Japanese Encephalitis Live Virus Vaccine for Swine

By YUJIRO FUJISAKI

Second Research Division, National Institute of Animal Health

Production of piglets in Japan has been obstructed markedly due to the swine stillbirth caused by Japanese encephalitis virus (JEV). Swine stillbirth can be prevented by inoculation with a large amount of inactivated high-titer vaccine prepared from the brains of mice infected with JEV.

It has been considerable difficult, however, to carry out such inoculation program on account of cost and labor. Therefore, it has been necessary to develop a live virus vaccine which can replace the conventional inactivated vaccine.

Early in the 1960's, Hammon & Rhim,[4] Hammon et al.,[5] Inoue[6] and others began studies on the attenuation of JEV by using tissue cultures. In the latter half of the 1960's, studies on the application of attenuated variant strains to a live virus vaccine have been carried out actively.

At present four attenuated strains, S^-,[1),2),3)] "m",[7] "at",[9] and ML-17, have been developed and used as live virus vaccine for the prevention of swine stillbirth in field. These strains were attenuated by serial passages of a field strain in bovine, swine, hamster, or monkey kidney cell cultures, respectively.

The present paper deals with the process of production, properties and efficacy of the atlenuated S^- strain which was developed at the National Institute of Animal Health.

Production of the S^- strain

Sazawa et al.[8] made an attempt to produce an attenuated variant virus for the purpose of developing a live virus vaccine and succeeded in preparing an attenuated S′ strain. The S′ strain was originated from a field strain, its name was the Sagara strain. When this strain was subjected serially to 21 passages in primary bovine kidney (BK) cells at 30°C, its name was changed to the S′ strain.

When a young pig was inoculated with the S′ strain, it presented viremia and might possibly be a source of infection for potential hosts through the intermediary of mosquitoes. Consequently, it was necessary to attenuate this strain further before the strain could be used as vaccine.

When the S′ strain was allowed to form plaques in embryonic swine kidney established (ESK) or green monkey kidney established (Vero) cell cultures, two kinds of plaques, medium and small in size, were differentiated among those formed.

Accordingly, it was presumed that the S′ strain might be a mixture of viruses showing the difference in plaque size. Then BK cells were used to carry out cloning of the S′ strain with a limiting dilution at 30°C.

As a result, two clones showing the different range of temperature for propagation were obtained. Of them, a newly obtained clone was called S^-, which was presumed to be the best adapted one for the cultivation at low temperature.

Properties of the S^- strain

1) *Range of temperature for propagation and plaque size*

The Sagara strain propagated well in

cultivation at any temperature of 40, 37, and 30°C, and formed large-sized plaques in ESK and Vero cells at 37°C. The S⁻ strain propagated in cultivation at 30°C but little at 37, and 40°C, and it formed small-sized plaques. The properties of S⁻ strain did not return to that of the field strain even after the strain had undergone passages in ESK cell culture, mouse brain or swine. Then, it is assumed that they can be used as makers of attenuation.

2) Pathogenicity for mice

The Sagara strain had the high peripheral infectivity to suckling mice and intracerebral infectivity to adult mice, but infectivities were reduced remarkably in the S⁻ strain.

3) Pathogenicity for swine

To study the pathogenicity of the S⁻ strain for newborn piglets, hysterectomy-produced, colostrum-deprived (HPCD) piglets 3 to 5 days old were inoculated subcutaneously or intranasally with the Sagara and S⁻ strain and held under clinical observation.

