

## Ecological Studies on Japanese Encephalitis Virus

Survey of virus dissemination in Nagasaki area, Japan, in 1966 and 1967

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(Received for Publication September 9, 1968)

### Abstract

Serial survey on the ecology of Japanese encephalitis (JE) virus was made in 1966 and 1967. In spring in the both years, wild caught females of *Culex tritaeniorhynchus* and many other species including a great number of hibernated females were examined for JE virus. In 1967, besides the above, wild caught females in spring were forced to engorge blood from susceptible pigs, and some days after, the engorged ones were examined for the virus and the pigs for HI antibody. However, no virus was found in all the mosquitoes examined, and no antibody was detected in the pigs. Nevertheless, in mid-spring in 1967 2-ME sensitive antibody was found in a few of slaughtered pigs. This fact is very important and it seems necessary to set forward the program of investigations. Japanese common snakes of six species were examined for the reservoir of JE virus in nature, but it was concluded that snakes play minor role as a natural source of the virus transmission even in the epidemic season. Despite of the fact that the starting time of epizootic in pigs and of the occurrence of infected mosquitoes was nearly the same in 1966 and 1967, the duration and size of the infection in mosquitoes and of the epidemic in men were shorter and smaller in 1967 than in the previous year. The reason seems that the larger number of infected mosquitoes at the starting time in late June, 1967 caused

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This work was supported in part by Research Grant CC00208 from Public Health Service, National Communicable Disease Center, Atlanta, Georgia, U. S. A.

Contribution No. 527 from the Institute for Tropical Medicine, Nagasaki University and No. 175 from the Department of Medical Zoology, Nagasaki University School of Medicine

earlier and more rapid rise in the HI antibody possessing rate of pigs at the early days of epizootic in them reducing the remaining rate of susceptible pigs as faster as about a half-month than in 1966, and this, in turn, caused subsequent infections in mosquitoes and then in men to be shorter in duration and smaller in size.

### Introduction

It is a well known fact that in the epidemic season of Japanese encephalitis (JE) the most important vector mosquito is *Culex tritaeniorhynchus* and a main amplifier of the virus is pigs. However, the aspect of virus dissemination will vary by year and area. For example, the mosquito infection with JE virus in Nagasaki area was found from June 8 to August 7 in 1964 and from May 30 to September 6 in 1965, and the period of occurrence of mosquito infection becomes gradually later in other places of Japan, especially in Kanto area than in Nagasaki area. For getting a more complete understanding of JE epidemiology, further investigations in Nagasaki area were carried out in 1966 and 1967 on the relation among the mosquito

prevalence and infection, the swine infection and human encephalitis cases.

Another subject to be studied is the question of overwintering of JE virus. To solve the question, JE virus isolation was attempted in 1965 from wild caught mosquitoes in early spring, but it was unsuccessful. However, as it was thought necessary to carry out the same examination persistently, the virus isolation from mosquitoes collected in early through late spring was repeated in 1966 and 1967. And, the examinations were made with Japanese common snakes for the possibility of becoming the natural reservoir of JE virus. The results obtained will be given and discussed in the following.

### Materials and Methods

*Mosquito collection:* Mosquitoes were collected at 11 places from March 11 to October 11 in 1966 and at 7 places from February 21 to September 22 in 1967. The places of mosquito collection are located near Nagasaki city and the method of collection was described in the previous paper (Hayashi et al., 1965).

*Virus isolation:* Isolation of virus from mosquitoes was made by the inoculation of mosquito suspension into suckling mice intracerebrally as described by Hayashi et al. (1965b, 1966). In addition to the above method the suspension of wild caught mosquitoes in early through mid-spring was inoculated into the

amniotic sack of 9 or 10 days embryonated eggs and blind passages of all specimens were carried out carefully for the virus isolation.

*Hemagglutination inhibition test:* Hemagglutination inhibition antibody in swine and human sera was examined as previously described by Hayashi et al. (1965b, 1966). Hemagglutination inhibition antibody sensitive to 2-mercaptoethanol was tested by the technique described by Konno et al. (1966). Each antibody and its possessing rate will be referred simply as HI-A and 2-ME-SA, and HI-A positive rate and 2-ME-SA positive rate respectively in this paper.

