Ecological Studies on Japanese Encephalitis Virus. Isolation of Japanese Encephalitis Virus from Mosquitoes Collected in Nagasaki and Kagoshima districts, Japan, in 1965.

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ABSTRACT : Continual survey in Nagasaki prefecture were established at Kuromaru village since last year. In this season, 13 places in Nagasaki prefecture and the suburbs of Taniyama city, Kagoshima prefecture, were selected additionally for the collection of mosquitoes. The hibernated female of *Culex tritaeniorhynchus* collected in Nagasaki destrict from 16th March to 30th April in this season have been made, however, no virus could be isolated. During the period from 30th May to 6th September, 47 of 56 strains were isolated from *Culex tritaeniorhynchus* mosquitoes, 5 of them from *Culex vishnui* and 2 of them from *Culex pipiens pallens* respectively. In addition, 2 strains regarded as Japanese encephalitis virus (hereinafter referred to as JE) were isolated from *Aedes vexans*. The first isolation of JE virus from *Culex tritaeniorhynchus* mosquitoes was made on 30th May in Nagasaki area, while in Kagoshima area it was made on 17th June.

Comparing the patterns of JE virus isolations, it was demonstrated that there were remarkable difference of JE virus dissemination by area and by periods. The ecological investigation of JE viruses has been stated on the bases of the JE virus isolations from mosquitoes, the rising of haemagglutination inhibition antibodies in swine sera and the occurrences of human encephalitic cases.

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Introduction

In the previous report described by HAYASHI et al. (1965) it was demonstrated that the evidence of the virus isolation from mosquitoes in Nagasaki area was an unexceptional matter in Japan and it was performed so early as in Taiwan, furthermore, it was supported that there were remarkable variation by area the basis of the first rising of antibody titre in rabbit sera in haemagglutination inhibition and in the occurrence of the first human Japanese encephalitic case serologically confirmed.

For the survey of the ecology of JE virus, the virus isolation from mosquitoes collected at several places in Nagasaki prefecture and one place in Kagoshima prefecture was carried out again in this season. In addition, as the experimentaly infected mosquitoes of *Culex tritaeniorhynchus* with JE virus could survive over the winter and transmit the virus to susceptible pigs as published by MIFUNE, K. (1965) and OMORI, N. et al, (1965), it suggests that there will be a possibility of surviving of JE virus in mosquitoes through the winter. Furthermore, OMORI, N. et al (1965) have succeeded to collect a lot of number of the female mosquitoes of *Culex tritaeniorhynchus* just emerged from hibernation. Consequently, the virus isolation from them has been carried out in early spring. On the other hand, in order to find indirectly the information of JE virus disseminarion in nature, the haemagglutination inhibition antibodies in swine sera collected at slaughterhouse in Nagasaki city were examined. The result of investigation of virus isolation from mosquitoes, antibody response in swine sera and the occurrence of human enceqhalitic cases in Nagasaki and Kagoshima areas will be described in this paper.

Materials and Methods

The areas for collection of mosquitoes : Continual survey in Nagasaki prefecture was established at Kuromaru village where has been investigated the dissemination of Japanese encephalitis virus since last year. In addition, this year, Kaizu, Mogi and Tomachi villages where are located at a distance of 23Km north-east, 6Km south-east and 4Km south from Nagasaki city respectively have been selected for the collection of mosquitoes. Moreover, mosquitoes were collected at some other places as shown in Table 1 and Fig. 1. There are rice fields and farmers are raising cows, pigs, hens and other domestic animals. In Mogi village, however, some inhabitants are also carried on fishery.

In Kagoshima prefecture, the suburbs of Taniyama city near Kagoshima city were selected for the collection of mosquitoes as shown in Fig. 1. The area have been located as same as outline of Kuromaru village in Omura city, Nagasaki prefecture, described previously by Hayashi et al. (1965).

Collection of Mosquitoes : For collection of mosquitoes in Nagasaki prefecture the survey has been carried out from 16th March through the end of October in 1965 by the Department of Medical Zoology and the Department of Pathology, Research Institute of Endemics, Nagasaki University, and the survey at the suburbs of Taniyama city, Kagoshima prefecture, had been made by the Department of Medical Zoology, Kagoshima University.

