

## Studies on Japanese Encephalitis of Animals in Hokkaido

### III. Serological Observation on Horses Naturally Affected with the Enzootic in 1966\*

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### 北海道における動物日本脳炎に関する研究

#### III. 馬の自然感染例における血清学的観察

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### Introduction

In the first paper of this series (GOTO *et al.*, 1969) the authors reported that the prevalence of Japanese encephalitis (JE) of horses is decreasing markedly in numbers, and that this may be attributed to the decreasing number of horses and also to the effect of strict enforcement of the JE vaccination law. Owing to this, in recent years it is becoming increasingly difficult to conduct serological investigations on horses naturally infected with JE virus.

The present report is concerned with some antibody responses and its immunoglobulins in the sera of horses affected with the enzootic of JE infection.

### Materials and Methods

The JaGAR #01 (JaGAR), Nakayama-NIH (Nakayama) and Orukestoru strains of JE virus, the techniques of hemagglutination-inhibition (HI) test, complement-fixation (CF) test and also the 2-mercaptoethanol (2-ME) treatment of serum for HI test used in this study were described in our earlier report (GOTO *et al.*, 1970).

Serum: The serum samples of horses used in the present study were collected from five horses raised in the affected herds. And the additional four sera

\* Part of this study were presented at the 69th meeting of the Japanese Society of Veterinary Science in April, 1970.

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were obtained from apparently healthy horses in several different herds raised in the neighbourhood of affected areas. The sera were stored in a deep freezer at  $-20^{\circ}\text{C}$  until use.

Hyperimmune sera were prepared from one rabbit per strain by immunizing with each of the above-described viral strains. The animals were inoculated twice intravenously and twice subcutaneously, one week apart, with 2.0 ml of 10 percent suspension (W/V) of the infected mouse brains. The animals were bled on the 14th day after the last inoculation.

Gel filtration: Filtrations of the infected horse sera were performed following the method of URASAWA *et al.* (1968). Serum samples of 1.2 ml was applied to a Sephadex G-200 column ( $1.5 \times 80$  cm) and elution was carried out with phosphate buffered saline solution pH 7.3. But only 0.6 ml of serum was used in horse No. 1, because additional serum sample was not available. The flow rate was 8 ml/hr under hydrostatic pressure, and the effluent was collected with a fraction collector in 2.0 ml portions. These samples were determined for their protein concentrations by measuring the optical density at  $280\text{ m}\mu$ , and were also used for the HI and CF tests.

## Results

### 1. Description of the enzootic of JE infection in horses

In the Tokachi district of Hokkaido, the occurrence of JE infection in horses was not recognized for 4 years following the outbreak in 1961, with the exception of a single case of the disease reported in 1964 (GOTO *et al.*, 1969). As indicated in Table 1, however, on September 11, 1966 JE infections of horses occurred in A and C farm in Ikeda, Tokachi district. Following this, the infections spread to the B and D farms on the 13th and 24th of September,

**Table 1.** Clinical and serological surveys on the horses affected by the enzootic in 1966

Horse			Date of outbreak	Clinical signs	Termination	Antibody titers	
No.	Age (year)	Farm				HI	CF
1	1	A	Sep. 11	Yes	Death	1:320	1:16
2	16	B	Sep. 13	Yes	Recovery	1:320	1:32
3	1	C	Sep. 11	Yes	Recovery	1:320	1:32
4	12	D	Sep. 24	Yes	Recovery	1:640	1:64
5	6	D	—	No	—	1:160	1:16
6	1	E	—	No	—	1:640	1:16
7	1	F	—	No	—	1:1,280	1:64
8	1	G	—	No	—	<1:10	<1:4
9	8	G	—	No	—	<1:10	<1:4

respectively. All the horses (Nos. 1-4) showed clinical signs, and one out of four died on the 4th day after the onset of illness. The remaining three recovered from the disease on the 3rd and the 4th day after the initial appearance of the symptoms. The clinical signs included high fever of 39.2 to 40.3°C, depression, loss of appetite, circular movements, sensoparalysis and spastic paralysis of the legs. In the death case, further additional signs such as excitement and astasia were observed.

