LABORATORY TRANSMISSION OF JAPANESE B ENCEPHALITIS VIRUS BY SEVEN SPECIES (THREE GENERA) OF NORTH AMERICAN MOSQUITOES*

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(Received for publication, November 19, 1945)

In view of the present importance of, and widespread interest in diseases of the Pacific Area, it would seem at this time to be of particular value to report the results of laboratory studies made over the past three years on the ability of several of the common species of North American mosquitoes to act as vectors of Japanese B encephalitis virus. Japanese and Russian worker have reported that *Culex pipiens* var. *pallens* Coq., *Culex tritaeniorhynchus* Giles, *Aedes japonicus* Theo., *Aedes albopictus* (Skuse), and *Aedes togoi* Theo. could be infected by feeding on virus suspensions and could act as laboratory vectors (1–3). In Japan and Russia, *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens* have been found infected in nature repeatedly (3, 4). Moreover, it is reported from Japan that mosquitoes reared in the laboratory from larvae or eggs collected in an endemic area are occasionally infected (5, 6). However, since all reported "isolations" from such materials were accomplished by serial blind passage, and since Japanese B virus, or one closely similar to it, has been isolated from colony mice by research workers in Japan (7) we are inclined to await further evidence before accepting transovarian infection as proven. According to Japanese and Russian reports, virus can be detected with relative ease in the blood of human beings (5, 8) and of naturally or experimentally infected mammals (9, 5).

The available evidence in support of mosquito transmission is very similar to that advanced in recent years for the St. Louis and Western equine types of encephalitis in North America: the virus has been isolated from mosquitoes collected in endemic areas (10–12), the same species of mosquitoes have been demonstrated to be vectors of virus in the laboratory (13, 14), and virus can be detected with relative ease in the blood of certain common vertebrate animals (15, 16).

* This investigation was carried out in collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, Preventive Medicine Division, Office of The Surgeon General, U. S. Army; and under a contract, recommended by the Committee on Medical Research between the Office of Scientific Research and Development, and the University of California. Aided by a grant from The National Foundation for Infantile Paralysis.

In the present study we have tested many of those species of mosquitoes which have been demonstrated to be natural or laboratory vectors of the arthropod-borne virus encephalitides of Western North America. These studies have been undertaken with the possibility in mind that the Japanese B encephalitis virus might appear on the North American mainland, particularly in the West, in which event a knowledge of the vector ability of our common mosquitoes would be of inestimable value in the immediate establishment of adequate control measures.

Methods and Materials

The techniques employed in these studies are in general the same as those reported in our previous papers on experimental transmission (13, 14). The virus employed was the Nakayama strain obtained through the courtesy of Lieutenant Colonel A. B. Sabin. For most experiments mosquitoes were infected by feeding on a pledget of cotton moistened with a mixture of a 10 per cent mouse brain virus suspension and defibrinated rabbit blood in proportions varying in the different experiments from 1:50 to 1:1000. A little granulated sugar was then sprinkled on the virus-blood-soaked cotton. The cotton pledget was placed on top of a bobbinet-covered pint jar containing approximately 50 mosquitoes which had been fasted for 24 hours. The mosquitoes fed very readily through the netting. By this method of feeding it was possible to get most of the mosquitoes to engorge and to be certain of their taking an infective meal. Following the infective meal all mosquitoes were held at a temperature averaging 80°F. or above in a room with a high relative humidity (average of 80 per cent or more). After an extrinsic incubation of 4 to 6 days, feedings were begun on 21 to 28 day old Webster strain Swiss mice. As routine the mice were anesthetized for mosquito feedings with a mixture composed of sodium pentobarbital 0.25 gm., sodium barbital 1.5 gm., benzyl alcohol 1.0 cc., propylene glycol 30.0 cc., and enough distilled water to bring the quantity to 50.0 cc., administered intraperitoneally in doses of 0.15 cc. The anesthetized mice were laid on the top of a bobbinet-covered pint jar containing the test mosquitoes, and the mosquitoes fed on the mice through the cloth. The number engorging was observed and recorded. If no specimens were engorged yet some were observed to insert their mouth parts or to probe about, it was considered that salivary gland secretions may have been inoculated, and the mice were held for observation. When feeding was terminated the mice were removed to holding cages and observed for a period of 21 days. Any mouse developing signs of encephalitis was sacrificed and the brain passed to 3 other mice. If bacterial cultures were sterile and all 3 of the second passage mice developed typical encephalitis and died between the 4th and 10th days, transmission by mosquito bite was accepted as demonstrated.

In two experiments mosquitoes were fed on chickens with a demonstrated viremia following subcutaneous inoculation with a 10^{-2} dilution of virus. In a single experiment infected mosquitoes were fed on a normal 6 weeks old chicken. This bird was bled by cardiac puncture 24 and 48 hours after the mosquito feeding and serum from this blood was tested for virus content by intracerebral inoculation into mice.

