

Isolation of Japanese Encephalitis and Getah Viruses from
Culex tritaeniorhynchus and Slaughtered Swine Blood Using
Aedes albopictus Clone C6/36 Cells in Nagasaki, 1981

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Abstract: In summer of 1981, virus isolation was performed from 16,600 female *Culex tritaeniorhynchus* in 166 pools and blood specimens of 300 slaughtered swines using *Aedes albopictus* clone C6/36 cells. Japanese encephalitis (JE) virus was first isolated from the mosquitoes collected on July 27, and was continuously isolated until September 8, totaling from 27 pools with highest infection rate of 9/20 on August 11. Inoculation to suckling mouse brains, on the other hand, detected JE virus from 20 pools of the mosquitoes. From blood specimens of slaughtered swines collected on August 4, JE virus was isolated by C6/36 cells with isolation rate of 6/30. 2-Mercaptoethanol-sensitive anti-JE virus antibodies were first detected on August 4, with its highest rate on August 18 (24/30). On August 25, all the blood specimens possessed anti-JE antibodies as revealed by the hemagglutination-inhibition test. Getah (GET) virus was isolated from 8 out of 30 swine blood collected on July 7. Concerning the isolation of these viruses from heparinized swine blood, plasma-platelet fraction appeared to be suitable material for inoculation to C6/36 cells.

INTRODUCTION

Japanese encephalitis (JE) virus circulates in nature by alternative growth in *C. tritaeniorhynchus* as main vector mosquito and in swine as main amplifier vertebrate host during epidemic seasons in Japan (Mitamura *et al.*, 1938; Hammon *et al.*, 1949;

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Buescher *et al.*, 1959; Scherer *et al.*, 1959; Matsuyama *et al.*, 1960; Konno *et al.*, 1966; Fukumi *et al.*, 1971). Virus isolation from vector mosquitoes and antibody surveillance among pig population were routinely used as indicators to monitor the activity and spread of the virus (Oya and Okuno, 1972; Oya, 1978). However, detection of viremia in pigs was not so commonly attempted, although Morita *et al.* (1971) and Ura (1976) isolated JE virus from slaughtered swine blood in Oita and Okinawa Prefecture, respectively. In order to solve the problem of overwintering of JE virus in temperate regions like in Japan, it appears important to examine critical timing of the appearance of the virus in vector mosquitoes and in vertebrate hosts in early epidemic season (Hayashi *et al.*, 1965; 1970). Since clone C6/36 of *A. albopictus* cells, as developed by the senior author, was shown to be a sensitive host cell to detect JE and Getah (GET) viruses from field-caught *C. tritaeniorhynchus* as well as from postmortem human brains (Buei *et al.*, 1979; Igarashi, 1980; Igarashi *et al.*, 1981a; b), we tried to apply the virus isolation using C6/36 to blood specimens of slaughtered swines in order to establish the isolation method.

MATERIALS AND METHODS

Mosquito collection and virus isolation: Mosquitoes were collected at a pigpen in Aino Town, Minamitakaki-gun, Nagasaki Prefecture (32°48'N, 130°09' E) from July 2 until September 8, 10 times almost at weekly interval using light traps operating overnight. Female *C. tritaeniorhynchus* was pooled (100 mosquitoes/pool) and was processed as described before (Buei *et al.*, 1979; Igarashi, 1980; Igarashi *et al.*, 1981 a; b). Briefly, homogenate was prepared from each pool and was centrifuged. The resulting supernatant was inoculated into brains of suckling mice (SMB) and the remainder was passed through Millipore HA filter followed by the inoculation into tube culture of C6/36 cells from which growth medium had been removed, using 0.1 ml per tube. These steps were performed at Nagasaki Prefectural Institute (NPI), and the remainder of the filtrate was transported to Institute for Tropical Medicine, Nagasaki University (ITM) and was inoculated into another tube culture of C6/36 cells. After adsorption of the virus for 2 hours at 28°C, the cells were covered by the maintenance medium and incubated at 28°C for 7 days. Presence of the virus in the inoculated culture fluids was detected by (1) hemagglutination test and (2) inoculation to C6/36 cells on 8-chamber slides, which were harvested after 3 days incubation at 28°C in order to detect intracellular virus antigen by immunoperoxidase staining (Okuno *et al.*, 1977).

