BIKEN JOURNAL Vol. 24 47-67, 1981

ESTABLISHMENT OF AN ATTENUATED ML-17 STRAIN OF JAPANESE ENCEPHALITIS VIRUS

IWAO YOSHIDA, MITSUO TAKAGI, ETSUO INOKUMA, HIDEO GODA, KOICHI ONO, KEISUKE TAKAKU and JUICHI OKU

Kan-onji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kan-onji, Kagawa 768, Japan

NOBUHARU KUNITA

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

TSUNEHISA AMANO and YOSHIOMI OKUNO[1]

Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka 565, Japan

(Received December 9, 1980)

SUMMARY An attenuated Japanese encephalitis virus strain, ML-17, was established through step-wise adaptation of a virulent strain, JaOH 0566, to monkey kidney cells using temperatures of 37 C, 33 C, 30 C, 27 C and then 25 C for growth of the virus and repeated plaque cloning. This new strain, though cloned at lowered growth temperature, can grow in chick embryo fibroblast culture at 37 C giving almost the same titers as that of the parent wild strain.

ML-17 showed low virulence to 5 g mice and suckling mice when administered subcutaneously and this character was retained after one passage in suckling mouse brain or one passage in *Culex* mosquitoes plus one passage in suckling mouse brain.

During the process of low temperature adaptation, ML-17 lost its ability to cause viremia in newborn and 1-month-old piglets when administered subcutaneously. Administration of the strain to pregnant swine was also quite safe and did not affect delivery. No infection of the placenta or embryos of the swine was detected.

Monkeys inoculated intrathalamically with the strain developed no clinical symptoms and no histopathological changes were found in their central nervous system.

Culex tritaeniorhynchus fed with blood meal containing ML-17 scarcely supported virus growth, and no virus antigens were detected by fluorescent antibody staining in the salivary glands of virus-fed mosquitoes. This lowered infection rate in mosquitoes did not increase even after one passage through viremia of the ML-17 strain virus in one-day chicks.

The ML-17 strain was further adapted to quail embryo fibroblasts at 30 C with-

1 Professor Emeritus of Osaka University.

out changes in the above characteristics. This adaptation seems to be highly beneficial for the development of live attenuated vaccine of the ML-17 strain.

A test in swine showed much higher potency of ML-17 than of a commercial killed JE vaccine for veterinary use in preventing viremia after challenge with a large dose of a virulent strain.

INTRODUCTION

Japanese encephalitis (JE) is an epizootic disease in summer in Japan and JE virus is disseminated in July mainly through the swine-*Culex tritaeniorhynchus* (*CT.*) mosquito cycle (Scherer et al., 1959a; Konno et al., 1966). It is important in prophylaxis of JE to stop this cycle as well as to immunize humans, since the over-wintering mechanism of JE virus has not yet been elucidated. There are two possible methods to achieve this: 1) eradication of *CT.*, and 2) immunization of swine sufficiently to prevent viremia after invasion of the virus. The former method is very difficult since no satisfactory chemicals are available for mosquito control. For the latter method immunization with attenuated virus seems suitable.

Several attempts have been made to attenuate JE virus (Hammon et al., 1963; Hammon et al., 1966; Inoue, 1964; Kodama et al., 1968; Sazawa et al., 1969; Fujisaki et al., 1975; Takehara et al., 1969a; Takehara et al., 1969b). The attenuation, however, is usually followed by decrease in immunizing potency, which tends to allow viremia after infection. Attempts were made in this laboratory to obtain an attenuated strain with sufficient immunizing potency to prevent viremia of swine during natural infection.

A strain of JE virus, JaOH 0566, isolated from the brain of a JE patient, has been adapted to primary monkey kidney cells in this laboratory (Takaku et al., 1971). Starting with this strain, an attenuated strain, designated as ML-17, was obtained by adaptation to a lowered growth temperature (25 C) and repeated plaque cloning. ML-17 is sufficiently attenuated and stable and is able to prevent viremia in swine.

This paper describes the procedure for isolation of ML-17 and the characteristics of the strain, including its safety and immunizing potency in swine.

MATERIALS AND METHODS

1. *JE virus strains*

JaOH 0566 strain was isolated in the Public Health Institute of Osaka Prefecture from the brain of a JE patient who died in the summer of 1966. This strain, identified as a JE virus, was given to us by courtesy of the above Institute and was used as the original (parent) strain. Virulent strains, JaGAr 01, Nakayama-Yakuken and Nakayama-Yoken, passaged in mouse brain, were also used for comparison.

2. *Mice*

Suckling mice of 1–3 days old and 5 g mice (about 2 weeks old) of the dd-strain were used. For determination of virus titers, doses of 0.01 ml and 0.03 ml of virus were injected intracerebrally (ic) into suckling mice and 5 g mice, and doses of 0.1 ml of samples were inoculated subcutaneously (sc) into each mouse. The mouse LD_{50} titers were calculated by the method of Reed-Muench after observation for 14 days and are expressed as logarithmic values.

3. *Swine*

All the swine used were non-immune to JE virus. Newborn 1-week-old piglets (bottle- and breast-fed), 1-month-old piglets and pregnant swine (9 months old, F1 hybrid of male Great Yorkshire and female Landrace) were used. Pigs were usually inoculated with 1.0 ml of virus suspension via ear lobes.

4. *Monkeys*

Healthy, quarantined *cynomolgus* monkeys, non-immune to JE virus and weighing 2.5–3.9 kg were used.

5. *Mosquitoes*

A colony of *CT.*, OK-7, was obtained by courtesy of the Public Health Institute of Osaka Prefecture. Mosquitoes were bred in wooden chambers with glass windows at 28 C, and fed with 0.5% sucrose as routine meal and non-immune defibrinated fresh rabbit blood for layings eggs. The membrane feeding method was used for feeding with blood (Doi, 1970). Small plastic tubes were constructed with an outer plastic jacket and equipped with inlet and outlet plastic tubings for circulation of water at 37 C through the jacket. The tube was filled with blood meal with or without virus, and then the mouth of the tube was tightly covered with fine nylon gauze, which did not allow blood to penetrate. Mosquitoes could suck the blood meal through the gauze when the tube was inverted with its opening downwards.

6. *Cell cultures*

1) Monkey kidney cells (MK cells)

Minced pieces of monkey kidney were trypsinized and cultured in medium 199 containing 5% normal calf serum (NCS). Medium 199 containing 2% NCS was used as maintenance medium for virus cultures.

2) Chick embryo fibroblasts (CEF)

Chick embryos at day 9 of development were minced and trypsinized, and cells were grown in YLH medium containing 5% NCS as monolayer cultures for plaque assay.

3) Quail embryo fibroblasts (QUEF)

Quail embryos were treated like CEF and grown in Eagle's MEM containing 5% NCS. For virus production, MEM containing 2% NCS was used.

7. *Plaque formation*

1) On CEF

Plaque formation on CEF for virus titration was performed at 37 C throughout the study. Volumes of 7 ml of cells suspended in culture medium (3×10^6 cells/ml) were distributed in plaque bottles, and incubated at 37 C. After 24 h, cell sheets were washed twice with 4 ml volumes of Earle's medium, and inoculated with 0.2 ml of virus sample. After incubation at 37 C for 90 min, 6 ml of overlay medium were added to each bottle, and when the layer solidified, the bottles were incubated at 37 C for 4 days. Then plaques were counted.

The overlay medium consisted of 2.2% Bacto-agar 96 ml, double concentrated medium 199 100 ml, 5% lactalbumin hydrolysate 4 ml, 0.25% neutral red 5 ml and 7.5% $NaHCO_3$ 5 ml.

2) On MK cells

Plaque formation on MK cells was performed at 25 C for virus cloning in this study. Cell sheets cultured in plaque bottles for 72 h at 37 C were washed twice with 4 ml volumes of Earle's medium, and 0.2 ml of virus inocula of appropriate dilutions were added to bottles and incubated at 37 C. After 90 min, 6 ml of the lst overlay medium were added to each bottle and, when it had solidified, the bottles were incubated at 25 C. After 10 days, 2 ml of 2nd overlay medium were added, and incubation was continued at 25 C until plaques appeared.

