Stability of St. Louis Encephalitis Viral Antigen Detected by Enzyme Immunoassay in Infected Mosquitoes

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The use of enzyme immunoassay to detect St. Louis encephalitis (SLE) viral antigen in vector mosquitoes enhances the effectiveness of surveillance because infected mosquitoes can be identified more rapidly than with conventional virus isolation systems and because it is a simple and accessible procedure. Infectivity among mosquitoes experimentally infected with SLE virus was lost within 24 h after the mosquitoes were stored at 27°C and 80% relative humidity; however, viral antigen remained stable under these conditions and could be detected by enzyme immunoassay 2 weeks later. Desiccation further extended the period during which antigen could be detected to 6 weeks. Absorbances were higher in infected mosquitoes stored at 27°C than in mosquitoes frozen continuously. Absorbances in infected mosquitoes also increased after repeated freezing and thawing and sonication. Both phenomena may be related to the release of antigen from decaying or disrupted cells. The relative stability of SLE viral antigen at ambient temperatures lends flexibility to schemes which use direct antigen detection to identify infected vectors. Surveillance systems can be designed without regard to collecting living mosquitoes, and a cold chain is unnecessary to preserve specimens, thus reducing the cost of surveillance and expanding the geographic areas to which it is accessible.

Major epidemics of St. Louis encephalitis (SLE) occur infrequently in the United States, but periodically, as in 1975, epidemics have produced hundreds or occasionally thousands of cases in a single season (7, 11a, 12). The potential for SLE outbreaks of this scale has led many state and local health agencies to survey SLE virus activity in nature as a means of predicting the probability of epidemic transmission. In epidemic years, viral transmission in the enzootic cycle among *Culex* mosquitoes and birds is amplified, resulting in an expanded population of infected vectors and an increased risk of epidemic transmission (1, 6–8, 11a). The SLE viral infection rate in vectors is an important surveillance instrument by which the impending risk of epidemic transmission can be measured and the application of interdictory mosquito control measures can be guided (1, 6–8, 11a, 12).

The principal limitation of this approach to surveillance has been the 5- to 10-day delay between the collection of mosquitoes in the field and the isolation and identification of viruses in suckling mice or cell cultures. Furthermore, local health and mosquito abatement agencies, which have the responsibility for surveillance of vector mosquitoes, often are unable to undertake viral isolation because they lack facilities to maintain cell cultures and mice. The isolation of viruses from mosquitoes has necessitated that specimens be maintained frozen in the field, with dry ice or liquid nitrogen, until they can be transferred to mechanical freezers. Often a cold chain cannot be maintained in distant surveillance sites, limiting the geographic range of areas that can be surveyed.

All of these obstacles to monitoring vector infection rates can be overcome by using assays that measure viral antigen in mosquitoes in place of viral isolation techniques (3–5, 9, 11). We previously showed that a rapid, simple and inexpensive antigen capture enzyme immunoassay (EIA) was ade-

quately sensitive to detect SLE viral antigen in infected mosquito pools (11). In this paper, we show that SLE viral antigen in infected mosquitoes, measured by EIA, did not decline even after the infected specimens had been stored for 2 weeks at 27°C and 80% relative humidity. When infected mosquitoes were desiccated in silica gel packets, antigen could be detected after 6 weeks of storage at 27°C. These results indicate that a cold chain may be unnecessary to preserve specimens for SLE viral surveillance, thus opening the way for expanding the geographic range accessible to viral surveillance.

MATERIALS AND METHODS

Infected mosquitoes. The 76v15838 strain of SLE virus originally isolated from *Culex pipiens* collected in Memphis, Tenn. (11), was propagated in suckling mice. Stock viral inocula were prepared as 10% (wt/vol) suspensions of infected mouse brain in phosphate-buffered saline (pH 7.4). Colonized *Culex tarsalis* mosquitoes (Montoya strain) were inoculated intrathoracically with approximately 0.2 μl of the infected-brain suspension. Infected mosquitoes were contained in double-screened cages at 27°C and 80% relative humidity for 10 days of extrinsic incubation.

