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EXPERIMENTAL TRANSMISSION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS BY *DEINOCERITES PSEUDES* DYAR AND KNAB, 1909[1]

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Abstract: In 5 different experiments, adult female laboratory-reared *Deinocerites pseudes* mosquitoes, which engorged on infected guinea pigs circulating $10^{5.2}$ LD_{50}'s or more of Venezuelan equine encephalitis virus in the blood, transmitted infections to normal guinea pigs or hamsters 7 to 27 days after exposure to the donor animals. The infection rate in 50 mosquitoes tested 16 to 20 days after feeding on a viremic guinea pig was 96%. Virus titers in 18 of the specimens tested ranged from $10^{4.2}$ to $10^{7.2}$ LD_{50}'s. The transmission rate in 12 infected individuals, each of which engorged on a single hamster 15 or 16 days after ingestion of an infective blood meal, was 42%. The potential role of this species in natural transmission cycles of Venezuelan equine encephalitis in Panama is discussed.

Venezuelan equine encephalitis (VEE) virus was first shown to be arthropod-borne by Gilyard (1944) during the investigation of an equine epizootic which occurred in Trinidad from 1943 to 1944. In studies of this outbreak, *Mansonia titillans* was found naturally infected with the virus and wild-caught mosquitoes of this species transmitted a fatal infection to a healthy donkey.

Subsequently, several species of anopheline and culicine mosquitoes were found naturally infected with VEE virus in Colombia (Groot et al. 1961), Venezuela (Sellers et al. 1965) and Guatemala (Sudia et al. 1971), countries in which extensive human and equine outbreaks have occurred. Viruses of the VEE complex have also been isolated from sabethine mosquitoes in Brazil (Shope et al. 1964).

In field studies carried out in Panama (Grayson & Galindo 1968), Trinidad (Jonkers et al. 1968) and Florida (Chamberlain et al. 1969), VEE was shown to be endemic in certain regions. Although the virus was detected in a variety of anopheline, culicine and sabethine species from these areas, infections were most prevalent in mosquitoes of the *Culex* (*Melanoconion*) complex.

In Mexico, where endemic foci of VEE have also been found (Scherer et al. 1964), a crab-hole breeding mosquito, *Deinocerites pseudes*, was found harboring the virus (Scherer, pers. commun.). This species, which was described from material collected in Panama, is distributed along the Pacific and Atlantic coasts of Middle America from eastern Panama north to Texas (Peyton et al. 1964). It is very abundant in the Pacific coastal flats of Panama where it has been found infected with St. Louis encephalitis (SLE) virus (Grayson et al. 1967). This natural occurrence of VEE and SLE in *D. pseudes*, a common but little-known culicine, prompted us to investigate the ecology and vector potential of this mosquito in Panama.

Accordingly, *D. pseudes*, collected from an area of Panama devoid of VEE, was established as a colony at the Gorgas Memorial Laboratory, where studies on the bionomics of this species were carried out (Galindo 1967). The results of transmission experiments with VEE virus and laboratory-reared *D. pseudes* mosquitoes from this colony are presented here.

MATERIALS AND METHODS

Mosquitoes used in these experiments were obtained from a *D. pseudes* colony in the 10th generation. This colony was established at the Rand Insectary of Gorgas Memorial Laboratory from 150 larvae collected at Nueva Gorgona, District of Chame, Province of Panama, Republic of Panama. The #3880 strain (Johnson et al. 1968) of VEE virus was used at the 9th passage level for infection of donor animals, all of which were adult guinea pigs (*Cavia porcellus*). Recipient vertebrates used in these studies were either guinea pigs or golden hamsters (*Mesocricetus auratus*).

Female mosquitoes were obtained from pupae isolated individually in shell vials. Newly emerged specimens were transferred in groups of 200 to plaster-of-Paris-lined glass beakers with a small hole in the bottom and a taped cardboard cover containing an aperture in the center for introduction and removal of insects. A slit in the plastered wall permitted vision inside the container. Beakers with mosquitoes were held in pans containing just enough water for capillary rise to occur along the plastered walls, thus maintaining a high humidity inside the container. Females were given access to sugar solution for 2 days by means of a soaked dental wick inserted through the opening in the top, after which they were starved for 3 days before exposure to the donor host.

