Virological and Epidemiological Studies on Encephalitis in Chiang Mai Area, Thailand, in the Year of 1982

VII. Mosquito collection and virus isolation

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Abstract: Totalling 15513 mosquitoes composed of *Culex tritaeniorhynchus*, *Cx. gelidus* and *Cx. fuscocephala* were collected in Chiang Mai Area from July 13 to August 12, 1982, and processed for virus isolation, using *Aedes albopictus*, clone C6/36, cells. JE virus was not isolated, although two unidentified flavivirus and 59 strains of unknown filtrable agents producing cytopathic changes in C6/36 cells were detected. Host preference of the above three species of *Culex* mosquitoes showed that they fed mostly on swine, followed by smaller percentages of bovines. Daily survival rate observed for *Cx. tritaeniorhynchus* females in Chiang Mai did not differ significantly from those observed in Japan.

Key words: Chiang Mai, Thailand, Encephalitis, Mosquito.

Since 1969, encephalitis due to Japanese encephalitis (JE) virus infection continued to occur every rainy season in Northern Thailand, Chiang Mai Area. Our study team

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aimed to obtain information about present status of JE virus infection and circulation in this area from virological and epidemiological point of view (Igarashi *et al.*, 1983a). As a part of this study, we tried to collect mosquitoes including possible vectors of JE and other virus around Chiang Mai City. Attempts were made to isolate of JE viruses from 3 species of *Culex* mosquitoes by inoculation to *Aedes albopictus*, clone C6/36, cell. Blood-meal identification was performed on limited number of engorged females of these 3 species of mosquitoes in order to know the host preference. Limited number of *Culex tritaeniorhynchus* females were examined for their parous states to estimate daily survival rates. Based on these results, daily survival rates of females of *Cx. tritaeniorhynchus* in Thailand were compared with those rates of the mosquitoes in Japan. The results of these studies are described in this paper.

MATERIALS AND METHODS

Mosquito collection: Mosquitoes were collected using by light traps (Fujihira super light trap, Fujihira Industry Co., Ltd.) at 4 collection sites, Hang Dong, San Pathong, Doi Saket and Mae Rim, as shown in Fig. 1. These sites were located in rural area around Chiang Mai City within 13 to 24 km, and are surrounded by ample rice fields. Collections were made at or near pigpens, and in the case of Hang Dong, the pigpen is located near the cowsheds and also several chicken, dogs and humans were present near by (Fig. 2). Collections were performed for 3 hours after sunset and collected mosquitoes were brought into Department of Parasitology in Chiang Mai University. On the next day, mosquitoes were anesthetized with carbon dioxide and then chilled to reduce activity for identification. *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. fucocephala* were sorted out and pooled according to the date, sampling place and engorgement, so that each pool contained mosquitoes not exceeding 200.

Virus isolation: The procedure is described before (Igarashi *et al.*, 1981a). Each pool of mosquitoes was homogenized with 2 ml of PBS (phosphate buffered saline, pH 7.2) containing 0.2% bovine plasma albumin (Armour, Fraction V) and was centrifuged at 2500 rpm for 15 min. The resulting supernatant was filtered through Millipore type HA filter.

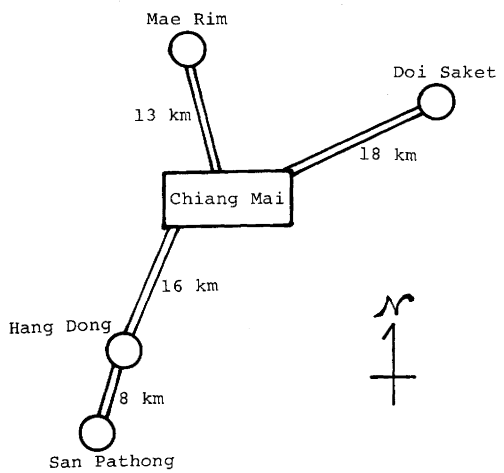

Fig. 1. Map of mosquito collection sites in Chiang Mai Area, Thailand, 1982.

