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FIELD EXPERIMENTS ON LIVE ATTENUATED JAPANESE ENCEPHALITIS VIRUS VACCINE FOR SWINE[1]

NOBORU UEBA, TOMOAKI KIMURA, SADAO NAKAJIMA, TAKASHI KURIMURA[2] and TOSHIYUKI KITAURA

Virus Laboratory, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-ku, Osaka 537, Japan

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SUMMARY The efficacy of a live attenuated Japanese encephalitis virus (JEV) vaccine was examined in swine under conditions where natural infection could occur. The pigs immunized with the vaccine produced antibodies within one week after vaccination, and the antibody was retained until the end of the experiment, i.e. 36 days. However, the antibody titers in this group were lower than that in control group naturally infected with JEV. No virus was isolated from the five vaccinated pigs, but virus was isolated from all four untreated control pigs after natural infection, i.e., viremia was detected in all these animals. The duration of viremia in control pigs varied from one to four days.

From these findings, it is concluded that immunization of swine with live attenuated JEV vaccine is useful in control of Japanese encephalitis (JE) in humans and some susceptible domestic animals.

INTRODUCTION

Since the first isolation of JEV in 1935, ecological studies on the virus have been performed by many investigators. The vector was shown to be mosquitoes by Mitamura et al. (1938) and the term "amplifier" was advocated for pigs and some birds by Scherer et al. (1959a) and Buescher and Scherer (1959). The virus multiplies in many hosts, such as man, domestic animals including pigs, birds and arthropods, with or without clinical signs. Swine do not show obvious symptoms after infection with the virus unless they are pregnant, and thus infection of swine with JEV is not an important problem in animal husbandry. But, since pigs act as amplifiers JEV infection in pigs is important from the standpoint of public health. The conception of amplifiers has been confirmed by epidemiological studies (Konno et al., 1966; Oya, 1967; Buei, et al., 1968; Kusuda et al., 1968; Ishida et al., 1969; Otsuka et al., 1969; Yamamoto and Manako, 1970; Yamada et al., 1971; Fukumi et al.,

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2 Present address: Department of Virology, Tottori University School of Medicine, Yonago, Tottori.