## Results

### I. Mosquito and swine infections and human encephalitis cases in 1966

#### (1) Virus isolation from mosquitoes

JE virus isolation from *C. tritaeniorhynchus* and other mosquitoes by inoculation into suckling mice in 1966 is presented in Tables 1, 2. As shown in Table 1, though quite many mosquitoes of *C. tritaeniorhynchus* including a great number of hibernated females were examined, no virus was isolated until late June. On June 24 the first isolation of the virus was made at 1.4 of isolation effici-

ency, and afterwards the virus continued to be isolated up to August 27, with the maximum virus isolation efficiency of 3.7 in mid-July. The number of *C. tritaeniorhynchus* pools from which JE virus was isolated was 71 out of 373 pools tested. During the epidemic season, 2 pools of *C. vishnui* and one of *Armigeres subalbatus* were also found positive for JE virus, as seen in Table 2.

With the mosquitoes collected in early to mid-spring, besides the intracerebral inoculation into suckling mice, the inoculation into amnio-

Table 1. JE virus isolation from *C. tritaeniorhynchus* in Nagasaki area, 1966.

Month	No. of mosquitoes	No. of pools	No. of pools positive	Isolation efficiency	Isolation rate(%)
March	M   L 1,432 ※	8	0		
April	E M L 15,670 ※ 2,390 ※ 675 ※	54 8 3	0 0 0		
May	E M L 8,108 ※ 5,141 ※ 1,556 ※	34 18 6	0 0 0		
June	E M L 6,436 1,222 720	26 6 4	0 0 1	1.4	25.0
July	E M L 7,833 10,753 16,527	32 41 55	7 40 14	0.9 3.7 0.8	21.9 97.6 25.5
August	E M L 8,434 3,036 3,386	29 11 12	6 1 2	0.7 0.8 0.6	20.7 9.1 16.7
September	E M L 4,197 2,425 284	15 9 1	0 0 0		
October	E 102	1	0		
Total	100,327	373	71		

- Remarks: 1. The signs of E, M, and L mean the early, middle, and late part of a month.  
2. The suspensions of 34,972 females (※) were used partly for inoculation into suckling mouse by pool and partly (those of most females) for inoculation into the amniotic sack of chick embryos (see Table 3, for *C. tritaeniorhynchus*).

**Table 2.** JE virus isolation from mosquitoes by species in Nagasaki area, 1966.

Species	Collection period	No. of mosquitoes	No. of pools	No. of pool positive
<i>C. tritaeniorhynchus</i>	10 Mar. —11 Oct.	100,327	373	71
<i>C. vishnui</i>	3 Apr. —15 Sep.	5,303	20	2
<i>C. whitmorei</i>	20 Apr. —21 May	2,395	9	0
<i>Ae. vexans nipponii</i>	2 Apr. — 8 Sep.	5,476	23	0
<i>Ar. subalbatus</i>	10 Apr. — 2 Aug.	2,032	10	1
<i>An. sinensis</i>	10 Mar. —11 Oct.	15,347	70	0
Total		130,880	505	74

**Table 3.** Attempt of JE virus isolation in spring, in Nagasaki area, 1966 from mosquitoes including hibernated females by inoculation into amniotic sack of chick embryo.

	March	April	May	Total
<i>C. tritaeniorhynchus</i>				
No. of mosquitoes	14,321	17,074	14,074	32,580
No. of pools	8	59	55	122
<i>C. vishnui</i>				
No. of mosquitoes		817	328	1,145
No. of pools		3	2	5
<i>C. whitmorei</i>				
No. of mosquitoes		1,913	432	2,345
No. of pools		7	2	9
<i>Ae. vexans nipponii</i>				
No. of mosquitoes		1,493	1,912	3,405
No. of pools		6	7	13
<i>An. sinensis</i>				
No. of mosquitoes	985	2,861	2,911	6,757
No. of pool	6	14	12	32
Total				
No. of mosquitoes	2,473	24,102	19,657	46,232
No. of pools	14	89	78	181
No. of pools positive	0	0	0	0

tic sack of embryonated chick eggs was made, as shown in Table 3. In total, 46,232 mosquitoes belonging to five species were examined by this method, but no virus was

isolated from them.

