A PROSPECTIVE FIELD EVALUATION OF AN ENZYME IMMUNOASSAY: DETECTION OF EASTERN EQUINE ENCEPHALOMYELITIS VIRUS ANTIGEN IN POOLS OF *CULISETA MELANURA*

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ABSTRACT. A prospective field study was conducted to determine the sensitivity and specificity of an enzyme immunoassay (EIA) compared to virus isolation in cell culture for the detection of eastern equine encephalomyelitis (EEE) virus in naturally infected mosquitoes. A total of 10,811 adult female *Culiseta melanura* were collected in light traps during 1985 from four locations in Maryland. Eastern equine encephalomyelitis virus was isolated from 5 of 495 mosquito pools in African green monkey kidney and baby hamster kidney cell cultures. All five virus-infected pools were detected by the EIA, and all 490 uninfected pools were correctly scored as not containing virus. The EIA did not produce false positive or false negative results. Results support the assertion of previous researchers that the antigen detection EIA is a rapid, sensitive, specific, and simple alternative to traditional bioassays for the detection of EEE virus in mosquitoes.

INTRODUCTION

Mosquito-transmitted viruses can cause significant morbidity and mortality in humans and domestic animals (Theiler and Downs 1973). The threat of disease transmission or the effectiveness of control measures for these viruses is often assessed by monitoring the infection frequencies and abundance of mosquito vectors (Bowen and Francy 1980).

Bioassays currently used to isolate and identify arboviruses from mosquitoes are time-consuming and require specialized facilities (Scott and Olson 1986). The few laboratories that are equipped to conduct bioassays examine a large number of specimens from broad geographic areas, which can result in a backlog of specimens. Unfortunately, this constraint extends the time required to test specimens and disseminate results. For mosquito control personnel, this delay means that too often data on mosquito infection frequencies are only of historical value and of little use in preventing disease through vector control.

Several investigators have shown enzyme immunoassays (EIA) to be rapid, sensitive, specific, simple, and cost-effective alternatives to traditional bioassays for the isolation of arboviruses (Hildreth 1984[7], Scott and Olson 1986, Hildreth and Beaty 1987). In laboratory (Hildreth and Beaty 1984) and retrospective (Hildreth et al. 1984) studies, Hildreth and co-workers demonstrated that their EIA detected eastern equine encephalomyelitis (EEE) virus in pools of mosquitoes. More recently, Scott and Olson (1986) evaluated an EIA for the detection of EEE virus antigen in avian blood and brain tissue. Results from the latter study support Hildreth and Beaty's (1984) assertion that the EIA is a useful addition to current surveillance techniques for EEE virus.

Eastern equine encephalomyelitis virus is transmitted by the mosquito *Culiseta melanura* (Coquillett) in the eastern United States and can cause severe illness in humans, horses and game birds. These infections are characterized by rapid onset of symptoms, a high fatality rate, and a low inapparent-to-apparent infection ratio (Monath 1979). If the EIA can be satisfactorily applied to the surveillance and diagnosis of EEE virus, it might reduce the time required to examine specimens, disseminate results and initiate effective control measures.

The next logical phase in the evaluation of the EIA for detecting EEE virus is to conduct prospective field studies and to compare results from the EIA with those of traditional assays.

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[7] Hildreth, S. W. 1984. Detection of arbovirus antigens in mosquitoes by enzyme immunoassays: a new method for surveillance. Ph.D. Dissertation. Yale University, School of Medicine, New Haven, CT.

Therefore, the purpose of this investigation was to conduct such a longitudinal study with naturally infected mosquitoes.

MATERIALS AND METHODS

Mosquito collection and processing. Mosquitoes were collected during 1985 at four sites in Maryland. The Pocomoke Cypress Swamp site has been described (Muul et al. 1975) and is known as an enzootic area of EEE virus transmission. The Willards site is located in a hardwood forest near the town of Willards on the eastern shore of Maryland. Historically, Willards is an area where horses have been infected with EEE virus, and during 1961–64 Joseph and Bickley (1969) collected large numbers of *Cs. melanura* from this site. The Patuxent Wildlife Research Center and Fort George G. Meade sites are adjacent to each other near Laurel, Maryland. These sites were included in the study because 7 of 39 Whooping Cranes (*Grus americana*) at the Patuxent Wildlife Research Center died from EEE virus infections during 1984 (Dein et al. 1986, Carpenter et al. 1987). These sites, therefore, were potential areas of EEE virus transmission during 1985.

