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Transmission of Japanese Encephalitis Virus to Susceptible Pigs by Mosquitoes of *Culex tritaeniorhynchus* After Experimental Hibernation

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Abstract : Adult female mosquitoes of *Culex tritaeniorhynchus* were infected with Japanese encephalitis virus in the end of October, 1964. They were exposed to the winter conditions at three different places. Although their mortality was very high from the middle of November 1964 till the middle of February 1965, a small number of them could survive over the winter till at least March 24th in 1965. Japanese encephalitis virus persisted in the overwintering infected mosquitoes of *Culex tritaeniorhynchus* so long as they were alive. The transmission tests of Japanese encephalitis virus to susceptible pigs were carried out by confirming the presence of the virus in the mosquitoes of *Culex tritaeniorhynchus* which had overwintered after infection. The experimental results show that the level of infectivity in the blood of the pigs infected with such mosquiotes was enough to infect other mosquitoes. The present study suggests the possibility of overwintering of Japanese encephalitis virus in the mosquitoes of *Culex tritaeniorhynchus* in nature, and confirms the significance of the pig as an amplifier for Japanese encephalitis virus.

Introduction

Many investigators have reported the isolation of Japanese encephalitis (JE) virus from the mosquitoes of *Culex tritaeniorhynchus* and *Culex pipiens var. pallens* collected in nature during the epidemic period of Japanese encephalitis. Furthermore, the experimental transmission of JE virus by these two species of mosquitoes have been confirmed by MITAMURA et al. (1938) and HAMMON et al. (1949).

However, little is known of the ecology of JE virus during the interepidemic period. Some hypotheses for explaining this problem have been : (1) survival of the virus in the mosquito or other arthropod vector(s) (2) latent infection in vertebrate hosts, probably avian, (3) reintroduction of the virus from other areas by infected migratory birds every summer. HURLBUT (1950) reported that JE virus could survive in

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mosquitoes of *Culex quinquefasciatus* for 82 days at 8° to 13°C and suggested that the mosquitoes might possibly carry JE virus through the winter.

The present study at first was performed to make sure the possibility of overwintering of JE virus in the *Culex tritaeniorhynchus* mosquito and the possibility of transmission of JE virus to susceptible pigs which were considered to be the amplifier of JE virus during the epidemic period. Secondly, in order to inquire into whether this fact could be really found in nature, isolation of JE virus was attempted from the overwintered mosquitoes of *Culex tritaeniorhynchus* collected in nature from April 9th through April 30th in collaboration with the Department of Medical Zoology in our institute. From the results of these experiments, JE virus persistence in nature during the winter period was discussed.

Materials and Methods

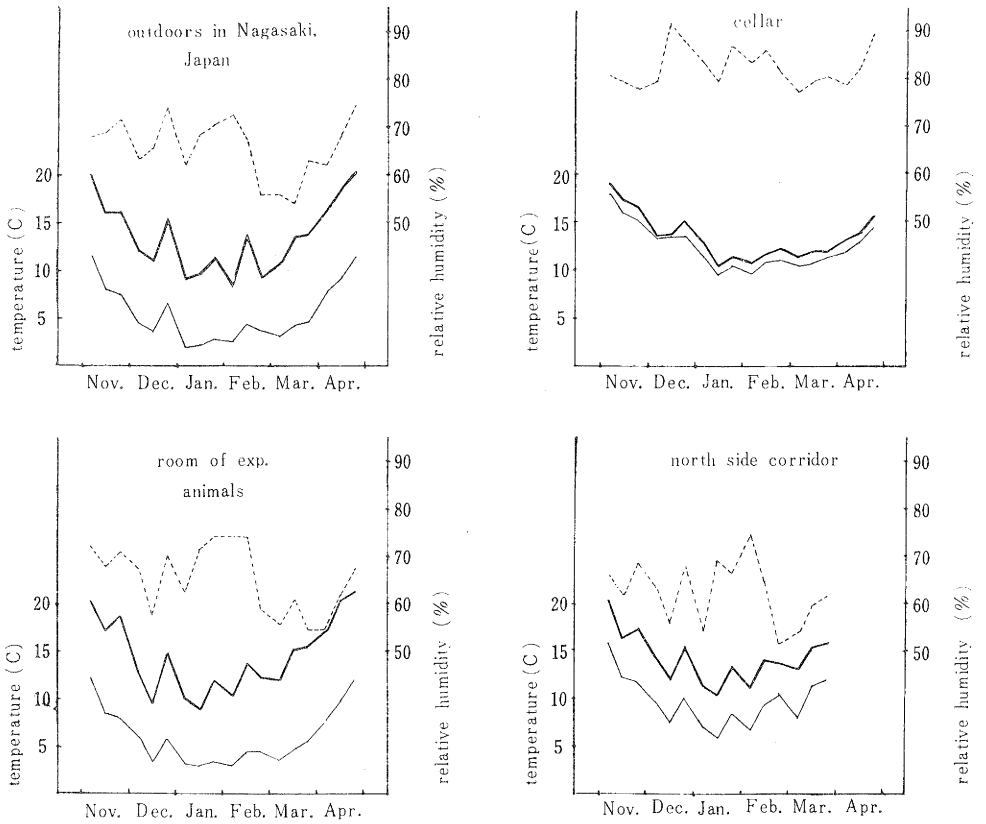
Mosquitoes : Adult female *Culex tritaeniorhynchus* mosquitoes (hereinafter referred as to only mosquitoes) tested were reared from egg rafts of the mosquitoes which had been collected on 6th October 1964 in Omura and Matsushima Districts, Nagasaki Prefecture, Japan. They were kept at 27°C to 28°C at a relative humidity (R.H.) of 70 to 80 per cent.

