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NATURAL INFECTION OF SWINE BY JAPANESE ENCEPHALITIS VIRUS AND ITS MODIFICATION BY VACCINATION

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SUMMARY The natural infection of swine with Japanese encephalitis virus (JEV) and its modification by vaccination were studied with the following results: 1) After natural infection with JEV all the swine showed detectable viremia and the duration of viremia was 3 to 6 days, (average 3.8–4.7 days). 2) When the swine were immunized with inactivated vaccine, the rate of detection of viremia caused by natural infection and its duration were much less than in non-immunized controls. 3) Even in a single group, there was about two weeks difference in the onset of natural infection of individual swine. Therefore, the period during which JEV is transmissible in a swine population in a restricted area can be longer than two weeks. 4) It is suggested that the virus coexists with its hemagglutination-inhibition (HI) antibody in the blood during the latter half of the period of viremia. 5) Viremia observed on natural infection with JEV was less frequent in rabbits, used as indicator animals, than in swine, and it seems that antibody production against JEV in rabbits has a longer time lag than in swine. 6) The rate of virus isolation from vector mosquitoes in a single pig pen varied significantly with the method used to collect mosquitoes. Thus it is necessary to consider the methods used to collect mosquitoes in tests on the infection rate of vectors with JEV.

INTRODUCTION

In 1959, Scherer and his collaborators gave the term " amplifier " to animals which are susceptible to Japanese encephalitis virus without showing symptoms (Scherer et al. 1959a). The conception of amplifiers has opened up a new epoch in ecological studies on Japanese encephalitis virus and there have been many studies on which is the most important amplifier.

The characters needed for a good amplifier are as follows; (1) the population size of the amplifier must be large enough, (2) the amplifier must pass through repeated generations, (3) the vectors must engorge the blood of the amplifier and (4) viremia in the amplifier must last long enough to allow transfer of virus to mosquitoes. Scherer pointed out that swine are the most probable amplifiers in Japan (Scherer et al., 1959a). This was confirmed in 1963 by Nakamura and his co-workers

(Nakamura et al., 1963) under well-planned experimental conditions. More recently, from the ecological standpoint, swine have been regarded as the most important amplifiers of JEV (Konno et al., 1966; Ueba et al., 1971). Thus vaccination of swine should suppress infection of vector mosquitoes. In several districts in Japan swine have been vaccinated against Japanese encephalitis to protect pregnant animals from abortion caused by JE infection (Kawakubo et al., 1966). Suppression of JE infection of swine by vaccination also seems important from the point of view of public health. Reduction in the extent of amplification in swine should result in modification of the course of natural infection. The effect of immunization of swine on the morbidity of man is uncertain and this type of indirect protection method has not yet been used for viral diseases. An experiment to test these expectations was first done by Oya but insufficient results were obtained (Oya, 1967). Recent studies by Takahashi et al. (1968) and Tsuchiya et al. (1971) suggested that vaccination of the amplifier is a useful way to reduce the density of infected mosquitoes. We have also reported similar results showing the effectiveness of vaccination of swine (Ueba et al., 1971). The present paper reports serological and virological results of our study on natural infection with JEV in vaccinated and non-vaccinated groups of swine. JEV infections of vector mosquitoes in the environment and of indicator animals were also examined to consider the effectiveness of vaccination of the amplifier in prevention of the disease.

MATERIALS AND METHODS

1. *Study areas and pigs*

Studies were made in Yamamoto, Yao city, Osaka Prefecture, which is situated on a plain about 2 km west of the Ikoma hills. As shown in Fig. 1, paddy fields and houses make a mosaic in this area. Studies were made from June 12 to September 20. The pigs examined were Yorkshire and Landrace breeds. We chose two pig pens, A and B about 0.5 km apart. All the pigs in pen B were vaccinated while those in pen A served as controls. Initially pen A contained about 200 pigs including a few procreative ones and pen B had about 60 pigs but the numbers changed slightly because adult pigs were sold for slaughter and for supply of weanlings.

2. *Vaccination*

The pigs in group B were vaccinated four times at about 7-day intervals between June 12 and July 7 using " High Titer Killed Vaccine " for veterinarian use (Kaketsuken Lot. 23). The vaccine was administered subcutaneously in the neck region.