As for the result of serological tests on JE virus, four horses revealed clinical signs with titers of 1:320 to 1:640 in the HI test, and titers of 1:16 to 1:64 in the CF test, respectively. On the other hand, three (Nos. 5-7) of the five healthy control horses raised in the neighbourhood of affected farms also showed antibodies of 1:160 to 1:1,280 and 1:16 to 1:64 in each of the HI and CF tests. These results apparently indicate that the horses showing positive titers were infected apparently or inapparently with JE virus throughout the period of this enzootic. However, the remaining two control horses showed completely negative responses to both tests.

## 2. Responses of HI titers to the three different strains of JE virus in the infected horses

The purpose of this article is to determine the immunotype of the causal agent of this enzootic through the antibody responses of infected horses to JE virus strains, because the virus isolation experiments from the dead horse-brain resulted in a failure, in spite of use of the intracerebral inoculation method of suckling mouse. At present the immunotypes of JE virus are classified into two groups, i.e. JaGAR-type and Nakayama-type (OKUNO *et al.*, 1968). In the present study, prototype strains of JE virus —JaGAR and Nakayama— were used as the viral antigens for HI test. Moreover, the strain Oukestoru is also included in the comparative study because this strain originated in a horse from Hokkaido (YANAGAWA, 1949). Table 2 gives the result of HI tests on the three strains in

Table 2. HI titers of the horse sera against three JE virus strains

Sera	HI titers against		
	JaGAR (1959)	Oukestoru (1948)	Nakayama (1935)
Horses infected in JE enzootic, 1966			
No. 4	1:640	1:320	1:80
No. 5	1:160	1:80	1:40
No. 6	1:640	1:1,280	1:160
No. 7	1:1,280	1:1,280	1:160
Rabbit antisera			
JaGAR	1:5,120	1:2,560	1:640
Oukestoru	1:1,280	1:1,280	1:80
Nakayama	1:320	1:320	1:160

the infected horse sera and the antisera of hyperimmunized rabbits by each of the strains. The infected horses had HI titers of 1:160 to 1:1,280 against the JaGAR strain, while the HI titers against Nakayama strain were from 1:40 to 1:160. It was 4- to 8-fold lower than those of JaGAR strain. This result strongly suggests that the immunotype of the causative virus strain in this enzootic was more closely related to the JaGAR immunotype.

As to the Oukestoru strain of horse origin, the four infected horses had HI titers of 1:80 to 1:1,280. The values showed almost the same titers as JaGAR strain, and were also higher than the titers of Nakayama strain. The same tendency was recognized in the HI titers of the antisera of rabbits immunized with JaGAR or Oukestoru strain, but the three strains showed almost the same lower titers against the rabbit serum immunized with the Nakayama strain. Thus, it can be said from this result that the Oukestoru strain belongs to the JaGAR immunotype of JE virus.

In this study, furthermore, an attempt was made to confirm the immunotype of Oukestoru strain through the antibody responses of vaccinated human groups with inactivated JaGAR or Nakayama virus. These serum samples were kindly supplied by Dr. M. KANAMITSU of the Department of Hygiene, Sapporo Medical College. The results are shown in Table 3. All vaccinated persons with the JaGAR strain had HI titers of 1:20 to 1:80 against both of the JaGAR and Oukestoru strains, with an almost the same values in each of the cases. However, there were quite negative titers against the Nakayama strain. In contrast to this, each of the persons vaccinated with the Nakayama strain showed almost the same value in HI titers against the three strains. These results also revealed that the Oukestoru strain has a close resemblance to the JaGAR immunotype of JE virus.