Mosquito preparation. One infected mosquito was pooled with 19 uninfected female *C. tarsalis* mosquitoes. The pooled mosquitoes were ground with alundum in 1.5 ml of BA-1 diluent (M199 with 1% bovine serum albumin, buffered to pH 7.4 with Tris). The clarified suspensions were tested by EIA and by infectivity in cell culture.

Infectivity titration. Suspensions were inoculated neat and in successive 10-fold dilutions onto monolayers of Vero cells grown in 6-well panels. After absorption, the cell sheets were overlaid with nutrient agarose. Plaques were enumerated 5 to 7 days later, after the addition of neutral red.

Antigen capture enzyme immunoassay. Polystyrene 96-well panels (Immulon II; Dynatech Laboratories, Inc., Alexan-

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dria, Va.) were sensitized with 4A4C-4, a SLE virus-specific monoclonal antibody directed at the envelope glycoprotein (10, 11). After they were washed, plates were blocked with phosphate-buffered saline containing 20% fetal calf serum. Mosquito pools, positive controls, and diluent controls were added and incubated overnight. After several washes, a flavivirus group-reactive monoclonal antibody directed at the envelope glycoprotein (6B6C-1), conjugated to horseradish peroxidase, was added (10, 11). Enzyme activity was developed with tetramethylbenzidine. Freshly diluted chromogen and substrate were made by adding 0.3 ml of tetramethylbenzidine solution (a concentrated solution in absolute methanol [3.5 mg/ml]) and 1.5 µl of 30% hydrogen peroxide to 9.7 ml of sterile citrate-acetate buffer (pH 6.0). The working solution of chromogen and substrate was added to washed plates, and after 3 to 5 minutes the reaction was stopped with 2 N H_2SO_4. A_{450} readings were determined in an automated reader.

Absorbance ratios were calculated from the sample absorbances and the mean of absorbances associated with diluent wells. Ratios greater than 2.0 were regarded as positive.

Effects of storage conditions on infectivity and EIA reactivity. In the initial experiment, infected *C. tarsalis* mosquitoes were divided into two groups. After an extrinsic incubation period of 10 days, mosquitoes serving as controls were frozen immediately at −70°C. Mosquitoes in the experimental group were killed and held at 27°C and 80% relative humidity in an open vial in an insectary bioclimatic room simulating summertime conditions. Pools of 1 infected and 19 uninfected mosquitoes were tested by EIA and for infectivity.

In the second experiment, infectivity and EIA reactivity were compared in control mosquitoes and in two groups of infected mosquitoes held in the insectary for 3 or 7 days; however, the mosquitoes were kept in sealed foil pouches containing silica gel (Dri-Pax; W. R. Grace Co., Baltimore, Md.) (2).

Uninfected mosquitoes (10 in each group) were held under the same conditions in the insectary (overnight without desiccation, and desiccated for 3 or 7 days) to determine if storage under these conditions would lead to an increase in background absorbance.

Desiccation and temperature effects on EIA reactivity. After it had been determined in the above experiment that antigen was detectable in desiccated mosquitoes after 1 week of storage at 27°C, four more groups of mosquitoes were studied to determine how long antigen would remain stable under these conditions. Additional comparisons were made with mosquitoes held desiccated at room temperature (20°C) and at 27°C.

