IN VITRO TRANSMISSION OF YELLOW FEVER VIRUS BY GEOGRAPHIC STRAINS OF *AEDES AEGYPTI*

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ABSTRACT. An *in vitro* feeding technique was developed to detect arbovirus transmission by mosquitoes. The technique was demonstrated to be as sensitive as engorgement upon suckling mice for the detection of yellow fever virus transmission by *Aedes aegypti* mosquitoes. In studies to determine the ability of geographic strains of *Ae. aegypti* to transmit yellow fever virus, the Caribbean strain was the most efficient transmitter; the African strain was intermediate; and the Asian strain was the least efficient.

INTRODUCTION

Arbovirus vector competence studies can be handicapped if there is no suitable laboratory host to induce vector engorgement or if the laboratory host does not sicken or die from viral disease induced through peripheral infection. Many arboviruses do not kill laboratory animals until virus adaptation has occurred through multiple laboratory passages. In vector competence studies, however, it is preferable to use low or unpassaged virus strains in order to approximate more closely natural circumstances. Such difficulties may be overcome sometimes by utilization of *in vitro* techniques to collect arthropod saliva which subsequently can be inoculated into a permissive system for assay.

A recently developed capillary feeding technique (Aitken 1977)[1] has proven to be an efficient mechanism for the collection of mosquito saliva, and inoculation of virus suspensions into noninfected mosquitoes has proven to be a sensitive method for arbovirus assay (Rosen and Gubler 1974). Since arbovirus infections in mosquitoes do not cause apparent morbidity or mortality, the heads of the inoculated mosquitoes are severed after an appropriate incubation period and squashed on slides. The resultant smears are examined by immunofluorescence for the presence of viral antigen (Kuberski and Rosen 1977).

An *in vitro* system using the capillary feeding technique with subsequent mosquito inoculation of the meal for viral assay was hypothesized to be an effective substitute for an *in vivo* susceptible laboratory host as a means to detect viral transmission. After development, the *in vitro* technique was used to determine the relative ability of 3 geographic strains of *Ae. aegypti* mosquitoes to transmit yellow fever (YF) virus.

MATERIALS AND METHODS

MOSQUITO STRAINS. The geographic strains of *Ae. aegypti* used were as follows:

a. Amphur (Bangkok), Thailand, F_{13} generation at Yale (received February, 1972) from the University of Notre Dame where it was colonized since 1970. This is presumably a house-frequenting strain.

b. Santo Domingo (Dr. Betances Street), Dominican Republic, F_{11} generation (eggs received 16 October 1972). This is a house-frequenting strain.

c. Kampala, Uganda, F_5 generation (eggs received 12 July 1973). This is a sylvan strain.

INFECTION OF MOSQUITOES WITH YF VIRUS. Since the purpose of this experiment was to determine transmission rates and not necessarily infection rates, mosquitoes were infected by intrathoracic inoculation of approximately 600 $SMICLD_{50}$ per ml of the Asibi strain of YF virus.

[1] An artificial feeding technique overlooked in the original paper is P. F. Mattingly, Nature 158:751, 1946.

MOSQUITO FEEDING AND TRANSMISSION. Mosquitoes were fed utilizing glass capillaries as related previously (Aitken 1977). In brief, capillaries are drawn to a fine point in a tiny flame. The point is severed so that the orifice is large enough to receive the proboscis of the mosquito. This capillary is charged with a predetermined amount of the meal suspension. In these studies the capillaries were charged with either 1) mechanically defibrinated chicken blood, 2) human blood, or 3) a 10% fetal calf serum (FCS)—10% sucrose solution. Capillaries were precharged with the feeding suspension and the meniscus point marked on the glass with indelible ink. Mosquitoes were deprived of sugar water 24 hrs. before feeding. After cold anesthetization, the anterior 4 legs were removed to prevent the insect from dislodging itself. Grasping firmly the base of the insect's wings with forceps, the proboscis was inserted into the pipette. Capillaries with mosquitoes were suspended from a styrofoam rack in a screened retaining chamber (Figure 1). The mosquitoes were permitted to engorge for approximately 15 min. before they were removed and frozen at −70°C for later infectivity studies. Meals were either inoculated directly or stored frozen until they were later inoculated into noninfected recipient mosquitoes for virus assay.