**Table 3.** HI titers of the vaccinated human sera against three JE virus strains

Sera from human vaccinated with	HI titers against		
	JaGAR (1959)	Oukestoru (1948)	Nakayama (1935)
JaGAR vaccine			
No. 168	1:20	1:20	<1:10
No. 171	1:40	1:20	<1:10
No. 174	1:80	1:40	<1:10
No. 175	1:20	1:20	<1:10
No. 176	1:20	1:40	<1:10
Nakayama vaccine			
No. 338	1:40	1:20	1:40
No. 347	1:40	1:40	1:40
No. 348	1:160	1:160	1:160
No. 349	1:20	1:20	1:40
No. 352	1:10	<1:10	1:10

### 3. The 2-ME sensitiveness and gel-filtration of serum antibody from horses infected with JE virus

As well known in several virus diseases of animals, the antibody of the early stage of the disease was reduced greatly in titer after treatment with 2-ME, whereas the antibody in the late stage resisted the treatment. Furthermore, gel filtration of the serum revealed that the former activity was located mainly in the IgM fraction, and the latter belongs to the IgG fraction of immunoglobulin. Moreover, it is well known that HI activities were found in both the IgM and IgG fractions, and CF activity was present only in IgG fraction in the sera of JE patients. As regards the JE infections of horses, no reports were found on analytical studies of the immunoglobulin in the serum antibodies. Thus, experiments were undertaken to study the physico-chemical properties of the serum antibody from the infected horses of this enzootic.

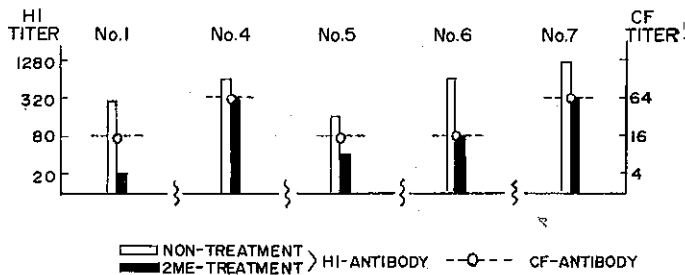


Chart 1. Sensitivity to 2-ME of antibodies in the sera of horses infected with JE virus in the enzootic

Chart 1 represents the sensitivity to 2-ME of antibodies in five sera of infected horses by means of HI and CF tests. In the HI antibodies, although one (No. 4) out of the five showed a resistance to the 2-ME treatment, all remaining horses revealed the significant reduction 4- to 16-fold in titers after the treatment. Thus, it is quite possible that the horses were in an earlier stage of the infection. As to the CF antibody, all these horses already showed titers of 1:16 to 1:64, in spite of the fact that the horse sera were obtained on the 2nd to 3rd clinical days, especially in foal No. 1 and No. 3 with no previous vaccination. The fact indicates an interesting fact on the CF antibody responses of the horses, in contrast to the relationship between the course of illness and the response of CF antibody in the human cases (OGATA *et al.*, 1967; ISHII *et al.*, 1968). Then an attempt was made to elucidate the CF activity in the sera of horses, from a viewpoint of immunoglobulin. Gel filtration and serological tests on each fraction were carried out on the serum samples of horse Nos. 7 and 1 (Chart 2). As indicated in the upper chart, in the horse No. 7 HI activities were demonstrated in the first and second among three peaks in the fractions of serum. But the CF activity was located only in the second peak of the three. This result was essentially similar to those obtained from the late

course of human infection.

As described in Chart 1, horse No. 1 showed highly sensitive HI antibody responses to 2-ME, which indicate a marked reduction of titers from 1:320 to 1:20 through the treatment. In addition to this fact, the horse already showed CF antibody titers of 1:16. But this CF activity could not be demonstrated in any of the fractions, as can be seen from the lower part of Chart 2, while the HI activity was found only in the first peak of the fractions. Then, the first and second peaks of the fractions were pooled respectively, and concentrated by Carbowax (Polyethylene Glycol #6,000) to a fourth part of the original volume, and were used for CF tests again. However, the demonstration of the CF activity resulted in a failure.