Swine blood: Blood specimens from slaughtered swines were collected at a slaughter house in Isahaya City (32°50'N, 130°03'E) from July 2 until September 17, 11 times at weekly interval except that the last specimen was collected 16 days after. Each time 30 individual specimens were collected from swines raised at several pigpens as

shown in Fig. 1. These places were Ohmura, Ono, Konagai, Ura, Yue, Miura, Taisho, Ohmisaki, Moriyama, Aino, Chijiwa, Imori, and Isahaya itself, and are located within 26 km from Aino, where mosquitoes were collected. Approximately 5 ml each of the blood was transferred into a heparinized tube and brought to ITM in an ice box. The blood was diluted 1:2 with phosphate buffered saline, pH 7.2, and one-tenth ml of the diluted blood (B) was inoculated into a tube culture of C6/36 cells covered by 2 ml of the cell growth medium. Two ml of the diluted blood was layered onto 2 ml of Ficoll-Pack (Pharmacia, Uppsala, Sweden) in 12×105 mm test tubes and was centrifuged at 400×g for 30 min at 20°C in order to separate into lymphocyte fraction (L) and plasma-platelet fraction (P) as described (Boeyum, 1968; Harris and Ukaejiofo, 1970). One-tenth ml each from L and P fractions was inoculated into separate tube cultures of C6/36 cells as described above. Following overnight incubation at 28°C, media were removed from the inoculated tubes and the cells were covered by the maintenance medium and incubated at 28°C for another 6 days. Presence of the virus in the culture fluids was examined as described above.

Cells: Origin and cultivation of *A. albopictus* clone C6/36 cells have been described (Igarashi, 1978; 1980).

Antisera and serological tests : Hyperimmune rabbit sera against JE and GET viruses were used in the immunoperoxidase staining. Hemagglutination-inhibition (HI)