3. *Collection of mosquitoes*

Mosquitoes were collected from July through August at several locations, as shown in Fig. 1. Mosquitoes were collected for one hour just after sunset using a modified " new dry ice baited trap " (Kato et al., 1966) in paddy fields and just outside the pig pens (#1, #2D, #3, #4D and #5 in Fig. 1 and Table 2) and with a net or by aspiration in the pens (#2N and #4N). The mosquitoes collected by each method were pooled separately. First the mosquitoes were kept to allow digestion of the blood of the pigs, and then they were anesthetized with chloroform and pooled by sex and species, and stored at −70 C. Then pools of 200 mosquitoes were used for inoculation of suckling mice.

4. *Virus isolation*

Isolation from swine : Blood specimens were obtained from one litter of 4 month old pigs in each pen (10 in A, 11 in B). Three ml of blood were taken from an ear vein using a syringe containing 1.0 ml of 0.002% heparin solution. The blood was stored in an ice bath until inoculation to suckling mice and centrifuged within one hour after being taken. The plasma was frozen at −20 C to avoid contamination with microorganisms.

One litter of 3 to 5-day old suckling mice (ICI strain) were inoculated intraperitoneally with 0.2 ml individual whole blood specimens using Simonsen's method (Simonsen et al., 1959). Isolation from the vector: Virus was isolated from vectors using pools of 200 female mosquitoes, (*Culex tritaeniorhynchus*). Homogenates were made by grinding the mosquitoes in a mortar with 2 ml of buffered saline (9.0 ml of 0.02 M $NaHPO_4$, 488.5 ml of physiological saline, 500 units/ml of penicillin, 500 μg/ml of streptomycin and 2% normal inactivated chicken serum (CSS)). The homogenate was stood for 1 hr in an ice bath, and then centrifuged at 10,000 rev/min for 30