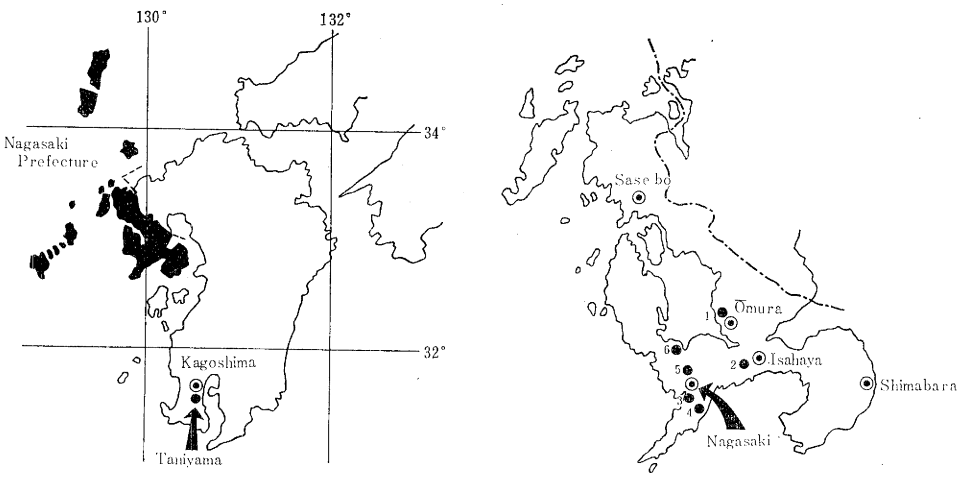
In Nagasaki districts, during the period from 16th March through the end of April, hibernated female mosquitoes of *Culex tritaenio-*

rhynchus have been collected as previous report published by OMORI et al. (1965). From lst May to the end of October, the collection of mosquitoes has been carried out by the dry ice technique described by OMORI et al. (1965) and also by the technique with the aid of livestockpen as previously described by HAYASHI et al. (1965). Mosquitoes collected in Nagasaki districts have classified by the Department of Medical Zoology, Na-

Table 1. Stations for collection of mosquitoes

Village	Distance from Nagasaki City	Times of Collections
Kuromaru	42km, NNE	18
Kurogi	53km, NNE	1
Kaizu	23km, NE	28
Mogi	6km, SE	19
Tomachi	4km, S	26
Kawabira	8km, NNE	8
Togitsu (Hotachime, Hinami Sasoko, Kobazaki)	11–13km, NNW	12
Hongōchi	East side of Nagasaki city	2
Yasakamachi	Mid-area of Nagasaki city	1
Minamiyamate	South side of Nagasaki city	1
Takenokubo	West side of Nagasaki city	1
Michinoo	North side of Nagasaki city	1
Fukuda	Far west side of Nagasaki city	3
Tanakamyo	Far east side of Nagasaki city	1

Fig 1. Outline of Kyushu island and survey stations in Nagasaki and Kagoshima prefectures.



Remarks : ⊙ mark means the city.
• mark shows the place of collection of mosquitoes;
1…Kuromaru village, 2…Kaizu village, 3…Tomachi village, 4…Mogi village, 5…Kawabira villege, and 6…Togitsu village

gasaki University. At the suburbs of Taniyama city, Kagoshima prefecture, resting mosquitoes in pigs, cows and fowls sheds have been picked up with a livestockpen after sunset and classified mosquitoes have been kept in dry ice acenton and transported by air for virus isolation from the Department of Medical Zoology, Kagoshima University to the Department of Pathology, Research Institute of Endemics, Nagasaki University.

Virus isolations and identifications : Virus isolations and identifications have been carried out by the Department of Pathology, Research Institute of Endemics, Nagasaki University. The procedure had been previously descriced by HAYASHI et al. (1964). A pool of mosquitoes is made 200 specimens in number and the suspension of triturated tissues of mosquitoes were centrifuged usually at 10,000 rpm for 15 minutes in this study.

Haemagglutination inhibition test for swine sera and serological confirmation of human encephalitic cases : The haemagglutination inhibition antibodies in swine sera collected at a sloughterhouse in Nagasaki city from the beginning of April 1965 through the end of February 1966 have been examined. Human encephalitic cases occurred in this season in Nagasaki prefecture have been also detected serologically by the test of haemagglutination inhibition and complement fixation. These serological examination will be published in detail in the following Bulletin by KAWASOE, one of authors.

Results

Number of mosquitoes by species collected and virus isolations from them : Virus isolations from hibernated female of *Culex tritaeniorhynchus* mosquitoes collected in Nagasaki districts, from 16th March to 30th April in 1965 have been made unsuccessfully as described previously by OMORI, et al. (1965). During the period from the beginning of May through the end of October in 1965, in total, 56 strains of JE virus could be isolated as shown in Table 2. Most of them were detected from *Culex tritaeniorhynchus* mosquitoes except the hibenarted female one, and no virus from *Culex whitomorei* and *Armigels sub-*

Table 2. Number of mosquitoes by species collected and virus isolations from them in Nagasaki in 1965.

Species	Number of mosquitoes	Number of pools	Isolated viruses
C. tritaeniorhynchus	90,552	544	47
C. vishnui	14,777	110	5
C. P. pallens	562	6	2
Ae. vexans	4,824	33	2
Ar. subalbatus	1,479	13	0
C. whitomorei	88	2	0
Total	112,282	708	56

Table 3. Number of moequitoes by species collected and virus isolation from them in the suburbs of Taniyama city, Kogoshima prefectnre, 1965.

