

Isolation of Japanese Encephalitis Virus from Mosquitoes Collected in Omura District, Nagasaki Prefecture, Japan, in 1964.

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Abstract : Isolation of Japanese Encephalitis virus from mosquitoes collected at Kuromaru village in Omura city Nagasaki prefecture had been made on May 26th thru October 13th 1964. The total 19 arboviruses could be isolated from 13442 in 144 pools of *Culex tritaeniorhynchus* mosquitoes, and no virus isolation from other species. Nineteen isolations had been identified as Japanese Encephalitis virus. Considering the occurrences of encephalitis cases during several years in Nagasaki prefecture, rabbits, free from Japanese Encephalitis virus infection, had been arranged at seven places selected to be exposed to mosquitoes. The information of Japanese Encephalitis virus dissemination in investigated area, especially in north and southwest area in Nagasaki prefecture, could be found from the evidence of antibody response of rabbit sera in haemagglutination-inhibition and from the evidence of Japanese Encephalitis virus isolation from mosquitoes. Japanese Encephalitis virus dissemination, however, have to be investigated by area and by stage during pre- and post-epidemic season for the ecological study of Japanese Encephalitis virus.

Introduction

The available evidence has supported that, during epidemic periods, Japanese Encephalitis (JE) viruses have been transmitted to human being and animals by the mosquitoes of *Culex tritaeniorhynchus* which play significant role of virus dissemination. The ecology of JE virus, however, is not yet clear; where JE virus

can survive during interepidemic periods, where the mosquitoes can obtain their virus and whether or not JE virus is disseminated among human being and animals yearly or only during epidemic which have occurred at varying and unpredictable intervals. There are many investigations of the JE virus

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isolation from *Culex tritaeniorhynchus* mosquitoes in Kanto, Osaka, Fukuoka, Nagasaki and Okinawa in Japan and in foreign islands of Taiwan. From the results of these investigations, the period and efficiency of JE virus isolation quite varied from year to year. However, it does not seem to be caused only by the variation in number of offspring or collection of the mosquitoes as previously described (1965). On the stand point of epidemiological view, attention has been focused on the possible forecast of the spread of JE virus dissemination on the basis of isolation of the viruses from mosquitoes prior to an

epidemic among human being. However, for the exploration of biological infection with JE virus in nature including the problems of overwintering of JE virus, it is important matter to investigate more in details as to how the variation of periods and efficiencies in JE virus isolation caused. The present study has been undertaken to obtain the information of JE virus dissemination during epidemic season 1964, in Nagasaki prefecture. The results obtained will become to be established the foundation for the future research plan.

Material and Method

The area for survey : As shown in Fig. 1 and 2, the area selected for the 1964 collecting program was Kuromaru village which face a bay to the west and is located slightly north of Omura city, almost the middle point in Nagasaki prefecture. Kuromaru village was selected because it was isolated from other villages and located in the middle of wide rice and vegetable field. Moreover, every farmer has run many cows, cattles, pigs and chicken raising as side jobs. Population of domestic animals in this village which is suspected to be exposed to mosquitoes are shown in Table 1. It is noticeable fact that 321 pigs had been born during from January thru October 1964 and 200 of them except adult female one had been transferred to other place for sale.

Collection of mosquitoes : Mosquito collection was performed on May 26th thru October 13th 1964. After sunset until midnight and sometime in the early morning before sunrise, pigs, cows and fowls sheds were visited periodically, resting mosquitoes were picked up with an aspirator with the aid of flashlight and transferred into holding cages. Anesthetized mosquitoes with CO₂ gas obtained from dry ice were classified as soon as