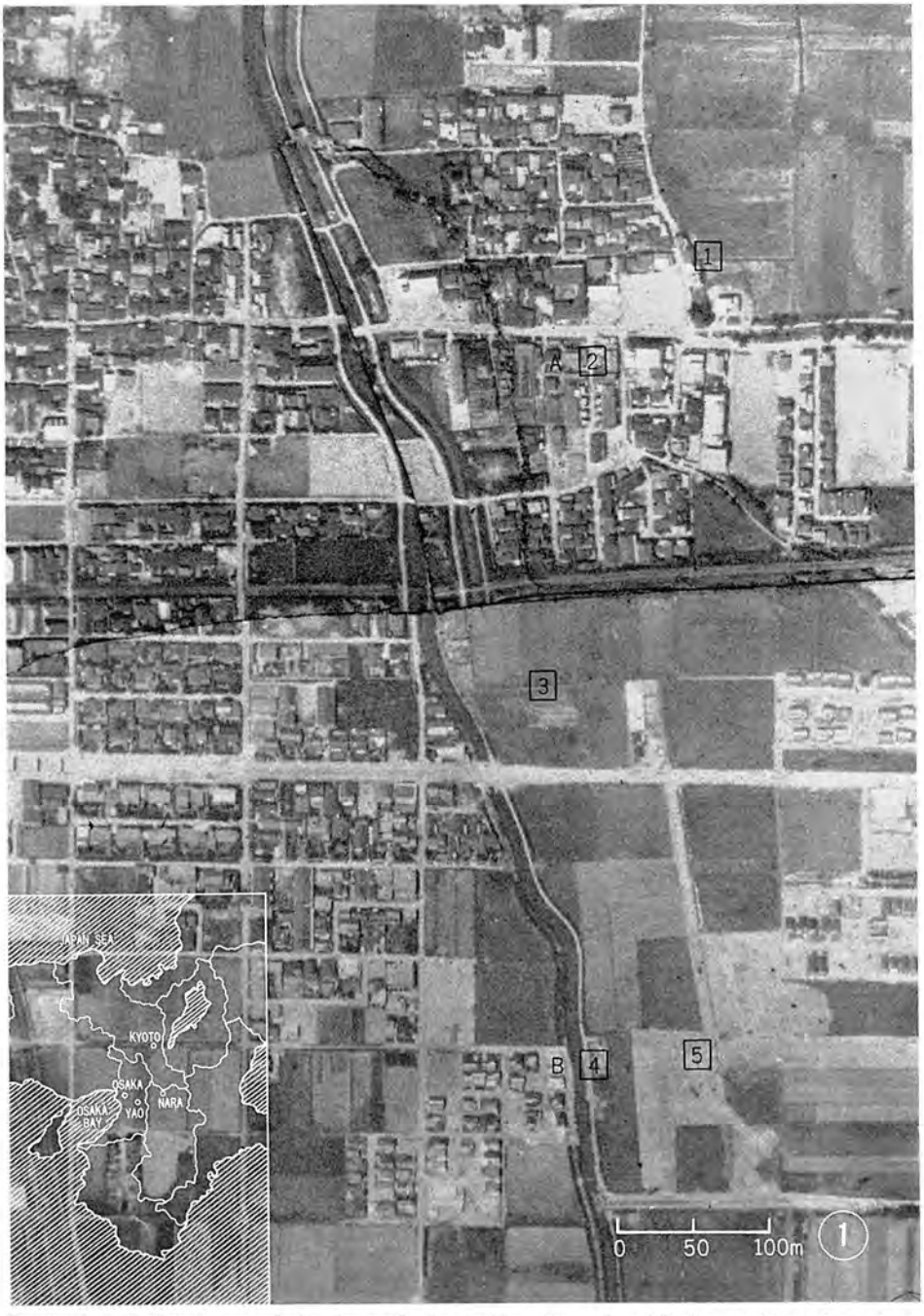

FIGURE 1. *Aerial photograph showing study sites in Yao city. A and B show positions of pig pens. Mosquitoes were collected at sites 1, 2, 3, 4 and 5. The location of this region in Osaka prefecture is shown in the inset.*

min in a refrigerated centrifuge. The resulting supernatant was injected intraperitoneally into a litter of mice as described above.

After inoculation mice were observed daily for 10 days. The brains of mice which became ill or died were ground in CSS to give 10% homogenates. The homogenates were centrifuged under the same conditions used for homogenates of mosquitoes and the supernatants were diluted to 10^{-3} and inoculated intracerebrally into suckling mice for serial trsanfer of virus. At the same time, the emulsion was also checked for bacterial and fungal contamination.

5. *Identification of the virus*

Isolated viruses were identified by the hemagglutination (HA) and hemagglutination inhibition (HI) tests, using the techniques described by Clarke and Casals (Clarke et al., 1961). For the HA test antigen was prepared from infected mouse brain at the second virus passage level by extraction with acetone-ether. For the HI test, mouse sera of anti-JaGAr O1 and anti-Nakayama NIH strains were used. The red blood cells used for the tests were obtained from one day old chicks. Sometimes virus was identified by the fluorescent antibody test (FAT) using the methods described elsewhere (Kimoto et al., 1968).

6. *Determination of antibody in plasms*

Plasma of both swine and rabbits were tested with standard HA antigen from mouse brain infected with JaGAr O1 strain. The plasma were also subjected to the HI test. Standard antigen and antisera were kindly provided by Dr. Oya, National Institute of Health, Japan, or were prepared by standard methods in his laboratory. The antibody titer is expressed as the reciprocal of the highest dilution of serum causing detectable inhibition of hemagglutination. Aliquots of some of these sera were treated with 2-mercapto ethanol (2-ME) to estimate the time of infection following the method of Konno et al. (Konno et al., 1967). When the HI titer of the serum was reduced to 1: 3 or less after treatment with 2-ME, it was regarded as 2-ME sensitive (19S) serum containing antibody.

RESULTS

1. *Isolation of virus from swine*

Table 1 shows results on the isolation of virus from 21 pigs in pens A (non-vaccinated) and B (vaccinated). Unexpectedly, virus was first isolated in the vaccinated group (B). In group B, 3 of 11 pigs showed viremia on July 5. In pig No. 4 in pen B the high antibody titer of 1: 1280 was recorded on July 3 so it seemed that the virus spread in the vaccinated group about 10 days earlier than in the control group.

In the control group viremia was detected in all pigs, but in the vaccinated group, 6 of 11 pigs did not show viremia and even when viremia was detected, its duration was shorter than in the control group. Some pigs, such as Nos. 6 and 9 in pen B were infected with JEV 2 or 3 weeks later than other pigs in the same group. Moreover, in spite of the presence of an HI antibody titer of 10, virus was isolated from the blood of pig No. 6 in pen B.

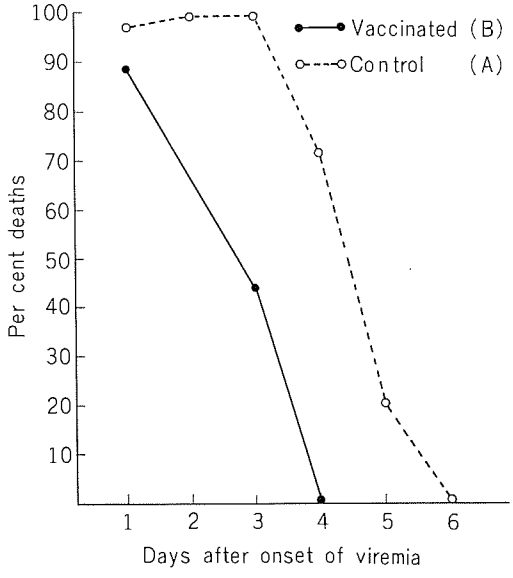

FIGURE 2. *Mortality of suckling mice. Mice were inoculated intraperitoneally with blood from swine with viremia*

