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## DISTRIBUTION OF ANTIBODIES IN ANIMALS AGAINST INFLUENZA B AND C VIRUSES

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The sera of 2656 animals (horses, cattle, swine, dogs, cats, mink and rats) and 704 birds (chickens, pekin ducks, pigeons and wild birds) were examined by the hemagglutination-inhibition (HI) test to detect antibodies against influenza B and C viruses. Sixteen out of 504 (3.2%) horses and one out of 1030 (0.1%) swine sera were positive to influenza B virus. The positive horses were born exclusively in 1976. Four out of 721 (0.6%) cattle sera were positive to influenza C virus. All of the positive cattle were raised on the same farm. Although 31 out of 38 (82%) rat sera were positive to influenza C virus, these positive reactions were considered to be caused by an inhibitor, since the HI activity was found mostly in the fractions moving faster than the albumin in electrophoresis, and the virus isolation from the rats was negative.

### INTRODUCTION

The influenza B virus has not been isolated from animals, with the exception of one report of isolation from horses.<sup>1)</sup> The antibodies against influenza B virus have been found in horses,<sup>2)</sup> swine<sup>11)</sup> and dogs.<sup>12)</sup> The influenza C virus has not yet been isolated from animals. The antibodies against influenza C virus have been found in horses<sup>2)</sup>. Whether or not animals are concerned with the human infection of influenza B and C viruses requires further study. The present report describes the distribution of antibodies against influenza B and C viruses in animals and birds in Japan.

### MATERIALS AND METHODS

**Sera** The sera were collected mainly in Hokkaido, Japan. (1) 504 horses, 2-months- to 25-years-old; sera were collected in 1977 in Hidaka subprefecture. (2) 812 cattle, mainly adults; sera were collected from 1975 to 1977 in Yakumo and Hiroshima towns and in Sapporo. (3) 1030 swine sera were collected mostly in Ebetsu and Asahikawa abattoirs in 1977, others were collected in the Takikawa Animal Husbandry Experiment Station of Hokkaido from 1968 to 1977, and the rest were found in Hiroshima in 1977. (4) 158 dogs including puppies; sera were collected from 1969 to 1973 in Tokyo and in Sapporo. (5) 52 cats, nearly half of them kittens; sera were collected

in 1977 in Sapporo. (6) 62 mink, approximately 6-months-old; sera were collected in 1977 in Sapporo. (7) 38 rats caught in a zoo and in a farm in Sapporo in 1977. (8) 389 chickens; sera were collected in 1976 and 1977 in Shiraoui town and in Sapporo. (9) 10 pekin ducks; sera were collected in 1976 in Sapporo. (10) 250 pigeons; sera were collected from 1975 to 1977 in the cities of Iwamizawa, Obihiro, Otaru and Sapporo. (11) 55 wild birds; sera were collected in 1976 and 1977. They included 44 seabirds accidentally netted with fish and drowned in the North Pacific, 9 wild free-flying ducks shot in Moseushi town, and 2 kites which struck moving cars in Sapporo. All of these animals and birds were apparently healthy, except for 25 chickens in Shiraoui town, which showed signs of past respiratory illnesses.

**HI test** The HI test was carried out by using the microtiter method.<sup>8)</sup> The influenza B virus (B/Gifu/2/76) and influenza C virus (C/AA/JJ/50) propagated in chorioallantoic fluid and amniotic fluid of chicken embryos, respectively, were used as antigens after inactivation with 0.05% formalin and the addition of equal amount of glycerol. All of the sera were treated with RDE before screening by the HI test. The sera which were positive at 1:32 serum dilution in the screening test were further treated with 0.8% trypsin and heated at 56°C for 30 min. KIO<sub>4</sub> was then added,<sup>6)</sup> and the sera were examined by the HI test. An HI titer of 1:32 or above was considered to be positive.

**Cellulose-acetate electrophoresis** Electrophoresis was carried out on a cellulose acetate membrane (SELECTA, Nakarai Chemicals, Ltd.) for 3 hours at 0.6 mA/cm. The acetate buffer pH 4.6 of 0.021 ionic strength was used as the electrolyte.<sup>4)</sup> 0.02 ml of serum was placed on strips measuring 3×7 cm. Two strips were inserted into a wet chamber each time. After separation was completed, one of the electrophoretograms was cut into transverse strips of 0.5×3 cm. The strips were eluted 1~3 days in the test tubes at 4°C with 0.1 ml of saline. The eluates were tested for HI activity. Another electrophoretogram was dyed by ponceau 3 R.

**Immunization of Wistar rats** Two 6-month-old Wistar rats were injected twice intramuscularly with amniotic fluid containing influenza C virus (hemagglutination (HA) titer at 1:256) mixed with Freund's complete adjuvant, with a two-week interval between the injections. The amniotic fluid was inactivated with 0.05% formalin beforehand. Two weeks after the second injection, the rats were bled and the sera were stored at -20°C.