At various intervals after an infective meal, pools of mosquitoes were killed, triturated, and tested for virus content by methods previously reported (13, 14). This included light chloroform anesthesia, occasionally freezing at −70°C. for a period of a few days or weeks, grinding with alundum in 30 per cent serum-broth, and then centrifugation for 15 minutes at a speed of approximately 18,000 r.p.m. in an International centrifuge, multispeed head. The supernatant was finally inoculated intracerebrally in mice.

In several experiments tests were made for transovarian transmission of virus from infected females to their progeny. In one experiment eggs were tested for virus, and, in other tests

the eggs were hatched, reared through the immature stages, and the adults were tested for virus content by mouse inoculation.

RESULTS

Experiments with Culex pipiens Linn.— Three tests have been made of the ability of *Culex pipiens* to act as a vector, and a total of 5 transmissions has resulted. Two different colonies of this species were employed.[1] The first two experiments were made with colony 1 from Oakland, California (Table I).

TABLE I

*Experiments with Culex pipiens Colony 1**

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
26 (1943)	Suspension	1:50	7	1	Mouse brain	+
			9	1	Mouse brain	0
			16	—‡	Mosquito	+
			48	183	Mosquito adult progeny	0
44 (1943)	Suspension	1:50	11, 20	1, 5	Mouse brain	+
			10, 12, 13, 14, 15	1, ?§, 1, 2, ?	Mouse brain	0
			16, 17	?, 2	Mouse brain	0
			3	10	Mosquito	+
			37	9	Mosquito adult progeny	0

* See footnote 1.

‡ Number of specimens not recorded.

§ Indicates that no specimens engorged with blood, but one or more specimens were seen to insert their mouth parts into or to probe at the skin of the experimental animal.

+ = transmission effected.

In Experiment 26, transmission occurred at 7 days' incubation from the bite of a single mosquito. In Experiment 44, transmission occurred at 11 and 20

[1] Mr. Pedro Galindo, Division of Entomology and Parasitology, University of California, furnished the parent stock for colony 1. The origin of this colony was Oakland, California. Mr. Galindo's unpublished laboratory studies indicated this was *Culex pipiens* var. *molestus* Forskal. In the laboratory he demonstrated this colony to be stenogamous and autogenous and it interbred readily with *Culex quinquefasciatus*.

The material for Experiment 23, colony 2, originated from the Yakima Valley, Washington, and probably represents *Culex pipiens* var. *pipiens*. In the laboratory this strain proved to be anautogenous and eurygamous. In addition, field studies indicated that in the Yakima Valley this species preferred to feed on birds and it was not recorded as feeding on man.

days' incubation. All attempts to demonstrate virus in 192 adults reared from eggs of infected females were negative.

In Experiment 23, mosquitoes of colony 2, from Yakima, Washington, were used (Table II). At 20 days' incubation transmission was made to a mouse and a chicken.

TABLE II

*Experiments with Culex pipiens Colony 2, Source: Yakima, Washington**

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
23 (1945)	Suspension	1:1000	14, 18, 19	2, 1, 1	Mouse brain	0
			20	1	Mouse brain	+
			20	4	Chicken serum	+
			21	6	Mosquito	+

* See footnote 1.

TABLE III

Experiments with Culex quinquefasciatus

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
17 (1943)	Suspension	1:50	11	5	Mouse brain	+
			9, 14	5, 1	Mouse brain	0
			23	3	Mosquito	0
			23	147	Mosquito adult progeny	0
			38	62	Mosquito adult progeny	0
45 (1943)	Suspension	1:50	6, 7, 8, 9, 12, 13	1, 1, 2, 4, 1, 2	Mouse brain	0
			27	47	Mosquito adult progeny	0
3 (1944)	Suspension	1:50	14, 22, 25	?, 5, 9	Mouse brain	+
			18, 20, 21, 23	1, 1, 1, 2	Mouse brain	0
			7	50	Mosquito (male)	+
			22, 26	141, 148	Mosquito adult progeny	0
			26	2	Mosquito	+
21 (1945)	Chicken		9, 11, 13	9, 5, 9	Mouse brain	0

Experiments with Culex quinquefasciatus Say.—Four experiments have been performed with this mosquito, in two of which (Experiments 17 and 3) transmission occurred, a total of 4 times (Table III). These mosquitoes were demonstrated to be infective by their bite at 11, 14, 22, and 25 days' incubation. Infection persisted in the vector up to 26 days. The one attempt (Experiment

21) to transmit the virus with mosquitoes which had fed on an inoculated chicken, at a time virus was present in its blood, was negative. Repeated attempts to recover virus from the adult progeny of infected females were negative (545 tested).

