

Short Communication

HEMAGGLUTINATION BY CALF DIARRHEA CORONAVIRUS.

K. SATO, Y. INABA, H. KUROGI, E. TAKAHASHI, K. SATODA, T. OMORI and
M. MATUMOTO*

National Institute of Animal Health, Kodaira, Tokyo (Japan)

**Kitasato Institute, Minato-ku, Tokyo (Japan)*

(Received 17 November 1976)

ABSTRACT

Sato, K., Inaba, Y., Kurogi, H., Takahashi, E., Satoda, K., Omori, T. and Matumoto, M., 1977. Hemagglutination by calf diarrhea coronavirus. *Vet. Microbiol.*, 2: 83-87.

The virus was grown in BEK-1 cells, a stable cell line from bovine embryo kidney, and tested for hemagglutination (HA) with erythrocytes of a variety of species at 4°C, room temperature and 37°C. HA was observed at all temperatures with chicken, mouse, rat, and hamster erythrocytes but not with erythrocytes of human (O), cattle, horses, sheep, guinea pigs, geese, ducks, pigeons and 1-day-old chicks. Chickens showed an individual variation in agglutinability of their erythrocytes, requiring selection of birds to obtain erythrocytes for HA. HA reaction was inhibited by specific antiserum. Some factors involved in HA and HA inhibition (HI) were investigated and standard HA and HI tests were worked out.

Calf diarrhea coronavirus has been recognized as one of the etiologic agents of neonatal calf diarrhea (Stair et al., 1972; Mebus et al., 1972, 1973 a and b; Sharpee et al., 1976). Recently the virus was reported to agglutinate erythrocytes from rats, mice and hamsters (Sharpee et al., 1976). Independently we also observed that the virus agglutinated mouse, rat, hamster and chicken erythrocytes. This paper describes our observations of hemagglutination (HA) with the virus and HA-inhibition by specific antiserum.

Calf diarrhea coronavirus passaged in bovine embryo kidney cell cultures (Mebus et al., 1973 a) was supplied by Dr C.A. Mebus, University of Nebraska. In our laboratory the virus was passaged serially in cultures of BEK-1, a continuous cell line derived from bovine embryonic kidney (Inaba et al., 1976), and used at the 7th or later BEK-1 passage level in the present study. Cultures of BEK-1 cells were prepared as described previously (Inaba et al., 1976). The growth medium used was Eagle's minimum essential medium (MEM) containing 10% tryptose phosphate broth (TPB) and 10% calf serum, and the maintenance medium was MEM containing 10% TPB, 0.05% yeast extract, 0.5% sodium glutamate and 0.1% glucose.

Culture fluid harvested from infected BEK-1 cells was used as HA antigen after centrifugation at 600 *g* for 10 min to remove coarse debris, and stored at -20 and -70°C . Blood was obtained in Alsever's solution and stored at 4°C . Chicken erythrocytes (0.5%) were used, unless otherwise stated. HA and HA inhibition (HI) tests were carried out by the microtiter method. The following is the standard method established in the present study. In the various experiments presented in this paper this technique was followed, unless otherwise specified.

Serial two-fold dilutions of HA antigen were prepared in 0.05-ml amounts and mixed with 0.025 ml of erythrocyte suspension. The mixtures were then incubated at room temperature ($20-25^{\circ}\text{C}$) for 1 h, and the tests were read. The HA titer was expressed as the reciprocal of the highest antigen dilution showing complete HA. For HI tests, 0.2 ml of the serum and 0.5 ml of phosphate buffer solution (PBS) were mixed, inactivated at 56°C for 30 min and mixed with 0.4 ml of 25% kaolin. After shaking for 1 h at room temperature, kaolin was removed by centrifugation, and the supernatant fluid was mixed with 0.2 ml of packed erythrocytes. After incubation at 37°C for 1 h, the cells were removed and the supernatant fluid was used for HI test. Eight units of HA antigen in 0.025 ml and 0.025 ml of serial two-fold dilutions of the treated serum were mixed, incubated at room temperature for 1 h, and mixed with 0.025 ml of erythrocyte suspension. The mixtures were then incubated at room temperature for 1 h and the tests were read. The HI antibody titer was expressed as the reciprocal of the highest serum dilution showing complete HI. The diluent used for HA and HI tests was veronal buffered saline (pH 7.0) containing 0.1% bovine serum albumin (Armour) and 0.001% gelatin.