TABLE 1. *Viremia and antibody responses in vaccinated and control swine*

	Swine No.	Jun 12	Jun 21	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16 (Jul)
Control	A-1	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-2	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		+	+	
	A-3	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-4	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-5	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-6	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-7	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-8	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		+	+	
	A-9	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	A-10	<10				<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
		↓	↓			↓				↓									
Vaccinated	B-1	10	<10			<10		<10		<10			320		320		20	160	
						—		+		+			—		—		—	—	
	B-2	20	10			<10		<10		<10			<10		20		80	40	
						—		—		—			—		—		—	—	
	B-3	10	<10			<10		<10		<10			160		320		640	320	
						—		—		+			—		—		—	—	
	B-4	<10	<10			1280		1280		1280			160		320		160	320	
						—		—		—			—		—		—	—	
	B-5	<10	<10			<10		<10		<10			<10		160		160	320	
						—		—		—			—		—		—	—	
	B-6	10	10			<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	B-7	10	10			<10		<10		<10			80		80		160	80	
						—		—		—			—		—		—	—	
	B-8	<10	<10			<10		<10		<10			160		80		80	160	
			—			—		+		—			—		—		—	—	
	B-9	20	<10			<10		<10		<10			<10		<10		<10	<10	
						—		—		—			—		—		—	—	
	B-10	<10	<10			<10		<10		<10			160		640		640	160	
			—			—		+		—			—		—		—	—	
	B-11	<10	<10			<10		<10		<10			<10		<10		80	640	
						—		—		—			—		—		—	—	

The arrows indicate dates of administration of JE vaccine (3.0 ml/dose). Numbers indicate HI titers of sera. + and — indicates the presence and absence of viremia in swine.

TABLE 1. Continued

															Aug		
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1	8	25
<10 −	<10 −	<10 −	<10 −	<10 −	<10 −		<10 +	<10 +	<10 +	<10 +	40 −			640 −		640	640
<10 +	40 −	1280 −	640 −	1280 −			1280 −		1280 −		1280 −			2560 −		320	640
<10 −	<10 −	<10 −	<10 −	<10 −	<10 −		<10 +	<10 +	<10 +	<10 +	80 −			640 −		1280	1280
<10 −	<10 −	<10 −	<10 −	<10 −	<10 −		<10 +	<10 +	<10 +	<10 +	160 −			1280 −		640	1280
<10 −	<10 −	<10 −	<10 −	<10 −	<10 −		<10 −	<10 +	<10 +	<10 +	20 −			1280 −		320	1280
<10 −	<10 +	<10 +	<10 +	<10 +	20 +		1280 −	640 −	1280 −		1280 −			1280 −		1280	1280
<10 +	<10 +	<10 +	<10 +	80 −	160 −		1280 −	1280 −	2560 −		1280 −			1280 −		640	1280
<10 +	20 +	640 −	640 −	1280 −			1280 −		1280 −		640 −			1280 −		1280	640
<10 −	<10 +	<10 +	<10 +	<10 +	20 +		640 −	640 −	1280 −		640 −			1280 −		640	1280
<10 −	<10 −	<10 −	<10 −	<10 −	<10 +		<10 +	<10 +	80 −	40 −	1280 −			1280 −		640	1280
160 −		160 −		80 −			80 −		160 −		80 −			160 −		80	320
160 −		80 −		160 −			320 −		80 −		80 −			80 −		40	80
320 −		640 −		640 −			640 −		320 −		320 −			320 −		80	160
640 −		40 −		320 −			80 −		160 −		80 −			160 −		40	80
320 −		320 −		640 −			80 −		160 −		80 −			160 −		40	80
<10 −	<10 −	10 −	10 −	10 −			10 −		10 +		40 −			640 −		160	160
160 −	40 −	160 −		320 −			1280 −		80 −		80 −			160 −		40	40
320 −		640 −		320 −			320 −		160 −		80 −			160 −		160	
<10 −		<10 −	<10 −	<10 −	<10 −		<10 −	<10 −	<10 −	<10 −	20 −			80 −		80	40
640 −	1280 −	2560 −		1280 −			160 −		1280 −		1280 −			1280 −		640	
1280 −	1280 −	640 −		640 −			640 −		640 −		320 −			640 −		320	

