ISOLATION OF ARBOR VIRUSES FROM MOSQUITOES COLLECTED AT LIVE-STOCK PENS IN GUMMA PREFECTURE IN 1959*

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Hitherto Japanese B encephalitis (JBE) virus has been isolated in many instances from men, animals and mosquitoes and it has been thought probable that JBE virus is the only prevalent arbor virus in Japan. In the meantime, Ando *et al.* (1952) reported the isolation of a virus (Negishi virus) from the autopsied brain of a patient clinically diagnosed as JBE. By our recent studies, evidences have been accumulated which indicate that Negishi is a member of Group B virus bearing antigen closely related with Russian Spring Summer Encephalitis virus. However, neither virus isolation nor discovery of a human infection has been reported ever since 1949. More recently, Scherer (1958) reported that he could isolate a new Group A virus (Sagiyama virus) from mosquitoes in Saitama, Japan.

Taking those facts in account, it seems important to pursue the distribution of arbor viruses present in Japan over a period of years, hoping that such an investigation might give a clue to learn the ecology of arbor virus in nature. In a limited area, Gumma Prefecture, an investigation was started to gain detailed informations on the yearly appearance of arbor virus in arthropod. The present paper reveals the results obtained in the first year on the infection of mosquitoes in summer. The details of the isolation of 3 arbor viruses including a new one will be presented.

GEOGRAPHICAL, TOPOGRAPHICAL AND EPIDEMIOLOGICAL CHARACTERS OF GUMMA PREFECTURE

Gumma Prefecture occupies the north-west part of Kanto district, a hundred miles apart from Tokyo City. It extends in N. La. 36° — 37° (*i. e.* 150 km) and in E. Lo. $138^{\circ}15'$ — $139^{\circ}45'$ (*i. e.* 120 km). Mountains (3,000—6,000 ft in height) occupy 4/5 and plane 1/5 of the area. The plane includes mulberry plant, well irrigated rice field and swampy areas as seen in Fig. 1. The population in the prefecture is 1,608,000. Farmers commonly keep domestic animals, hens, pigs, cows and horses. A nesting point of egrets

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Fig. 1. Topography of Gumma prefecture.

and herons is found near Takasaki and thousands of birds migrate in summer every year. In the year 1958, 81 human cases were reported as JBE, mostly in the plane area. They occurred exclusively in summer from July to September. Among them 36 (44 %) were confirmed as JBE serologically (Fig. 2).

MATERIALS AND METHODS

Collection of mosquitoes: In the plane, 6 places were selected as sites of mosquito collection, *i. e.* Takasaki, Kiryu, Isezaki, Ota, Akabane and Itakura (Figs. 1 and 2). From the end of July to the middle of September, mosquitoes were caught by sucking tubes from walls of live-stock pens, keeping mainly cattles, pigs and horses at night from 7.30 to 8.30 pm. The collected mosquitoes were kept alive one night and were brought to the station laboratory at the Gumma Prefectural Institute, Maebashi. They were classified and stored in a dry ice box until virus isolation.

Isolation of virus: Mosquitoes of approximately 100 for each species were emulsified in a mortar by adding 2 % normal rabbit serum saline containing penicillin 500 units/cc and streptomycin 500 µg/cc. Volume of diluent varied for each species; for 100 mosquitoes, *C. tritaeniorhynchus* 2.0 cc, *C. pipiens* 4.0 cc, *C. bitaeniorhynchus* 4.0 cc, *Mansonia uniformis* 4.0 cc, *A. vexans* 6.0 cc to 8.0 cc, *Armigeres* 8.0 cc, and *Anopheles* 8.0 cc. Those concentrations of mosquitoes were found to avoid toxic effects upon suckling mouse brain. The suspensions were held at 4°C for 2 hours and centrifuged at 3,000 rpm for 10 minutes in cold room. Two hundredth cc of the supernate was inoculated *i. c.* into suckling mice. For each pool of mosquitoes, 2 litters of 2 to 4 day old suckling mice (dd strain) were used. The mice were observed daily for 10 days and brains

were harvested from sick mice when they became moribund. The harvested brains were tested for bacterial and mycotic contaminations with thioglycolate medium and stored at -70°C .

Identification of virus isolates: Identification studies were carried out in the National Institute of Health by hemagglutination-inhibition and complement fixation tests. JBE virus strains were identified at their 3rd passage levels. Details of the identification studies of viruses other than JBE virus will be described in a separate paper (Oya *et al.*, 1961).

Fig. 2. Incidence of Japanese B encephalitis patient in 1958. (reported cases).