### Discussion

A limited enzootic of JE infection of horses was recognized in the Tokachi district of Hokkaido, from the middle to late part of September, 1966. The authors fortunately had good chance to examine the immunological characteristics of JE virus strains in the horse populations. Because the nature of the JE virus was previously investigated mainly on the strains isolated from human brains and mosquitoes (*Culex tritaeniorhynchus*) (AIZAWA *et al.*, 1968; OKUNO *et al.*, 1968), no evidence concerning strains of other animal origins have so far been obtained. In this enzootic, however, virus isolation from the dead horse resulted in a failure. Thus, the authors tried indirect serological experiments and demonstrated that the enzootic strain possessed the JaGAR immunotype of JE virus, through the antibody responses of infected horses in HI tests on the JaGAR and Nakayama strains. On the other hand, Oukestoru strain of JE virus, which was isolated from the brain of affected horses in the severe epizootic of JE infection in Hokkaido, in 1948 (YANAGAWA, 1949), was also found to be related to the JaGAR immunotype. The findings were based on the results of HI tests by the three virus strains on the serum samples of the infected horses and the JE vaccinated humans. It seems likely that the JaGAR immunotype strain prevails chiefly among the horse populations of Hokkaido, although future

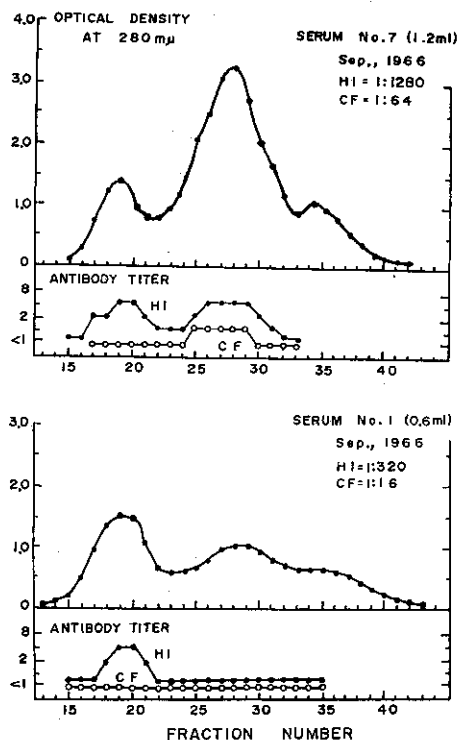


Chart 2. Distribution of antibodies in horse sera by gel filtration (Sephadex G-200 column)

work on other epizootic strains are required.

Many horses showed CF antibody titers of 1:16 to 1:64 in the early course of JE infection. Especially, horse No. 1 had a CF titer of 1:16 in spite of the fact that the horse showed a significant reduction of HI titers from 1:320 to 1:20 after the treatment with 2-ME. Next a detailed study on this activity was made by gel filtration method, because it is well known that CF activity appears only in the IgG fraction during the later period (1-5 weeks) following infection with JE virus in the majority of human cases (OGATA *et al.*, 1967; ISHII *et al.*, 1968). In the present paper, however, CF activity was not detected in any of the fractions of the serum of horse No. 1, since the activity was demonstrated in the IgG fraction of the No. 7 serum. As to the presence of CF antibodies in JE infected horses, SHIMIZU *et al.* (1952) mentioned the fact in their paper on the epizootic of JE infections in 1950. Namely, the CF test was conducted on the sera of 22 affected cases, which were bled during the period of the 1st to 26th day of illness. Most of the cases showed a positive reaction. Even on the first day of illness, 3 out of the 4 sera showed titers of 1:8 to 1:32. These findings suggest that the horses were previously exposed to JE virus, prior to the appearance of the first clinical sign, and/or this may be due to the multiple natures of horse immunoglobulins which have been identified to give as many as 8 unique classes in the recent work on the horse antihapten antibody (ROCKEY, 1967).