Virus and Diluents : JaGAr 01 strain of JE virus, originally isolated from mosquitoes was used in this study in the 7th and 8th mouse brain passages. It was stored in dry ice acetone as a 20 per cent suspension of infected mouse brain in a diluent consisting of two parts of normal rabbit serum, free from neutralizing antibody against JE virus and of eight parts of 0.5 per cent lact-albumin hydrolysate Hanks' solution. The meal for infecting the mosquitoes was prepared by mixing 20 per cent JE virus suspension with defibrinated rabbit blood in a proportion of 1 : 4. The titration of virus infectivity in the meal was made by intracranial inoculation into 2 to 3 week old weanling mice at the beginning of feeding of mosquitoes in each experiment.

Infection of mosquitoes : For the first 2 to 4 days after emergence, the mosquitoes were kept in rayon cages of 28 cm^3 in size, at 27°C and 70 to 80 per cent R.H. and were fed on 0.5 per cent sugar solution soaked in a cotton ball hanging from the top of cages. Before being given the infecting meal, the sugar solution was removed and the mosquitoes were starved at least for 2 days to stimulate the engorgement. Then, the mosquitoes were permitted to engorge the infecting meal soaked in a few cotton balls sprinkled with a little glucose powder. At intervals of 30 to 60 minutes, the infecting meal had to be entirely or partially replaced with the fresh one which had been kept in a frozen state as it took 1 to 4 hours until a large proportion of the mosquitoes had engorged.

Winter temperature conditions : Before the infected mosquitoes were exposed to winter conditions, a part of them was left as it was in a room at 27°C and 70 to 80 per cent R.H., while other parts of them were transferred and kept in a room at 24°C and 70 to 80 per cent R.H. for various periods. As winter temperature conditions, the north side corridor and the cellar of our laboratory and also the experimental animal room of our institute were selected. The temperature and relative humidity among these three experimental conditions and those of the outdoors in our region in the winter from October 1964 through April 1965 are illustrated in Fig. 1 for comparison.

Fig. 1. Average temperatures in winter (1964–1965)



Remarks : —— maximum temperature, —— minimum temperature.
····· relative humidity.

Virus recovery from mosquitoes : About the mosquitoes which had just ingested the infecting meal, or kept in a room at 27°C or 24°C and 70 to 80 per cent R.H. for various periods after infection, five mosquitoes were sacrificed and frozen in dry ice acetone. And after exposure to winter conditions, the mosquitoes which were apparently about to die, were picked out and frozen. These frozen specimens of mosquitoes were transferred to a chilled mortar and ground in a 20 per cent chicken serum phosphate buffer solution (pH 7.4) containing 500 units of penicillin and 500γ of streptomycin per ml. The volume of diluent used was based on the number of mosquitoes in a test pool at the rate of 1 ml, 1 to 10 mosquitoes; 1.5 ml, 11 to 20 mosquitoes; and 2.0 ml, 21 to 30 mosquitoes. The suspension was centrifuged for 15 minutes at 15,000 r.p.m. at 0° to 4°C in a refrigerated centrifuge. The supernatant fluid was removed, and inoculated into suckling mouse brain for the recovery of virus. Isolated viruses were identified by haemagglutination inhibition tests with specific antisera.

For the determination of virus titer in an individual mosquito, five sacrificed mosquitoes were triturated in 1.0 ml of diluent and supernatant fluid was inoculated with 0.02 ml or 1/50 of the mosquito suspension into 2 to 3 week old weanling mice by intra-

cranial injection. To estimate the value of weanling-mice-intracranial LD_{50} per single mosquito, the experimental titer per 0.02 ml must be multiplied by 10. The calculation of virus infectivity was based on the method of REED AND MUENCH.

Transmission of JE virus by mosquitoes to susceptible pigs : Before biting susceptible pigs, the mosquitoes were removed from the places where they had overwintered, and kept in a room at 18° to 20°C for one day and deprived of 2 per cent sugar solution which was their usual food. The pigs, Y 39 -9 and Y 39—12, Yorkshire, which had been born on September 27th, the last year, were about 6 month old and free from neutralizing antibody against JE virus. These pigs were bitten at their lateral abdomen which had been shaved, by the infected mosquitoes as the rayon cage (5 cm^3 in size) was held against the abdomen. After the pigs were exposed to the mosquitoes, their blood was obtained daily from the ear veins with syringes wetted with 0.02 per cent heparin solution and was tested for the detection of viremia by intracranial inoculation into 2 to 4 day old suckling mice. Furthermore, for the determination of titer of viremia, 2 to 3 week old weanling mice were used.