#### (2) Swine infection and human cases

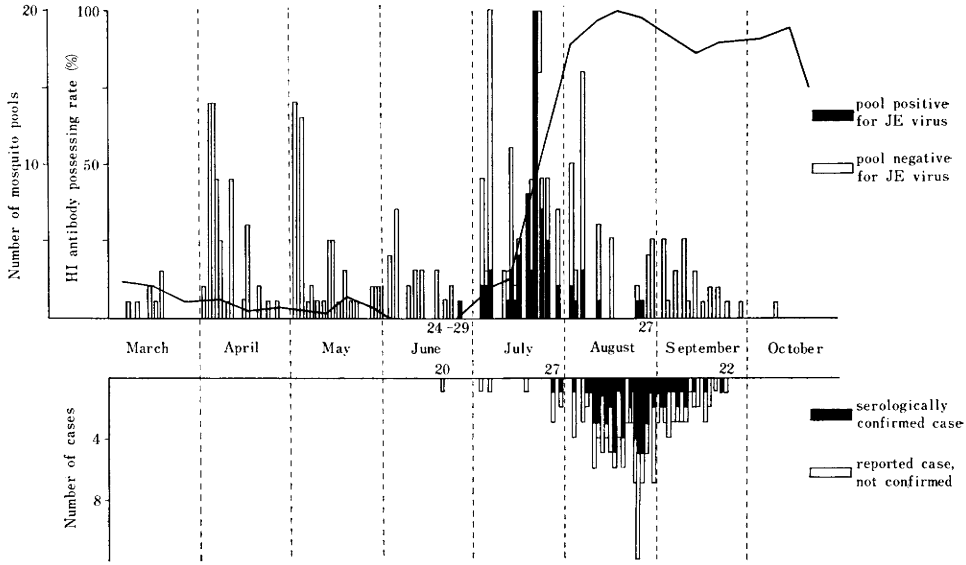
Fig. 1 shows the HI-A positive rate in slaughtered pigs, the occurrence of human encephalitis cases, and the JE virus isolation from *C. tritaeniorhynchus* in 1966. Fig. 2 shows the relation between the increase in HI-A positive rate in the pigs and the seasonal prevalence of *C. tritaeniorhynchus* in 1966.

HI-A in slaughtered pigs was detected at 3 to 8 per cent in spring. It is assumed that the antibody detected must have developed after the infection with JE virus in the last epizootic season in pigs and remained at low level.

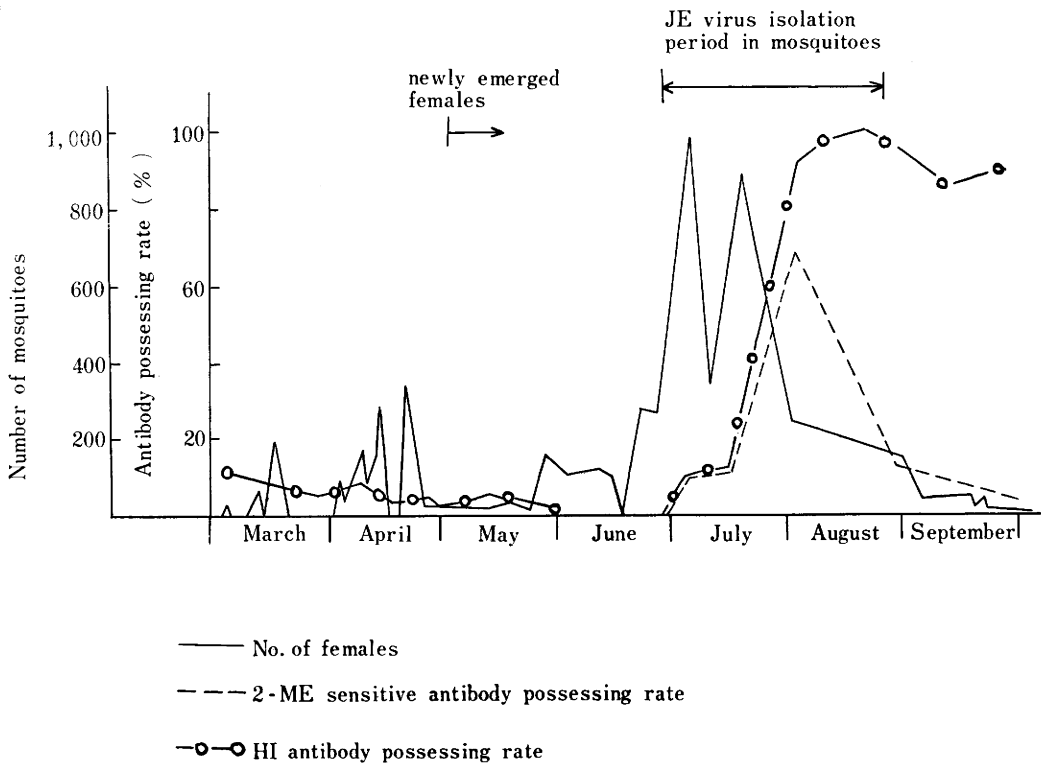
In spring, 1966, 2-ME-SA was not detected. This antibody develops in pig sera, as stated by Konno et al. (1967) and Otsuka et al. (1967), soon after the natural infection with JE virus, but it disappears in a few weeks and the antibody becomes resistant to 2-ME. Consequently, negative occurrence of 2-ME-SA in pigs in this season means negative occurrence of new infection in the pigs.