**Virus isolation** The organs of the rats which were positive to the HI test were ground with sterile sand to give a 10% suspension (weight/volume) in a phosphate buffered saline containing 1% bovine serum albumin and antibiotics. The suspension was inoculated in 10-day-old chicken embryos by amniotic route.

## RESULTS

The incidence of antibodies against influenza B and C viruses in animals and birds is shown in table 1.

The sera positive in the HI test to influenza B virus were found in 16 (3.2%) out of 504 horses and one (0.1%) out of 1030 swine. None of the remaining animals and birds were positive. The HI titer of the positive sera was not decreased after the treatments with RDE, trypsin, heating at 56°C for 30 min., and KIO<sub>4</sub>, indicating that the HI activity was due to the antibodies. The HI titer of the positive horses and swine ranged from 1:32 to 1:64. The positive horses were exclusively born in 1976, and 7 of them were raised on the same farm. The positive swine was slaughtered in October 1977, in Ebetsu.

The sera positive in the HI test to influenza C virus were found in 4 (0.6%) out of 721 cattle, and 31 (82%) out of 38 rats. None of the remaining animals and birds were negative. Of the positive sera, those of cattle did not change their HI titers after the treatments with RDE, trypsin, heating at 56°C for 30 min., and KIO<sub>4</sub>. The positive cattle, therefore, were considered to possess the antibodies against influenza C virus. The HI titer of the positive cattle ranged from 1:32 to 1:64. All of the positive cattle, aged 5- to 23-months-old, were raised on the same farm in Hiroshima town. The positive rate in the farm was 4/61, which was significantly different from that

TABLE 1 *Incidence of antibodies against influenza B and C viruses in animal sera*

ANIMAL	INFLUENZA B VIRUS	INFLUENZA C VIRUS
Horse	16/ 504 (3.2)* <sup>1</sup>	0/504 ( 0 )
Cattle	0/ 812 ( 0 )	4/721 ( 0.6)
Swine	1/1030 (0.1)	0/792 ( 0 )
Dog	0/ 158 ( 0 )	0/128 ( 0 )
Cat	0/ 52 ( 0 )	0/ 52 ( 0 )
Mink	0/ 62 ( 0 )	0/ 62 ( 0 )
Rat	0/ 33 ( 0 )	31/ 38 (82 )* <sup>2</sup>
Chicken	0/ 389 ( 0 )	0/347 ( 0 )
Pekin duck	0/ 10 ( 0 )	0/ 10 ( 0 )
Pigeon	0/ 250 ( 0 )	0/191 ( 0 )
Wild birds	0/ 55 ( 0 )	0/ 50 ( 0 )

\*<sup>1</sup> Number of positive reactor/numbers tested  
Percentages are shown in parentheses.

\*<sup>2</sup> HI activity found in rat sera was considered to be due to an inhibitor, as shown in the text.

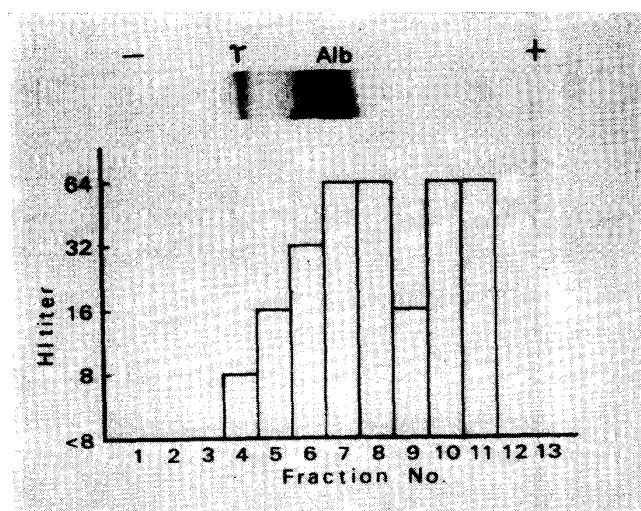
(0/660) in the remaining farms (The probability value determined by Fisher's exact test was  $4.7 \times 10^{-5}$ ).

Concerning the rats, an extraordinary high percentage of the positive reactors, showing considerably high HI titers (1:128 to 1:512), and the report of STYK<sup>10</sup> describing that normal rat serum contained a thermostable inhibitor which was resistant to RDE,  $KIO_4$  and 0.5% trypsin, indicated that further investigation was necessary. Whether the HI activity of the rat sera was due to an antibody or an inhibitor was examined in the following experiments.