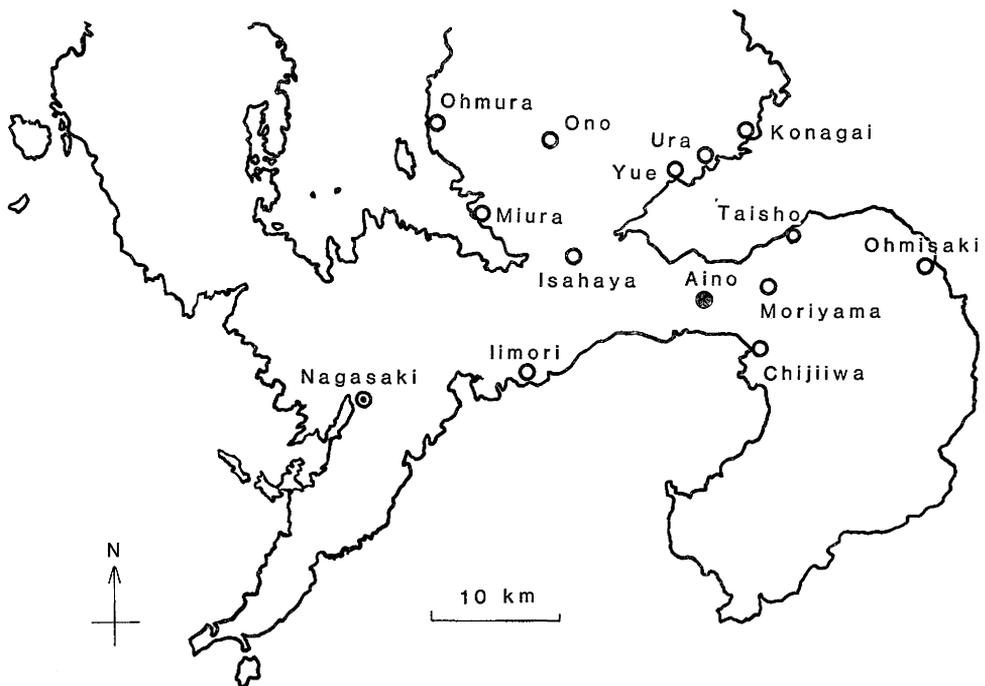


Fig. 1. Production sites of swines and mosquito collection site in Nagasaki Prefecture.

test was performed at NPI according to the method of Clarke and Casals (1958) with and without 2-mercaptoethanol (2ME) treatment (Oya, 1978).

RESULTS

Isolation of JE virus from *C. tritaeniorhynchus*: Totalling 16,600 female *C. tritaeniorhynchus* in 166 pools were processed, of which 32 pools were engorged and others were unengorged. JE virus was first isolated from one of the 20 pools collected on July 27 both by C6/36 and by SMB. Afterwards the virus was continuously isolated until September 8, the last day of mosquito collection. The number of JE-positive pools by C6/36 was 1 on July 27, 2 on August 4, 9 on August 11, 8 on August 18, 4 on August 25, and 3 on September 8, respectively. Since on each collection day after July 14, 20 pools were processed, the rate of JE virus isolation went up to its maximum on August 11 followed by gradual decrease as shown in Fig. 2-A. Except 3 pools collected on September 8, all the other JE-positive pools were unengorged. Table 1 compares JE virus isolation by SMB and by C6/36 cells, and the latter was performed both at NPI and at ITM except 6 pools collected on August 11 which were not tested at ITM. All the 20 pools which yielded JE virus in SMB were also positive by C6/36 cells. On the other hand, there were 6 pools which were JE-negative by SMB but positive by C6/36 cells. Three out of these 6 pools turned out to be positive both at NPI and at ITM, 2 were positive at ITM only, and one was positive only at NPI. The result confirms out previous observations that C6/36 cells have similar or even better efficiency to isolate JE virus from field materials, such as *C. tritaeniorhynchus* or postmortem human brains. Result of JE virus isolation by C6/36 cells at NPI and at ITM agreed quite well, except 3 specimens which were positive only at ITM, and one specimen positive only at NPI. Thus the coincidence rate was 88%.

Isolation of JE virus from and anti-JE HI antibodies in slaughtered swine blood: In contrast to long duration of JE virus isolation from *C. tritaeniorhynchus* as shown above, the virus was detected from blood of slaughtered swines only on a single day of August 4, with 6 positive out of 30 specimens collected (isolation rate of 20%). Origin of these 30 specimens were 10 each from Miura, Ura, and Ohmura, and JE virus was isolated from each 2 of the 10 blood specimens collected from each of these 3 sites. These 3 sites located within 26 km from mosquito collection site at Aino (Fig. 1). All the JE-positive blood specimens did not possess detectable HI antibodies against JE virus. On the previous collection day of July 27, each 10 blood specimens were obtained from swines produced in Yue, Moriyama, and Konagai, and on the following day of August 11, each 10 blood specimens from Ohmura, Ohmisaki, and Ura were collected for the examination. Antibodies against JE virus, as revealed by the HI test, were first detected in one of the 30 sera collected on July 27, with minimal titer of 1:10, which

