Incidence, Diagnosis and Control of Japanese Encephalitis in Pigs in Japan, With Reference to Stillbirth

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Incidence of Japanese encephalitis in pig

Japanese encephalitis is a mosquito-born viral disease which occurs in Japan and several Far East countries, from east Siberia to Indonesia and from Japan to India. It has been found that Culex tritaeniorhynchus is one of the principal vectors of the disease. The disease has a summer-fall seasonal incidence and has assumed epizootic form which starts from south west and spreads to north east in Japan.

Japanese encephalitis virus is one of the ribonucleic acid (RNA) containing agents (Nakamura and Ueno, 1963; Igarashi et al., 1963) classified as Group B arbovirus and based on its antigenic properties (Casals and Brown, 1954). The size of the virus particle has a diameter of 38 $m\mu$ with a dense center and a distinct membrane, as observed in ultrathin section by electron microscopy (Higashi et al., 1963). The infective particle is sensitive to the action of ethyl ether, deoxycholate and lipase as well as some proteolytic enzymes (Takehara and Hotta, 1961).

The Japanese encephalitis virus (JEV) is cultivable in various kinds of tissue culture cells with or without manifesting cytopathic effect (CPE), such as chick embryo cell, hamster kidney cell, bovine kidney cell, monkey kidney cell (Vello cell), porcine kidney cell and established line of porcine kidney cell.

Most of the mammalians and birds are susceptible to JEV. A high incidence of subslinical infection with JEV was demonstrated among them by serological surveys. Humans and horses are the most susceptible hosts manifesting the symptoms of encephalitis following natural infection with this virus, whereas the others show only subsclinical infection with the exception of swine stillbirth caused by JEV.

After conduction of the vaccination on horses, the incidence of equine encephalitis has decreased very much, and now it is limited within 10 cases a year; however, the current vaccine is not so effective for the prevention of swine stillbirth. Therefore, swine stillbirth is now the most important among the JEV infections in animals and birds from the economical point of view.

Shimizu and Kawakami (1949) demonstrated a high incidence of inapparent 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). Prior to these reports, Takenouchi et al. (1938) and Mitamura et al. (1938) demonstrated a high percentage of swine positive reactors to JEV by means of neutralization tests. However, little emphasis was placed on the swine's possible role in the yearly ecology of JEV.

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 develop 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.

These four observations have already been reported by Japanese workers prior to this report. However, Scherer et al. (1959) strongly suggested that pigs were major natural sources of JEV for the vector mosquitoes, Culex tritaeniorhynchus.

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.

A nation-wide serological survey of 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 HI-antibody to JEV in a total of 4,542 blood samples collected throughout Japan by use of filter paper strip during the nonepizootic 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 guggested that the former pigs received the maternal antibody through colostrum of dams, but the latter ones developed antibodies through 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 antibodies to JEV. Such antibody negative pigs as well as young pigs which had recidual maternal antibody become positive during the following summer season. Stillbirths are obsered among these primarily infected pigs with JEV.

As mentioned before, a nation-wide serological survey of pigs during the summer season has been conducted these years for the purpose of predicting the outbreak of patients suffering from JE on the basis of a hypothesis that a pig will serve as an amplifier of the infection. Generally speaking, this hypothesis seems to be certain, but additional observations were obtained from the serological surveys by several workers.

As far as the surveys of Sazawa et al. (1957) are concerned, horse and cattle seem to be the other major amplifiers in some areas as the pig. The difference of amplifier seems to depend upon the difference of the place and the year. For example as shown in Fig. 1, a bovine po-

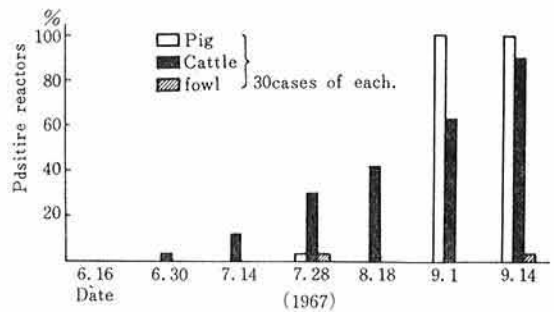

Fig. 1. Appearance of positive reactors to JEV in pigs, cattle and fowl.

sitive reactor to HI test appeared in Tochigi Prefecture on June 30, 1967, and then the positive reactors increased gradually before the appearance of swine positive reactors on September 1. In this case cattle seems to be a major amplifier instead of pig. In addition to this, Kitaoka et al. (1951, 1953), Buescher et al. (1959), and 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. Thus, further experiments are needed for final conclusion on main amplifiers,

Diagnosis for swine Japanese encephalitis

Diagnosis for JE in pigs are conducted by means of clinical, serological and histopathological examinations. For the serological tests, HI test, CF test, neutralization test and fluorescent anibody technique are used.