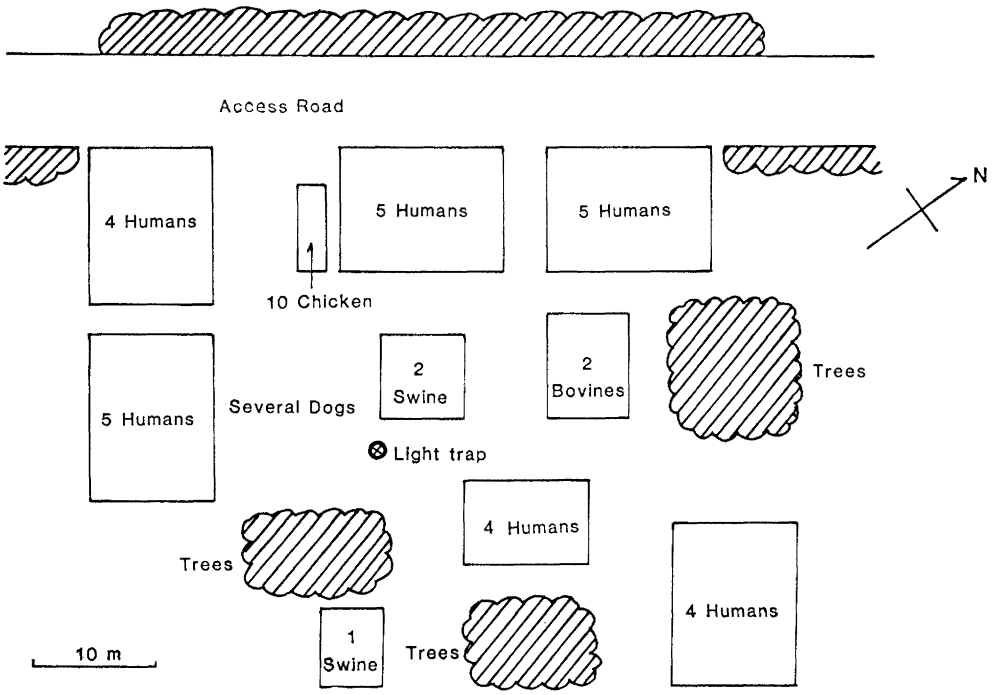

Fig. 2. Location of several vertebrates at mosquito collection site in Hang Dong, Chiang Mai.

One-tenth ml of the filtrate was inoculated to tube culture of *Ae. albopictus*, clone C6/36, cells after removing cell growth medium. After 2 hours of adsorption, the cells were refed with maintenance medium and incubated at room temperature for 7 days. Presence of the virus in the infected fluid was detected as described in the accompanying paper (Igarashi *et al.*, 1983b). Virus isolation from swine sera was performed similar to the procedure described for patient sera in the accompanying paper (Igarashi *et al.*, 1983b).

Cell cultures: Origin and cultivation of *Ae. albopictus*, clone C6/36, cells were as described before (Igarashi, 1978) and in the accompanying paper (Igarashi *et al.*, 1983b).

Swine sera: Slaughtered swine sera were collected at a slaughter house in Chiang Mai City and were tested by the hemagglution-inhibition (HI) to detect anti-JE antibodies (Ogata *et al.*, 1983). The specimens with low or negative HI antibodies were used to inoculate C6/36 cells for virus isolation.

Blood-meal identification: The principle described by Weitz (1956) was followed with agar gel diffusion system (Ouchterlony, 1958), which has been used by Sullivan *et al.* (1971), Gould *et al.* (1974), and Karoji *et al.* (1980). Agar plate for micro gel diffusion was prepared with 1.2% Purified Agar (Difco, Mich, USA) molten in PBS containing 0.1% NaN_3. The agar was poured over 50 × 70 mm horizontal glass plate

to make agar layer of 1 mm thickness. After the agar was solidified, round holes of 2.5 mm in diameter were punched with distance of 4 mm apart from each other and each hole was surrounded by 6 holes. Each engorged mosquito was triturated in a well on plastic U-shaped microplate with 0.1 ml of PBS containing 0.1% of NaN_3 using a small disposable wooden stick. The plate was incubated at 4°C overnight to extract blood meals. Ten microliter of the extract was placed in a hole punched on the agar plate, and surrounding holes were filled with 10μl each of the rabbit antisera against whole serum of the following animals; human, swine, bovine, dog and chicken. These antisera were the products of Miles Laboratories, Ill. USA, and were used at 1:10 dilution. The antiserea could detect homologous antigen of each serum at 1:1000 dilution without showing any cross-reactions. One of the 6 holes surrounding test specimen was used as a control with PBS. After several hours of incubation at room temperature, precipitin lines became visible and the results were recorded for each test specimen.

Estimation on daily survival rate: Dissections were made with limited number of unfed females of *Cx. tritaeniorhynchus* to find their parity by examining trachole changes in an ovary (Detinova, 1962). Following the description by Davidson (1954), daily survival rate (P) was estimated from parous rate (p) and gonotrophic cycle (q) using the equation;

