JAPANESE ENCEPHALITIS IN DOMESTIC ANIMALS

MORIMATSU WATANABE
National Institute of Animal Health, Kodaira, Tokyo, Japan

Incidence

Most of mammalians and birds are susceptible to Japanese encephalitis virus (JEV). A high incidence of latent infection with JEV was demonstrated among them by serological surveys. However, humans and horses are the most susceptible hosts which manifest the symptoms of encephalitis following natural infection with JEV.

Shimizu et al. (1949) demonstrated a high incidence of latent infection with JEV in pigs by serological survey, and also demonstrated that swine stillbirth was caused by JEV infection. They observed a high titer of viremia in pigs experimentally infected with JEV, and suggested that pigs might play an important role for the epizootics of Japanese encephalitis (JE).

Scherer et al. (1959) strongly suggested that pigs near Tokyo could serve as sources of JEV for the vector mosquito, Culex tritaeniorhynchus, on the basis of four observations: (1) Under natural conditions swine are frequently infected and developed viremia. (2) Swine viremia lasts long enough (2–4 days) for mosquitoes to become infected. (3) Laboratory reared C. tritaeniorhynchus transmit virus from pig to pig. (4) C. tritaeniorhynchus bite pigs in large numbers in nature.

Otsuka et al. (1965) demonstrated that the appearance of antibodies to JEV in pigs were correlated with the occurrence of JEV-infected mosquitoes.

Konno et al. (1966) insisted that the pig was one of the hosts most likely to serve as an amplifier of the infection which was passed by mosquito in a cycle involving pig-mosquito-pig-mosquito-man transmission.

For the collection of a large number of blood samples from animals and birds for serological diagnosis, filter paper strips are used by means of Nobuto (1965), a modification of Karstad's technique (1957). A nation-wide serological survey for pigs during the summer season was conducted for the purpose of predicting JEV infection in humans by research workers at various laboratories in Japan.

Kurata et al. (1965) examined the presence of hemagglutination-inhibition (HI) antibody to JEV in total of 4,542 blood samples collected through Japan by use of the filter paper strips during the non-epizootic season, such as in winter season. High percentage of the presence of the antibody was observed in pigs both under three months and over seven months of age. It is suggested that the former pigs received the maternal antibody through colostrum of dams, but the latter ones developed the antibodies through the natural infection during the summer season of the previous year. The remaining pigs, which were born and raised during the off-season of JEV epizootics, had few antibody to JEV. Such antibody negative pigs as well as young pigs, which had residual antibody become positive during the following summer season. Fetal death and stillbirth are observed among these primarily infected pigs with JEV.
As mentioned above, a nation-wide serological survey of pigs during the summer season had been conducted these years for the purpose of predicting the outbreak of patients suffering from JE on the basis of hypothesis that a pig will serve as an amplifier of the infection. Generally speaking, this hypothesis seems to be certain, however, Scherer et al. (1959) suggested that a migratory bird, black-crowned night heron (Nycticorax nycticorax), was one of the major sources of virus for vector mosquitoes, and also Sazawa et al. (1967) found that HI positive pigs were preceded about two months by positive cattle in a limited place (Fig. 1). These data may suggest that animals other than the pigs will serve as amplifiers sometimes depending on the difference of the place and the year. Thus, further experiments are needed for final conclusion on amplifiers.

There are two possible hypothesis about the overwinter of JEV. One is that JEV will hybernate in pig, lizard etc in Japan, and the other is that JEV will pass over the winter time of Japan in southern warm countries. Japanese research workers showed the possibility of overwinter of JEV in lizard and swine fetus, while research workers in Okinawa (Ura et al., 1967) detected swine positive reactors to JEV in the winter time of Japan. Watanabe et al. (1968) demonstrated the presence of positive reactors to JEV in cattle, goat and sheep in Indonesia in the winter time of Japan (Table 1). These data suggest that JEV will pass over the winter time of Japan in southern warm countries. Ura et al. found the 2-mercaptoethanol sensitive antibodies in their serum samples, but not yet Watanabe et al. Therefore, for the latter samples, final conclusion can not be made until the 2-mercaptoethanol (2-ME) test is performed.