RESULTS

Number and Species of Mosquitoes Collected

In total, 52,641 mosquitoes were collected at the whole study sites; 13,815 at Itakura, 10,680 at Takasaki, 8,790 at Kiryu, 7,212 at Ota, 7,059 at Akabane and 5,085 at Isezaki. Mosquito species were as follows:

- Culex (Culex) tritaeniorhynchus* Giles
- Culex (Culex) pipiens* (Linnaeus)
- Culex (Culex) bitaeniorhynchus* Giles
- Culex (Lutzia) vorax* Edwards
- Aedes (Aedimorphus) vexans nipponii* (Theobald)
- Aedes (Stegomyia) albopictus* (Skuse)
- Armigeres (Armigeres) subalbatus* (Coquillett)
- Anopheles (Anopheles) spp.*
- Mansonia (Mansonioides) uniformis* (Theobald)

Table 1. Number and species of mosquitoes collected at live-stock pens in Gumma in 1959

Date collected	<i>Culex</i>				<i>Aedes</i>		<i>Armi-geres</i>	<i>Ano-pheles</i>	<i>Man-sonia</i>
	<i>tritaen.</i>	<i>pipiens</i>	<i>bitaen.</i>	<i>vorax</i>	<i>vexans</i>	<i>albo</i>			
July 28 1959	702	120(12)	0	0	249(1)	2	51	361	0
29	1,174	275(9)	8	0	474	0	25(10)	601	0
30	1,821	338(59)	45	0	470	2	15	902	0
Aug. 11	646	374(49)	30	0	167	0	18	257	0
12	1,278(1)	279	22	0	234	1	10	689	0
13	324(3)	167(44)	10	0	33	0	20	270	0
18	254	124(18)	2	0	288	0	30(4)	75	0
19	966	223(23)	9	0	866	0	25(6)	127	0
20	854	362(30)	4	0	1,761	2	55(2)	280	0
25	680	287(7)	3	1	742	2	75(1)	327	0
26	989	282(4)	9	0	829	0	49	838	9
27	1,299	232(49)	10	1	435	1	70(5)	211	10(1)
Sept. 1	1,248	287(15)	18(1)	0	677	1	65(1)	1,116	16
2	1,582	275(13)	3	0	749	1	50(4)	1,177	4
3	1,680	356(6)	9	0	404(1)	3	124(65)	570(4)	18
8	1,102(1)	124(3)	5	0	1,680	0	120(36)	1,789	9
9	1,072	128(16)	4	0	1,262	0	85(9)	2,306	12
10	1,129	273(29)	19	0	1,641	2	33(7)	2,683	15(1)
Total	18,805	4,895	211	2	12,963	17	1,070	14,583	95

Grand total 52,641

Note: Numbers in parenthesis denote the numbers of male mosquitoes.

Number of mosquitoes collected at each period are listed in Table 1. These numbers do not seem to reflect the real distribution of mosquitoes of live-stock pens, since some selection was made by collectors when they found a large number of mosquitoes.

Properties of New Arbor Virus Isolates

In general, 47 strains of arbor virus were isolated from 407 pools of mosquitoes. Among them, 39 were identical to JBE virus. The other 8 strains had biological characters remarkably different from that of JBE virus. Six strains out of 8 were found to be virulent to adult mice as well as to suckling mice showing an i. c. infective titer of $10^{7.2}$ in the adult and $10^{7.6}$ in the suckling. The incubation period found in the adult mice was 3 to 5 days and that in the suckling 2 to 3 days. Identification studies furnished evidences that those 6 were identical with each other and neither belonged to Group A or Group B virus nor related with any other ungrouped arbor viruses tested. On the other hand, they were found to have such common properties of arbor virus as agglutination of one day chick or goose erythrocytes, inactivation with desoxycholate (Theiler, 1957) and resistance against precipitation by protamine sulphate (Warren *et al.*, 1949). They were temporarily designated as Akabane virus.

The remaining two strains showed a very low virulence even in the suckling mouse

brain ($10^{2.0}$ to $10^{3.0}$). Incubation period was around 7 days after inoculation. Adult mice inoculated i. c. with this virus manifested no symptom, while the immune serum against this virus contained common antibody against Group A virus, and finally this virus was considered to be closely related with Sagiyama (Scherer, 1958) and AMM 2021 (Buescher, 1959) viruses, though possessing a different pathogenicity to the mouse.

Table 2. Isolation of Japanese B encephalitis virus from mosquito-pools

Date collected	<i>Culex</i>		<i>Aedes</i>	Other mosquitoes
	<i>tritaen.</i>	<i>pipiens</i>	<i>vexans</i>	
July 28, 29, 30	*7/41 (17.1%)	0/6	0/12	0/16
Aug. 11, 12, 13	3/19 (15.8%)	0/7	0/5	0/9
18, 19, 20	6/18 (33.3%)	1/6 (16.7%)	0/27	0/5
25, 26, 27	8/27 (29.6%)	0/4	0/20	0/11
Sept. 1, 2, 3	8/35 (22.9%)	0/4	0/16	0/15
8, 9, 10	6/29 (20.7%)	0/5	0/40	0/28
Total	38/169 (22.5%)	1/32 (3.1%)	0/120	0/84

Note: Denominators show number of mosquito-pools tested. Numerators mean number of virus-positive pools. One pool consisted of about 100 mosquitoes.

Isolation of JBE Virus

Thirty eight out of 39 strains of JBE virus were isolated from *C. tritaeniorhynchus* and only one strain from *C. pipiens*. No JBE virus was obtained from other species. As indicated in Table 2, JBE virus was recovered throughout the collection period. The peak of mosquito infection was demonstrated in the middle of August when a portion as high as 33 % of the pools was found positive. In addition, JBE virus was obtained at all study sites except Akabane.