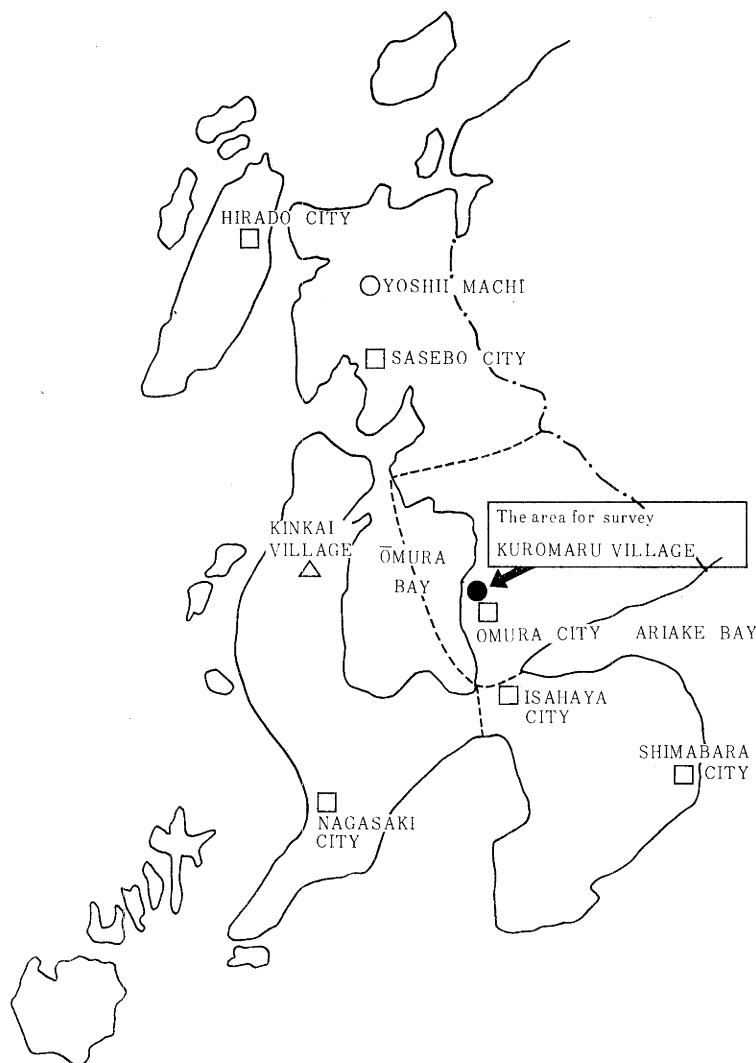
possible with the aid of glass of 20 magnification. The classification of mosquitoes were carefully undertaken; only mosquitoes which could be definitely identified were selected and pooled into glass tubes not exceeding 100 in number. These specimens of mosquitoes

Table 1. Population of Domestic Animal in Kuromaru Village (25th. Oct. 1964)

Domestic animal	Adult	Animal born from 1st January 1964 on ward	Total
Japanese black cattle	25	4	29
Milk cow	53	19(-11)	61
Pig	60	312(-200)	172
Fowl	320	34	354
Rabbit	0	2	2
Dog	17	5	22
Cat	21	13(-6)	28
Goat	0	0	0
Sheep	3	0	3
Dove	0	1	1

Remarks ; The Numbers of parenthesis denote the numbers of transferred animals from survey station to other area.

Fig 1. Outline of Nagasaki Prefecture and the Survey Station

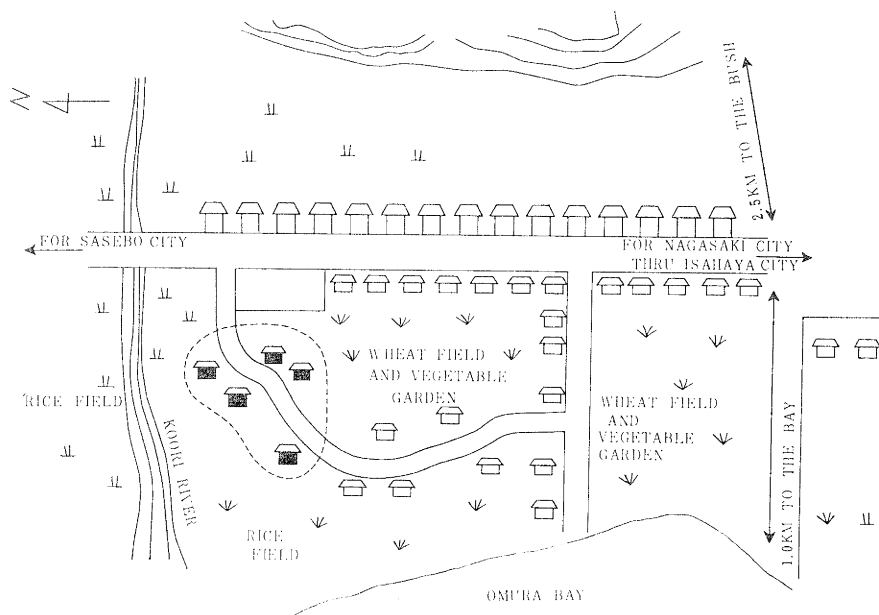


in tubes were kept in dry ice acetone till just before virus isolation.