Table 1. Range of temperature for propagation and plaque size of the Sagara and S⁻ strain

Strain	Virus titer at			Plaque*2 size
	30°C	37°C	40°C	
Sagara	7.1*1	7.6	6.9	L
S⁻	6.9	<2.3	<1.5	S

*1 $TCID_{50}$/ml (log)
*2 L and S indicate large-, and small-sized plaques (about 5 mm, and 1 mm in diameter), respectively

Table 2. Pathogenicity of the Sagara and S⁻ strain for mice

Strain	Virus titer in suckling mice		Virus titer in adult mice
	ic*1	sc*2	ic
Sagara	9.7*3	9.1	8.3
S⁻	7.1	<1.5	<1.4

*1 Intracerebral inoculation
*2 Subcutaneous inoculation
*3 LD_{50}/ml (log)

Table 3. Pathogenicity of the Sagara and S⁻ strain for HPCD piglets

Virus inoculation			Days Post-infection	Virus distribution								Lesions*5 of brain tissue
Strain	Route*1	Inoculum (−Log $TCID_{50}$)		Brain	Spinal cord	Lymph node	Lung	Liver	Kidney	Spleen	Blood	
Sagara	SC	5.2	2	2.5*2	1.7	3.7	3.0	4.7	4.2	4.3	4.5	—
			4	2.8	1.7	3.5	3.3	3.0	3.3	4.2	4.0	##
			6	2.5	3.0	1.7	1.8	—*3	2.0	1.6	—	###
	in	5.2	2	1.6	—	2.0	1.7	+*4	2.0	2.0	3.0	—
			4	—	1.6	2.0	3.2	2.5	1.6	1.7	3.7	##
			6	—	—	—	—	—	1.7	2.6	—	###
S⁻	SC	6.8	2	—	—	—	—	—	—	—	—	—
			4	—	—	+	—	—	—	—	—	—
			6	—	—	—	—	—	—	—	—	—
	in	6.8	2	—	—	—	—	—	—	—	—	—
			4	—	—	—	—	—	—	—	—	—
			6	—	—	—	—	—	—	—	2.0	—

*1 sc=subcutaneous; in=intranasal
*2 Virus titer in log ($TCID_{50}$ per g or ml)
*3 Virus demonstration was negative in ESK cell cultures and suckling mice inoculated with 10% organ emulsion or undiluted blood plasma
*4 Virus demonstration was negative in ESK cell cultures inoculated with 10% organ emulsion. Clinical infection was apparent in several of 10 suckling mico inoculated
*5 These lesions include glial reaction, infiltration of neutrophils, enlargement and hyperplasia of vascular adventitial cells, enlargement of endothelial cells, and degeneration of nerve cells. The minus sign indicates the absence of changes. The plus signs, +～++++, indicate the severity and degree of distribution of pathological changes

Some of them were sacrificed 2, 4, and 6 days after inoculation and examined for the amounts of virus in organs. Histopathological examination was carried out on the brain tissue stained with hematoxylin and eosin.

Piglets inoculated with $10^{5.2}$ $TCID_{50}$ of the Sagara strain manifested such clinical symptoms as pyrexia of more than 40°C, tremor, and anorexia. A large amount of virus was recovered from their blood and organs. Severe changes were seen in the brain tissue.

Piglets inoculated with $10^{6.8}$ $TCID_{50}$ of the S^- strain showed no clinical abnormalities. Virus was recovered only from some lymph nodes 4 days after subcutaneous inoculation and blood 6 days after intranasal inoculation. It was difficult to recover virus from organs. No changes were found at all in the brain tissue.

Six pigs 30 days of age were inoculated subcutaneously with $10^{6.2-6.7}$ $TCID_{50}$ of the S^- strain, but none of them showed viremia.

Four sows were inoculated subcutaneously at the early stage of pregnancy with $10^{6.8-7.7}$ $TCID_{50}$ of the S^- strain.

All the sows, however, remained free from any abnormal clinical symptom or viremia. Of them, two sows were sacrificed 38 days after inoculation and the other two 10 days after inoculation for autopsy. All the fetuses exhibited normol development in every sow.

An attempt was made to isolate virus from the placenta and fetuses by the method of intracerebral inoculation of suckling mice, but in vain.

Five sows were inoculated subcutaneously at early stage of pregnancy with $10^{6.5-7.8}$ $TCID_{50}$ of the S^- strain. All of them were allowed to give birth spontaneously to young, which were examined for effect of inoculation. None of them presented any abnormality during the pregnancy period. The newborn young were all normal.