As seen in Fig. 2, from just after the first isolation of JE virus in mosquitoes in late June, the 2-ME-SA positive rate in slaughtered pigs rose rapidly reaching a peak at the beginning of August. Concurrently, HI-A

**Fig. 1.** JE virus isolation from mosquitoes of *C. tritaeniorhynchus* in relation to antibody rising in swine sera and to outbreak of encephalitis cases in Nagasaki area, 1966.



**Fig. 2.** Relation between antibody rising in swine sera and population of *C. tritaeniorhynchus* mosquitoes in Nagasaki area, 1966.



positive rate rose sharply and reached nearly 100% level nearly at the same time as the 2-ME-SA positive rate reached the peak, implying that the dissemination in pigs with JE virus must have occurred very intensely within about a month from late June in 1966. After the beginning of August, the HI-A positive rate remained at the same high level, while the 2-ME-SA positive rate decreased quickly from the peak toward the end of August suggesting that after the peak new infections in pigs must have scarcely occurred.

## II. Mosquito and swine infections and human encephalitis cases in 1967

### (1) Virus isolation from mosquitoes

Table 4 shows virus isolation from *C. tritaeniorhynchus* mosquitoes in 1967. The virus was isolated from June 23 to July 27. The duration of the virus isolation was shorter than in 1966 when it was from June 24 to August 27. Table 5 shows virus isolation from mosquitoes by species in 1967. As shown in Table 5, 25 strains of JE virus were isolated from *C. tritaeniorhynchus*. It is interesting that a strain of JE virus on July 14 and 3 strains of non-JE arbovirus on June 15, July 6 and 28 were isolated from *Ae. vexans nipponii*. The three strains of non-JE arbovirus were sensitive to ether and sodium deoxycholate, and the antigens prepared from acetone-ether

**Table 4.** JE virus isolation from *C. tritaeniorhynchus* in Nagasaki area, 1967.

Month	No. of mosquitoes	No. of pools	No. of pools positive	Isolation efficiency	Isolation rate(%)	
March	M					
	L	1,517 *	13	0		
April	E	5,757 *	24	0		
	M	6,537 *	24	0		
	L	198 *	2	0		
May	E	929	7	0		
	M	2,950	12	0		
	L	3,546	18	0		
June	E	3,282	18	0		
	M	2,788	15	0		
	L	2,667	11	7	2.6	63.6
July	E	2,238	11	8	3.5	72.7
	M	5,506	23	9	1.6	39.1
	L	3,507	17	1	0.3	5.8
August	E	1,357	6	0		
	M	121	2	0		
	L	353	4	0		
September	E	81	1	0		
	M	377	3	0		
	L	278	2	0		
Total	43,989	213	25			

Remarks: 1. The sings of E, M, and L mean the early, middle, and late part of a month.

2. The suspensions of 14,009 females (\*) in 63 pools were used partly for the inoculation into suckling mouse by pool and partly for the injection into pig No. 1 (see Fig. 3) after keeping at a deepfreezer and making them two pools.

**Table 5.** JE virus isolation from mosquitoes of different species in Nagasaki area, 1967.

Species	Collection period	No. of mosquitoes	No. of pools	No. positive pools
<i>C. tritaeniorhynchus</i>	18 Mar.—22 Sep.	43,989	213	25
<i>C. vishnui</i>	10 Apr.—14 Aug.	594	8	0
<i>C. whitmorei</i>	23 May—10 Aug.	24	2	0
<i>C. pipiens pallens</i>	20 Apr.—27 July	514	8	0
<i>Ae. vexans nipponii</i>	13 Apr.—5 Sep.	23,751	92	1(3)
<i>Ar. subalbatus</i>	20 Apr.—3 Oct.	3,246	14	0
<i>An. sinensis</i>	21 Feb.—11 Sep.	4,558	34	0
<i>An. sineroides</i>	18 Apr.—7 July	48	4	0
<i>C. bitaeniorhynchus</i>	8 May—12 July	95	2	0
<i>C. vorax</i>	30 May—4 July	6	1	0
<i>M. uniformis</i>	30 June—13 July	4	1	0
<i>Ae. albopictus</i>	23 June—12 July	3	1	0
Total		76,832	380	26(3)

( ) non-JE arbovirus

extract or sucrose-acetone extract of infected suckling mouse brains have no hemagglutination activity at any pH range. Exact identification of these isolates are being carried out and the result will appear in the series of this paper. No virus was detected from other species of mosquitoes than the above two species in this year.