Species	Number of mosquitoes	Number of pooles	Isolated viruses
C. tritaeniorhynchus	§ 10,182 (8,081)	47	4
C. p. pallens	22	not tested	
C. whitomorei	40	〃	
Ae. vexans	1,910	〃	
An. hyr. sin.	778	〃	
Ar. octurbans	142	〃	
Total	13,074	47	4

Remarks: Virus isolations were carried out from 8,081 of 10,182 mosquitoes of C. *tritaeniorhynchus* transported by air from Department of Medical Zoology, the faculty of Medicine, Kagoshima University to Department of Pathology, Research Institute of Endemics, Nagasaki University.

albatus. It can be pointed out as the noteworthy evidence that two strains regarded as JE virus have been isolated from the mosquitoes of *Aedes vexans* as in the following descriptions. On the other hand, 47 pools of 8.081 specimens of *Culex tritaeniorhynchus* mosquitoes transported by air were examined for virus isolations and 4 strains of JE virus could be detected as shown in Table 3.

Seasonal appearance of JE viruses in mosquitoes by species are summarized in Table 4 and 5. In Nagasaki districts, JE viruses could be isolated from mosquitoes of *Culex tritaeniorhyuchus* from 30th May to 6th Septemper. The highest isolation efficiency indicated the 5.2 value of index during the first period of 10 days of July. During the period of concentrated dissemination of JE viruses in epidemic season, 5 strains from *Culex vishnui* and each 2 strains from *Aedes vexans* and *Culex pipiens pallens* collected at pigs or cows sheds or outside could be isolated respectively. Through the investigation which have been made by HAYASHI et al. (1964), TAKAHASHI et al. (1964) and in this paper, it can be considerable findings that JE virus dissemination in mosquitoes in Nagasaki districts may occurre usually during from the end of May to the first of June. The isolation of JE virus from mosquitoes of *Culex tritaeniorhynchus* at Taniyama area, Kagoshima prefecture, could be detected from 17th June through 26th July.

Table 4. Virus isolations from mosquitoes by species collected in Nagasaki districts in 1965.

	C. tritaeniorhynchus				*C. vishnui*			*C. p. pallens*			*Ae. vexans*		
	No. of mosquitoes	No. of pools	Isolated viruses	Isolation efficiency	No. of mosquitoes	No. of pools	Isolated viruses	No. of mosquitoes	No. of pools	Isolated viruses	No. of mosquitoes	No. of pools	Isolated viruses
Apr. F	67	1											
Apr. M	2,444	29											
Apr. E	13,036	85			660	8							
May F	2,870	19			167	4					119	1	
May M	3,730	23			68	2					579	4	
May E	3,180	19	1	0.3	28	1					94	1	
June F	6,893	41	7	1.0	264	3					409	3	
June M	9,130	48	7	0.8	282	4		241	1		2,371	13	1
June E	2,358	13	5	2.2	258	2	1	46	1	1	147	1	
July F	1,927	11	10	5.2				69	1	1			
July M	3,129	17	4	1.3	321	3	1				339	3	1
July E	16,898	87	11	0.7	1,614	10	1	181	2		592	4	
Aug. F	4,796	26			594	4							
Aug. M	9,621	52	1	0.1	3,855	20	2				63	1	
Aug. E	7,077	40			3,842	23					68	1	
Sep. F	2,187	15	1	0.5	1,758	14		25	1				
Sep. M	228	4			119	2							
Sep. E	660	6			786	8							
Oct. F	275	7			161	2							
Oct. M	46	1									43	1	
Total	90,552	544	47		14,777	110	5	562	6	2	4,824	33	2

Remarks: The signs of F, M and E mean the first, middle and end of month.

Table 5. Isolations of Japanese encephalitis viruses from *Culex tritaeniorynchus* collected in the suburbs of Taniyama city, Kagoshima prefecture, in 1965.

Date collected	No. of mosquitoes	No. of pools	Isolated viruses
June 8	495	3	
17	962	5	1
24	981	5	1
July 4	625	4	
13	501	3	
19	1,298	7	1
26	1,050	6	1
Aug. 3	467	3	
9	685	4	
16	147	1	
23	471	3	
Sep. 1	206	1	
13	110	1	
27	83	1	
Total	8,081	47	4

From point of view of geographical location, the periods and frequencies of JE virus isolations in Kagoshima area may not far different from those in Nagasaki area. The pattern of JE virus isolations from *Culex tritaeniorhynchus* mosquitoes collected at five places as the representation of the survey had distingushing characteristics by areas and periods as illustrated in Fig. 2.

JE virus isolations have been made ealier in Tomachi village (30th May), Kaizu village (1st June) and Mogi village (2nd June) than in Kuromaru village (29th June) or Taniyama area (17th June). However, it can be also seen in Fig 2 that the periods and frequencies of virus isolations have been varied by areas in which the investigation carried out.