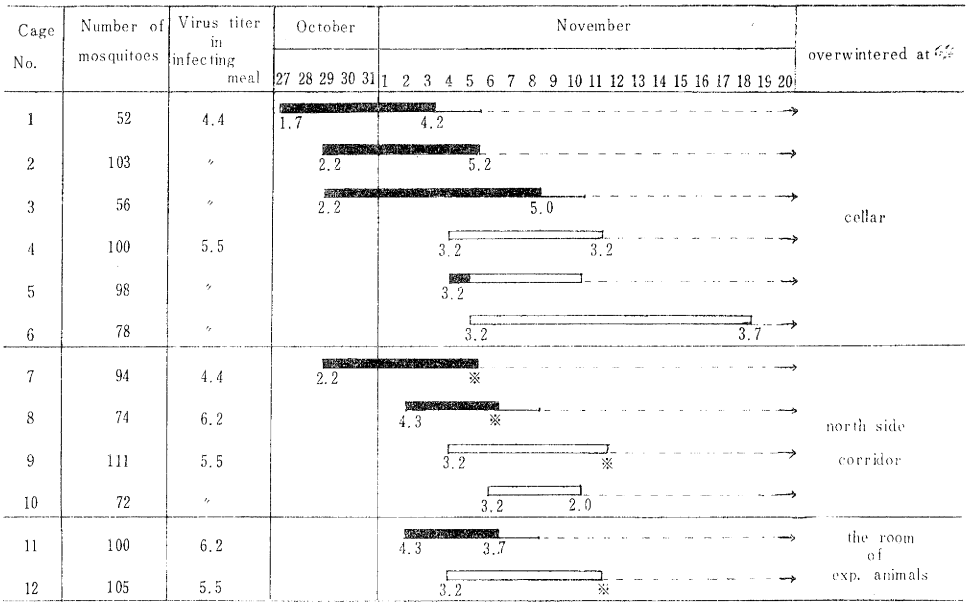
Sera from the pigs were tested before and after the exposure to the mosquitoes for the antibody against JE virus by the haem-agglutination inhibition (HI) test of acetone extracted plasma and by the serum-dilution-neutralization test employing the tissue culture method. The cells used for this purpose were a stable line of porcine kidney cells (PS cells) and had continuously been cultivated in the growth medium consisting of 10 parts of bovine serum and 90 parts of 0.5 per cent lactalbumin hydrolysate Earle solution and as a maintenance medium, 4 per cent rabbit serum free from neutralizing antibody against JE virus in 0.5 per cent lactalbumin hydrolysate Earle solution was used. The virus used in this method was JaGAr OI strain in its 24th PS cell passages. The test serum was heated at 56° C for 30 minutes and diluted by 2-fold serial dilution with the maintenance medium. Following admixing the test serum with an equal volume of 100 TCD_{50}/ 0.1 ml of virus in each dilution, the mixture was incubated at 37°C for 60 minutes and inoculated into cell culture with 0.2 ml per tube. Cytopathogenic effect of virus in PS cell was effectively prevented by neutralizing antibody. The results of neutralization test were given as the reciprocal of the maximum serum dilution by which the cytopathogenic effect of virus was completely inhibited.

Results

JE virus multiplication in mosquitoes : The 1043 mosquitoes infected as described in the experimental methods were divided into 12 cages and 6 of them were kept in a room at 27°C and 70—80 per cent R.H. and the other 6 were transferred to a room at 24°C and 70—80 per cent R.H. for various periods, respectively. As it was the purpose of this study to keep alive as many infected mosquitoes as possible and to make them overwinter as long as possible, the concentration of virus in the mosquitoes was not frequently examined in all cages.

However, the outline of the difference of virus multiplication in mosquitoes under these two conditions could be presumed. The virus concentration of infecting meal at the time of feeding and the results of virus multiplication in the mosquitoes at various periods after infection are shown in Fig. 2. In the infected mosquitoes in Cages Nos. 2 and 3 which were allowed to feed on

Fig. 2. Incubation of infected mosquitoes before exposure to winter conditions and virus multiplication in mosquitoes.



Remarks : 1) Virus titers in the signs of ※ are substituted by the titer of Cage 4 for Cage 9 and Cage 12, by the Cage 2 for Cage 7 and by the Cage 11 for Cage 8.

2) The numbers under the black and white bars show the virus titers per mosquito expressed as the negative logarithm of the LD_{50} in weanling mice.

3) The signs of ■, □ and —— indicate the incubation of mosquitoes at 27°C, 24°C and 21°C, respectively.

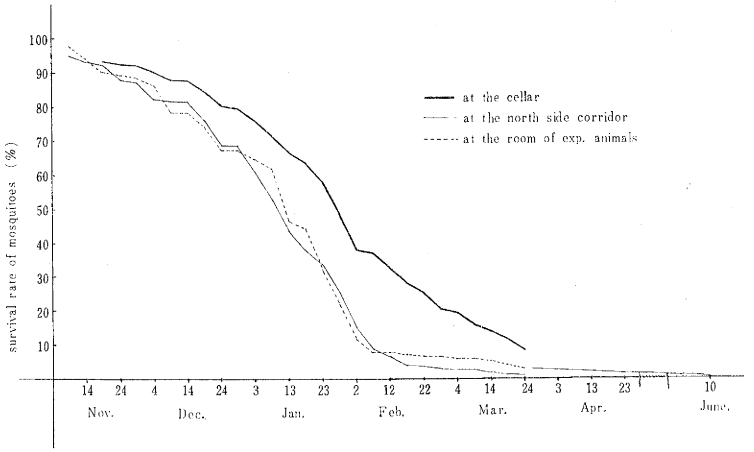
the infecting meal contaminated with virus of $10^{4.4}$ mouse IC LD_{50} and were incubated in a room at 27°C for seven days and ten days after infection, respectively, the virus concentration increared markedly from $10^{2.2}$ to $10^{5.2}$ and $10^{5.0}$. In the cases of mosquitoes in Cages Nos. 4 and 6 which were incubated in a room at 24°C for seven days and thirteen days, the virus concentration became from $10^{3.2}$ to $10^{3.2}$ and $10^{3.7}$, respectively. While, the virus titer in mosquitoes in Cages Nos. 10 and 11, which were incubated for 4 days at 24°C and 27°C, decreased from $10^{3.2}$ and $10^{4.3}$ to $10^{2.0}$ and $10^{3.7}$, respectively.

