Ecology of Japanese Encephalitis Virus in Japan

I. Mospuito and pig infection with the virus in relatien to human incidences

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ABSTRACT : The ecological studies on Japanese encephalitis (JE) virus were performed from 1964 to 1973 in Nagasaki district, southern part of Japan. The summarized results during epidemic periods demonstrated that *Culex tritaeniorhynchus* is a main vector of JE virus and the extensive virus infections in vector mosquitoes are generally seen before or in the peak of the seasonal prevalence of mosquito population density and that the size of human epidemics seems to be mainly influenced with the vector mosquito population density at the start of epizootic in swine. Some particular attempts to isolate JE virus from fairly large number of overwintered *C. tritaeniorhynchus* and the tests for their history of the oviposition in last autumn of these overwintered mosquitoes suggested that the possibility of the virus to overwinter in the adult female of *C. tritaeniorhynchus* is unlikely as far as southern parts of Japan are concerned.

Many studies have been performed on the ecology of Japanese encephalitis (JE) virus in an epidemic season (Buescher *et al.*, 1959; Buescher *et al.*, 1959; Konno *et al.*, 1966; Oya *et al.*, Scherer *et al.*, 1959; Yamamoto *et al.*, 1968; Yamamoto *et*

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to year mainly due to the changes of the environmental conditions, the study was continuously undertaken at two villages of Kaizu and Mogi after 1965. These two villages have good rice fields, pigsties and cowsheds and are surrounded by low hills studded with copses.

Collection of mosquitoes:

Mosquitoes for virus isolation were collected by hand at pigsties and cowsheds and also by dry ice method (Omori *et al.*, 1965) at the sides of rice fields.

Virus isolation from mosquitoes:

Collected mosquitoes were kept at room temperature for a few days to allow the engorged blood to be digested. Then, these mosquitoes were anesthetized with carbon dioxide and identified under a stereoscope. Identifed mosquitoes were pooled into test tubes not to exceed 200 specimens in an ordinary way and stored at −75°C until processing for virus isolation.

Virus isolation was carried out by intracranial inoculation of the supernatant of mosquito suspension into 3− to 4−day-old suckling mice (gpc strain from 1964 to 1971, ICR strain after 1972). During the observation period of 2 weeks, the mice which manifested sickness were sacrificed and their brains were removed. Virus isolation was confirmed by inoculating again of the 10 % brain suspension into suckling mice.
The diluent used for mosquito suspension and brain suspension was phosphate buffered saline solution (pH 7.4) supplemented with 0.75% bovine serum albumin and antibiotics. The supernatants of these suspensions were obtained by centrifugation at 10,000 rpm for 15 minutes at 4°C

Identification of virus isolates:

In most cases, virus isolates were identified by means of hemagglutination (HA) and hemagglutination-inhibition (HI) test against anti-JaGAr 01 and anti-Nakayama NIH rabbit or mouse sera of Japanese encephalitis (JE) virus. In some cases, the combinations of HI test and complement-fixation (CF) test or HI test and indirect fluorescent antibody (FA) technique were used for identification.

The techniques of HI test were based on the method described by Clarke and Casals (1958). HA and CF antigens were prepared by sucrose acetone extraction technique, however the crude extraction method with trichlorotrifluoroethane and aceton-ether extraction method were used in some case. The procedures of CF test were essentially the same as described by Lennette and Schmidt (1964).

Collection and HI test of swine sera and virological confirmation of human encephalitis cases:

Swine sera were collected at a slaughter house of Nagasaki city at which the pigs raised in the suburbs of Nagasaki city including study sites were killed. In an epidemic season from May through October, more than 20 specimens of swine sera were collected

et al., 1970) and the evidences accumulated so far have revealed that the mosquito of *Culex tritaeniorhynchus* is a main vector of JE virus in Japan and constitutes an infectious cycle of JE virus usually with pigs and in some areas, with some kinds of bird (Buescher *et al.*, 1959) but not with other domestic animals and human beings although they can be infected by infected *C. tritaeniorhynchus*.

