EPIDEMIOLOGICAL ANALYSES OF JAPANESE ENCEPHALITIS VIRUS SPREAD FROM MOSQUITOES TO PIGS THROUGH 5 YEARS

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SUMMARY: The results from a five year survey on the antibody rise in summer against JE virus in pigs in Natori District of Miyagi Prefecture were analysed with regard to the time of antibody rise, detection rate of 2-ME sensitive antibodies, level of antibody titers, and duration of high-titered antibodies. These serological results obtained in pigs were finally correlated to the isolation rate of JE virus from mosquitoes collected in the same area and to the incidence of JE patients among 1,750,000 residents in Miyagi Prefecture.

On the basis of this limited 5 year survey, one can conclude the following:
1. A hundred percent infection among pigs and positive isolation of the virus from mosquitoes have consistently been observed during these 5 years. Thus the cyclic outbreak between mosquitoes and pigs existed every year, even in the absence of the spread to humans.
2. The earlier the appearance of the initial antibody rise and the longer the duration of high-titered antibody among pigs, the higher the incidence of infection among humans.
3. The time of the initial and subsequent isolation of virus from mosquitoes coincided completely with the time of antibody rise among pigs, as was pointed out by us 5 years ago.
4. A minor pre-outbreak and minor post-outbreak of infection among pigs were noticed before and after the main outbreak among pigs. The fact was clearly illustrated by pursuing 2-ME sensitive antibodies.
5. The height of antibody titers in pig sera was assumed to be a reliable indicator to predict the amount of virus harbored by mosquitoes. The titer in pig sera was lowest in a year of the least incidence of human infection, irrespective of the fact that 100% of pigs were infected every year.
6. An assumption was proposed to predict the appearance of virus harboring mosquitoes almost 3 weeks in advance by setting the threshold number of 150 of the candidate mosquitoes trapped per night under a defined condition.

INTRODUCTION

The Japanese encephalitis virus is found in practically all geographic sections in Japan except for Hokkaido. In early summer, the epidemics among humans start from southern islands and the wave of epidemics moves from south to north.
through Honshu Island. Our study was performed in Miyagi Prefecture which is located in the northern part of Honshu Island and is close to the northernmost limit of Japanese encephalitis outbreaks among humans (Oya, 1969).

The cyclic transfer between pigs and humans at an interval of 18 days was first suggested by us (Konno et al., 1966) and the fact was later confirmed by others (Nakamura et al., 1968, Oda et al., 1969 and many other reports).

This paper is concerned chiefly with the fate of hemagglutination inhibition (HI) antibodies among pigs in Miyagi Prefecture examined through 5 years, starting from 1964 up to 1968. The time of antibody rise, the fate of 2-mercaptoethanol (2-ME) sensitive antibodies and the level of HI titers among pigs, considered together with the virus isolation results from mosquitoes, were analyzed and all of the results obtained were correlated to the extent of prevalence of Japanese encephalitis among humans.

**MATERIALS AND METHODS**

*Collection of Pig Sera:* From 1964 to 1968, serum specimens were collected from pigs of about 6 months old at the County Slaughterhouse in Natori, Miyagi Prefecture. Samples were obtained once a week during the epidemic period (July, August and September) and once or twice a month in the other months. As a rule, 30 to 50 pigs were bled at once from a jugular vein.

*Titration of HI Antibody:* Pig sera were tested for HI antibody against Japanese encephalitis (JE) virus. HI antibody titration was performed according to the method of N. I. H. of Japan, which is a slight modification of the original technique described by Clark and Casals (1958). Acetone treatment of sera, adsorption of sera by goose blood cell and the procedure for HI test was detailed previously (Konno et al., 1967). Standard antigens were purchased from Takeda Chemical Industries, LTD. In 1964, Nakayama-NIH strain was used as the HI antigen, but since April 1965 JaGAr;01 strain was used. With the former antigen, the HI titer was almost one half to that obtained by the latter. Pig sera were simultaneously tested for the presence of 2-ME sensitive antibodies. Detailed techniques to titrate 2-ME sensitive antibody were described previously (Konno et al., 1967).

*Collection of Mosquitoes:* Mosquito-trapping was carried out at a station about 3 km north to the County Slaughterhouse in Natori (Fig. 1). The station was located between paddy fields and vegetable gardens. Two window type mosquito traps made of funnel-like wire screen (Kato et al., 1966) were set to a pigsty in which one young pig was placed. Mosquitoes flying into the pigsty were captured 3 to 5 times a week from 6:00 in the evening to 6:00 in the next morning. The captured mosquitoes were sorted after chloroform anesthesia. Then female *C. tritaeniorhynchus* were stored in small glass tubes with rubber stoppers tightly sealed with adhesive plaster and kept in a dry ice box until the virus isolation.

