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Transmission of African Horse-Sickness by Means of Mosquito Bites and Replication of the Virus in Aedes aegypti (*)

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Various insects such as *Stomoxys calcitrans, Anopheles spp.*, have been suspected as possible carriers of African horse-sickness (AHS) virus. Such suspicions, however, have not been supported with adequate experimental evidence.

Nieschulz and Du Toit[4] reported that ASH virus remained alive only in exceptional cases up to seven days in certain mosquitoes, *Aedes* and *Anopheles* species. Normally, AHS virus was destroyed more quickly, and it never could be transmitted by permitting infected mosquitoes to feed on susceptible animals. They concluded that mosquitoes were probably not the transmitters of AHS virus.

Du Toit[2] investigated *Culicoides* as possible vectors of the virus and recovered AHS virus by injecting a horse with a suspension of *Culicoides* caught in the field. Since the appearance of this report, *Culicoides spp.* have been believed to be the vectors of AHS, and recently Andrews[1] classified AHS virus as a *Culicoides*-borne arbovirus.

In our experiments, AHS (type 9) virus was transmitted experimentally to horses by artifically infected mosquitoes, *Anopheles stephensi, Culex pipiens,* and *Aedes aegypti.* This paper presents a summary of positive transmission experiments and the results of investigations on AHS virus in *Aedes aegypti.*

Materials and Methods

Culex pipiens caught in Teheran in 1963 were maintained at the insectarium of the Razi Institute, and the 16th generation was used in the experiment.

Anopheles stephensi were also caught in Iran. A part of the colony was obtained from the Institute of Parasitology and Malariology of Teheran University through the courtesy of Doctor Mofidi. Eight additional breedings were made before their use in this experiment.

Aedes aegypti Linnaeus, originally collected in 1956 at Orlando, Florida, was also obtained from Teheran University. At least 15 successive breedings were made at the Razi Institute before using them in experiments.

The temperature range of the insectarium during the experiments was approximately 21° to 29.5° C, and the range of relative humidity was 52 to 81 percent.

Horses. Non-vaccinated healthy horses, approximately 12 months old, were

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obtained during the winter season from an isolated village by the Caspian Sea. They were treated with appropriate anthelmintics and kept under close observation for at least one week before the experiment commenced. They were maintained in an insect-proof stable throughout the experiment.

Virus.—Infectious horse blood was obtained from a horse which had been experimentally infected with the fifth mouse brain passage of Iranian strain, 10/60 (type 9). The blood was taken from the horse at the height of fever, one day before it died of horse-sickness. It was defibrinated and stored at 4° C. This blood was used to infect *Culex pipiens* and *Anopheles stephensi* for transmission experiments.

The virus used for the experiment with *Aedes aegypti* was the same Iranian strain 10/60, but adapted to monkey kidney stable (MS) cells. The fifth or sixth passage of the virus fluid was mixed with an equal volume of hemolysed horse blood suspension prepared as follows: normal horse blood cells were washed three times with phosphate-buffered saline without magnesium and calcium ions (PBS) by centrifuging ten minutes at 500 g.: two milliliters of the sedimented cells were hemolysed in 10 ml. of distilled water: to this hemolysed blood, 1 gm. of glucose was added: the mixture was centrifuged again and the supernatant fluid was mixed with an equal volume of tissue-culture fluid containing 10/60 virus. The mosquitoes were starved overnight and then allowed to engorge the hemolysed blood. They were left in the cage containing the dish for six hours and the specimens which had engorged were then transferred to a clean breeding cage. In some experiments with *A. aegypti*, the same strain 10/60 passaged once (MPI) or three times (MPIII) through *A. aegypti* was used.

Transmission Experiment.—For transmission experiments, a special small cage was constructed for virus carrying mosquitoes. A strong, metal frame was covered with organdies. 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 and wire mesh. This safety cage was tightly fitted to the horse with belts and springs.

Isolation and Titration of Virus.—Virulent virus was isolated or titrated by inoculating appropriate materials in brains of four to five-day-old mice. Virus adapted to MS cells was isolated or titrated in MS cell cultures prepared in the manner described previously.[5]

To determine rise and fall of AHS virus titers in *A. aegypti*, a large number of female mosquitoes were fed with virus-containing tissue culture fluid mixed with hemolysed blood.