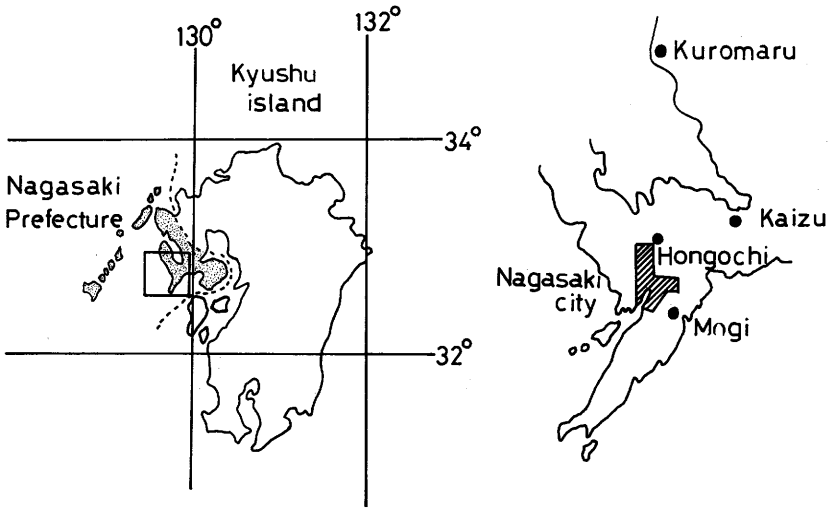
However, the ecology of JE virus in an interepidemic season is still unclear. Reeves (1974) summarized the studies reported on this problem and proposed some hypotheses and possible reservoirs for explaining the persistence of JE virus in winter season.

This series of studies have been performed to know firstly the epidemiology of JE in Nagasaki distict in an epidemic season and secondly the possibility of overwintering of JE virus in adult female of *C. tritaeniorhynchus* among the proposed reservoirs. In the present paper, we summarized the results from 1964 to 1973 which have been annually published previously (Hayashi *et al.*, 1965; Hayashi *et al.*, 1966; Hayashi *et al.*, 1970; Hayashi *et al.*, 1973; Shichijo *et al.*, 1968) and discuss the epidemiology of JE virus in epidemic season and the possibility of overwintering of JE virus in the vector mosquitors in Nagasaki district, southern part of Japan.

MATERIALS AND METHODS

Study sites:

At the beginning of the study, Kuromaru village of Omura city about 42 km from Nagasaki city was chosen as the study site. From 1965, Kaizu, Mogi and Tomachi villages which are located at 23 km, 6 km and 4 km from the center of Nagasaki city respectively, and other small villages added as the study sites. Fig. 1 shows these study sites. Although there were some changes of the study sites from year



Remarks: Black circles show villages for mosquito collection.

Fig 1. Study sites and Kyushu island of Japan.

every week, however, the frequeney of the collection was reduced to 2 to 3 times per month in an interepidemic season.

HI antibody titers of swine sera were examined by the method of Clarke and Casals (1958) against HA antigen of JaGAr 01 strain after treatment of the sera with cold aceton twice to remove the non-specific inhibitors of HA.

Serological identification of human cases was made as a rule by comparing the HI titers in paired sera. The cases whose HI antibody titers increased more than 4 times of the initial titers were considered sero-positive. In the cases whose paired sera were not obtained, serological confirmation was judged collectively from the HI and CF antibodies level and also from the presence of 2-mercaptoethanol sensitive antibody.

RESULTS

(1) *Virus isolations from various species of mosquitoes*:

Table 1 shows the summarized results of virus isolation from mosquitoes by species from 1964 through 1973. A total of 232 strains of JE virus were isolated among which 217 (93.5%) strains were from *C. tritaeniorhynchus*, 8 (3.4%) strains from *C.*

Table 1. JE virus isolations from various mosquitoes in Nagasaki from 1964 to 1973

Year	*C.trit.**	*C.p.p.***	*C.pseudo-vishnui*	*C.bit.****	*C.whit-morei*	*Aehes. vexans nipponii*	*Anopheles. sinensis*	*Anopheles. sineroides*	*Armigeres. subalbatus*
1964	13,442a (19)b	418					2,913		
1965	90,552 (47)	562 (2)	14,777 (5)		88	4,824 (2)			1,479
1966	100,327 (71)		5,303 (2)		2,395	5,476	15,347		2,032 (1)
1967	43,989 (25)	514	594	95	24	23,751 (1)-3c	4,558	48	3,246
1968	7,272 (6)	628	152 (1)	17	52	4,038 (1)	5,964	94	1,668
1969	23,762 (20)	323	183	40	45	5,124	7,878	22	2,558
1970	8,277 (14)	75				963 (0)-1			130 (0)-1
1971	7,036 (8)	159	3	34		484	1,138		170
1972	2,475 (2)	76				164			104
1973	2,085 (5)	28		7		102			279
Totals	299,217 (217)	2,783 (2)	21,012 (8)	193	2,604	44,926 (4)-4	37,798	164	11,666 (1)-1

a: Number of mosquitoes tested
b: Number of JE viruses isolated
c: Number of non-JE arboviruses isolated

