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TRANSMISSION OF AFRICAN HORSE-SICKNESS BY A SPECIES OF MOSQUITO, *AEDES AEGYPTI* LINNAEUS (*)

Y. Ozawa, G. Nakata, F. Shad-del, S. Nvai

SUMMARY

African horse-sickness was transmitted by means of the bites of *Aëdes aegypti* which had been fed with virus suspension. The body temperature of the horse increased on the 6th day, and the horse died on the 18th day after exposure. African horse-sickness virus remained infectious in *Aëdes aegypti* for more than 5 weeks after engorgement.

Various views have been expressed about the vectors of African horse-sickness (AHS) virus. *Stomoxys calcitrans, Lyperosia minuta, Anopheles spp., Aëdes spp.*, and *Simulium spp.* have been suspected as possible carriers of AHS virus. Such suspicions, however, have been raised without adequate experimental evidence.

Nieschulz *et al.*[5] considered that *Aëdes* mosquitoes were not significant vectors, because these mosquitoes could harbor AHS virus for only one week after experimental feeding. Since further investigations by Nieschulz and Du Toit[6] on mosquitoes as potential vectors of AHS virus gave negative results, these authors concluded that mosquitoes were not involved in natural transmission.

Du Toit[3] extended this work and recovered AHS virus by injecting horses with a suspension of *Culicoides* caught in the field. It was not determined how long *Culicoides* may carry the virus or whether these insects may transmit the disease.

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Since the appearance of this report *Culicoides spp.* have widely believed to be the major vectors of AHS, and recently, Andrewes [1] classified AHS virus as a *Culicoides*-borne arbovirus.

In our previous work, [9] however, horse-sickness virus was transmitted experimentally to horses by mosquito bites. Mosquitoes used in successful transmission experiments were *Anopheles stephensi* and *Culex pipiens*. The present report concerns experiments carried out with *A. aegypti*.

Materials and Methods

Mosquitoes.—*Aëdes aegypti* Linnaeus, originally collected in 1956 at Orlando, Florida, was kept at the insectarium of the Institute of Parasitology and Malariology of Teheran University. A part of the colony at the 54th generation was obtained.* At least 15 successful breedings were made at the insectarium of the Near East Animal Health Institute, Razi Institute, before using them in experiments. Batches of these mosquitoes were tested for the absence of AHS virus. During this experiment, the temperature of the insectarium ranged between 21.9 and 27.2 C., and the range of relative humidity was between 52 and 81%.

Horses.—Approximately 12-month-old, nonvaccinated healthy horses were obtained during the winter season from an isolated village in the mountainous area by the Caspian Sea. They were kept in an insect-proof stable throughout the experiment.

Virus.—The virus used in this experiment was viscerotropic type 9 AHS virus (strain 10/60) isolated in Iran. The virus was cultivated in monkey kidney stable (MS) cells, [4] with the methods described by Ozawa and Hazrati. [7] The identity of the virus was confirmed by a neutraliaztion test in MS-cell cultures using the method previously described. [7,8]

In a positive transmission experiment with *A. aegypti*, the tissue culture virus fluid mixed with healthy horse blood was used in place of infectious horse blood [9] to feed the mosquitoes. The supernatant fluid of the 5th passage of the virus in MS cells was mixed with an equal volume of hemolized horse blood suspension prepared as follows: Normal horse blood cells were washed 3 times in phosphate buffered saline solution (pH 7.2) by centrifugating 10 minutes at 500 g. Two milliliters of the sedimented cells were hemolized in 10 ml. of distilled water. This material was centrifuged again, and the supernatant fluid was mixed with 1 Gm.

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* Mofidi, Ch.; Institute of Parasitology and Malariology, Teheran University, Teheran, Iran.

of glucose and 10 ml. of tissue-culture fluid containing $10^{6.5}$ TCID50/ml. of virus. This material was placed on absorbent cotton in a petri dish and the mosquitoes, fasted overnight, were allowed to engorge the hemolyzed blood (diurnal feeding). They were left in the cage containing the dish for 6 hours and the specimens which had engorged were then transferred to a clean breeding cage.