Virus isolation and identification : Pools of mosquitoes were ground in a mortar and the cooled diluent was added slowly to make suspension of triturated tissue. The volume of diluent was used varying due to the mosquito species; 4.0 ml to a pool of *Culex tritaeniorhynchus* mosquitoes, 6.0 ml to *Culex pipiens pallens*, 8.0 ml to *Anopheles sinensis*. The diluent was made in phosphate buffered saline solution (PBS) at pH 7.4 contained 20% one day chicken sera and 500 units of penicillin, 500

mg of streptomycin per ml. The suspension had been kept in ice water for 30 minutes. The initial suspension and the second supernatant were centrifuged at 3,000 rpm for 10 minutes and at 10,000 rpm for 40 minutes in a refrigerated centrifuge, respectively. The last supernatant of 0.02 ml was inoculated intracerebrally into a litter of 2-4 days suckling mice of gpc family. A part of the last supernatant was also inoculated into thioglycolate medium to find whether or not there were bacterial contamination, and remains of it had been stored again in dry ice

Fig 2. Outline of the Survey Station, Kuromaru Village in Omura City in the middle Area of Nagasaki Prefecture.



action. The mice were observed daily the sign of illness caused by viruses for 10–14 days. If a mouse showed the sign of illness, the brain was removed and passed again intracerebrally to other 10–14 days mice. The transmissible agents were identified by means of verification of hemagglutinating activities, and by hemagglutination inhibition test and neutralization test using immune rabbit sera or mice sera against JE virus of JaGAR OI or Nakayama strain. The antigens of transmissible agents were prepared from mouse brain infected with the agents by the method of acetone-ether or sucrose-acetone extractions.

Rabbits exposed to mosquitoes: Four to six months rabbits which were born in late autumn thru early spring were used. It was

confirmed that rabbit sera had been free from antibodies against JE virus by using hemagglutination inhibition and neutralization test. A cage kept in two rabbits was arranged at outdoor of the selected place to be exposed to mosquitoes. The rabbit sera were obtained every 10 days from May 20th thru September 20th 1964. After the sera were pretreated with acetone, they were used for hemagglutination inhibition test.

Serological confirmation of encephalitis cases: Acute and convalescent or sometime single sera of cases were pretreated with acetone. Serum dilution were used in the test of hemagglutination inhibition. The method will be described in detail in this Bulletin by KAWASOE, one of the authors.

Results

Isolation of viruses: A summary of the numbers of mosquitoes tested, virus isolation efficiency is presented in Table 2. The numbering totaled 13442 in 144 pools of *Culex*

tritaeiorhynchus were tested for the isolation of viruses. As the mosquitoes of *Culex pipiens pallens* and *Anopheles sinensis* were excepted intentionally, in addition, a few of these two

Table 2. Isolation of virus from Collected Mosquitoes

		<i>C. tritaeniorhynchus</i>			<i>C. pipiens pallens</i>			<i>A. sinensis</i>		
Date		Collected Number	Virus Isolation	Isolation Efficiency	Collected Number	Virus Isolation	Isolation Efficiency	Collected Number	Virus Isolation	Isolation Efficiency
May	26	157	0	0	254	0	0	277	0	0
June	8	954	2	2.096	45	0	0	84	0	0
	23	1500	6(1)	4.000	80	0	0	0	0	0
July	3	1578	3(1)	1.901	39	0	0	200	0	0
	9	1600	1	0.625	a few			300	0	0
	17	1700	4(1)	2.353	"			300	0	0
	30	1600	0	0	"			300	0	0
August	7	2000	3	1.500	"			300	0	0
	18	600	0	0	"			300	0	0
	27	242	0	0	"			227	0	0
September	4	700	0	0	"			300	0	0
	15	800	0	0	"			300	0	0
	28	6	0	0	"			25		
October	13	5	0	0	0			a few		
Totaled Number		13,442	19	1.414	418	0	0	2888	0	0

Remaks ; Numbers in parenthesis indicate that the isolated virus had lower or no hemagglutination activity in the beginning of isolation from mosquitoes.