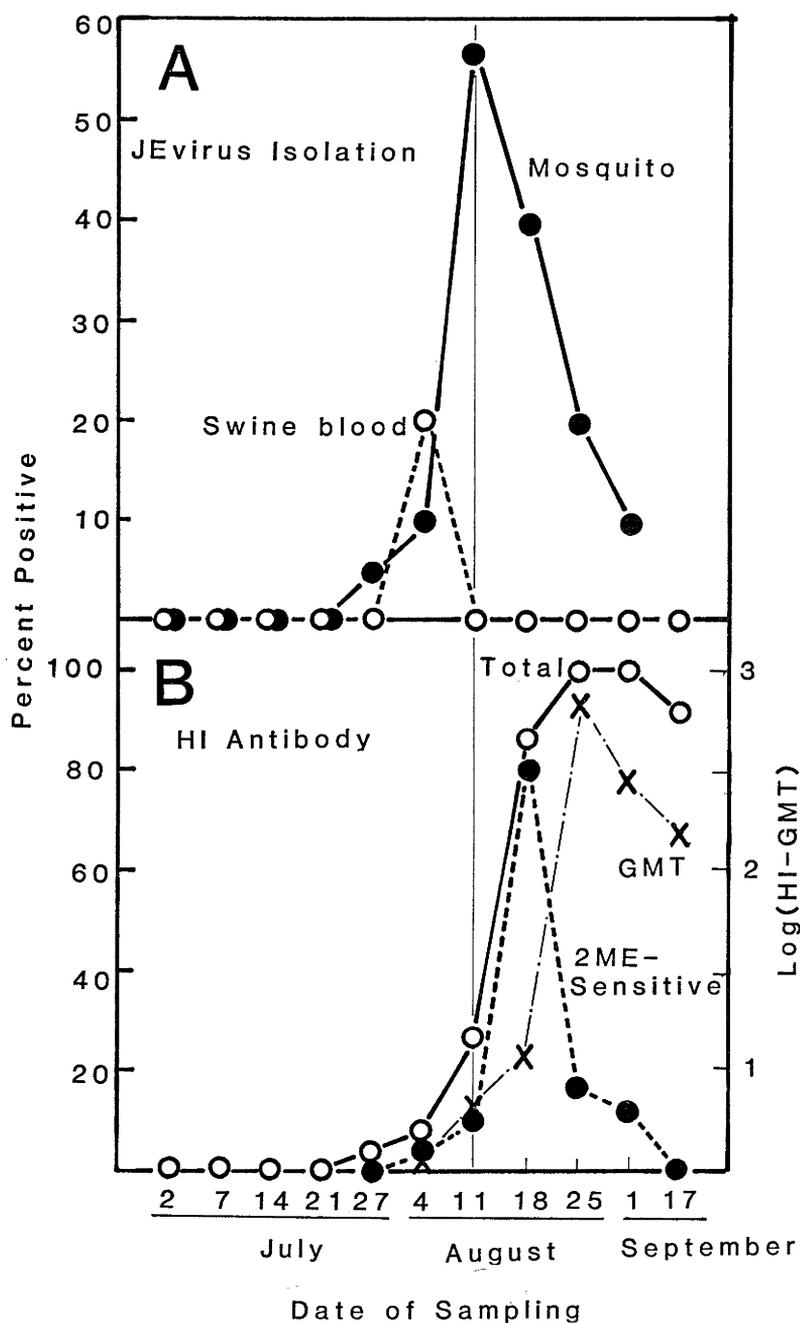


Fig. 2. Isolation of JE virus from *C. tritaeniorhynchus* and swine blood and appearance of anti-JE antibodies in sera of slaughtered swines. (A) Isolation of JE virus from *C. tritaeniorhynchus* (●—●) and blood specimens from slaughtered swines (○—○) according to the sampling date as shown by the percent positives. (B) Appearance of anti-JE antibodies in sera of slaughtered swines as shown by percent positives of total (○—○) and 2ME-sensitive (●—●) HI antibodies, as well as by the geometrical mean titer (GMT) in logarithmic scale (×—×).