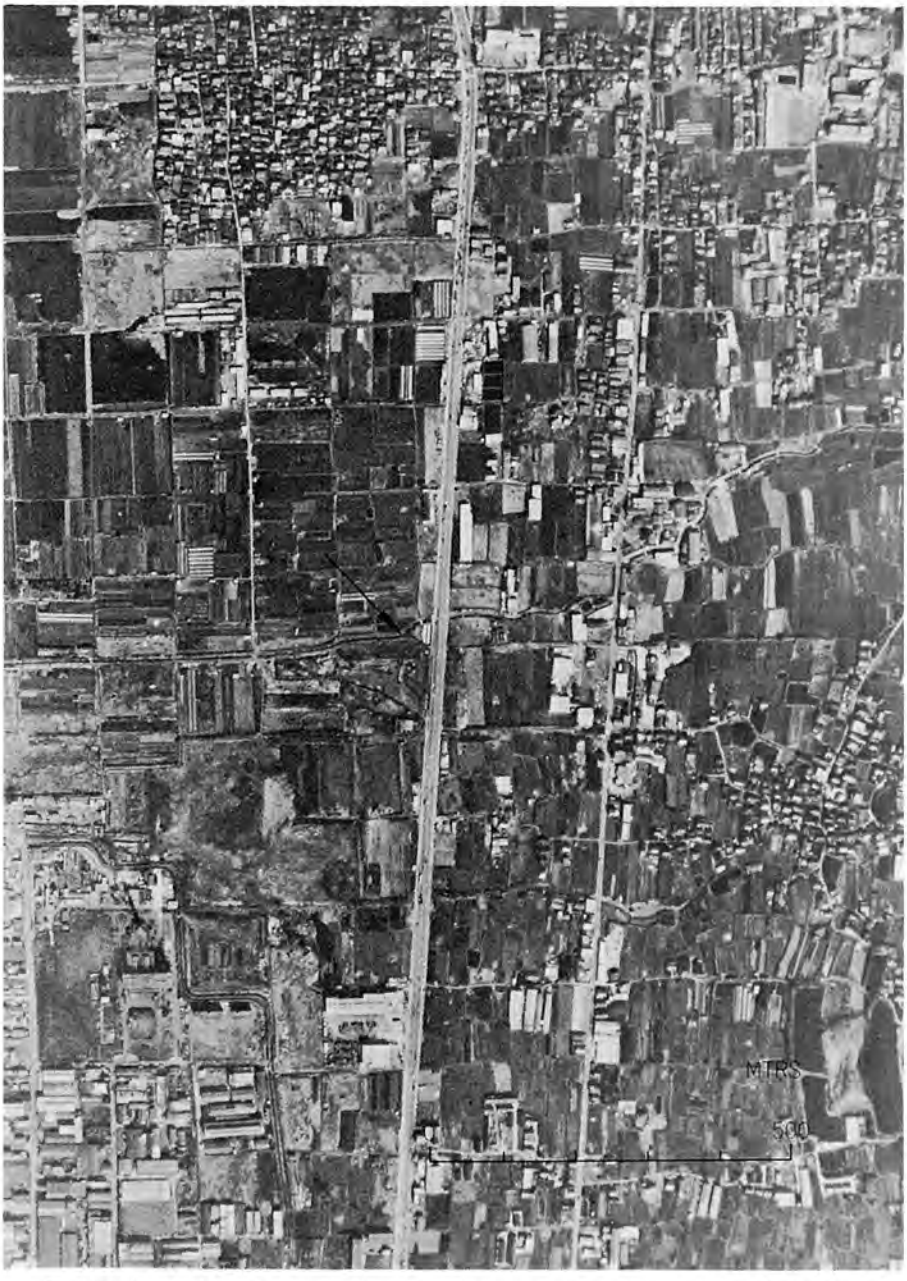

FIGURE 1. Aerial photograph showing the pig pen (arrow) in Higashi-Osaka City.

1975). We have also shown that the infection rate of humans, rabbits and hens, is closely related to the population density of swine (Ueba et al., 1971), and it is now generally accepted that swine are the most important amplifiers of JEV during the epidemic season in Japan. Therefore, experiments on immunization of swine have been conducted using formalin-treated or live JEV vaccines in the hope of reducing the number of infected mosquitoes (Oya, 1967; Takahashi et al., 1968; Tsuchiya et al., 1970; Ueba et al., 1972). However, there are no direct proofs that live JEV vaccine prevents viremia of pigs. In this work we examined whether a live attenuated JEV vaccine could prevent viremia after natural infection.

MATERIALS AND METHODS

1. *Study site and pigs*

Tests were made on pigs on Yokosyoji, Higashi-Osaka City, Osaka Prefecture. As shown in Fig. 1, the pigs were in a pen surrounded by paddy fields and houses, and there were about 150 pigs in this pen. Nine weanling litter-mates of four months old of a Yorkshire-Landrace hybrid pig were chosen for experiments, and studies were made from July 14 to September 1, 1975.

2. *Vaccination*

Five of the 9 pigs were vaccinated once on July 21 with live attenuated JEV vaccine. The vaccine was a commercially available lyophilized vaccine, Lot L-4, produced in monkey kidney cell culture by the Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa. One milliliter of this vaccine was injected subcutaneously in the neck region of the swine. The other 4 pigs served as controls. Vaccinated and untreated pigs were ear marked and bred in the same box of the pen.

3. *Virus isolation from swine*

Specimens of 3 ml of blood were taken from an ear vein of the pigs using a syringe containing 1.0 ml of 0.002% heparin solution. The blood samples were stored in an ice bath until inoculated into suckling mice. After inoculation, plasma specimens of pigs for antibody tests were separated by centrifugation in the cold and then frozen at −20 C until use.