As stated above, virus isolation was unsuccessful from mosquitoes collected in nature in the pre-epizootic season which were examined by inoculating the suspension of them into suckling mice intracerebrally in 1965 through 1967, and besides, in 1966 into amniotic sack of embryonated eggs.

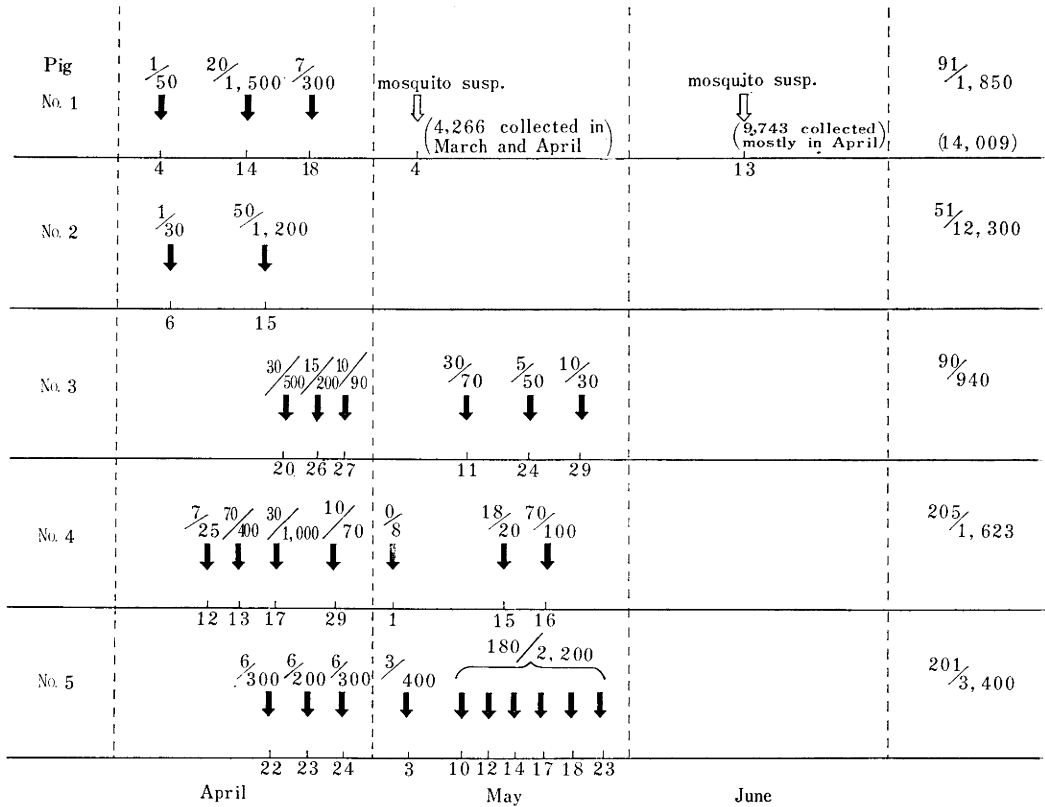
Despite of the above facts, however, with the hope that the possibility of causing the infection by the bite of females collected in nature in the pre-epizootic season on the susceptible pigs, the following biting experiments

were conducted as shown in Fig. 3. Wild caught 16,713 females of *C. tritaeniorhynchus* and 3,400 of *Ae. vexans nipponii* were allowed to feed on the susceptible pigs and 437 of the former and 201 of the latter species were engorged from five pigs No. 1..... No. 5. And in addition, suspension of 14,009 wild caught females of *C. tritaeniorhynchus* from March to April in 1967 (see Table 4) were also injected into the pig No. 1. Virus isolations were tried from the blood of the pigs successively at a certain interval and from the engorged mosquitoes after rearing them for some days in the laboratory. However, no virus was isolated from the pigs and the mosquitoes, and no HI antibody was detected in the sera of the pigs.

#### (2) Swine infection and human cases

Fig. 4 shows the relation among the HI-A positive rate in slaughtered pigs, the occur-

**Fig. 3.** Attempt of JE virus isolation in spring from mosquitoes including hibernated females by using susceptible pig, in 1967.



