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Isolation of Japanese encephalitis and Getah Viruses by *Aedes albopictus* Clone C6/36 Cells and by Suckling Mouse Brain Inoculation, in Nagasaki, 1980

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Abstract : Virus isolation was performed from field-caught female *Culex tritaeniorhynchus* and from postmortem brain materials of an encephalitis patient by inoculation into *Aedes albopictus* clone C6/36 cells and into suckling mouse brains (SMB). From 18,000 mosquitoes in 180 pools, Japanese encephalitis (JE) virus was isolated from 17 pools by C6/36 cells, in contrast to 8 pools by SMB. Seven pools with positive JE virus isolation by SMB were also with positive results by C6/36 cells. There was a single pool with positive JE virus isolation by SMB with negative result by C6/36 cells. Inoculation into SMB could not detect JE virus from 10 mosquito pools and postmortem brain materials which yielded JE virus by C6/36 cells. Getah (GET) virus was detected from 5 mosquito pools by C6/36 cells, and 2 out of the 5 pools also yielded GET virus by SMB. A single pool which produced JE and GET viruses by C6/36 cells produced only JE virus by SMB. No significant difference was observed between the virus strains isolated from mosquito pools which yielded the virus by both methods and the virus strains isolated only by C6/36 cells, in terms of neutralization test and of plating efficiency on C6/36 cells at 28°C and that on BHK21 cells at 37°C. The results indicate that C6/36 cells have higher sensitivity than SMB to detect JE and GET viruses from field materials.

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INTRODUCTION

Japanese encephalitis (JE) is an acute infectious disease caused by JE virus and is prevalent not only in Japan but also in other parts of East, Southeast and South Asia (Miles, 1960), and in temperate regions like Japan the diesease shows seasonal incidence in summer to fall (Clarke and Casals, 1965). The indidence of human JE in Japan markedly decreased after the last epidemic in 1966 and was recently observed mostly in the West and South parts of this country including Nagasaki Prefecture (Health and Welfare Statistical Association, 1975). The Nagasaki Prefectural Institute of Public Health and Environmental Sciences has been monitoring the activity of JE virus in nature by virus isolation from main vector, *Culex tritaeniorhynchus*, (Mitamura et al., 1938; Hammon et al., 1949; Buescher et al., 1959), and by antibody surveillance among pig populations as main amplifier of the virus (Scherer et al., 1959; Konno et al., 1966). The isolation of JE virus has routinely been performed by inoculation of the materials into brains of suckling mice (SMB) as described (Work, 1964; Fukumi et al., 1971; Oya and Okuno, 1972). Recently senior suthor of this paper isolated clone C6/36 (Igarashi, 1978) from a line of Singh's *Aedes albopictus* cells (Singh, 1967) and, in collaboration with his colleagues in Osaka, proved it as a sensitive and better alternative to SMB for the isolation of JE and GET viruses from field-caught *C. tritaeniorhynchus* (Buei et al., 1979 a, b, 1980; Igarashi, 1980; Igarashi et al., 1981 a). This method of virus isolation was applied to the field materials collected in Nagasaki Prefecture in 1980 in order to verify the validity of the method in other part of this country.

MATERIALS AND METHODS

Mosquito collection: Mosquitoes were collected at a pigpen in Aino Town, Minamitakagi-gun, Nagasaki Prefecture (32° 48′N, 130° 09′E). The site is surrounded by rice fields surving as breeding sites of *C. tritaeniorhynchus*. In 1980, collection was performed from June 30 until September 1, by light traps operating overnight. Mosquitoes were transported to Nagasaki Prefectural Institute and identified. Female *C. tritaeniorhynchus* was pooled using 100 mosquitoes/pool according to the state of engorgement and was processed without freezing as described below.

Virus isolation and identification: Pooled mosquitoes were homogenized on the next day after their collection and were centrifuged. The resulting supernatant was inoculated into SMB using a litter for each pool as described (Oya and Okuno, 1972). The remainder of the supernatant was transported in an ice bath to the Institute for Tropical Medicine and was passed through Millipore HA filter and was inoculated into tube cultures and Petri dish cultures of C6/36 cells as described before (Buei et al.,

1979 a, b, 1980; Igarashi, 1980; Igarashi et al., 1981 a). Cells in tube cultures were covered by the maintenance medium and those in Petri dish cultures with agar overlay to detect plaque-forming agents. Presence of viruses in SMB was detected by extracting hemagglutinins from moribund mouse brains (Clarke and Casals, 1958; Oya and Okuno, 1972), however, presence of viruses in the infected C6/36 cell culture fluid was detected by hemagglutination using the fluid itself and by immunoperoxidase staining of C6/36 cells inoculated with the fluid (Okuno et al., 1977). Preliminary identification was performed by the hemagglutination-inhibition (HI) test (Clarke and Casals, 1958; Oya and Okuno, 1972) and the final identification was by the neutralization test using focus-reduction method on BHK21 cells (Okuno et al., 1978).