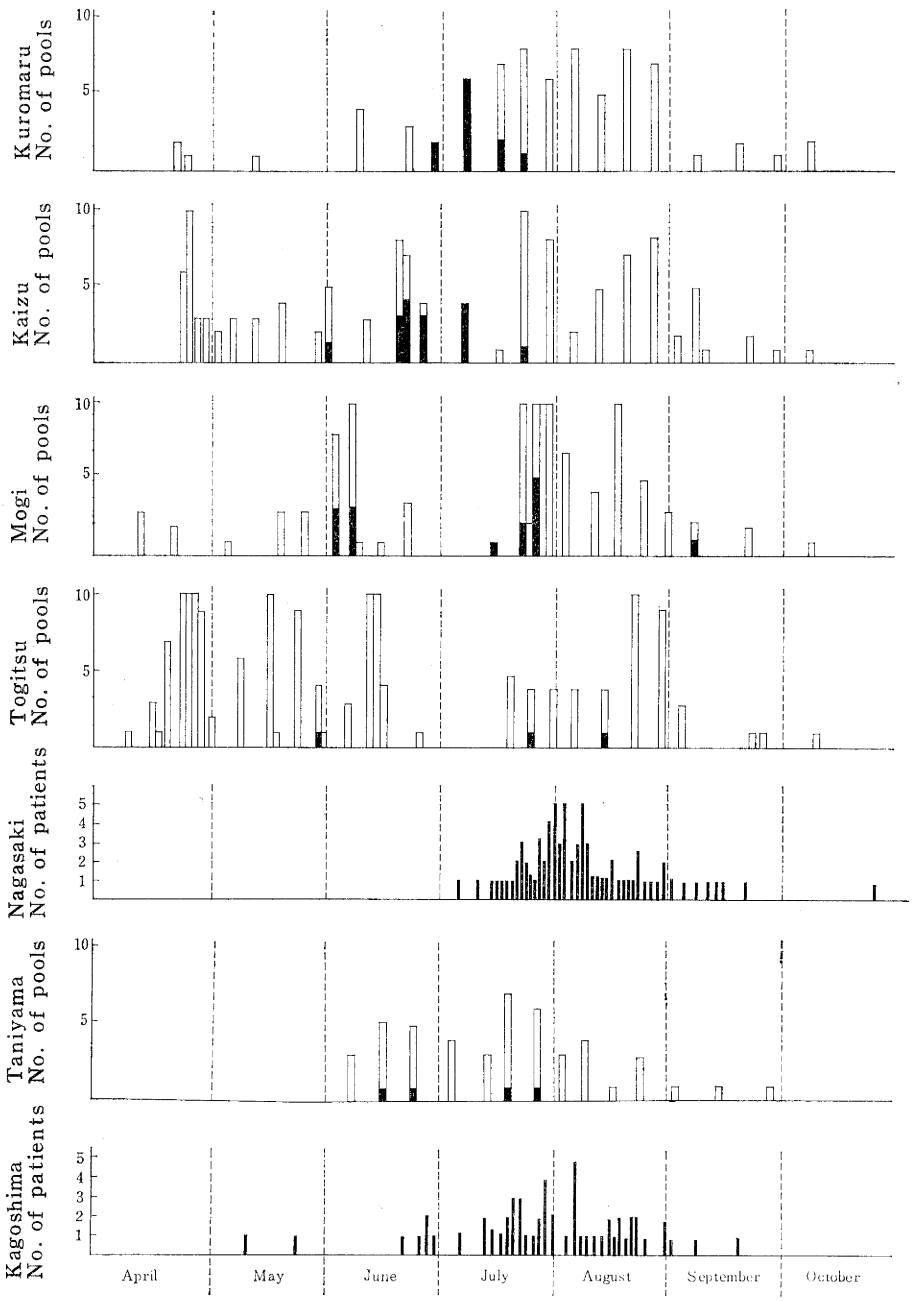
These finding have indicated the pattern of JE virus dissemination in nature as similar as the results on the antibody response of rabbits arranged at certain places in Nagasaki prefecture in the previous paper published by HAYASHI et al. (1965).

Occurrences of human encephalitic cases : As shown in Fig. 2, 3, and 4, during from 5th July to 23rd October, 82 encephalitic cases clinically diagnosed have outbroken in Nagasaki prefecture and during from 1st May to 19th September, 62 cases have occurred in Kagoshima prefecture. In both districts, the peak of encephalitic incidents have revealed in the period during from the end of July to the first of August. Serological examinations were carried out by the haemagglutination inhibition test and complement fixing test for the cases occurred in Nagasaki prefecture. Consequently, 37 of 82 cases have been confirmed serologically as Japanese encephalitis. The first confirmed case was found on 5th July and the last case was on 20tn September.

