

Isolation of Japanese Encephalitis and Getah Viruses from Mosquitoes Collected in Nagasaki City in the Year of 1980 and 1981, Using *Aedes albopictus* Clone C6/36 Cells

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Abstract: Isolation of viruses was performed from various species of mosquitoes collected in Mogi area in Nagasaki City during the summer of 1980 and 1981, by inoculation to *Aedes albopictus* clone C6/36 cells. In 1980, five strains of Japanese encephalitis (JE) and one strain of Getah (GET) viruses were isolated from five pools of female *Culex tritaeniorhynchus*. One of the pools yielded both JE and GET viruses. Another strain of GET virus was isolated from a pool of engorged *Armigeres subalbatus*. In 1981, two JE and one GET strains were isolated from three pools of *C. tritaeniorhynchus*.

INTRODUCTION

Japanese encephalitis (JE) is a serious disease of central nervous system caused by infection with JE virus, a flavivirus of *Togaviridae* (Melnick, 1974), or group B arbovirus (Clarke and Casals, 1965). The virus is transmitted by *Culex tritaeniorhynchus* as main vector mosquitoes (Mitamura *et al.*, 1938; Hammon *et al.*, 1949; Buescher *et al.*, 1959), while pigs served as major amplifier vertebrate hosts (Scherer *et al.*, 1959; Konno *et al.*, 1966) during epidemic seasons in Japan. The incidence of human JE

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in Japan has markedly decreased after the last large epidemic in 1966 and are now observed mostly in the West and South parts of this country (Health and Welfare Statistical Association, 1975). However, the disease is still one of the major public health problems in other parts of Asia. As shown by several investigators, the number of human JE cases is dependent on the density of the vector, *C. tritaeniorhynchus* (Buei, *et al.*, 1968; Ishida *et al.*, 1969; Yamamoto, 1970; Wada *et al.*, 1975), and the decrease of the human JE cases appears to be mainly due to the lowered density of the vector mosquitoes. One of the authors, T. Oda., together with his colleagues, has performed ecological surveillance on the mosquito population and environmental factors at a field area of Mogi in Nagasaki City for about ten years. In order to better understand the relationship between the levels of mosquito population and JE virus dissemination, attempts were made to isolate JE virus from various species of mosquitoes collected at Mogi in the year of 1980 and 1981. Inoculation of the test materials to *Aedes albopictus* clone C6/36 cells was used as the primary isolation procedure because of its proven sensitivity equal or even better than the classical inoculation into suckling mouse brains (Buei, *et al.*, 1979; Igarashi, 1980; Igarashi *et al.*, 1981a; b). Screening for Getah (GET) virus, another arbovirus belonging to alphavirus of Togaviridae (Melnick, 1974), was done along with JE virus, because of its occasional detection from several species of mosquitoes collected in Japan (Scherer *et al.*, 1962; Matsuyama *et al.*, 1960; 1968; Hurlbut and Nibley, 1964; Ueba *et al.*, 1967; 1968; Shichijo *et al.*, 1970; Fukumi *et al.*, 1975; Yamamoto, 1980).

MATERIALS AND METHODS

Mosquito collection: Mosquitoes were collected at Mogi (32°43'N, 129°55'E): see Fig. 1 by Fukumi *et al.* (1975), using dry-ice traps operated 1 to 1.5 hours after the sunset followed by 20 minutes' collection by aspirators at pigpens. In 1980, collection was performed from May 6 to October 3, and in 1981 from May 21 to October 6, usually with weekly intervals. Mosquitoes were identified and live females were pooled, not exceeding 100 per pool, according to the date and species, and were kept frozen until processed.

Cell cultures: Origin and cultivation of *A. albopictus* clone C6/36 cells were described before (Igarashi, 1978; 1980). The cells were grown at 28°C with cell growth medium (GM) consisting of 10% heat-inactivated fetal calf serum (FCS) in Eagle's medium (Eagle, 1959) in Earle's saline supplemented with 0.2mM each of nonessential amino acids. Maintenance medium (MM) contained 2% of FCS instead of 10% in GM.