Cells: Origin and cultivation of clone C6/36 of *A. albopictus* cells have been described before (Igarashi, 1978; 1980), as well as the BHK21 cells (Igarashi et al., 1981).

Antiserum and serological tests : Hyperimmune mouse antiserum against GET virus and hyperimmune chicken antiserum against a JE virus isolate, JaOAr 363−70, were kindly supplied by Dr. N. Ueba, Osaka Prefectural Institute of Public Health. Rabbit antisera against JE or GET virus used in the immunoperoxidase staining were prepared in rabbits. Most of the serological tests were performed in a microtiter system (Sever, 1962).

Virus strains, infectivity assay, temperature-sensitivity test and the neutralization test: Standard strains of JE virus and GET virus were obtained from National Institute of Health, Tokyo, and were maintained in this Institute by suckling mouse brain passages. The infectivity was assayed by focus-counting method on BHK21 cells grown on 8-chamber slides (Lab-Tek, Miles, Ill. USA) as described elsewhere (Igarashi, 1978) using 24 hours and 16 hours of incubation at 37°C for JE and GET virus, respectively, with tragacanth gum overlay for GET virus. Neutralization test was performed by the constant virus varying serum dilution system incubating the serum-virus mixture at 28 °C for 2 hours before inoculating to BHK21 cells. Temperature-sensitive virus isolates were screened by comparing the efficiency of plating on BHK 21 cells at 37°C with that on C6/36 cells at 28°C assaying the infectivity by plaque formation under agar overlay as described before (Igarashi 1979).

RESULTS

Isolation of JE virus from *C. tritaeniorhynchus*: The number of female *C. tritaeniorhynchus* processed was 18,000 in 180 pools, of which 13,800 mosquitoes in 138 pools were unengorged and others were engorged states as shown in Table 1. Results of JE virus isolation from these specimens were shown in Table 2. The first isolation in 1980 was from a mosquito pool collected on July 28, both by SMB and by C6/36 cells. The virus was continuously detected until September 1 by C6/36 cells with isolation rates of

Table 1. Number of mosquito pools processed for virus isolation

Date of collection	Number of pools*	
	Unengorged	Engorged
Jul. 2	7	6
Jul. 5	0	7
Jul. 9	20	0
Jul. 18	5	15
Jul. 22	10	10
Jul. 28	20	0
Aug. 4	20	0
Aug. 12	20	0
Aug. 21	20	0
Sept. 1	16	4
Subtotal	138	42
Total	180	

* Each pool consists of 100 mosquitoes of female *Culex tritaeniorhynchus*

Table 2. Isolation of JE Virus from *C. tritaeniorhynchus* by C6/36 cells and by SMB inoculation, in Nagasaki, 1980

Code of mosquito pools	Date of collection	C6/36		SMB	
		Isolation	Plaques ¶	Isolation	Dead/Inoculated
95	Jul. 28	+	300 L	+	8/8
114	Aug. 4	+	35 L	−	
121	Aug. 12	+	20 L	+	1/6
122	Aug. 12	+	30 L	+	2/7
124	Aug. 12	+	105 L	+	4/7
125	Aug. 12	+		−	
128	Aug. 12	+	116 L	−	
131	Aug. 12	+	170 L	−	
144	Aug. 21	+	64 L	−	
147	Aug. 21	+	12 L	−	
153	Aug. 21	+	2 L	−	
156	Aug. 21	+	250 m	+	5/5
157	Aug. 21	−§		+	4/4
159	Aug. 21	+	300 m	+	5/6
160	Aug. 21	+	80 L	+	3/8
171	Sept. 1	+		−	
177	Sept. 1	+	7 L	−	
182	Sept. 1	+	12 L	−	

¶ L and m mean large and medium sized plaques, respectively, and the figures represent number of plaques.

§ Unsuccessful result because of bacterial contamination

1/20, 1/20, 6/20, 6/20, and 3/20, for Jul. 28, Aug. 4, Aug. 12, Aug. 21, and Sep. 1 collections, respectively. On the other hand, isolation rates of JE virus by SMB was 1/20, 3/20, and 4/20, for Jul. 28, Aug. 12 and Aug. 21 collections, respectively, and JE virus was not detected by SMB from mosquito pools collected on Aug. 4 and Sep. 1. Altogether, JE virus was isolated from 17 pools of mosquitoes by C6/36 cells, in contrast to 8 pools by SMB. There was a single pool which yielded JE virus by SMB but not by C6/36 cells, the result possibly due to bacterial contamination of the cell culture. On the other hand, there were 10 pools of mosquitoes from which JE virus was detected by C6/36 cells but not by SMB. All the JE virus isolates were obtained from unengorged mosquito specimens. Except for pool 157 which yielded JE virus only by SMB, the higher the mouse mortality, the larger the number of large or medium sized plaques formed on C6/36 cells, but not *vice versa*. This may be due to heterogeneity of the virus population in terms of affinity to SMB and to C6/36 cells or due to the presence of some plaque-forming agents which are not JE or GET virus.