ASSAY OF VIRUS TRANSMISSION IN MOSQUITOES. The meal containing the saliva was ejected into a 0.01 ml drop of a 10% FCS diluent on a glass slide. After mixing, the suspension was drawn into a fine capillary and inoculated into noninfected recipient mosquitoes (Rosen and Gubler 1974). Recipient mosquitoes were maintained for 10 days after inoculation at approximately 27°C before their heads were squashed and the resultant smears were examined by immunofluorescence for the presence of YF viral antigen (Kuberski and Rosen 1977).

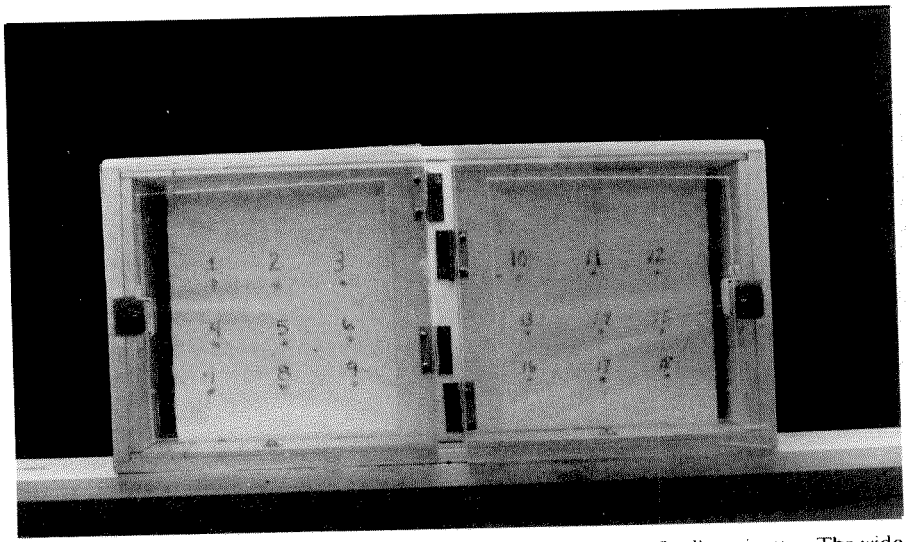

Figure 1. Enclosed chamber to restrain mosquitoes dislodged from feeding pipettes. The wide-mesh nylon permits easy observation of engorging mosquitoes and does not create air currents when the doors are opened or closed.

EXPERIMENTAL DESIGN. a. Assessment of the *in vitro* technique.

Three experiments were conducted to determine if the *in vitro* technique is a satisfactory substitute for a susceptible laboratory host in the detection of YF virus transmission by *Ae. aegypti* mosquitoes. In all studies, infected sibling mosquitoes were randomly separated, and each group was exposed concurrently to one of the feeding regimens. After feeding, mosquitoes were stored at −70°C for later virus assay. Capillary meals, from the *in vitro* technique, were either inoculated into recipient mosquitoes or stored frozen until inoculated later. Engorgement upon suckling mice was used as the standard by which to assess the accuracy of other techniques to direct virus transmission. One mosquito was permitted to engorge upon one mouse which was then observed for signs of YF infection.

Experiment 1. In the original description of the capillary feeding technique (Aitken 1977), a suspension of red blood cells (RBC's) was used to charge the pipettes. An artificial meal is advantageous because it would be no longer necessary to maintain laboratory animals as a source for RBC's. Previous studies (Beaty and Aitken unpublished data) to determine the optimal concentrations of FCS-sucrose for the artificial meal revealed a 10% FCS-10% sucrose solution to be the most effective in stimulating engorgement by *Ae. aegypti*. Since this sucrose meal would be directed to the diverticulum instead of the midgut, an experiment was conducted to compare the artificial and the "normal" RBC meal in their sensitivity to detect viral transmission.

Experiment 2. The *in vitro* technique using the 10% FCS-10% sucrose meal was compared to the *in vivo* technique of engorgement upon suckling mice. The sensitivity of the two techniques to induce engorgement and to detect transmission was compared.