4) Infectivity to mosquitoes

To prevent a pig inoculated with vaccine virus from being an amplifier, it is desirable not only that no viremia will occur to the pig, but also that vaccine virus has infectivity reduced to mosquitoes. Then, mosquitoes of *Culex tritaeniorhynchus summorosus* were allowed to suck the blood containing the S^- strain by the membrane-feeding technique which was examined for infectivity.

The rates of infection were 0.7 and 1.3% among mosquitoes having allowed to suck $10^{1.6}$ and $10^{2.0}$ $TCID_{50}$ of the S^- strain per mosquito, respectively. The rate was 26.3% among mosquitoes having allowed to suck $10^{1.7}$ $TCID_{50}$ of the field (AS-6) strain per mosquito. Therefore, it was presumed that the S^- strain might have been reduced in ability to propagate in the mosquito body as compared with the field strain.

Table 4. Infection of the mosquito, Culex tritaeniorhynchus summorosus, with S^- and AS-6 strains as tested by membrane feeding technique

Strain	Virus titer ingested per mosquito	No. of mosquitoes ingested	Mosquito infection	Mosquito infection rate (%)
S^-	1.6*1	140	1/20*2	0.7*3
	2.6	160	2/16	1.3
AS-6	1.7	210	20/21	26.3

*1 Log $TCID_{60}$ per 0.002 ml as determined with suckling mice
*2 No. of positive pools/No. of pools tested
*3 Estimated from the formula of Chiang and Reeves

5) Pathogenicity for monkey

Four green monkeys were inoculated with $10^{5.0-6.0}$ $TCID_{50}$ of the S^- strain into the thalamus and clinical observation and histopathological examination of the central nervous system were carried out. As a result, no monkey manifested any abnormal clinical symptom.

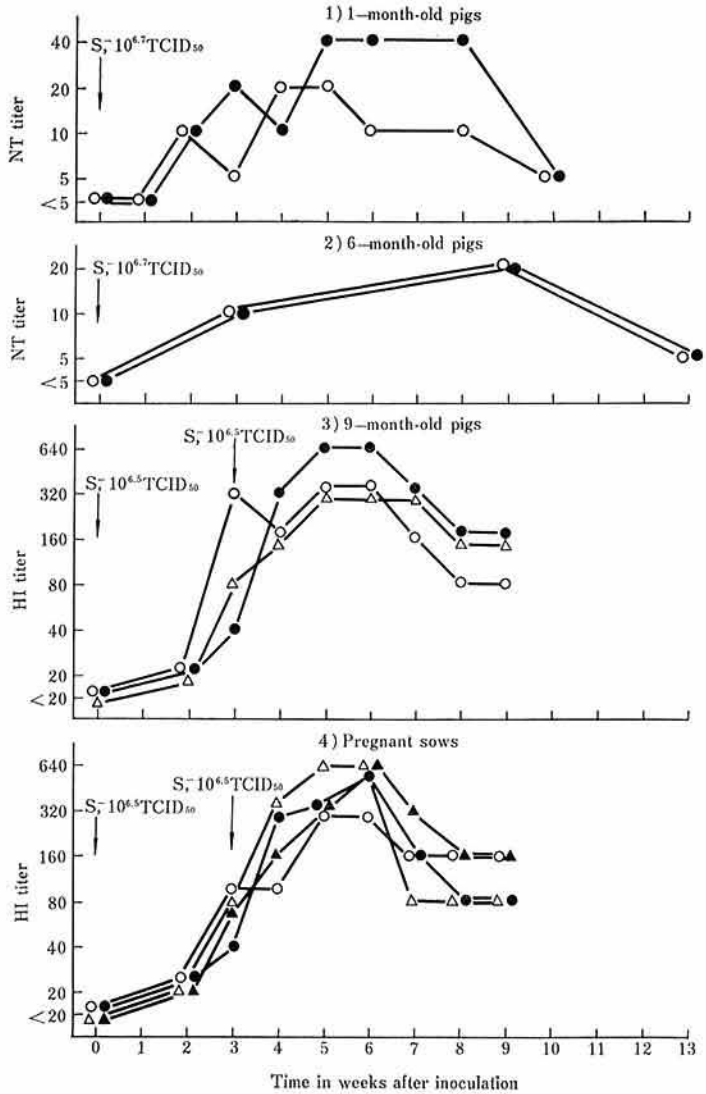
One of them inoculated with $10^{6.0}$ $TCID_{50}$ presented mild histophathological changes, but the other three were perfectly free from any microscopical change. These results suggested that the S^- strain might also have been reduced markedly in pathogenicity for monkeys.[10]