One litter of 3- to 5-day-old suckling mice were inoculated intracerebrally with 0.025 ml-aliquots of whole blood.

4. *Identification of the agents*

Isolated agents were identified by the hemagglutination (HA), hemagglutination inhibition (HI), complement fixation (CF) and neutralization tests. The HA, HI and CF tests were done as described by Clarke and Casals (1958). The antigens for the HA and CF tests were prepared from infected mouse brain at the second virus passage level by extraction with acetone-ether. Two laboratory strains, Nakayama NIH and JaGAr Ol, were used as standard strains of JEV. Hyperimmune sera to standard or wild JEV strains were prepared in mice, and were used for the HI, CF and neutralization tests. Fresh guinea pig serum at a final dilution of 1 : 24 was used as complement in the neutralization test.

5. *Determination of antibody in plasma*

Plasma samples of swine were treated with cold acetone and absorbed with red blood cells from one-day-old chicks to avoid non-specific reactions of inhibitors. The treated samples were heated at 56 C for 30 min before use. Red blood cells from one-day-old chicks were used as indicator cells. Commercial HA antigens (Takeda Chemical Industries, Ltd. Osaka, Japan) of the standard JEV strains were used in the HI test. The antibody titer is expressed as the reciprocal of the highest dilution of plasma causing detectable inhibition of hemagglutination.

RESULTS

1. *Effect of previous vaccination on viremia caused by natural infection*

Blood specimens were taken daily from vaccinated and control pigs to test for viremia and the antibody response to natural infection. Table 1 shows the results on isolation of virus from the blood of pigs in the two groups. In all four pigs, p-1, p-4, p-5 and p-9, in the unvaccinated control group, virus was found for one to four days, and a total of eight specimens gave positive results for virus. On

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

Vaccine	Pig	JUL 14	21	28	29	30	31	1	2	3	4	5	6	7
No	p-1	<10[b]		—[a] <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10
	p-4	<10		+ <10	— <10	— <10	— 80	— 160	— 640	— 640	— 640	— 640	— 640	— 640
	p-5	<10		+ <10	— <10	— <10	— 20	— 40	— 80	— 320	— 160	— 160	— 320	— 320
	p-9	<10		— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10	— <10
Yes	p-2	<10	↓[c]	— 40	— 40	— 80	— 80	— 40	— 40	— 80	— 40	— 40	— 40	— 40
	p-3	<10	↓	— 40	— 20	— 40	— 40	— 40	— 40	— 80	— 80	— 80	— 160	— 160
	p-6	<10	↓	— 80	— 20	— 40	— 80	— 40	— 40	— 40	— 40	— 40	— 80	— 80
	p-7	<10	↓	— 20	— 20	— 20	— 20	— 20	— 20	— 20	— 20	— 20	— 40	— 40
	p-8	<10	↓	— 80	— 80	— 40	— 80	— 80	— 80	— 160	— 80	— 80	— 320	— 160

the other hand, no virus was isolated from five pigs, p-2, p-3, p-6, p-7 and p-8 immunized with the vaccine before natural infection. One pig in the vaccinated group, p-2, died in an accident during the experiment. The difference in the rates of virus isolation in the two groups is clearly due to vaccination.