Supernatant fluid harvested from BEK-1 cell cultures infected with the virus was tested for HA with erythrocytes from a variety of species at 4°C , room temperature and 37°C . Chicken, mouse, rat and hamster erythrocytes were agglutinated at all three temperatures, whereas erythrocytes from human (O), cattle, horse, sheep, guinea pig, goose, duck, pigeon and 1-day old

TABLE I

HA titers with erythrocytes from individual chickens

HA titer	Number of birds
<2	7
2	5
4	2
8	6
16	7
32	3
Total	30

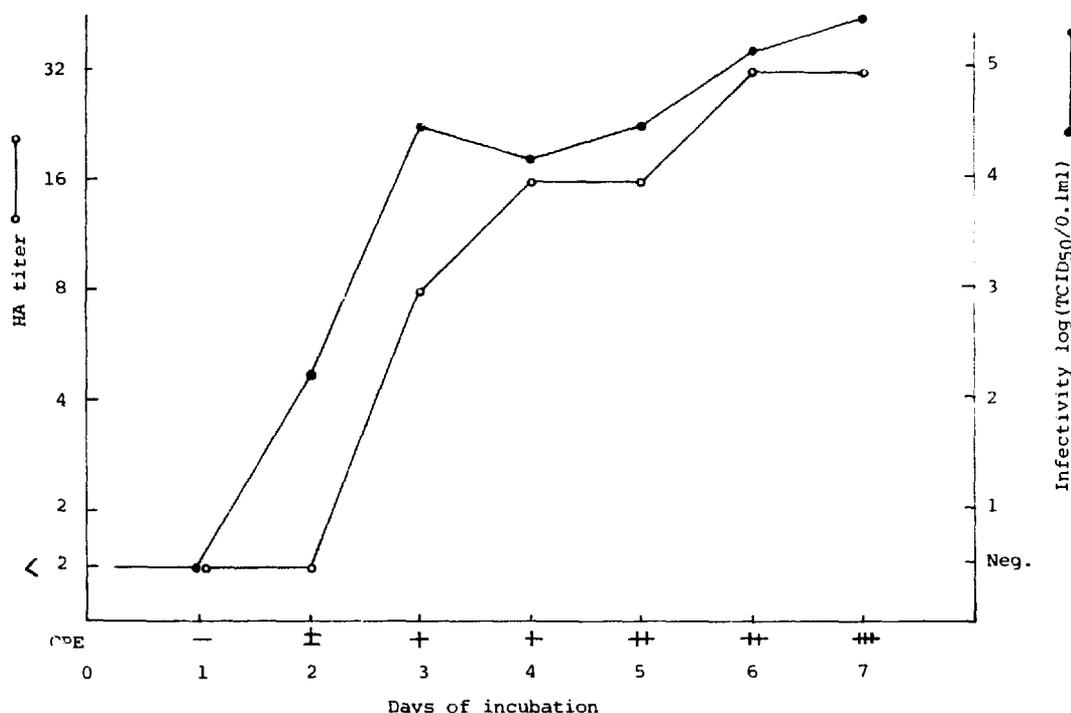


Fig.1. Production of hemagglutinin and active virus in BEK-1 cell cultures infected with calf diarrhea coronavirus.

chicken failed to agglutinate under the test conditions. Sharpee et al. (1976) also reported HA with mouse, rat and hamster erythrocytes. However, contrary to our result, they observed no HA with erythrocytes from chickens. These contradictory results were explained by our subsequent observation that erythrocyte samples from individual birds showed a wide variation in their agglutinability with the virus as shown in Table I. Therefore, a selection of birds is required to obtain erythrocytes for HA.

Culture fluid harvested from infected BEK-1 cells showing cytopathic changes has HA titers of between 8 and 32. Fig. 1 illustrates a representative example of HA antigen production as well as that of viral infectivity in the fluid phase of roller tube cultures of BEK-1 cells incubated at 34°C after infection at an input multiplicity of 0.1 50% tissue culture infectious doses (TCID₅₀)/cell. The HA titer rose rapidly between 2 and 3 days after inoculation with a subsequent gradual rise to a plateau of 32 at 6 days post infection. The curve of viral infectivity resembled that of HA production although it rose somewhat earlier.

The HA activity of calf diarrhea coronavirus was very stable compared with the infectivity of the virus. For example, an infectious culture fluid with an HA titer of 32 showed no change in titer for at least 7 days at 37, 4 and -20°C. Even at 56°C no change in titer was shown for 6 h, a two-fold decline in 12 h, and no titer remained after 24 h of incubation. On the other hand,

TABLE II

Filtration of calf diarrhea coronavirus through Sartorius membrane filters

Filtration (pore size)	HA titer	Infectivity log(TCID ₅₀ /0.1 ml)
Before	64	5.2
450 nm	64	5.2
200 nm	64	5.2
100 nm	8	2.2
50 nm	<2	0*

* Negative with undiluted material.

the viral infectivity was rapidly lost at 56°C; the original titer of 10^{4.5} TCID₅₀/ml declined to 10^{3.2} in 10 min and no infectivity was detected after 20 min of incubation.

Treatment at 25°C for 2 h with 20% ether, 5% chloroform and 1% Tween-80 plus 20% ether completely destroyed HA activity as well as infectivity. Fluorocarbon precipitated neither HA activity nor infectivity. Treatment with sodium deoxycholate (1%, 37°C, 30 min) partially destroyed HA activity, and that with formalin (0.05%, 4°C, 168 h) completely destroyed HA activity and infectivity.

The results of filtration shown in Table II indicate that HA activity is associated with infectious virus particles. This is further supported by the results of CsCl equilibrium density gradient centrifugation (K. Sato et al., unpublished data) that showed a peak of infectivity which coincided in position with the peak of hemagglutinin, and electron microscopic examination of these peak fractions by the phosphotungstic negative staining technique revealed numerous spherical virions.