*Virus Isolation from Mosquitoes:* Virus isolation was made with each pool consisting of 50 mosquitoes of non-blood sucking female *C. tritaeniorhynchus*. Mosquitoes in a pool were suspended in 2 ml of phosphate buffered saline (pH 7.2) containing 10% inactivated calf serum, 500 units penicillin and 500 µg streptomycin per ml. After keeping in an ice water bath for 90 min, the mosquito
suspension was centrifuged at 10,000 rpm for 10 min at 4°C. A 0.02 ml each of the supernatant was injected intracerebrally into 2 litters of dd strain suckling mice, 3 to 5 days old. Death of mice with typical symptoms was taken as positive isolation and the isolated viruses were identified both by the neutralization test in suckling mice and by the HI test. The HI antigens for virus identification were prepared from infected mouse brains at the third passage by acetone-ether extraction. Standard antisera supplied from N.I.H. of Japan was used for the identification.

Detection of human cases: Clinically diagnosed cases of JE virus infection in Miyagi Prefecture was legally reported to 14 Health Centers in this prefecture and serum specimens were immediately collected and tested for the 2-ME sensitive HI antibodies. When patients survived, paired serum specimens were obtained. Either the presence of 2-ME sensitive antibodies or the rise of antibody titer was used as a criterion of positive serodiagnosis. The population of Miyagi Prefecture is almost 1,750,000 and the number of serologically confirmed cases was 58 in 1964, 2 in 1965, 10 in 1966, 11 in 1967 and 0 in 1968, respectively.

RESULTS

A Hundred Percent Infection of Pigs Irrespective of the Non-incidence of Patient

Results of a 5 year survey on the HI antibodies among pigs in Natori Slaughterhouse in the epidemic season are illustrated in Fig. 2. In the figure, curves written by the solid line illustrate the positive rate, where the HI titer over 1:10 was taken as positive infection. As described in Materials and Methods, the mother population of serum specimens was 30 to 50 every week in the epidemic season. Looking through the figure, it is evident that a 100% infection of pigs occurred every year, irrespective of the spread of infection to humans. As men-
Fig. 2. Development of HI antibodies against JE virus among pigs from 1964 to 1968.

Solid lines represent the positive rates of antibody (≥1:10) possessing pigs, based on groups of 30 to 50 pigs.
Each bar represents the positive rate of serum specimens containing 2-ME sensitive antibodies.

mentioned already, although serologically confirmed human cases were only 2 in 1965 and nil in 1968, a 100% infection was evident among pigs. This was further confirmed by testing serum specimens obtained from the other 3 County Slaughterhouses in the same Prefecture in the epidemic season (Konno et al., 1966). The result may indicate that an enough amount of JE virus which is able to infect all pigs in Miyagi Prefecture was harbored by mosquitoes during the epidemic period.
The initial time of infection among pigs throughout these 5 years can also be seen in Fig. 2. There was a remarkable difference in the time of the initial antibody rise and the earliest antibody rise was found in 1964 (the first week of August), and concomitantly we experienced the highest infection rate among humans in this year. The second early rise was observed in 1967 (the second week of August), when the prevalence of JE outbreak was outstanding in South West of Japan, although the number of patients in Miyagi Prefecture was limited to 11.

When the antibody positive rate of pigs reached 100%, it continued as such for almost 5 months. The rate declined slowly thereafter and became stabilized in the next May under 20% (Fig. 3). These antibodies in early spring were all 2-ME resistant. The pattern of the disappearance of HI antibodies was practically the same through the 5 years' survey.

Detection Rate of 2-ME Sensitive Antibodies

Detection of 2-ME sensitive antibodies was first introduced into the epidemiological analysis of JE virus infection among pigs by the authors (Konno et al., 1967). This method made it possible to discriminate whether HI titers found in late spring or early summer were due to antibodies produced by infection in the previous year (maternal antibodies) or in the current year (self-producing antibodies). Since the half-life of this antibody was very short (Konno et al., 1967), the appearance of 2-ME sensitive antibodies gave us a reliable information on the occurrence of an early infection among pigs. Thus the appearance and disappearance of 2-ME sensitive antibody gave us the information when antigenic stimuli were given. As a result, the detection of 2-ME sensitive antibodies enabled us to demonstrate the
incidence of minor outbreaks. Results obtained on the ratio of antibody containing 2-ME sensitive antibodies are shown in Fig. 2 by black bars, where the specimen was judged to contain 2-ME sensitive IgM, when the reduction in the titer was more than 8 times after 2-ME treatment. In Fig. 2, the rise and fall of 2-ME sensitive antibodies revealed the waves of antigenic stimuli were given to the population of pigs.