Cellulose-acetate electrophoresis of the sera of 5 rats which were positive in the HI test against influenza C virus was carried out at pH 4.6. The HI activity of fractions of a rat serum is shown in figure 1. HI activity was very low in the  $\gamma$ -globulin fraction. It was much higher in the albumin fraction and in the fractions moving faster than the albumin. The results obtained from the sera of 4 rats were similar. The substance in the rat sera showing high HI activity, therefore, was not considered to be due to an antibody, but to an inhibitor.

In an additional experiment using two Wistar rats immunized with influenza C virus, which possessed a high inhibitory activity (HI titers, 1:2048~1:4096), the anti-influenza C virus antibody appeared in the  $\gamma$ -globulin fraction (tab. 2). This result may indicate that the rat possessing a high level of the inhibitor still produced, after antigenic stimulation, the specific anti-influenza C virus antibody in the  $\gamma$ -globulin fraction.

FIGURE 1 *Distribution of HI activity of the fractions of a rat serum*



$\gamma$ :  $\gamma$ -globulin fraction  
Alb: Albumin fraction

HI titer is expressed as a reciprocal of a dilution showing complete inhibition of 4 HA units of influenza C virus.

TABLE 2. *Distribution of HI activity of the fractions of the serum of a Wistar rat before and after immunization with influenza C virus*

SAMPLING OF SERUM	FRACTIONS OF SERUM												
	1	2	3	4* <sup>1</sup>	5	6	7* <sup>2</sup>	8	9	10	11	12	13
Before immunization					8* <sup>3</sup>	8	8	8	8	8			
After immunization			8	16	16	8	8	8	8	8			

\*<sup>1</sup>  $\gamma$ -globulin fraction

\*<sup>2</sup> Albumin fraction

\*<sup>3</sup> HI titer is expressed as shown in the footnote of figure 1.

An additional attempt was carried out to isolate influenza C virus from 5 rats which were positive in the HI test. The tracheae, lungs, spleens, livers, kidneys, hearts and intestines of the rats were examined. Neither influenza C virus nor any hemagglutinating virus was isolated.

#### DISCUSSION

The HI test is a simple method for detection of antibodies against influenza viruses. However, there have been numerous reports describing the occurrence of inhibitors and the difficulty of completely removing them from the sera of animals.<sup>6)</sup> The difficulty of distinguishing inhibitors from antibodies arises, therefore, in sero-epidemiological studies of influenza. Of the methods for removing inhibitors, it was said that the best results seemed to be obtained by a method combining effects of trypsin, heating and  $KIO_4$ .<sup>6)</sup> By applying the combination method, NAKAMURA<sup>7)</sup> detected the anti-swine influenza virus antibodies in swine sera. GOTO et al.<sup>8)</sup> used the method combining the treatments with  $KIO_4$ , RDE and heating, and detected the antibodies against equine influenza viruses in horse sera. HI antibodies against influenza B virus were found in the swine sera<sup>11)</sup> treated with heating and  $KIO_4$ . Referring these reports, the present authors treated the sera with RDE, trypsin, heating and  $KIO_4$ , and detected the antibodies against influenza B virus in horses and swine.

Our findings may support the view<sup>5)</sup> that influenza B virus may infect some animals. The influenza B virus prevailed in man throughout Hokkaido in 1976 (SAKURADA, Hokkaido Institute of Public Health, personal communication). It was noticed, in the present study, that the positive horses were exclusively born in that year. The fact that there were some horses which were positive against influenza B virus might be due to the infection of this virus from man.

There have been no evidences of infection of influenza C virus in animals except that the antibodies against influenza C virus were found only in horse sera by complement-fixation test.<sup>2)</sup>

Ordinary inhibitors against influenza C virus in the cattle sera, if they were present, were considered to be removed by the treatments employed in the present study, since most of the cattle sera (717/721) were negative in the HI test.

It is worthy of note that all of the positive cattle were raised on the same farm. The positive rate in the farm was 4/61, which was significantly different from that (0/660) in the remaining farms.

With regard to the cattle sera, the above findings might suggest that the positive cattle were infected with influenza C virus. However, further studies, including the virus isolation from cattle and the demonstration of antibodies by means of other serological methods such as neutralization test and immunoelectrophoresis, are necessary to define whether or not influenza C virus infects cattle.

Concerning the rats which were positive in the HI test under the treatment of sera with RDE, trypsin, heating and KIO<sub>4</sub>, the results of electrophoresis of the sera and of the virus isolation indicated that the HI activity was due to an inhibitor.

STYK<sup>9,10)</sup> reported that normal rat serum contained an inhibitor to influenza C virus hemagglutination, which was thermostable and resistant to the action of RDE and KIO<sub>4</sub> and was not completely destroyed by trypsin. HANA & STYK<sup>9)</sup> further found that the inhibitor was a glycoprotein and moved faster than the albumin on paper electrophoresis at pH 4.6. These properties of the inhibitor remarkably resemble those of the inhibitor found in the rat sera in the present study, indicating that these inhibitors are probably identical.

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