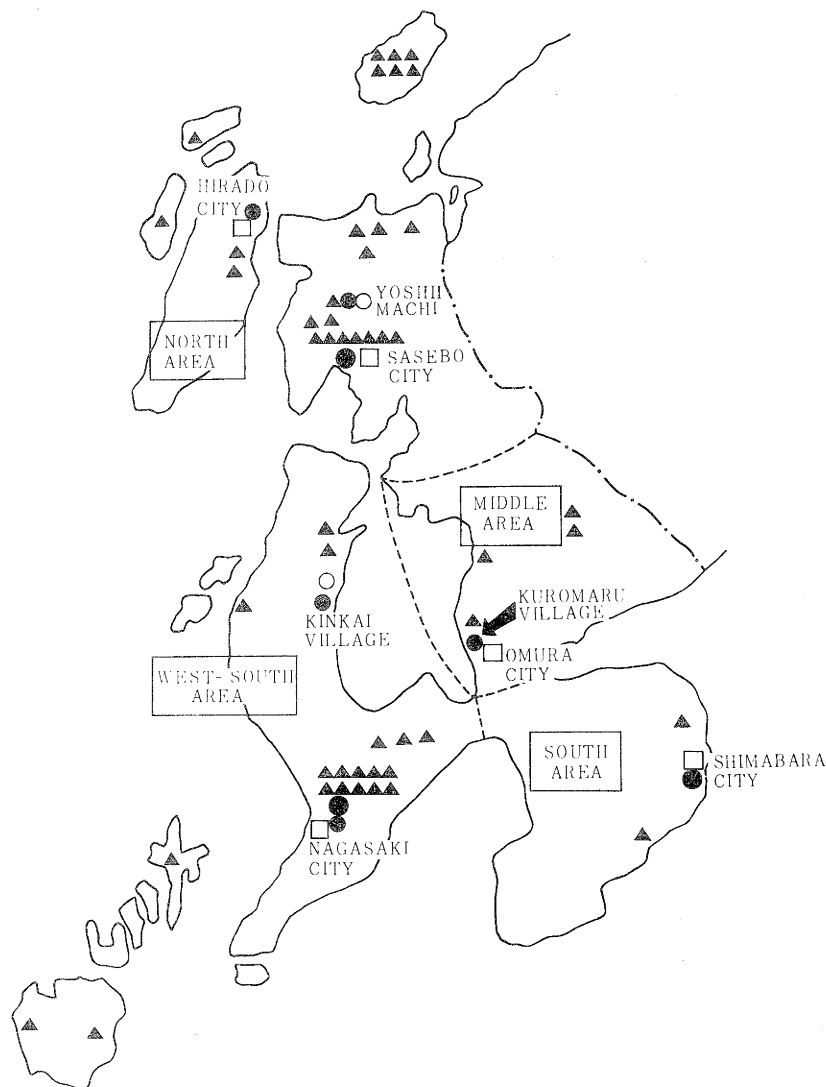
species could be found occasionally in cows and pigs sheds, they were less than *Culex tritaeniorhynchus* mosquitoes in number as given in Table 2. The consideration of the prevalence of *Culex tritaeniorhynchus* is still only inside presumption, because the mosquitoes were collected by the aid of aspiration only in domestic animals sheds. However, from the evidence that 157 mosquitoes of *Culex tritaeniorhynchus* could be already found on May 26th in the sheds, it was deemed to be on their way of raising in number. The rising of *Culex tritaeniorhynchus* mosquitoes had seemed to continue from June 23rd thru August 7th and slightly decrease in number from August 18th thru September 15th. Since September 28th, *Culex tritaeniorhynchus* mosquitoes in the

sheds were on the decrease in number rapidly.

The total 19 arboviruses could be isolated from only *Culex tritaeniorhynchus* mosquitoes from June 8th thru August 7th in the survey station. The isolation efficiency had indicated 0.6-4.0 in index of value. The most highest isolation efficiency was obtained on June 23rd. Consequently, it was presumed that the transmission of JE virus had been made with most effective frequency in this period.