* Culex tritaeniorhynchus
** Culex pipiens pallens
*** Culex bitaeniorhynchus

pseudovishnui, 4 (1.7%) strains from *Aedes vexans nipponii*, 2 (0.9%) strains from *C. pipiens pallens* and one strain (0.4%) from *Armigeres subalbatus*, respectively. Although JE virus was isolated from these 5 species of mosquitoes, the constant isolation from *C. tritaeniorhynchus* every year with high isolation efficieney suggested that *C. tritaeniorhynchus* is a main vector of JE virus as established well and that other 4 species of mosquitoes are not playing an important role on the dissemination of JE virus.

Table 2 summarized the JE virus isolations from *C. tritaeniorhynchus* by month and by year during the studies. Although the numbers of virus isolates fluctuated every year, JE virus began to be isolated from *C. tritaeniorhynchus* usually in June or July, followed by continuous isolations until the first of September.

As shown in Table 1, 4 strains and 1 strain of non-JE arbovirus were isolated from *Aedes vexans nipponii* and *Armigeres subalbatus*, respectively. Three of 4 strains from *Aedes vexans nipponii* were isolated in 1967 and one in 1970 in an epidemic season. These non-JE arbovirus strains were identified as Getah virus, one of the group A arboviruses, by cross CF and cross neutralization test (Shichijo *et al.*, 1970). One

Table 2. JE virus isolations from *C. tritaeniorhynchus* in Nagasaki by year and by month

Year		March	April	May	June	July	August	September	October	Total
					a					
1964	early				954(2)	3,178(4)	2,000(3)	700(0)		
	middle					1,700(4)	600(0)	800(0)	5(0)	13,442(19)
	late			157(0)	1,500(6)	1,600(0)	242(0)	6(0)		
1965	early		67(0)	2,870(0)	6,893(7)	1,927(10)	4,796(0)	2,187(1)	275(0)	
	middle		2,444(0)	3,730(0)	9,130(7)	3,129(4)	9,621(1)	228(0)	46(0)	90,552(47)
	late		13,036(0)	3,180(0)	2,358(5)	16,898(11)	7,077(0)	660(0)		
1966	early	1,432(0)	15,670(0)	8,108(0)	6,436(0)	7,833(7)	8,434(6)	4,197(0)	102(0)	
	middle		2,390(0)	5,141(0)	1,222(0)	10,753(40)	3,036(1)	2,425(0)		100,327(71)
	late		675(0)	1,556(0)	720(1)	16,527(14)	3,386(2)	284(0)		
1967	early	1,517(0)	5,757(0)	929(0)	3,282(0)	2,238(8)	1,357(0)	81(0)		
	middle		6,537(0)	2,950(0)	2,788(0)	5,506(9)	121(0)	377(0)		43,989(25)
	late		198(0)	3,546(0)	2,667(7)	3,507(1)	353(0)	278(0)		
1968	early	56(0)	308(0)	186(0)	648(0)	476(0)	1,619(5)			
	middle		236(0)	153(0)	189(0)	462(0)	163(0)	91(0)		7,272(6)
	late		62(0)	843(0)	282(0)	772(1)	696(0)	30(0)		
1969	early	49(0)	83(0)	104(0)	1,132(0)	77(0)	3,344(10)	505(0)		
	middle		6,453(0)	0	2,030(0)	289(0)	1,937(7)	142(0)		23,762(20)
	late		1,023(0)	1,013(0)	1,701(0)	2,277(0)	1,603(3)			
1970	early			31(0)	270(0)	895(0)	905(3)			
	middle		1,038(0)	112(0)	803(0)	629(1)	477(2)			8,277(14)
	late		44(0)	195(0)	792(0)	1,674(8)	412(0)			
1971	early		1,516(0)		391(0)	508(0)				
	middle		731(0)	205(0)	299(0)	1,373(6)	269(0)			7,036(8)
	late	217(0)	75(0)	41(0)	890(0)	504(2)	17(0)			
1972	early		1,935(0)		159(0)			29(1)		
	middle	22(0)					137(1)			2,475(2)
	late			5(0)		197(0)				
1973	early		372(0)	234(0)		405(1)	117(0)			
	middle				22(0)	46(1)				2,085(5)
	late					759(3)	130(0)			
Total		3,293(0)	60,650(0)	35,289(1)	47,549(35)	86,139(135)	52,849(44)	13,020(2)	428(0)	299,217(217)