Isolation of JE virus from a patient with fatal encephalitis: We happened to have a chance to isolate virus from postmortem materials from a patient with fatal encephalitis. The patient was 61 years old male showing acute encephalitis on Aug. 26 dying on Aug 30. Serum specimens taken on the day of admission did not have detectable HI antibody against JE virus, however, the specimen taken just before his death showed HI titer of 80, which was completely destroyed by treatment with 2-mercaptoethanol, showing the presence of IgM antibody. Brain specimens were taken as well as cerebrospinal fluid and virus isolation was attempted both by SMB and by C6/36 cells. As shown in Table 3, in spite of extensive effors to isolate the virus in SMB, the virus was isolated from brain materials only by C6/36 cells and not by SMB. All the isolates as shown in Tables 2 and 3 were identified as JE virus by the neutralization test as shown in Table 4. Because there were several specimens which yielded JE virus only in C6/36 cells and not in SMB, these JE virus isolates were screened for the presence of possible temperature-sensitive (ts) virus by comparing the plating efficiency on C6/36 cells at 28°C with that

Table 3. Isolation of JE virus from postmortem materials of a patient by C6/36 cells and by SMB inoculation

Materials for virus isolation	Isolation by		
	C6/36	Plaque§	SMB
Cerebrospinal fluid	−		−
Pons	+		−
Cerebrum	+		−
Cerebellum	+	2L	−

§ Number of plaques. L means large plaque.

Table 4. Temperature-sensitivity test and neutralization of JE virus isolates

Code of isolates	Log (PFU/ml) assayed by			Log (FR_{50})
	C6/36 (A) at 28°C	BHK21 (B) at 37°C	A−B	
95	8.9	7.4	1.5	6.4
114	9.1	7.7	1.4	6.2
121	8.7	7.6	1.1	5.8
122	8.7	7.7	1.0	5.7
124	8.7	7.5	1.2	6.2
125	8.7	7.5	1.2	6.0
128	8.8	7.6	1.2	6.5
131	8.9	7.8	1.1	6.4
144	9.2	7.5	1.7	6.0
147	8.8	7.0	1.8	6.0
153	8.7	7.6	1.1	6.1
156	8.7	7.8	0.9	6.0
159	8.3	8.8	-0.5	5.9
160	8.8	7.5	1.3	6.2
171	8.9	7.5	1.4	6.0
177	8.8	7.5	1.3	6.1
182	8.9	7.8	1.1	5.7
Pons	8.5	7.2	1.3	5.5
Cerebrum	8.8	7.2	1.6	6.3
Cerebellum	8.8	7.2	1.6	5.4
Nakayama	8.8	7.7	1.1	5.9

on BHK21 cells at 37°C. However, there was no positive indication that these isolates, as strains, are ts virus. Thus, the difference between the isolation efficiency by C6/36 cells and that by SMB is probably due to the difference in the sensitivity between these 2 host systems against JE virus.

Isolation of GET virus from *C. tritaeniorhychus*: Infected culture fluids of C6/36 cells inoculated with the mosquito homogenates were tested for the presence of GET virus and 5 pools of the mosquitoes were shown to produce GET virus in C6/36 cells. Two out of the 5 pools also yielded GET virus by SMB but not from the rest 3 pools as shown in Table 5. From pool 159, JE virus was also detected together with GET virus as described above, and these 2 kinds of virus could be separated from each other by plaque formation on C6/36 cells in the presence of specific antibody against each virus. These isolates were identified as GET virus by the neutralization test as shown in Table 6. Possibility of the presence of "ts" GET virus isolates was tested as in the case with JE virus and the result is shown in Table 6. There was not much difference between the GET virus strains isolated both by C6/36 cells and by SMB and those isolated only by C6/36 cells in terms of their plating efficiency on C6/36 cells at 28°C and that on

Table 5. Isolation of GET virus from *C. tritaeniorhynchus* by C6/36 cells and by SMB inoculation, in Nagasaki, 1980

Code of mosquito pools	Date of collection	C6/36		SMB	
		Isolation	Plaques ¶	Isolation	Dead/Inoculated
9	Jul. 2	+	50 s	+	1/8
103	Aug. 5	+	40 h	−	
123	Aug. 12	+	3 L	+	3/7
133	Aug. 12	+	1 L	−	
159	Aug. 21	+	300 m	−§	

¶ Number of plaques. L and s mean large and small plaques, respectively. h means hazy appearance of the plaques.
§ JE virus was isolated from this pool by SMB and by C6/36 cells.