From these results, it was suggested that JE virus infectivity titer in mosquitoes decreased temporarily after ingesting the infecting meal, and subsequently began to increase rapidly. Furthermore, the multiplication of JE virus in mosquitoes was more rapid and larger at 27°C than at 24°C.

The overwintering of infected mosquitoes and JE virus survival in them : The mosquitoes, which were infected with the virus during the period of October 27th to November 11th, 1964 and which were confirmed of virus multiplication after having been incubated at 27°C and 24°C for various periods as shown in Fig. 2, were transferred to the winter conditions and observed daily.

As given in Fig. 1, with respect to the changes of maximum and minimum temperatures and the differences between the both temperatures and relative humidity, the

Fig. 3. Survival rate of overwintering infected mosquitoes.



conditions in the room of experimental animals was the most similar to the outdoor conditions. While the north side corridor and the cellar were less similar to outdoor in that order. In the cellar, it was noted that the differences of maximum and minimum temperatures were within 0.5°C to 1.4°C and the relative humidity was maintained constantly more than 75 per cent during the observation periods.

Fig. 3 shows the survival rate of overwintering infected mosquitoes in three different winter conditions. The curves do not show the accurate survival rate because, in this experiment, the mosquitoes which seemed about to be dead were picked out of the cages and frozen in dry ice acetone for the examination of virus survival in the mosquitoes. However, the survival rate was calculated by regarding the dying mosquitoes as dead ones.

As shown in Fig. 3, the mortality of the mosquitoes was very high in any places from the middle of November 1964 till the middle of February 1965, the survival rate in the cellar was the highest among these three places. The survival rate in the north side corridor and that in the room of experimental animals were similar to each other but they were significantly lower than that in the cellar. The survival rate on March 24th, 1965 was 8.0 per cent in the cellar, 2.5 per cent in the room of experimental animals and 0.2 per cent in the north side corridor. Though the differences of the survival rate between the overwintering mosquitoes incubated at 27°C and at 24°C were not shown in this figure, the survival rate in the north side corridor and in the room of experimental animals were higher at 24°C than at 27°C throughout the observation periods. On the other hand, it was lower at 24°C than 27°C, in the cellar, however, after March 9th, 1965 it showed a similar tendency to those of other two places. It might be presumed that such variation was caused by the difficult conditions of the mosquito, i. e. starvation periods before engorgement and incubation periods after infection. As a matter of fact, however, no reliable information was available as to how the results should be understood. At a certain rate, a part of infected mosquitoes could survive till March 24th, 140th to 149th day after infection, in any place in winter conditions. After March 25th, as the mosquitoes which had survived through the winter were tested for the virus transmission to susceptible pigs, the survival

rate in each place was not taken into consideration for calculation and after the transmission test, they were collected together and kept in the cellar again. The last survivor died on June 10th, 1965, 216th to 225th day after infection.

The results of the test for the virus survival in the mosquitoes are shown in Table 1. JE virus survived in the mosquitoes which were tested from January 7th through May 15th and the isolated viruses were highly pathogenic for mice. The virus isolation was not carried out per each surviving mosquito in present study, consequently, a question

Table 1. Virus survival in overwintering mosquitoes.

Date	overwintering at	incubation before exposure to winter conditions	incubation periods (days) in winter conditions	identification of virus	
				mosquitoes pooled	
				number in a pool	virus isolation
Jan. 25th	cellar	27°C	89—91	13	+
〃	〃	24°C	82—83	13	+
〃	north side corridor	27°C	85—89	14	+
〃	〃	24°C	81—83	15	+
〃	room of exp. animal	27°C & 24°C	83—85	23	+
Feb. 5th	cellar	27°C	100—102	9	+
〃	north side corridor	24°C	92—94	7	+
〃	room of exp. animal	27°C & 24°C	94—96	14	+
Feb. 20th	cellar	27°C	115—117	5	+
〃	〃	24°C	108—109	5	+
〃	north side corridor	27°C	112—116	15	+
〃	〃	24°C	107—109	12	+
Mar. 5th	cellar	27°C	128—130	5	+
〃	〃	24°C	121—122	6	+
〃	north side corridor	27°C	125—129	2	+
〃	〃	24°C	120—122	2	+
〃	room of exp. animal	27°C & 24°C	122—124	2	+
Mar. 20th	cellar	27°C	143—145	5	[3.5]
〃	〃	24°C	136—137	5	[3.2]
〃	north side corridor	27°C	140—144	2	+
〃	〃	24°C	135—137	5	[3.2]
〃	room of exp. animal	27°C & 24°C	137—139	5	[3.3]
Mar. 26th	cellar	27°C	149—151	4	+
May 15th	altogether		191—201	2	+

Remark : The number in the brackets in the lines of March 20th means the negative logarithm of virus titer in mosquito.