2. *Antibody responses of vaccinated and naturally infected pigs*

The HI antibody responses against JEV in vaccinated and control pigs are shown in Table 1 and Fig. 2. HI antibody titers of 1:20 to 1:80 were found in vaccinated pigs 7 days after vaccination and the titers tended to increase slightly during the experiment. Antibody production in control pigs was closely related with viremia: antibody was first detected soon after the development of viremia. The antibody titers of naturally infected pigs rapidly rose to high levels, which persisted throughout the experiment. The mean HI an-

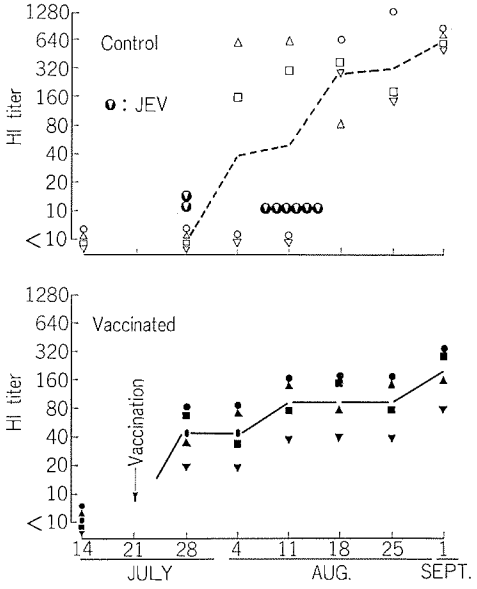

FIGURE 2. Antibody responses in naturally infected and vaccinated swine. Solid and dotted lines indicate the mean antibody titers in each group. Each different symbol in the figure represents results from different swine at the indicated time.

AUG														SEP
8	9	11	12	13	14	15	16	18	19	20	21	22	25	1
— <10	— <10	— <10	+ <10	+ <10	+ <10	+ <10	— 10	— 640	— 1280	— 1280	— 640	— 640	— 1280	640
— 640	— 320	— 640	— 640	— 640	— 320	— 320	— 160	— 80	— 	— 320	— 	— 320	— 	640
— 320	— 320	— 320	— 160	— 320	— 320	— 160	— 320	— 320	— 320	— 320	— 160	— 160	— 160	640
— <10	+ <10	+ <10	— <10	— 40	— 160	— 320	— 320	— 320	— 640	— 320	— 320	— 160	— 160	640
— 10	†[d]													
— 160	— 160	— 160	— 160	— 160	— 160	— 160	— 160	— 80	— 80	— 160	— 80	— 160	— 160	160
— 80	— 40	— 80	— 80	— 80	— 80	— 80	— 80	— 160	— 80	— 80	— 80	— 80	— 80	320
— 40	— 80	— 40	— 40	— 80	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 40	— 80
— 320	— 160	— 160	— 160	— 160	— 160	— 160	— 160	— 160	— 80	— 80	— 80	— 80	— 160	320

[a] Virus isolation.
[b] HI titer of plasma.
[c] Administration of JEV vaccine.
[d] Death.

tibody titer in the unvaccinated control group was higher than that in the vaccinated group. These data suggest that the antibody in vaccinated pigs was effective for protection against natural infection and antigenic stimulation by naturally occurring virus.

3. *Identification of virus in blood from unvaccinated pigs*

HA, HI, CF and neutralization tests were carried out to identify the virus in the eight samples of blood. The HA activities of the newly isolated virus and the standard JEV strain are shown in Table 2. The highest HA activity of the isolated virus and a standard JEV strain, JaGAr 01, were exhibited at pH 6.6 to 7.0 and the titers were 1: 800 to 1: 3200. The HA titer of the other JEV strain, Nakayama NIH, was 1: 12800 at the optimal pH of 6.4. Among the strains tested this virus gave the highest titer.

These HA activities were specifically inhibited by standard anti-JEV mouse sera. The data are shown in Table 3. The CF test was also carried out on the isolated virus, and the results are shown in Table 4. Cross-reactions were observed between the newly isolated strains and anti-standard JEV sera.

