

## Replication of Bovine Coronavirus in Cell Line BEK-1 Culture

### Brief Report

By

Y. INABA<sup>1</sup>, K. SATO<sup>1</sup>, H. KUROGI<sup>1</sup>, E. TAKAHASHI<sup>1</sup>,  
Y. ITO<sup>1</sup>, T. OMORI<sup>1</sup>, Y. GOTO<sup>2</sup>, and M. MATUMOTO<sup>3</sup>

<sup>1</sup> National Institute of Animal Health, Kodaira, Tokyo, Japan

<sup>2</sup> National Veterinary Assay Laboratory, Kokubunji, Tokyo, Japan

<sup>3</sup> Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan

With 1 Figure

Accepted December 4, 1975

### Summary

Bovine coronavirus readily multiplied and induced marked cytopathic effect in BEK-1 cultures, thus providing a sensitive, practical assay system for viral infectivity and neutralizing antibody and a satisfactory source of the virus.

\*

MEBUS and his associates (4—6) have demonstrated an agent with morphologic features of coronavirus by electron microscopy in fractions prepared by density gradient ultracentrifugation from diarrheal feces of calves with natural and experimentally produced neonatal diarrhea. The agent multiplied but failed to induce readily recognizable cytopathic effect in bovine embryonic kidney cell cultures (3). The virus has been assayed therefore in these cultures by a rather cumbersome method in which the presence of the virus was determined by microscopic examination for syncytia of cultures stained with gentian violet, or by immunofluorescent technique (3).

This paper describes briefly our recent observation that the virus readily replicates and induces a marked cytopathic effect in cultures of a continuous cell line, BEK-1, derived from bovine embryonic kidney (2), thus providing a sensitive, practical assay method and a satisfactory source of the virus.

BEK-1 cells were grown at 37° C in Eagle's minimum essential medium (MEM) containing 10 per cent tryptose phosphate broth (Difco) (TPB), 10 per cent bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 µg/ml fungizone. The bovine coronavirus (3), at the 29th passage level in bovine embryonic kidney cells, was kindly supplied by Dr. C. A. Mebus, University of Nebraska, and was passaged twice in primary calf kidney cell cultures in our laboratory before use in the present study. BEK-1 cell cultures prepared in 10 × 110 mm tubes were

inoculated with 0.1 ml amounts of virus dilution, and incubated in a roller drum at 34° C. The maintenance medium after virus inoculation was MEM containing 10 per cent TPB, 5 per cent sodium glutamate, 1 per cent glucose, 0.1 per cent yeast extract, 0.1 per cent bovine serum albumin (Armour) and antibiotics.

The virus readily multiplied and induced marked cytopathic effect. The effect was first recognized 3 days after inoculation as rounded cells scattered in the cell sheet. As incubation progressed, round cells increased in number, fused to form syncytia, disintegrated and sloughed off the glass surface. These changes destroyed cell sheets within 4 or 5 days after inoculation (Figs. 1a and 1b). Further passages were readily made with supernatant fluid from infected cultures developing cytopathic effect, and the viral yield attained ranged from  $10^4$  to  $10^5$  TCID<sub>50</sub>/0.1 ml.

The cytopathic effect was specifically inhibited by antiserum to the bovine coronavirus. The antiserum used was prepared in rabbits, which received an intravenous dose of 1.0 ml of virus suspension followed at 3 and 6 weeks intervals, by two intramuscular doses of 1.0 ml each of equal volume mixture of the virus suspension and Freund's complete adjuvant. The virus suspension was prepared by resuspending, in  $1/100$  volume of phosphate buffered saline, pellets obtained by centrifugation of infected BEK-1 culture fluid at  $100,000 \times g$  for one hour. Serum was obtained from the animals 3 weeks after the last dose. The neutralization test was carried out by the method described elsewhere (1). The serum neutralized the virus, the antibody titer being 1:512, while the preimmunization serum was negative.

Infection of BEK-1 cells with the bovine coronavirus was also proved by immunofluorescent staining. Coverslip cultures of BEK-1 cells inoculated with the virus were examined after 2 or 3 days of incubation at 34° C. The cultures were fixed with acetone at -20° C for 10 minutes, and stained with the fluorescein isothiocyanate-conjugated antibody for the bovine coronavirus which was kindly donated by Dr. C. A. Mebus. Many immunofluorescent mono- and multi-nucleated cells were observed; the cytoplasm contained granular fluorescence, in some cells most of the cytoplasm fluoresced (Fig. 1c). As MEBUS *et al.* (3) we prepared fractions from infectious culture fluid by density-gradient ultracentrifugation, and examined them by phosphotungstic negative staining in an electron microscope. Numerous particles, about 130 nm across, with morphologic features of coronavirus were observed (Fig. 1d).

These results prove BEK-1 cells to be a good medium for propagation and assay of bovine coronavirus, rendering consistent work with the virus feasible.

### References

1. INABA, Y., KUROGI, H., TAKAHASHI, E., SATO, K., TANAKA, Y., GOTO, Y., OMORI, T., MATUMOTO, M.: Isolation and properties of bovine parvovirus type 1 from Japanese calves. *Arch. ges. Virusforsch.* **42**, 54—66 (1973).
2. MATUMOTO, M., INABA, Y., TANAKA, Y., ITO, H., OMORI, T.: Behavior of bovine ephemeral fever virus in laboratory animals and cell cultures. *Japan. J. Microbiol.* **14**, 413—421 (1970).
3. MEBUS, C. A., STAIR, E. L., RHODES, M. B., TWIEHAUS, M. J.: Neonatal calf diarrhea: propagation, attenuation and characteristics of a coronavirus-like agent. *Amer. J. vet. Res.* **34**, 145—150 (1973).