Experiments with Culex tarsalis Coq.—Seven experiments have been completed with this species (Table IV). Transmission was achieved in only Experiment

TABLE IV

Experiments with Culex tarsalis

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
36 (1943)	Suspension	1:50	6, 8, 9, 10, 12, 14, 22, 21	?, ?, ?, ?, ?, ?, ?, ?	Mouse brain	0
			25	9	Mosquito	+
35 (1943)	Suspension	1:50	6, 10, 11, 12, 14, 16, 24, 25	?, ?, ?, ?, ?, ?, ?, ?	Mouse brain	0
			27	9	Mosquito	0
8 (1944)	Suspension	1:50	6, 8, 10, 12, 14	2, 1, 3, 4, 4	Mouse brain	0
			18, 20	4, 8	Mosquito	+
25 (1944)	Suspension	1:50	5	7	Mosquito (male)	+
26 (1945)	Suspension	1:1000	8, 10, 12, 14, 16, 18	1, 12, 2, 7, 6, 2	Mouse brain	0
			20	59	Mosquito	+
48 (1945)	Suspension	1:100	6, 10	3, 2	Mouse brain	0
52 (1945)	Suspension	1:100	6–8*, 8–10	?, 3	Mouse brain	+
			15–17	6	Mosquito	+
			6–8, 8–10, 11–13, 13–15	?, 4, 2, 5	Mouse brain	0

* In this experiment the mosquitoes were infected in three lots over a 3 day period. The entire group then was combined for feeding tests. It is necessary for this reason to express the incubation period as extending over a possible time of 3 days.

52 (2 mice infected). However, in earlier experiments it was demonstrated repeatedly that virus survived in this species for times ranging up to 25 days. In only one instance were specimens tested at a later period (27 days) with negative results. Failure to achieve transmission in several of the early experiments may be explained by the almost complete refusal of this species to feed on mice. Virus persisted in male mosquitoes of this species for at least 5 days.

Experiments with Aedes dorsalis (Meigen).—In Experiments 14 and 18 (Table

V) no transmission occurred from the bite of *A. dorsalis* nor could virus be recovered from the small number of mosquitoes tested. In Experiment 27, however, a single transmission was attempted and effected at 16 days' incubation. Ten specimens fed and the mouse subsequently developed typical encephalitis.

Experiment with Aedes nigromaculis (Ludlow).—In a single experiment (Table VI) *Aedes nigromaculis* transmitted the virus at 8, 10, 12, and 14 days' incubation. Transmission was not achieved at 16 days but the mosquitoes themselves were demonstrated to be infected at this time. The experiment was then terminated. Virus was not isolated in a test of 100 eggs from these infected mosquitoes.

TABLE V

Experiments with Aedes dorsalis

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
14 (1943)	Suspension	1:50	19	2	Mouse brain	0
			19	2	Mosquito	0
18 (1943)	Suspension	1:50	7, 9, 12, 14	4, 4, 5, 2	Mouse brain	0
			15	2	Mosquito	0
27 (1944)	Suspension	1:50	16	10	Mouse brain	+

TABLE VI

Experiments with Aedes nigromaculis

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
6 (1943)	Suspension	1:50	8, 10, 12, 14	26, 17, 31, 21	Mouse brain	+
			16	11	Mouse brain	0
			16	11	Mosquito	+
			16	100	Mosquito eggs	0

Experiments with Aedes vexans (Meigen).—Four experiments with this species have all been negative both for transmission and persistence of infection (Table VII).

Experiments with Aedes varipalpus (Coq.).—In two experiments *Aedes varipalpus* failed to transmit (Table VIII). Virus was shown to survive for at least 8 days in males of this species and 14 days in the females.

TABLE VII

Experiments with Aedes vexans

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
14 (1943)	Suspension	1:50	19	4	Mouse brain	0
			19	12	Mosquito	0
30 (1943)	Suspension	1:50	10, 11	1, 2	Mouse brain	0
			27	21	Mosquito adult progeny	0
16 (1945)	Chicken		8, 10, 14, 16	2, 3, 4, 3, 3	Mouse brain	0
			18	5	Mosquito	0
17 (1945)	Suspension	1:1000	8, 10, 12, 14	14, 18, 3, 3	Mouse brain	0

TABLE VIII

Experiments with Aedes varipalpus

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
12 (1944)	Suspension	1:50	6, 8, 12	22, 8, 1	Mouse brain	0
			8, 12	10, 6	Mosquito	0
			8	20	Mosquito (male)	+
4 (1945)	Suspension	1:1000	6, 8, 10, 12, 14	17, 14, 27, 1, 1	Mouse brain	0
			14	26	Mosquito	+

TABLE IX

Experiments with Culiscta incidens

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
46 (1943)	Suspension	1:50	6, 7	1, 1	Mouse brain	0
			17	1	Mosquito	+
1 (1944)	Suspension	1:50	8, 12, 14	12, 1, 2	Mouse brain	+
			4, 6, 10, 18	15, 22, 8, 1	Mouse brain	0
			20	10	Mosquito	+

Experiments with Culiseta incidens (Thomson).—In Experiment 46 (Table IX) transmission did not occur, but virus was shown to survive for 17 days. In

Experiment 1, transmission was effected three times at 8, 12, and 14 days' incubation. At 20 days' incubation the mosquitoes were still infected and the experiment was terminated.