At intervals, groups of mosquitoes fed with virus were killed for virus titration. They were macerated in a Ten Broeck tissue grinder, to which 1 ml. of PBS containing 5 percent inactivated calf serum, 200 units of penicillin and 200 ug of streptomycin were added. Each mosquito emulsion was centrifuged at 2,000 g. for 30 minutes at 4° C. Ten-fold dilutions of the supernatant fluid were made with maintenance medium and titrated in MS tube cultures inoculating 0.1 ml. of each virus dilution per tube. The end points were calculated by the method of Reed and Muench.[8] In every experiment, the identity of the virus was confirmed by neutralization test.

Identity of virus isolated either from horses or mosquitoes was confirmed by neutralization tests in mice or tissue cultures, respectively.

Antibody levels in blood collected from infected and control horses were determined before and after infection. All serums were heated at 56° C. for 30 minutes and the antibody titers measured by the method described previously.[3]

Results

Transmission Experiments.—*Anopheles stephensi*, which had engorged infectious blood 15 to 18 days previously, were placed on a normal horse (No. TE3). Fourteen of 30 mosquitoes sucked blood from the horse. Sixteen days after exposure, the body temperature of the horse rose to 40.5° C. High fever lasted the following few days then the temperature returned to the normal range. The horse, however, became weak and lost a considerable amount of weight during the course of the disease.

During the last two days, before death, the horse could not remain standing.

The clinical symptoms and post-mortem findings were indistinguishable from those of AHS. The virus isolated from the horse during the febrile period was identified as the same type 9 virus by neutralization tests in mice. A slight antibody response was demonstrated in the serum of the horse collected 24 days after exposure.

Culex pipiens, which had engorged infectious blood 15-22 days previously, were placed on a normal horse (No. TE5). Two of 19 mosquitoes placed on the horse sucked blood. The horse showed a febrile reaction which commenced on the 13th day after exposure. Twenty days later, the horse succumbed with symptoms similar to horse TE3. It is interesting to note that the yellow gelatinous infiltrate in ventral muscles was most pronounced in the area below the site where the mosquito cage had been placed. Acute hemorrhage and necrotic inflammation was found in the intestine.

Other post-mortem findings were indistinguishable from those of AHS. A slight antibody response (N.I. = 0.7) was shown in the serum of the horse collected 24 days after exposure.

Aedes aegypti that had engorged virus suspension in hemolysed blood 19 days previously, were placed on a horse (No. 60). Nine of ten mosquitoes engorged on the horse. Six days later the animal showed a febrile reaction which lasted until death. Swelling of the eyelids became evident.

The horse died 18 days after exposure. The post-mortem findings were characteristic of AHS with pronounced changes in the lungs. A yellow, gelatinous edema was present in the subcutaneous tissue underlying the area where the mosquito cage had been placed. The identity of the virus isolated from the blood of this horse collected at the onset of fever was confirmed by neutralization test.

The details of these transmission experiments have been reported previously.[6, 7]

Frequency of Persistence of AHS Virus in Mosquitoes.—In previous experiments, AHS virus disappeared rapidly from the majority of the mosquitoes within a week after feeding, but it remained for a long period in a limited number of mosquitoes. To investigate the percentage of mosquitoes harboring the virus after a certain incubation period, starved *A. aegypti* were fed with virus suspension ($10^{5.5}$ TCID50/0.1 ml.). Mosquitoes that engorged medium amounts of virus suspension were maintained at the insectarium and 22 to 24 days later, 69 of them were titrated.

For titration, groups of two individuals were macerated in 1 ml. of diluent, and the supernatant fluid of the emulsions was used. The result is shown in Table 1. Five of 34 groups tested carried virus at high titer. The probability of having a positive mosquito is approximately 1/10.5 to 1/13.6. The probability of infection is, therefore, considered to be less than 10 percent.

Single-Passage Growth Pattern of AHS Virus in Mosquitoes.—Several experiments were carried out to determine the growth cycle of AHS virus in Aedes mosquitoes.

Groups of 15 engorged mosquitoes were macerated in 1 ml. diluent. Ten-fold dilutions of the supernatant fluid of the emulsion were made in maintenance medium, and each of eight tube cultures was inoculated with 0.1 ml. of each dilution.

The results of titration expressed as log TCID50/0.1 ml. of emulsion, are summarized in Table 2. It is noted that the titers of virus in mosquitoes shortly after engorgement were similar and that the titers of the virus samples used for feeding were also similar (between $10^{4.8}$ and $10^{5.0}$ TCID50/0.1 ml. of emulsion).

TABLE 1—Virus Titers Recovered from Infected *A. aegypti* that had been Fed with Type 9 AHS Virus 22 to 24 Days Previously.