TABLE 2. *HA tests with eight isolates from blood of swine and standard JEV strains*

	Name of strain or isolate	Optimal pH	Titer
Standard strain	Nakayama NIH	6.4	12800
	JaGAr 01	6.8	3200
Newly isolated virus	JaOAr-17-75	6.8	3200
	JaOAr-21-75	6.6	800
	JaOAr-24-75	7.0	1600
	JaOAr-36-75	6.8	800
	JaOAr-51-75	7.0	3200
	JaOAr-52-75	6.8	800
	JaOAr-107-75	6.8	3200
	JaOAr-110-75	6.6	3200

The CF titers on cross-reaction with the standard JEV strain were similar with those of standard strains.

Results of the neutralization test are shown in Table 5. The infective titers of isolated virus were reduced from 2.25 to 5.00 log values by anti-standard and wild JEV sera. The neutralization indices of the sera with the standard JEV strains and newly isolated ones

TABLE 3. *Titers obtained in HI tests using standard antisera and antigens prepared from newly isolated and standard JEV strains*

	Strain	HI antibody titer	
		anti-Nakayama NIH	anti-JaGAr 01
Standard strain	Nakayama NIH	5120	320
	JaGAr 01	2560	1280
Newly isolated virus	JaOAr-17-75	2560	640
	JaOAr-21-75	2560	1280
	JaOAr-24-75	2560	1280
	JaOAr-36-75	1280	640
	JaOAr-51-75	1280	1280
	JaOAr-52-75	1280	640
	JaOAr-107-75	5120	1280
	JaOAr-110-75	2560	1280

TABLE 4. *Complement fixation tests with eight strains isolated from blood of swine and standard JEV strains*

	Antigen	Antiserum	
		Nakayama NIH	JaGAr 01
Standard strain	Nakayama NIH	64[a]/128[b]	128/ 64
	JaGAr 01	64 / 64	256/ 64
Newly isolated virus	JaOAr-17-75	16 / 64	128/ 32
	JaOAr-21-75	64 / 32	128/ 32
	JaOAr-24-75	64 / 64	128/ 32
	JaOAr-36-75	32 / 32	128/ 32
	JaOAr-51-75	64 /128	128/ 32
	JaOAr-52-75	32 / 64	128/ 32
	JaOAr-107-75	64 / 64	128/ 32
	JaOAr-110-75	64 /128	128/128

[a] Antigen titer to four units of antisera.
[b] Antibody titer to four units of antigen.

TABLE 5. *Neutralization tests with eight samples isolated from blood of swine, two JEV standard strains and a wild strain isolated from mosquitoes*

	Strain	Log neutralization index with			
		anti-Nakayama NIH	anti-JaGAr 01	anti-JaOAr-121-71	normal serum
Standard strain	Nakayama NIH	5.00	4.00	5.00	0.50
	JaGAr 01	3.50	2.50	3.75	0.75
	JaOAr-121-71[a]	4.00	3.75	3.75	0.50
Newly isolated virus	JaOAr-17-75	2.75	2.25	3.00	0.00
	JaOAr-21-75	3.75	3.50	4.75	0.00
	JaOAr-24-75	3.50	3.00	3.50	0.50
	JaOAr-36-75	3.50	3.50	3.50	0.25
	JaOAr-51-75	3.75	3.25	5.00	0.50
	JaOAr-52-75	3.75	3.50	4.25	0.25
	JaOAr-107-75	3.50	3.25	3.25	0.00
	JaOAr-110-75	3.50	3.25	3.50	0.25

[a] This strain was isolated from a pool of vector mosquito, *Culex tritaeniorhyncus*, collected in 1971 at Izumi city, Osaka prefecture.

were similar. Normal mouse serum did not reduce the infective titer of these strains.

From these virological and serological tests, it was concluded that all the virus specimens isolated from unvaccinated swine were JEV.

DISCUSSION

JEV infection in swine has been reviewed by Fujisaki (1971). Pigs do not show any clinical signs after infection with JEV. However, when pregnant pigs are infected with the virus, abnormal delivery, such as stillbirth, abortion or premature delivery, often occurs (Shimizu and Kawakami, 1949; Shimizu et al., 1954). Consequently JEV vaccines have mainly been used to prevent JEV infection of pregnant pigs (Kawakubo et al., 1966; Hsu et al., 1972).