Transmission Experiment.—For transmission work, a special small cage was constructed for virus-carrying mosquitoes. A strong, metal frame was covered with organdie. The bottom of the cage was closely attached to the shaved skin of the horse's back. This small cage was covered with another stronger cage made of metal frame and wire mesh, and this safety cage was tightly fitted to the horse with belts and springs. The cages were covered with layers of thick cloth to protect the insects from cold.

Titration of Virus in Mosquitoes.—To determine how long AHS virus persists in *A. aegypti*, a large number of female mosquitoes were fed with hemolysed blood mixed with the virus-containing tissue culture fluid prepared in the manner described. The virus used was the 6th passage in MS-cell cultures of a viscerotropic type virus (strain 10/60). The tissue culture fluid contained $10^{6.6}$ TCID50/ml. before mixing with hemolysed horse blood. At intervals, groups of 10 mosquitoes fed with virus were killed for virus titration. They were macerated in a tissue grinder, * to which 1 ml. of maintenance medium [7] containing 2% inactivated calf serum was added. Each suspension was centrifuged at 2,000 g for 30 minutes at 4 C. The supernatant fluid was removed, with a syringe with a long needle to avoid a cloudy layer which had formed on top of the liquid. Tenfold dilutions of the supernatant fluid were made with maintenance medium and titrated in the manner previously described.[7] In every case, the identity of the virus was confirmed by neutralization tests.

Results and Discussion

In a positive transmission experiment, 10 mosquitoes that had engorged virus suspension 19 days previously were placed in a cage, and this cage was fitted to a susceptible horse. During a 7-hour exposure period, 9 of the mosquitoes engorged on the horse. Six days later the body temperature of the horse was increased to 39.4 C. Swelling of the eyelids was evident. The horse died 18 days after exposure. The necropsy findings were characteristic of AHS with pronounced changes in the

* Ten Broeck tissue grinder, Kontes Glass Co., Vineland, N.J.

lungs. A yellow, gelatinous edema was in the subcutaneous tissue underlying the area where the mosquito cage had been placed.

TABLE 1 — Titers of Virus Recovered from Aëdes aegypti after Experimental Infection with African Horse-Sickness Virus Type 9 (Strain 10/60)

Days after feeding virus	Virus titer TCID50/ml.
1	$10^{2.1}$*
8	$10^{3.0}$
12	0
14	$10^{2.7}$
16	0
18	$10^{2.7}$
20	$10^{2.0}$
24	0
28	$10^{2.0}$
32	0
36	$10^{1.3}$
42	0

* Titer determined in suckling mice (LD50/ml.). The remainder were titers in monkey kidney stable tuce cultures.

The identity of the virus isolated from the blood of this horse collected at the onset of fever was confirmed by a neutralization test in MS-cell cultures.

In this experiment, hemolysed rather than unhemolysed horse blood was used to avoid any inconsistent results due to reactions between AHS virus and various horse blood cells.

The final concentration of AHS virus in the suspension after mixing with hemolysed blood was approximately $10^{6.2}$ $TCID_{50}$/ml. It is difficult to conclude whether this titer was higher or lower than the titer expected in the blood of a typical donor horse, since there is no information regarding the relationship between TCID, horse infective dose, and suckling mice infective dose of nonattenuated virus.

The AHS virus was detected in *Aëdes* mosquitoes as long as 36 days after feeding with virus (Table 1). This period is much longer than that reported by Nieschulz *et al.*,[5] who found that *Aëdes* mosquitoes could carry AHS virus for not more than 1 week and concluded that mosquitoes were no vectors of horse-sickness.

In nearly half of the experimentally infected mosquito groups, virus could not be detected. Therefore, the actual number of *Aëdes* mosquitoes harboring virus ap-

pears to have been limited. It is conceivable that AHS virus could multiply or persist only in a certain number of mosquitoes in which there were optimum conditions for the survival or multiplication of the virus, such as those mentioned by Chamberlain and Studia.[2] These conditions should be investigated. Since the number of mosquitoes which harbored virus was limited and the titers were inconsistent, it is not possible to conclude from the present results that AHS virus actually multiplies in these insects.

However, the fact that the virus remained infectious in *A. aegypti*, for more than 5 weeks after engorgement and that 3 species of mosquitoes (*A. aegypti*, *A. stephensi*, and *C. pipiens*) were able to transmit the disease to horses 2 to 3 weeks after feeding with virus are difficult to reconcile with purely mechanical transmission.

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