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DETECTION OF ST. LOUIS ENCEPHALITIS AND WESTERN EQUINE ENCEPHALOMYELITIS RNA IN MOSQUITOES TESTED WITHOUT MAINTENANCE OF A COLD CHAIN

LAURA D. KRAMER,[1] ROBERT E. CHILES,[2] THUY D. DO[2] AND HESHAAM M. FALLAH[2]

ABSTRACT. Western equine encephalomyelitis and St. Louis encephalitis viral RNA can be detected 20 days after death of infected *Culex tarsalis* in the absence of a cold chain. Viral RNA was detected with the reverse transcription–polymerase chain reaction in mosquitoes infected either parenterally or perorally in the laboratory and then killed and held for up to 20 days at 27°C. Cell culture assay and in situ enzyme immunoassay did not detect infectious virus in the same mosquitoes.

KEY WORDS Arbovirus, St. Louis encephalitis, western equine encephalomyelitis, *Culex tarsalis,* surveillance

INTRODUCTION

Mosquito-borne arboviruses represent a public health threat in the USA through sporadic viral encephalitis outbreaks. Accurate and timely detection of these viruses in field-collected mosquito vectors is a vital component of surveillance and control programs. In California, current surveillance activities rely on 2 techniques: sentinel chicken flocks are bled periodically to monitor St. Louis encephalitis virus (SLE) and western equine encephalomyelitis virus (WEE) seroconversion rates, and vector infection is monitored in mosquitoes collected in Centers for Disease Control (CDC)-style (CO_2-baited) traps. Viral infection rates are based on enzyme immunoassay (EIA) identification after viral amplification in Vero cell culture. Mosquitoes are collected alive in dry ice–baited CDC-style traps, sorted, and sent frozen to a central laboratory for viral detection (Reeves et al. 1990). Mosquitoes collected and killed in New Jersey light traps (NJLT) are enumerated to provide population estimates.

Recent development of rapid, sensitive reverse transcription–polymerase chain reaction (RT-PCR) assays to detect arboviral RNA in mosquito pools (Howe et al. 1992, Sellner et al. 1992, Chang et al. 1994, Armstrong et al. 1995) is a possible approach that would incorporate molecular methods into current surveillance activities. If a cold chain, although preferable, was not required to maintain viral RNA, viral surveillance would no longer absolutely require the use of dry ice or −80°C freezers, facilitating studies in locations without ready access to dry ice. In addition, after mosquitoes killed in NJLT are identified and counted, they could be retained for detection of viral RNA, thereby providing additional information on viral infection.

[1] The Arbovirus Laboratories, Wadsworth Center, New York State Department of Health, 5668 State Farm Road, Slingerlands, NY 12159.

[2] Center for Vector-borne Disease Research, University of California, Davis, Davis, CA 95616.

MATERIALS AND METHODS

Mosquito infection: Colonized *Culex tarsalis* Coquillett (high–virus-producing strain genetically selected to replicate WEE to high titers) were infected by intrathoracic inoculation or per os by pledget feeding (Kramer et al. 1998) with WEE (BFS 1703) or SLE (BFS 1750). In experiment 1, inoculated mosquitoes were incubated for 5 days and engorged females were incubated for 10 days at 27°C, then killed with an overdose of triethylamine (Kramer et al. 1990). In experiment 2, perorally infected mosquitoes were killed with No-Pest Strips® (NPS; 19.2% 2,2-dichlorovinyl dimethyl phosphate, Texize, Greeenville, SC), which currently are being used to rapidly kill collections in NJLT. Some infected mosquitoes were killed and frozen immediately and tested to determine infection rates in each experiment. The remaining mosquitoes were killed and held at 27°C for 2–30 days after death, then frozen individually and placed at −80°C until assayed for viral RNA and infectious virus.