Table 2. Transmission of JE virus to susceptible pigs by overwintered *Culex tritaeniorhynchus*.

Experiment number	Date of bite	Extrinsic incubation periods (days)	pigs tested	mosquitoes engorged / No. of exposed	virus in mosquito (−log)	Mosquito transmission to susceptible pig		pre-bleed	1	2	3	4	5	6	7	8	9	10	12	14	17	19	31	48	51
						viremia & antibody response		days after infection																		
1	March 25.26th	141−150	Y39−9 50.8kg male	16/26	4.2	viremia	suckling mouse mortality						8/8	9/8	9/8	9/8	9/8	9/8								
							titers in weanling mice	not tested																		
						antibody response	HI	<10							1280			2560		640		320		160	
							Neut.	<10							2560			1280		1280		1280		640	
2	April 12th	159−168	Y39−12 51.5kg male	1/12	4.4	viremia	suckling mouse mortality			9/6	7/7	8/8	7/7	2/7												
							titers in weanling mice			0	1.75	2.25	1.75	0.25												
						antibody response	HI	<10				<10				320			2560		1280		320		160
							Neut.	<10				<10				80			640		1280		1280		640

Remarks : 1 Virus titer per mosquito is expressed as the negative logarithm of the LD_{50} of virus supension upon inoculation intracranially into 2 to 3 week-old weanling mice.

2 Viremia is expressed as the negative logarithm of the LD_{50}/0.02 ml of blood upon inoculation intracranially into weanling mice.

arises whether or not the active virus could remain in all mosquitoes throughout the winter. However, considering the virus isolations from two mosquitoes kept in the north side corridor tested on March 5th and March 20th and from two other mosquitoes tested on May 15th and, moreover, considering the virus transmission experiment by using a single mosquito which will be stated later, it was supposed that in such experimental condition, JE virus could survive as long as the mosquitoes were alive.

The numbers in the brackets in the lines of March 20th in Table 1 showed no significant differences of virus infectivity titer in a single mosquito could be found after overwintering in any cases. Besides, there was no apparent reduction of the virus in the overwintering mosquitoes compared with the virus infectivity before exposure to the winter condition.

Transmission of JE virus to susceptible pigs : Of the 1,043 infected mosquitoes, 42 mosquitoes survived the winter from October 1964 till March 24th, 1965. These mosquitoes were incubated for one day in a room at 18°C to 20°C before exposure to susceptible pigs.

Experiment 1 : As shown in Table 2, 26 mosquitoes were exposed to the pig, No. Y 39--9, on March 25th and 26th, 141th to 150th day survival after infection and 16 of them ingested the blood of the pig. The virus infectivity in an individual mosquito at that time was $10^{4.2}$ in average titer. For the demonstration of the transmission of virus, the virus isolation from the pig blood and the test for the development of haemagglutination- inhibition (HI) and neutralizing antibody in the pig were employed. Following the bites of these mosquitoes, the pig was infected with JE virus, as evidenced by the viremia on the 5th day and by the development of HI and neutralizing antibodies in the pig serum on the 7th day after infection.

Experiment 2 : This was carried out on April 12th. There were 12 mosquitoes, 159th to 168th day survival after infection and possessed the virus infectivity of $10^{4.4}$ individually. One of them ingested the pig, No. Y 39—12. During the next two days, virus was not demonstrable when the swine blood was inoculated into 2 to 4 day old suckling mice intracranially. On the 3rd day, the pig became viremic and it continued for 4 days ranging in titer from $10^{2.25}$ to $10^{0.25}$ in weanling mice. The problem whether or not this viremia is enough to infect the other overwintered or new born mosquitoes when they feed on this pig, will be discussed later.

The antibodies against JE virus in the pig seemed to begin to develop at least on the 7th to 8th day following the bites of infected mosquitoes. HI antibody developed and declined rather rapidly, while neutralizing antibody was maintained in fairy high titers during the observation periods.

Isolation of JE virus from hibernated *Culex tritaeniorhynchus* mosquitoes collected in Nagasaki : The total of 20,277 mosquitoes collected from April 9th through April 30th were confirmed to have overwintered with detail and precise observations in the Department of Medical Zoology in our institute (OMORI et al. 1965). 16,174 mosquitoes in 122 pools of them were tested for virus isolation. Pools of mosquitoes, usually not exceeding 200 specimens in number, were transferred to a chilled mortar and ground in a 20 per cent chicken serum in PBS solution (pH 7.4) containing 500 units of penicillin and 500 γ of streptomycin per ml. These suspensions were centrifuged at 15,000 r. p. m. for 15 minutes in a refrigerated centrifuge. The supernatant fluid was removed and inoculated into 2 to 4 day old suckling mice with 0.02 ml intracranially. The mice were observed daily for 14 days for signs of illness or death caused by the

virus.

In 3 pools, as the mice revealed signs of illness, secondary passage was carried out with brain suspensions into suckling mice. However, no virus could be isolated from these specimens.