Antibody response and prevention from infection in pigs inoculated with S⁻ strain

Eight pigs 1 to 6 months old were inoculated subcutaneously with a single dose of $10^{6.5-7.5}$ $TCID_{50}$ of the S⁻ strain. In these pigs, the neutralizing (NT) titer or hemagglutination-inhibiting (HI) titer increased to 1:10~1:320. An antibody titer exceeding 1:10 was mainteined for 2 to 9 weeks.

Nine pigs 5 to 9 months old were inoculated subcutaneously with $10^{6.5-7.0}$ $TCID_{50}$ of the S⁻ strain twice at 3 weeks interval. In them, HI titer increased 1:80~1:640. In many of these pigs, HI titers of 1:80~1:160 persisted for more than 6 weeks.

There was a tendency that pigs which advanced in age in months showed a better



HI : Hemagglutination-inhibiting antibody
HT : Neutralizing antibody

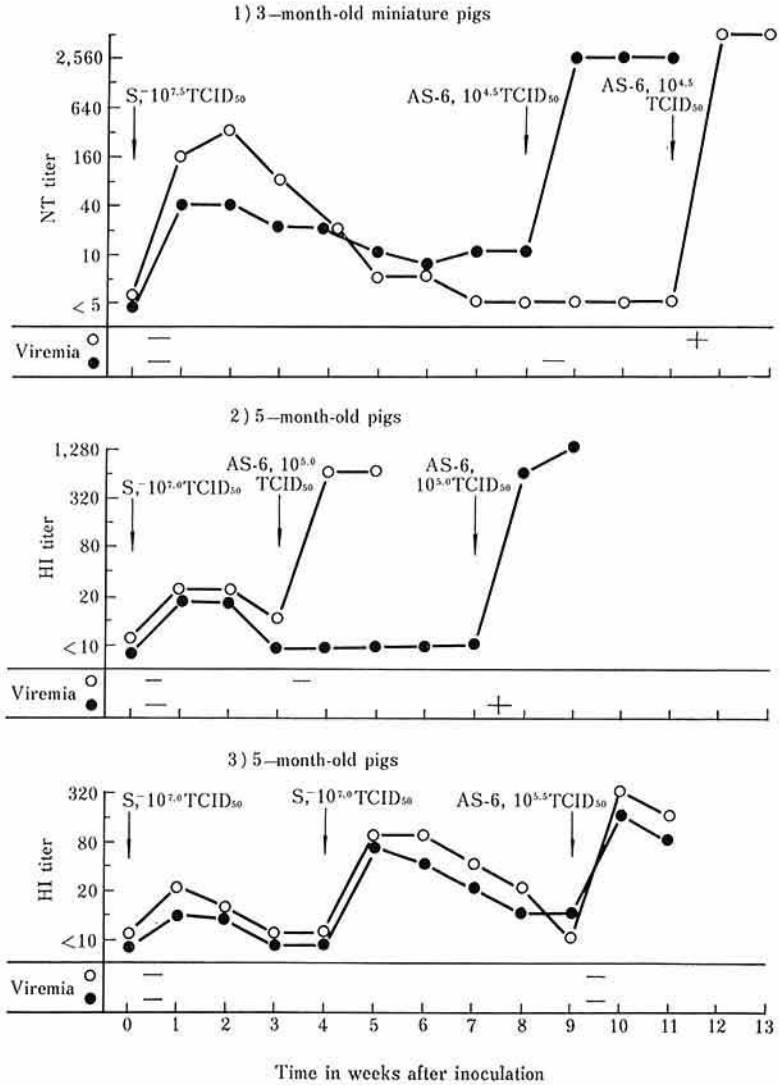
Fig. 1. Antibody response in pigs inoculated with S⁻ strain

antibody response than infant pigs and piglets.