Haemagglutination inhibition (HI) antibodies against JE virus in swine sera : In total, 773 sera were obtained from six to eight months old swine during from 15th February 1965 to the end of October 1965.

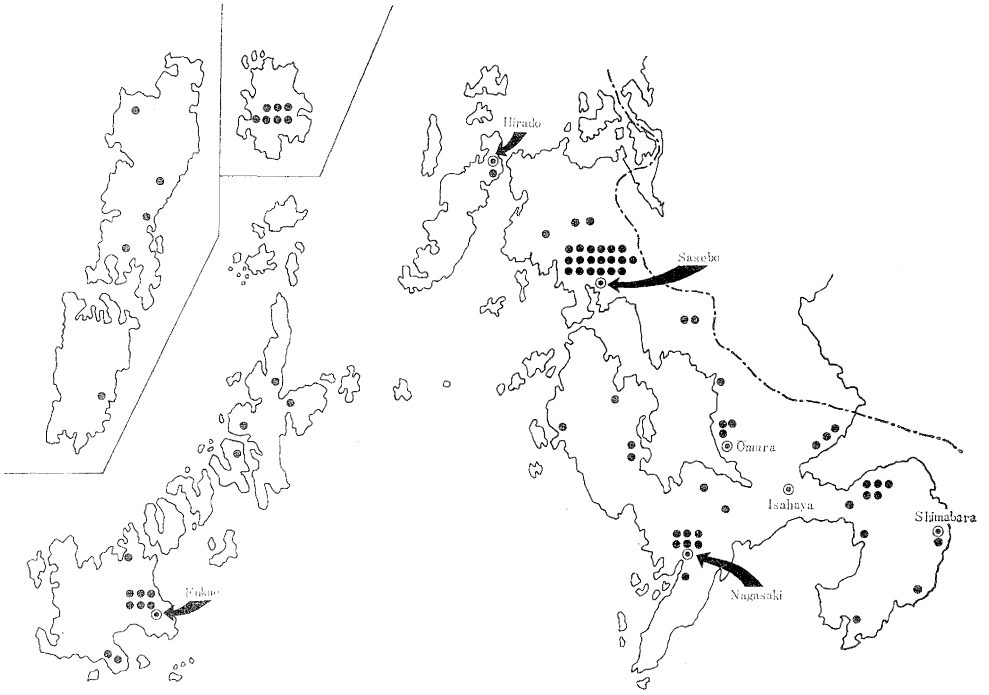
The swine transported to the slaughterhouse had been raised in the southwest area of Nagasaki prefecture where the places for collecting mosquitoes in this study. As shown in Fig. 6, the rate of infected pigs indicated by the HI antibody was fairly high in the middle of April, but it decreased to naught during from the end of April to the end of May. The number of infected pigs, however, increased rapidly in the middle-June and on 28th July, and reached to be counted by one hundred per cent. The highest HI antibody possessing rate continued untill the middle-September. subsequeutly it decreased slowly.

Fig 2. Isolations of JE viruses from mcsquitoes in different five places and outbreak of human encephalitic cases in Nagasaki and Kagoshima prefecture.



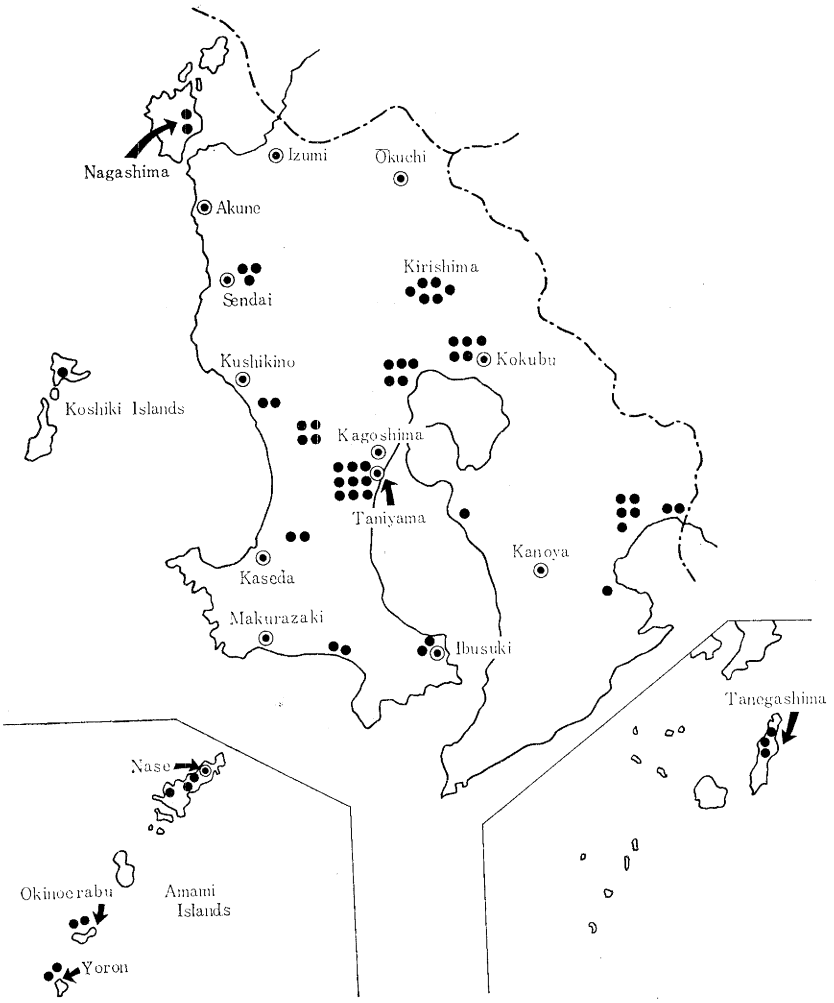
Remarks : The white sticks indicate the number of pools which could not isolate JE virus from *C. tritae*. mosquitoes and the black sticks indicate the number of pools which could isolate the viruses from them.