Nineteen isolations could be identified as JE virus, however, 3 of them have less or no hemagglutination activity at any suitable pH ranges for JE virus at the beginning of isolation. These 3 viruses were obtained from only *Culex tritaeniorhynchus* mosquitoes; one of

Fig 3. Arrangement of Rabbit's Exposed to Mosquitoes and Occurrence of Encephalitis Cases



Remarks ; The sign of ● indicate arranged stations, which rabbits were exposed to mosquitoes. The sign of ▲ indicate encephalitis cases. The sign of □ indicate city. The sign of ○ indicate Machi and the sign of ◇ indicate Village.

them was isolated on June 23rd, one of them on July 3rd, and the last one on July 17th, respectively. These isolations had be carried out more detailed study on their properties. Consequently, after 5 passages of these viruses into wealing mice brain, it could be identified as JE virus due to the appearance of

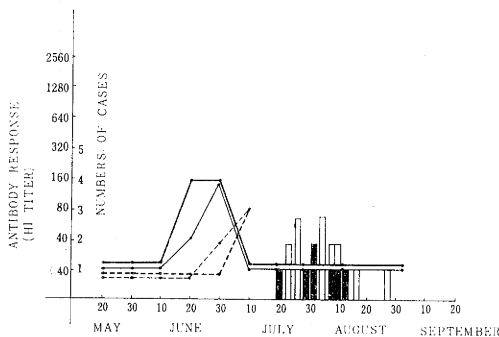
their hemagglutination activity.

In order to possibly forecast how JE virus dissemination will be turn out, some investigators had tried to pursue viremia and antibody response of susceptible animals exposed to mosquitoes comparing with virus isolation from mosquitoes collected in nature. In this

study, young rabbits, free from JE virus infection, were arranged at seven places selected in Nagasaki prefecture as given in Fig. 3.

As shown in Fig. 4-6, the information of JE virus dissemination could be found before occurrence of encephalitis cases, especially serologically confirmed cases. Antibody response in rabbit sera against JE virus in the test of hemagglutination inhibition had appeared on June 20th in north area (Yoshii-machi and Sasebo city) before outbreak of the cases by one month, and on July 10th and 20th in west-south area (Kinkai village) and in middle area (survey station: Kuromaru village) before outbreak of the cases by nearly one week.

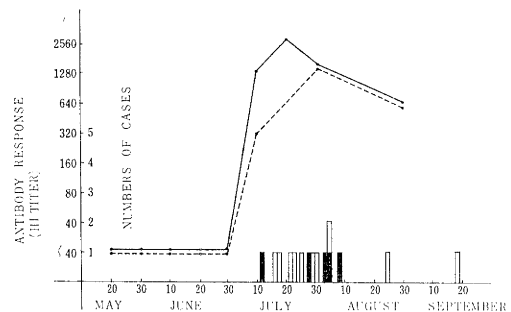
Fig 4. Antibody Response of Rabbits Arranged at Yoshii-Machi and Sasebo City and Outbreak of Encephalitis Case in North Area



Remarks : The solid line indicate HI antibody titers in two rabbit sera. In this case, the rabbits were arranged at the outdoor of the animal room of Infections Disease Hospital in Sasebo city. The dotted line also indicate the HI antibody titers in rabbits sera. These rabbits were arranged at the outdoor of the animal room of the Public Health Service Centre in Yoshii-Machi as shown in Fig. 3. The black sticks denote the serologically confirmed cases as caused by JE virus, and white sticks indicate the serologically unconfirmed one.

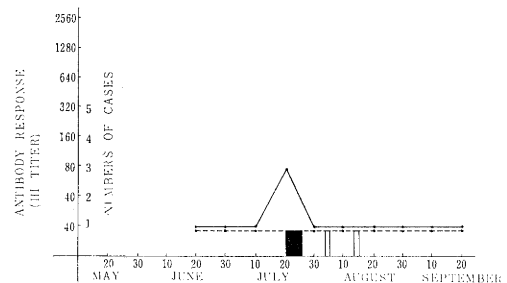
Despite of the outbreak of the cases serologically confirmed, however, no antibody response in rabbit sera could be definite in one of north places (Hirado city) and in south area (Shimabara city) as given in Fig 7-8.

Fig 5. Antibody Response of Rabbits Arranged at Kinkai Village and Outbreak of Encephalitis cases in southwest Area



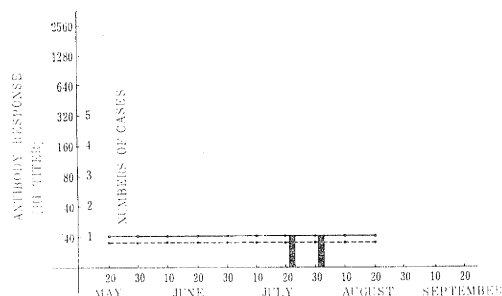
Remarks ; The solid and dotted line show antibody titers in two young rabbits sera. These two rabbits were arranged at the outdoor of the animal room of primary school in Kinkai village in southwest area as shown in Fig. 3. The black and white sticks see Table 4.