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Guinea pigs used as donors in these transmission experiments were injected intraperitoneally with 0.2 ml of VEE virus suspension containing $10^{6.0}$ or $10^{4.5}$ median lethal doses ($LD_{50's}$) for weanling mice, the inoculum being titrated immediately prior to injection of the guinea pig. Two to 3 days after inoculation of the donor vertebrate, starved adult female *D. pseudes* mosquitoes, hatched within 24 hr of each other, were transferred in groups of 50 or 100 to a small round plastic cage, with a screen disk inserted in the cover. One cage was attached to each side of the infected guinea pig with the screened top in contact with the clipped skin of the immobilized animal. One hundred or 200 mosquitoes were allowed to feed through the screen disks on the donor host for 4 to 5 hr. Blood for infectivity titrations was obtained from the donor animal near the end of or shortly after the exposure period.

The following morning fully engorged mosquitoes were transferred to the original beaker and held for the desired incubation period at 26.5°±1.2°C with 85–100% relative humidity, during which time fresh sugar solution was made available to them every 2 days. Mosquitoes were deprived of all food 3 or 4 days before exposure to recipient hosts. Only mosquitoes which engorged upon an infective donor guinea pig were given access to recipient animals. Guinea pigs were exposed to groups of infected mosquitoes in the manner described for exposure of the donor vertebrates. Hamsters used as recipients were exposed to the bites of single mosquitoes by means of a small covered plastic box with a screened bottom taped to the shaved skin of the hamster. Infected mosquitoes were allowed to feed on recipient hosts for 4 to 5 hr.

All attempts to isolate virus were made in Swiss white mice, 3 to 4 days of age, using 0.02 ml of inoculum per mouse for each route of injection employed. Healthy mosquitoes were sacrificed at specified intervals following exposure by quick-freezing. Moribund or dead mosquitoes were removed from beakers twice a day, quick-frozen and stored at −65°C. Mosquito pools of 1 to 10 specimens were triturated in 1.0 ml of 10% rabbit serum saline (RSS) solution (Grayson & Galindo 1968), adding 0.1 ml of diluent for every additional 5 mosquitoes. Suspensions of sacrificed mosquitoes were centrifuged at 2000 rpm for 30 min. at 4°C whereas suspensions of dead mosquitoes were spun at 4000 rpm for 60 min. in the cold. All clarified mosquito suspensions were inoculated without dilution in a litter of baby mice by the combined intracerebral and intraperitoneal routes. Blood sera from guinea pigs were diluted with 9 volumes of RSS and injected intracerebrally in infant mice. Samples of liver, heart and brain tissue from moribund or dead animals were collected aseptically, pooled by individual, and processed for virus isolation attempts. The procedures used for processing tissue samples and for the serial passage of suspected virus isolates are described elsewhere (Grayson & Galindo 1968).

Virus titrations were performed in male weanling mice, 3 to 4 weeks of age. Inocula were injected intraperitoneally, using 0.02 ml per mouse. Titration end-points, based on the number of mice surviving on the 8th post-inoculation day, were determined by the method of Reed & Muench (1938). Deaths occurring on the 1st or 2nd day of the test as well as those resulting from cannibalism were considered non-specific and were therefore excluded from the results.

Virus isolates were identified by complement-fixation (CF) technique (Kerr 1952). A crude antigen was prepared for each isolate using 1st or 2nd passage infected mouse brain. Antigens were tested at a dilution of 1: 8 with serial dilutions of antiserum prepared by 2 injections of VEE virus (# 3880) in adult mice. Some of the isolates from mosquitoes, donors and recipients were compared with the reference VEE virus strain in cross grid titrations employing one-injection antisera. Attempts to detect antibody conversions in surviving recipient animals were made by testing sera collected at specified intervals during the first 6 weeks after exposure for hemagglutination-inhibition (HI) antibodies to VEE virus. Serum specimens were titrated with 4 units of VEE (# 3880) hemagglutinating antigen, utilizing the technique of Clarke & Casals (1958). The preparation of reagents employed in the CF and HI tests and modifications thereof are described in another report by Grayson & Galindo (1968).