Discussion

The reason why the present investigation was undertaken was to make clear whether the mosquito would play a role of reservoir of JE virus during the interepidemic period. If the mosquito carries JE virus through the winter, it must take an infecting meal in the fall, survives over the winter, refeeds, and transmits the infection in the following spring.

Although regarding the cycle of infection of mosquito-borne virus during the interepidemic period, any actual proof has not been given yet, there are several reports which show the possibility of carrying the virus over the winter in the mosquito. HINDLE (1930) reported that yellow fever virus had persisted in *Aedes aegypti* for at least one month at 10°C to 18°C, but reincubation of the mosquitoes at 28°C and 36°C was necessary before they could transmit the infection. It was demonstrated by BELLAMY et al. (1958) that WEE virus survived up to 113 days in experimentally infected *Culex tarsalis* incubated in an unheated cellar in winter and it was transmitted to chicken after 97 and 109 days of incubation in such a condition.

About JE virus, HURLBUT (1950) recognized that the virus persisted in *Culex quinquefasciatus* for 82 days in a refrigerator in complete darkness where the temperature ranged from 8°C to 13°C, and no prolonged posthibernation incubation of mosquitoes was necessary to infect the mice by their biting and he indicated the possibility of the overwintering of JE virus in female mosquitoes which overwintered as adult. LA MOTTE (1963) reported the virus multiplication in *Culex pipiens var. pallens* infected with JE virus by feeding upon viermic chicken, and indicated that there was no apparent reduction in JE virus infectivity titer in the mosquitoes which were exposed to a low temperature following the incubation at 26.5°C for 25 days, but the virus multiplication was inhibited when the mosquitoes were held at 10°C soon after they had taken an infecting meal, but the virus infection persisted in these mosquitoes and the virus multiplied rapidly when they were placed again at 26.5°C. It is suggested that low temperature in nature could exert an effect upon JE virus transmission by inhibiting virus multiplication in the mosquito vector.

In present study, female mosquitoes of *Culex tritaeniorhynchus* playing a main role in the epidemic of Japanese encephalitis were experimentally infected with JE virus in the fall, and they were exposed to low temperature through the winter after recognizing the virus multiplication in them to some extent, and examined for the virus survival so long as they lived. From the results shown in Table 1, these overwintered mosquitoes carried the virus sufficiently pathogenic to mice throughout the experiment and, furthermore, there was no apparent reduction of virus as compared with the virus infectivity after several days following the infection (Fig. 2). Besides, the overwintered mosquitoes could transmit the virus to susceptible pigs only one day after removal of the mosquitoes from the hibernation temperature. These facts suggest that once JE virus infection is firmly established in mosquitoes, the subjection to cold temperature will not cause sufficient reduction of the virus in the

infected mosquitoes, and moreover, the virus will persist in active state in them.

It is considered to be very important epidemiologically to know the difference of virus multiplication in mosquitoes when they are incubated at several temperatures. The present report deals with the test at no more than 24°C and 27°C with little variation in virus multiplication. The difference between the results in the two temperature degrees may be presumed only in outline, but more detailed experiments will be required for this problem.

The experimental transmission of JE virus by mosquitoes has been reported by many investigators: to mice by *Culex tritaeniorhynchus* (MITAMURA et al. 1938; HAMMON et al. 1949); to chicks by *Malaysian Culex tritaeniorhynchus* (HALE et al. 1957); chicks or mice by *Culex quinquefasciatus* which had been kept at low temperature (HURLBUT, 1950; LA MOTTE, 1963). HAMMON et al. (1949) also stated that transmission of JE virus by *Culex tritaeniorhynchus* to unnatural hosts such as mice did not necessarily prove that *Culex tritaeniorhynchus* was a vector of the virus in nature.

In present study, the overwintered infected *Culex tritaeniorhynchus* mosquitoes were applied to the transmission of the virus to susceptible pigs. The pig became viremic for four days from 3rd day through the 6th day following the bite of a single mosquito, ranging in titer from $10^{2.25}$ to $10^{0.25}$ mouse LD_{50}/0.02ml of blood.

GRESSER and HARDY et al. (1958) made an experiment in detail about the factors influencing transmission of JE virus using swine and birds as natural hosts. He indicated that the infection of mosquito and transmission followed ingestion of blood containing as little as 3 mouse LD_{50} of JE virus/0.04ml blood (0.15 LD_{50}/mosquito), and the concentration of virus in blood of the hosts depended upon the numbers of mosquitoes to be infected and consequently, the overall transmission rate. Considering from these facts, if some mosquitoes become infected in the fall and survive to infect swine next spring by chance, these pigs will be adequate infective sources for other overwintered *Culex tritaeniorhynchus*, some of them take their first blood from the pigs.

According to the isolation pattern of JE virus from *Culex tritaeniorhynchus* collected in Nagasaki District in 1964 (HAYASHI et al. 1965, TAKAHASHI et al. 1965), the infected mosquitoes in nature began to detected at first on May 19th and the isolation rate became maximum in June and no virus could be found after August 8th. Furthermore, in 1965, JE virus was isolated at first on May 29th, too. (HAYASHI and OMORI et al. 1965). These facts are noticeable compared with the findings presented by OYA et al. (1964) that JE virus could be isolated in the mosquito from the middle of July through early in September in Kanto District. In Nagasaki District, JE virus can be detected in mosquitoes extremely early compared with the OYA et al. findings, therefore, if a bold imagination is allowed, it may suggest that the possibility of the overwintering of JE virus in mosquitoes is larger than in Kanto District.