Fig 6. Antibody Response of Rabbits Arranged at Kuromaru Village, The Survey Station in Omura City and Outbreak of Encephalitis Cases in Middle Area



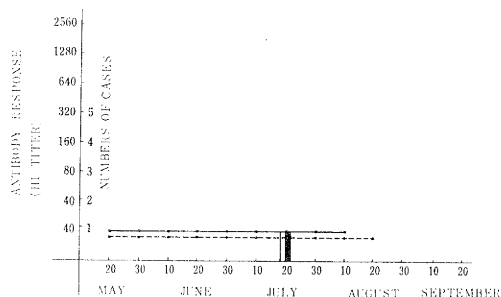
Remarks ; The solid and dotted line indicate the HI antibody titers of the rabbits sera. These rabbits were exposed to mosquitoes at the outdoor of the survey station. The black and white sticks see in Table 4.

Fig 7. Antibody Response of Rabbits Arranged in Hirado City in North Area and Outbreak of Encephalitis Cases



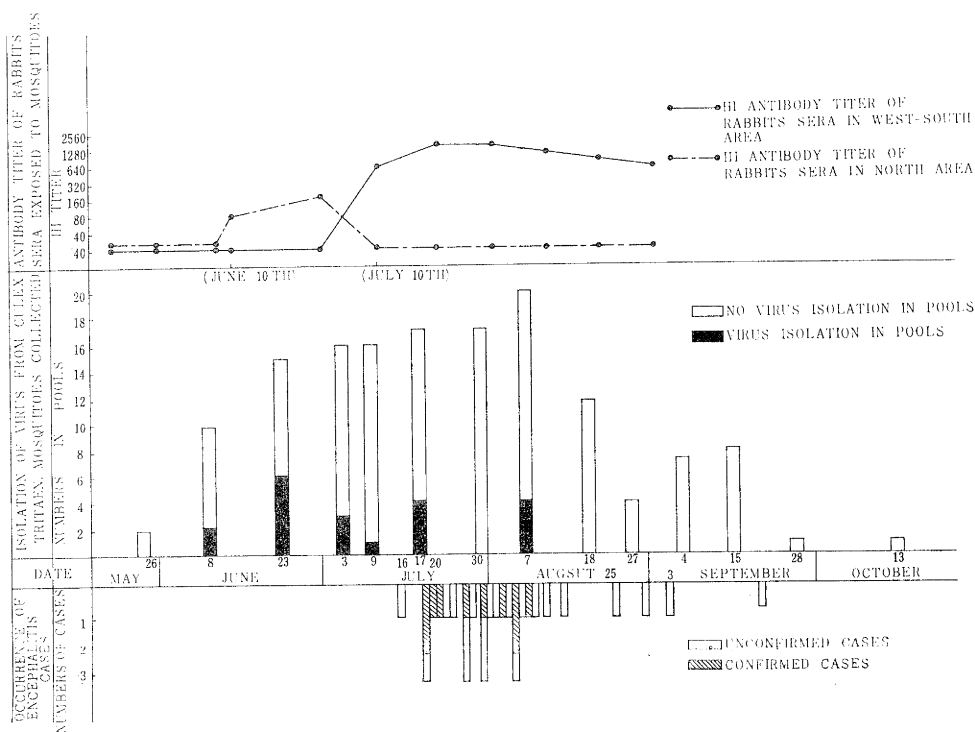
Remarks ; See the Table 6, however, the sera were obtained from rabbits arranged at the outdoor of the animal room in the Public Health Service Centre in Hirado city.

Fig 8. Antibody Response of Rabbits Arranged in Shimabara City in South Area and Outbreak of Encephalitis Cases



Remarks ; See the Table 6, however, the sera were obtained from rabbits arranged at outdoor of the animal room in the public Health Service Centre in Shimabara city.

Fig 9. Issues of The HI Antibody Response in Rabbit Sera Exposed to Mosquitoes, and of Virus Isolation from Mosquitoes of *Culex Tritaentorhynchus* Collected and of Occurrence of Encephalitis Cases Which Could be Applied for The Serological Confirmation.