- Remarks :
1. Black and white arrows show exposure and injection respectively.
  2. Pigs signed with No. 1 to No. 4 were exposed to *C. tritaeniorhynchus* mosquitoes and No. 5 to *Ae. vexans nipponii* respectively; pig No. 1 was additionally injected with suspensions of females (number of which is shown in parentheses; cf. Table 4) of *C. tritaeniorhynchus*.
  3. Denominator and numerator show the number of mosquitoes exposed to the pigs and the number of mosquitoes engorged respectively.

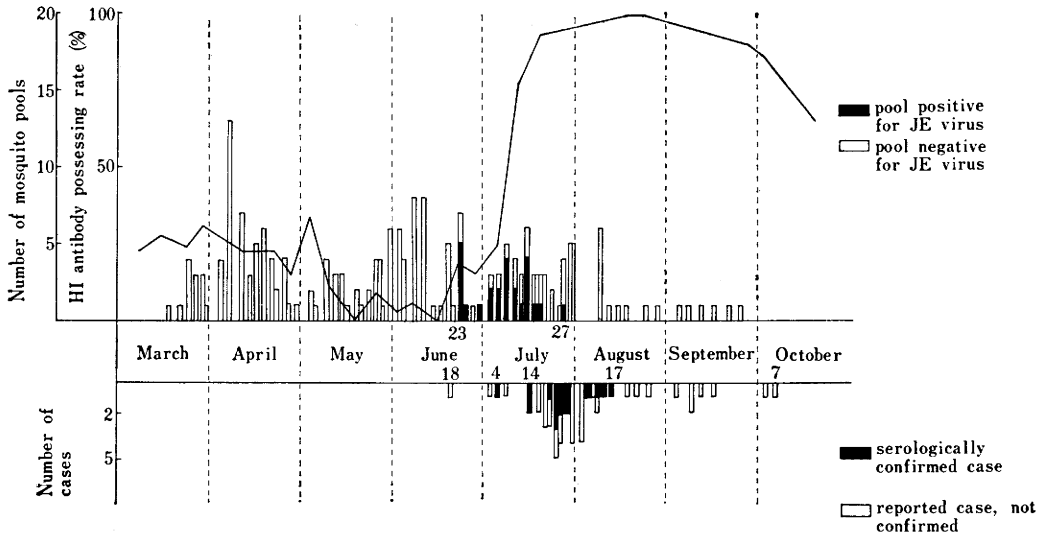
rence of human encephalitis cases, and the seasonal change of the JE virus isolation from *C. tritaeniorhynchus* in 1967. As seen in Fig. 4, the rise in the HI-A positive rate in pigs and the occurrence of human encephalitis cases appeared roughly similar as those observed in 1966, but the rise in the HI-A positive rate in pigs in 1967 was earlier and quicker and the human cases were smaller in number than in the previous year. In 1967, only 74

cases were reported and 24 out of them were confirmed serologically as genuine cases, when compared with 149 reported and 77 genuine cases in 1966.

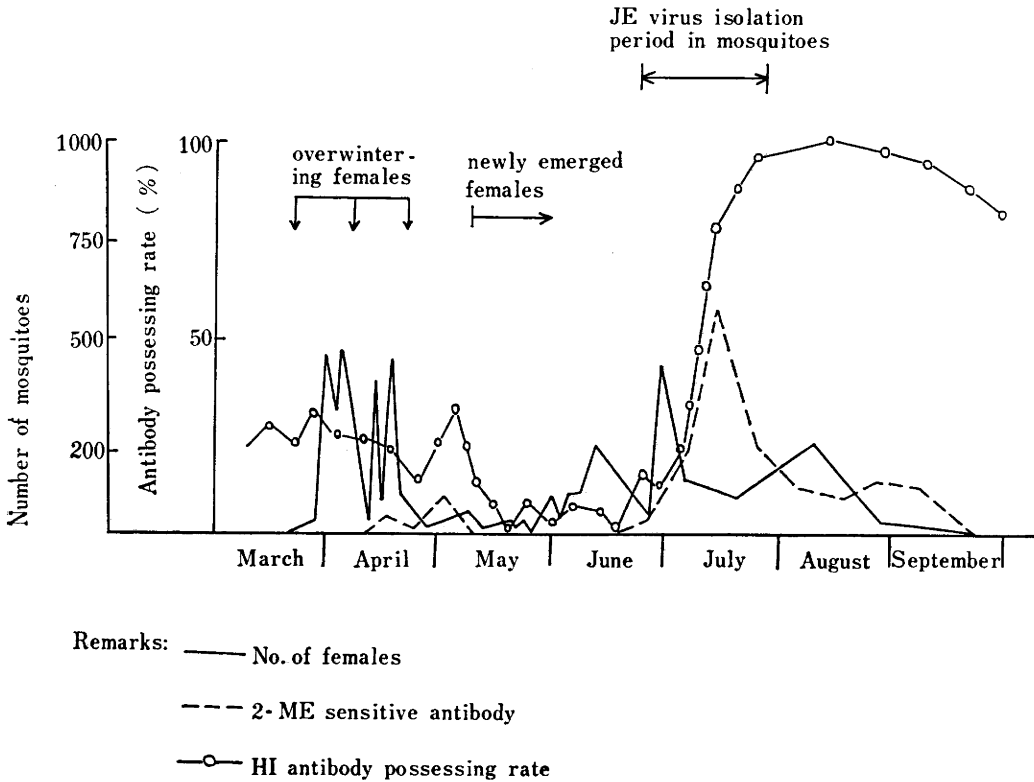
Fig. 5 shows the relation between the increase in HI-A positive rate in slaughtered pigs and the seasonal prevalence of *C. tritaeniorhynchus* in 1967. The 2-ME-SA positive rate rose very much sharply from late June reaching a peak at mid-July, and concurrently,



