*Journal of the American Mosquito Control Association*, 12(2):334–336, 1996


# STABILITY OF EQUINE INFECTIOUS ANEMIA VIRUS IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE), *STOMOXYS CALCITRANS* (DIPTERA: MUSCIDAE), AND *TABANUS FUSCICOSTATUS* (DIPTERA: TABANIDAE) STORED AT −70°C

B. E. GREEN,[1] L. D. FOIL,[1] S. D. HAGIUS[2] AND C. J. ISSEL[3]

ABSTRACT. Equine infectious anemia virus (EIAV) was injected intrathoracically into *Aedes aegypti*, *Stomoxys calcitrans*, and *Tabanus fuscicostatus*, and fed to *Ae. aegypti* in suspensions of either artificial blood or Eagle's Minimum Essential Medium. Insects were stored at −70°C for up to 9 months before testing for the presence of EIAV. The viral tissue culture titers detected from stored insects were similar to those from insects tested at time 0.

Equine infectious anemia virus (EIAV) is a retrovirus with worldwide distribution that can produce a fatal, acute disease or a chronic infection in horses. The EIAV is transmitted mechanically either by man or by hematophagous Diptera (Foil and Issel 1991). There is no experimental evidence to indicate that retroviruses survive for long periods in arthropods. Foil et al. (1987) quantified the amount of blood remaining on the mouthparts of horse flies following an interrupted feeding and used these data to correctly estimate the number of horse flies required to transmit bovine leukemia virus and EIAV. However, such predictions of the probability of mechanical transmission of agents by insects are made with the assumption that there is no propagation or degradation of the agent. There have been indications that rapid degradation of EIAV may occur on mosquito mouthparts. Williams et al. (1981) found that EIAV survived only 1 h on mosquito mouthparts as compared to 96 h on needles. Differences in the results of 2 laboratory studies on the biological transmission of human immunodeficiency virus (HIV) were thought to be due to storage of HIV in insects at −70°C in one of the studies (Jupp and Lyons 1987, Webb et al. 1989). As a measure of quality control for future studies on survival of EIAV in insects, the objective of this study was to measure the effect of storage at −70°C on titers of EIAV in intrathoracically injected and orally fed insects.

Primary fetal equine kidney cells (FEK) were grown by standard techniques in Eagle's Minimum Essential Medium (EMEM) with Hepes buffer, penicillin, streptomycin, and amphotericin B. Cells were maintained by weekly addition of EMEM containing penicillin, streptomycin, and 3% fetal bovine serum (FBS). Cells were split using a 0.05% trypsin solution, dispersed to 75-cm$^2$ or 25-cm$^2$ flasks, and grown in EMEM with penicillin, streptomycin, and 10% FBS (Amborski et al. 1979).

Two EIAV strains (135-1 and F1LC) that were isolated by Rwambo et al. (1990) were used in the experiments. Stock solutions of isolate 135-1 and F1LC contained $10^{8.0}$ and $10^{6.5}$ $\log_{10}$ median tissue culture infective doses per milliliter ($TCID_{50}$/ml), respectively.

Mosquitoes, *Aedes aegypti* (Linn.) black-eye strain, were obtained from a colony in the Department of Veterinary Microbiology and Parasitology at the Louisiana State University School of Veterinary Medicine. Horse flies, *Tabanus fuscicostatus* Hine, were captured at the Thistlewaite Wildlife Management Area in St. Landry Parish, LA, with $CO_2$-baited canopy traps (Foil et al. 1989). Stable flies, *Stomoxys calcitrans* (Linn.), were obtained from a colony maintained at the U.S. Livestock Insects Laboratory at Kerrville, TX.

Mosquitoes were injected intrathoracically with 0.33 µl ($10^{3.0}$ $TCID_{50}$) of F1LC using an ISCO Microapplicator. Horse flies and stable flies were anesthetized at −20°C and injected with 2.5 µl ($10^{3.9}$ $TCID_{50}$) and 1.0 µl ($10^{3.5}$ $TCID_{50}$) of stock F1LC, respectively. All insects were placed in a Revco Ultralow™ freezer (−70°C) in groups of 5 either whole or homogenized in 0.7 ml of phosphate-buffered saline (PBS; $Ca^{2+}$- and $Mg^{2+}$-free, pH 7.4).

Artificial membranes were prepared by the methods of Butler et al. (1984), and feeding chambers were placed on a magnetic stirrer in a 37°C incubator. A 3-µl volume was used as an estimate for the amount ingested by each engorged mosquito (Klaus et al. 1993). Mosquitoes

[1] Department of Entomology, 402 Life Sciences Building, Louisiana Agricultural Experiment Station, LSU Agricultural Center, Baton Rouge, LA 70803.
[2] Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.
[3] Department of Veterinary Science, University of Kentucky, Lexington, KY 40546.