Much further experiments are needed for the final conclusion about the overwinter of JEV.

**Diagnosis**

Diagnosis for the swine Japanese encephalitis is conducted by means of clinical, serological and histopathological findings.

For serological tests, HI test, complement fixation (CF) test, neutralization test and fluorescent antibody technique are used.

HI test is conducted by the method of Clark and Casals in pig (Sazawa et al.,
Acetone is used to remove non-specific inhibitor in serum, while it can not be used in case of sera collected by filter paper strips, but can be used Kaolin acid washed (Catalogue No. K-5, Fisher Sci. Co., USA) to remove the inhibitor. Sazawa (1965) found an inhibition of elution of the antibody from the filter paper when it was kept in a place containing a trace of formalin gas. Therefore, the samples should be kept very carefully.

Otsuka et al. (1966) found that the antibody in early stage of infection was 2-ME sensitive, and then it transferred to 2-ME resistant antibody. Appearance of 2-ME sensitive antibody to JEV means a primary infection.

CF test in pig can be conducted by the procedure of Sazawa et al. (1958). A direct CF test is successfully conducted by using 8 units of antigen and two-fold diluted serum in physiological or phosphate buffer solution which are heated at 60°C for 30 minutes prior to the test.

Neutralization test in pig can be conducted by a plaque reduction method by Sazawa et al. (1964) from Porterfield's method (1960).

Fluorescent antibody technique is available for diagnosis (Kusano et al., 1965). For the sero-diagnosis in the field, HI test is used because of simplicity.

The dams infected with JEV are symptomless during pregnancy, but produce fetal death and stillbirth. The fetuses are delivered in the states of mummy, stillbirth and normal appearance, but hydrocephalus. In the histopathological findings, a number of still-born fetuses revealed the lesions of non-prulent encephalitis.

A quite similar swine fetal death and stillbirth were produced experimentally with HVJ (Sendai virus) by Sasahara (1955), so that a differential diagnosis for swine fetal death and stillbirth is needed.

**Control**

JE vaccine for domestic animals has been produced from the material of 1.0% infected mouse brain saline suspension inactivated with an addition of formalin at the rate of 0.5%.

The potency test has been carried out by the method of Fujie and Watanabe (1953), that is, on the 1st and 4th day, an intraperitoneal injection of 0.1 ml of

### Table 1. Positive HI Reactors in Animals in Indonesia (1967-1968)

<table>
<thead>
<tr>
<th>Region</th>
<th>Animals</th>
<th>HI titers</th>
<th>Pos./exam.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Timor</strong></td>
<td>Cattle</td>
<td>71</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>55</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td><strong>Bali</strong></td>
<td>Cattle</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Java</strong></td>
<td>Cattle</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><strong>Lombok</strong></td>
<td>Cattle</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Bovine sera were examined by neutralization tests to JEV and also showed positive reaction.
the vaccine is given to 30 mice of 3-4 weeks old, and then on the 8th day, the test animals as well as 30 mice in control are respectively challenged with 0.2 ml of 10^{-1} dilution of mouse brain saline suspension infected with Nakayama strain of JEV, mouse brain passaged line. On the 22nd day, the test animals must survive over 50% under the conditions of the titer of the i. p. challenge virus used ranging from 10^2 to 10^3 LD_{50}, and the mortality of control mice inoculated with the same virus is over 90%. When the test animals survive over 50%, the vaccine is good. National veterinary assay has adopted this method.

By using this potency test, Watanabe et al. (1954) found the antigenic variation of JEV. Mouse brain passage line of Nakayama strain of JEV (M) was serially passaged through embryonated eggs, and the virus of the 2nd, 10th and 20th generation was designated as E2, E10 and E20 respectively. The vaccine prepared from E2, E10 or E20 was designated as E2, E10 or E20 Vaccine. The virus of egg passaged line of the Nakayama strain of JEV was given by the Chiba Prefecture Serum Research Institute which had been given by Kitaoka, National Institute of Health, Tokyo, and thereafter it had been kept in serial passage in eggs. This strain was serially passaged through mouse brain, and the virus of the 2nd, 10th or 20th generation of passage was designated as CM2, CM10 or CM20. The vaccine prepared from CM2, CM10 or CM20 was designated as CM2Vac, CM10Vac or CM20Vac. The vaccine prepared from M was designated as MVac.