Virus isolation and identification: The method was similar as described previously (Buei *et al.*, 1979; Igarashi, 1980; Igarashi *et al.*, 1981 a; b). Each pool of mosquitoes was homogenized in a motor-driven glass homogenizer using 2ml of phosphate-buffered

saline (Dulbecco and Vogt, 1954) containing 0.4% of bovine plasma albumin fraction V (Armour, III, USA). and was centrifuged at 2,500rpm for 15 min. at 4°C. The resulting supernatant was filtrated through Millipore HA filter (25mm diameter). One tenth ml of the filtrate was inoculated to a tube culture of C6/36 cells from which GM had been removed. After 2 hours of adsorption, the cells were covered by 2ml of MM and incubated at 28°C for 7 days. Presence of the virus in the culture fluid was screened by inoculation to C6/36 cells grown on 8-chamber slides, which were harvested 3 days later in order to detect intracellular virus antigens by the immunoperoxidase staining (Okuno *et al.*, 1977) using anti-JE and anti-GET rabbit serum. The specimens showing positive reactions were identified by the neutralization test (Okuno *et al.*, 1978), and all the isolates were examined for neuropathogenicity to mice by intracerebral inoculation to suckling mice.

Antisera: Hyperimmune rabbit serum against JE or GET virus was prepared by 2 successive intramuscular inoculations with 1 week interval of purified virus preparations mixed with an equal volume of Freund's complete adjuvant. Rabbits were bled 1 week after the 2nd immunization, and the sera were separated and used at 1: 1000 dilution for immunoperoxidase staining. Anti-rabbit IgG goat serum was the product of Research Foundation for Microbial Diseases of Osaka University and was used at 1: 100 dilution. Peroxidase-antiperoxidase rabbit IgG complex was the product of Cappel Laboratories, Pa, USA and was used at 1: 1000 dilution.

RESULTS

Number of mosquitoes collected and processed for virus isolation is 1,744 mosquitoes in 147 pools in 1980, and 1,197 in 137 pools in 1981. The species composition of mosquitoes is shown in Table 1. *Culex tritaeniorhynchus* was the most dominant species (70.4% in 1980 and 70.9% in 1981) followed by *Anopheles sinensis* (20.0% in 1980 and 19.1% in 1981). Other species were less than 7% of total population.

Results of virus isolation from these mosquitoes were shown in Table 2. In both years, JE virus was isolated only from *C. tritaeniorhynchus*. The same species yielded one strain each of GET virus in each year. Both JE and GET viruses were detected from pool No.69 in 1980 and these 2 viruses were separated from each other by plaque formation on C6/36 cells under agar overlay containing antiserum against each of the different viruses. Peculiar finding is the isolation of one GET strain from a pool of 2 engorged *Armigeres subalbatus* collected on July 29, 1980. All the isolates were identified by the neutralization tests and were proven to be pathogenic to sucking mice by intracerebral inoculation.

Table 1. Species composition of mosquitoes collected and processed for virus isolation in 1980 and 1981

Mosquito species	Number of females (pools)*						Total of 2 years [%]
	1980			1981			
	UF	F	Subtotal [%]	UF	F	Subtotal [%]	
<i>Culex tritaeniorhynchus</i>	865(42)	362(12)	1227[70.4]	603(38)	246(13)	849[70.9]	2076[70.6]
<i>Anopheles sinensis</i>	231(25)	117(13)	348[20.0]	162(25)	66(11)	228[19.1]	576[19.6]
<i>Armigeres subalbatus</i>	97(25)	16(4)	113[6.5]	44(19)	8(6)	52[4.3]	165[5.6]
<i>Aedes albopictus</i>	39(12)	3(3)	42[2.4]	53(15)	2(1)	55[4.6]	97[3.3]
<i>Aedes japonicus</i>	6(4)	0	6[0.3]	8(5)	1(1)	9[0.8]	15[0.5]
<i>Culex bitaeniorhynchus</i>	3(2)	0	3[0.2]	3(2)	0	3[0.3]	6[0.2]
<i>Culex pipiens pallens</i>	4(4)	1(1)	5[0.3]	1(1)	0	1[0.1]	6[0.2]
Total	1245(114)	499(33)	1744[100]	874(105)	323(32)	1197[100]	2941[100]

* F: engorged, UF: unengorged mosquitoes.