Mosquito collections were attempted with miniature CDC light traps augmented with dry ice at the four study sites at least once each week. The collection period at the Pocomoke Cypress Swamp was April through November, and at the other three sites July through October. Traps were collected in the morning following overnight operation. Live mosquitoes were returned to the laboratory and killed by freezing. Each light trap collection was transferred to a chilled surface, and *Cs. melanura* were identified, pooled, and frozen at −70°C until trituration at a later date. *Culiseta melanura* from the Pocomoke Cypress Swamp were pooled into groups of ≤50; those from the other three sites were sorted into pools of ≤25. None of the mosquitoes tested contained visible amounts of blood in their abdomen.

At the University of Maryland, each mosquito pool was ground with a mortar and pestle in 1.0 ml of minimum essential medium (Eagle) cell culture medium supplemented with 20% heat-inactivated fetal calf serum, 500 units penicillin/100 ml, 500 μg streptomycin/100 ml, and 1 ml fungizone/100 ml. Mosquito suspensions were centrifuged for 2 min at room temperature (about 21°C) and then frozen at −70°C until assayed for virus. Methods for titration of infected pools are described by Scott and Olson (1986).

Bioassays. Each mosquito suspension was assayed for infectious virus in two different kinds of cell culture. Assays were conducted in 24-well plates (2 wells/pool) by examining each suspension for plaque forming units in African green monkey kidney (Vero) cell cultures with an agar overlay (Scott et al. 1983a) and for cytopathic effects in baby hamster kidney cell cultures with a liquid overlay (Scott et al. 1984).

Virus isolates were identified by a constant antibody, virus dilution plaque reduction neutralization test in Vero cells (Scott et al. 1983b). Hyperimmune mouse ascitic fluids directed against EEE virus were used for virus identification. To minimize freezing and thawing, which might reduce infectious virus titers to undetectable levels, all mosquito suspensions were assayed in the two cell culture systems before the EIA was conducted.

Results from the cell culture assays and the EIAs were compiled separately. Thus personnel who conducted the EIAs did not know which pools were virus positive by the cell culture assay, or vice versa, until all assays had been completed.

Enzyme immunoassay. The EIA procedure and reagents used are reviewed in Fig. 1 and are described by Scott and Olson (1986). They also described how reagents and procedures for the test were selected, and defined the test's relative sensitivity and specificity. In brief, polyvinyl EIA plates were coated with mouse antibody directed against EEE virus, plates were blocked with a horse serum solution, undiluted mosquito suspensions were added, rabbit antibody di-

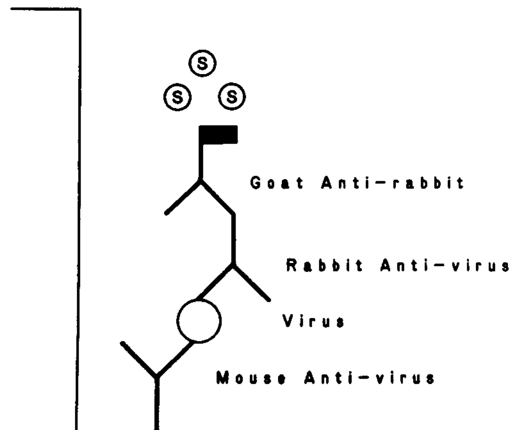

Fig. 1. Diagram of the antigen captive enzyme immunoassay for detection of eastern equine encephalomyelitis virus in mosquito pools. Capture antibody was produced in mice and coated onto polyvinyl microtiter plates. Detection antibody was produced in rabbits. Indicator antibody was commercially prepared in goats and conjugated to peroxidase, which is denoted by the black flag. Substrate (ABTS) is denoted by the circled S.

rected against EEE virus was added, goat anti-rabbit IgG conjugated to peroxidase was added, the substrate 2.2′-azino-di[3-ethyl-benzthiazoline sulfonate (6)] (ABTS) was added, and finally absorbance values were determined on an EIA plate reader. Plates were washed 3–5 times between each step, and suspensions were incubated for 2 hr at 37°C followed by overnight incubation at 4°C. Each plate contained positive and negative control specimens; positive controls were a stock of EEE virus (ME-77132, see Scott et al. 1984); negative controls were uninfected *Cs. melanura* from a laboratory colony. All specimens were examined in triplicate using only the inner 60 wells on the microtiter plate. Mosquito suspensions were considered to contain EEE virus antigen if optical density of all three wells for a given suspension exceeded the mean plus 3 standard deviations of the negative controls.

