Virus-Dependent Mortality in Rift Valley Fever, Eastern Equine Encephalomyelitis, and Chikungunya Virus-Inoculated Mosquito (Diptera: Culicidae) Larvae

MICHAEL J. TURELL

Department of Epidemiology, Disease Assessment Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702

J. Med. Entomol. 29(5): 792–795 (1992)

ABSTRACT The effect of inoculation of mosquito larvae with Rift Valley fever (RVF) virus on survival to the adult stage was evaluated in *Aedes aegypti* (L.), *Ae. fowleri* (Charmoy), *Ae. mcintoshi* Huang, *Ae. taeniorhynchus* (Wiedemann), *Ae. triseriatus* (Say), *Eretmapodites quinquevittatus* Theobald, *Anopheles albimanus* Wiedemann, and *Culex pipiens* L. Pupation rates were similar for RVF virus-inoculated and diluent-inoculated larvae of all mosquito species tested except *Cx. pipiens*. However, with the exception of *An. albimanus* and *Ae. triseriatus*, virtually all pupae derived from RVF virus-inoculated larvae failed to emerge successfully as adults. In contrast, both pupation and emergence rates were similar for diluent-inoculated and either La Crosse or St. Louis encephalitis virus-inoculated larvae of *Ae. taeniorhynchus*. There was also poor survival to the adult stage of *Ae. taeniorhynchus* inoculated with either eastern equine encephalomyelitis (EEE) or chikungunya (CHIK) virus. The high mortality rates observed under laboratory conditions of pupae derived from larvae inoculated with either RVF, EEE, or CHIK virus may be responsible for the lack of laboratory confirmation of vertical transmission of these viruses.

KEY WORDS Insecta, Culicidae, inoculation, arboviral, mortality

ALTHOUGH RIFT VALLEY FEVER (RVF) VIRUS has been recovered from adult *Aedes mcintoshi* Huang (reported as *Ae. lineatopennis* (Ludlow)) reared from field-collected larvae and pupae (Linthicum et al. 1985), we have been unable to demonstrate vertical transmission of this virus in the laboratory (M.J.T. & L. A. Patrican, unpublished data). In an experiment to determine if RVF virus could be transmitted transstadially from *Culex pipiens* L., *Ae. aegypti* (L.), or *Ae. taeniorhynchus* (Wiedemann) larvae to adults, infection with this virus was lethal to mosquito larvae (Turell et al. 1985). These species are not associated with enzootic RVF virus in nature; therefore, the mortality associated with RVF virus infection may have been because of the use of inappropriate mosquito species. To determine whether the virus-associated mortality reported for *Cx. pipiens* was due to the unnatural virus–vector relationship, we inoculated larvae of several mosquito species with RVF virus, including *Ae. mcintoshi*, a species known to transmit RVF virus vertically in Kenya (Linthicum et al. 1985). In addition, to determine if other viruses also caused excess mortality, *Ae. taeniorhynchus* larvae were inoculated with several different viruses, including some known to be vertically transmitted.

Material and Methods

Mosquitoes. *Anopheles albimanus* Wiedemann and *Ae. taeniorhynchus* (VERO BEACH) were provided by S. Dobson of the U.S. Army Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, Md., and *Ae. aegypti* (BLACK EYE) and *Ae. triseriatus* (Say) (WALTON) were provided by A. Spielman of the Harvard School of Public Health. Information was not available on the strain designation for the *Anopheles* species, and the number of generations of colonization was not known for these species.

Culex pipiens (EL GABAL), derived from specimens collected in an RVF virus enzootic area in Egypt in 1981 (Gargan et al. 1983), were used in the 38th to 94th generations of colonization. This species was implicated as a vector during the 1977 epidemic of RVF in Egypt (Meegan et al. 1980). *Ae. mcintoshi* were the F_1 progeny of adult females collected near a dambo in Ruiru,

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities at USAMRIID are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Table 1. Source and passage history of viruses used

Virus[a]	Strain	Source	Passage history[b]
CHIK	1455/75	Human	Mosq-2, C6/36-2
EEE	MP-42	*Culiseta melanura*	LLC-1
LAC	WIS-78-40	*Aedes triseriatus*	Mosq-4
RVF	ZH501	Human	FRL-2
SLE	Parton	Human	?, SMB-1

[a] CHIK, chikungunya virus; EEE, eastern equine encephalomyelitis virus; LAC, La Crosse virus; RVF, Rift Valley fever virus; SLE, St. Louis encephalitis virus.
[b] Mosq, mosquito; LLC, LLC-MK_2 is a rhesus monkey kidney cell line; C6/36, an *Aedes albopictus* cell line; FRL, fetal rhesus lung; SMB, suckling mouse brain passage.