After 10 days of extrinsic incubation, infected mosquitoes were killed by freezing and were stored under various conditions of humidity and temperature for up to 6 weeks. After each interval, mosquitoes were transferred to a −70°C freezer, until all samples could be tested simultaneously by EIA. For group A, 20 mosquitoes were frozen continuously at −70°C (control group); for group B, five sets of 10 mosquitoes each were held in open vials in the insectary (27°C and 80% humidity) for 3, 7, 14, 21, or 42 days; for group C, five groups of 9 or 10 mosquitoes each were held in the insectary for the same intervals, but the mosquitoes were stored desiccated in Dri-Pax; and for group D, four groups of 10 mosquitoes each were desiccated in Dri-Pax but were held in an air-conditioned room (20°C) for 7, 14, 21, or 28 days.
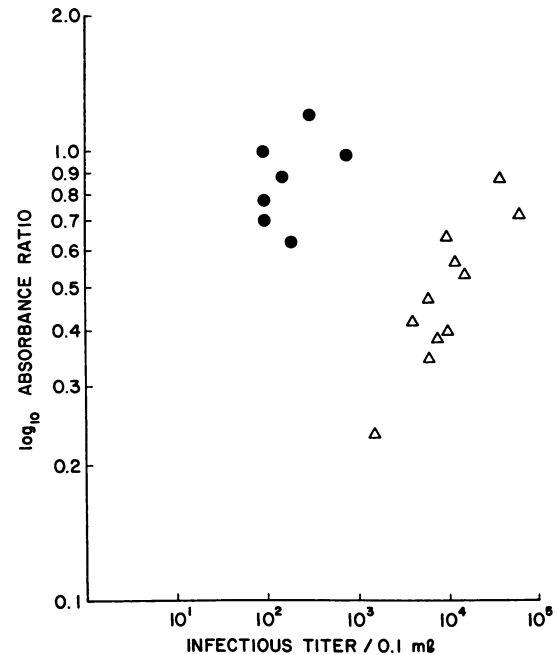

FIG. 1. Effects of storing SLE virus-infected mosquitoes at 27°C and 80% relative humidity overnight on infectivity and reactivity in an antigen capture EIA. Symbols: ●, experimental group; △, control mosquitoes kept frozen.

Freeze-thaw and sonication effects. We tested the effects of mechanical means of disrupting infected mosquito cells and disaggregating antigen on EIA reactivity. Infected mosquitoes from the above experiment were repeatedly frozen and thawed and finally sonicated. Mosquito suspensions (pellet and supernatant) were alternately frozen at −70°C and then thawed, three times. After each thaw cycle, the pellets were resuspended, the suspensions were clarified by centrifugation, and the supernatants were tested by EIA. After the last freeze-thaw cycle, the suspensions also were sonicated before they were tested by EIA.

Statistical analyses. Tests for significance were determined on a microcomputer with True Epistat (Epistat Σ Services, Richardson, Tex.).

RESULTS

Effects of storage on infectivity and EIA reactivity. After infected mosquitoes had been stored overnight at 27°C and 80% humidity, the infectious titer of the group declined significantly, from dex 4.0 ± 0.26 (2 standard errors) in the control group to dex 2.3 ± 0.24 in the experimental group ($t_{[16]}$ = 8.8446, $P < 10^{-6}$) (Fig. 1). EIA absorbance ratios, however, were significantly higher in the experimental group (mean absorbance ratios were 8.31 and 3.48 in experimental and control groups, respectively; $t_{[7]}$ = 2.8031, $P < 0.03$). A correlation of infectious titer with EIA signal was observed in the control mosquitoes, which were frozen continuously [y = −0.953657 + 0.364428(x); r = 0.87248, $F_{(1,9)}$ = 28.69239, $P < 0.0005$]; however, in the experimental mosquitoes held overnight at 27°C and 80% humidity, infectivity was dissociated from EIA signal and no correlation remained.