Table 1. Comparative titers ($log_{10}$ $TCID_{50}$/ml) of EIAV from mosquitoes, stable flies, and horse flies injected and homogenized or frozen intact at time 0 and at various intervals after storage at −70°C.

| Species | Time at −70°C | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 h | 24 h | 1.5 months | 2 months | 3.5 months | 8 months | 9 months |
| *Aedes aegypti* homogenized[1] | 1.3 | Negative | 1.4 | ND[3] | 1.5 | ND | 0.5 |
| *Aedes aegypti* whole[2] | 2.5 | 3.5[4] | 2.5 | ND | 2.0 | ND | 1.5 |
| *Stomoxys calcitrans* homogenized | 3.0 | 3.0 | ND | 3.5[4] | ND | 1.5 | 1.5 |
| *Stomoxys calcitrans* whole | 2.5 | 2.5 | ND | 2.5 | ND | 1.5 | ND |
| *Tabanus fuscicostatus* homogenized | 3.0 | 3.5 | ND | 2.5 | ND | 2.5 | 3.0 |
| *Tabanus fuscicostatus* whole | 4.0[4] | 4.5[4] | ND | 4.5[4] | ND | 4.0[4] | 3.5 |

[1] Insects homogenized immediately after injection and before storage.
[2] Insects stored intact at −70°C.
[3] ND = not done.
[4] Not different from the highest expected recovery (*Aedes aegypti,* 3.67; *Stomoxys calcitrans,* 4.2; *Tabanus fuscicostatus,* 4.6).

were fed suspensions of artificial blood (Kogan 1990) with $10^{8.2}$ $TCID_{50}$ 135-1 (approximately $10^{5.7}$ $TCID_{50}$/mosquito) or EMEM plus 5% FBS with $10^{6.2}$ $TCID_{50}$ of F1LC (approximately $10^{3.7}$ $TCID_{50}$/mosquito). Engorged mosquitoes were placed in vials and stored whole at −70°C in 3 groups of 5 for each sample period.

For virus titration, insects stored whole were homogenized in 0.7 ml of PBS. All homogenates were centrifuged at 12,800 × *g* for 2 min. A 0.5-ml sample of the supernatant was used to make dilutions for titration. Samples were diluted in 1.5 ml of EMEM containing penicillin, streptomycin, and amphotericin B, and 5% FBS. Tenfold dilutions were made to $10^{-4}$ or $10^{-5}$ for F1LC or to $10^{-6}$ for 135-1. One-half milliliter of the first dilution, and 1.0 ml of the remaining dilutions were placed on duplicate cultures of FEK in 25-$cm^2$ flasks with one control flask receiving no homogenate. The flasks were incubated at 37°C for 30 min to allow for viral adsorption, rinsed with PBS, and maintained as described. The culture media were tested 3 and 5 wk later for the presence of EIAV antigen using the methods described by Amborski et al. (1979). The infectivity was estimated using the Reed–Muench method (Reed and Muench 1938). A difference in titer of one $log_{10}$ or less was not considered significant (Amborski et al. 1979). For control, virus titration of appropriate virus stocks was conducted at each time point.

When insects were injected intrathoracically and stored at −70°C intact for up to 9 months, the virus titers varied less than one log from those sampled 1 h after freezing (Table 1). The $TCID_{50}$s from stable flies homogenized before storage and those stored for 2 months were equivalent, but the titers were lower after storage for 8–9 months (Table 1). The titers from mosquitoes and horse flies homogenized and then stored were equivalent to those at 1 h (Table 1).

The $TCID_{50}$s detected from mosquitoes fed either F1LC in EMEM + 5% FBS or 135-1 in artificial blood and then frozen whole at −70°C were equivalent to those from mosquitoes processed at time 0 (Table 2). The objective of this study was to determine if storage of insects at −70°C significantly affected recovery of EIAV, but there were indications that the relative amount of virus recovered was dependent upon route of exposure and insect species. The virus recovery from mosquitoes fed artificial blood or EMEM containing EIAV was consistently lower than expected (Table 2). The volume ingested was estimated for these insects, but all individuals were visibly replete before they were designated as engorged. Therefore, low recovery of EIAV from mosquitoes that have ingested EIAV may indicate antiviral activity in the mosquito digestion process.