The lst overlay medium consisted of 2.2% Noble agar 92 ml, double concentrated medium 199 100 ml NCS 4 ml, tryptose phosphate broth 4 ml and 7.5% $NaHCO_3$ 3.8 ml. The second overlay medium consisted of 2.2% Noble agar 87 ml, double concentrated medium 199 100 ml, NCS 4 ml, 0.25% neutral red 5 ml and 7.5% $NaHCO_3$ 3.8 ml.

8. *Detection of virus in porcine organs and in mosquitoes*

Slices of organs of infected animals were homogenized with medium 199 containing 0.2% bovine plasma albumin to obtain 10% homogenates, and these were centrifuged at 10,000 rpm for 30 min. The supernatants were filtered through a 0.45 μ membrane filter. Pooled insects or individual mosquito, paralyzed at -20 C, were treated similarly with 2 ml of the same medium. The filtrates were tested for virus. Samples of 0.01 ml were injected ic into suckling mice for LD_{50} determination. For plaque assay on CEF cultures, 0.2 ml of each sample were inoculated per plaque bottle.

9. *Infection rate of Culex mosquitoes*

The infection rates were calculated from the following formula (Chiang and Reeves, 1962):

$$P = 1-\left(\frac{n-x}{n}\right)^{1/m}$$

where P, n, x and m represent the infection rate, number of mosquito pools examined, number of virus-positive pools and the pool size (number of mosquitoes in pool), respectively.

10. *Detection of viremia*

Animals (monkeys, swine and chicks) treated with virus samples were bled daily until day 7 after infection. Heparin (0.01% final concentration) was used

as anticoagulant. The blood specimens obtained were tested for virus in suckling mouse brain (LD_{50}) and CEF cultures (PFU) to detect viremia.

11. *Fluorescent antibody (FA) technique*

1) FITC labeled antibody was prepared by the method of Kawamura (1969) from JE hyperimmune rabbit sera. Direct FA stain was used throughout this study.

2) Staining of infected animal organs

Pieces of infected animal organs were frozen at −70 C and sectioned in a cryotome at −18 C to −20 C. Sections were fixed with acetone at −20 C and stained by FA. Blocking for proof of specificity was performed using unlabeled antisera for JE virus.

3) Staining of infected mosquitoes

Mosquitoes were killed at −20 C by freezing and their legs and wings were removed. Their bodies were washed with 1% sodium lauryl sulfate and put into a warm mixture of 3% agarose and 2% gelatin for embedding (Doi, 1970). The embedded blocks were brought to −70 C in dry ice-acetone mixture. Sagittal serial sections of about 4 μ thickness were prepared in a cryotome. Forty sections were cut serially from each mosquito: 30 were stained with FA and the other 10 were used for blocking tests as described above.

12. *Hemagglutination and HI antibody titration*

The hemagglutination and HI antibody were measured according to Clarke and Casals (1958) using goose erythrocytes at a definite pH, usually pH 6.6. Normal serum inhibitors were removed by cold acetone treatment.

13. *Miscellaneous*

Bacto-agar, Noble agar, Bacto-gelatin, Medium 199 and tryptose phosphate broth were products of Difco Laboratories. Lactalbumin hydrolysate, bovine plasma albumin and Agarose A-45 were purchased from Nutritional Biochemicals Corporation, Sigma Chemicals and Nakarai Chemicals, respectively. MEM (Eagle, 1959) was prepared in this laboratory according to the original description.

Trypsin (1: 250) was a product of Difco Laboratories and fluorescein isothiocyanate (crystal) was obtained from Baltimore Biochemical Laboratories. Neutral red and sodium bicarbonate (both of " special analytical grade ") were obtained from Wako Pure Chemicals. NCS free from JE virus antibody was specially selected for this work in this laboratory. Hen eggs were obtained from our own flocks free from JE virus antibody. All other chemicals were of " special analytical grade ".

RESULTS

1. *Establishment of an attenuated JE virus strain, ML-17*

The procedures for establishment of attenuated JE virus strain ML-17 and its derivatives, are summarized in Fig. 1.

Tubes of MK cell culture ($1.5—3.0\times10^5$ cells / tube) were inoculated with 0.2 ml aliquots of 1: 1—1: 10^7 dilutions of the centrifuged supernatant of a 10% suspension of JaOH 0566 infected suckling mouse brain and, after addition of 1 ml of maintenance medium, they were incubated at 37 C for 3 days. Culture fluids of tubes were assayed for infective virus on CEF at 37 C by plaque counting. After the assay, the fluid with the highest virus titer was serially diluted 1: 10. Aliquots of 0.2 ml of each dilution, including the undiluted solution, were passaged in MK cell cultures as described in the " METHODS " and incubated at 37 C for 3 days. This passage procedure was repeated until the 40th passage, when CPE was first detected (Takaku et al., 1971). At that passage level, the virus titer of the culture fluid reached a constant level of about 10^7 PFU/ml.

Subsequently, the culture fluid showing the highest virus titer in every passage level was diluted 1: 10^3–10^4 and 0.2 ml aliquots were inoculated into the next MK cell cultures and incubated at 37 C for 1 week. Virus passage was continued in this manner until the 68th passage (MK68). At this stage, MK68 had already lost the capacity to cause viremia in 1-month-old piglets on subcutaneous inoculation (Table 1).

Aliquots of 0.2 ml of the undiluted culture fluid of the highest virus titer of MK68 were inoculated into 10 MK cell cultures and incubated at 33 C for 4–7 days. Subcultures of the undiluted culture fluids were made at 33 C

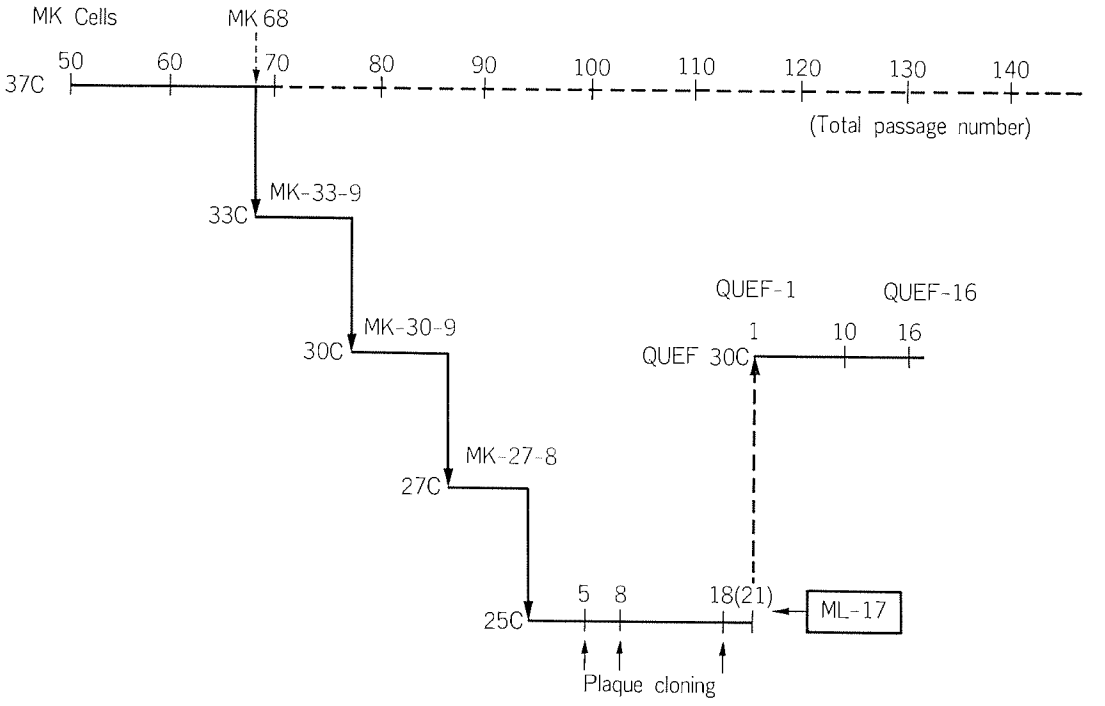


FIGURE 1. Passage history of ML-17 and its QUEF derivatives

until the 9th passage (MK-33-9). At this stage, infectivity by the sc route against 5 g mice disappeared but that by the ic route still remained (Table 1).

Aliquots of 0.2 ml of the culture fluid of the MK-33-9 showing the highest plaque titer on CEF at 37 C were seeded into 10 MK cell cultures and incubated at 30 C for 10–14 days until CPE became apparent. The same procedure was repeated for one more passage (MK-30-2). Then 0.2 ml of 1: 10 dilution of the culture fluid was seeded for the next passage. This procedure was repeated until the 9th subculture (MK-30-9).