When infected mosquitoes were stored in a desiccated condition for 3 or 7 days, infectivity was lost in 89% of the
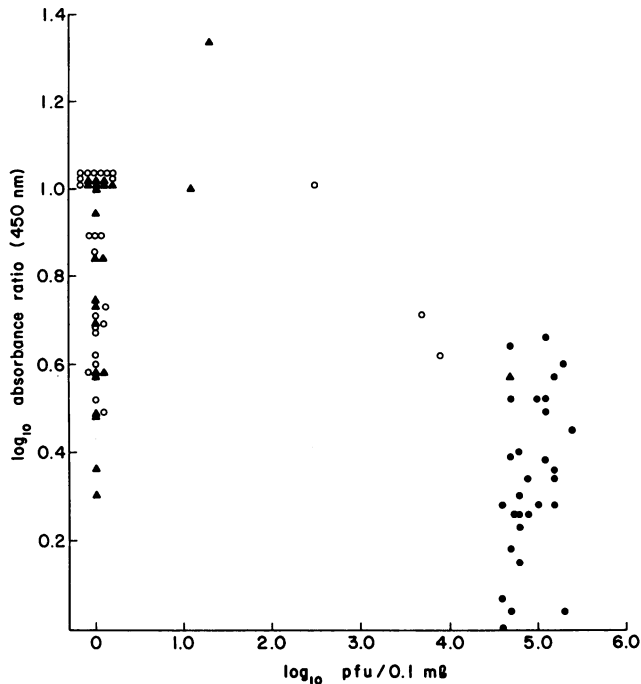
FIG. 2. Dissociation of infectivity from reactivity in antigen capture EIA in SLE virus-infected mosquitoes stored desiccated at 27°C for 3 (▲) or 7 (○) days or in frozen control mosquitoes (●).
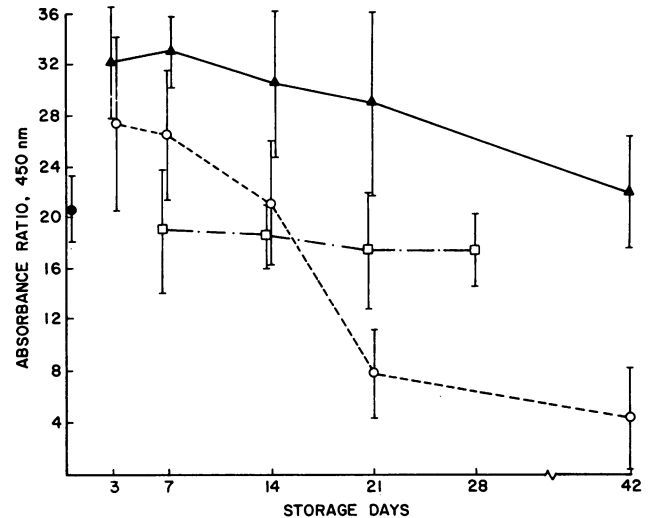

FIG. 3. Effects of storing SLE virus-infected mosquitoes under various conditions of temperature and humidity on reactivity in antigen capture EIA (shown as mean of absorbance ratios with two standard errors). Symbols: ●, group A (frozen controls); ○, group B (stored at 27°C and 80% relative humidity); ▲, group C (stored desiccated at 27°C); □, group D (stored desiccated at 20°C).

specimens (24 of 27 and 23 of 26, respectively) (Fig. 2). The EIA reactivities of the desiccated mosquitoes, however, were higher in both groups compared with those of the frozen controls ($t_{[42]}$ = 7.448, $P < 10^{-7}$; and $t_{[53]}$ = 9.120, $P < 10^{-7}$) (Fig. 2). The $\log_{10}$ absorbance ratios of mosquitoes desiccated for 3 and 7 days were 0.83 and 0.85, respectively; the $\log_{10}$ absorbance ratio for the controls was 0.34 (Fig. 2).

Storage under various conditions did not lead to increased background absorbance in any of the uninfected control mosquitoes.

Desiccation and temperature effects on EIA reactivity. When mosquitoes were stored under conditions simulating a summer environment (27°C and 80% humidity) (group B), EIA detected no significant loss of reactivity for storage periods up to 14 days (Fig. 3 and Table 1). Compared with those of controls, EIA signals in mosquitoes stored under these conditions were significantly lower after day 21, but signals were higher after storage for only 3 or 7 days.

Mosquitoes kept desiccated in Dri-Pax (group C) but held side by side with mosquitoes in the insectary chamber gave significantly higher EIA signals than controls for storage periods up to 21 days; after 42 days under these conditions, there was no significant difference in the reactivities of mosquitoes in group C and in controls (Fig. 3 and Table 1).

Desiccated mosquitoes held at 20°C for various periods up to 28 days retained a similar level of reactivity in the EIA; absorbance ratios were similar to those of the controls (Fig. 3 and Table 1).

Freeze-thaw and sonication effects. The EIA signals in mosquito suspensions repeatedly frozen and thawed rose after each cycle (Fig. 4) except in mosquitoes that had been stored for 21 days at 27°C and 80% humidity. After the fourth freeze-thaw cycle, when the mosquito suspensions were also sonicated, absorbance ratios rose dramatically in all groups, to approximately threefold the reactivity signals obtained after the preceding freeze-thaw cycle.