$$p=P^q.$$

RESULTS

Table 1 shows the number of identified possible vectors of JE virus, which were processed for virus isolation, according to the date and site of their collection. Total number of mosquitoes processed was 15513, which consisted of 4935 *Cx. tritaeniorhynchus*, 7052 *Cx. gelidus* and 3526 *Cx. fuscocephala*. Besides these species, many *Mansonia uniformis*, *Aedes vexans*, *Ae. lineatopennis* and *Armigeres* sp. were collected and small number of *Cx. hutchinsoni*, *Cx. annulus*, *Cx. pseudovishnui*, *Cx. bitaeniorhynchus* and *Cx. sinensis* were caught. These number and proportion do not necessarily reflect actual species composition of the collected mosquitoes, because not all the materials were identified due to the limited capacity of the processing. A total of 125 pools were tested, however, JE virus was not isolated. Two strains each from *Cx. tritaeniorhynchus* and *Cx. gelidus* collected at Mae Rim on August 26, cross reacted with JE virus by the immunoperoxidase staining of the infected cells, however, they were not neutralized efficiently by anti-JE serum to the same extent as standard JE virus. Fifty nine mosquito pools were found to contain some filtrable agents which produced various degrees of cytopathic effects on C6/36 cells. Isolation from swine sera did not yield JE virus also.

In spite of many apparent cases of encephalitis, isolation of JE virus from field-caught mosquitoes turned out to be negative. This result is strong contrast to the findings in Japan, where, there are few apparent encephalitis patients, still the isolation of JE

Table 1. Number of *Culex tritaeniorhynchus*, *Culex gelidus* and *Culex fuscocephala* females collected and processed for virus isolation in Chiang Mai, 1982

Collection		Mosquito species*			
Site	Date	C. tri.	C. gel.	C. fus.	Total
Hang Dong	Jul. 15	262	81	11	354
	Jul. 22	283	325	279	887
	Jul. 27	223	1104	118	1445
	Aug. 5	500	1023	183	1706
	Aug. 12	235	536	326	1097
	Total	1503	3069	917	5489
San Pathong	Jul. 14	135	140	107	382
	Jul. 21	460	916	596	1972
	Jul. 29	564	800	323	1687
	Aug. 4	87	175	156	418
	Total	1246	2031	1182	4459
Doi Saket	Jul. 13	85	55	25	165
	Jul. 20	91	41	106	238
	Jul. 28	476	729	319	1524
	Aug. 3	27	27	8	62
	Aug. 10	335	415	739	1489
	Total	1014	1267	1197	3478
Mae Rim	Jul. 26	1024	623	187	1834
	Aug. 2	28	13	6	47
	Aug. 9	120	49	37	206
	Total	1172	685	230	2087
Total		4935	7052	3526	15513
(%)		(31.8)	(45.5)	(22.7)	(100.0)

* C. tri. : *Culex tritaeniorhynchus*
C. gel. : *Culex gelidus*
C. fus. : *Culex fuscocephala*

Table 2. Identification of blood-meals of *Culex* mosquitoes collected by light traps at Hang Dong, Chiang Mai, 1982

Mosquito species	No. tested	No. of blood source			
		Porcine	Bovine	Porcine & Bovine	Negative
Cx. tritaeniorhynchus	224(100.0)	204(91.1)	16(7.1)	3(1.3)	1(0.5)
Cx. gelidus	53(100.0)	43(81.1)	6(11.3)	0(0.0)	4(7.6)
Cx. fuscocephala	28(100.0)	21(75.0)	4(14.3)	1(3.6)	2(7.1)

Number in parenthesis: Percentage of mosquitoes fed on blood meal of each animal.

virus from field mosquitoes is quite frequently observed. In order to understand the reason of this discrepancy, we tried to know the feeding preference of collected mosquitoes and also daily survival rate of *Cx. tritaeniorhynchus*. The results are shown in Tables 2 and 3. *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. fuscocephala* collected at Hang Dong were found to have fed preferentially on swine (66−91%), and less frequently on bovines (4−14%). Some mosquitoes were found to have fed both on swine and bovine, however, no one fed on dogs, chicken or humans, as far as tested. Estimation on the daily survival rate (P) showed that the value P was estimated as 0.5997 assuming the gonotrophic cycle (q) is 3. P-value of 0.6815 was obtained assuming q-value is 4. These P-value estimated for Chiang Mai *Cx. tritaeniorhynchus* did not differ so much from the daily survivai rates of 0.42−0.74 estimated by Buei and Ito (1982) for the same species mosquito in Osaka, Japan by the same method, or the daily survival rate of 0.4888 estimated by Wada *et al.* (1969) for the same species by dispersal experiment in Nagasaki.