Isolation of Akabane Virus

Among 6 strains of Akabane virus, 4 were obtained from *A. vexans* and 2 from *C. tritaeniorhynchus*. None of other species yielded Akabane virus in the present study. Mosquito infection was limited to the middle and end of August (Table 3). The places yielding Akabane virus were; Akabane (2 strains), Itakura, Ota, Kiryu and Takasaki (1 strain from each).

Isolation of Itakura Virus

Two strains of Itakura virus were isolated from *A. vexans* and all were obtained from Itakura (Table 4).

DISCUSSION

As expected beforehand, JBE virus was isolated most frequently from mosquitoes. Isolation was successful through the period of from the end of July to the middle of

Table 3. Isolation of Akabane virus from mosquito-pools

Date collected		<i>Culex</i>		<i>Aedes</i>	Other mosquitoes
		<i>tritaen.</i>	<i>pipiens</i>	<i>vexans</i>	
July	28, 29, 30	*0/41	0/6	0/12	0/16
Aug.	11, 12, 13	0/19	0/7	0/5	0/9
	18, 19, 20	1/18 (5.6%)	0/6	1/27 (3.7%)	0/5
	25, 26, 27	1/27 (3.7%)	0/4	3/20 (15.0%)	0/11
Sept.	1, 2, 3	0/35	0/4	0/16	0/15
	8, 9, 10	0/29	0/5	0/40	0/28
Total		2/169 (1.2%)	0/32	4/120 (3.3%)	0/84

* The same to Table 2.

Table 4. Isolation of Itakura virus from mosquito-pools

Date collected		<i>Culex</i>		<i>Aedes</i>	Other mosquitoes
		<i>tritaen.</i>	<i>pipiens</i>	<i>vexans</i>	
July	28, 29, 30	*0/41	0/6	0/12	0/16
Aug.	11, 12, 13	0/19	0/7	0/5	0/9
	18, 19, 20	0/18	0/6	0/27	0/5
	25, 26, 27	0/27	0/4	1/20 (5.0%)	0/11
Sept.	1, 2, 3	0/35	0/4	0/16	0/15
	8, 9, 10	0/29	0/5	1/40 (2.5%)	0/28
Total		0/169	0/32	2/120 (1.7%)	0/84

* The same to Table 2.

August. The infection rate of *C. tritaeniorhynchus* was fairly high from the beginning of the present study and seemed to have attained a peak toward the end of August. At the peak, one out of 300 *C. tritaeniorhynchus* was thought to be infected with JBE virus. Such a high incidence of infected mosquitoes seems quite adequate to explain the remarkably high incidence of antibody presence in the sera of residents in the epidemic area. An interesting result was noted on the relation of virus to the species of mosquito. With only one exception, JBE virus was isolated exclusively from *C. tritaeniorhynchus* confirming the results obtained by previous investigators (Mitamura *et al.*, 1938; Hammon *et al.*, 1949). As far as the rural district is concerned, it is no doubt that *C. tritaeniorhynchus* is a main vector of JBE virus in Japan. However, it is not quite correct to conclude that only a single type of virus transmission mode is responsible for all epidemics occurring in Japan. JBE virus has been isolated heretofore from autopsied materials even in the center of big cities as Tokyo or Yokohama, where *C. tritaeniorhynchus* seems scanty.

On the other hand, the favorite host of Akabane and Itakura viruses seems to be different from that of JBE virus. Akabane virus was isolated from *A. vexans* as well

as *C. tritaeniorhynchus*. Itakura virus was isolated only from *A. vexans*, but a similar virus was obtained from *C. tritaeniorhynchus* by Scherer (1958). No other species of mosquito yielded viruses. The reason why there is a limited relation between virus and mosquitoes is still obscure. The difference of the susceptibility or difference of zoophily of mosquitoes might account for this fact.

It is obvious that Akabane virus is a new arbor virus isolated in Japan. As far as we know, nobody succeeded in isolating this virus before. Then a question may arise as to how it appeared in 1959. Also, it is interesting to see whether it would reappear in the following years. There may be many speculations to explain the problem. Anyhow, it is apparent that newly described virus may furnish a good tool for the understanding of the ecology of arbor viruses in Japan. The facts that Itakura virus was isolated only from a swampy area where the migrating birds used to be in plenty and that this virus has close immunological similarity with Malayan virus (AMM 2021) appear to support the idea there must be a close relation between Japan and other countries located in South Asia with regard to arbor virus exchange.

SUMMARY

A description was made on the arbor virus isolation from mosquitoes collected in summer of 1959 at Gumma, Japan. Out of 47 isolates, 39 were Japanese B encephalitis virus, 2 were Sagiya-like virus of Group A and 6 were considered to be a new agent. All Japanese B encephalitis virus strains were isolated from *C. tritaeniorhynchus* but one from *C. pipiens*. Group A virus strains came from *A. vexans* collected at swampy area. A new agent was isolated from both *C. tritaeniorhynchus* and *A. vexans*. No other species of mosquito yielded arbor virus.

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