An antibody response to natural infection with JEV was seen in all 10 pigs in group A. Table 1 shows several interesting phenomena, such as the variation in the time of infection with virus within a litter and the simultaneous detection of virus and antibody in pigs Nos. 6, 8 and 9 in group A. Fig. 2 shows the mortality of suckling mice inoculated with blood obtained from pigs after the onset of viremia. Mortality was less among mice inoculated with

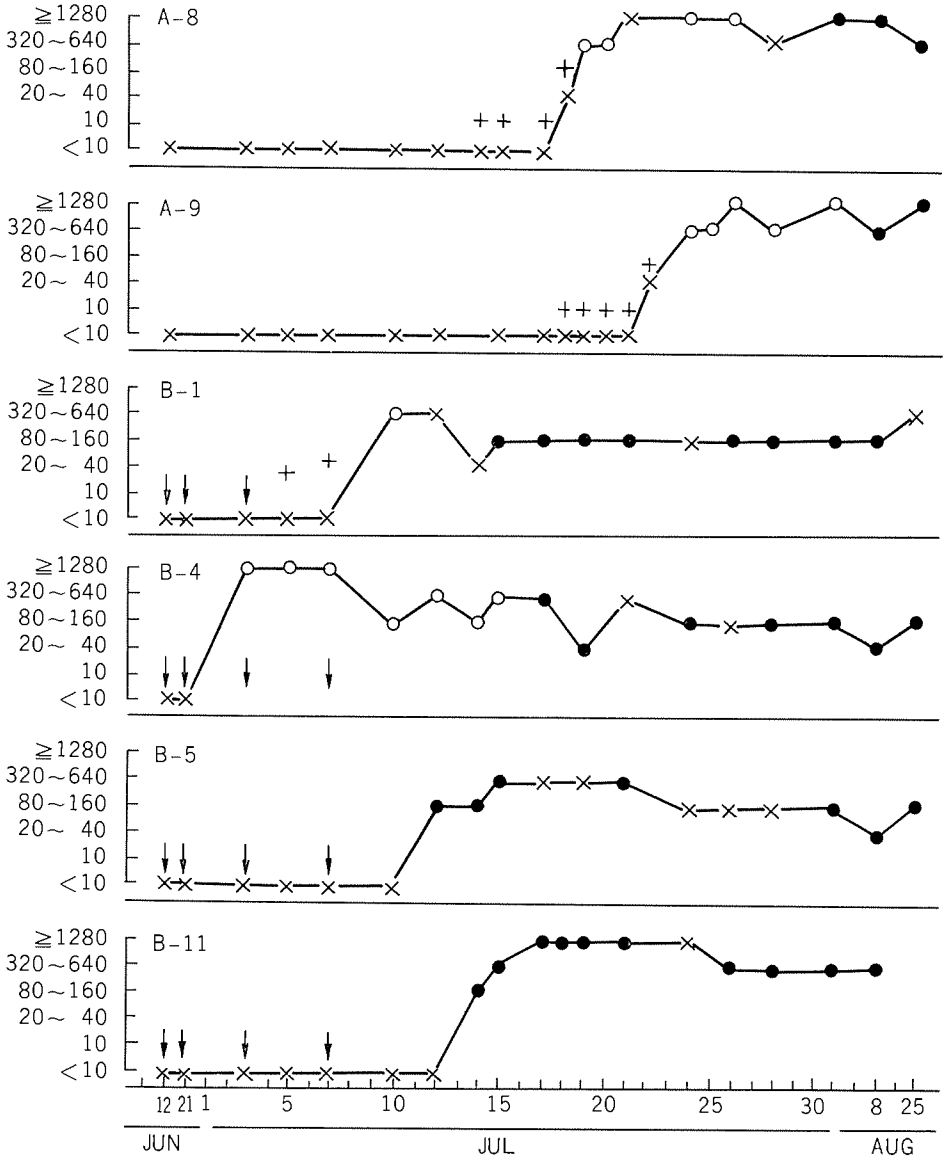

FIGURE 3. *Nature of antibody in individual pigs naturally infected with JEV. The numbers of pigs are the same as in Table 1. The arrows indicate times of administration of JE vaccine.*
+, *virus positive;* ○, *2-ME sensitive;* ●, *2-ME resistant;* ×, *not tested with 2-ME.*

blood from vaccinated pigs than among those inoculated with blood from controls. After recovery from viremia, the antibody titer of group A was generally higher than that of group B. Some sera of pigs in groups A and B were treated with 2-ME and results are shown in Figure 3. The effects of 2-ME on the antibody of pigs in the two groups differed. Antibody of 19 S remained for one to two weeks in group A while, in group B, it soon shifted from 19 S in all but one serum (No. 4).