In Japan, actually, JE virus has never been recovered in mosquitoes after the end of September. Though little is known about the ecology of *Culex tritaeniorhynchus*, if the mosquitoes begin to hibernate at about the end of September, some of the infected mosquitoes at that time are deemed to possess the virus sufficiently, survive over the winter, refeed on the susceptible hosts in the next spring and will reestablish the infection.

Nevertheless, no JE virus could be found from the mosquitoes of *Culex tritaeniorhynchus* collected in nature in Nagasaki District

from April 9th through April 30th. The attempts by other investigators to recover JE virus from naturally infected mosquitoes during the winter have been unproductive (MITAMURA AND KITAOKA, 1950; 406th MEDICAL GENERAL LABORATORY, 1955). What to these facts mean? Some questions arise whether the virus may possibly be found if more overwintered populations are collected, whether or not JE virus survival can depend upon solely the mosquitoes in the critical period, or there are any other complicated mechanisms. In the case of WEE virus, BELLAMY et al. (1958) stated that one could not ignore the possibility that virus in mosquitoes may be reactivated by a blood meal in spring. REEVES et al. (1958) stated that the strains isolated from mosquitoes in winter had some characteristics which were mainly expressed by virulence attenuation as they were nonpathogenic for mice. In this study, active JE virus could be recognized in the mosquitoes throughout the experiment in spite of not being fed on a blood meal. However, it must be pointed out that there remain important problems to study on the attenuation of virulence or changes in characteristics of JE virus when the virus are associated for long periods with mosquitoes.

Anyhow, there is a possibility in Japan of surviving of JE virus in mosquitoes through the winter months and of transmitting of virus to susceptible hosts in the next spring. However, to establish the original purpose, further studies on the ecology of *Culex tritaeniorhynchus* will be required that (1) from when do they begin to hibernate? (2), from what host species do they take a first blood meal in spring? (3) what parts of overwintered mosquitoes took prewinter blood meal in the last fall? Simultaneously, it must be required to investigate on the susceptibility to JE virus of those hosts at the time when the mosquitoes begin to feed after hibernation.

Summary

1. At various conditions in the winter, JE virus persisted in experimentally infected *Culex tritaeniorhynchus* for at least 140 to 149 days after the mosquitoes were incubated at 27°C or 24°C for certain periods and the virus multiplication in the mosquitoes was recognized.

2. In the mosquitoes which had survived for 141 to 168 days in winter conditions, JE virus could persist and be transmitted to susceptible pigs by their biting. A pig had the virus in its circulating blood, ranging in titer $10^{2.25}$ to $10^{0.25}$ mouse-LD_{50}/0.02 ml.

3. No JE virus could be isolated from a total of 16,174 overwintered *Culex tritaeniorhynchus* collected in nature from April 9th through April 30th.

4. There is a possibility that infected *Culex tritaeniorhynchus* can carry JE virus through the winter months in Nagasaki, probably in the southern part of Japan, and transmit the virus to susceptible hosts in next spring. However, further studies will be required to confirm this possibility in nature.

Acknowlegment

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Literatures

1) BELLAMY, R. E., REEVES, W. C. et al. : Relationships of mosquito vectors to winter survival of encephalitis viruses. 2. under experimental conditions. Am. J. Hyg., 67 : 90–100, 1958.

2) CHAMBERLAIN, R. W., KISSLIMG, R. E. et al. : Virus of St. Louis encephalitis in three species of wild birds. Am. J. Hyg., 5 : 110–118, 1957.

3) CHAMBERLAIN, R. W., SUDIA, W. D. : Multiplication of eastern equine encephalitis virus in mosquitoes following intrathoracic inoculation. Proc. Soc. Exp. Med. & Biol., 95 (2) : 261–262, 1957.

4) GRESSER, I., HARDY J. L. et al. : Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of Culex tritaeniorhynchus Giles, from infected pigs and chicks to susceptible pigs and birds. Am. J. Trop. Med. & Hyg., 7 (4) : 365–373, 1958.

5) HALE, J. H., COLLESS, D. H. and LIM, K. A. : Investigation of the Malaysian form of Culex tritaeniorhynchus as a potential vector of Japanese B encephalitis virus on Singapore Island. Ann. Trop. Med. & Parasitol., 51 : 17–25, 1957.

6) HAMMON, W. McD., REES, D. M. et al. : Experimental transmission of Japanese B encephalitis virus by Culex tritaeniorhynchus and Culex pipiens, var. pallens, suspected natural vectors. Am. J. Hyg., 50 : 46–50, 1949.

7) HAMMON, W. McD., TIGERTT W. D. et al. : Isolations of Japanese B encephalitis virus from naturally infected Culex tritaeniorhynchus collected in Japan. Am. J. Hyg., 50 : 51–56, 1949.

8) HAMMON, W. McD., REEVES, W. C. : Laboratory transmission of St. Louis encephalitis virus by three genera of mosquitoes. J. Exp, Med., 78 : 241–253, 1943.

9) HAYASHI, K., MIFUNE, K. et al : Isolation of Japanese encephalitis virus from mosquitoes collected in Omura District, Nagasaki Prefecture, Japan in 1964. End. Dis. Bull. Nagasaki Univ., 7 (3) : 155–164, 1965.