Table 2. Comparative titers ($log_{10}$ $TCID_{50}$/ml) of EIAV from mosquitoes (*Aedes aegypti*) fed artificial blood with 135-1 or Eagle's Minimum Essential Medium with F1LC. Titers were less than the highest expected recovery (135-1, 6.2; F1LC, 4.7).

| Replicate | Time at −70°C | | | |
|---|---|---|---|---|
| | Time 0 | 4 wk | 7 wk | 9 wk |
| 1 135-1 | 3.5 | ND[1] | 3.5 | 5.0 |
| 2 135-1 | 4.0 | ND | 3.5 | 4.5 |
| 3 135-1 | 4.0 | ND | 4.5 | 4.0 |
| 1 F1LC | 1.5 | 3.5 | ND | ND |
| 2 F1LC | 2.5 | 2.5 | ND | ND |
| 3 F1LC | 2.75 | 2.5 | ND | ND |

[1] ND = not done.

The EIAV is stable at −70°C within insects. Thus, insects can be exposed to virus suspensions and stored intact at −70°C for subsequent testing. Studies comparing the kinetics of survival of EIAV in insects relative to ambient conditions would not be compromised by storing insects at −70°C.

This research was partially funded by the Grayson–Jockey Club Research Foundation, Inc. We are grateful to Kerney Sonnier and the staff of the Department of Wildlife and Fisheries, District 6, and the Thistlethwaite heirs for allowing us to use the facilities at the Thistlethwaite Wildlife Management Area. We thank Lacey Wieser-Schimpf for technical support. We thank T. R. Klei and Sharon Coleman for providing mosquitoes, and the ARS U.S. Livestock Insects Laboratory at Kerrville, TX, Sidney L. Kunz, Director, for providing stable flies. Approved for publication by the Director of the Louisiana Agricultural Experiment Station as Manuscript 92-17-6037.

## REFERENCES CITED

Amborski, G. F., G. Jeffers, R. L. Amborski and C. J. Issel. 1979. Equine infectious anemia virus: development of a simple reproducible method for titrating infectivity of the cell-adapted strain. Am. J. Vet. Res. 40:302–304.

Butler, J. F., W. R. Hess, R. G. Endris and K. H. Holscher. 1984. *In vitro* feeding of *Ornithodoros* ticks for rearing and assessment of disease transmission. Acarology VI 2:1075–1081.

Foil, L. D. and C. J. Issel. 1991. Transmission of retroviruses by arthropods. Annu. Rev. Entomol. 36: 355–381.

Foil, L. D., D. J. LePrince and G. E. Church. 1989. Changes in the parity rate of *Tabanus fuscicostatus* (Diptera: Tabanidae) populations associated with controlling available hosts. J. Med. Entomol. 26: 306–310.

Foil, L. D., W. V. Adams, J. M. McManus and C. J. Issel. 1987. Blood meal residues on mouthparts of *Tabanus fuscicostatus* (Diptera: Tabanidae) and the potential for mechanical transmission of pathogens. J. Med. Entomol. 24:613–616.

Jupp, P. G. and S. F. Lyons. 1987. Experimental assessment of bed bugs (*Cimex lectularius* and *Cimex hemipterus*) and mosquitoes (*Aedes aegypti formosus*) as vectors of human immunodeficiency virus. AIDS 1:171–174.

Klaus, R. M., L. D. Foil, C. J. Issel and D. J. LePrince. 1993. Insect blood meal studies using radiosodium $^{24}$Na and $^{22}$Na. J. Am. Mosq. Control Assoc. 9:264–268.

Kogan, P. H. 1990. Substitute blood meal for investigating and maintaining *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 27:709–712.

Reed, L. J. and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27:493–497.

Rwambo, P. M., C. J. Issel, K. A. Hussain and R. C. Montelaro. 1990. *In vitro* isolation of a neutralization escape mutant of equine infectious anemia virus (EIAV). Arch. Virol. 111:275–280.

Webb, P. A., C. M. Happ, G. O. Maupin, B. J. B. Johnson, C. Ou and T. P. Monath. 1989. Potential for insect transmission of HIV: experimental exposure of *Cimex hemipterus* and *Toxorhynchites amboinensis* to human immunodeficiency virus. J. Infect. Dis. 160:970–977.

Williams, D. L., C. J. Issel, C. D. Steelman, W. V. Adams, Jr. and C. V. Benton. 1981. Studies with equine infectious anemia virus: transmission attempts by mosquitoes and survival of virus on vector mouthparts and hypodermic needles, and in mosquito tissue culture. Am. J. Vet. Res. 42:1469–1473.