a: Number of mosquitoes tested. Figure in parenthesis means the number of JE virus isolates.

strain from *Armigeres subalbatus* was isolated in 1970. This virus was sensitive to ether and sodium deoxycholate and considered to be arbovirus, however, detailed identification has not been accomplished.

(2) *Attempts to isolate JE virus from overwintered mosqnitoes*:

Since it has been demonstrated that JE virus can persist in winter season in experimentally infected adult *C. tritaeniorhynchus* and can be transmitted to susceptible pigs (Mifune, 1965), some particular attempts were made to make sure this possibility in nature. Overwintered mosquitoes were mainly collected by dry ice method in early spring. Most of *C. tritaeniorhynchus* were processed for virus isolation after keeping them for 3–4 days at room temperature, while the rest were examined for their history of the oviposition in last autumn by inspecting the dilatation of ovariole follicle and unwinding of tracheole skein of the ovary. Other species of mosquitoes, *C. pseudovishnui*, *C. pipiens pallens* and *Aedes vexans nipponii* collected at the same time were also examined for JE virus infection, although *Aedes vexans nipponii* does not overwinter as an adult.

Table 3 shows the numbers of *C. tritaeniorhynchus* which were processed for virus isolation by year and describes the kinds of virus isolation techniques. In 1966

Table 3. Attempts to isolate JE virus from over wintered *Culex tritaeniorhynchus*

Year	March	April	May	Total	Methods and animals used for virus isolation
1965	0	15,547[a]	533	16,080	SMB, ic, blind passage, twice.
1966	1,432	18,735	14,805	34,972	SMB, ic, blind passage, twice.
					10-day-old chickembryo, ammiotic cavity
1967	1,517	12,492	0	14,009	SMB, ic, blind passage, twice.
					biting experiment, subcutaneous
					inoculation of mosquito suspension
					into susceptible pigs.
1968	56	606	186	848	SMB, ic, blind passage, twice.
1969	49	7,559	104	7,712	SMB, ic, 〃
1970	0	1,082	143	1,225	SMB, ic, 〃
1971	217	2,322	205	2,744	SMB, ic, 〃
1972	22	1,935	0	1,957	SMB, ic, 〃
1973	0	372	234	606	SMB, ic, 〃
Total	3,293	60,650	16,210	80,153	

a: number of mosquitoes tested
ic: intracranial inoculation
SMB: suckling mouse brain

and 1967, as the host animals for virus isolation, 10-day-old chick embryo and susceptible pigs, respectively, were used in addition to the suckling mice. Chick embryos were inoculated into their amniotic cavity with 0.04 ml of mosquito suspensions (Shichijo *et al.*, 1968), and the pigs were inoculated subcutaneously with mosquito suspension and also were exposed for the bites of the mosquitoes, followed by the virus isolation from the blood and by detecting HI antibody to JE virus in their sera (Shichijo *et al.*, 1968).

As can be seen, no virus was isolated from the total of 80,153 of *C.* tritaeniorhynchus caught in early spring in spite of some particular attempts to isolate the virus. Despite of above facts, there might be the possibility of overwintering of JE virus in other mosquitoes alternate to *C. tritaeniorhynchus*, the main vector in an epidemic season. Thus, the attempts with *C. pseudovishnui*, *C. pipiens pallens* and *Aedes vexans nipponii* caught in nature in early spring were made, however, virus isolation was unsuccessful as shown in Table 4.