Table 2. Survival of selected mosquito species after inoculation as larvae with either diluent or RVF virus[a]

Mosquito species	Pupation rate[b]		Emergence rate[c]	
	Diluent	RVF virus	Diluent	RVF virus
Ae. aegypti	76 (33)	85 (40)	80 (25)	0 (34)
Ae. fowleri	79 (24)	71 (24)	58 (19)	0 (17)
Ae. mcintoshi	95 (20)	80 (30)	53 (19)	4 (24)
Ae. taeniorhynchus	89 (74)	90 (81)	64 (66)	4 (73)
Ae. triseriatus	92 (104)	87 (86)	76 (96)	55 (75)
An. albimanus	82 (62)	80 (95)	75 (51)	25 (76)
Cx. pipiens	78 (90)	57 (136)	73 (70)	0 (77)
Er. quinquevittatus	94 (32)	77 (22)	77 (30)	6 (17)

[a] Fourth instars were inoculated with ≈0.2 μl of inoculum containing diluent or $10^{1.7-2.0}$ PFU of RVF virus.
[b] Percentage pupating (number of larvae).
[c] Percentage successfully emerging as adults (number of pupae).

Kenya, in 1985. *Aedes fowleri* (Charmoy) were derived from adult females collected near a dambo in Senegal in 1983 (Turell et al. 1988) and were used in the fourth generation. *Eretmapodites quinquevittatus* Theobald, derived from specimens collected in South Africa, were provided by T. Zavortink of the University of San Francisco and were used in the 11th generation. All larvae were reared under standard conditions at 26°C (Gargan et al. 1983).

Viruses and Virus Assays. Table 1 indicates the strains and passage histories of the viruses used in this study. Individual specimens (larvae, pupae, or adults) were triturated in 1 ml of diluent (10% fetal bovine serum in Medium 199 (GIBCO Laboratories, Grand Island, NY) with Hanks' salts and antibiotics) and frozen at −70°C until plaque-assayed for infectious virus in Vero cell monolayers. Procedures described by Gargan et al. (1983) were followed, except the time before the addition of the second overlay, containing neutral red, was dependent on the virus being assayed. Virus titers were expressed as $\log_{10}$ plaque-forming units (PFU) per specimen.

Experimental Design. In experiment 1, fourth instars of selected mosquito species were inoculated intrathoracically (Turell et al. 1985) with 0.2 μl of either diluent or diluent containing $\approx 10^2$ PFU of RVF virus. The next day, all pupae and dead larvae were discarded, and the active fourth instars were counted and transferred to clean water and provided with ground Tetramin (TetraWerke, Melle, Germany) as food. Pupae and dead larvae were counted and removed daily. Upon removal, pupae were washed in tap water and placed in ≈50 ml of water in a plastic container inside a 0.5-liter cardboard container with netting at one end. These pupae were observed daily, and when all of the pupae had either emerged or died, adult mosquitoes (1–7 d after emergence) were removed, triturated individually in 1 ml of diluent, and frozen at −70°C until they were assayed for virus. For purposes of this study, a mosquito must have emerged and left the water surface to be considered as a successfully emerged adult. For each species, the percentage of fourth instars that pupated (pupation rate) and the percentage of pupae that successfully emerged as an adult (emergence rate) were calculated.

In a second experiment, fourth instars of *Ae. taeniorhynchus* were inoculated with either diluent or one of the viruses listed in Table 1. Each virus suspension was color-coded so that the person inoculating the larvae was unaware of the contents of each inoculum. There were two replicates of this experiment. As in experiment 1, active fourth instars on the day after inoculation were used to initiate the experiment. In addition to the fourth instars used to monitor pupation and adult emergence, a second group of inoculated fourth instars was assayed daily to determine the growth curves of each virus.

Results

Comparison of Selected Mosquito Species. The pupation rate for *Cx. pipiens* larvae inoculated with RVF virus was significantly ($\chi^2 = 9.8$, df = 1, $P = 0.002$) lower than that for larvae inoculated with diluent. For each of the remaining species, however, pupation rates for larvae inoculated with RVF virus were not significantly ($\chi^2 \leq 1.85$, df = 1, $P \geq 0.17$) different than those of larvae inoculated with diluent (Table 2). In contrast, emergence rates for all species tested were significantly lower (Fisher's exact test, $P < 0.005$) for adults that had been inoculated with RVF virus as larvae than for those that had been inoculated with diluent as larvae. In fact, with the exception of *An. albimanus* and *Ae. triseriatus*, virtually none of the RVF virus-inoculated larvae emerged successfully as adults. Virus was recovered from all of the adults derived from the RVF virus-inoculated larvae.