2. *Isolation of virus from mosquitoes*

A total of 97,000 female mosquitoes (*Culex tritaeniorhynchus-vishnui* complex) were obtained from the five sites and were examined for the presence of virus. The results are summarized in Table 2. JE virus was first isolated from a pooled sample of mosquitoes caught by in a net on July 5 in pen B after pigs had been vaccinated. Then virus were isolated on July 19 at site No. 3, on July 21 at No. 5, on July 24 at No. 2 and on July 26 at No. 1. In all, JEV was found in 38 of the 486 pools of mosquitoes examined. The isolation rate at site No. 4 throughout the observation period was 21.0 per cent, being higher than that at site No. 2 (isolation rate, 10.6%). The rates at sites No. 1, No. 3 and No. 5 on rice farms similar ranging from 5.5 to 6.8%. July 5 is the earliest date that we have isolated JEV from vector mosquitoes since the beginning of our field works in 1963. Usually, we isolated viruses during the period from late July to mid-

TABLE 2. *Isolation of Japanese encephalitis virus from vector mosquitose collected at five sites*

Date	Site 1	Site 2		Site 3	Site 4		Site 5	Total	Isolation rate
	D	D	N	D	D	N	D		
Jul. 5			0/1			1/3		1/4	25.0
7			0/1			0/2		0/3	0.0
14	0/1	0/1			0/4			0/6	0.0
17	0/2	0/1		0/3	1/15		0/12	1/33	3.1
19	0/11	0/8	0/10	1/10		2/9	0/9	3/57	5.3
21	0/1	0/1	0/1	0/3	0/5	4/6	1/10	5/27	18.5
24	0/2	0/3	1/1	1/4	0/2	0/4	0/10	2/26	7.7
26	1/12	0/10	3/5	1/11		3/9	0/10	8/57	13.8
28	1/11	0/3	2/6	2/12		3/9	3/9	11/50	22.0
31	2/10	0/11	1/9	0/12		0/10	2/9	5/61	8.2
Aug. 4		0/10	0/10	0/9			0/2	0/31	0.0
8	2/6	0/6	0/12					2/24	8.3
11	0/8							0/8	0.0
15	0/6			0/10		0/10	0/10	0/36	0.0
18	0/12							0/12	0.0
23	0/12							0/12	0.0
29	0/7		0/10		0/4		0/10	0/31	0.0
Sep. 5	0/8							0/8	0.0
Total	6/109	0/54	7/66	5/74	1/30	13/62	6/91	38/486	7.8
Isolation rate	5.5	0.0	10.6	6.8	3.3	21.0	6.6	7.8	

D: Dry ice bait trap including light. N: Netting or sweeping.
/: Virus positive pools number of mosquito pools.

TABLE 3. *Natural infection of rabbits in pig pens with JEV*

Pig pen	Rabbit No.	July						August						
		17	19	21	24	28	31	4	8	11	15	18	23	25
A	91	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	<10 +	80 −	40 −
	95	<10 −	<10 −	<10 −	D									
	96	<10 −	<10 −	<10 −	<10 −	<10 −	<10 +	<10 −	20 −	80 −	640 −			320 −
B	87	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	40 −	80 −	160 −	320 −			160 −
	88	<10 −	<10 −	<10 −	D									
	89	<10 −	<10 −	<10 −	<10 −	<10 −	<10 −	<10 +	<10 −	160	640			640
	94							<10 −	20 −	160 −	320 −			320 −
	97	<10 −	<10 −	<10 −	<10 −	<10 +	<10 −	160 −	80 −	160 −	1280 −			1280 −

D: Death. Symbols and numerals are as in Table 1.

August in Osaka prefecture.

It is also interesting that mosquitoes caught by different methods showed significantly different rates of virus isolation: mosquitoes collected in a " new dry ice baited trap " showed a lower isolation rate than mosquitoes caught by netting or sweeping ($\chi^2=12.9$, $\alpha<0.01$).

3. *Infection of indicator animals with JEV*

Table 3 shows the rates of virus isolation and HI antibodies in blood of rabbits which were kept in pens A and B. All the rabbits in both pens were infected with JEV at the same time in late July and produced antibody. In some instances, virus was isolated from the blood, as in the case of swine. The period of infection of rabbits with JEV was shorter than that of pigs.