Six pigs 3 to 5 months old inoculated once or twice with $10^{7.0-7.5}$ $TCID_{50}$ of the S^- strain were challenged by inoculation with $10^{4.5-5.5}$ $TCID_{50}$ of a field strain and examined for the occurrence of viremia. As a result, an ability to protect from infection was demonstrated in pigs which showed an antibody titer surpassing 1:10 at the time of challenge.

Prevention of fetuses from infection in sows inoculated with S^- strain

Four sows negative for antibody were mated with a boar on the same day. Of them, two were inoculated with $10^{7.0}$ $TCID_{50}$ of the S^- strain 4 days after mating and two served as uninoculated controls. The



HI : Hemagglutination-inhibiting antibody
HT : Neutralizing antibody

Fig. 2. Antibody respones and protection from challenge with AS-6 strain in pigs inoculated with S^- strain

Table 5. Protection by S⁻ strain from fetal infection

Pregnant sow		Inoculation with S⁻ strain					Challenge with AS-6 strain							Days between challenge and autopsy	Change of fetuses			
No.	Age in Years	Route	Dose	Days of gestation	Viremia	Symptom	Days of gestation	Route	Dose	Antibody titer at challenge NT	Antibody titer at challenge HI	Viremia	Symptom		Alive	Dead	Mummified	Virus recovery
1	2	SC	$10^{7.0}$	4	—	—	28	iv	$10^{7.0}$	16	16	—	—	14	11	0	0	—
2	2	SC	$10^{7.0}$	4	—	—	28	iv	$10^{7.0}$	128	64	—	—		11	0	0	—
3	2						28	iv	$10^{7.0}$	<10	<10	+	—		2	2	7	+
4	2						31	iv	$10^{7.0}$	<10	<10	+	—		12	1	0	+

HI : Hemagglutination-inhibiting antibody
HT : Neutralizing antibody

inoculated sows manifested no abnormal clinical signs and were negative for viremia.

The four sows were challenged by intravenous inoculation with $10^{7.0}$ $TCID_{50}$ of the field (AS-6) strain 24 days after inoculation with the S⁻ strain. The two sows immunized against the S⁻ strain had an NT titer of 1:16 and 1:128 and HI titer of 1:16 and 1:64, respectively, at the time of challenge. Both NT and HI titers were less than 1:10 in the two control sows at the time of challenge.

Pyrexia or any other abnormal clinical sign was not noticed in the 4 sows after challenge. Viremia was not found in either sow immunized with the S⁻ strain, but was seen in both control sows for 4 days after challenge.

The 4 sows were sacrificed for autopsy 2 weeks after challenge. In them, the placenta and fetuses were examined and an attempt was made to recover the virus from the fetal brain. Nothing abnormal was recognized in the placenta or fetuses in either sow immunized with the S⁻ strain.

In the control sows, however, hyperemia, hemorrhage and necrosis were noticed in the placenta. Of 11 fetuses of sow No. 3, seven were affected with mummification and four with hyperemia and hemorrhage. Of 13 fetuses of sow No. 4, one was found dead with hyperemia and hemorrhage.

The virus was not recovered from any fetus of the sows immunized with the S⁻ strain, but was from 2 surviving and 2 dead fetuses of control sow No. 3 and 6 surviving and 1 dead fetuses of control sow No. 4.

Field trials of S⁻ strain for prevention of stillbirth among sows

In 1970, in several districts in Hiroshima and Fukushima prefectures, field trials for the prevention of swine stillbirth by application of the S⁻ strain were conducted in gilts with no antibodies against JEV.