Virus and RNA assay: One experimentally infected *Cx. tarsalis* was added to 49 uninfected *Cx. tarsalis* to make pools of 50 individuals for testing. This is the pool size used routinely in mosquito surveillance programs. Pools were triturated in 2 ml of phosphate-buffered saline containing 20% fetal bovine serum plus antibiotics (100 U penicillin, 100 U streptomycin, and 200 U nystatin) with a mixer mill (Spex CertiPrep, Metuchen, New Jersey). Data from plaque assays on Vero cell culture (Reisen et al. 1997, Kramer et al. 1998) and in situ EIAs (Graham et al. 1986) were used to compare the level of virus detection with the RT-PCR results.

Unlike EIAs, which detect antibody, the in situ EIA is dependent on viral amplification in cell culture. In this assay, mosquito pool suspensions are inoculated onto 96-well plates and allowed to incubate for 4–7 days. This incubation period is followed by fixation in cold methanol and plates are washed and incubated with hyperimmune mouse ascitic fluids (polyclonal antibody). This is followed by a secondary anti-mouse conjugate and the utilization of a substrate 3,3′-diaminobenzidine te-

Table 1. Detection of Western equine encephalomyelitis virus in perorally infected mosquitoes maintained without a cold chain after death.

		Number of pools positive/number of pools tested (% positive)[1]		
Mode of death	Day after death	RT-PCR	Plaque assay	In situ EIA
Triethylamine	0	10/11 (91)	8/11 (73)	9/11 (82)
	3	3/3 (100)	0/3 (0)	0/3 (0)
	6	3/3 (100)	0/3 (0)	0/3 (0)
	8	2/3 (67)	0/3 (0)	0/3 (0)
	10	3/3 (100)	0/3 (0)	0/3 (0)
	13	2/3 (67)	0/3 (0)	0/3 (0)
	15	0/2 (0)	0/3 (0)	0/3 (0)
No Pest Strips®[2]	0	8/10 (80)	4/10 (40)	4/10 (40)
	5	6/10 (60)	0/10 (0)	0/10 (0)
	9	4/10 (40)	0/10 (0)	0/10 (0)
	14	8/10 (80)	0/10 (0)	0/10 (0)
	16	3/8 (38)	0/10 (0)	0/10 (0)
	20	4/8 (50)	0/10 (0)	0/10 (0)

[1] Pools of 50 mosquitoes each, including 1 infected mosquito. RT-PCR, reverse transcription–polymerase chain reaction; EIA, enzyme immunoassay.
[2] 19.2% 2,2-dichlorovinyl dimethyl phosphate.

trahydrochloride, which renders the antigen (bound viral-infected tissue) and the antibody reaction visible. The positive reacting product is read in a light microscope and has distinct areas of brownish red stain that is indicative of a positive reaction.

Reverse transcription–polymerase chain reaction: Viral RNA was extracted from triturated mosquito pools with Trizol (Gibco BRL, Rockville, MD) according to the manufacturer's protocol, modified by the addition of silica (Harris et al. 1998). Reverse transcription was conducted at 42°C for 50 min with random hexanucleotides (Pharmacia, Piscataway, NJ) and Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's protocol with 9 μl total RNA. Viral cDNA was amplified in an Amplitron II thermocycler (Thermolyne, Dubuque, IA) with primers from the envelope region of the viral genomes and Taq polymerase (Qiagen, Valencia, CA). The oligonucleotide primers, synthesized by Genemed Biotechnologies (San Francisco, CA), were WEE forward, 2471V (5′ TGT TAT TCT GTT CCG CTG CTT T 3′) and reverse, 3055C (5′ CCC CTT TCT GAT GAC GAC CTT 3′) primers, yielding a product of 584 bases; and SLE forward, 1916V (5′ AGG GCA CGG GAC AGT GAT T 3′) and reverse, 2272C (5′ CTG AAC GCT CCT CCG AAA ACT T 3′) primers, yielding a product of 356 bases. Amplification consisted of a 2-min denaturation step at 95°C, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, with a final extension of 5 min at 72°C.

