Sero-Epidemiological Study on Equine Influenza in Japan

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A serological survey was conducted on horse sera collected for 7 years just before the first outbreak of equine influenza (EI) infection in Japan in 1971. No antibodies against the A/Equi-1/Prague/56 (equi-1) and A/Equi-2/Miami/63 (equi-2) strains of EI virus were detected in any of the sera of 452 native horses when employing hemagglutination inhibition (HI) and complement fixation (CF) tests against viral (V) antigen. On the contrary, of the 80 imported horses, 48 (60.0%) had HI titers of 1:8 or higher against equi-1 and 23 (28.8%) against equi-2. In the CF-V test 42.6% of the horses showed titers of 1:4 or higher against equi-1 antigen and 42.9% against equi-2 antigen. However, all the test sera of the native and imported horses were negative (less than 1:4) in CF tests against soluble human influenza antigen. Epidemiological analysis was carried out to clarify the relationship between the history and the presence of serum antibody against EI viruses in individual imported horses.

Equine influenza (EI) is apparently a common disease in horses and has a wide distribution throughout Europe and the Americas. A number of papers on epidemiological and virological studies of the disease have been published in these countries (2, 3, 7, 8, 9, 11, 12). Also, the immunological relationship between animal and human influenza is being investigated very actively and reported because of the possible role of animals in the epidemiology of human influenza. Although the importation of animals from these countries to Japan is increasing yearly (14), we found no literature relating to the natural occurrence or the epidemiological study of influenza infections in animals in this country before 1971 (1, 6). In such an immunologically virgin soil, it is quite possible that the serological responses of animals may provide epidemiological data which cannot be obtained by examining animals raised in European and American countries, since both natural and artificial infections of animals with influenza viruses are prevalent in these countries. The purpose of this study was to attempt to elucidate the epidemiological features of the antibody responses to EI viruses in the sera collected from horses raised in Japan for the period October 1965 to April 1971.

MATERIALS AND METHODS

Sera. The horse sera used in this study were collected at random from animals in a large number of herds during the years 1965 to 1971. The animals were all adult, ranging in age from 2 to 21 years.

Both males and females were used. The serum samples were stored at -20°C until examined. When used for serological tests, the samples were treated with either commercial Receptor Destroying Enzyme (3 volumes:1 volume of serum) at 37°C overnight or M/90 potassium periodate (2 volumes:1 volume of serum) at 4°C overnight. The excess periodate was neutralized with 1% glycerol saline (1 volume) for 30 min at room temperature. All the treated sera were inactivated at 56°C for 30 min and absorbed with chicken erythrocytes to remove natural agglutinins against the cells.

Viruses. The strains of EI virus used were A/Equi-1/Prague/56, Heq1 Neq1 (equi-1) and A/Equi-2/Miami/63, Heq2 Neq2 (equi-2). The strains were obtained from the collection of the World Influenza Center, London, and have since been maintained by serial passages in 10-day-old embryonating chicken eggs.

Hemagglutination inhibition (HI) test. Serial twofold dilutions of the treated serum were prepared with 0.01 M phosphate-buffered saline, pH 7.2, in the wells of plastic trays. To 0.2 ml of each diluted serum was added an equal amount of 8 hemagglutinating units of virus. After an incubation period of 1 h in a refrigerator at 4°C , 0.4 ml of 0.5% chicken erythrocytes was added to the wells using an automatic pipette. The plate was allowed to stand at room temperature for 1 h. Titers of the test sera were expressed as reciprocals of the highest initial dilution of serum completely inhibiting hemagglutination.

Complement fixing (CF) test. CF tests were performed by microtitration in plastic trays employing a twofold dilution of the serum inactivated at 56°C for 30 min, 2 U of antigen in 0.025-ml volumes, 4 U of complement, and 1% sensitized sheep erythrocytes in 0.05-ml volumes. Mixtures of serum, anti-

gen, and complement were incubated at 4 C overnight before adding the sensitized cells. Virus concentrated from infected allantoic fluid by high-speed centrifugation at 28,000 rpm for 1 h was used as CF-viral (V) antigen. CF-soluble (S) antigen was prepared from a suspension of the chorioallantoic membranes infected with the A/Kumamoto/67 (H2 N2) strain of human influenza virus. Supernatant hemagglutinin-free fluid prepared by high-speed centrifugation of the membrane suspension at 28,000 rpm for 1 h was used as the antigen. End points were recorded as reciprocals of the highest initial dilution of serum showing 75 to 100% inhibition of hemolysis.

RESULTS

Occurrence of HI and CF antibodies against influenza viruses in horse sera. The 532 serum samples used for the present study were collected from 452 native and 80 imported horses. Table 1 shows the antibody titers demonstrated in the HI test against equi-1 and equi-2 strains of EI virus. In the native horses, no titers of 1:8 or higher were seen against either strain. On the contrary, of the 80 imported horses, 48 showed HI titers of 1:8 or higher against equi-1 strain and 23 against equi-2 strain. The titers ranged from 1:8 to 1:512.