DISCUSSION

The times of infection of swine with JEV and of isolation of virus from mosquitoes in pens A (control) and B (vaccinated) are summarized in Fig. 4. Unexpectedly virus was isolated from mosquitoes caught in pen B, and a high HI antibody titer was observed in swine in this pen earlier than in pen A. From these results the efficacy of vaccination seems doubtful. However, vaccination seems to be effective in blocking the spread of JEV in swine for the following reasons: (1) viremia was detected in all the pigs in group A but in only 45 per cent of those in group B, (2) viremia lasted 3 to 6 days in group A, but less in group B, (3) after virus isolation the average HI antibody titers of swine in group B were lower than those in group A, and (4) as shown in Fig. 3, the HI antibody in the sera in groups A and B after natural infection responded differently to 2-ME treatment. The titers of virus in the blood specimens were not estimated, but Fig. 2 shows that there was an apparent difference between the virus titers in groups A and B. Vaccination did not prevent the occurrence of viremia in swine but reduced their capacity to amplify JEV.

On July 3, at the time of the third vaccination, pig No. 4 in group B had a high HI antibody titer. This was probably due to natural

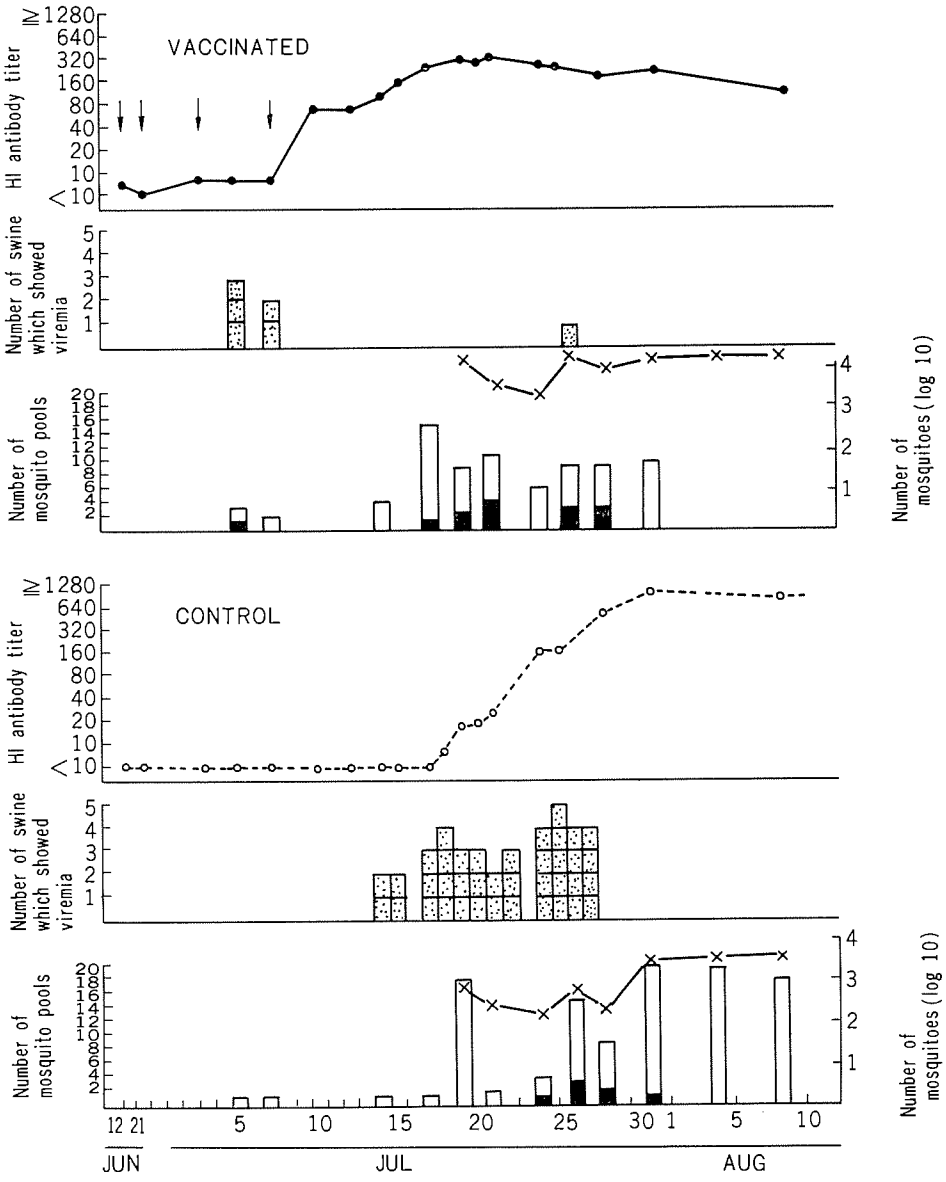

FIGURE 4. *Relationship of times of JEV infection of swine and vector mosquitoes in control (A) and vaccinated (B) pig pens. open circles (○) and closed circles (●) indicate geometric mean HI titers in the control group and vaccinated group, respectively. Crosses (×) show the number of vector mosquitoes collected (females of Culex tritaeniorynchus) ▒, individuals with viremia; □, number of pools tested; ■, numbers of pools with JEV; Arrows indicate times of administration of JE vaccine.*

infection with JEV, since an antibody titer of as high as 1: 1280 can not usually be obtained by vaccination with inactivated JEV and the response of the antibody in this pig to 2-ME was quite similar to that of antibody from animals in group A (Fig. 3).

Various investigators have suggested that swine are the most important amplifiers (Scherer et al., 1959) in Japan but their response at natural infection with JEV, (e.g., the duration of viremia, virus titer in the blood and antibody titer) have not been studied in detail. The data show that the chance of infection extends over a period of two to three weeks, even in pigs in the same litter in the same pen, that viremia lasts for 3 to 6 days and that production of antibody reduces the virus in the blood.

The average duration of viremia was found to be 3.8–4.7 days. This is one or two days longer than the period of 2.6 days reported by Nakamura (Nakamura et al., 1964). Scherer reported that viremia lasts for 4 days in swine on experimental infection with JEV (Scherer et al., 1959b). It has been considered that JEV infection of swine in a certain area all occurs within a short period, but it seems to be a sporadic phenomenon in some areas.