RESULTS

VEE virus infections in mosquitoes. Five infected guinea pigs, circulating at least $10^{7.2}$ $LD_{50's}$ of VEE virus per ml of blood, were exposed to the bites of 900 *D. pseudes* females, of which 207 (23%) engorged. Twenty-two mosquitoes, found dead on post-exposure days 6 through 33, were tested in 10 pools for the presence of virus and found positive. A single specimen found dead 2 days after exposure to an infected guinea pig gave negative results. Four additional pools containing 26 live mosquitoes sacrificed on post-exposure days 9, 16, 20 and 32 were also positive for virus.

TABLE 1. Transmission of Venezuelan equine encephalitis virus to guinea pigs by experimentally infected *Deinocerites pseudes* mosquitoes.

Donor feeding			Recipient feeding		
Experiment number	Virus titer	Engorged / Exposed	Days after infective feeding	Engorged / Exposed	Virus titer
1	7.2*	40/200	7	12/16	5.3*
1	7.2*	40/200	10	11/13	7.9
2	7.3	65/200	15	34/56	7.9
3	8.3	8/200	19	7/7	7.3
4	8.7	25/100	19	19/23	8.3

*Log_{10} $LD_{50's}$/ml of guinea pig blood serum injected intraperitoneally in weanling mice.

For estimation of the infection rate, 25 live mosquitoes, which engorged on an infected donor guinea pig with a serum titer of $10^{7.9}$ $LD_{50's}$ of virus, were sacrificed 16 to 18 days after exposure and tested individually for the presence of virus. VEE virus was isolated from 24 of these. Another 25 mosquitoes, sacrificed 20 days after feeding on the blood of an infected guinea pig with a titer of $10^{7.3}$ $LD_{50's}$, were also processed singly for virus isolation attempts. Twenty-four were positive. Thus, the infection rate in 50 mosquitoes sacrificed 16 to 20 days after ingestion of an infective blood meal was 96%. Eighteen of the infected mosquitoes selected for titration had virus concentrations ranging from $10^{4.2}$ to $10^{7.2}$ $LD_{50's}$, with a mean titer of $10^{5.7}$.

Transmission of VEE virus to guinea pigs. The results of attempts to transmit VEE virus to guinea pigs through the bites of infected *D. pseudes* mosquitoes are summarized in TABLE 1. Virus titers in the blood sera of 4 donor guinea pigs near the end of the exposure period ranged from $10^{7.2}$ to $10^{8.7}$ $LD_{50's}$. All of the donor animals became ill and were sacrificed or found dead on the 3rd or 4th post-injection day. Of the 700 mosquitoes exposed to 4 infected guinea pigs, 138 (19.7%) were observed to take a blood meal.

Following the infective feeding, engorged mosquitoes were held 7 to 19 days before exposure to normal adult guinea pigs. In these experiments, 83 (72.2%) of 115 mosquitoes given access to 5 recipient guinea pigs engorged. VEE virus was transmitted to each of the recipients which exhibited viremias with titers ranging from $10^{5.3}$ to $10^{8.3}$ $LD_{50's}$ on the 2nd or 3rd post-exposure day. All 5 recipient guinea pigs succumbed to infection 3 to 7 days after exposure to mosquitoes, and tissue samples from the 3 animals tested yielded VEE virus.

Transmission of VEE virus to hamsters. In Experiment 5, an infected guinea pig was exposed to the bites of 200 *D. pseudes* mosquitoes and 69 engorged. Blood serum obtained from this donor during the latter part of the exposure period had a titer of $10^{7.9}$ $LD_{50's}$ of virus. The animal was found dead 2 days later at which time VEE virus was also isolated from tissue samples. Fifteen days after this donor feeding, 30 golden hamsters were each exposed to the bites of a single *D. pseudes* female, of which 8 engorged. VEE virus was transmitted to 6 of the hamsters in this group. The following day, 14 additional mosquitoes were allowed to feed individually on another group of golden hamsters. Six of these mosquitoes took a blood meal and 6 of the recipients in this group were also positive for VEE virus.