HI test is conducted by a modification of Casals' method (Sazawa et al. 1958). Aceton is used to remove nonspecific inhibitor in serum.

For the collection of a large number of blood samples from animals and birds, filter paper strips are used by means of Nobuto, (1965) a modification of Karstad's technique (1957). In this case, aceton can not be used to remove the

nonspecific inhibitor, but kaolin acid washed (Catalogue No. K-5, Fisher, Scientific Co., U. S.A.) is available for it. Sazawa et al. (1965) found an inhibition of elution of the antibody from the filter paper which was kept in a place containing a trace of formalin gas. Therefore, much attention should be taken to keep the filter paper strips after collecting the blood samples in order to get correct results.

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

Complement fixation (CF) test for pig is conducted by the procedure of Sazawa et al. (1958); the direct CF test is successfully carried out by using two-fold diluted swine serum in physiological or phosphate buffer saline solution which is heated at 60 C for 30 minutes.

Neutralization test in pig is conducted by the plaque reduction method modified from Porterfield's method (Porterfield, 1960; Sazawa et al. 1964).

Fluorescent antibody technique is used only at the laboratory.

For the sero-diagnosis in the field, HI test is used routinely because of simplicity.

The dams infected with JEV are symptomless during pregnancy, but produce stillborn or abnomal progeny. The fetuses are delivered in the state of mummy or normal size with hydrocephalus.

In the histological findings a number of stillborn fetuses revealed the lessons of non-prulent encephalitis.

A quite similar swine stillbirth was produced experimentally with HVJ by Sasahara (1955), so that a differential diagnosis for swine stillbirth is needed to make it sure. Therefore, HVJ will be also described in this paper as appendix.

Control

JE vaccine for domestic animals has been produced from the material of 0.2% infective mouse brain saline suspension inactivated with an addition of formalin at the rate of 0.5%.

The potency test is carried out by use of the Fujie and Watanabe method (1953). This method is as follow: on the 1st and 4th day, an intraperitoneal injection of 0.1 cc of the vaccine is given to 30 mice of 3 to 4 weeks old, and then on the 8th day, the test animals as well as 30 mice in control are respectively inoculated intraperitoneally with 0.2 cc 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 virus infectivity in control, being that LD_{50} is over 10^{-3}, for example $10^{-2.6}$, and also the mortality in 10^{-1} is over 90%. When LD_{50} is under 10^{-4}, the test must be retested. When the test animals survive over 50%, the vaccine is good.

This method has been used for National Veterinary Assay for Japanese encephalitis Vaccine.

By using this potency test, Watanabe et al. (1954) found the antigenic variation of JEV.

Mouse brain passaged line of Nakayama strain of JEV (M: standard line) was rerially passaged through embryonated eggs, and the virus of the 2nd, 10th, and 20th generation was respectively designated as E2, E10 and E20. The vaccine prepared from E2, E10 or E20 was designated as E2Vac, E10Vac or E20Vac. The virus of egg passaged line given by the courtesy of Kitaoka at the National Institute of Health in Tokyo was serially passaged through mouse brain, and the virus of the 2nd, 10th or 20th generation of passage was degignated 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 vaccines and viruses.

In the 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 potency decreased in parallel with the increase of passage generation in egg.

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

On the contrary, the potency of MVac was the highest in the case of challenge with M or CM20, the lowest in the case of challenge with CM2 and intermediate in the case of challenge with CM10. CM20Vac showed the same tendency as MVac.

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

From the results mentioned above, it is concluded as follows: the antigenicity of mouse brain passaged line of Nakayama strain of JEV varies by serial passage through embryonated eggs, but the variation is reversible within 20 generations of passage.

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

After the application of the vaccine in the field, the incidence of equine JE 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 stillbirth caused by JEV. Hard work has been conducted for the development of effective vaccine to JEV infection in Japan.