The temperature for virus growth was again shifted down to 27 C. For the first culture, 0.2 ml aliquots of the undiluted culture fluid of MK-30-9 with the highest plaque titer on CEF at 37 C were seeded into 10 MK cell cultures and incubation was continued for 10–14 days until CPE appeared (MK-27-1). From the second subculture, 1: 10 dilution of the culture fluid showing the highest CEF plaque titer was used for seeding and the passages were continued until the 8th passage (MK-27-8).

Here, the incubation temperature for virus growth was again shifted down to 25 C. The undiluted culture fluid with the highest CEF plaque titer at 37 C was used as seed for further passage. The incubation period to obtain recognizable CPE was much longer, being 2–3 weeks at this stage. From the second subculture (MK-25-2), 1: 10 dilution of the culture fluid, with the highest CEF plaque titer at 37 C was used as seed for the next generation.

Plaque cloning was done using culture fluid with the highest CEF plaque titer at 37 C, of the 4th subculture (MK-25-4). Volumes of 0.2 ml of $1:10^5$ dilution of the culture fluid were inoculated onto MK monolayer cultures. The monolayers were kept at 25 C for 90 min and then washed twice with 4 ml of Earle's medi-

TABLE 1. *Changes in characters of strains derived by adaptation to lower temperature*

Virus strain	Passage history			$\log_{10}$ PFU/ml on CEF	CPE on MK cell		
					25 C	30 C	37 C
JaOH 0566		9 passage		8.1	−	−	−
	MK cell	37 C	68 p. (68)[a]	7.2	−	+	+
		33 C	9 p. (77)	8.0	−	+	+
		30 C	9 p. (86)	6.5	−	+	+
		27 C	8 p. (94)	6.6	+	+	+
		25 C	4 p. (98)	7.1	+	+	+
		25 C	5 p. (99)	7.0	+	+	+
		25 C	9 p. (103)	7.1	+	+	+
		25 C	10 p. (104)	7.2	+	+	+
		25 C	12 p. (106)	7.2	+	+	+
		25 C	17 p. (111)	7.4	+	+	+
		25 C	18 p. (112)	7.3	+	+	+
		25 C	19 p. (113)	7.4	+	+	+
ML-17		25 C	21 p. (115)	7.8	+	+	+
ML-17 QUEF	QUEF	30 C	1 p. (116)	7.3	+	+	+
		30 C	10 p. (125)	7.5	+	+	+
		30 C	16 p. (131)	8.1	+	+	+
Nakayama-Yoken		mouse brain		8.2	−	−	−
Nakayama-Yakuken		mouse brain		8.1	−	−	−
JaGAr 01		mouse brain		8.0	−	−	−

[a] Total passage number.

um. After addition of 7 ml of the first overlay medium, the infected cultures were incubated at 25 C for 10 days. Incubation at 25 C was continued for 1 day more after addition of 2 ml of the second overlay medium until plaques appeared. These plaques were marked with contour lines on the bottom of the culture bottle and incubated further at 37 C for 5 days. Then 20 small plaques that did not show marked increase in size at 37 C were individually picked up, inoculated into tubes of MK cell culture and incubated at 25 C until CPE appeared. A volume of 0.2 ml of each supernatant was transferred to fresh MK cell cultures for successive passages. At the 7th passage, plaque cloning was performed using $1:10^4$ dilution of each culture fluid in the same manner as in the first cloning. The small plaques, which did not markedly enlarge in size after temperature-shift to 37 C, were picked up and these clones were grown in MK cells in tubes at 25 C (MK-25-9).

The ratio of the ic LD_{50} titer to that on sc inoculation in suckling mice (ic/sc ratio) was considered as another criterion of attenuation at this stage. Therefore, 20 clones with high ic/sc ratios in MK-25-9 were selected and grown successively on MK cells at 25 C up to the 17th subculture (MK-25-17). Plaque cloning was again performed at $1:10^5$ dilution of the culture fluid of MK-25-17.

Then lower infectivity to *CT.* on feeding and a lower infective titer for 5 g mice by the ic route were used as criteria for selection of at-

Mouse virulence[b]				Viremia in 1-month-old piglets	Infection rate in *CT.* (%)	Monkey virulence	Optimal pH of HA
5 g mice		suckling mice					
ic	sc	ic	sc				
7.4	5.3	8.0	6.8	+	100	+	6.6
5.2	0	5.2	3.7	−	100		6.6
3.5	0			−			6.6
3.4	0			−			6.6
3.2	0			−			6.6
3.3	0			−			6.6
3.2	0	4.3	1.2	−	20.6		
3.5	0	4.4	1.4	−	8.6		
3.3	0	4.5	2.4	−	13.8		
3.8	0	5.0	2.3	−	10.0		
3.6	0	5.4	3.2				
2.1	0	5.6	3.5	−	4.1		
0.7	0	5.0	3.8	−	0		
0.6	0	5.6	3.6	−	0–1.0	−	6.6
0.9	0	5.4	3.2	−	0–2.2		6.6
0.5	0	4.8	1.7	−	1.1–3.2	−	6.6
0.7	0	4.6	1.5	−	0	−	6.6
7.2	2.5	7.6	4.7				6.4
7.3	3.0	7.0	5.1	+	100	+	6.6
7.5	5.7	7.3	6.5	+	100	+	6.6

[b] Log_{10} LD_{50}.

tenuated clones in addition to the criteria described above.

One clone at the MK-25-19 passage level, which had an infective titer of 0.7 LD_{50} per 0.03 ml for 5 g mice by the ic route and almost no infectivity in mosquitoes, was selected and passaged until the 21st passage using 0.2 ml of $1:10^5$ dilutions of culture fluid of each generation as inoculum.

The virus of this clone at the 21st passage (MK-25-21) satisfied all the selection criteria (markers) as shown in Table 1; i.e. 1) no sc infectivity to 5 g mice, 2) very low encephalitogenicity (0.6 LD_{50} per 0.03 ml) on ic injection into 5 g mice, 3) no enlargement of plaque size on MK cells after temperature-shift to 37 C, 4) no viremia in 1-month-old piglets after infection, 5) an (ic LD_{50}) / (sc LD_{50}) ratio of 2.0 in suckling mice, in contrast to the ratio of 1.2 of the parent virus, 6) very low infectivity (0–1.0% infection rate) in *CT.*, 7) no encephalitogenicity in monkeys by ic inoculation.

This virus clone had the same optimal pH of 6.6 as the parent JaOH 0566 in hemagglutination and was neutralized to the same extent as the parent strain by antiserum to the parent strain.

The clone was designated as the ML-17 strain. Attempts were made to grow ML-17 on QUEF at 30 C for efficient virus production. As growth of the virus was not good in early stages, undiluted culture fluid was transferred to the next culture. After 10 passages, the clone showed much better growth in this

culture system. After this, $1:10^3$ diluted culture fluids were transferred until the 16th passage (QUEF-16).

All the markers of ML-17 were still retained at this passage level as shown in Table 1.

2. *Pathogenicity of ML-17 strain in swine*

As swine are known to be amplifiers of JE virus in Japan (Scherer et al., 1959b; Konno et al., 1966), measures for immunization of swine have to be sufficiently effective to break the swine-*CT.* mosquito infection cycle. Therefore, ML-17, a possible live virus vaccine, was examined for pathogenicity in swine.

1) Safety in newborn and 1-month-old piglets

Four newborn (one-week-old) piglets were injected sc with $10^{8.0}$ PFU of QUEF-1 and observed for 2 weeks in isolators. They showed no physical symptoms, such as anorexia, diarrhoea, retarded growth, fever or neurological signs, and no viremia.

Of three 1-month-old piglets, two were injected sc with $10^{7.1}$ PFU of ML-17 and one was injected sc with the same dose of QUEF-1 virus. The piglets were kept in quarantine for 20 days. As shown in Fig. 2, they did not show any pathological symptoms or viremia. As a control, 1 piglet of the same age was infected by the sc route with $10^{7.4}$ PFU of virulent JaOH 0566. Marked viremia was seen from day 1 to 4 after inoculation and fever from day 4 to 7. After inoculation of the virulent strain the HI antibody titer increased rapidly to 320 in one week and 640 in two to three weeks, whereas after injection of the attenuated strains ML-17 and QUEF-1 the HI antibody titer increased slowly, being 10 in one week, 40 in two weeks and 80 in three weeks after the inoculation.