Suspensions that contained detectable EEE virus antigen were re-examined with an inhibition test to verify specificity of the EIA. Except for two modifications, methods for the inhibition test were identical to those described by Scott and Olson (1986). First, in this analysis suspensions were incubated with a different anti-EEE virus mouse polyclonal ascitic fluid prior to testing; coating antibody was not used to block antigenic sites. Second, specimens were incubated with one of three, not two, solutions prior to testing. Those solutions included the mouse anti-EEE antibody mentioned above, mouse anti-St. Louis encephalitis (SLE) virus polyclonal ascitic fluid, or mosquito diluent without antibody. The negative control was an uninfected pool of *Cs. melanura*; the positive control was a stock of inactivated EEE virus (ME-77132).

RESULTS

A total of 10,811 adult female *Cs. melanura* in 495 pools was examined for virus (Table 1). Five virus-infected mosquito pools were detected in Vero and baby hamster kidney cell cultures (Tables 1 and 2). All five virus isolates were subsequently identified as EEE virus by the plaque reduction neutralization test.

Results from the EIA analysis agreed with the cell culture bioassays (Table 2). All five EEE virus-infected mosquito pools were positive for EEE virus antigen as determined by the initial EIA assay and the subsequent inhibition test. Of the 490 pools without detectable infectious virus, none were positive by EIA. No other viruses were isolated in cell culture, nor were any other virus antigens detected by the EIA. Thus, there were no false positive or false negative results.

All virus isolates were recovered from *Cs. melanura* collected in the Pocomoke Cypress Swamp (Table 1). Table 2 summarizes optical density values from the EIA, virus titers of suspensions, and collection dates of pools that contained detectable EEE virus.

DISCUSSION

Results from this prospective field study support the previously conducted laboratory and

Table 1. *Culiseta melanura* collected in Maryland with CDC light traps during 1985 and assayed for eastern equine encephalomyelitis virus in cell culture (CC) and by enzyme immunoassay (EIA).

	Pocomoke Swamp (50)[a]				Willards (25)				Patuxent Wildlife Research Center (25)				Fort George G. Meade (25)			
			Virus assay				Virus assay				Virus assay				Virus assay	
Month	No. mosq.	No. pools	CC[b]	EIA	No. mosq.	No. pools	CC	EIA	No. mosq.	No. pools	CC	EIA	No. mosq.	No. pools	CC	EIA
April	93	7	0	0	NC[c]				NC				NC			
May	498	24	0	0	NC				NC				NC			
June	1,071	29	0	0	NC				NC				NC			
July	669	24	0	0	1,077	49	0	0	2	2	0	0	63	7	0	0
Aug.	1,117	33	1	1	732	33	0	0	5	5	0	0	501	51	0	0
Sept.	1,218	28	2	2	1,848	76	0	0	13	8	0	0	267	37	0	0
Oct.	722	22	2	2	804	33	0	0	2	2	0	0	57	21	0	0
Nov.	52	4	0	0	NC				NC				0	0	0	0
Total	5,440	171	5	5	4,461	191	0	0	22	17	0	0	888	116	0	0
Grand Total	10,811	495	5	5												

[a] Maximum number of mosquitoes per pool.
[b] All mosquito suspensions were assayed in Vero and baby hamster kidney cell cultures. The lower limit of detection in both cell cultures was 10^1.
[c] NC denotes that no mosquito collections were made during that month.

Table 2. Summary of *Culiseta melanura* pools collected in the Pocomoke Cypress Swamp, MD, during 1985 that contained eastern equine encephalomyelitis virus.

Sample identification number	Optical density in enzyme immunoassay (range)[a]	Log_{10} BHK $TCID_{50}$ titer of mosquito pools	Collection date
187-85	1.047–1.257	6.05	Aug. 29
215-85	1.365–1.411	7.30	Sept. 4
219-85	1.520–1.652	6.05	Sept. 4
318-85	0.695–0.749	6.80	Oct. 20
323-85	0.121–0.128	4.30	Oct. 20

[a] Mean and standard deviation of optical densities for negative controls were as follows: $\bar{X} = 0.087$ and SD = 0.008.

retrospective evaluations of EEE virus antigen detection by EIA (Hildreth and Beaty 1984, Hildreth et al. 1984, Scott and Olson 1986). Sensitivity and specificity of the EIA with naturally infected mosquitoes were excellent. All five pools of *Cs. melanura* that contained detectable infectious EEE virus were correctly identified by the EIA. No false positive or false negative results were recorded. Nor did the EIA detect noninfectious EEE virus antigen, something that Scott and Olson (1986) described in their study of EEE virus in birds and Tsai and co-workers (1987) reported in their EIA study of SLE virus in mosquitoes. Only in other studies when high concentrations of a stock of Highlands J virus were examined was cross-reactivity observed (Hildreth and Beaty 1984, Hildreth et al. 1984, Scott and Olson 1986). Based on this observation and other EIA studies (Monath and Nystrom 1984, Tsai et al. 1987), it would not be surprising if the EEE-EIA would cross-react with high concentrations of other closely related alphaviruses. To date, however, there have been no false positive results with uninfected specimens or with specimens collected from animals infected with Highlands J, western equine encephalomyelitis, or SLE viruses (Hildreth et al. 1984, Scott and Olson 1986).