Fig 3. Occurrences of human encephalitic cases in Nagasaki prefecture in 1965.



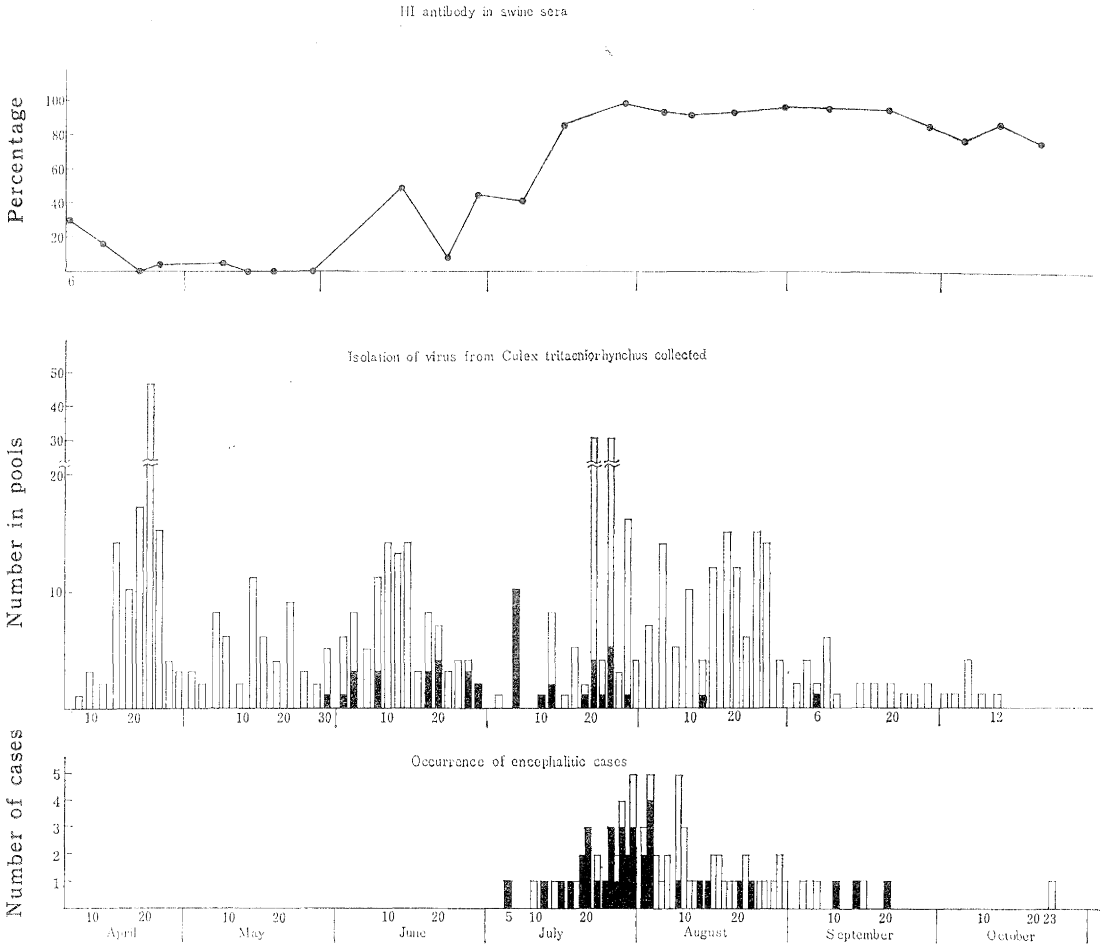
Remarks : ◉ mark means one patient of huma Japanease encephalitic cases.
● mark shows the city.
Twenty-three of 80 cases have occurred in north area, 9 of them middle area, 10 of them south area, 13 of them southwest area, 12 of them north islands and 13 of them south west islands respectively.