After sonication, the mean absorbance ratios among group C mosquitoes, which ranged from 77.6 to 85.3, did not differ significantly; however, they were significantly higher than the mean absorbance of the sonicated controls (67.1; F = 2.816, P = 0.02) (Fig. 4). The reactivity of group D and control mosquitoes did not differ significantly after sonication (F = 1.053, P > 0.35). The mean absorbance ratio of mosquitoes stored 21 days at 27°C and 85% relative humidity (20.2) after sonication remained significantly lower than the mean of the controls ($t_{[23]}$ = 5.926, $P < 10^{-4}$) (Fig. 4).

DISCUSSION

The approach to surveillance and control of epidemic vector-borne diseases, of which SLE is an example, differs from the strategy employed in controlling other infectious diseases, because the focus of surveillance is not on monitoring human cases but on measuring viral activity in nature (1, 6–8, 11a, 12). It is generally held that SLE virus activity in birds and in vector mosquitoes must exceed a threshold before the virus spills out of its maintenance cycle and is transmitted to humans. In principle, by monitoring infection rates in vectors, epidemics can be anticipated and control measures can be instituted before any human cases occur (1, 6–8, 11a, 12).

It is in the interval between the initial identification of infected vectors and the onset of epidemic transmission that emergency mosquito control measures, in the form of adulticides, can be applied to abort or limit the extent of an outbreak. The effectiveness of this approach depends on the use of rapid systems to identify infected vectors from surveillance collections, to maximize the period in which control measures can be mobilized.

The chief limitation of measuring infection rates in vectors has been the necessity to use cell culture or mice to recover and identify viral isolates. These systems are relatively slow, requiring 5 to 10 days before results are obtained, and they usually are unavailable in the field stations from which surveillance collections are submitted. The direct detection

TABLE 1. Temperature and humidity effects on detectability of SLE viral antigen in infected mosquitoes as determined by capture EIA

Treatment group[a]	Day of storage	No. of mosquitoes	Mean A_{450} ratio ± SD	$t_{(n)}$	P	F	P
A (control)		20	20.25 ± 5.280				
B	3	10	27.37 ± 10.994	$1.939_{(11)}$	0.08		
	7	10	26.46 ± 8.118	$5.233_{(28)}$	$<10^{-3}$		NS^b
	14	10	20.20 ± 6.946		NS		
	21	10	7.78 ± 5.573	$7.255_{(28)}$	$<10^{-7}$		
	42	10	4.39 ± 6.346	$5.989_{(28)}$	$<10^{-5}$		
C	3	10	32.22 ± 7.047	$2.532_{(28)}$	<0.02		
	7	10	33.00 ± 6.377	$5.821_{(28)}$	$<10^{-5}$		
	14	9	30.49 ± 8.731	$3.927_{(28)}$	$<10^{-3}$	2.808	<0.04
	21	9	29.02 ± 10.972	$2.28_{(9)}$	<0.05		
	42	10	21.99 ± 7.281		NS		
D	7	10	18.92 ± 7.856		NS		
	14	10	18.56 ± 4.300		NS		NS
	21	10	17.35 ± 7.407		NS		
	28	10	17.40 ± 4.556		NS		

[a] SLE virus-infected mosquitoes were killed after an extrinsic incubation period of 10 days. Mosquitoes were treated as follows: group A, frozen immediately at $-70°C$ (controls); group B, kept at 27°C and 80% humidity; group C, desiccated in silica gel packets at 27°C; and group D, desiccated at 20°C. All mosquitoes were kept frozen at $-70°C$ until they were tested simultaneously in the EIA.
[b] NS, Not significant.

of SLE viral antigen in mosquitoes by EIA is a means of circumventing these obstacles to monitoring infected vectors. The antigen capture EIA is a sensitive and specific procedure; the rapidity with which results become available and the accessibility of the procedure to laboratories with limited physical facilities should improve the responsiveness of surveillance (1, 6–8, 11, 11a, 12).