An attenuated mutant of JEV has been developed by Inoue (1964) by means of serial passage of a parental virus, Mukai strain, in mouse embryonic skin cultures. Kodama et al. (1966) demonstrated that this strain did not cause any clinical signs with little viremia in colostrum-derived pigs inoculated with.

Sazawa et al. (1967) has also developed an attenuated mutant by serial passage of Sagara strain which had been isolated from an abnomal baby pig of stillbirth, in bovine kidney cell culture at 30°C. This strain does not grow at 37°C in bovine kidney cell culture, whereas it grows rather well at 30°C without CPE. At 25°C, it grows very well with CPE. The method for the intermediate growth, the cultivation at 30°C, has been adopted for the attenuation, however, the serial passage of the virus at 25°C has also been continued.

When this strain is inoculated subcutaneously into specific pathogen-free colostrum-derived baby pigs, one day of age, a slight viremia is observed, but these baby pigs remain symptomless. Older pigs inoculated do not show any viremia and any symptoms.

This strain has characteristics of no growth at 42°C and smaller plaque in size than that of parental virus on swine embryonic kidney cell: ESK-No. 1.

References

1) Buescher, E. L., Scherer, W. F., McClure J. T., Rosenberg, M. Z., Yoshii, M. and Okada, Y.: Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. Amer. J. Trop. Med. and Hyg. 8, 678-688, 1959.
2) Casals, J. and Brown, L. V.: Hemaggultination with arthrod-born viruses. J. Exp. Med. 99, 429-449, 1954.
3) Fujie, N. and Watanabe, M.: Studies on potency test against Japanese encephalitis vaccine. I. A test for the potency of the encephalities vaccine by using young mice 3-4 weeks old. Med. and Biol. 29, 140-147, 1953.
4) Higashi, N., Inoue, Y., Matsumoto, A., and Fujiwara, E.: Electron microscopic studies on Japanese encephalitis visus grown in tissue cultue cells. 11th. Annual Meet. Soc. Japanese Virologists. 1963, pp. 14.
5) Igarashi, A., Kitano, H. and Fukai, K.: An infectious and ribonuclease-sensitive fraction from mouse brain infected with Japanese B encephalitis virus. Biken J. 6, 21-23, 1963.
6) Karstad, L., Spalatin, J. and Hanson, R. P.: Application of the paper disc technique to the collection of whole blood and serum samples on Eastern equine encephalomyelitis. J. Inf. Dis. 101, 295-299, 1957.
7) Kitaoka, M., Okubo, K., Miura, T. and Nakamura, N.: A latent infection of Japanese encephalitis virus in birds. Jap. J. Bact. 6, 293-295, 1951.
8) ———, ———, ———, and ———.: Relationship between Japanese B encephalitis and Russian Spring-Summer encephalitis and birds. Jap. J. Med. Sci. and Biol. 6, 247-256, 1953.
9) Kodama, K. and Sasaki, F.: Experimental infection of colostrum-derived piglets with Japanese encephalitis virus. Jap. J. Vet. Sci. 8 Suppl. Proc. 62nd Meet. Jap. Soc. Vet. Sci. 475-476, 1966.
10) Konno, J., Endo, K., Agatsuma, H. and Ishida,