**Fig. 4.** JE virus isolation from mosquitoes of *C. tritaeniorhynchus* in relation to antibody rising in swine sera and to outbreak of encephalitis cases in Nagasaki area, 1967.



**Fig. 5.** Relation between antibody rising in swine sera and population density of *C. tritaeniorhynchus* mosquitoes in Nagasaki area, 1967.



**Table 6.** JE virus isolation and HI antibody detection from the sera of snakes in Nagasaki area, 1967.

Species	Virus isolation		HI test against JE virus	
	No. of sera tested	No. of sera positive	No. of sera tested	No. of sera positive
<i>Rhabdophis tigrinus tigrinus</i>	213	0	186	3(10x, 10x, 40x)
<i>Elaphe climacophora</i>	28	0	24	0
<i>Elaphe quadrivirgata</i>	49	0	47	2(10x, 20x)
<i>Natrix vibakari</i>	6	0	4	0
<i>Elaphe conspicillata</i>	4	0	4	0
<i>Agkistrodon halys</i>	5	0	5	1(10x)
Total	305	0	270	6(2.2%)

the HI-A positive rate also rose similarly reaching nearly 100% level shortly after the 2-ME positive rate having reached the peak. After mid-July, the HI-A positive rate remained at the same high level, while the 2-ME-SA positive rate decreased toward the beginning of August. The above seems to show that the dissemination in pigs with JE virus occurred very intensely within only a few weeks after late June, and that in and after late July there remained no susceptible pigs.

It is interesting that some pigs were found positive for 2-ME sensitive antibody from mid-April to early May. This means that the pigs were infected in spring when the new generation of *C. tritaeniorhynchus* had not yet appeared. It is important to determine whether the pigs were infected by overwintered females of *C. tritaeniorhynchus*, or by other mosquitoes, or by other blood sucking insects, or by any other means. A program to in-

vestigate the subject seems necessary to be set forward in near future.

### III. Natural infection of common snakes

Table 6 shows the results of JE virus isolation and HI antibody detection made respectively with 305 and 270 sera of common snakes belonging to six species caught in Nagasaki area during the period from April 14 to September 28, in which the epidemic season of JE was being included. As indicated in Table 6, the virus was not isolated from any of the 305 snakes, and the HI antibody was only found at low titers in the sera from 3 specimens of *Rhabdophis tigrinus tigrinus*, 2 of *Elaphe quadrivirgata* and one of *Agkistrodon halys*. Thus it seems that snakes play minor role as a reservoir or as an amplifier of the virus even in the epidemic season. The results on the experimental infection of common snakes with JE virus will be published elsewhere.

### Discussion

A smaller mosquito population and consequently a smaller number of infected *C. tritaeniorhynchus* females at the start of epizootic in

pigs in late June, in 1966, seems to have caused slower rises of the 2-ME-SA positive rate for reaching a peak and of the HI-A

positive rate to reach nearly 100% level as later as about a half-month than in 1967, in spite of the greater breeding number of the mosquito in July. The slower rise in the rates in July, conversely speaking, the slower decrease in the remaining rate of susceptible pigs must have induced the longer duration of the occurrence of infected mosquitoes and consequently of human cases; and the greater breeding number of the mosquito in July seems to have taken effect on promoting the intensity of epizootic in mosquitoes and epidemic in men.