Since it has been shown that pigs are the most important animals as sources of infection of vector mosquitoes, immunization of swine with JEV vaccines should be effective in preventing JEV infection of humans. The first field tests on this possibility were conducted by Oya in 1964 (Oya, 1967). Later similar tests were made by Takahashi et al., (1968) and Tsuchiya et al., (1970) in Nagasaki and Kyoto, respectively. In these experiments the efficacy of vaccination was shown by the antibody response in pigs and the rate of infection of vector mosquitoes. We have also reported a similar test with inactivated vaccine (Ueba et al., 1972). In this test we detected antibody production in vaccinated pigs. We also found that the rate of isolation of virus from vaccinated pigs was lower than that from control pigs and that the rate of infection of vector mosquitoes in the vaccinated area was significantly lower than that in a control area. But five of eleven vaccinated pigs showed viremia for one to three days after natural infection. In the present experiment with live attenuated JEV vaccine, no virus was isolated from immunized pigs, whereas viremia was demonstrated in all control pigs.

Swine immunized with live JEV vaccine produced antibodies soon after a single vaccination, and the titers were higher than those in our previous test with killed vaccine (Ueba et al., 1972). The antibody titers in immunized pigs were slightly lower than those of controls after natural infection and a booster effect of natural infection was recognized in the vaccinated group without any manifestation of viremia (Fig. 2). The results on the antibody response after natural infection are similar to the data reported by Takahashi et al. (1968) and our own previous data (Ueba et al., 1972). The extent of the antibody response in pigs seems to be related to the extent of viremia. When pigs were infected with JEV, viremia persisted for 3 to 5 days (Nakamura et al., 1964; Ueba et al., 1972). Scherer et al. (1959b) reported that viremia lasted for 4 days in swine after experimental infection with JEV. In p-4 and p-5 viremia lasted 3 and 4 days, respectively, but in p-1 and p-9 it lasted one day. The blood samples collected on July 29 and 30 were stored at -70 C for 3 to 4 days, because no suckling mice were available for virus isolation at that time, and this storage may have reduced the isolation rate. Antibodies were usually produced in pigs at the time when viremia disappeared, but in one sample early antibody was found with infectious virus (Ueba et al., 1972).

All eight virus specimens isolated from unvaccinated control pigs were identified as JEV by various virological and serological tests. The HA patterns of these isolated specimens resembled those of the JaGAr 01 strain more closely than those of the Nakayama NIH strain. The optimal pH for HA activity was 6.6 to 7.0.

Now that farm-machinery is widely used in Japan, few domestic animals such as horses and cattle are kept on the farms. However, there are still many pigs in suburban and rural areas. The turnover of the swine population is rapid, because most pigs are slaughtered for meat at the age of 5 to 6 months. *Culex tritaeniorhyncus*, the main vector of JEV in Japan, shows a host preference for pigs (Oya, 1962; Wada, 1969) and after infection, viremia

in pigs lasts 2 to 5 days. Thus swine are the main source of infection of mosquitoes with JEV. Therefore, immunization of swine with live attenuated JEV vaccine is a useful procedure for preventing infection of vector mosquitoes.

A theoretical model of the prevalence of JE was proposed by Wada (1975). He pointed out that the density of vector mosquitoes had an important influence on the prevalence of JE, and that when the number of mosquitoes increased over a certain limit, vaccination of swine would be ineffective in control of JEV. Another problem about vaccination is that when maternal antibody is present in the blood vaccination with killed or live vaccine has little or no effect on production of antibody (Ogata et al., 1970; Ueba et al., unpublished data). Although there are these problems about vaccination of swine with live vaccine, this method, as well as vector control and vaccination of humans, is a useful procedure for eradicating JE from regions where the virus is endemic.

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