Sample No.	*Virus titer*	*Sample No.*	*Virus titer*	*Sample No.*	*Virus titer*	*Sample No.*	*Virus titer*
1	—	10	—	19	—	28	—
2	—	11	—	20	$10^{3.5}$	29	—
3	—	12	$10^{2.5}$	21	—	30	—
4	—	13	—	22	—	31	—
5	—	14	—	23	—	32	—
6	—	15	—	24	—	33	$10^{3.0}$
7	$10^{3.0}$*	16	$10^{2.3}$	25	—	34	—
8	—	17	—	26	—		
9	—	18	—	27	—		

— = No virus detected.
*TCID50/0.1 ml. of emulsion.

It is possible to calculate from these data the average titer of virus engorged by each individual mosquito, approximately $10^{2.4}$ TCID50/mosquito. Furthermore, the average amount of virus fluid engorged by each mosquito (0.26-0.4 mm^3) can be estimated. A decrease in virus titer in mosquitoes during the first few days after engorgment was evident.

Taking the result of the pervious experiment into consideration, the virus recovered on the seventh day after feeding and thereafter appears to be virus which multiplied in a limited number of infected mosquitoes (less than 10 percent).

In most cases, 15 mosquitoes were emulsified as a group, and the virus recovered from positive samples can be considered in most instances as the virus content of one infected mosquito since the probability of having two positive mosquitoes in one group is less than 25 percent. Therefore, virus titers in individual insects may be considered to be ten times as high as the titers recorded in Table 2.

The frequency of negative samples among those tested more than seven days after feeding is also within a statistically reasonable range if it is accepted that only one out of 10.6 to 13.6 mosquitoes becomes infected and this is applied to all experiments recorded in Table 2.

Based on this interpretation of the data presented in Table 2, the growth cycle of AHS virus in artifically infected *A. aegypti* is illustrated graphically in Figure 1. The solid line (C) indicated the estimated growth curve of AHS virus in each infected mosquito.

TABLE 2—Titers of Virus in *A. aegypti* Fed With Type 9 AHS Virus Mixed With Hemolysed Blood. (Log $TCID_{50}$/0.1 ml. of emulsion)

Days after engorgement	*Experiment No.*							
	1[a]	2	3		4		5	
0	2.7	2.5	2.6	2.5[a]			2.7	2.5
1	1.0		1.8	0				
2		0	0.4	0			0	0
3	0		1.0	0				
4		1.6			0*	0*		
5			0.8	0	0*	0*		
6	0						0	0
7	1.5	1.6	0.8	0.5				
8					0*	0*		
9			2.2	0	1.5*	0*	2.5	0
10		2.7						
11	0				2.8	2.0		
12		0.6	0	0	2.5	2.2	2.3	2.0
13	0*							
14		2.5						
15			2.8	2.5			2.4	0
16		2.5						
18		0						
19			3.0	1.0			2.0	0
21		3.3					0	0
25							3.0	2.6
26		2.8						
36							0	0
37		2.3						

a: MS cell adapted 10/60 virus was used. In the rest of experiments MPI was used.

*: Ten mosquitoes/group (15 mosquitoes/group in the rest of samples).

0: No virus detected.

The virus fluid used for these experiments (MPI) was dispensed in small hemagglutination tubes fitted with rubber stoppers. They were stored in the insectarium where the engorged mosquitoes were placed. At intervals specified in the test, samples were taken for the virus titration. The results are shown in Table 3. Infectivity of the virus had completely disappeared after 37 days storage at the insectarium.

Effect of Storage of Sacrificed Mosquitoes on the Virus Titer.—In the above experiments, sacrificed mosquitoes were often stored in a refrigerator for up to about one hour before they were emulsified for titration. To investigate whether or not this was the cause of a sudden drop in virus titer, *A. aegypti* were fed with the same MPI virus ($10^{4.8}$ TCID50/0.1 ml. The engorged mosquitoes were kept at the insectarium and samples were taken on the fifth, ninth and 12th days after feeding. Half of them were kept alive until just before maceration. The virus titers are shown in Table 4. It appears

that storage of killed mosquitoes at 4° C for 1 hour has no effect on virus titers.

Effect of Volume of Virus Engorged by Mosquitoes on the Frequency of Infection.—Aedes aegypti were fed with MPI virus suspended on hemolysed blood ($10^{5.5}$ TCID50/0.1 ml.). After feeding, they were divided into 3 groups, (A) mosquitoes fully engorged, (B) mosquitoes having engorged small amount only, and (C) mosquitoes indistinguishable from non-engorged ones.