2) Safety in pregnant swine

JE virus infection often causes death of embryos in pregnant swine, especially in the early stage of pregnancy (Shimizu et al., 1954).

Pregnant swine in the early stage of pregnancy were injected sc with $10^{8.0}$ PFU of ML-17 (two pigs), $10^{7.7}$ PFU of QUEF-1 (one pig). Two pregnant swine were treated with $10^{7.9}$ PFU of virulent JaOH 0566 strain as controls. As shown in Table 2, viremia was not detected in the 3 swine treated with ML-17 or QUEF-1 virus, and they remained quite healthy. Two of them were autopsied on day 15 (HI antibody titers: 40 and 80) and the other on day 16 (HI antibody titer: 320). Their embryos were quite normal in development, and no JE virus could be isolated by injecting the supernatants of homogenates of organs of the embryos into suckling mouse brains. FA staining of various organs of the embryos also gave negative results.

In the control swine, viremia was clearly detected. One pig was autopsied on day 14 (HI antibody titer: 80). JE virus was isolated from 4 of 5 of its embryos and viral antigen was detected by FA staining. The other pig was autopsied on day 16 (HI antibody titer: 160). Virus was isolated from one of its 2 embryos and viral antigen was detected in both embryos by FA staining.

In another experiment, 9 pregnant swine of 1–50 days pregnancy, including 6 of 20–40 days pregnancy, the time when abortion is most frequent on JE infection (Shimizu et al., 1954), were injected sc with $10^{8.7}$ PFU of ML-17, and kept in quarantine throughout pregnancy. In addition, 7 female swine at 1–40 days before pregnancy were injected with the same amount of ML-17. All these swine remained healthy and their newborn piglets did not show any pathological signs. These results are shown in Table 3.

These results indicate that injection of ML-17 is quite safe in swine, including pregnant swine and their embryos.

3. *Infectivity of ML-17 in CT. mosquitoes*

1) Infection rate of ML-17 in mosquitoes

Mosquitoes of the OK-7 colony of *CT.* were infected by being fed on fresh defibrinated non-immune rabbit blood with $10^{5.0}$ PFU per ml of ML-17; in this way each mosquito received about 10^2 PFU of virus. QUEF-1 was also tested, and as controls MK68, JaOH 0566 adapted to MK cells at the 68th passage level

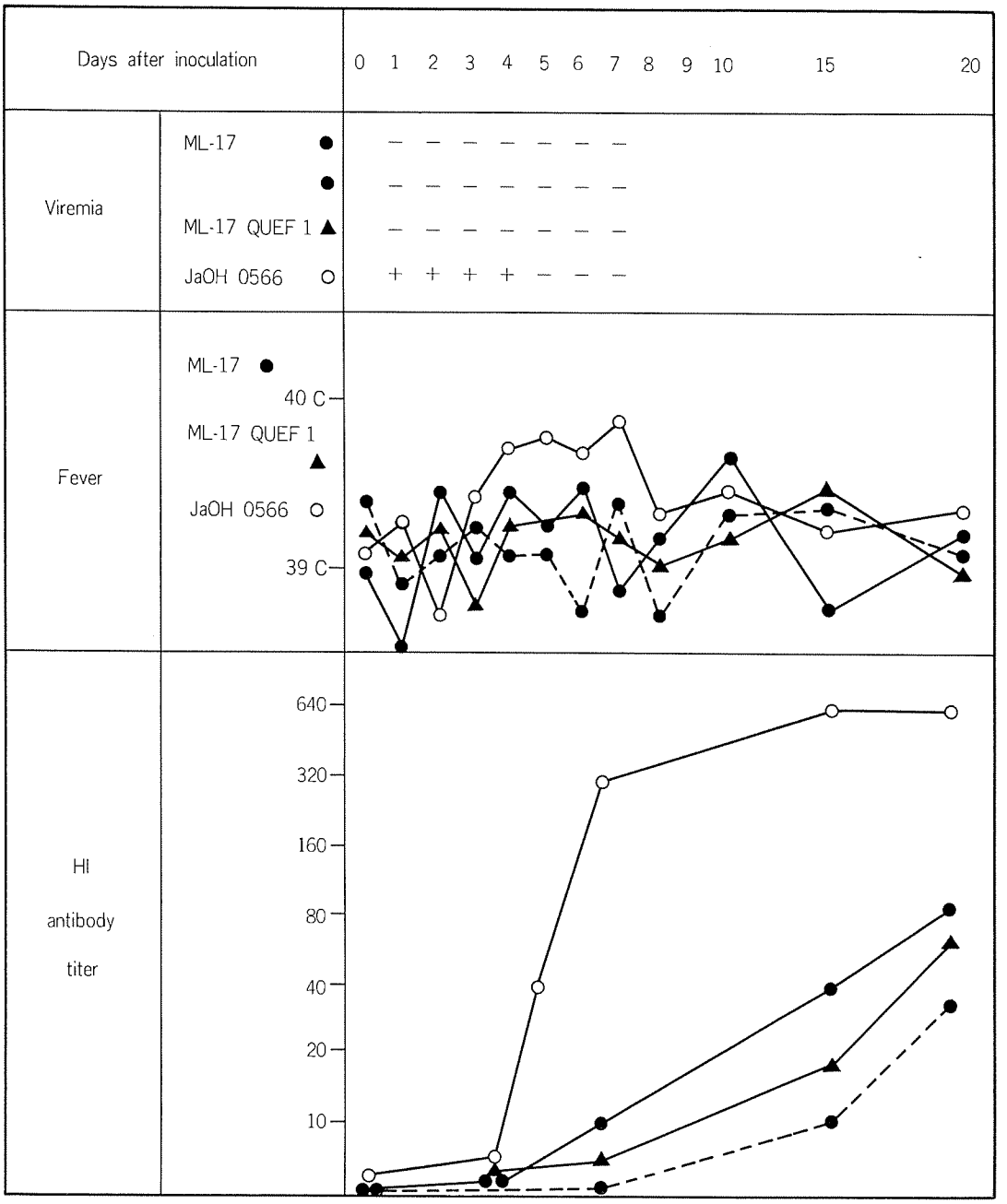


FIGURE 2. Viremia, fever and HI antibody response in 1-month-old piglets
Inoculation doses:
● ML-17 $10^{7.1}$ PFU / piglet
▲ ML-17 QUEF1 $10^{7.1}$ PFU / piglet
○ JaOH 0566 (MB) $10^{7.4}$ PFU / piglet

TABLE 2. *Safety for pregnant swine*

Virus strain	Pig No.	Inoculum dosis PFU/swine	Day of pregnancy	Viremia	Autopsy day after inoculation
ML-17	2	$10^{8.0}$	31	−	15
	5	$10^{8.0}$	46	−	16
QUEF1	7	$10^{7.7}$	36	−	15
JaOH 0566	3	$10^{7.9}$	39	+	14
(virulent)	4	$10^{7.9}$	34	+	16

[a] Inoculation in to suckling mouse brain: No. of virus positive embryos/total No. of embryos.

TABLE 3. *Effects of ML-17 inoculation on pregnant swine and their embryos*

Time of inoculation day(s)		No. of swine tested (a)	No. of normal neonates (b)	No. of stillborn neonates (c)	b/a	c/a
During pregnancy	1–10	1	7	0	9.7	0.10
	11–20	1	13	0	(87/9)	(1/9)
	21–30	2	24	0		
	31–40	3	29	0		
	41–50	2	14	1[a]		
Before pregnancy	1–10	1	8	0	7.7	0.14
	11–20	2	17	0	(54/7)	(1/7)
	21–30	1	7	0		
	31–40	− 2	15	1[a]		
	41–50	1	7	0		
Total		16	141	2[a]	8.8	0.13
					(141/16)	(2/16)

[a] Accidental death at delivery.

at 37 C, and the parent strain JaOH 0566 passaged in mouse brain were also included.

Each experimental group consisted of about 300 mosquitoes. Thirty of each group were taken soon after feeding, and homogenized individually, and their JE content was estimated by PFU titration on CEF. The mean value for each group was calculated. In addition, 100 mosquitoes were taken from each group 10–21 days after feeding and divided into 10 pools (10 mosquitoes / pool). Homogenates prepared from the pools were assayed for virus by plaque formation on CEF and by ic injection into suckling mice to calculate the infection rate. Of the remaining mosquitoes, about 40 from each group were examined for viral antigens by FA staining 10 and 21 days after feeding. The results are shown in Table 4.