Table 6. Temperature-sensitivity test and neutralization test of GET virus isolates

Code of isolates	Log (PFU/ml) assayed by			Log (FR_{50})
	C6/36 (A) at 28°C	BHK21 (B) at 37°C	A−B	
9	8.3	5.7	2.6	4.3
103	7.2	7.0	0.2	4.7
123	7.4	6.0	1.4	4.6
133	7.8	6.2	1.6	4.3
159	8.6	7.6	1.0	4.3
GET	8.1	8.5	-0.4	4.3

BHK21 cells at 37°C. Although isolate from pool 9 showed more than 100 fold higher plating efficiency on C6/36 cells than on BHK21 cells, this strain could be detected by SMB also. Thus, the difference of isolation efficiency by C6/36 and that by SMB was possibly due to the different sensitivity of the 2 methods against GET virus also.

DISCUSSION

In the previous attempts performed by the senior author and his colleagues in Osaka Prefecture, the efficiency of JE virus isolation by C6/36 cells showed at least the same or even better results than by classical SMB inoculation (Buei et al., 1979a, b, 1980; Igarashi, 1980; Igarashi et al., 1981). In our present results, the method by C6/36 could detect more than twice the number of JE virus isolates than by SMB method. The higher effiency than the previous results may partly be due to the difference in the

mouse strains used, because ICR mice were used in Osaka and gpc mice in Nagasaki, respectively. Another reason may be the different method of JE virus detection in mouse brains, because fluorescent antibody staining was used in Osaka and extraction of hemagglutinin was used in Nagasaki as the screening methods. Also mosquito specimens had been kept frozen for several days until processed in Osaka, in contrast to immediate processing of the mosquitoes without freezing in Nagasaki. Effects of these variables should be considered individually in order to explain significantly higher efficiency to detect JE virus by C6/36 cells in Nagasaki. In the experiments performed in Osaka also, GET virus could be detected only by C6/36 cells but not by SMB, thus the virus apparently has higher affinity to C6/36 cells than to SMB as shown by the results in Nagasaki again. Present result indicated also that the virus isolation by C6/36 cells could be used for the isolation of virus from postmortem specimens as well as from field-caught mosquito specimens. Higher efficiency of JE and GET virus isolation by C6/36 cells as compared with SMB inoculation is probably due to the higher sensitivity of C6/36 cell system than SMB and no direct evidence was obtained that the difference is due to the presence of possible "ts" virus strains which could be detectable by C6/36 cells and not by SMB. However, this does not exclude the presence of small number of such "mutant" virus in the mosquitoes that could be detectable by plaque isolation on C6/36 cells (Igarashi et al., 1981b).

In the previous experiments in Osaka Prefecture, many unidentified, plaque-forming agents were detected on C6/36 cells from field-caught *C. tritaeniorhynchus*. These agents are probady insect visuses of mosquitoes. In the present study in Nagasaki Prefecture, the prevalence of such plaque-forming agents were much less than in the case of Osaka which was briefly described before (Igarashi et al., 1981a). Probably the distribution of such mosquito insect viruses may be different in various parts of Japan. This problem should wait for further studies.

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ヒトスジシマカ培養細胞クローンC6/36と乳呑マウス脳内接種法による日本脳炎ウイルスおよびゲタウイルスの分離．1980年長崎における成績．

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18,000匹の野外採集コガタアカイエカ180プールと，１例の脳炎患者死后脳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と乳呑マウス脳内接種（SMB）により実施した．日本脳炎（JE）ウイルスは17プールの蚊材料と脳炎患者死后脳材料からC6/36で分離されたが，SMBでは８プールの蚊材料から分離された．SMBで分離陽性でC6/36での分離陰性の１プールに対して，C6/36で分離陽性，SMBで分離陰性プールは10存在し，脳材料からのウイルス分離もSMBでは陰性であった．ゲタ（GET）ウイルスは５プールの蚊材料からC6/36で分離され，このうちの２プールからはSMBでも分離された．C6/36とSMBの両方で分離陽性であった株と，C6/36でのみ分離陽性であった株の間でウイルスの中和反応および，C6/36とBHK細胞での平板効率に関する限り有意の差を認めることは出来なかった．C6/36によるウイルス分離成績がSMBに比して良い結果が得られたのは，C6/36がSMBよりも高い感度でこれらのウイルスを野外材料から検出できる結果であると考えられる．

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