In this series of experiments we showed that SLE viral antigen remained stable in infected mosquitoes stored at elevated temperatures for an extended period. Infected mosquitoes kept up to 2 weeks under temperature and humidity conditions mimicking a summertime environment could be detected by EIA without a significant loss of sensitivity (Fig. 3). Desiccation extended this period to 6 weeks (Fig. 3). Reactivity in the antigen detection EIA was nearly completely dissociated from the infectivity of the mosquito suspension (Fig. 1 and 2).

Infectivity declined by 2 dex in mosquitoes stored overnight at 27°C and 80% relative humidity (Fig. 1). Although desiccation protected against the loss of infectivity, protection was limited and of a variable degree: 89% of specimens stored desiccated for 3 to 7 days lost all infectivity, and a >10-fold decline in infectious titers was seen in five of the six mosquitoes that retained some infectivity (Fig. 2).

While infectivity declined in mosquitoes kept at 27°C and 80% relative humidity for 1 to 7 days, reactivity in the EIA increased significantly over that of the frozen control mosquitoes (Fig. 1 to 3 and Table 1). After 21 days under these conditions, however, reactivity in the EIA declined significantly (group B, Fig. 3). This observation suggests that elevated temperature contributed to the decay of infected mosquito cells, releasing viral antigens and making them more accessible to detection by EIA. The integrity of antigen persisted until day 14, after which antigen also became subject to decay under these conditions. The EIA signals of infected mosquitoes which were kept desiccated at this temperature (group C) remained elevated for a longer period (4 weeks). Evidently desiccation, despite playing no role in the processes leading to increased accessibility of antigen, nevertheless contributed to its stabilization.
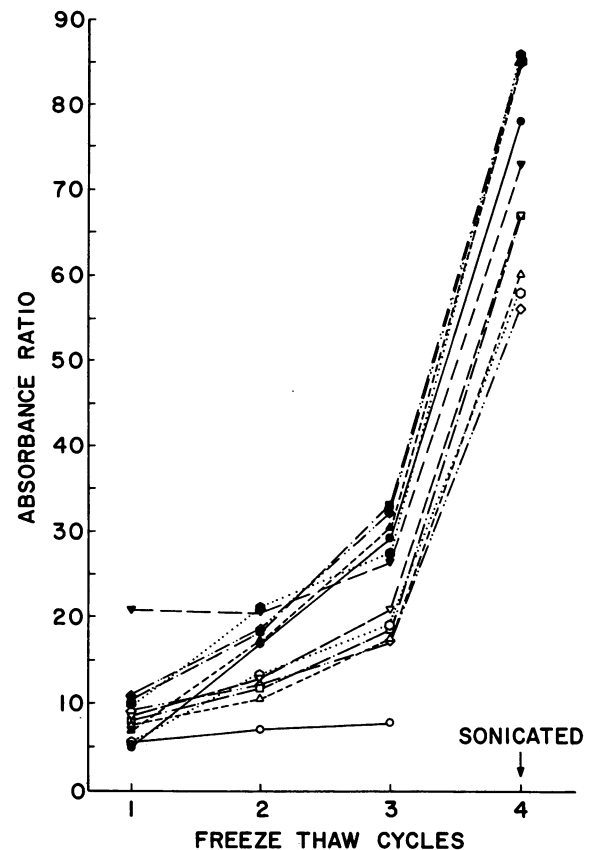

FIG. 4. Effects of freezing, thawing, and sonicating SLE virus-infected mosquitoes on reactivity in antigen capture EIA (shown as absorbance ratios) for group A (controls) (▽), group B (stored at 27°C and 80% relative humidity for 3 [●], 7 [▼], or 21 [○] days), group C (stored desiccated at 27°C for 3 [◆], 7 [■], 14 [▲], or 21 [●] days), and group D (stored desiccated at 20°C for 7 [○], 14 [□], 21 [◇], or 28 [△] days).

Infected mosquitoes which were kept desiccated at room temperature (group D) for up to 4 weeks gave EIA signals indistinguishable from those of the frozen controls. Reactivities in mosquitoes from group D were not elevated as they were in group C. Desiccation at the lower temperature may have created an effect simulating lyophilization, preserving antigen reactivity to the same extent as freezing; however, the effects of cellular disruption or antigen disaggregation that may have occurred at the higher temperature, leading to increased EIA signals, were not observed at room temperature.