No ML-17 was detected in mosquitoes (infection rate: 0%) 10 or 21 days after feeding by plaque formation or by ic inoculation into suckling mouse brain. On FA staining, only one of 20 mosquitoes examined 10 days after feeding gave a positive staining reaction in the mid-gut; its salivary glands gave no staining reaction as shown in Table 5. Of 29 mosqui-

HI antibody at autopsy (HA)	Number of embryos and findings	Virus isolation from:		Positive FA stain
		Organs of embryos	Placenta	
40	8 normal	0/8[a]	–	0/8[c]
320	7 normal	0/7	–	0/7
80	6 normal	0/6	–	0/6
80	5 affected[c]	4/5	+	4/5
160	2 affected[c]	1/2	+	2/2

[b] No. of FA positive embryos/total No. of embryos. [c] Petechia on head, hips and navel area.

toes examined 21 days after feeding, none gave a positive result. The infection rate of QUEF-1 was 1.04% in samples examined 10 and 21 days after feeding by plaque formation on CEF and was negative (0%) by ic inoculation into suckling mouse brain. No viral antigen could be detected by FA staining of specimens 10 and 21 days after feeding.

The infection rate of MK68 was 100% by both assay methods after 10 and 21 days, but on FA staining of only 6 of 17 individual mosquitoes after 10 days and 12 of 24 after 21 days gave a positive result.

The data on MK68 show that the possibility of obtaining a pool composed of 10 virus-negative mosquitoes is very low, so that all 10 pools should contain more than one virus-positive mosquito. On the other hand, MK68 was attenuated to some extent as regards infectivity in mosquitoes when compared with the parent strain, which showed 100% infection of mosquitoes estimated by both virus assay and FA staining.

2) Location of viral antigens in mosquitoes deduced by FA staining

The location of viral antigen in individual mosquitoes examined by FA staining is described here in detail. The results are shown in Table 5.

In the case of ML-17, 10 days after feeding the antigen was found only in the mid-gut with none in other organs, and no antigen was detected in 29 mosquitoes examined 21 days after feeding.

In the case of QUEF-1, no antigen was found in 40 mosquitoes examined 10 and 21 days after virus feeding.

In the case of MK68, 6 of 17 mosquitoes examined 10 days after feeding had antigen only in the mid-gut. After 21 days, 12 of 24 insects had antigen in the mid-gut and fat bodies, 11 in the salivary glands, 9 in thoracic ganglia, 3 in Malpighian tubes, 2 in ovariole sheaths and 1 in the compound eyes.

In the case of the parent strain JaOH 0566, all 5 insects examined 10 days after feeding had antigen in the mid-gut, 4 had antigen in fat bodies, 2 in salivary glands, 1 in thoracic ganglia and 1 in ovariole sheaths. After 21 days, the antigen was demonstrated in the mid-gut and fat bodies of all 25 mosquitoes examined, in the salivary glands of 23, in the thoracic ganglia of 21, in the compound eyes of 19, in ovariole sheaths of 24 and in Malpighian tubes of 9.

Thus when about 10^2 PFU of virus was fed ML-17 and QUEF-1 were attenuated as regards infectivity to *CT*.

3) Multiplication of ML-17 in *CT*. mosquitoes

Although ML-17 showed markedly decreased infectivity in mosquitoes, further studies seemed necessary on its multiplication in mosquitoes after its ingestion at higher doses.

The mosquitoes were given various amounts of ML-17 virus (136, 640 and 4600 PFU per mosquito on the average) and kept alive for 21 days. At 1, 3, 5, 7, 10, 15 and 21 days after

TABLE 4. *Infection of Culex tritaeniorhynchus*

Virus strain	Estimated amount of virus given/mosquito	Days after virus feeding	No. of *CT.*	No. of pools tested
ML-17	a) 186±61	10	100	10
		21	100	10
	b) 195±87	10	100	10
		21	100	10
QUEF1	187±90	10	100	10
		21	100	10
JaOH 0566 MK68	265±131	10	100	10
		21	100	10
JaOH 0566 MB	171±57	10	100	10
		21	100	10

[a] One litter (7–10 suckling piglets) was used for a pool.
[b] ND : not done.

feeding, several mosquitoes were examined individually for virus by plaque formation on CEF. As a control, the parent strain (800 PFU per mosquito) was given to another group of mosquitoes.

The results are shown in Fig. 3. As can be seen, on feeding 136 and 640 PFU of ML-17, the virus did not multiply and disappeared from the mosquito body, and on feeding 4600 PFU, less than 100 PFU was detected in 2 of 6 mosquitoes examined on day 21.

On the contrary, in mosquitoes given the parent strain, the virus grew soon after ingestion and after 10 days 10^4—10^5 PFU was found per mosquito and more than 10^5 PFU of the virus remained until day 21.

These data also indicate that the ML-17 strain had lost infectivity to *CT.*, except when an extraordinarily large amount of virus was ingested.

4. *Pathogenicity of ML-17 and its QUEF derivatives in monkeys*

As ML-17 had been adapted to MK cell cultures, the pathogenicity of ML-17 and its QUEF derivatives was next examined in monkeys, by injecting 0.5 ml volumes of various concentrations of virus into the right and left thalami. The series of monkeys inoculated with ML-17 were kept in quarantine for 28 days after the inoculation, except Nos. 20, 21, 28, 29 and 31, which were kept in quarantine for 18 days. No pathological symptoms or viremia was seen and all the monkeys showed a good HI antibody response, except 2 of 3 administered with low doses of $10^{5.0}$—$10^{5.4}$ PFU of the viruses, as shown in Table 6.

All the monkeys in the series were autopsied and the central nervous system was examined microscopically for pathological changes. No histopathological change was found, as shown in the Table.

As a control, the virulent strains, JaOH 0566, Nakayama-Yakuken and JaGAr 01 were each injected into 2 monkeys. As also shown in Table 6, 4 monkeys administered with Nakayama-Yakuken and JaGAr 01 died after 7–9 days showing paralysis but 2 monkeys administered with $10^{8.1}$ PFU of JaOH 0566 survived until day 28. Irrespective of whether they died or survived during the experiment, all these monkeys showed anorexia, loss of weight, fever and neurological symptoms. At autopsy on day 28, remarkable histophathological

Test by plaque formation		Test on suckling mouse brain		Positive FA stain
No. of virus positive pools	Infection rate (%)	No. of virus positive pools	Infection[a] rate (%)	
0	0	0	0	ND[b]
0	0	0	0	ND
0	0	0	0	1/20
0	0	0	0	0/29
1	1.04	0	0	0/20
1	1.04	0	0	0/20
10	100	10	100	6/17
10	100	10	100	12/24
10	100	10	100	5/5
10	100	10	100	25/25

changes were seen in almost all locations in the central nervous system of control monkeys, as shown in Table 6. Thus it is clear that ML-17 and its QUEF derivatives are attenuated as regards virulence in monkey brain, although ML-17 was adapted to MK cells.

5. *Stability of markers of ML-17*

The stabilities of the markers, used as selection criteria for establishment of the strain were examined.

1) Pathogenicity in mice

As shown in Table 7 ML-17 or its QUEF derivatives, were tested to see whether their ic and sc virulence in 5 g mice could be recovered after one passage through suckling mouse brain or one passage through *CT.* at high dosage plus one passage in suckling mouse brain. As controls, virulent strains JaOH 0566, JaOH 0566-MK68 and Nakayama-Yakuken were tested after similar passages.

As shown in the Table, after passage through suckling mouse brain or mosquitoes, ML-17 and its derivatives, recovered ic virulence slightly but not sc virulence in 5 g mice. In contrast, JaOH 0566–MK68 which had lost sc virulence in 5 g mice regained sc virulence by this treatment.

Thus it is clear that the avirulence of ML-17 and its derivatives in mice when given by the subcutaneous route is stable even after passage through suckling mouse brain and mosquitoes.