Experiment 3. The three systems (RBC, FCS-sucrose and mice) were compared simultaneously.

b. Vector competence study.

The *in vitro* technique was used to determine the transmission capability of the three geographic strains of *Ae. aegypti* after infection with YF virus by intrathoracic inoculation. At predetermined intervals post-infection, selected mosquitoes from each strain were assayed for transmission capability. Each tested mosquito was also examined for infectivity by the head squash technique.

RESULTS

The *in vitro* technique utilizing a 10% FCS-10% sucrose meal to charge the pipette was a satisfactory substitute for red blood cells in the detection of viral transmission (Table 1). The 10% FCS-10% sucrose solution was as sensitive in the detection of virus transmission as the RBC solution, and it resulted in an increased amount of engorgement. The fact that the artificial meal was directed to the diverticulum instead of the midgut did not result in detectable differences in transmission rates. It is noteworthy that even mosquitoes with no visible evidence of feeding were still capable of salivating and transferring virus to the meal. The *in vitro* feeding technique using capillaries charged with the FCS-sucrose meal was also a satisfactory substitute for engorgement on a susceptible laboratory host in the detection of virus transmission (Table 2). Feeding on mice did result in a greater degree of engorgement, but this was not reflected in the sensitivity for detection of transmission; 80% of each group transmitted.

In the 3rd study comparing the 3 systems, feeding on mice was again superior in inducing engorgement, but the artificial meal appeared equally sensitive in the detection of transmission. The FCS-sucrose and mouse engorgement methods resulted in 67% and 80% transmission rates, respectively. Inexplicably, in this study the blood meal was clearly inferior in both inducing engorgement and detecting transmission.

The three strains of *Ae. aegypti* differed

Table 1. Comparison of YF virus transmission rates of sibling *Aedes aegypti* mosquitoes feeding on either defibrinated chicken blood or 10% FCS–10% sucrose.

Defibrinated Chicken Blood			10% FCS and 10% Sucrose		
Mosquito	Degree of Engogrement	Transmission	Mosquito	Degree of Engorgement	Transmission
1	Empty	Yes	1	Empty	No
2	Empty	Yes	2	Empty	No
3	Empty	Yes	3	Empty	No
4	Empty	No	4	Empty	No
5	Empty	Yes	5	Trace	Yes
6	Empty	No	6	Trace	Yes
7	Empty	No	7	Trace	Yes
8	Empty	Yes	8	¼	No
9	½	No	9	⅓	Yes
10	Full	Yes	10	⅓	No
			11	½	Yes
			12	½	Yes
			13	½	No
			14	½	Yes
			15	¾	No
			16	¾	Yes
			17	¾	Yes
			18	Full	Yes
			19	Full	Yes
			20	Full	Yes
	2/10=20% Engorged	6/10=60% Transmission		16/20=80% Engorged	12/20=60% Transmission

significantly ($X^2 = 10.31$, $P < 0.01$) in their ability to transmit YF virus at 26 days cumulative incubation (Table 4), but this was primarily due to the differences in transmission rates between the Amphur and the Santo Domingo strains (18% - vs - 88%, respectively). The transmission rates obtained with the Kampala strain did not differ statistically from those obtained with the Santo Domingo

Table 2. Comparison of YF virus transmission rates among engorging sibling *Aedes aegypti* mosquitoes exposed to either the *in vitro* capillary technique or suckling mice.

Capillary technique feeding (FCS/sucrose)			Mouse feeding		
Mosquito	Degree of Engorgement	Transmission	Mosquito	Degree of Engorgement	Transmission
1	½	Yes	1	Full	Yes
2	½	No	2	Full	No
3	½	Yes	3	Full	No
4	Full	Yes	4	Full	Yes
5	¾	No	5	Full	Yes
6	¾	Yes	6	Full	Yes
7	¾	Yes	7	Full	Yes
8	Full	Yes	8	Full	Yes
9	½	Yes	9	Full	Yes
10	Full	Yes	10	Full	Yes
Capillary technique transmitting 8/10–80%.			Mouse feeding transmitting 8/10–80%.		