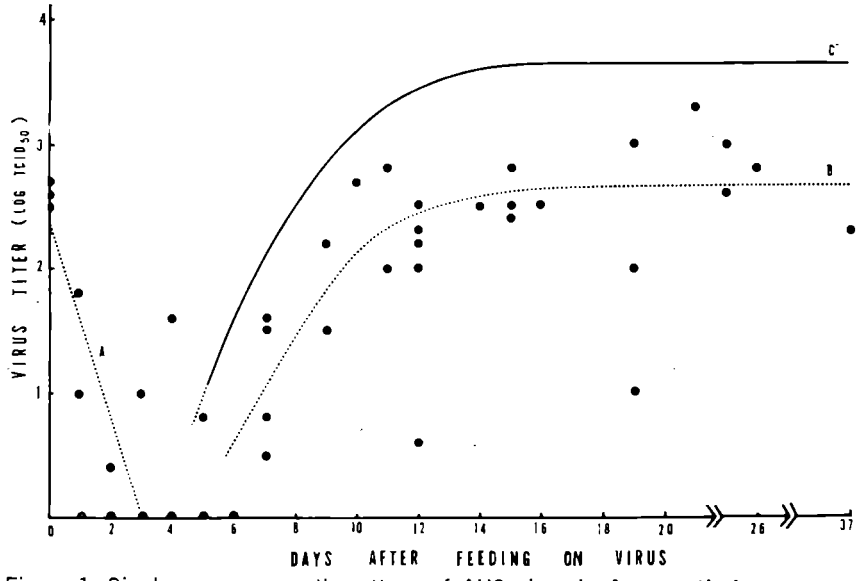

Figure 1: Single-passage growth pattern of AHS virus in **A. aegypti.** A: engorged virus remaining in the body of each mosquito, B: average titers of virus recovered from infected mosquitoes ($TCID_{50}$/0.1 ml. of emulsion), C: estimated growth curve of AHS virus in each mosquito.

On the 15th, 16th, and 17th days after feeding, 30 mosquitoes from each group were divided into groups of 15 for titration. The results of the titration are summarized in Table 5. There was no difference in virus titers and frequency of infection between groups A and B. The virus titers demonstrated in mosquitoes of group C were just as high as those in fully engorged mosquitoes.

Effect of the Age of Mosquitoes on Their Susceptibility to AHS Virus—Aedes aegypti of two different age groups, zero to three days and 10 to 13 days, were allowed to suck virus fluid. The titer of virus in hemolysed blood was $10^{4.5}$ TCID50/0.1 ml. On the 14th, 16th, 18th, 24th and 36th days after infection groups of two mosquitoes were emulsified for titration. The results are shown in Table 6. Virus was recovered from nine samples of 104 younger mosquitoes titrated. Of 104 older mosquitoes titrated, seven samples contained virus. The percentage of infection in the whole experiment is approximately the same as the previous one in which virus of high titer was used for feeding mosquitoes.

From this result it may be concluded that there is no significant difference in susceptibility between these two different age groups of *Aedes aegypti.*

TABLE 3—Rate of Inactivation of Type 9 AHS Virus (MPI) Stored at the Insectarium.

Days of storage	*$TCID_{50}$/0.1 ml.*
0	$10^{3.9}$
2	$10^{2.5}$
4	$10^{2.3}$
9	$10^{2.0}$
11	$10^{2.0}$
14	$10^{1.9}$
18	$10^{1.2}$
25	$10^{1.2}$
30	$10^{0.8}$
37	0

TABLE 4—Effect of Storage at 4° C. of Sacrificed Mosquitoes on the Virus Titers.

Days after Infection	*Virus titers ($TCID_{50}$/0.1 ml.)*			
	Mosquitoes kept alive		*Mosquitoes killed and kept at 4° C.*	
5	—	—	—	—
9	—	—	—	$10^{1.5}$*
12	$10^{2.8}$	$10^{2.5}$	$10^{2.0}$	$10^{2.2}$

—: No virus detected.

*: Virus titer in 10 mosquitoes; the rest in 15 mosquitoes.

TABLE 5—Virus Titers in A. aegypti Which Engorged Different Amount of AHS Virus Suspended in Hemolysed Blood.

Days after infection	*Virus titers (Log $TCID_{50}$/0.1 ml)*					
	Group A		*Group B*		*Group C*	
15	1.5	3.0	2.7*	3.0	—	—
16	2.7	—	3.0	2.3	3.3	3.0
17	2.3	2.8	—	2.5	2.7	—

Group A: Mosquitoes fully engorged.

Group B: Mosquitoes engorged a little.

Group C: Mosquitoes indistinguishable from non-engorged ones.

* Virus titers in 15 mosquitoes.

TABLE 6--Virus Titers in *A. aegypti* of Two Different Age Levels Infected with Type 9 AHS Virus (MPI).