Table 1. Isolation of JE virus from *C. tritaeniorhynchus* by SMB and C6/36 cells

Isolation of JE virus			No. of pools
NPI*		ITM**	
SMB	C6/36	C6/36	
+	+	+	19
-	+	+	3
-	-	+	2
-	+	-	1
+	+	nt***	1

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***Not tested

became undetectable after 2ME- treatment. This specimen was collected from a swine produced at Yue, however, because of its low titer, epidemiological significance of this specimen is rather uncertain. On August 4, one of the swine sera collected from Miura showed definite HI titer of 1: 80, which was reduced to less than 1: 10 after 2ME-treatment, indicating the first sign of recent JE virus infection. The rate of 2ME-sensitive HI antibodies rose up to 3/30 on August 11, and 14/30 on August 18, followed by sharp decrease, which is probably due to the appearance of IgG antibodies as shown by the increase in geometrical mean titer (GMT) with 100% positivity of the HI antibodies on August 25. Thus viremia period in slaughtered swines was observed one week after the first appearance of JE virus in *C. tritaeniorhynchus*, and the peak of 2ME-sensitive HI antibody followed 2 weeks after the viremia peak.

Detection of GET virus from swine blood: In contrast to many JE virus isolates from *C. tritaeniorhynchus*, no one GET virus was isolated from *C. tritaeniorhynchus* collected in Aino Town in 1981. However, 8 of the 30 swine bloods collected on July 7 produced GET virus by C6/36 cells, of which 2 specimens produced GET virus from lymphocyte (L) and 4 from plasma-platelet (P) and 2 from both L and P fractions, respectively (Table 2). Origin of the 30 swines examined on July 7 was 11 from Taisho, 9 from Ura, and 10 from Isahaya, and GET virus was detected from 2, 3 and 3 specimens from these production sites, respectively. On July 2, the preceding collection day, each 10 of the blood specimens were collected from swines produced at Konagai, Isahaya and Iimori, on the other hand, on July 14, the following collection day of July 7, each 10 from Taisho, Aino and Ura were examined. Thus, the viremia produced by GET virus in swines appeared also to be very brief in Nagasaki, 1981. As shown in Table 2, isolation of JE and GET viruses was most efficiently performed using plasma-platelet (P) fraction, since all the P-fraction of JE-positive specimens yielded JE virus.

Table 2. Isolation of JE and GET viruses from swine blood

Date of collection	Sample No.	Production site of swines	Sources of virus isolation		
			Total blood (B)	Lymphocyte (L)	Plasma & platelets (P)
July 7	32	Taisho	—	G*	—
	33	Taisho	—	G	—
	44	Ura	—	G	G
	45	Ura	—	G	G
	50	Ura	—	—	G
	53	Isahaya	—	—	G
	54	Isahaya	—	—	G
	60	Isahaya	—	—	G
August 4	156	Miura	—	—	J**
	158	Miura	J	—	J
	163	Ura	J	—	J
	168	Ura	J	J	J
	173	Ohmura	J	J	J
	174	Ohmura	—	—	J

*Getah virus

**JE virus

There is not preferential association of JE virus to lymphocyte fraction (L). On the other hand, GET virus was detected in 6 P-fractions of 8 GET-positive specimens, and 2 specimens yielded GET virus only from L-fraction, suggesting more frequent association of GET virus with lymphocytes compared with JE virus. Lower efficiency to detect these viruses from whole blood specimen (B) is probably due to toxic effects of swine red blood cells on C6/36 cells giving cell damage.

DISCUSSION

Morita *et al.* (1971) detected JE virus in swine blood before its detection in the mosquitoes during 3 out of 4 epidemic seasons from 1967 to 1970 in Oita Prefecture. Ura (1976) reported that JE virus was detected in mosquitoes before its appearance in swine blood during epidemic seasons from 1973 to 1976 in Okinawa, although he isolated the virus in swine blood earlier than in mosquitoes from 1969 to 1972. Previous analytical examination on JE virus infection in early epidemic seasons from 1964 to 1969 in Nagasaki Prefecture by Hayashi *et al.* (1970) indicated that the starting time of JE virus infection in mosquitoes appeared 4 to 37 days earlier than that in pigs. According to their description, the mosquitoes from which JE virus was first isolated on July 27, 1981, had probably been infected with the virus 2-12 days before, that is, between July