2) Infectivity against *CT.* after passage through one-day chicks

It has been shown that one-day chicks are very sensitive to JE virus, readily developing viremia on infection (Buescher et al., 1959; Takahashi et al., 1969). When 10 one-day chicks were injected sc with $10^{5.8}$ PFU of ML-17, viremia was seen in 3 of the chicks after two days and in 5 of the chicks after 4 days. In other 5 chicks it did not develop as shown in Fig. 4.

On day 5 of infection, 0.2 ml of blood was taken for estimation of viremia and then the 10 chicks were each exposed to 90 mosquitoes. The chick that had the highest viremia was determined by virus assay, and then the mosquitoes that had fed on this chick were kept alive for 21 days. On day 21, 70 mosquitoes were taken randomly and homogenized in 10 pools of 7 mosquitoes per pool for virus assay by plaque formation and inoculated into suckling mouse brain to calculate the infection rate.

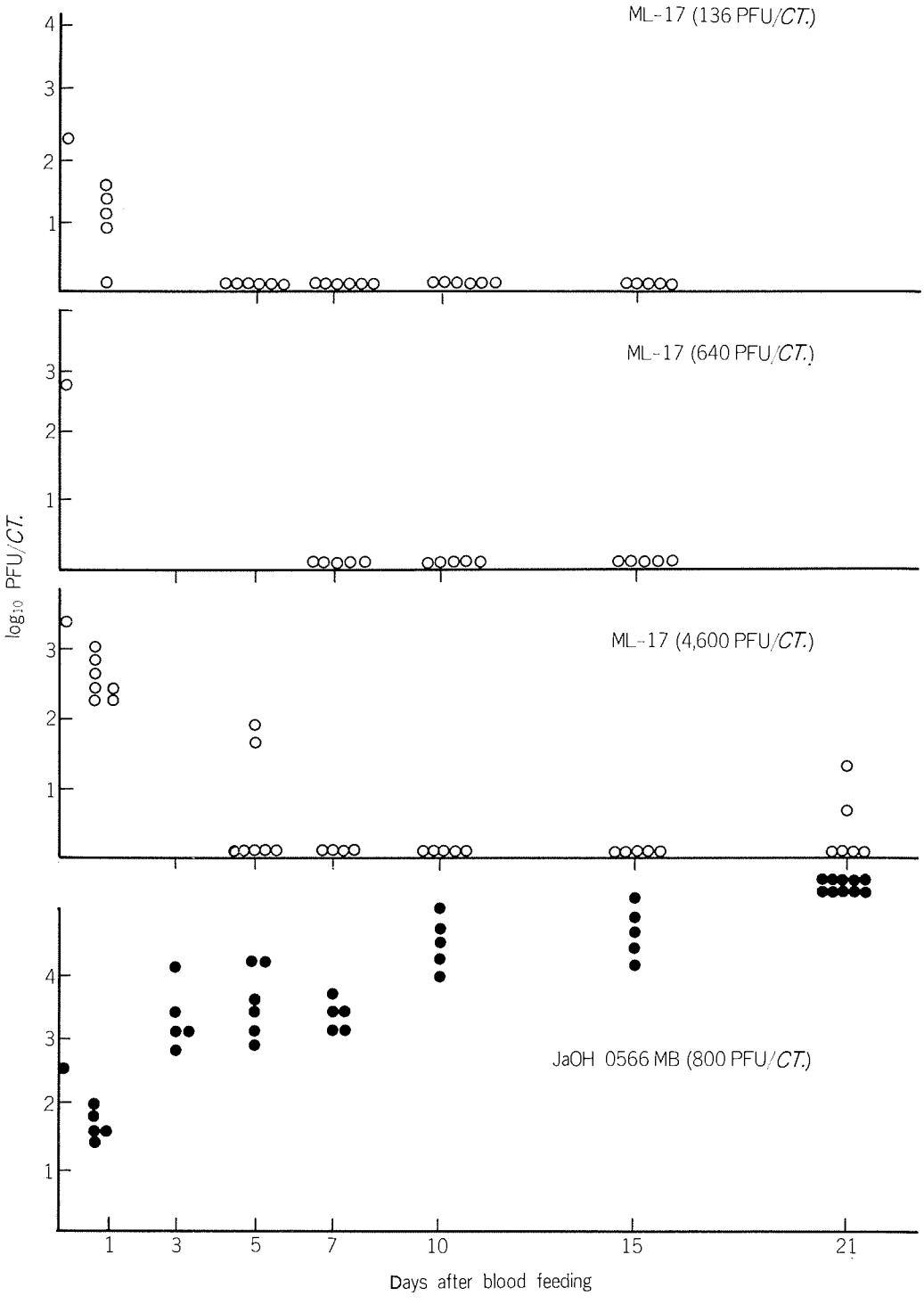

ML-17 (136 PFU/CT.)
ML-17 (640 PFU/CT.)
ML-17 (4,600 PFU/CT.)
JaOH 0566 MB (800 PFU/CT.)
log10 PFU/CT.
Days after blood feeding
1
3
5
7
10
15
21

TABLE 5. *Demonstration of virus antigen in mosquitoes by FA stain*

Virus	Days after virus feeding	No. of *CT.* examined	No. of positive *CT.*	Organ[b]						
				MG	FB	SG	TG	CE	OS	MT
ML-17	10	20	1	+	−	−	−	−	−	−
	21	29	0	−	−	−	−	−	−	−
ML-17 QUEF1	10	20	0	−	−	−	−	−	−	−
	21	20	0	−	−	−	−	−	−	−
JaOH 0566-MK68	10	17	6	6+[a]	−	−	−	−	−	−
	21	24	12	+	+	+	+	+	+	+
				+	+	+	+	−	+	+
				+	+	+	+	−	−	+
				+	+	+	+	−	−	−
				+	+	+	+	−	−	−
				+	+	+	+	−	−	−
				+	+	+	+	−	−	−
				+	+	+	+	−	−	−
				+	+	+	−	−	−	−
				+	+	+	−	−	−	−
				+	+	−	−	−	−	−
JaOH 0566	10	5	5	+	+	+	+	−	+	−
				+	+	+	−	−	−	−
				+	+	−	−	−	−	−
				+	+	−	−	−	−	−
				+	−	−	−	−	−	−
	21	25	25	25+[a]	25+	23+	21+	19+	24+	9+

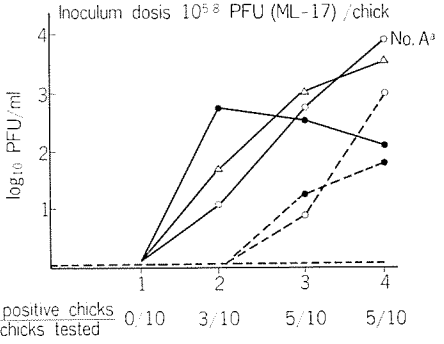
[a] No. of FA positive *CT.*

[b] MG: mid-gut, FB: fat body, SG: salivary gland, TG: thoracic ganglion, CE: compound eye, OS: ovariole sheath, MT: Malpighian tube

◀

FIGURE 3. Multiplication of ML-17 in *Culex tritaeniorhynchus*

○, ●: values for individual mosquitoes



▶

FIGURE 4. Viremia of ML-17 in one-day chicks

a No. A(○——○) chick used for mosquito infection test

TABLE 6. *Comparison of pathogenicities of various JE strains in monkey*

Virus	Inoculum $\log_{10}$ PFU	Monkey No.	Days observed	Clinical symptoms				
				Anorexia	Diarrhoea	Fever	Weight loss	Neurologic symptoms
ML-17	8.1	28	18	−	−	−	−	−
		29	18	−	−	−	−	−
		31	18	−	−	−	−	−
	7.4	4	28	−	−	−	−	−
		6	28	−	−	−	−	−
	6.4	15	28	−	−	−	−	−
		13	28	−	−	−	−	−
	5.4	17	28	−	−	−	−	−
		18	28	−	−	−	−	−
QUEF1	7.7	20	18	−	−	−	−	−
		21	18	−	−	−	−	−
QUEF10	7.0	22	28	−	−	−	−	−
		23	28	−	−	−	−	−
	5.0	24	28	−	−	−	−	−
QUEF16	7.5	30	28	−	−	−	−	−
		32	28	−	−	−	−	−
JaOH 0566	8.1	14	28	+	−	+	+	+
		49	28	+	−	+	+	+
Nakayama-	6.9	9	6p[a] 7D[b]	+	−	+	+	+
Yakuken	4.9	16	6p 7D	+	−	+	+	+
JaGAr 01	7.9	11	8p 8D	+	−	+	+	+
		12	9p 9D	+	−	+	+	+

[a] Days until paralysis.
[b] Days until death.
[c] ND: not done.