Comparison of Selected Viruses in Ae. taeniorhynchus. Except for chikungunya (CHIK) virus, pupation rates were similar for virus-inoculated and diluent-inoculated larvae (Table 3). The pupation rate for larvae inoculated with CHIK virus

Table 3. Survival of *Ae. taeniorhynchus* after inoculation of larvae with selected viruses[a]

Virus	No. larvae[b]	Pupation rate[c]	Emergence rate[d]
Diluent	67	91	64a
La Crosse	65	91	69a
St. Louis encephalitis	62	95	61a
Chikungunya	62	66	41b
Eastern equine encephalomyelitis	56	93	15c
Rift Valley fever	60	90	6c

[a] Fourth instars were inoculated with ≈0.2 μl of inoculum containing $10^{0.7-2.0}$ PFU of virus.
[b] Number of live larvae 24 h after inoculation.
[c] Percentage of larvae that pupated.
[d] Percentage of pupae that successfully emerged as adults. Emergence rates followed by the same letter were not significantly different (Fisher's exact test, $\alpha = 0.05$).

was significantly ($\chi^2 \geq 8.7$, $P < 0.005$) lower than that for those inoculated with diluent or any of the other viruses. Although emergence rates were similar for larvae inoculated with diluent, La Crosse (LAC) virus, or St. Louis encephalitis (SLE) virus, successful emergence as adults was significantly lower for larvae inoculated with CHIK, EEE, or RVF viruses (Table 3). In addition, the two viruses not associated with increased mortality, LAC and SLE viruses, did not replicate as rapidly as did the three viruses associated with reduced emergence rates (Fig. 1).

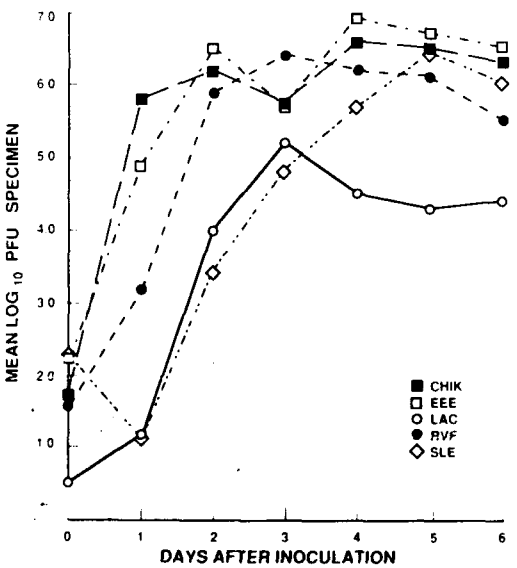

Fig. 1. Replication of selected viruses in *Ae. taeniorhynchus* after inoculation of fourth instars. Each point represents the mean viral titer ($\log_{10}$ PFU) recovered from an average of seven specimens. For each virus, titers recovered from larvae, pupae, or adults on the same day after virus inoculation were similar and were averaged together.

Recovery of virus from emerged adults indicated that they were infected with the appropriate virus.

Discussion

The present study confirms and expands the results of Turell et al. (1985), which showed that inoculation of fourth-instar *Ae. aegypti*, *Ae. taeniorhynchus*, *Ae. triseriatus*, and *Cx. pipiens* larvae with RVF virus was generally lethal to mosquitoes. With the possible exception of CHIK virus in *Ae. taeniorhynchus* and RVF virus in *Cx. pipiens*, inoculation of virus into fourth instars did not affect pupation rates. However, emergence rates were significantly reduced for those species that had been inoculated as fourth instars with CHIK, EEE, or RVF viruses. This mortality appeared to be caused by these viruses, not merely due to the inoculation procedure, as larvae of all of the species tested pupated and emerged successfully as adults after inoculation with diluent. Also, larvae of *Ae. taeniorhynchus* inoculated with either LAC or SLE viruses pupated and adults emerged successfully.

The mortality observed with inoculation of CHIK, EEE, and RVF viruses may be due to pathogenic effects of virus infection or may be the result of starvation as a consequence of the energy requirements associated with viral replication during the nonfeeding pupal stage. It is possible that although sufficient energy reserves were present in the larvae for them to pupate successfully, because of the timing of infection, these energy reserves were depleted before the mosquito could emerge successfully. If this is true, the mortality observed in this study would not apply to vertically infected larvae because most of the virus replication would have taken place before pupation, and the larvae would have had the opportunity to feed longer to replenish their energy reserves. In fact, for many of the viruses studied, vertically infected larvae take longer to develop from egg to pupal stage than do their uninfected siblings (Beaty et al. 1980, Tesh 1980, Turell et al. 1982b). Also, in the present study, virus titers in pupae 24–48 h after inoculation with LAC or SLE viruses were 100–1,000-fold lower than those in pupae inoculated with CHIK, EEE, or RVF viruses after the same time interval. It was not until mosquitoes were in the adult stage that titers recovered from mosquitoes inoculated with SLE virus attained levels reached in the pupal stage in mosquitoes inoculated with CHIK, EEE, or RVF viruses. Thus, the rapid replication associated with CHIK, EEE, and RVF viruses and the timing of infection 24–72 h before pupation may have been responsible for the reduced emergence rates observed in these mosquitoes.