As given in Fig. 3, the totaled 51 encephalitis cases had reported from July 13th thru September 3rd 1964 in Nagasaki prefecture according to authors' investigation. Most of cases had occurred from July 20th thru August 16th. Distribution of the cases by area during epidemic season was as follows: 25 of 51 cases in north area, 19 of them in south-west area, 5 of them in middle area and 2 of them in south area. Pre- and post-infection occasionally single sera of the cases were able to be obtained from 30 cases of the reported cases. From the evidence of serological examination, 11 of 30 cases had been confirmed to be caused by JE virus. The results of the

investigation of this cases will be published more in detail by KAWASOE, one of the authors. The occurrence of encephalitis cases and hemagglutination inhibition antibody response in rabbit sera exposed to mosquitoes at outdoor had been duly considered to be in coincidence with JE virus dissemination in epidemic season as shown in Fig. 3.

In conclusion, the hemagglutination inhibition antibody response in rabbit sera had appeared earlier than the outbreak of the first encephalitis case by one week or one month. Moreover, virus isolations could be found more earlier than the antibody response in rabbit sera.

Discussion

In this study, mosquitoes of *Culex tritaeniorhynchus* definitely identified had been mainly collected and other species of mosquitoes were excepted intentionally as previously described. Consequently, the isolation of 19 JE viruses could be performed only from *Culex tritaeniorhynchus* mosquitoes and no virus isolation from other species of mosquitoes. It is a matter for deep reflection that an attempt of JE virus isolation from other species except *Culex tritaeniorhynchus* mosquitoes in early or late period have been more significant than that in season. It is noticeable evidence that the first JE virus had been isolated from mosquitoes of *Culex tritaeniorhynchus* on June 8th in survey station. JE virus isolation from *Culex tritaeniorhynchus* mosquitoes had been made from June 28th thru September 5th 1960-1963 in Japan by Oya et al. and also from May 31st thru August 22nd 1958-1959 in Taiwan by Wang et al. The evidence of the virus isolation in this study is an unexceptional matter in Japan and it is performed so early as in Taiwan. However, in consideration of remarkable differences in climate condition between of Nagasaki prefecture and of Taiwan, it is not quite clear how should this fact be considered.

In consideration of the occurrence of encephalitis cases during several years, cages kept in rabbits, free from JE virus infection, had been arranged at outdoor of seven places selected in Nagasaki prefecture to be exposed to mosquitoes as shown in Fig. 3.

As shown in Fig. 4-6, the information of JE virus dissemination in nature could be found from the evidences of virus isolation from mosquitoes and antibody response in rabbit sera exposed to mosquitoes. However, the suitability of arranged places of rabbits exposed to mosquitoes and aptitude of rabbits for investigation of antibody response against JE virus have to be inquired closely as given in Fig. 7-8.

It is noticeable fact that there are remarkable variation by area in the first rising of antibody titer in rabbit sera in hemagglutination inhibition and in the outbreak of the first case serologically confirmed. Though there is a common view that Japanese Encephalitis cases have occurred at randomly in certain area during epidemic, it is believed that to pursue the focus of the variation caused by JE virus dissemination by area and by stage through the year is to study the ecology of JE virus.

Summary

The total 19 JE viruses were isolated from 13442 in 144 pools *Culex tritaeniorhynchus* mosquitoes collected in survey station. Three of them have less of no hemagglutination activity at any suitable pH ranges for JE virus at the beginning of first isolation, however, it reveals after 5 passages into wealing mice brains. The sera obtained from

rabbits exposed to mosquitoes had developed their antibody in hemagglutination inhibition before epidemic among human being. The information of JE virus dissemination could be found on the basis of antibody response in the sera and of isolation of the viruses in nature. However, it deem to remarkable variation in JE virus dissemination.

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1964年大村市近郷で捕集した蚊の日本脳炎ウイルス保有状況, 林 薫, 三舟求真人, 本村一郎, 松尾幸子, 川副広俊, 二木浩一, 長崎大学風土病研究所病理学部 (主任: 福見秀雄教授)

1964年5月26日から10月13日に至る間大村市近郷黒丸部落で捕集した13442個体144プールのコガタアカイエカから19株の日本脳炎ウイルスが分離された。このうち3株は分離当初ヒナ血球に対する凝集能が甚だ低かったがマウス脳内5代通過後、凝集能が認められJEウイルスと同定された。最初のウイルスは6月8日分離され従来日本各地におけるウイルス分離時期に比べ甚だ早いことが注目された。オトリ動物として使用したウサギの抗体上昇の時期は患者発生に先立つこと1週又は1ヶ月前に認められ、県下の患者発生状況と比較しウイルス汚染の程度を推察することが出来た。