The results of the examinations for the age compositions of overwintered *C. tritaeniorhynchus* which were performed at the sama time will be described and discussed in the

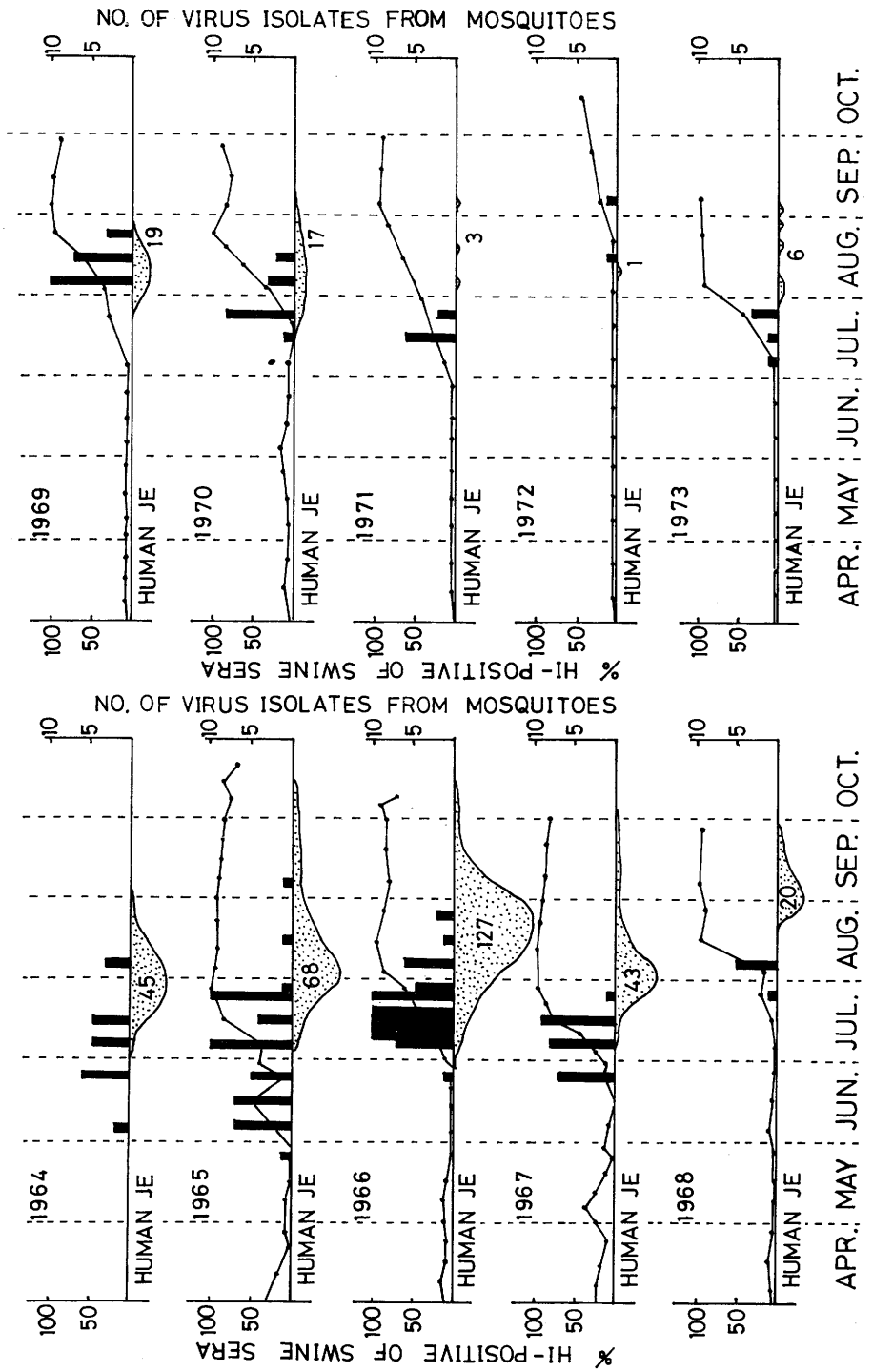
Table 4. Attempts to isolate JE virus from *C. pseudovishnui*, *C. pipiens pallens* and *Aedes uexans nipponii* caught in nature in spring

Year	Month	Species of mosquitoes		
		Culex pseudovishnui	*Culex pipiens pallens*	*Aedes vexons nipponii*
1965	April	660[a]	0	0
	May	263	0	792
1966	April	817	0	1,493
	May	328	0	1,912
1967	April	183	2	457
	May	53	106	8,376
1968	April	19	0	911
	May	26	0	310
1969	April	71	0	367
	May	0	30	2,263
1970	April	0	0	162
	May	0	15	109
1971	April	0	0	35
	May	0	63	31
1972	April	0	0	43
	May	0	0	50
1973	April	0	0	0
	May	0	28	0
Total		2,420	244	17,311

a: number of mosquitoes tested.

following paper.