In addition, 11 mosquitoes exposed in a previous experiment to a donor guinea pig circulating $10^{8.7}$ $LD_{50's}$ of VEE virus were each given access to a single golden hamster 27 days after ingestion of the infective meal. Four of these mosquitoes were observed to feed and virus transmission was obtained in 2 instances.

In summary, 55 *D. pseudes* mosquitoes were given access individually to normal golden hamsters 15 to 27 days after the initial infective feeding and 18 engorged. Fourteen of the 55 recipient hamsters were moribund or dead on the 2nd or 3rd day following exposure. In each case, organ pools of liver, heart and brain tissue yielded VEE virus. The 41 surviving hamsters remained serologically negative for 2 to 6 weeks following exposure, during which time they were sacrificed or died. Thus, 14 (26%) of the 55 exposed *D. pseudes* mosquitoes transmitted VEE virus to a recipient hamster. Transmissions were effected by 7 (39%) of 18 engorged and 6 (17%) of 36 unengorged mosquitoes, re-

TABLE 2. Transmission of Venezuelan equine encephalitis virus to hamsters by experimentally infected individual *Deinocerites pseudes* mosquitoes.

Specimen number	Virus titer in mosquito	Transmission to recipient hamster
TE 72	4.5*	−
TE 63	4.7	−
TE 75	4.9	−
TE 59	5.1	−
TE 74	5.4	−
TE 60	6.3	+
TE 61	6.3	+
TE 76	6.3	+
TE 73	6.4	+
TE 67	6.6	−
TE 52	6.9	−
TE 70	7.1	+

*Log_{10} $LD_{50's}$ for weanling mice injected intraperitoneally.

spectively. One *D. pseudes* female which was not classified with respect to engorgement also transmitted.

These results indicate that some of the mosquitoes which fed upon the recipient host either did not become infected during the donor feeding or were unable to transmit the infection under the conditions of the experiment. The fact that several unengorged mosquitoes also transmitted VEE virus to hamsters suggests that they probed or partially fed upon their host. Since it was not ascertained if all 55 females were infected as a result of the initial donor feeding or if all unengorged mosquitoes probed their respective recipient hosts, the rate of virus transmission was determined from the results of exposures employing only infected, engorged specimens. Accordingly, 12 mosquitoes, each of which had engorged upon a normal hamster 15 or 16 days after the donor feeding, were sacrificed 2 or 3 days after the recipient feeding and tested individually for the presence of virus. All yielded VEE virus in high concentration, with titers ranging from $10^{4.5}$ to $10^{7.1}$ $LD_{50's}$. The transmission rate in this group of mosquitoes, all of which were known to be infected and to have fed upon the recipient host, was 42%. As shown in TABLE 2, only those specimens containing $10^{6.3}$ $LD_{50's}$ or more of virus transmitted the infection. However, the ability to transmit was not correlated with the amount of virus in the latter group of mosquitoes.

DISCUSSION

In spite of numerous isolations of VEE virus from mosquitoes in Panama (Galindo et al. 1966), only 1 species, *Culex (Melanoconion) aikenii*, has been shown conclusively to transmit the virus in nature (Galindo & Grayson 1971). In Almirante, an endemic area of Panama, VEE virus infections in *Culex pipiens quinquefasciatus* and *Culex (Melanoconion) taeniopus* were found in close association with infections in humans and sentinel mice, respectively, but proof of transmission was not obtained (Grayson & Galindo 1968).

Although *D. pseudes* and several related species occur abundantly in Panama, members of this group have not yet been found infected with VEE virus there, a fact which may be due to their distribution, feeding habits, susceptibility to infection or lack of sufficient testing. Between 1964 and 1967, nearly 21,000 *Deinocerites* mosquitoes, of which *D. pseudes* comprised less than 5.0%, were collected from areas of central, eastern and western Panama for arbovirus surveys. VEE virus was not obtained from any of these specimens (unpubl. data).