In 1964, an existence of pre-outbreak among pigs was evident by the presence of 2-ME sensitive antibodies at an early weeks in July. It is consistent with the results obtained by pursuing the antibody positive rate, in which early rise was also clearly detected.

In 1965, 100 % HI antibodies became 2-ME sensitive at the week when more than 50 % of the pigs turned out to be positive in the HI test. The 2-ME sensitive antibodies continued to exist for about 4 weeks and then disappeared. Therefore, in 1965 the incidence of natural infection of JE virus among pigs was estimated to be only once.

In 1966, the detection rate of 2-ME sensitive antibodies reached the height of 100 % at the same period as in the preceding year. However, the detection rate declined at a slow pace and the existence of the antibodies lasted for about 2 months. Meanwhile, 2 small peaks followed at the end of September and in the middle of October. This was considered as a kind of minor post-outbreak among pigs.

In 1967, as was the case in 1964, there was a minor outbreak ahead of the major outbreak and the 2-ME sensitive antibodies lasted as long as 2 months followed by a rapid disappearance as were also the case in 1964 and 1965.

In 1968, 100 % 2-ME sensitive antibodies were found only in one week of August, when positive rate of HI test rose abruptly and the detection rate of the 2-ME sensitive antibody became almost 20 % by the next week, suggesting the short duration of the antigenic stimuli. The least distribution of JE virus among pigs was suggested in this year, not only on the basis of 2-ME sensitive antibody analysis but also on that of the level of antibody titers as will be described later.

**Mode of the Distribution of HI Titers**

On the basis of an idea that the level of antibody titers might be a reflection of the amount of virus introduced into pigs, the 5 year survey results were analyzed. This is illustrated by Fig. 4. One can first notice the fact that the time when the positive rate of HI test (curves in solid line) rises abruptly and the time when HI titer of individual pigs starts to increase are closely corresponding to each other. With one exceptional year of 1968, the mode of HI titer was over 1:640 with a narrow distribution when the positive rate of HI test reached a maximum. On the other hand, the mode in 1968 ranged between 1:320 and 1:640 with a rather wide distribution toward lower titers. As mentioned already, the 2-ME sensitive antibody was found only one week through the epidemic season and no human cases were found in this year.

The duration of high titered (≥1:640) antibody in more than 50 % population of pigs was compared in Table 1. This may again illustrate the fact that in 1964, the duration was as long as 12 weeks. The incidence of human epidemic was highest in this year (58 persons). A relatively longer duration was found in
1966 and 1967, in which the incidence of human epidemic was 10 and 11 persons, respectively. The fact that human epidemic was scarcely detectable in 1965 and not in 1968 was in accord with the finding that the duration of a high HI titer among pigs was shorter in the respective years.
Table 1. Duration of high-titered (≥1:640) antibody in more than 50% pigs

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* Counted from the first week of January.

Seasonal Prevalence of Culex tritaeniorhynchus in Correlation to the Virus Isolation

Analyses of HI antibodies in pigs described above gave an information on the amount of virus widespread through the prefecture in these 5 years. Initiation of infection among pigs, as well as the heaviness and duration of infection varied from year to year, although a 100% infection of pigs occurred through 5 years.

Next effort was made to correlate the above serological results to the seasonal appearance of the mosquitoes and also to the isolation results of JE virus from the mosquitoes. In discussing the former correlation, one of the difficulties encountered was that the bait trap-captured mosquitoes did not necessarily represent the real population of the mosquitoes endowed with the ability to transmit the virus. Having these considerations in mind, the seasonal prevalence of female Culex tritaeniorhynchus trapped at the same station in Natori was examined from 1964 to 1968. Results obtained are illustrated in Fig. 5, in which the number of mosquitoes captured per trap per night was given by the dotted line. One can notice the difference of the time of appearance and the absolute number of female C. tritaeniorhynchus captured in the same trap in each year. The earliest appearance was noticed in 1964, being accompanied with the early rise in HI antibody in pigs.

Evidently, one can not find any correlation between the peak of mosquito appearance (dotted line) and the antibody rise in pigs (solid line). However, if the time when the absolute number of mosquitoes reached a threshold value (150 mosquitoes per trap per night) is taken as an index of time, it was roughly 3 weeks ahead of the antibody rise. Although this was consistently found as far as the 5 year survey results are concerned, further evaluation must be necessary to draw a significant conclusion.