(3) *Relationships between mosquito infection, swine infection and human JE cases.*



Remarks: Black bar means the number of virus isolates from the mosquitoes. The figures in the lines of human JE mean the number of reported human Japanese encephalitis cases.

Fig. 2. Relationships between the virus isolation from *Culex tritaeniorhynchus*, the swine infection and the human epidemics in Nagasaki district from 1964 to 1973.

Fig. 2 shows the relatinships between the isolation of JE virus from *C. triaeniorhynchus*, the swine infection and the epidemic size of human JE cases. As can be seen, JE viruses were almost continuously isolated from the vector mosquitoes every year once the virus began to be isolated, although the start of JE virus infection in the mosquitoes varied from year to year. And, the isolation efficiencies were generally higher in the beginning than in the latter parts of the virus isolation period during which JE virus was almost constantly isolated from the mosquitoes. As shown in Table 5, the

Table 5. JE virus isolations from *C. tritaeniornynchus* and human encephalitis cases in Nagasaki from 1964 to 1973

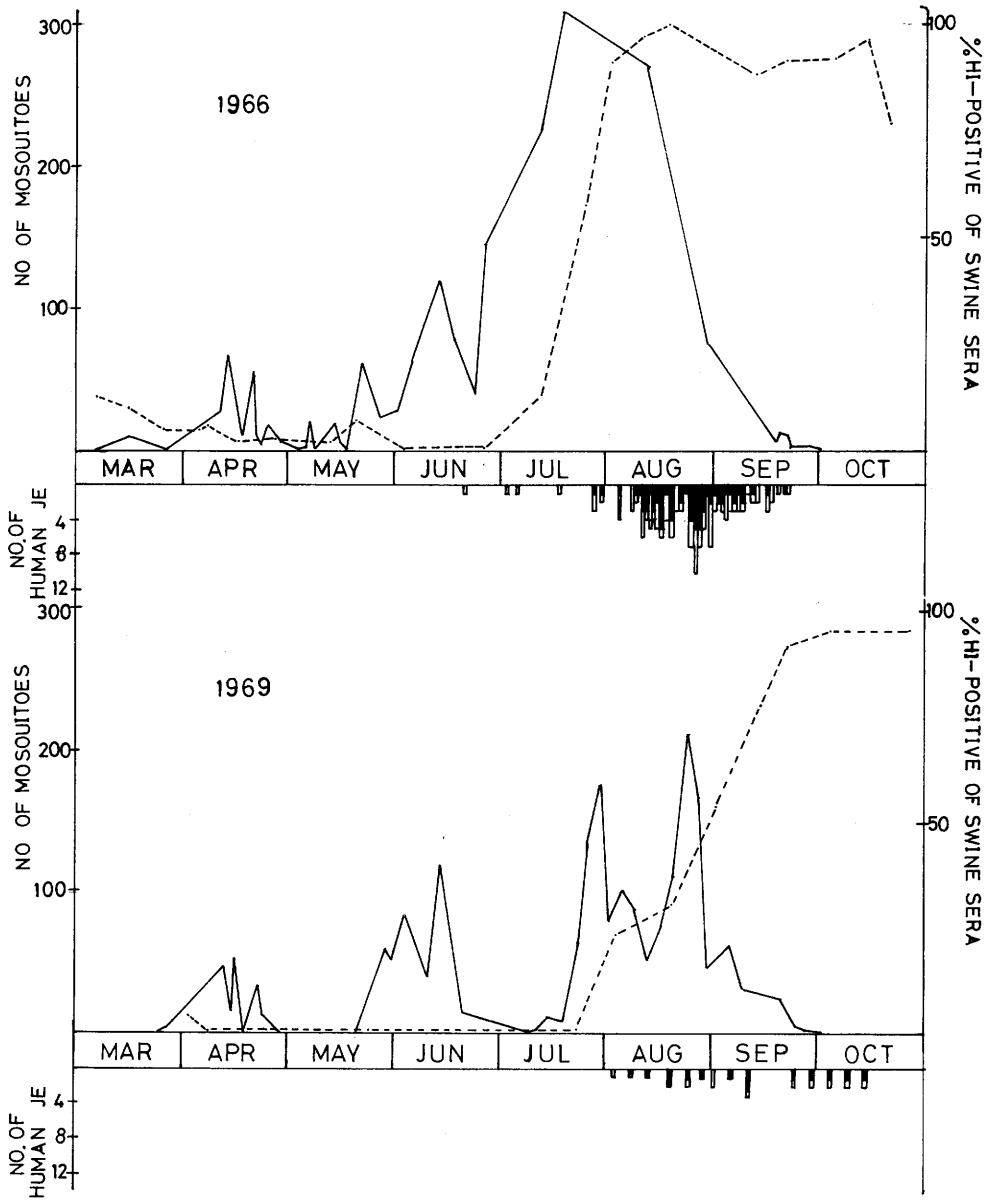
Year	JE virus from *C. tritaeniorhynchus*		Human encephalitis cases*			
	isolation periods (days)	No. of virus isolates	No. of reported cases	No. dead cases	No. virologically confirmed cases	periods
1964	June 8-Aug. 7 (61)	19	45	20	N.T	July 3-Sep. 10
1965	May 30-Sep. 6 (100)	47	68	22	34	July 5-Oct. 23
1966	June 24-Aug. 27 (65)	71	127	54	33	July 2-Nov. 26
1967	June 23-Jul. 27 (35)	25	43	21	11	June 18-Sep. 7
1968	July 22-Aug. 7 (17)	6	20	12	2	July 5-Sep. 30
1969	Aug.1-Aug. 26 (26)	20	19	12	4	July 22-Sep. 26
1970	July 19-Aug. 16 (28)	14	17	11	3	July 23-Sep. 18
1971	July 13-July 27 (15)	8	3	3	0	Aug. 8-Sep. 15
1972	Aug. 16-Sep. 9 (25)	2	1	0	0	Aug. 10
1973	July 10-July 24 (15)	5	6	2	4	July 30-Sep. 4