On the contrary, in 1967, the greater mosquito population and greater number of infected mosquitoes at the starting time of epizootic in pigs seems to have caused faster rises of the 2-ME-SA positive rate to reach a peak and of the HI-A positive rate for reaching about 100% level, as faster as about half a

month than in the previous year. The faster rise in the rates in early July, conversely speaking, the quicker decrease in the remaining rate of susceptible pigs must have reduced the duration of the occurrence of infected mosquitoes and consequently of human cases; and rather smaller breeding number of the mosquito in July seems to have reduced the intensity of epizootic in mosquitoes and of epidemic in men.

From these results, it seems that the duration and the size of JE epidemic is closely related to the population density of the vector mosquito at the start and then in the course of the epizootic in pigs. If the mosquito density is lower at the start and higher in the course of pig epizootic, then the duration and the size of human epidemic will be longer and larger.

### Summary

In March through May, 34,972 females of *C. tritaeniorhynchus* and 15,004 ones of other four species in 1966, and 21,434 of the former species and 12,817 of other nine species in 1967 were examined for JE virus by various methods. With all the experiments, no virus has been detected in these mosquitoes.

Nevertheless, in 1967 the rise of 2-ME-SA positive rate was observed in mid-spring, i. e., in mid-April to early May far prior to the epizootic in pigs. The fact is very important and it seems necessary to set forward the program of investigations.

Possibility of Japanese common snakes which were collected in Nagasaki area from April to September was examined for the natural reservoir of JE virus. As a result, the HI antibody of low titer was detected

only in 6 out of 270 snakes but no virus was isolated from 305 snakes, suggesting that they play minor role as a natural source of the virus transmission even in the epidemic season.

Despite of the fact that the starting time of epizootic in pigs and of the occurrence of positive mosquitoes for JE virus was nearly the same being in late June in both 1966 and 1967, the duration and size of the infection in mosquitoes and, in turn, of the epidemic in men were very shorter and smaller in 1967 than in the previous year. The reason seems to have been that the higher mosquito population density and larger number of infected mosquitoes at the starting time in 1967 caused more rapid rise of 2-ME-SA positive rate for reaching a peak in mid-July

and concurrently the HI-A positive rate to reach about 100% level shortly as faster as about a half-month than in 1966. The very rapid rise in the rates, conversely speaking, very rapid decrease in the number of susceptible pigs, in company with smaller breeding number of mosquitoes in July, reduced not only the duration but also the size of the infection in mosquitoes and consequently in men.

Among common mosquitoes in Nagasaki

area, natural infections with JE virus have been found in epizootic season in five mosquito species: In *C. tritaeniorhynchus*, *C. vishnui*, *C. pipiens pallens*, and *Ae. vexans nipponii* in 1965, in the first two species and *Armigeres subalbatus* in 1966, and in the first one and *Ae. vexans nipponii* in 1967.

It is noteworthy that from *Ae. vexans nipponii*, a strain of JE virus as stated above and three strains of non-JE arbovirus were isolated in 1967.

#### Acknowledgement

The authors wish to express their sincere appreciation to Hikari Branch of Takeda

Chemical Industry Ltd. for kind supply of pregnant mice.

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日本脳炎ウイルスの生態学的研究  
1966年及び1967年の野外調査成績

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摘 要

前年に引き続き、1966年及び1967年に日本脳炎ウイルスの生態学的調査を行つた。両年の春、野外で採集した、多数の越冬雌成虫を含むコガタアカイエカ及び他の数種の蚊からのウイルス分離を試みた。更に1967年の春には、野外採集蚊を感受性豚から吸血させ、数日後に吸血蚊からはウイルス分離を、吸血された豚からはウイルス分離とHI抗体の検出を試みた、しかし、上記の何れの蚊からもウイルスは発見されず、何れの豚からも抗体は認められなかつた。それにも拘らず1967年の春には少数のと場豚から2-ME感受性抗体が検出された。この事実は注目し、今後この方面からの研究が必要である。野外で採集した6種の蛇について調査した結果、蛇は日本脳炎ウイルスの伝搬源としては、日本脳炎の流行期においても、重要な役割を果していないものと結論される。豚における日本脳炎ウイルスの汚染及び感染蚊出現の開始時期は1966年と1967年とで殆んど同じであつたが、蚊における感染及び人における流行の期間と大きさは1966年には1967年におけるよりも長く、かつ大きかつた。従つて、これは1967年におけるように、流行の開始時期に感染蚊が多く、豚における汚染の上昇が前年に比較して半月ほど早く、かつ急激であつた。このことが感受性豚を少なくし、従つて蚊における感染及び人における感染を短かく、そして小さくしたものと思われる。