The effects of repeated freezing and thawing and sonication led to a general increase in reactivity in the EIA except in mosquitoes stored for 21 days at 27°C and 85% relative humidity. Although these mosquitoes were not sonicated, mechanical disruption did not lead to a further increase in reactivity in this group, indicating that antigen reactivity had declined under these conditions.

Simple mechanical means of disrupting mosquitoes and disaggregating antigen led to a considerable increase in the sensitivity of the EIA (11). These observations underscore the precept that the correlation between infectivity and reactivity in an antigen detection system is not constant and depends on the conditions under which the specimen is handled (11). In a previous report, repeated freeze-thaws led to no change in reactivity in an eastern equine encephalitis antigen capture EIA (4). These specimens had been stored frozen for a prolonged period, and disruptive effects may already have occurred to a significant degree.

The observation that SLE viral antigen in infected mosquitoes is stable under ambient summertime conditions may have important practical implications for vector surveillance. The standard protocol for collecting mosquitoes for virus isolation requires the inspection of light traps each morning, to ensure that freshly caught mosquitoes are collected and frozen before a significant decay in infectivity occurs. If the goals of surveillance are simply to monitor infection rates in vectors and the recovery of viral isolates is a secondary concern, it may be sufficient to identify infected mosquitoes by using a direct antigen detection assay. In this event, collections could be made without regard to collecting living mosquitoes and the traps could be inspected at less frequent intervals (11). Even if collections from traps were made at intervals of 2 to 3 days, EIA results could be obtained before the results from conventional isolation procedures became available from collections made daily. Furthermore, field collections could be stored unfrozen and shipped by ordinary mail to central laboratories for testing, reducing the costs associated with the need for dry ice. Indeed, our results indicate that the sensitivity of the EIA could be improved by holding infected mosquitoes at 27°C for 3 to 21 days. The introduction of direct antigen detection systems has the potential for increasing the geographic range accessible to surveillance and increasing the flexibility and responsiveness of vector surveillance systems.

Limited data are available on the operating characteristics of the EIA in evaluating field-collected mosquitoes (11, 13; G. S. Smith and T. F. Tsai, unpublished observations). A summary analysis of these studies indicates that the EIA may be more sensitive than conventional isolation procedures in identifying mosquitoes naturally infected with SLE virus (Table 2).

The relationship of mosquito infection rates to risk of epidemic transmission currently is based on infection rates determined by infectivity assays (1, 6–8, 11, 11a). The relationship of epidemic risk with vector infectivity rates

TABLE 2. Sensitivity and specificity of antigen capture EIA in identifying SLE virus from field-collected mosquitoes[a]

Cell culture result	No. of EIA results		
	Positive[b]	Negative	Total
Positive	18	9	27
Negative	18	2,141	2,159
Total	36[b]	2,150	2,186

[a] Mosquito pools from Imperial County, Calif.; Mesa County, Colo.; and Ohio, 1986 to 1987 (10, 13).

[b] The probability of finding ≥36 positive pools in 2,186 specimens is 0.055 if the expected number of positives is 27.

determined by EIA may differ quantitatively because the EIA technique is somewhat more sensitive (Table 2). There are no data on the operating characteristics of the EIA in evaluating naturally infected mosquitoes that have been desiccated in the field. These characteristics need to be established before guidelines can be promulgated for implementing this technique in epidemiologic assessments of risk of epidemic transmission.

Desiccation in silica gel is the standard procedure for preserving and transporting throat swabs collected for the purpose of isolating Streptococcus pyogenes and Corynebacterium diphtheriae (2). The observations that SLE viral antigen reactivity in mosquitoes is enhanced by sonicating the specimens and that antigen is stable upon desiccation may find practical applications in other systems that directly detect more fastidious infectious antigens. In epidemiologic and clinical situations in which it is unnecessary to recover an infectious isolate, antigen detection not only offers the well-recognized advantages of increased speed and reduced cost of performance but also obviates the need for fastidious conditions during specimen storage and transport (3).

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