Table 2. Isolation of Japanese encephalitis (JE) and Getah (GET) viruses from mosquitoes collected at Mogi in 1980 and 1981

Mosquitoes				Virus isolated
Date of collection	Pool No.	Species	Pool size*	
1980 Jul. 29	599	<i>Ar. subalbatus</i>	2 F	GET
Aug. 5	615	<i>Cx. tritaeniorhynchus</i>	100 UF	JE
Aug. 5	616	<i>Cx. tritaeniorhynchus</i>	70 UF	JE
Aug. 5	617	<i>Cx. tritaeniorhynchus</i>	46 F	JE
Aug. 13	629	<i>Cx. tritaeniorhynchus</i>	93 F	JE + GET
Aug. 19	635	<i>Cx. tritaeniorhynchus</i>	53 UF	JE
1981 Jul. 28	767	<i>Cx. tritaeniorhynchus</i>	54 UF	JE
Aug. 28	806	<i>Cx. tritaeniorhynchus</i>	42 UF	JE
Sep. 8	813	<i>Cx. tritaeniorhynchus</i>	33 UF	GET

* F: engorged, UF: unengorged mosquitoes.

DISCUSSION

In 1980, the first JE virus isolation from *C. tritaeniorhynchus* collected at Aino Town in Nagasaki Prefecture was July 28 with the last isolation on September 1 (Igarashi *et al.*, 1981b). JE virus isolation at Mogi appeared to be restricted to shorter period from August 5 to 19. In 1981, on the other hand, JE virus isolation at Aino was from July 27 to September 1 (Igarashi *et al.*, 1982), while the isolation at Mogi was sporadically between July 28 and August 28. The results may reflect smaller scale of pig-

mosquito cycle of virus transmission at Mogi compared with Aino.

In 1980, GET virus was sporadically isolated from *C. tritaeniorhynchus* collected at Aino between July 2 and August 21 (Igarashi *et al.*, 1981b). The period of GET virus isolation at Mogi also fell within this season. However, in 1981, GET virus was not isolated from *C. tritaeniorhynchus* collected at Aino, although the virus was detected from several swine blood collected on July 7. These swines were raised at 3 different sites in the central part of Nagasaki Prefecture (Igarashi *et al.*, 1982). GET virus isolation at Mogi in 1981 was from *C. tritaeniorhynchus* collected on September 8, far later than the virus isolation date from swines in the central part of the Prefecture. These results may indicate low level and sporadic nature of GET virus circulation among mosquito population as discussed by Yamamoto (1980). Although Fukumi *et al.* (1975) briefly described the isolation of non-JE arbovirus from *Ar. subalbatus* collected in 1970, the virus was not clearly identified. Since 2 *Ar. subalbatus* from which GET virus was isolated in 1980 were engorged, it may simply suggest viremic state of pigs. However, experiments now undergoing in our laboratories indicate that several strains of GET virus could grow to high titer in this species of mosquitoes.

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1980年と1981年長崎市において採集した蚊材料からヒトスジシマカ培養細胞クローン C6/36を用いた日本脳炎およびゲタウイルスの分離

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1980年と1981年の夏長崎市茂木地区で採集した蚊材料からのウイルス分離をヒトスジシマカ培養細胞クローン C6/36接種法により実施した。1980年には5プールのコガタアカイエカから5株の日本脳炎(JE)ウイルスと1株のゲタ(GET)ウイルスが分離された。この5プール中1プールからはJEとGETの両ウイルスが分離された。更に吸血したオオクロヤブカ1プールから1株のGETウイルスが分離された。1981年には3プールのコガタアカイエカから2株のJEと1株のGETウイルスが分離された。