10) HAYASHI, K., MIFUNE, K., OMORI, N., WADA, Y. et al. : unpublished data in 1965.

11) HINDLE, E. : The transmission of yellow fever. Lancet, 219 : 835–842, 1930.

12) HURLBUT, H. S. : The transmission of Japanese B encephalitis by mosquitoes after experimental hibernation Am. J. Hyg., 51 : 265–268, 1950.

13) HURLBUT, H. S., CARLYLE NIBLEY, JR. : Virus isolations from mosquitoes in Okinawa. J. Med. Ent., 1 (1) : 78–83, 1964.

14) LA MOTTE, L. C. JR. : Effect of low environmental temperature upon Japanese B encephalitis virus multiplication in the mosquito. Mosquito News, 23 (4) : 330–335, 1963.

15) LA MOTTE, L. C. JR. : Japanese B encephalitis virus in the organs of infected mosquitoes. Am. J. Hyg., 72 : 73–87, 1960.

16) LA MOTTE, L. C. JR. : Japanese encephalitis in bats during simulated hibernation. Am. J. Hyg., 67 : 101–108, 1958.

17) MITAMURA, T., KITAOKA, M. : Seasomal occurence of mosquito in Okayama 1946 and infectivity of the mosquito with Japanese B encephalitis virus. Transovary infection of the virus in the mosquito. Jap. Med. J., 3 : 141–148, 1950.

18) MITAMURA, T., KITAOKA, M. et al. : Isolation of the virus of Japanese epidemic encephalitis from mosquitoes caught in nature. Reports of the Ninth Meeting of the Committee on Encephalitis. Tokyo Iji Shinshi, 62 : 820–824, 1938.

19) OMORI, N., WADA, Y. et al. : Preliminary notes on the collection of hibernated

females of Culex tritaeniorhynchus in Nagasaki. End. Dis. Bull. Nagasaki Univ., 7 (2) : 147–153, 1965.

20) Oya, A., Takahashi, M. et al. : Ann. Report of National Institute of Health, Tokyo, 75–76, 1963.

21) Reed, L. F., Muench, H. : A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 27 : 493, 1938.

22) Reeves, W.C., Bellamy, R. E. et al. : Relationships of mosquito vectors to winter survival of encephalitis viruses. 1. under natural conditions. Am. J. Hyg., 67 : 78–89, 1958.

23) Scherer, W. F., Buesher, E. L. : Ecologic studies of Japanese encephalitis virus in Japan. Am. J. Trop. Med. & Hyg., 8 : 644–722, 1959.

24) Scherer, W. F., Moyer, J. T. et al. : Immunologic studies of Japanese encephalitis virus in Japan. V. Maternalantibodies, antibody responses and viremia following infection of swine. J. immunol., 83 : 620–626, 1959.

25) Takahashi, K., Matsuo, R. et al. : Studies on mosquito infection with Japanese encephalitisi virus in 1964 in Nagasaki Prefecture. End. Dis. Bull. Nagasaki Univ., 7 (3) : 165–177, 1965.

26) U. S. Army 406th Medical General Laboratory. Professional Report, 1954. Tokyo. 289 pp, 1955.

実験的日本脳炎ウィルス感染コガタアカイエカの越年と生残蚊による感受性豚へのウィルス伝播について，長崎大学風土病研究所病理部（主任：福見秀雄教授）三舟求真人

要　　約

日本脳炎ウィルスの流行閑期における生態については現在2，3の仮説があるが，いずれもその確証はなくその実態は全く不明である．著者は1964年10月下旬日本脳炎媒介の主役を演ずるコガタアカイエカ1,043個体を供試し，実験的に日本脳炎ウィルスを吸血感染せしめ日本脳炎ウィルスが蚊体内において越年し，更にこの越年蚊が翌春日本脳炎ウィルスの増幅動物（amplifier）と考えられる豚にそのウィルスを伝播しうるか否かについて実験を行ない次の様な結果を得た．1）日本脳炎ウィルス感染後蚊を27°Cに或いは24°Cに数日間飼育し，蚊体内においてウィルス増殖を確認したのちこれらの蚊をそれぞれ条件の異なる3ケ所に配置した．日本脳炎ウィルスはこれらの蚊体内で少なくとも140日－149日間は生き残ることができた．2）日本脳炎ウィルス感染後141日－168日の越年蚊を使用し，翌1965年春感受性豚にウィルス伝播実験を行なった．その結果豚は蚊の刺螫により$10^{2.25}$－$10^{0.25}$ mouse LD_{50}/0.02mlの感染価を示すウィルス血症を4日間示し，越年蚊によるウィルス伝播を確認した．一方この成績から豚は日本脳炎ウィルスの強力なamplifierであることをも追認した．3）これらの事実は日本脳炎ウィルスがコガタアカイエカと共に越年し，翌春感受性動物への伝播がおこなわれる可能性があることを示唆している．

従って実験的に得られた上記の可能性について自然界における実証を得んとして，1965年早春，本研究所衛生動物部で越年蚊と確認された野外捕集コガタアカイエカ16,174個体について日本脳炎ウィルス分離を試みた．その成績は陰性に終ったが，本実験の可能性はコガタアカイエカの越年の生態，越年蚊体内のウィルスの存在様式等を究明しつつ実証されねばならない．