Virus was isolated from the blood in spite of the presence of detectable HI antibody in 3 of 10 pigs in group A and 1 of 11 in group B. These results may be interpreted in terms of a quantitative relationship between antigen and antibody. The time relationship of the antibody titer to the clearance of virus suggests that antibody may be important in elimination of JEV from the blood stream. Barker also reported a time relationship between the appearance of humoral antibody and parasitemia in experiments with protozoa (Barker et al., 1971). At site No. 4 the rate of virus isolation was significantly higher than at other sites and the time of virus isolation was also earlier than elsewhere. Thus the virus probably spread from site No. 4 to sites No. 3, 2 and 1 by infected vectors, although, the speed of spread seems slow judging from reports on the spread of mosquitoes (Provost et al., 1952; Uemoto et al., 1967; Wada et al., 1969). It also seems likely that, spread of virus in groups A and B, respectively occurred independently.

Results obtained on collection of vector mosquitoes with light traps are shown in Table 2 and Fig. 4. Fewer mosquitoes were usually caught at site 2 than at site 4. Pig pen A is surrounded by houses, so some mosquitoes might not have been able to reach the shelter (Fig. 1). The rates of virus isolation differed significantly with different collection methods. Using the " new dry ice baited trap " method the isolation rate was usually less than that using sweeping or netting. Buei et al. reported that the physiological age of mosquitoes caught by different methods varied (Buei et al., 1968). We did not examine the age of the mosquitoes we caught but our results seem to support their data. This will be discussed in detail elsewhere.

Our results showed that the rate of virus isolation of mosquitoes in pig pen B was higher than in pen A. This result does not support the idea of the efficacy of vaccination of the amplifier. Recently we reported that vaccination of swine seems to be valuable in preventing the amplification of JEV, judging from the rate of virus isolation from mosquitoes (Ueba et al., 1971). This is incompatible with the results in this paper. However the most important findings in the present work were that the extent of viremia, the virus titer and the duration of viremia, were reduced remarkably by vaccination of swine, as shown in Table 1 and Fig. 2. To elucidate the results in detail, the following factors should be examined: the area of vaccination of swine, the potency of the vaccine used and the migration of vector mosquitoes.

All the rabbits bred in both pig pens were infected with JEV. Therefore these animals could not be used as indicators to study the effectiveness of vaccination of swine. However, interesting results were obtained on natural infection of rabbits by JEV: namely, viremia was detectable in rabbits and rabbits

were infected with JEV later than swine.

From the present results it seems that the best way to prevent JEV is to vaccinate swine before the epidemic season. Use of improved vaccine, such as live attenuated vaccine (Kodama et al., 1967; Tsuchiya et al., 1970) or adjuvant vaccine (Ando, 1971) and better procedures of vaccination should also be examined.

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