As shown in Table 8, the infection rate, though slightly increased, remained at ca. 3% (cf. Table 4 also). These results indicate that the low infectivity of ML-17 to *CT.* mosquitoes is stable even after passage by viremia in one-day chicks.

3) Stability of markers of ML-17 on passage in QUEF cultures at 30 C

The high efficiency of virus production of ML-17 in QUEF cultures at 30 C described above enabled us to obtain enough virus for further tests on the possibility of using this strain as live vaccine.

Even after 16 passages in these growth conditions, the markers of ML-17 were still retained, as shown in Table 7; i.e. i) lower pathogenicity against 5 g mice especially on sc inoculation, ii) an ic/sc infectivity ratio of more than 3.0 of $\log_{10}$ mouse LD_{50} values in suckling mice, iii) a very low infection rate in *CT.*, iv) no pathogenicity against swine or monkeys, and v) growth on MK cells at 25 C.

6. *Effectiveness of ML-17 inoculation of swine against challenge with a virulent strain*

Comparative studies were made on the effec-

Histological examination							HI antibody	
Cerebral cortex	Cerebral ganglia	Midbrain	Pons	Cerebellum	Medulla oblongata	Spinal cord	Before inoculation	After (at autopsy)
−	−	−	−	−	−	−	<10	160
−	−	−	−	−	−	−	<10	160
−	−	−	−	−	−	−	<10	40
−	−	−	−	−	−	−	<10	320
−	−	−	−	−	−	−	<10	80
−	−	−	−	−	−	−	<10	160
−	−	−	−	−	−	−	<10	40
−	−	−	−	−	−	−	<10	160
−	−	−	−	−	−	−	<10	<10
−	−	−	−	−	−	−	<10	160
−	−	−	−	−	−	−	<10	80
−	−	−	−	−	−	−	<10	320
−	−	−	−	−	−	−	<10	320
−	−	−	−	−	−	−	<10	<10
−	−	−	−	−	−	−	<10	80
−	−	−	−	−	−	−	<10	80
+	+	+	+	+	+	+	<10	160
+	+	+	+	+	+	+	<10	80
+	+	+	+	+	+	−	<10	ND[c]
+	+	+	+	+	+	−	<10	ND
+	+	+	+	+	+	+	<10	ND
+	+	+	+	+	+	+	<10	ND

tiveness of ML-17 and a commercial killed JE vaccine for veterinary use. Three groups of four pigs were used. The first group was injected sc with ML-17: 2 of the pigs were immunized sc once with $10^{7.5}$ PFU and the other 2 were immunized twice with $10^{7.5}$ PFU with a 2-week interval between immunizations. The second group was injected sc with killed vaccine; two pigs were immunized once and other two were immunized twice with a 2 week interval between injections. The third group was not immunized as a control.

Five weeks after the lst immunization, all the pigs, including the controls were challenged with $3.6\times10^{8.0}$ PFU of virulent JaGAr 01 virus. This challenge dose corresponded to more than 100 times the highest virus content of a single mosquito infected with JaGAr 01. Swine were bled on day 1, 3 and 5 after challenge and viremia was assayed by inoculation into suckling mouse brain. The HI antibody titer was estimated on the days of the lst and 2nd immunizations, on the day of challenge and 3 weeks after challenge. As shown in Table 9, the swine immunized with ML-17 once or twice showed no viremia, whereas

TABLE 7. *Stability of virulence markers of ML-17 and QUEF after passage in CT. and/or suckling mouse brain*

Virus	Control			One passage in suckling mouse brain			One passage in *CT.*, then one passage in suckling mouse brain		
	$\log_{10}$ PFU/ml	ic	sc	$\log_{10}$ PFU/ml	ic	sc	$\log_{10}$ PFU/ml	ic	sc
JaOH 0566	8.1	7.4[a]	5.3	8.5	7.5	5.4	8.3	7.8	5.6
JaOH 0566 37 C MK68	7.5	3.9	0	8.3	7.8	4.2	8.1	7.6	4.9
ML-17	7.9	0.6	0	8.7	5.9	0	8.4	5.1	0
QUEF1	7.3	0.7	0	8.5	5.5	0	ND[b]	ND	ND
QUEF10	7.5	0.5	0	8.4	5.4	0	8.3	5.5	0
QUEF16	7.5	0.7	0	8.3	5.1	0	8.2	4.9	0
Nakayama-Yoken	8.1	7.3	3.0	8.7	7.7	3.9	ND	ND	ND

[a] $\log_{10}$ LD_{50}
[b] ND : not done.

TABLE 8. *Infection rate of ML-17 in Culex tritaeniorhynchus fed with viremic blood of a one-day chick*

Chick		No. of *CT.* fed on the chick	No. of pools tested	No. of virus positive pools detected by :		Infection rate (%) calculated from data on	
No.	Viremia (PFU/ml) :			Plaque formation	Suckling mouse brain inoculation	Plaque formation	Suckling mouse brain inoculation
A	$10^{4.1}$	70[a]	10	2	2	3.13	3.13

[a] 70 mosquitoes were divided into 10 pools after feeding.

those immunized with killed vaccine and control pigs developed similar extents of viremia.

It can be assumed from these data that ML-17 is very effective as a live vaccine in preventing amplification of JE virus in swine.

DISCUSSION

A strain of attenuated JE virus, ML-17, was isolated by adapting virulent JaOH 0566 to MK cells by stepwise temperature shift from 37 C to 25 C. During this process, plaque cloning was carried out on MK cells at 25 C and plaques that did not enlarge after the temperature shift to 37 C were selected repeatedly. However, the clones grew on CEF at 37 C to a high titer comparable to that of the parent strain. Therefore, ML-17 is not a low-temperature mutant, but its range of permissible temperature for growth is widened to 25 C.

Unexpectedly, we found that JaOH 0566–MK68 had already lost the ability to evoke viremia in 1-month-old piglets before adaptation to lower temperature. Its pathogenicity on sc injection into 5 g mice was lost after adaptation to 33 C, whereas its ic virulence in 5 g mice was still retained at this stage. During adaptation to 25 C, several marked changes in pathogenicity occurred; namely, the virus showed 1) lower virulence on ic injection into 5 g mice, 2) a higher ic/sc ratio of LD_{50} titers in suckling mice, 3) very low infectivity in *CT.* and 4) no encephalitogenicity on ic injection into monkeys.

TABLE 9. *Protection of viremia in immunized swine challenged with virulent JaGAr 01 strain*[a]

	No. of injection	HI antibody titer				Viremia after challenge		
		Before 1st injection	Before 2nd injection	Before challenge	3 weeks after challenge	1	3	5[b]
ML-17	once[d]	<10	10	40	400	0/7	0/7	0/7[c]
		<10	<10	10	200	0/8	0/7	0/8
	twice[e]	<10	<10	40	50	0/6	0/7	0/7
		<10	40	80	400	0/8	0/7	0/8
Killed JE vaccine	once	<10	<10	<10	50	3/4	1/8	0/7
		<10	<10	<10	50	9/9	0/6	0/8
	twice	<10	<10	<10	100	9/9	5/10	1/7
		<10	<10	<10	200	8/8	0/5	0/7
Non-immunized control	—	<10	<10	<10	400	6/6	2/6	1/6
		<10	<10	<10	800	7/7	6/6	0/7
		<10	<10	<10	800	8/8	6/6	0/7
		<10	<10	<10	400	8/8	3/10	0/8

[a] Challenging dose: 3.6×10^8 PFU.
[b] Day(s) after challenge.
[c] No. of positive mice/No. of mice injected.
[d] Immunizing dose: $10^{7.5}$ PFU.
[e] Immunizing doses: $10^{7.5}\times2$ with 2-week interval.

These markers were still retained after growth on QUEF at 30 C. The good yield in this growing condition is a valuable characteristic of a candidate for a live vaccine.

These markers were quite stable even after growth in suckling mouse brain, or after growth in one-day chicks and / or growth in mosquitoes. In addition, the optimal pH for hemagglutination was 6.6 which is the same as that of recent epidemic strains of JE virus.