Some of the horse sera were examined by the CF test with S and V antigens of influenza virus (Table 2). None of the 129 native horses

tested had CF antibody in tests against any of the antigens employed. No CF-S antibody was seen in the imported horses. However, in 26 of 61 sera CF-V antibody was detected for equi-1 antigen, and in 27 of 63 sera CF-V antibody was detected against equi-2 antigen. The CF-V antibody titers ranged from 1:4 to 1:128.

These findings strongly suggest that the EI antibodies were of foreign origin, and that the imported horses did not cause EI infection among the horse population in Japan.

EI antibodies in imported horses. (i) Distribution of EI antibodies by years of importation. The titers of HI antibodies against the equi-1 and equi-2 strains are plotted against years of importation of horses in Fig. 1. In the upper part of the figure, HI titers of equi-1 antibody are shown over the period 1952 to 1969. Some of the horses imported in the years 1952 to 1965 had moderate titers ranging from 1:8 to 1:64. However, it is seen that significant titers of HI antibody against equi-1 virus were detected in the sera of four horses brought from the U.S. during the years of 1952 to 1955, and the first isolation of the virus was made in the year 1956. Although the horses brought to Japan in the period 1966 to 1969 had a wide range of titers (1:8 to 1:512), a large proportion of the titers were in the high range of 1:64 to 1:512. The distribution of HI titers of equi-2 antibody is shown in the lower part of the figure; all the horses imported in the period 1952 to 1965 had

TABLE 1. HI antibodies against EI viruses in native and imported horses

Group	No. of sera	Antigen	No. of sera with antibody at indicated dilution								≥8	%
			<8	8	16	32	64	128	256	512		
Native horses	452	Equi-1	452	0	0	0	0	0	0	0	0	0
		Equi-2	452	0	0	0	0	0	0	0	0	0
Imported horses	80	Equi-1	32	7	12	6	6	8	5	4	48	60.0
		Equi-2	57	5	4	3	7	2	2	0	23	28.8

TABLE 2. CF antibodies against influenza viruses in native and imported horses

Group	No. of sera	Antigen	No. of sera with antibody at indicated dilution							≥4	%
			<4	4	8	16	32	64	128		
Native horses	129	CF-S(A/Kumamoto/67)	129	0	0	0	0	0	0	0	0
	129	CF-V(equi-1)	129	0	0	0	0	0	0	0	0
	129	CF-V(equi-2)	129	0	0	0	0	0	0	0	0
Imported horses	63	CF-S(A/Kumamoto/67)	63	0	0	0	0	0	0	0	0
	61	CF-V(equi-1)	35	1	8	2	15	— ^a	—	26	42.6
	63	CF-V(equi-2)	36	4	4	10	4	4	1	27	42.9

^a —, Not tested.

FIG. 1. Distribution of equi-1 and equi-2 antibodies in horses imported from the U.S.A. (x), England (●), and France (○). Year of first isolation of each equine virus is indicated by vertical arrow.

titers of less than 1:8, whereas many horses imported in the years 1966 to 1969 had HI titers of 1:8 to 1:256.

These data show that the horses imported in 1966 or later had higher levels of EI antibodies than those imported in 1965 or earlier. The responses might have been elicited not only by natural infection with EI viruses, but also by the virus vaccines which became available in 1965.

(ii) Responses of EI antibodies of horses by native country. An attempt was made to determine whether the length of stay in the native country and/or in Japan after importation had an effect on the occurrence of EI antibodies (Table 3). The horses brought from the U.S. had almost the same length of stay in their native country as the horses from England. But the former showed a higher positive rate of antibody and higher mean HI titers of equi-1 and equi-2 antibodies, despite the fact that they had had a longer stay in Japan after importation. In the horses brought from France, the period of stay there was as short as 2 to 5 years with an average of 3, and the incidence of EI antibodies was also lower than that in animals from the other countries.

Table 4 shows the effect of the period of stay in the native country on occurrence of HI antibody against the equi-1 strain. The positive rate and the mean titer of antibody become higher with prolonged stay in the native country. As shown in Table 5, however, extended stay in Japan had no effect on the positive rate

TABLE 3. Percentage and HI titer level of EI antibodies in horses after their importation

Expt items	Native country		
	U.S.A.	England	France
Period of stay in native country (avg time in years)	6 (4) ^a	7 (4)	3 (2)
Period of stay in Japan after importation (avg time in years)	8 (3)	3 (2)	8 (3)
Equi-1 antibody			
No. positive horses/no. tested	10/11	31/45	7/24
Positive rate (%)	90.9	68.9	29.2
Mean HI titer ^b	89	47	19
Equi-2 antibody ^c			
No. positive horses/no. tested	4/6	14/35	4/12
Positive rate (%)	66.7	40.0	33.3
Mean HI titer	42	32	45

^a Parentheses show average time after 1963.

