The remainder had fed on birds (8%), other taxa (1%) including reptiles, or hosts that could not be determined (9%) because of insufficient quantities of blood or for other reasons. Among a sample of 172 *Ae. albopictus* collected at the Potosi waste-tire site during August–Sep-

tember 1989 and June–September 1990, 9 mammalian taxa were identified as hosts (Savage et al. 1993). The 3 hosts fed upon most frequently at the waste-tire site where infected *Ae. albopictus* were originally collected were rabbit (15.7%), deer (9.3%), and dog (8.7%). A high percentage (21.8%) of the mammal-positive samples could not be identified to a lower taxon.

Among other mosquito species found to be infected with POT virus, *Cq. perturbans* is considered to be a general feeder, but in many situations it feeds mainly on mammals, including deer, other ruminants, and rabbit (Edman 1971, Tempelis 1975). *Psorophora columbiae* and *An. punctipennis* are mammalophilic (Edman 1971, Tempelis 1975). Tempelis (1975) found that over 70% (n = 500) of *Cx. restuans* from Minnesota and Illinois had fed on passerine birds; however, this species also feeds on mammals (Murphey et al. 1967). McLean et al. (1996) found N antibody against POT virus in deer from Missouri, Iowa, Arkansas, and Colorado. Thus far, available evidence points to a POT virus transmission cycle involving deer, *Ae. albopictus,* and other mammalophilic mosquitoes. Critical studies on the viremic response of deer to POT virus infection currently are underway at the University of Notre Dame (R. G. McLean, personal communication).

In North America, BUN serogroup viruses have been isolated only from mosquitoes, with the exception of Lokern (LOK) and Main Drain (MD) viruses, which also have been isolated from *Culicoides* species (Karabatsos 1985). Both LOK and MD viruses replicate in hares, and MD virus also replicates in rabbits as does CV virus (Calisher et al. 1986). Since the discovery of POT virus, relatively few *Culicoides* have been tested from areas where the virus is known to occur. Although the weight of evidence suggests that POT virus is vectored by mammalophilic mosquito species, the possible involvement of other arthropods such as *Culicoides* cannot be ruled out. Further studies on the role of *Ae. albopictus* in the transmission cycle of POT virus, and other arboviruses with which it has been associated in nature in the United States or for which it has been shown to be a competent experimental vector (Mitchell 1991), are clearly warranted. Vector competence studies should also be conducted with the other mosquito species from which POT virus has been recovered in order to better assess their roles as vectors.

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