N.T: not tested

*: A part of this collum is referred to the annual reports of Nagasaki Prefectural Institute of Public Health

periods of virus isolation also varied from year to year ranging from 15 days in 1971 to 100 days in 1965. However, the length does not appear to be correlated with the size of human epidemics, since, for instance JE viruses were isolated for the longest 100 days in 1965, while the size of the human epidemics was not the largest. Also, the number of isolated virus does not appear to have the relationship to human epidemics, because this number of virus isolates does not reflect the real number of infected mosquitoes and because the numbers of the mosquito samples tested were also different by year. Thus the size of the epidemics of human JE does not appear to be directly related with the number of virus isolates, the length of virus isolation period and also with the starting time of mosquito infection.

Epizootic in pigs started almost simultaneously with the infection in mosquitoes throughout the study periods. The HI positive rates of swine sera began to increase



Remarks: Dotted line means the seasonal prevalence of *Culex tritaeniorhynchus* collected at pigsties at Mogi village and solid line means the percent of HI-positive of swine sera. Black bar means virologically confirmed human case.

Fig. 3. Relationships between the seasonal prevalence of the vector mosquito population, the swine infection and the human epidemics.

thereafter and reached more than 80% every year except in 1972 in which it did not reach 50% even in the middle of October.

Human epidemics was consitently observed after the epizootic in pigs although the periods from pig epizootic to human epidemics varied from year to year. The largest human epidemics was observed in 1966, however, the number of case gradually decreased thereafter and drastically reduced from 1971.

Fig. 3 shows the relationships between the seasonal prevalence of the mosquito population of *C. tritaeniorhynchus*, swine infection and human epidemics in 1966 and 1969 as the representatives of large and small human epidemics respectively. As can be seen, a larger mosquito population, consequently a larger number of infected mosquitoes, was observed at the start of epizootic in pigs in 1966 as compared with that in 1969. The size of human epidemic seems to depend upon the density of mosquito population at the start of swine infection.