^b Mean titer of positive sera (1:8 or higher).

^c Results of imported horses after 1963.

TABLE 4. Relation of period of stay in native country to equi-1 antibody of imported horses

Period of stay in native country (years)	No. of horses tested	Positive rate (%)	Mean HI titer ^a
1-3	19	36.8	26
4-6	37	62.2	31
7-9	10	80.0	75
Over 10	14	71.4	128

^a Mean titer of positive sera (1:8 or higher).

TABLE 5. Relation of period of stay in Japan to equi-1 antibody of imported horses

Period of stay in Japan (years)	No. of horses tested	Positive rate (%)	Mean HI titer ^a
0-1	30	70.0	77
2-4	13	53.8	47
5-7	11	45.5	18
8-11	14	50.0	14
Over 12	11	63.6	32

^a Mean titer of positive sera (1:8 or higher).

of equi-1 antibody, although there was an effect on the mean titer of antibody, especially when the period was 5 years or more.

The equi-2 antibody was not included in these tables, since the imported horses had too short a stay in their native countries, and be-

cause equi-2 virus was first isolated in the year 1963. Therefore, it was not possible to study the equi-2 antibody levels as fully as those described for the equi-1 antibody. However, the positive rate of the HI antibody increased from 0 to 61.9% in relation to the number of years of stay in the native land during the short period 1963 to 1968. The mean HI titer also increased from less than 1:8 to 1:35. Although the period of stay in Japan after the horses' importation was limited to the years 1966 to 1969 (Fig. 1), the percentage of positives and the mean titer of HI antibody were 53% and 1:35, respectively.

DISCUSSION

Although the prototype strain of equi-1 virus was first isolated in Prague, Czechoslovakia, in 1956 (13), related strains of the virus were widely disseminated among horse populations in European and American countries as early as 1956 (2, 4, 8, 9). The prototype equi-2 virus was initially isolated from an outbreak among race horses in Florida in 1963 (15), although no epizootics of EI infection due to the equi-2 related viruses had occurred in England or the U.S. before that year (2, 10). In Japan, the majority of imported horses have been brought from the U.S., England, and France. Infections with both the viruses are very prevalent among horses in those countries (2, 3, 4, 11, 12). Therefore, the animals in Japan are exposed to an invasion of the disease from abroad, though all the imported animals are kept and checked in quarantine. The first occurrence of EI infection was recognized in December 1971, when it was presumed that the disease was introduced into Japan by some of the imported horses (1, 6).

The present serological survey was carried out immediately before the first outbreak of EI infection in Japan and failed to demonstrate significant HI antibody against the strains of EI virus in any of the sera of the 452 native horses. HI antibodies were detected, however, in a large number of the serum samples from the 80 imported horses. In these horses CF-S antibody was not detectable in any of the sera tested, even though the sera showed positive titers of CF-V antibody against the equi-1 and equi-2 viruses. These findings suggest strongly that EI antibodies are of foreign origin and are attributable to considerably earlier infection with the viruses.

In the distribution of HI titers of equi-2 antibody, titers of 1:8 or higher were recognized in 23 (54.8%) of the 42 horses imported in the year 1966 or later. None of the 38 horses imported for the years 1952 to 1965 had positive titers of the antibody. These results are impossible to ex-

plain if the epizootic of equi-2 virus infections had occurred among the horse populations in our country. Thus, it is clear that these antibodies were acquired in their native countries, and their distribution reflects the epidemiological status in those countries. The same may be said of the equi-1 antibody. That is, the positive rate and the mean HI titers of the antibody increased in direct proportion to the extension of length of stay in the native countries, whereas the mean titers decreased with prolonged stay in Japan after importation. Our findings indicate that equi-1 or the related virus existed among horse populations in the U.S. prior to 1952, since the significant titers of HI antibody against equi-1 virus were demonstrated in the sera of four horses which were imported from the U.S. in 1952 to 1955. This finding is in accord with the hypothesis of other workers (4, 8, 9).

In the present study, the horses imported from the U.S. showed higher positive rates of EI antibodies and higher mean titers than the horses imported from England. It is of interest to speculate whether this is the result of EI infections among the horse population in the U.S. or is due to increased vaccination with EI viruses. There were low levels of EI antibodies in the imported horses from France, though it is known at the present time that the infections of equi-1 and equi-2 viruses have been widespread among horses in that country (5). This may be attributed to the fact that the horses were imported to Japan at the younger age of 3 years on the average.

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