N.: Cyclic outbreak of Japanese encephalitis among pigs and humans. Amer. J. Epidem. 84, 292-300, 1966.
11) Kurata, K., Nobuto, K., Sato, S. and Kaizuka, I.: Application of the filter paper method of the collection of whole blood samples for HI test in Japanese encephalitis. III. Nation-wide survey of HI antibodies in swine which were born and raised during the off season of mosquito in Japan of 1965. J. Vet. Sci. 27, Suppl. Proc. 60th Meet. Jap. Soc. Vet. Sci. 443, 1965.
12) Mitamura, T., Hazato, H., Kitaoka, M., Watanabe, S., Okubo, K., Ichikawa, O., Tenjin, C., Hayashi, T. and Tomisawa. T.: Virocidal action of sera harvested from healthy humans and animals in both endemic and nonendemic areas in Japan. Tokyo Iji-shinshi 62, 802-804, 1938.
13) Nakamura, M. and Ueno, Y.: Infectious ribonucleic acid of Japanese B encephalitis virus: Optimal conditions for its extraction and for plaque formation in chick embryo cell monolayers, and some biologic properties. J. Immun. 91, 136-143, 1963.
14) Nobuto, K.: Filter paper strip for collection of blood in a large scale. Modern Media. 11(9), 363-374, and 11(11), 440-445, 1965.
15) Otsuka, S., Masago, K., Kunihiro, H. and Motomura, I.: Studies of the effect of 2-mercaptoethanol on swine anti-sera against Japanese encephalitis virus. I. Jap. J. Bact. 21, 724-730, 1966.
16) Porterfield, J. S.: A simple plaque-inhibition test for the study of arthopod-born viruses. Bull. WHO 22, 373-380, 1960.
17) Sazawa, H., Kawamura, H., Chikatsune, M., Kataoka, T., Tsubahara, H, and Watanabe, M.: Studies on seroreaction of Japanese encephalitis in swine. Bull. Nat. Inst. Anim. Hlth. 34, 83-94, 1958.
18) Sazawa, H.: Application of the plaque technique to virus titration and neutralization test of Japanese encephalitis. Bull. O.I.E. 62, 851-854, 1964.
19) ———: Unpublished data. 1965.
20) Scherer, W. F., Buescher, E. L. and McClure, H. E.: Ecologic studies of Japanese encephalitis virus in Japan. V. Avian factors. Amer. J. Trop. Med. and Hyg. 8, 689-697, 1959.
21) Scherer, W.F., Moyer, J.T., Izumi, T., Gresser, I. and McCown: Ibid. VI. Swine infection. Ibid. 698-706, 1959.
22) Shimizu, T. and Kawakami, Y.: Studies on swine stillbirth, especially on its relation to Japanese encephalitis. Report of Government Experimental Station for Animal Hygine 22, 117-128, 1949.
23) ——— and ———: Experimental studies on swine artificially inoculated with Japanese encephalitis virus. Ibid. 151-158, 1949.
24) Takenouchi, M., Sato, J., Yamamoto, I., Nagahara, T., Takizawa, M. and Okada, Y.: Studies on the causative agent of endemic encephalitis (VI). Tokyo Iji-shinshi 62, 1155-1162, 1938.
25) Takehara, M. and Hotta, S.: Effect of enzyms on partially purified Japanese B encephalitis and related arbor viruses. Science 134, 1878-1880 1961.
26) Watanabe, M. and Fujie, N.: Experimental studies on antigenic variation of Japanese B encephalitis virus. Virus 4, 227-230, 1954.

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Appendix to JE:

HVJ in Pig Especially With Stillbirth in Japan

Incidence

HVJ (Hemagglutinating Virus of Japan) was isolated from pigs by Sasahara et al. (1954) and Watanabe et al. (1954) independently.

Fukumi et al. had recognized a new virus since 1950 when they had carried out serial passages of influenza virus in mice by an intranasal route in a contaminated condition, and they had called it HVM (Hemagglutinating Virus of Mice). Kuroya et al. (1953) had isolated this virus from newborn pneumonitis and called it Type Sendai, and now in foreign countries this virus is often called Sendai virus.

Thus, confusion arose for the nomination of this new virus. Therefore, Watanabe asked the Society of Japanese Virogists to bring the name of this virus into unity. The Society has standardized the name as HVJ in 1959. However, Andrews et al. (1959) suggested that HVJ should be designated as Myxovirus parainfluenza 1. HVJ and HA 2 viruses are included in Parainfluenza 1.

HVJ causes a slight respiratory symptom in natural and experimental infections and a still-

birth in experimental infection.

Ninety-eight cases (14%) of 712 serum samples collected at slaughter houses throughout Japan were positive by neutralization tests (Sasahara, 1955), but only four cases (0.8%) of 2185 serum samples collected at random throughout Japan from October 1957 to March 1958 were positive by complement fixation (CF) tests, however, in some limited area, such as in Kyoto Prefecture, positive reactors were found at the rate of 6.63 % of pigs tested (Yamamoto et al., 1959). In this case further survey by use of neutralization tests are needed, because CF antibody disappears much earlier than neutralizing antibody.

As mentioned before, HVJ has a capability of producing stillbirth in pregnant swine, but at the present time, field outbreaks of stillbirth in swine are unknown.

Diagnosis

Diagnosis is conducted by means of virus isolation and serological tests, such as HI, CF and neutralization (NT) tests.