In contrast to the extremely high rates of mortality observed for *Cx. pipiens* and *Ae. mcintoshi*

larvae inoculated with RVF virus in the present study, both of these species became infected, pupated, and emerged successfully as adults when fourth instars ingested RVF virus-infected tissue (Turell et al. 1990). Perhaps the delay in the development of a disseminated infection associated with oral exposure allowed for the completion of pupation before maximum viral replication occurred.

Additional studies, conducted at lower temperatures with larvae infected earlier (i.e., inoculated as second instars) and with orally exposed larvae, are needed to evaluate this phenomenon further. Although inoculation of larvae is an artificial means of infecting a mosquito, it may enable us to study vertical infection in certain mosquito–virus combinations. After a mosquito becomes infected as a result of either ingestion of a viremic blood meal or by intrathoracic inoculation, infection of the germinal tissue may be a rare event with some mosquito–virus combinations because of the various membranes involved. However, progeny infected vertically would already have a germinal tissue infection, thus would be able to transmit virus at a high rate. This type of stabilized infection has been described for San Angelo (Tesh & Shroyer 1980) and California encephalitis viruses (Turell et al. 1982a). Inoculation of mosquito larvae may be a means of initiating a stabilized infection for these viruses in the laboratory.

Acknowledgment

I thank J. Kondig and his insectary staff for their assistance in rearing the mosquitoes, C. Adams and J. Beaman for their technical assistance, and L. Durden and J. Freier for their critical reading of the manuscript.

References Cited

Beaty, B. J., R. B. Tesh & T.H.G. Aitken. 1980. Transovarial transmission of yellow fever virus in *Stegomyia* mosquitoes. Am. J. Trop. Med. Hyg. 29: 125–132.

Gargan, T. P., II, C. L. Bailey, G. A. Higbee, A. Gad & S. El Said. 1983. The effect of laboratory colonization on the vector pathogen interactions of Egyptian *Culex pipiens* and Rift Valley fever virus. Am. J. Trop. Med. Hyg. 32: 1154–1163.

Linthicum, K. J., F. G. Davies, A. Kairo & C. L. Bailey. 1985. Rift Valley fever virus (family Bunyaviridae, genus *Phlebovirus*). Isolations from Diptera collected during an inter-epizootic period in Kenya. J. Hyg. Cambridge 95: 197–209.

Meegan, J. M., G. M. Khalil, H. Hoogstraal & F. K. Adham. 1980. Experimental transmission and field isolation studies implicating *Culex pipiens* as a vector of Rift Valley fever virus in Egypt. Am. J. Trop. Med. Hyg. 29: 1405–1410.

Tesh, R. B. 1980. Experimental studies on the transmission of Kunjin and San Angelo viruses in mosquitoes. Am. J. Trop. Med. Hyg. 29: 657–666.

Tesh, R. B. & D. A. Shroyer. 1980. The mechanism of arboviral transovarial transmission in mosquitoes: San Angelo virus in *Aedes albopictus*. Am. J. Trop. Med. Hyg. 29: 1394–1404.

Turell, M. J., J. L. Hardy & W. C. Reeves. 1982a. Stabilized infection of California encephalitis virus in *Aedes dorsalis*, and its implications for viral maintenance in nature. Am. J. Trop. Med. Hyg. 31: 1252–1259.

Turell, M. J., W. C. Reeves & J. L. Hardy. 1982b. Transovarial and trans-stadial transmission of California encephalitis virus in *Aedes dorsalis* and *Aedes melanimon*. Am. J. Trop. Med. Hyg. 31: 1021–1029.

Turell, M. J., T. P. Gargan, II & C. L. Bailey. 1985. *Culex pipiens* (Diptera: Culicidae) morbidity and mortality associated with Rift Valley fever virus infection. J. Med. Entomol. 22: 332–337.

Turell, M. J., M. E. Faran, M. Cornet & C. L. Bailey. 1988. Vector competence of Senegalese *Aedes fowleri* (Diptera: Culicidae) for Rift Valley fever virus. J. Med. Entomol. 25: 262–266.

Turell, M. J., K. J. Linthicum & J. R. Beaman. 1990. Transmission of Rift Valley fever virus by adult mosquitoes after ingestion of virus as larvae. Am. J. Trop. Med. Hyg. 43: 677–680.

Received for publication 21 February 1991; accepted 30 March 1992.