Fig 4. Occurences of human encephalitic cases in Kagoshima prefecture in 1965.



Remarks : • mark means one patient of human Japanease encephalitic cases.
⊙ mark shows the city.

Fig 5. Results of isolations of JE virus from *Culex tritaeniorynchus* mosquitoes in relation to rising the rate of possessing HI antibody against JE virus in swine sera and to outbreak of human encephalitic cases in Nagasaki Prefecture in 1965.



Remarks : See Fig. 2 and 3.

Discussion

Since the establishment of experimental infection of JE virus to mosquitoes of *Culex tritaeniorhynchus* by MITAMURA, et al. (1935) and the isolation of the virus from them in nature by MITAMURA et al. (1938) or BUESHER, et al. (1959), it has been demonstrated that the mosquitoes of *Culex tritaeniohynchus* plays the important part of vector of JE virus in epidemic season. However, the activity of JE virus ceases during the off-season and no information are available even on the ecology of the *Culex tritaeniohynchus* mosquitoes. OMORI et al. (1965) had succeeded to collect a large number of hibernated female mosquitoes in early spring in the field, furthermore, experimentally infected mosquitoes of *Culex tritaeniohynchus* with JE virus could survive over the winter and transmit to susceptible pigs as published by MIFUNE (1965) and OMORI et al. (1965). It is

suggested that there is a possibility of survival of JE virus in mosquitoes through the winter. Consequently, an attempt to be verified the virus in hibernated female mosquitoes have been carried out. The total of 16080 mosquitoes in 119 pools of the mosquitoes of *Culex tritaeniorhynchus* collected during April and examined for virus isolation. However, no virus could be isolated from them. It might be considered that even through the suckling mice are the most useful animal for JE virus isolation, special regard might be paid to the changes in characteristics of the virus in the mosquitoes as stated on the Western equine encephalitis virus associated with mosquitoes by Reeves, W. C. (1958).

From the result of previous investigation made by HAYASHI et al. (1965), it has been pointed out that the JE virus isolation in Nagasaki area has been performed earlier than other areas in Japan including Okinawa islands and in Taiwan, moreover, there has been remarkable variation by area in the rising of HI antibody in rabbit sera exposed to mosquitoes.

As shown in Fig. 2, the first isolations of JE virus in this season have been made on 30th May, 1st and 2nd June, at Tomachi village, Kaizu village and Mogi village respectively. As the results of successive two years survey in Nagasaki area, the infected mosquitoes could be usually detected during from the end of May to the beginning of June. On the other hand, the first virus isolation in Kuromaru village, where have been the survey station since last year, were made on 29th June. It was later three weeks than last year.

At the suburbs of Taniyama city in Kagoshima prefecture, the first virus could be isolated on 17th June. It is supposed that there may be no discrepancy in virus dissemination in nature between in Nagasaki and Kagoshima prefecture, being different in climate and geographical condition.

As given in Table 2 and 4, fourty-seven strains of JE viruses have been isolated from the mosquitoes of *Culex tritaeniorhyn,chus*, 5 strains from *Culex vishnui* and 2 strains from *Culex pipiens pallens*. In addition to this, 2 strains of arboviruses which were very similar to JE virus in the biological and serological characteristics especially by the haemagglutination test and complement fixation test could be isolated from the mosquitoes of *Aedes vexans*. However, the identifications of the viruses isolated in this season have been making more exactly. Furthermore, the examination of JE virus multiplication in the body of the mosquitoes of *Aedes vexans* and the experimental transmission of the virus have been proceeding now. There have been no investigation that JE virus could be isolated from the mosquitoes of *Aedes vexans* up to now. The finding on JE virus isolation from the *Aedes vexans* mosquitoes will be offer the important problems in relation to JE virus dissemination in nature, because the mosquitoes of *Aedes vexans* can be found in the same season as many as the mosquitoes of *Culex tritaeniorhynchus* in some places of Japan such as Kanto area (middle of Japan) and Kagoshima prefecture (south of Japan).

JE virus isolations from mosquitoes, haemagglutination inhibition antibodies in swine sera and the occurrences of human Japanese encephalitis cases are summarized in Fig. 5. The JE virus isolation from mosquitoes in this season could be made from 30th May through 6th September. The pattern of JE virus isolations in Nagasaki area is deemed to be remarkable difference from other places in Japan. Considering the infection of swine with JE virus from the results of the hae-

magglutination inhibition antibody response in swine sera, infected swine with JE virus could be found almost simultaneously at the time of the first isolation of JE virus from mosquitoes, and infected swine had increase remakably in number to the end of June. It is evident that the swine is a good decoy animal to predict the epidemic of Japanese encephalitis in human being.