False negative results have, however, been reported by Hildreth and co-workers (1984). Their sensitivity rate was 0.97 for mosquito pools containing $>10^{3.2}$ PFU per ml. Sensitivity declined to 0.14 for pools containing $<10^{2.7}$ PFU per ml. The EEE antigen detection EIA described by Scott and Olson (1986) had a lower limit of detection of $10^{3.5}$ $TCID_{50}$ per 1.0 ml for a stock of EEE virus.

This level of sensitivity and specificity was obtained by using polyclonal reagents. Monoclonal reagents have the advantages of being well characterized, consistent in avidity, and available in essentially unlimited quantity. However, we are unaware of an EIA for detecting EEE virus antigen that uses monoclonal reagents and is more sensitive than our protocol (Scott and Olson 1986; T. F. Tsai, personal communication). The ease of obtaining reagents and thus of conducting the EEE-EIA would be improved if monoclonal reagents with increased sensitivity were developed.

Antigenicity and detectability of virus infected mosquito suspensions are apparently not reduced by freezing and thawing, something that can occur during the processing and testing of mosquito pools or if specimens are mishandled (Hildreth et al. 1982, Niklasson and Gargan 1985, Tsai et al. 1987). As mentioned above, normal processing of mosquitoes in this study did not reduce virus titers to undetectable levels (Table 2). Freezing and long-term storage, however, can apparently reduce antigenicity and thus detectability by EIA (Hildreth et al. 1984). Therefore, until this issue is more closely examined, mosquito pools should not be stored for more than a year prior to being tested by the EIA.

During the early phase of virus infection or for specimens with low virus titers, virus isolation in cell culture may be more sensitive than the EIA. Hildreth and Beaty (1984) were unable to detect antigen in 100% of their EEE virus-infected *Aedes triseriatus* (Say) pools until 3 days after mosquitoes had been intrathoracically inoculated. Tsai and co-workers (1987) observed the same kind of effect in their EIA study of SLE virus in orally infected mosquitoes. Similarly, for EEE virus-infected chick blood, an EIA was less sensitive than a cell culture assay at detecting virus during the first 24 hr after inoculation (Scott and Olson 1986). At 24- and 48-hr postinoculation, sensitivity was 100%. Finally in this study, the mosquito pool with the lowest virus titer had lower optical density readings than the other four virus positive pools (Table 2).

The number of mosquito pools containing EEE virus (n = 5) in this study was small, but the number of pools without detectable infectious virus or virus antigen was large (n = 490, Table 1). A larger sample of infected pools should be examined in future longitudinal studies. It would also be valuable to know if the EIA cross-reacts with naturally infected mosquitoes containing closely related viruses. In addition, the sensitivity and specificity for detecting EEE virus strains from different geographical locations, including Central and South America, should be evaluated. Future studies should also examine the effects of mosquito engorgement on virus detection. Our study was conducted with

unengorged mosquitoes to avoid assaying specimens that might have EEE virus-infected blood in their abdomen while not yet able to transmit virus biologically. We have not evaluated the EIA with engorged mosquitoes. Our protocol might be improved by using substrates other than ABTS, such as 3.3′, 5.5′-tetramethylbenzidine (TMB). In a SLE antigen detection EIA, TMB was four times more sensitive than ABTS (Tsai et al. 1987).

If investigators know precisely the kind of virus they seek to detect, the EIA technique provides significant advantages over traditional bioassays (Hildreth and Beaty 1983). The EIA requires less labor, time, specialized equipment, and in most cases costs less than do bioassays (Hildreth 1984, Scott and Olson 1986, Hildreth and Beaty 1987). Scott and Olson's (1986) protocol requires approximately 36 hr to test 108 mosquito suspensions. Without overnight incubation of mosquito suspensions, the EIA can be completed in approximately 8 hr. Under ideal conditions, traditional bioassays require 3–5 days for EEE virus isolation and identification; more often 7–14 days are required.

Because it is easier and more convenient to conduct than bioassays, the EIA is a substantial improvement for EEE virus surveillance, and the technique should be more widely used. As with any other newly developed diagnostic procedure, additional longitudinal field studies should be conducted. These analyses will help to accurately define, over an extended period of time, how results from the EIA compare to those obtained with traditional techniques.

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