In studies on the feeding habits of this genus in Panama, Tempelis & Galindo (1970) found that all of the 5 species tested showed a preference for avian and/or reptilian blood. *D. pseudes*, in particular, attacked reptiles, birds, mammals and amphibians in order of decreasing frequency. Host preferences of female *D. pseudes* in this study were correlated with the availability of vertebrate fauna in the immediate vicinity of the collecting site, a fact which is not too surprising in view of the short flight range exhibited by females of this species (Peyton et al. 1964). Lizards were extremely abundant around the crab-holes inhabited by these mosquitoes. Shore birds, wading birds, vultures, doves and numerous species of passeriform birds were present. Wild mammals were relatively scarce except for some species of rodents, opossums, sloths and bats. Domestic mammals, on the other hand, were quite common in the area, a detailed description of which is given by Tempelis & Galindo (1970). In this study, horses, cows and dogs provided most of the mammalian blood meals for *D. pseudes*. Avian blood was obtained largely from columbiform, ciconiiform and passeriform species while lizards constituted the sole source of reptilian blood for these mosquitoes.

Peyton et al. (1964) have also found that, given the opportunity, *D. pseudes* females readily bite man under certain atmospheric conditions. Thus, this species exhibits truly versatile feeding habits, attacking humans, domestic animals and a variety of terrestrial and arboreal wild vertebrates. As pointed out by Tempelis & Galindo (1970), no other species or group thus far studied has shown the host range exhibited by *D. pseudes*, *D. epitedeus* and *D. cancer*, each of which feeds upon animals in all 4 classes of land vertebrates.

Many of the vertebrate species which serve as hosts for *D. pseudes* have been found infected with VEE virus in the field (Grayson & Galindo 1968). Others have produced viremias of varying intensity and duration after experimental infection with the virus (Grayson & Galindo 1969). Several of these are of particular interest as potential donors of VEE virus to *Deinocerites* mosquitoes in nature.

The Green Heron (*Butorides virescens*), a common resident of the brackish swamps in which *D. pseudes* and related species breed, has been found naturally infected with VEE virus in Almirante. Virus was isolated from 2 nestling herons, one of which proved to be viremic (Grayson & Galindo 1968). A close relative, the Striated Heron (*B. striatus*), also appears to be a potentially good reservoir of VEE virus. After injection with low doses of the virus, these birds exhibited fairly high-titered viremias of

several days duration (Grayson & Galindo 1969).

Experimentally infected three-toed sloths (*Bradypus griseus*) circulated VEE virus for 1 to 2 weeks presenting high titers during periods of peak viremia (Grayson & Galindo 1969). This species is a common arboreal inhabitant of the mangrove swamp at Nueva Gorgona where *D. pseudes* was collected for host-feeding studies (Tempelis & Galindo 1970). Some of the blooded females in this study were found to have fed upon sloths.

Very little information has been accumulated with regard to VEE virus infections in lizards, which are apparently the preferred hosts of *D. pseudes* mosquitoes. In the field, low-titered antibodies to VEE virus have been encountered in the common iguana (*Iguana iguana*) and, recently, low-titered viremias have been experimentally produced in this species (unpubl. data).

The results of transmission experiments presented in this report indicate that *D. pseudes* females, after feeding on a viremic host circulating high levels of virus, are highly susceptible to infection with VEE virus and readily transmit it under laboratory conditions. Thus, the potential for transmission of VEE virus in nature by at least 1 species of *Deinocerites* exists. However, additional field and laboratory studies, particularly transmission experiments with natural reservoirs such as herons, are indicated before the vector potential of *D. pseudes* and its relatives in Panama can be accurately assessed.

LITERATURE CITED

Chamberlain, R. W., W. D. Sudia, T. H. Work, P. H. Coleman, V. F. Newhouse & J. G. Johnston, Jr. 1969. Arbovirus studies in south Florida, with emphasis on Venezuelan equine encephalomyelitis virus. *Amer. J. Epidemiol.* **89:** 197–210.

Clarke, D. H. & J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Amer. J. Trop. Med. Hyg.* **7:** 561–73.