HI test: In case of swine Japanese encephalitis, removing the nonspecific inhibitor to hemagglutination is necessary for the HI test by using aceton or kaolin (Sazawa et al.. 1958). On the contrary, in case of HVJ infection in swine, pretreatment of the swine serum by aceton or or kaolin is not needed for the HI test, because there is no nonspecific inhibitor to HVJ in swine serum. When chicken blood cells are used for the HI tests, however, 5% of chicken red blood cells should be added to the test serum inactivated at 56°C for 30 minutes in order to remove the normal agglutinin. The mixture is placed at 37°C for one hour or at 4°C for overnight, and then is centrifuged. The resulting supernatant is used for the HI test.

The HI test is a simple technique and used for a routine diagnosis of this disease. A significant rise of HI antibody titer two-four days after inoculation with HVJ and fall of the titer within five weeks are observed in pigs. Therefore, the HI test is actualy used for the diagnosis in the early stage of this disease. However, the HI test is not useful to survey the incidence.

Neutralizing antibody appears later than the HI antibody, but lasts much longer than the latter. The neutralization test is usually carried out by using swine kidney cell cultures with observation of CPE.

CF antibody appears at about the same time with NT antibody, but disappears earlier than the latter and later than HI antibody. CF test is carried out by the Kolmer's method.

Standards for serological diagnosis are as follows:

	HI*	CF*	NT**
−	8		1.0
±	1:16		1.1−1.9
+	>1:32	>1:2	>2.0

* Dilution of serum

** Log index of neutralization

Virus isolation: Virus isolation for diagnosis is performed by inoculation of the material into the allantoic cavity or yolk sac of the embryonated hens' egg, or onto the monkey's kidney cell or the swine's kidney cell culture.

Differential diagnosis of stillborn fetuses.

Hydrocephalus or cerebromalacia which was marked in experimental and natural cases of stillborn fetuses produced by Japanese encephalitis virus was not observed in these cases of stillborn fetuses experimentally produced by HVJ (Sasahara, 1955). In the former, nonprulent encephalitis is observed, but not in the latter, on the basis of histological findings.

Control

No vaccine has been developed yet.

A hyper immune serum showed a favorable effect for prevention of fever, clinical symptoms, and decrease of body weight in pigs when the serum was given one day prior to the inoculation of a virulent strain of HVJ (Sasahara et al., 1960).

References

1) Andrews, C. H., Bang, F. B., Chanock, R. M., and Zhdanov, V. M.: Para-influenza viruses 1, 2, and 3: suggested names recently described myxovirus. Virology 8, 129-130, 1959.
2) Fukumi, H., Nishikawa, T.: A pneumotropic virus from mice causing hemagglutination. Jap. J. Sci. and Biol. 7, 345-364, 1954.
3) Kuroya, M., Ishida, N., and Shiratori, T. Newborh virus pneumonitis (Type Sendai). II. The isolation of a new virus possessing hemagglutinin activity. Yokohama Med. Bull. 4, 217-

233, 1953.
4) Sasahara, J., Hayashi, S., Kumagai, T., Yamamoto,T., Hirasawa, N. and Kato, K. : On the influenza-like new virus from the swine. Jap. J. Vet. Sci. 15 Suppl. 142, 1954 (March).
5) Sasahara J. : Studies on the HVJ (Hemagglutinating Virus of Japan) newly isolated from the swine. Exp. Report No. 30 of Nat. Inst. Anim. Hlth. pp. 13-38, 1955.
6) Sasahara J., Kato, K. and Watanabe, M. : Stu dies on immune serum of Para-influenza 1. Bull. Nat. Inst. Anim. Hlth. No. 38, pp. 1-9, 1960.
7) Sazawa, H., Kawamura, H., Chikatsune, M., Kataoka, T., Tsubahara, H. and Watanabe, M. : Studies on seroreaction of Japanese encephalitis in swine. Bull. Nat. Inst. Anim. Hlth. 34, 83-94, 1958.
8) Watanabe, M., Sato, U., Nishimura, Y. and Narita, R. : A virus disease of Pig. I. Clinical symptoms and the isolation of the causal agent. Jap. J. Vet. Sci. 16, Suppl. pp. 48, 1954 (December).
9) Watanabe, M., Sato, U. and Nishimura, Y. : Ibid. II. Hirst's phenomenon and its variation. Ibid. pp. 48-49, 1954.
10) Yamamoto, K., Morita, S., Harada, K., Kuroki, Y., Sasahara, J. and Omori, T. : Survey on HVJ in pig by use of complement fixation test. J. Jap. Vet. Med. Assoc. 12, 170-174, 1959.

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