DISCUSSION

The results of virus isolations from various kinds of mosquitoes collected during epidemic periods demonstrated that *C. tritaeniorhynchus* is a main vector of JE virus in Japan and the virus isolation pattern is a almost same as that reported for western part of Japan (Yamamoto, 1968), in which the extensive virus disseminations in vector mosquitoes generally occur before or in the peak of the seasonal prevalence of mosquito population density (Table 2, Fig. 3) not after the peak as observed in Kanto plain Tohoku district of Japan (Buescher *et al.*, 1959; no *et al.*, Kon 1966; Oya *et al.*, 1963). Continuous virus isolations from vector mosquitoes were also observed after the start of virus infection in the mosquitoes, which was generally seen in June to July in Nagasaki district although it was observed in late May as the earliest case in 1965 (Table 2). These results are essentially identical with those of the studies which were undertaken in other areas in Japan.

In addition to *C. tritaeniorhynchus*, JE virus was isolated from *C. pseudovishnui*, *C. pipiens pallens*, *Aedes vexans nipponii* and *Armigeres subalbatus* (Table 1). These species of mosquitoes were demonstrated to be infected experimentally (Mitamura and Kitaoka, 1947), however the role of these mosquitoes on the epidemiology of JE virus is doubtful since virus isolates were incomparably few in number and were not consistently isolated every year.

Attempts to isolate JE virus from fairly large number of overwintered *C. tritaeniorhynchus* and also from overwintered *C. pseudovishnui* and *C. pipiens pallens* were unsuccessful. However, there still remains the possibility that the mode of the presence and the affinity to cells of the virus were changed during winter and the virus might not be isolated by conventional methods. However, it was demonstrated in our previous studies (Mifune, 1965; Shichijo *et al.*, 1972) that once the virus infection was established in the mosquitoes, the infectivity of the virus does not decrease significantly during the hibernation perids and even if the virus content in the mosquitoes was very low at the beginning of the hibernation, the virus can multiply quickly after hibernation only by transfer to the

temperature conditions of early spring and the virus can be easily recovered by routine technique. The overwintered mosquitoes tried to isolate the virus in the study were kept for certain periods at room temperature before processing for virus isolation and also the animal hosts other than suckling mice were used to isolate the virus. Therefore, the failure to isolate the virus from overwintered moquitoes appears to represent the absence of the virus in the mosquitoes.

In addition to above data, it was indicated that the feeding rate of *C. tritaeniorhynchus* which had been reared as adults under the outdoor natural or indoor experimental conditions of the short day-length, that is, under the condition to induce the winter diapause in the mosquitoes, is very low (Oda Wade, 1973; Shichijo *et al.*, 1972) and almost all of *C. tritaeniorhynchus* enter hibernation in the nulliparous and unfed status from about mid-September in Nagasaki district (Kawai, 1969). Thus, the possibility of the virus to overwinter in the adult female of *C. tritaeniorhynchus* seems to be unlikely as far as southern parts of Japan are concerned.

In the present studies, the direct relationships were not observed between the size of human epidemics and the number of virus isolates, the periods of virus isolation, the starting time of epizootic in vector mosquito. The production of infected vctor mosquitoes seems to depend upon the seasonal prevalence of vector mosquito population and the rate of susceptible pigs, in other words, if the peak of breeding number of vector mosquito coincides with the time at which the greater number of susceptible pigs remain, it must induce the extensive infections in pigs, resulting in the production of greater number of infected vector mosquitoes.

Thus, if the immune status of human population is not taken into consideration, the size of human epidemics seems to be closely related with the vector mosquito population density at the start of epizootic in swine.

The largest human epidemics in the past ten years was in 1966, however gradual decreae in the number of human cases was observed after that and none was confirmed virologically in 1972. The reason for explanation of this phenomenon will be analysed and discussed in the following paper.

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日本における日本脳炎ウイルスの生態学. Ⅰ. 蚊，豚の日脳ウイルス感染と人の流行との関係

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長崎地方における日本脳炎（日脳）ウイルスの生態学的研究のうち1964年から1973年に至る10年長間の調査成績を総括し解析を加えた．日脳ウイルスの主媒介蚊であるコガタアカイエカのウイルス感染の拡がりは媒介蚊の密度が最高に達する以前か或いはその時間に一致しているのが例年の様相である．また，人の日脳流行は主に豚の日脳感染が始まる頃の媒介蚊の密度によって影響されるようである．過去10年，相当の大量の越年コガタアカイイカ雌成虫から日脳ウイルスの分離を試みたが，いずれも不成功に終った．このことは蚊体内におけるウイルスの越年の可能性は南方諸地域とは異ることが推察される．

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