Galindo, P. 1967. Preliminary observations on the colonization and bionomics of the crab-hole breeding mosquito *Deinocerites pseudes* Dyar and Knab, 1909. *Mosquito News* **27:** 187–90.

Galindo, P. & M. A. Grayson. 1971. *Culex (Melanoconion) aikenii:* natural vector in Panama of endemic Venezuelan encephalitis. *Science* **172:** 594–95.

Galindo, P., S. Srihongse, E. Rodaniche & M.A. Grayson. 1966. An ecological survey for arboviruses in Almirante, Panama, 1959–1962. *Amer. J. Trop. Med. Hyg.* **15:** 385–400.

Gilyard, R. T. 1944. Mosquito transmission of Venezuelan virus equine encephalomyelitis in Trinidad. *U.S. Army Med. Dep. Bull.* **75:** 96–107.

Grayson, M. A. & P. Galindo. 1968. Epidemiologic studies of Venezuelan equine encephalitis virus in Almirante, Panama. *Amer. J. Epidemiol.* **88:** 80–96.

1969. Ecology of Venezuelan equine encephalitis virus in Panama. *J. Amer. Vet. Med. Assoc.* **155:** 2141–45.

Grayson, M. A., S. Srihongse & P. Galindo. 1967. Isolation of St. Louis encephalitis virus from *Deinocerites pseudes* in Panama. *Mosquito News* **27:** 204.

Groot, H., A. Morales & H. Vidales. 1961. Virus isolations from forest mosquitoes in San Vicente de Chucuri, Colombia. *Amer. J. Trop. Med. Hyg.* **10:** 397–402.

Johnson, K. M., A. Shelokov, P. H. Peralta, G. J. Dammin & N. A. Young. 1968. Recovery of Venezuelan equine encephalomyelitis virus in Panama. A fatal case in man. *Amer. J. Trop. Med. Hyg.* **17:** 432–40.

Jonkers, A. H., L. Spence, W. G. Downs, T. H. G. Aitken & C. B. Worth. 1968. Arbovirus studies in Bush Bush Forest, Trinidad, W. I., September 1959–December 1964. VI. Rodent-associated viruses (VEE and agents of groups C and Guamá): Isolations and further studies. *Amer. J. Trop. Med. Hyg.* **17:** 285–98.

Kerr, J. A. 1952. Studies on certain viruses isolated in the tropics of Africa and South America. Immunological reactions as determined by cross complement-fixation tests. *J. Immunol.* **68:** 461–72.

Peyton, E. L., J. F. Reinert & N. E. Peterson. 1964. The occurrence of *Deinocerites pseudes* Dyar and Knab in the United States, with additional notes on the biology of *Deinocerites* species of Texas. *Mosquito News* **24:** 449–58.

Reed, L. J. & H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27:** 493–97.

Scherer, W. F., R. W. Dickerman, C. Wong Chia, A. Ventura, A. Moorhouse, R. Geiger & A. Diaz Najera. 1964. Venezuelan equine encephalitis virus in Veracruz, Mexico, and the use of hamsters as sentinels. *Science* **145:** 274–75.

Sellers, R. F., G. H. Bergold, O. M. Suarez & A. Morales. 1965. Investigations during Venezuelan equine encephalitis outbreaks in Venezuela—1962–1964. *Amer. J. Trop. Med. Hyg.* **14:** 460–69.

Shope, R. E., O. R. Causey, A. H. Andrade & M. Theiler. 1964. The Venezuelan equine encephalomyelitis complex of group A arthropod-borne viruses, including Mucambo and Pixuna from the Amazon region of Brazil. *Amer J. Trop. Med. Hyg.* **13:** 723–27.

Sudia, W. D., R. D. Lord, V. F. Newhouse, D. L. Miller & R. E. Kissling. 1971. Vector-host studies of an epizootic of Venezuelan equine encephalomyelitis in Guatemala, 1969. *Amer. J. Epidemiol.* **93:** 137–43.

Tempelis, C. H. & P. Galindo. 1970. Feeding habits of five species of *Deinocerites* mosquitoes collected in Panama. *J. Med. Ent.* **7:** 175–79.