Cross immune tests were carried out by use of these viruses and vaccines. In case of challenge with M, the potencies of the vaccines were as follows: E2Vac was the highest, E20Vac the lowest and E10Vac intermediate. That is, the potencies of the vaccines originated from mouse brain passed virus decreased in parallel with the increase of passaged generation in eggs when challenged with the mouse brain passaged virus.

In case of challenge with E2, E10 or E20, however, the potencies of E2Vac, E10Vac and E20Vac are almost the same.

On the contrary, the potencies of MVac was the highest when challenged with M or CM20, the lowest with CM2 and intermediate with CM10.

CM20Vac showed the same tendency as MVac.

The potency of CM2Vac was the highest for the challenge with CM2, but decreased in the order of CM10, CM20 and M.

From the results mentioned above, it is concluded that the antigenicity of mouse brain passaged line of Nakayama strain of Japanese encephalitis virus varies by serial passages in embryonated eggs, but the variation is reversible within 20 generations of passage.

After these findings, the vaccine made from infected chick embryo disappeared, because the mouse brain passaged virus has been used for the challenge against JE vaccine at National Veterinary Assay. However, no one knows what strain is the best for the prevention of natural infection with JEV.

After application of this vaccine in the field, the incidence of equine JE has decreased very much, such as 3,678 diseased horses in 1948, 200 in 1960, 77 in 1961, 2 in 1962, 1 in 1963, 0 in 1966 and 9 in 1967. However, this vaccine is not so effective for the prevention of swine fetal death and stillbirth caused by JEV as for equine JE.
Fig. 2. Chang of Antibody Titers in Pigs after Epizootic Season

Neutralization

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hemagglutination-Inhibition

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N₁ & N₂: Negative cont. without VAC.; P₁ & P₂: Positive control without VAC.
V₁₁ & V₁₂: Three successive repeated shots of 10% VAC.; V₂: Two shots of 10% VAC.; V₁₃: Three Shots of half-dose of 10% VAC. (5ml each).

Fig. 3. Difference of Virus Distribution between Parent Strain Sagara and its Attenuated Mutant "T37-" in Hysterectomized Colostrum-Deprived Pigs Inoculated by Subcutaneous Route

<table>
<thead>
<tr>
<th>Days examined</th>
<th>2 days after inoc.</th>
<th>4 days</th>
<th>6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCID₉₀/g</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>Virus distribution</td>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphnode</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note T37- was detected only in lymphnode on day 4.

Fig. 4. Difference of Virus Distribution between Parent Strain Sagara and Its Attenuated Mutant "T37-" in Hysterectomized Colostrum-Deprived Pigs Inoculated by Intranasal Route

<table>
<thead>
<tr>
<th>Days examined</th>
<th>2 days after inoc.</th>
<th>4 days</th>
<th>6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCID₉₀/g</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>Virus distribution</td>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphnode</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note T37- was detected only in blood on day 6.
The committee of studies on the prevention of swine fetal death and stillbirth caused by JEV, which was consisted of the members of Animal Hygiene Section, Ministry of Agriculture and Forestry; National Institute of Animal Health; National Veterinary Assay Laboratory; The Chemo-Serotherapeutic Research Institute; The Institute of Microbiological Chemistry; The Nihon Institute of Biological Science; Kitasato Institute; The Nihon Vaccine Institute of Research; and the Prefectures of Miyazaki, Kyoto, Kanagawa, Ibaragi, and Fukushima, conducted field experiments on the effect of formalinized JE vaccines.