Table 3. Estimation on daily survival rate of *Culex tritaeniorhynchus* collected at Hang Dong, Chinag Mai, Thailand on August 12, 1982

Parou srate	0.216	0.216
Gonotrophic cycle*	3 days	4 days
Daily survival rate	0.600	0.682

* Based on the data by Kawai (1969)

DISCUSSION

In spite of many apparent encephalitis patients, and also positive isolation of JE virus from a postmortem brain material and serological evidence of JE infections of humans and several vertebrate animals as shown in the accompanying papers (Igarashi *et al.*, 1983b; Fujita *et al.*, 1983; Uzuka *et al.*, 1983; Fukunaga *et al.*, 1983; Ogata *et al.*, 1983), isolation of JE virus from possible vector mosquitoes turned out to be negative. This is a striking contrast to our experience in Japan as described in the text. *Cx. tritaeniorhynchus* as main vector of JE virus in Japan has been well-established(Mitamura *et al.*, 1938; Hammon *et al.*, 1949; Buescher *et al.*, 1959). This species and also 2 other species of *Culex* mosquitoes were known vector of JE virus in Southeast Asia (Wang *et al.*, 1962; Simpson *et al.*, 1970; Simasathien *et al.*, 1972; Thoa *et al.*, 1974), including Chiang Mai Area(Gould *et. al*, 1974). The latter authors also showed that infection rates of these mosquitoes with JE virus are much lower than the value in Japan reported by Buescher *et al.* (1959), because they could isolate 13 JE strains from more than 400,000 possible vectors. Our study in Nagasaki, 1981, showed that *Cx. tritaeniorhynchus* was infected from July 27 to September 1 with minimum infection rate between 0.05 to 0.55% with its maximum on August 11 (Igarashi *et al.*, 1981b). Assuming the same infection rate in Chiang Mai, 4935 *Cx. tritaeniorhynchus* should have yielded at least 2 JE virus strains. Therefore, low infection rate of JE vectors appears to be an ecological characteristic of

JE virus circulation in Chiang Mai Area, Several possibilities could be considered about this phenomenon. Gould *et al.*(1974) showed that possible JE vectors of *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Cx. fuscocephala* and *Cx. vishnui* fed preferentially on bovines (78-85%) and they did not fed on swine so much (6-10%). These authors discussed that the strong preference of the vector species for bovines may explain the relatively low infection rate with JE virus, as Carey *et al.* (1968) hypothesized that bovines serve to dampen the spread of JE virus. However, our study revealed that the most preferred blood meal host of these mosquitoes was swine, and bovines were not so frequently bitten. As reported in the accompanying paper (Ogata *et .al*, 1983), swine population in Chiang Mai Province in 1982 was more than the sum of cattle and water buffaloes, similar to the figures reported by Johnsen *et al.* (1974) in the year of 1970 in Chiang Mai. It may be that the discrepancy between our result and the result by Gould *et al.* (1974) is the result of sampling place, however, our result together with the serological data on swine sera (Ogata *et al.*, 1983) shows that *Culex*-swine cycle of JE virus transmission is active in Chiang Mai. The second possibility would be that Chiang Mai mosquitoes have shorter life span than those in Japan, resulting in higher turnover and lower infection rate with JE virus. However, examination on the daily survival rate of *Cx. tritaeniorhynchus* captured in Chiang Mai showed that the value was almost similar to those obtained in Japan by Wada *et al.* (1969) and Buei and Ito (1982). Another possibility would be that too many mosquitoes were emerging in Chiang Mai, resulting in the dilution of infected vectors. Also it should be considered that the timing and place of sampling were not adequate. The encephalitis patient, who died on the 27th of July and yielded JE virus from his brain, developed encephalitis symptoms starting from July 21 (see the accompanying paper by Igarashi *et al.*, 1983b). So that he could probably be infected with the virus around July 14. His home address was Mae Ai, the northern part of Chiang Mai Province (see the accompanying paper by Uzuka *et al.*, 1983), and mosquito collection was not performed around this area. The northern most collection site in our study was Mae Rim, and the collection at this place was started from July 26. Also it may be considered that in Chiang Mai the proportion of swine which are susceptible to JE virus in the pre-epidemic season is relatively low because of the climatic condition which allows mosquito activity and circulation of the virus through the year, on the other hand, most of the swine in Japan are susceptible to JE virus in the pre-epidemic season, resulting in high and efficient infection of the swine with the virus. These situations could result in the low infection rate of the mosquitoes with JE virus in Chiang Mai compared with the infection rate of *Cx. tritaeniorhynchus* in the epidemic season in Japan.

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1982年タイ国チェンマイ地区における脳炎のウイルス学的疫学的調査　Ⅶ. 蚊の採集とウイルス分離

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1982年7月13日より8月12日までの間，タイ国チェンマイ市の近郊4カ所の豚舎にライトトラップを設置し，飛来する蚊を採集した．採集された蚊のうち日本脳炎媒介蚊として知られているコガタアカイエカ4935匹，*Cx. gelidus* 7052匹，*Cx. fuscocephala* 3526匹からヒトスジシマカ培養細胞クローンC6／36を用いてウイルスの分離を試みた．その結果2株の未同定のフラビウイルスと59株の未知濾過性因子が分離されたが，日本脳炎ウイルスは分離されなかった．この3種のイエカ属の蚊はブタからよく吸血しており日本脳炎の伝搬に大きな役割を果しているものと思われる．

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