In the JE infection of horses, the relationship between the course of illness and the appearance of CF antibody was considerably different from that of JE patients.

### Summary

A serological investigation was conducted on the horses naturally affected by the enzootic of JE infection in the Tokachi district of Hokkaido, in 1966.

In this enzootic four horses showed clinical signs of JE infection, and one out of four died. In addition three horses had inapparent infection with JE virus from evidence provided by serological tests. All the horses had HI antibody titers of 1:160 to 1:1,280, and also CF antibodies of 1:16 to 1:64. Although the isolation experiment of the causal virus from the dead horse-brain resulted in a failure, it was revealed the enzootic virus strain was more closely related to the JaGAR immunotype of JE virus through HI tests on the JaGAR and Nakayama strains in the infected horse sera. Oukestoru strain of JE virus, which was isolated from the affected horse of the severe epizootic in 1948, was also related to the JaGAR immunotype. This result was concluded from the antibody responses of the affected horses and the JE vaccinated humans in comparative HI tests on the JaGAR, Oukestoru and Nakayama strains.

In this enzootic, many infected horses had CF antibody titers of 1:16 to 1:64, in spite of the early course of JE infection. Although in one out of two

cases the CF activity could not be demonstrated in any of the immunoglobulins in the gel filtration of serum, this result was quite different when compared with the CF antibody responses of JE patients.

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### 摘 要

馬の日本脳炎に関する血清学的な研究は人ならびに他動物のそれに比較して著しく少ない。現在わが国では、馬の飼育頭数の減少と予防接種の普及によって、本病の発生数が年々激減している。たまたま、われわれは1966年9月、北海道の十勝地方において馬日本脳炎のまれな発生例に遭遇したので、そのときの自然感染馬について血清学的な研究を

行ない、次の成績を得た。

1. この発生では4頭において反射機能減退、知覚麻痺、旋回運動および後軀蹠踉などの臨床症状がみられた。そのうち1頭 (No. 1) は発病から4日目に斃死したが、他の3頭 (No. 2, 3, 4) は発病から3~4日目に回復した (第1表)。

2. これら4頭では、発病中に採取された血清について JaGAr #01 株に対する HI, CF 抗体を測定したところ、HI 価は1:320~1:640, CF 価は1:16~1:64に保持されていた。またその対照群としてその周辺の農家に飼育されていた健康馬5頭についても血清抗体を測定した結果、そのうち3頭 (No. 5, 6, 7) が HI 抗体を1:160~1:1,280, CF 抗体を1:16~1:64に保持した (第1表)。なおこの HI 抗体は2-メルカプトエタノール (2-ME) に対して感受性を示すところから (第1図)、この3頭は今回の発生において不顕性に感染したものと推察される。しかし、他の健康馬2頭では各抗体とも陰性であった。

3. この発生では斃死馬からの原因ウイルスの検出は不成功に終わったが、感染馬血清の JaGAr #01 株と中山株に対する HI 抗体の反応態度から、流行ウイルスは免疫学的に JaGAr 型に属することが推察された (第2表)。また1948年北海道における馬日本脳炎の大流行の際に分離されたオルケストル株も、今回の感染馬血清ならびに日本脳炎ワクチンを接種した人血清の各ウイルス株に対する HI 抗体の反応態度から、JaGAr 型に属するような成績を得た。

4. 感染馬血清 (No. 1, 7) の Sephadex G-200 によるゲルろ過試験において、No. 7 では人血清と同様に HI 抗体は IgM と IgG の両分画に、CF 抗体は IgG 分画のみで検出された。次に感染馬 No. 1 では、そのほとんどの抗体が 2-ME 感受性でありながら CF 抗体の検出された例で、この CF 活性がどの免疫グロブリンに属するかを検討したが実証できなかった (第2図)。

しかし、馬の日本脳炎ウイルス感染に伴う免疫グロブリンの推移と各種抗体活性との関係は、人における本病感染例のそれに比較し、少しく異なることを考察した。