Swine are important as amplifiers of JE virus, mainly in June-August, in Japan, because almost all of them are born after epidemics in the previous summer and hence are not immune to JE virus. When these swine are infected with JE virus by the vector, they do not develop encephalitic symptoms, or die from infection, but show viremia. Thus the swine-mosquito cycle plays a major role in spreading JE virus. For this reason, immunization of swine is a useful means of preventing spread of the virus.

As shown in Table 9, ML-17 was more effective for immunizing swine than killed virus vaccine, and could prevent viremia of swine inoculated with $10^{8.5}$ PFU of the virus, which is at least 100 times the maximal amount of virus contained in the whole body of a single infected mosquito.

For immunization of swine with live vaccine, the vaccine strain should satisfy the following two criteria: 1) it should be safe for swine, especially pregnant swine and their embryos, and 2) it should have no probability of transmission through viremia from immunized swine to *CT.* mosquitoes.

The sc injection of an ML-17 derivative, QUEF-1, into 1-week-old or 1-month-old non-immune piglets evoked no pathologic symptoms or viremia, and caused slower but definite increase of HI antibody. Even pregnant swine, whose embryos often die or are aborted after injection of wild type JE virus remained healthy after injection of ML-17 without vi-

remia and their piglets were delivered normally. ML-17 could not be recovered from the newborn piglets and no viral antigen was detectable in any of their organs. Immunization of swine shortly before pregnancy also had no unfavourable effects.

For use as live vaccines, ML-17 and its QUEF derivatives should have almost completely lost the capacity to grow in *CT.* mosquitoes. Since the ability to elicit viremia in swine and the ability to grow in *CT.* were lost at different stages during the course of attenuation of ML-17, it can be assumed that at least two mutations occurred at different sites of the viral genom. Therefore, even if the progeny of ML-17 or its QUEF derivatives were to mutate and regain the ability to elicit viremia after administration to swine, these progeny would probably still not be able to grow in mosquitoes and so would not spread through the swine-mosquito cycle. For this reason, we tried to obtain a clone that was almost incapable of growing in mosquitoes.

ML-17 showing these characters was successfully isolated; it did not grow in mosquitoes ingesting an average dose of 136 or 640 PFU, and no viral antigens were found in the salivary gland of the infected mosquitoes. Even in 34 mosquitoes that ingested an average of 4600 PFU, only 2 of 7 insects examined on day 5 retained about 10^2 PFU and 2 of 6 examined on day 21 retained about 10 PFU. The other insects examined on days 7, 10 and 15 retained no virus. In all these insects the amount of virus detected was far less than 4600 PFU, proving that the virus did not grow in the mosquitoes. Since a mosquito can suck only 2 μl of blood (Takahashi et al., 1969), the blood meal ingested contained 2.3×10^6 PFU per ml in the experiment on 4600 PFU feeding. As it is known that the highest level of swine viremia is $10^{2.5-2.6}$ mouse LD_{50}/0.03 ml (Scherer et al., 1959b), the virus concentration used in this feeding experiment corresponds to the maximal level in viremia. Thus it can be stated that ML-17 had lost its ability to multiply in *CT.* mosquitoes.

All these data clearly indicate that the two requisits for use of ML-17 and its QUEF derivatives as live vaccines for swine were satisfied.

A safety test was performed on monkeys with favorable results even though ML-17 was adapted to MK cells. However, we have no intention of using this strain for humans, because we heard a rumor of a sad case caused by misbelief in an " attenuated tick encephalitis vaccine " which was safe in a monkey test, and because humans and monkeys respond quite differently to viruses such as Marburg virus.

JE live vaccine for swine immunization was officially approved in Japan in 1972 by the Ministry of Agriculture, Forestry & Fisheries and has since been used for pregnant swine only from an economical basis to prevent abortion or still-birth. No cases of harmful effects have been reported. The ML-17 strain could be used for this purpose and also as a new, powerful and safe tool for control of JE by breaking the swine-mosquito cycle. However, immunization of the general population of swine has not yet been widely adopted, because less than 100 human cases of JE have been reported yearly since 1970 in this country.

ACKNOWLEDGMENT

The authors express their thanks to the Public Health Institute of Osaka Prefecture for kindly donating the JE virus strain, JaOH 0566, and a colony of *Culex tritaeniorhynchus.*

REFERENCES

Buescher, E. L., Scherer, W. F., Rosenberg, M. Z., McClure, H. E. 1959. Immunologic studies of Japanese encephalitis virus in Japan. III. Infection and antibody responses of birds. J. Immunol. 83: 605–613.

Chiang, C. L., Reeves, W. C. 1962. Statistical

estimation of virus infection rates in mosquito vector populations. Am. J. Hyg. 75: 377–391.

Clarke, D. H., Casals, J. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. 7: 561–573.

Doi, R. 1970. Studies on the mode of Japanese encephalitis virus in some groups of mosquitoes by the fluorescent antibody technique. Jpn. J. Exp. Med. 40: 101–115.

Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130: 432–437.

Fujisaki, Y., Sugimori, T., Morimoto, T. 1975. Development of an attenuated strain for Japanese encephalitis live virus vaccine for porcine use. Natl. Inst. Anim. Health Q. 15: 15–23.

Hammon, W. McD., Rhim, J. S. 1963. Progress report on Japanese B encephalitis OCT-541 attenuated virus strain. Am. J. Trop. Med. Hyg. 12: 616–619.

Hammon, W. McD., Darwish, M. A., Rhim, J. S., Sather, G. E., Yohn, D. S., Mittelman A., Deverson, E., Soyka, J. P. 1966. Studies on Japanese B encephalitis virus vaccines from tissue culture. J. Immunol. 96: 518–524.

Inoue, Y. K. 1964. An attenuated mutant of Japanese encephalitis virus. Bull. WHO 30: 181–185.

Kawamura. A., Jr. (Editor) 1969. Fluorescent antibody techniques and their applications. Univ. Tokyo Press and Univ. Park Press.

Kodama, K., Sasaki, N., Inoue, Y. K. 1968. Studies of live attenuated Japanese encephalitis vaccine in swine. J. Immunol. 100: 194–200.

Konno, J., Endo, K., Agatsuma, H., Ishida, N. 1966. Cyclic outbreaks of Japanese encephalitis among pigs and humans. Am. J. Epidemiol. 84: 292–300.

Sazawa, H., Sugimori, T., Morimoto, T., Miura, Y., Watanabe, M. 1969. Response of swine to an attenuated strain of Japanese encephalitis virus obtained by passage in bovine kidney cell cultures. Natl. Inst. Anim. Health Q. 9: 74–82.

Scherer, W. F., Moyer, J. T., Izumi, T., Greseer, I., McCown, J. 1959a. Ecologic studies of Japanese encephalitis virus in Japan. VI. Swine infection. Am. J. Trop. Med. Hyg. 8: 698–706.

Scherer, W. F., Moyer, J. T., Izumi, T. 1959b. Immunologic studies of Japanese encephalitis virus in Japan. V. Maternal antibodies, antibody responses and viremia following infection of swine. J. Immunol. 83: 620–626.

Shimizu, T., Kawakami, Y., Fukuhara. S., Matumoto, M. 1954. Experimental stillbirth in pregnant swine infected with Japanese encephalitis virus. Jpn. J. Exp. Med. 24: 363–375.

Takahashi, M., Yabe, S., Okada, T. 1969. Effects of various passages on some properties of an attenuated strain of Japanese encephalitis virus with special regard to mosquito infectivity. Jpn. J. Med. Sci. Biol. 22: 163–174.

Takaku, K., Yamashita, T., Yoshida, I., Akashi, M., Inokuma, E., Goda, H., Hirota, T., Takamizawa, A., Sugahara, Y., Osame, J., Kato, M., Amano, T., Kunita, N., Inoue, K. 1971. Immunization for Japanese encephalitis: 185–191. Igaku Shoin Ltd, Tokyo.

Takehara, K., Mitsui, T., Nakamura, H., Nakamura, J. 1969a. Studies on Japanese encephalitis live virus vaccines. I. Development of an attenuated strain of virus by serial passage in hamster kidney cell cultures. Nibs Bull. Biol. Res. VIII: 11–22.

Takehara, K., Mitsui, T., Nakamura. H., Fukusho, K., Kuramasu, S., Nakamura, J. 1969b. Studies on Japanese encephalitis live virus vaccines. II. Immunization of pigs with an attenuated strain of virus. Nibs Bull. Biol. Res. VIII: 23–37.