As shown in Fig. 2, three successive repeated inoculations of 10 ml of 10% vaccine induced the highest antibody titers after vaccination, and showed the lowest rate of titer increase and the highest rates of no change and decrease of the titers after epizootic season of JE among the experimental groups. These data indicate that this group took the wild virus at the minimum rate during the epizootic season. In this group, the incidence of fetal death and stillbirth caused by JEV was the lowest.

On the other hand, control pigs which had not received the vaccine showed the highest rate of the antibody titer increase and the incidence of fetal death and stillbirth after the epizootic season.

The vaccination of 1.0% vaccine, which was conducted by the same method as that of 10% vaccine, was not so effective as that of 10% vaccine. In this group. The incidence of fetal death and stillbirth was in the middle between the 10% vaccine group and control group.

These formalinized vaccines are effective when they are used in a large amount of the vaccine and in three successive repeated inoculations at the intervals of one week and 4 weeks, but they are too expensive and troublesome to be applied in the field, and hard works have been conducted for the development of less expensive and more effective vaccine.

An attenuated mutant of JEV has been developed by Inoue (1964) by means of serial passage of a parent virus, Mukai strain, in mouse embryonic skin culture. Kodama et al. (1966) demonstrated that this strain did not cause any viremia and clinical symptoms in pigs which were deprived colostrum after natural birth.

Sazawa et al. (1967) has also developed an attenuated mutant by means of serial passage of Sagara strain, which had been isolated from an abnormal baby pig of stillbirth, in bovine kidney cell culture at 30°C. This strain did not cause any viremia and any clinical symptoms in specific pathogen-free miniatr ur pigs originated from USA, 3 weeks of age, and also in young pigs born in Japan. Morimoto et al. (1968) have selected a less virulent mutant, T37-, from this strain, and demonstrated a trace of viremia in hysterectomized produced colostrum deprived baby pigs (Figs. 3 and 4).

Pigs received each of these strains respectively demonstrated no viremia after challenge with a virulent strain of JEV at laboratories, however, the results in the field were something different from those at laboratories.

Takahashi et al. (1968) inoculated the Inoue's m-strain into all pigs which had been raised in Iki Island which is isolated far away from the Main Island and is the best place for the field tests like these, under the project of control against amplification of the vector mosquito infected with Japanese encephalitis virus by pig vaccination. They insisted that this m strain was very effective on
the prevention of the appearance of infective mosquitoes which should be originat-
ed from the amplifier (pig), however, almost all pigs vaccinated revealed the
antibody titer increase after the epizootic season. These data indicate two things:
one is that the m-strain is not so effective on the control of infective mosquitoes
as they have insisted, and the other is that there must be other amplifiers than
the pigs, because almost all pigs received the m strain took the wild JEV during
the epizootic season, and revealed the antibody titer increase.

As far as preliminary experiments of Miura et al. (1967) are concerned, the
grade of the immunity obtained by vaccination with live or killed vaccine is dif-
ferent due to the difference of pigs (Figs. 5 and 6). Generally speaking, two
shots of vaccines are much more effective than one shot, although the 2nd shot
is not effective when the antibody exists (Figs. 5b, 8 and 9). This phenomenon
is similar to that of inactivated vaccine which was reported in Bull. Nat. Inst.

Fig. 5. Repeated Inoc. with Live Attenuated Virus in Pig

(a)

Titer increase after 2nd shot

(b)

No increase after the 2nd shot

when antibody exhists

Fig. 6. A Good Response to One Shot

Note: S': An attenuated mutant of JEV.
K: Formalinized vaccine

A simultaneous inoculation of JE and hog cholera vaccines showed a good
production of both antibodies in specific pathogeb-free pigs (unpublished data by
Kodama et al., 1968; and Sazawa et al., 1968). However, much further experi-
ments are needed before the field application, because the immunity obtained by
JE live virus vaccine does not last so long as that by Hog cholera live vaccine
in pigs, and furthermore the live virus vaccine is not taken by pigs which have
had maternal antibody to JEV.
Conclusion

Further experiments are needed for final conclusion for the amplifier, overwinter in Japan, and the control of amplifier and viremia of JEV, and also for the prevention of swine fetal death.
REFERENCES