The first serologically confirmed case of human Japanese encephalitis has outbreak on 3th July in this season. It is pointed out that the first confirmed case occurred later one month moreover than the first isolation of JE virus from mosquitoes made on the end of May.

On the bases of the results of virus isolation from mosquitoes in this season, it will be concluded that for the ecology of JE virus in nature especially the problems on overwintering of JE virus must be more classified the mode of infection of mosquitoes as well as the ecology of *Culex tritaeniorhynchus*.

Summary

A total of 112,252 mosquitoes of 6 species (*Culex triteianorhynchus*, *Culex vishnui*, *Culex pipens palleus*, *Aedes vexans*, *Armigeres subalbatus*, *Culex whitomorei*) were collected from 9th April to 6th October in Nagasaki prefecture and 8,081 mosquitoes of *Culex tritaeniorhynchus* were collected in Kagoshima prefecture for virus isolations from them. On the other hand, the sera of pigs which were killed at the slaughterhouse in Nagasaki city were collected from 15th February 1965 to 2nd February, 1966 for the examination haemagglutination inhibition antibody againist JE virus. Results were obtained as follows.

(1) The total of 16080 mosquitoes in 119 pools of the hibernated female of *Culex tritaeniorhynchus* mosquitoes have been coll ected during April in this season, however,no virus could be isolated from them.

(2) Fifty-six strains and four strains of Japanese encephalitis virus were isolated from the mosquitoes in Nagasaki and Kagoshima prefecture, respectively.

(3) Forty-seven strains of 56 Japanese encephalitis virus were isolated from *C. tritaeniornynchus* and 5 strains were from *C. vishnui* and 2 strains were from *C. p. pallens* in Nagasaki prefecture respectively. Four strains of Japanese encephalitis virus were isolated from *C. tritaeniorhynchus* in Kagoshima prefecture.

(4) The first virus from *C. tritaeniorhynchus* was is olated of 30th May in Nagasaki prefecture. While, in Kagoshima prefecture, the first virus was detected on 17th June. From this finding of the data reported by HAYASHI et al. (1965) last year, the infected mosquitoes in nature might be found early in the end of May or beginning of June every year in Nagasaki prefecture.

(5) Two strains of arboviruses were also isolated from *Aedes vexans*. These two viruses are closely akin to Japanese encephalitis virus by haemagglutination inhibition and complement fixation test. But more detailed identification is being carried out now.

(6) From the results of virus isolation, it was demonstrated that the remakable variation by periods and by areas eraisted in JE virus dissemination in nature.

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日本脳炎ウイルスの生態学的研究，1965年長崎及び鹿児島地方における捕集蚊からの日本脳炎ウイルス分離状況，林　薫，三舟求真人，七条明久，川副広俊，松尾幸子，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），大森南三郎，和田義人，伊藤寿美代，河合潜二，西垣定治郎，長崎大学医学部医動物学教室及び長崎大学風土病研究所衛生動物部（主任：大森南三郎教授），阿部康男，真喜屋清，上園善隆，鹿児島大学医学部医動物学教室（主任：阿部康男教授）

総　　括

昨年に続いて長崎市周辺部，諫早市，大村市で捕集したコガタアカイエカ90522個体，シロハシイエカ14777個体，アカイエカ562個体，キンイロヤブカ4824個体，オオクロヤブカ1479個体，セジロイエカ88個体からウイルス分離を試みた結果，コガタアカイエカから47株，シロハシイエカから5株，アカイエカから2株，キンイロヤブカから2株のウイルスが分離された．ウイルス保有蚊は5月30日に出現し昨年の調査の場合と大差がなかった．これは関東，関西地区に比較すると異常に早いことを確認する資料である．このようなウイルス撒布状況を地理的に長崎県より南に位置する鹿児島地方と比較検討するため，鹿児島大学医学部医動物学教室で捕集し．ドライアイス，アセトンで冷却し空輸されたコガタアカイエカ8081個体についてウイルス分離を行ない4株の日本脳炎ウイルスを分離したが，最初のウイルス保有蚊の出現は6月17日であった．

また4月9日から4月30日までに捕集され衛生動物部で越年蚊であることが確認されたコガタアカイエカ16174個体からウイルス分離を試みたが不成功に終った．またキンイロヤブカから分離されたウイルスはその性状が日本脳炎ウイルスに極めて類似していることが注目され，詳細な同定試験と実験的感染並びに伝播実験を実施中である．屠場に集まる豚血清中の日本脳炎ウイルスＨＩ抗体保有状況を年間を通じて調査した．その結果，豚の日本脳炎抗体保有率は野外蚊からウイルスが分離される状況と密接な関係が認められ豚は日本脳炎ウイルスの増幅動物としての従来の所見を確認したが，蚊からのウイルスの分離状況，豚血清中のＨＩ抗体保有状況は地域的にも時間的にもかなりの差があることが認められた．

1966年6月6日受付