The gilts were divided into 3 groups. In the first and second groups, the S⁻ strain was inoculated once and twice in 2-ml doses containing $10^{6.2-7.5}$ $TCID_{50}$ of this virus, respectively. The third served as the control group without vaccination.

In each group, the state of NT and HI antibody response and the results of delivery after the epidemic period were studied. The following results were obtained:

1) In the 2 vaccinated groups 51 to 75% production of antibody with a titer of more than 1:20 was demonstrated before the epidemic season. The average titer of pre-epidemic NT antibody of the sows was about 1:60. After the epidemic season, the rate of possession of antibody was changed to 71 to 98%. In the unvaccinated control group antibody developed in all animals after the epidemic season.

2) In the vaccinated groups the results of delivery of mother sows were obviously better than in the control group. The average number of normal young delivered was larger by 1.9~3.5 per litter, and the stillbirth rate

Table 6. Results of delivery in field trial of S⁻ strain for the prevention of swine stillbirth

Group*1		N	V_1	V_2
Number of mother sows	Total	35	47	42
	Farrowed stillborn piglets	17	17	8
Number of farrowed young	Total	285	416	378
	Average	8.1	8.9	9.0
Number of normal young	Total	178	331	365
	Average	5.1	7.0	8.6
Number of stillborn piglets	Total	107	85	15
	Average	3.1	1.8	0.4
Rate of stillbirth (%)	Mother sows*2	48.6	36.2	19.0
	Farrowed*3 young	37.5	20.4	4.0

*1 N: Unvaccinated
V_1, V_2: Administrated in one dose and two doses of the S⁻ strain, respectively
*2 Number of mother sows which farrowed stillborn piglets/Total number of mother sows
*3 Total number of stillborn piglets/Total number of farrowed young

of the farrowed young was smaller by 1/2~1/9 than in the control group. Particularly, the finest results of delivery were obtained in the second group. The stillbirth rate of this group was 4%. Accordingly, it was presumed that stillbirth caused by JEV might be prevented almost completely by the inoculation of two doses of the S⁻ strain.

Discussion and conclusion

In 1970, the Japanese Society of Veterinary Science established the minimum requirements for the safety of Japanese encephalitis live virus vaccine.

By that time various experiments had been performed independently to develop vaccine from 3 attenuated strains, S⁻,[1),2),3)] "m",[7)] "at",[9)] and some variant strains which had been obtained in the process of production of the attenuated strains.

The results of these experiments were discussed from a veterinary and medical point of view and used as basic data for the establishment of these requirements.

In the development of a Japanese encephalitis live virus vaccine for swine, it is necessary to take its safety for human beings directly or indirectly into consideration, in addition to its safety for swine which are objects of inoculation with such vaccine. The following principles were introduced into these requirements from this point of view.

(1) No clinical symptoms are manifested by piglets inoculated subcutaneously with the respective vaccine within 10 days after birth.

(2) No placental or fetal infection or abnormal parturition occurs to female swine inoculated subcutaneously at about 1 month of pregnancy.

(3) No viremia is observed in swine inoculated subcutaneously at 1 month of age.

(4) Infectivity to *Culex tritaeniorhynchus summorosus* is attenuated in the respective vaccine.

(5) Pathogenicity for monkeys is attenuated in this vaccine.

(6) The vaccine has stabilized markers different from those of the field strain.

Of these principles, the first two were set up, taking the safety for swine into consideration. The other principles, except item (6), were adopted to prevent swine inoculated with the vaccine virus from being amplifiers, and verify the safety for human beings.

In item (6), it is required that the respective vaccine must be provided with markers which can be used to check whether the vaccine virus has regained pathogenicity or not.

The S⁻ strain possesses properties which meet all the items of the requirement mentioned above. Accordingly, the S⁻ strain is presumed to have safety for use as Japanese encephalitis live virus vaccine.

The results of the experiment on the immunization of swine and field trials for the prevention of swine stillbirth seem to indicate that the attenuated S⁻ strain can be used as live virus vaccine for the prevention of stillbirth in swine.

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