Days after infection	*Age of mosquitoes (day-old)*	*Virus titers in groups of 2 mosquitoes (Log $TCID_{50}$/0.1 ml)*
14	0-3	- - - - 2.5 - - - - 3.0 - 2.0 - -
	10-13	- - - - - 2.3 - - - - - - - - - - -
16	0-3	- - 1.0 - - - - - 0.7 2.3 - - -
	10-13	- 1.0 2.0 - - - - - - - - - - - - -
18	0-3	- - - - - - - - - 2.0 - - - - - - - -
	10-13	- - - - - - - 2.5 - - - - - - - - -
24	0-3	- - - 2.0 - - -
	10-13	- - 1.7 - - - -
36	0-3	- - - - - - 2.0 -
	10-13	- 2.7 - 3.0 - - -

In a separate experiment, however, older mosquitoes (21-24 days) were fed in the same manner with MPI and MPIII viruses. The titers of these viruses suspended in hemolysed blood were adjusted to $10^{5.3}$ TCID50/0.1 ml. Two weeks after feeding, a total of 38 individuals fed with MPI virus and 48 individuals fed with MPIII virus were titrated. In spite of the fact that these mosquitoes were fed with virus of higher titer, no virus was recovered from any of these insects. This may indicate that the susceptibility of *A. aegypti* to AHS virus is lost at the age between two and three weeks. This observation must be substantiated by further tests.

DISCUSSION

Three positive transmission experiments summarized here refer to the transmission of AHS virus to horses by artificially infected mosquitoes. Transmission of the virus from horse to horse via mosquitoes may be less easy to achieve since virus titers in the blood of infected horses are not consistent and are usually lower than the titers of the virus suspensions fed to mosquitoes in these experiments. Therefore, the minimum titer of virus required to infect adequate numbers of susceptible mosquitoes needs to be determined.

Although *Aedes aegypti* are not common in Iran, they were employed to study the relationship between AHS virus and mosquitoes, because they suck horse blood well and the frequency of microbial contamination in tissue cultures inoculated with mosquito macerates was least. They can be fed during day (diurnal feeding following overnight starvation).

The amount of virus engorged by each mosquito was calculated from virus titers in mosquitoes and virus solutions fed to them. The amount (0.26-0.4 mm^3) is fairly close to the average amount of virus fed by means of capillary tubes (0.34 mm^3).

Virus suspended in such a small quantity of fluid engorged by a mosquito will be rapidly inactivated by heat or washed away by subsequent drinking of water and glucose solution, unless the virus is adsorbed onto susceptible cells.

It is usual that the virus in the mosquitoes disappears within three to four days after feeding. Occasionally virus may persist in them as long as seven days as described by Nieschulz and du Toit.[4]

However, when the engorged virus multiplies in a limited number of mosquitoes, the virus titers start increasing within a week after infection and reach a plateau within two weeks after infection. Average virus titers between $10^{3.0}$ and $10^{4.0}$ TCID50/mosquito are maintained during the following few weeks or longer.

There is a good chance that the virus may even persist for life in infected mosquitoes. If so, it is possible that infected-over-wintering mosquitoes may act as reservoirs of AHS virus.

It was noticed that virus titers and the frequency of virus recovery from mosquitoes fed with mosquito-passed (MPI) virus were higher than those in mosquitoes fed with original viscerotropic virus. This is indicated by the results shown in Table 2 and those of previous experiment.[7]

Possibly some sort of adaptation or selection of virus takes place during passage through mosquitoes. This may be one of the factors which decide the percentage of infection.

Individual differences in mosquitoes may be major factor influencing their susceptibility to AHS but other possible factors such as age level, concentration of feeding virus, and temperature of incubation should also be studied.

SUMMARY

Anopheles stephensi, *Culex pipiens*, and *Aedes aegyptic* transmitted African horse-sickness virus to horses under experimental conditions.

Using *A. aegypti*, the relationship between African horse-sickness virus and mosquitoes was investigated. Virus titers in mosquitoes which engorged the virus emulsion decreased markedly within a few days after engorgement. When these mosquitoes were kept in an insectarium at 26.3 $\pm$ 3° C. virus titers in infected mosquitoes became higher than the average amount of virus engorged by each mosquito approximately seven and nine days after feeding the virus. titers in infected mosquitoes reached maximum approximately two weeks after engorgement, and titers between 10^3 and 10^4 TCID50 per mosquito were maintained for the following three weeks or longer.

It was found that the virus multiplies only in a limited number of *Aedes aegypti*. Some of the factors possibly causing this were investigated.

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