Experiments with Culiseta inornata (Williston).—In Experiments 2 and 5 (Table X) transmission occurred three times at 10, 12, and 20 days' incubation.

Experiments with Anopheles maculipennis freeborni Aitken.—In two experiments this species failed to transmit the virus (Table XI) but a few mosquitoes remained infected up to 8 days after an infective meal.

TABLE X

Experiments with Culiseta inornata

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
2 (1944)	Suspension	1:50	20	2	Mouse brain	+
			4, 6, 8, 10, 12, 14, 18	12, 9, 4, 3, 1, 2, 1	Mouse brain	0
			20	8	Mosquito	+
5 (1945)	Suspension	1:100	6, 8, 14, 16, 18, 20	1, 4, 5, 1, 6, 4	Mouse brain	0
			10, 12	9, 6	Mouse brain	+
			23	4	Mosquito	0

TABLE XI

Experiments with Anopheles maculipennis freeborni

Experiment No. and year	Infectious virus-blood meal		Days after infective meal when transmission or isolation was attempted	No. mosquitoes feeding or tested	Material tested for virus	Result
	Type	Mouse brain dilution				
40 (1943)	Suspension	1:50	0, 1, 3, 8	10, 10, 10, 23	Mosquitoes	+
			2, 4, 5, 6, 7	10, 10, 10, 10, 10	Mosquitoes	0
53 (1944)	Suspension	1:100	10, 12, 14, 16	4, 6, 2, 1	Mouse brain	0
			16	3	Mosquitoes	0

DISCUSSION AND SUMMARY

In the present studies ten common species of Western North American mosquitoes have been tested for their ability to act as vectors of Japanese B encephalitis virus (see summary Table XII). The strain of Japanese B encephalitis virus which was used was adapted to direct mouse brain passage, probably a disadvantage, but no freshly isolated strain was available. Of the ten species of mosquitoes tested, seven were demonstrated to be laboratory vectors. These seven species represent three genera (*Culex*, *Aedes*, and *Cu-*

liseta). In previously reported work Japanese and Russians had only incriminated five species of two genera (*Aedes* and *Culex*) (1–3). Transmission was made to mice 21 times and to a chicken once. Two attempts to infect mosquitoes from an infected chicken were unsuccessful, but no significance is attached to so few experiments. Repeated tests for virus in the eggs, or in imagines reared from eggs of infected female mosquitoes have been negative. In this we failed to confirm results claimed by Japanese investigators (5, 6).

These data, in addition to the published accounts by Japanese and Russian workers of the natural epidemiology of this disease lead us to believe that this virus might well establish itself in North America, especially if introduced in those areas where our native encephalitides are now endemic. These studies also indicate that species of mosquitoes (*Culex tarsalis*, *Culex pipiens*,

TABLE XII

Summary of Mosquito Transmission Experiments with Japanese B Virus

Mosquito	Source of infection	Days after onset when demonstrated to be infectious	No. times transmission effected	Days virus demonstrated to persist in mosquitoes
Culex pipiens var. molestus	Virus blood suspension	7–20	3	20
Culex pipiens var. pipiens	Virus blood suspension	20	2	21
Culex quinquefasciatus	Virus blood suspension	11–25	4	26
Culex tarsalis	Virus blood suspension	6–10*	2	25
Aedes nigromaculis	Virus blood suspension	8–14	4	16
Aedes varipalpus	Virus blood suspension	—	0	14
Aedes dorsalis	Virus blood suspension	16	1	16
Aedes vexans	Virus blood suspension	—	0	0
Culiseta incidens	Virus blood suspension	8–14	3	20
Culiseta inornata	Virus blood suspension	10–20	3	20
Anopheles maculipennis freeborni	Virus blood suspension	—	0	8

* See footnote Table IV.

Aedes dorsalis, and *Culiseta inornata*) now known to be fully incriminated vectors of the Western equine or St. Louis encephalitis viruses can also serve as laboratory vectors of the Japanese B virus. Methods for the effective abatement of these species should be further developed and put into practice if future epidemics of encephalitis of the Western equine, St. Louis, or Japanese B types in Western North America are to be prevented or brought under control.

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