RESULTS

Western equine encephalomyelitis and SLE RNA were detected by RT-PCR in *Cx. tarsalis* intrathoracically inoculated with 10 plaque-forming units (PFU) of virus per mosquito, then held 1–12 days at 27°C after death (data not shown). After peroral infection with 10^4 PFU per mosquito, WEE RNA was detected in *Cx. tarsalis* for 13 days in experiment 1, in which mosquitoes were killed with triethylamine, and for 20 days in experiment 2, in which mosquitoes were killed with NPS (Table 1). St. Louis encephalitis RNA was detected by RT-PCR 15 and 9 days after mosquitoes were killed with triethylamine and NPS, respectively (Table 2). Infectious virus assays, both plaque assay and in situ EIA, detected virus on the day the mosquitoes were killed and not at 3–20 days after death, even though the same pools were positive by RT-PCR. The titer of virus in the mosquitoes at the time of death was 5.0 $\log_{10}$ PFU for WEE, and 5.3 $\log_{10}$ PFU for SLE.

DISCUSSION

LaCrosse viral RNA was shown to remain stable at room temperature for 7 days (Wasieloski et al. 1994) and dengue viral RNA can be detected in infected mosquitoes maintained at 27°C for at least 24 h after death (Harris et al. 1998). The ability to test mosquitoes found dead in CDC traps and mosquitoes captured in NJLT (which are killed in the process of trapping) increases the potential number of individuals available for testing in a surveillance program. In addition, the ability to test mosquitoes for viral RNA after they have been held at ambient temperature allows for shipment without dry ice. This method also could be useful in field situations where dry ice is not available, as long as the investigator is searching for a particular virus or viruses, because PCRs can be made specific for the test virus. Variants with nucleotide alterations at the 3′ end of the sequence where the primers anneal might be missed. Such a procedure would not be useful if an infectious isolate is required for further study.

The use of NPS adversely affected the detection of virus. In the WEE study, the percent of mosqui-

Table 2. Detection of St. Louis encephalitis virus in perorally infected mosquitoes maintained without a cold chain after death.

Mode of death	Day after death	Number of pools positive/number of pools tested (% positive)[1]		
		RT-PCR	Plaque assay	In situ EIA
Triethylamine	0	6/14 (43)	6/14 (43)	6/14 (43)
	3	1/4 (25)	0/4 (0)	0/4 (0)
	6	2/4 (50)	0/4 (0)	0/4 (0)
	8	2/4 (50)	0/4 (0)	0/4 (0)
	10	3/4 (75)	0/4 (0)	0/4 (0)
	13	1/4 (25)	0/4 (0)	0/4 (0)
	15	3/3 (100)	0/3 (0)	0/3 (0)
No.-Pest Strips®[2]	0	1/5 (20)	1/5 (20)	1/5 (20)
	2	1/4 (25)	0/4 (0)	0/4 (0)
	9	1/4 (25)	0/4 (0)	0/4 (0)
	14	0/4 (0)	0/4 (0)	0/4 (0)
	19	0/3 (0)	0/3 (0)	0/3 (0)

[1] Pools of 50 mosquitoes each, including 1 infected mosquito. RT-PCR, reverse transcription–polymerase chain reaction; EIA, enzyme immunoassay.

[2] 19.2% 2,2-dichlorovinyl dimethyl phosphate.

toes infected on the day of death, that is, day 0 was 80% as determined by RT-PCR but only 40% as determined by plaque assay or in situ EIA. When NPS was used to kill SLE-infected *Cx. tarsalis,* the percent infected was one half of that seen after the use of triethylamine.

Surveillance protocols that freeze specimens at −80°C immediately after death will always be preferred. However, detection of SLE and WEE in mosquito populations with RT-PCR assays on dead mosquitoes collected as a part of the population monitoring program could supplement the spring–summer CDC-style trap collection of live specimens for virus isolation and assay. An expanded program to detect virus activity and to target specific areas where the potential for mosquito-borne virus transmission is greatest would benefit a vector control program. However, the interpretation of RT-PCR–positive mosquito pools without the benefit of confirmatory cell culture assays for infectious virus must be made with caution. The mosquitoes in which RNA is detected should be considered to be potential vectors until the species is demonstrated to transmit infectious virus.

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