In the same figure, positive virus isolation are illustrated with solid circles and negative one with open circles. One circle represents a pool consisting of 50 mosquitoes. At the time when the initial antibody rise was recognized, the increase of positive isolation rate became quite apparent. This conclusion first drawn from the study performed in 1964 (Konno et al., 1966) was confirmed by
Fig. 5. Number of mosquitoes trapped in a pigsty and positive virus isolation.

Dotted lines represent the numbers of female Culex tritaeniorhynchus captured in one night.

Circles are the isolation results of 50 mosquito pools. Solid lines represent the positive rates of antibody (≥1:10) possessing pigs based on groups of 30 to 50 pigs.
the additional 4 years’ survey.

In 1964 and 1968, JE virus was isolated from mosquitoes almost 2 weeks ahead of the major antibody rise among pigs and this must be the footprint of a minor preoutbreak noticed from the HI antibody analysis in pigs.

**DISCUSSION**

In the previous paper (Konno et al., 1966), it was suggested that a high incidence of JE virus antibody in pigs heralds an outbreak of JE among humans in the same geographic locality. The data also revealed the fact that the time when the prevalence of JE virus is highest among mosquitoes well corresponds to the time of apparent antibody rise among pigs. The latter fact has been confirmed by many other authors in Japan (Kato et al., 1966). Since our works were published, the detection of the initial antibody rise among pigs has currently been used for the prediction of JE virus spread in humans. This decision was made by the Ministry of Welfare as a public health policy for the warning and prediction of JE outbreaks, because of its reliableness.

A possible way of overwintering of the virus in lizards has been suggested experimentally by Doi et al. (1968). After hibernation over the winter in lizards (detectable only under simulated natural condition), the virus may be transmitted to a kind of mosquitoes, amplified in pigs (Scherer, 1959) and finally transferred to humans by *Culex tritaeniorhynchus*.

In this paper we extended the original works made in 1963 and 1964 to confirm the previous findings. Although the overwintering mechanism of JE virus has not been established yet, it is apparent from our study that the time of appearance of mosquitoes, that of virus harboring mosquitoes and the time of the initial antibody rise among pigs are different from year to year. The ecological study made by Kato et al. (1970) on the initial appearance and the prevalence of *C. tritaeniorhynchus* has already established their correlation to the weather conditions of the preceding winter and spring. However, these results cannot be correlated to the initial appearance of JE virus. In addition, difficulties still exist in performing such a study since we have no sensitive method to detect JE virus in mosquitoes in the early phase of epidemics. As a compensation for this disadvantage, the serological analysis of the blood ingested by early appearing female *C. tritaeniorhynchus* was made to know the blood source and the natural host to JE virus (Ishida, 1969). As far as examined, however, all the ingested blood came only from vertebrates, chiefly from pigs, but did not from the species of lizards, snakes or frogs living in the same geographic section. Such circumstances restrict our study to the epidemiological analysis of JE virus spread only after the initial antibody rise among pigs.

With no exceptional cases through the 5 years’ survey, when the positive rate of antibody abruptly rose up to 100 %, the rate of virus isolation from mosquitoes apparently increased. Thus the close correlation between the highest virus isolation among mosquitoes and the abrupt HI antibody rise in pigs is established. As far as the number of vertebrates is concerned, the population of pigs in Miyagi Prefecture (almost 100,000) in the next to that of humans (almost 1,750,000). Therefore, the role of pigs as an amplifier of JE virus is
thought to be most important, still leaving a possibility of the other vertebrates to play important roles on the ecology of JE virus.

The levels of antibody (Fig. 4) and the duration of high titered antibody (Table 1) among pigs were different from year to year, showing differences in the prevalent amount of JE virus from year to year. Moreover, by pursuing the detection rate of 2-ME sensitive antibody, the integral analysis of cyclic outbreaks was achieved. The intervals between the pre- and main outbreaks were almost 18 days in 1964 and 1967.

As for human epidemics, an earlier isolation of JE virus from mosquitoes may be the most critical factor to determine its widespreadness. However, it should be noticed that the assumption was made from this limited 5 years’ study in the northern part of Honshu Island.

For the earlier prediction purpose of JE outbreak in humans, an ecological analysis on the prevalence of candidate mosquitoes was also paralleled in this study. When the number of mosquitoes get to the threshold number of 150 per trap per night under the defined condition described here in at the same station, JE virus harboring mosquitoes appeared after the interval of almost 3 weeks. This kind of correlation may exist, but again we have to keep in mind that the assumption is based upon the results of the 5 years’ limited survey.

REFERENCES