The HA reaction was shown to be virus specific, since it could be inhibited by specific antiserum (Table III). Sera from 20 adult cattle were tested for HI and neutralization (NT) antibodies. NT tests were carried out in tube cul-

TABLE III

HI tests on antisera against calf diarrhea coronavirus

Antiserum		HI titer	NT titer
Rabbit number 3	pre	< 10	< 2
	post	> 1 280	4096
Guinea pig number 2	pre	< 10	< 2
	post	> 1 280	8192

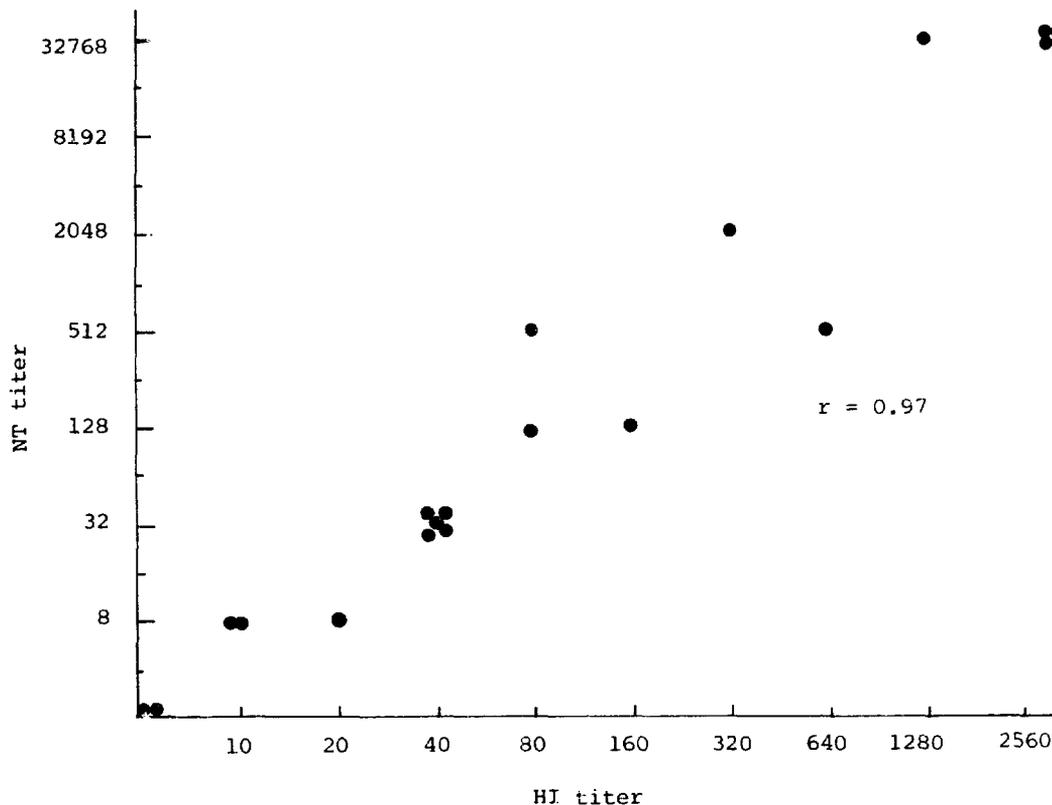


Fig. 2. HI and NT antibody titers of individual adult cattle.

tures of BEK-1 cells as described previously (Inaba et al., 1976). As shown in Fig. 2, HI titers were closely correlated with NT titers.

The HA and HI tests developed in this study seem very useful and will find a wide application in studies on the virus and its disease.

REFERENCES

- Inaba, Y., Sato, K., Kurogi, H., Takahashi, E., Ito, Y., Omori, T., Goto, Y. and Matumoto, M., 1976. Replication of bovine coronavirus in cell line BEK-1 culture. *Arch. Virol.*, 50: 339-342.
- Mebus, C.A., White, R.G., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J., 1972. Neonatal calf diarrhea: Results of a field trial using a reovirus-like virus vaccine. *Vet. Med. Small Anim. Clin.*, 67: 173-178.
- Mebus, C.A., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J., 1973 a. Neonatal calf diarrhea: Propagation, attenuation and characteristics of a coronavirus-like agent. *Am. J. Vet. Res.*, 34: 145-150.
- Mebus, C.A., Stair, E.L. and Twiehaus, M.J., 1973b. Pathology of neonatal calf diarrhea induced by a coronavirus-like agent. *Vet. Pathol.*, 10: 45-64.
- Sharpee, R.L., Mebus, C.A. and Bass, E.P., 1976. Characterization of a calf diarrheal coronavirus. *Am. J. Vet. Res.*, 37: 1031-1041.
- Stair, E.L., Rhodes, M.B., White, R.G. and Mebus, C.A., 1972. Neonatal calf diarrhea: Purification and electron microscopy of a coronavirus-like agent. *Am. J. Vet. Res.*, 33: 1147-1156.