15 to 25. On the other hand, the first 2ME-sensitive HI antibody detected on August 4 indicates JE virus infection of the pig at least 5 days before, that is, before July 30. Since 2-3 days are required before the appearance of viremia in pigs following JE virus infection, the first pig with 2ME-sensitive HI antibody is hard to serve as the virus source to infect the first JE virus-positive mosquitoes. Rather, the first JE virus-positive mosquitoes could infect the virus to the pig(s) making them produce 2ME-sensitive antibodies detected on August 4. The pig(s) could be the first sign of viremia peak among pig populations observed on August 4. Konno *et al.* (1966) reported cyclic outbreaks of JE virus infection among pig populations in Miyagi Prefecture, Northern part of Mainland Japan. However, spread of JE virus in the central part of Nagasaki Prefecture in 1981 appeared to be rather rapid with single large epidemic in swines initiated by a small number of infective mosquitoes followed by a high infection level in mosquitoes.

Although not detected from the mosquitoes collected in Aino in 1981, GET virus was isolated from one of the *C. tritaeniorhynchus* pools collected at Mogi in Nagasaki City on September 8, 1981 (Igarashi *et al.*, in preparation) and from 5 pools of the mosquitoes collected at Aino in 1980 (Igarashi *et al.*, 1981 b). Thus, GET virus appears to circulate among pigs and *C. tritaeniorhynchus* in Nagasaki Prefecture. However, GET virus infection among the mosquitoes did not appear to reach a high level as discussed by Yamamoto (1980). Viruses of GET complex have been isolated from *C. tritaeniorhynchus* as well as from other species of mosquitoes in Japan (Matsuyama *et al.*, 1960; Scherer *et al.*, 1962; Hurlbut and Nibley, 1964; Ueba *et al.*, 1967; Shichijo *et al.*, 1970). However, in northern part of Kyushu, *C. tritaeniorhynchus* appears to be the only principal vector (Yamamoto, 1980). Isolation of GET complex from swine blood has been reported by Matsuyama *et al.* (1968) and Morita *et al.* (1971). However, pathological significance had remained unknown. Recently Kumanomido *et al.* (1980) reported that the virus caused severe febrile exanthematous illness with respiratory symptoms among race horses, thus indicating potential hazards to some live stocks.

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ヒトスジシマカ培養細胞クローン C6/36 を用いた コダカアカイエカおよびと場豚血液からの日本脳炎ウイルスおよびゲタウイルスの分離, 1981年長崎県における成績
五十嵐章, 森田公一, 分藤桂子, 松尾幸子, 林 薫 (長崎大学熱帯医学研究所ウイルス学部門)
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1981年夏, 16,600のコダカアカイエカ雌166プールと, 300匹のと場豚血液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 を用いて実施した. 日本脳炎 (JE) ウイルスは7月27日採集の蚊材料から最初に分離され9月8日に至るまで合計27プールから分離された. 最も多く分離されたのは8月11日採集の材料で20プール中9プールが陽性であった. 乳呑マウス脳内

接種では合計20プールの蚊からJ Eウイルスが分離され、マウスで陽性のプールはすべて C6/36でも分離陽性であった。と場豚血液からは8月4日採取の30検体中6検体から C6/36 を用いてJ Eウイルスが分離された。豚血清中のJ Eウイルスに対する血球凝集抑制抗体は8月4日に2メルカプトエタノール感受性抗体が検出され8月18日には30検体中24検体に陽性となったが、以後その検体率は低下した。J Eウイルスに対する血球凝集抑制抗体陽性率は8月25日には100%に達し抗体価の幾何平均値も最高となった。ゲタウイルスは7月7日に採取した30のと場豚血液中8検体から C6/36 を用いて分離された。ヘパリン化と場豚血液を用いた C6/36 によるこれらウイルスの分離にはプラズマ血小板分画が全血およびリンパ球分画よりも適していると思われる。

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