Table 3. Comparison of engorgement and YF transmission rates of sibling *Aedes aegypti* mosquitoes fed by the *in vitro* and *in vivo* techniques.

Defibrinated human blood			10% Fetal Calf Serum & 10% Sucrose			Suckling Mice		
Mosquito	Engorgement	Transmission	Mosquito	Engorgement	Transmission	Mosquito	Engorgement	Transmission
1	Trace	No	1	Empty	No	1	Full	Yes
2	Empty	No	2	Full	Yes	2	Full	No
3	Empty	No	3	Full	Yes	3	Full	Yes
4	Empty	No	4	Full	No	4	Full	Yes
5	Empty	Yes	5	¾	Yes	5	Full	Yes
6	Empty	Yes	6	¾	Yes	6	Full	No
7	Empty	No	7	¼	Yes	7	Full	Yes
8	Empty	No	8	Full	No	8	Full	Yes
9	Empty	No	9	Full	Yes	9	Full	Yes
10	Empty	No	10	½	Yes	10	Full	Yes
11	Empty	No	11	Full	Yes	11	Full	Yes
			12	Full	No	12	Full	No
						13	Full	Yes
						14	Full	Yes
						15	Full	Yes
	1/11=9%	2/11=18%		11/12=92%	8/12=67%		15/15=100%	12/15=80%

Table 4. Transmission rates of geographic strains of *Aedes aegypti* mosquitoes intrathoracically inoculated with YF virus.

Mosquito strain	Incubation (inclusive days post infection) 26	33	75	Total
Amphur	2/11* (18%)	—		2/11 (18%)
Kampala	5/8 (62%)	6/12 (50%)	7/25 (28%)	18/45 (40%)
Santo Domingo	8/9 (88%)	10/13 (77%)		18/22 (82%)

* Numerator—Number females transmitting virus.
Denominator—Number females infected.

strain. Essentially the same results were obtained in a replicate trial. Santo Domingo strain was the first to transmit virus at 4 days incubation; the first Amphur and Kampala transmissions were at 9 days and 7 days, respectively. All inoculated females were later examined by the headsquash-FA procedure to determine infection rates. With the exception of one Amphur strain female, all examined mosquitoes contained detectable YF viral antigen. Therefore, any observed differences in transmission rates are not attributable to differences in infection rates. The longevity of the Kampala strain is noteworthy.

DISCUSSION

The *in vitro* feeding technique using a 10% FCS-10% sucrose meal coupled with mosquito inoculation seems to be a satisfactory substitute for a susceptible laboratory host as a mechanism for detecting virus transmission. Although it would seem that manipulations of extremely small quantities of mosquito saliva would result in a decreased virus titer, perhaps the use of the extremely sensitive mosquito inoculation technique for virus assay compensates for any decrease in titer. An additional benefit of the method is that moribund mosquitoes, which are incapable of engorgement by themselves, can frequently be induced to engorge passively and thus assayed for virus transmission using this technique. For *Ae. aegypti* the 10% sucrose-10% FCS meal was found to be optimal for inducing engorgement, but this composition may differ for other mosquito species.

Since the artificial meal does not coagulate around the proboscis of the mosquito as well as serum (Aitken 1977), some mosquitoes were able to extricate themselves from the capillary and to fly away. The screened chamber (Figure 1) restrained such mosquitoes. Severance of all but the basal portion of the wings also prevents mosquitoes from dislodging themselves from the capillaries (Elaine Vier, Yale Arbovirus Research Unit, personal communication).

The present differences in vector competence corroborate those observed previously (Aitken et al. 1977). Since these mosquitoes were infected by intrathoracic inoculation, it would appear that more than the mesenteron is operative in determining vector competence. The Amphur strain of *Ae. aegypti* from Asia is not only a less efficient transmitter of YF virus when infected *per os* (Aitken et al. 1977) but also when infected by intrathoracic inoculation. Santo Domingo strain is the most efficient transmitter by either route. This fact may partially explain the apparent lack of YF in Asia; Asian strains of *Ae. aegypti* may be less competent vectors. The hypothesis needs further investigation with other strains of *Ae. aegypti* from various geographic areas, especially other Asian strains.

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