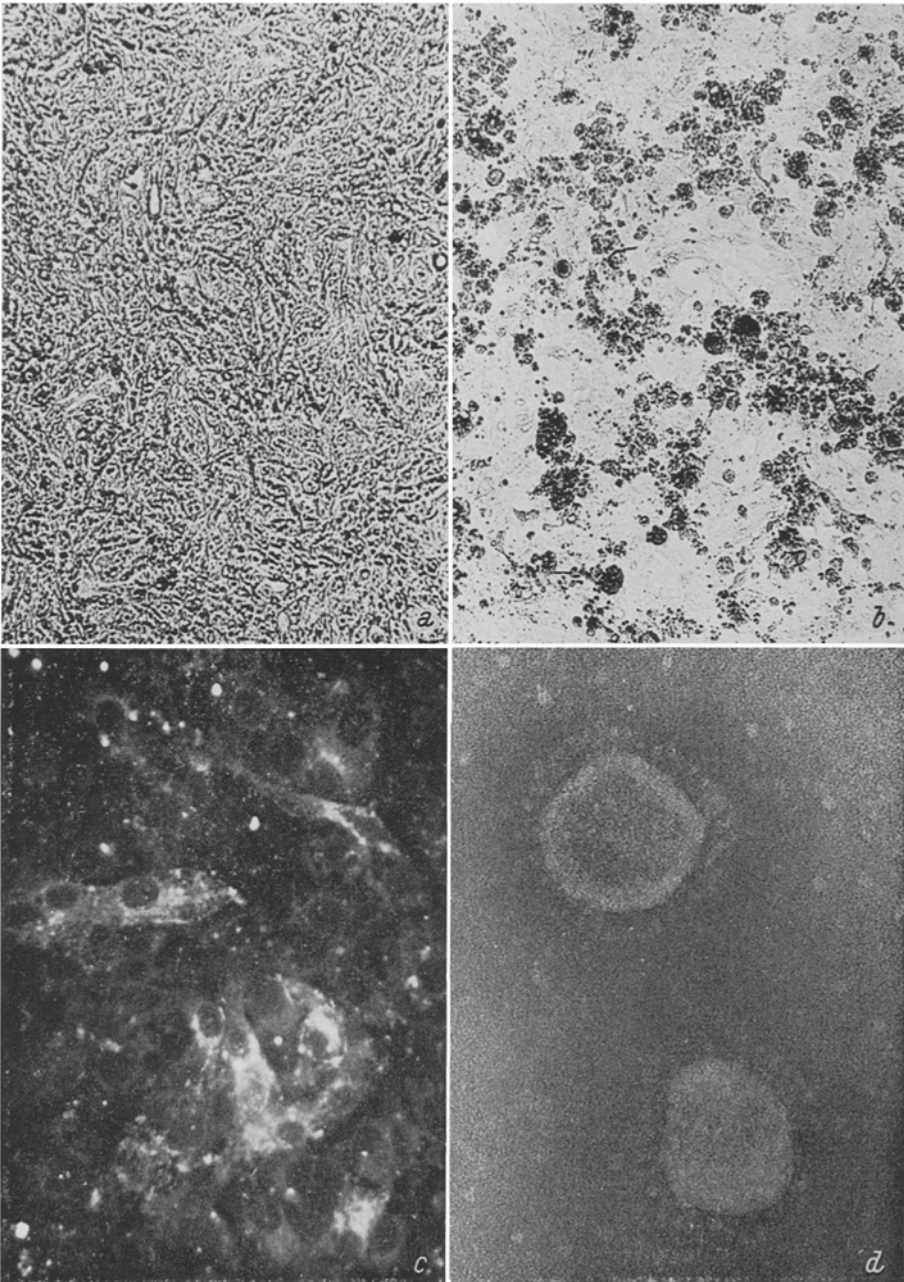


Fig. 1. Cytopathic effect of bovine coronavirus in BEK-1 cell culture (unstained preparation): Cells uninfected (a), and 5 days after infection with the virus (b) Immunofluorescence of BEK-1 cells 48 hours after inoculation with bovine coronavirus (c).  $\times 250$

Negatively stained virus particles purified from BEK-1 cell culture fluid (d).  $\times 175,000$

4. MEBUS, C. A., STAIR, E. L., RHODES, M. B., TWIEHAUS, M. J.: Pathology of neonatal calf diarrhea induced by a coronavirus-like agent. *Vet. Path.* **10**, 45—64 (1973).
5. MEBUS, C. A., WHITE, R. G., STAIR, E. L., RHODES, M. B., TWIEHAUS, M. J.: Neonatal calf diarrhea: results of a field trial using a reo-like virus vaccine. *Vet. Med./Small Anim. Clin.* **67**, 173—174, 177—178 (1972).
6. STAIR, E. L., RHODES, M. B., WHITE, R. G., MEBUS, C. A.: Neonatal calf diarrhea: purification and electron microscopy of a coronavirus-like agent. *Amer. J. vet. Res.* **33**, 1147—1156 (1972).

Authors' address: Dr. Y. INABA, National Institute of Animal Health, Kodaira, Tokyo, Japan.

Received November 25, 1975