

## PROPERTIES OF A CORONAVIRUS ISOLATED FROM A COW WITH EPIZOOTIC DIARRHEA

H. AKASHI, Y. INABA, Y. MIURA, S. TOKUHISA, K. SATO and K. SATODA

*National Institute of Animal Health, Tsukuba, Ibaraki (Japan)*

(Accepted 21 May 1980)

### ABSTRACT

Akashi, H., Inaba, Y., Miura, Y., Tokuhisa, S., Sato, K. and Satoda, K., 1980. Properties of a coronavirus isolated from a cow with epizootic diarrhea. *Vet. Microbiol.*, 5: 265—276.

A coronavirus (Kakegawa isolate) isolated from a cow with epizootic diarrhea was grown in BEK-1 cells and examined for biophysical and biochemical properties. The Kakegawa isolate was able to replicate in the presence or absence of 5-iodo-2'-deoxyuridine, indicating that its viral nucleic acid was RNA. It was highly sensitive to ether and chloroform, and moderately sensitive to trypsin and heat. It was, however, readily stabilized by treatment with cation at 50°C for 1 h. Its infectivity was slightly reduced at pH 3.0. The virus passed through a membrane filter of 200 nm pore size, but not through one of 100 nm pore size. The buoyant density of the virus was determined in a sucrose density gradient. The peak of infectivity and hemagglutinin activity was found at a density of 1.182. Neutralization and hemagglutination inhibition tests showed a close serological relationship between the Kakegawa isolate and the American strain of calf diarrhea coronavirus.

### INTRODUCTION

Bovine coronaviruses causing neonatal calf diarrhea have been isolated in the U.S.A. (Stair et al., 1972; Doughri et al., 1976) and in England and Denmark (Bridger et al., 1978b). The presence of the virus has also been reported in Belgium on the basis of a serological survey (Zygraich et al., 1975) and by immunofluorescence (Wellemans et al., 1977a, b), and in Canada by electron microscopic and immunofluorescence observations (Acres et al., 1975; Morin et al., 1976). In contrast to the condition in calves, the etiological agents of diarrhea in adult cattle have been reported in New Zealand (Horner et al., 1976; Durham et al., 1979), and recently Takahashi et al. (1980) isolated a coronavirus-like agent associated with epizootic diarrhea in a cow in Japan.

This report deals with the physico-chemical characteristics of the Japanese isolate, the identification of the virus as a member of the coronavirus family, and an investigation of a possible serological relationship between this virus and the American strain of calf diarrhea coronavirus.

## MATERIALS AND METHODS

### *Cell cultures and media*

BEK-1 cells (Inaba et al., 1976) were propagated in a growth medium consisting of 80% Eagle's minimum essential medium (MEM), 10% tryptose phosphate broth (TPB) and 10% fetal bovine serum. Maintenance medium (MM) used was MEM containing 10% TPB, 0.05% yeast extract, 0.5% sodium glutamate and 0.1% glucose. Added to each medium were 100 I.U. penicillin/ml, 100  $\mu$ g streptomycin/ml, 60  $\mu$ g kanamycin/ml and 1  $\mu$ g fungizone/ml.

### *Viruses*

The Kakegawa isolate (Takahashi et al., 1980) was used at the ninth or tenth passage level in primary bovine kidney cells. The American strain of calf diarrhea coronavirus that had been passaged in cell cultures of fetal bovine kidney was supplied by Dr. C.A. Mebus, University of Nebraska, NE, U.S.A. This virus was passaged 11 or 12 times in BEK-1 cells before it was used.

The C-121E strain of bovine enterovirus (BEV) (Kurogi et al., 1976), and the Los Angeles strain of bovine herpesvirus type 1 (BHV-1) (Madin et al., 1956) were used as reference viruses.

### *Infectivity assay*

Serial ten-fold dilutions of viral material were made in MM and each was inoculated in 0.1 ml amounts into three tubes of BEK-1 cells. After the virus had been adsorbed to cells at 37°C for 90 min, 0.5 ml of MM was added. The infected cells were incubated in a roller drum at 37°C for 7 days and the infectivity titer expressed as TCID<sub>50</sub>/0.1 ml was calculated.

### *Nucleic acid determination*

Nucleic acid type was determined by the use of 5-iodo-2'-deoxyuridine (IUDR). Virus diluted to contain 1,000 TCID<sub>50</sub>/0.1 ml was inoculated into BEK-1 cells and adsorbed at 37°C for 90 min. After washing the infected monolayers three times with MEM, to one half of each set of cultures was added 0.5-ml amounts of MM containing 50  $\mu$ g IUDR/ml. To the other half serving as controls, 0.5 ml of MM was added. All the cultures were incubated at 37°C. At daily intervals, four cultures were taken from each group, and the infective fluids were pooled after low-speed centrifugation. Infectivity titers were determined by the method mentioned above. As reference viruses, BEV and BHV-1 were also tested.

### *Sensitivity to lipid solvent*

To determine the ether sensitivity of the virus, 1.6 ml of each virus suspension was mixed with 0.4 ml of anesthetic ether. The mixture was vigorously shaken, left to stand at room temperature for 5 min, and then centrifuged at 2,000 rpm for 5 min, and the water phase assayed immediately for infectivity. For treatment with chloroform, 1.9 ml of each virus suspension was mixed with 0.1 ml of chloroform. The mixture was treated in the manner mentioned above, and the resulting water phase was assayed for infectivity.

### *Susceptibility to acid pH*

Virus suspensions were diluted ten-fold in McIlvaine's buffer solution and adjusted to pH 3.0, 5.0 or 7.0. Controls were diluted with PBS (pH 7.2). Thereafter, incubation was carried out at room temperature for 1 h.

### *Sensitivity to heat*

Virus suspensions were incubated in a water bath at 50, 60, 70 or 80°C for 1 h. Others were kept in a water bath at 50°C for 5, 10, 15, 30, 60 or 180 min.

### *Cationic stabilization*

Virus suspensions were mixed with an equal volume of 2 M MgCl<sub>2</sub> solution, MM and distilled water. The mixtures were incubated in a water bath at 50°C for 1 h.

### *Sensitivity to trypsin*

Doubling dilutions of trypsin (Sigma Type III) ranging from 2 to 0.125% were prepared in PBS. One milliliter of the virus suspension was mixed with an equal volume of each trypsin dilution and the mixtures placed in a water bath at 37°C for 1 h. To each was then added 2 ml of trypsin inhibitor (Sigma Type 1-S).

### *Filtration*

Virus suspensions were filtered through a series of Sartorius' membrane filters of 200, 100 and 50 nm pore size.

### *Sucrose density gradient centrifugation*

Infective culture fluid, from which cell debris had been removed by low-speed centrifugation, was centrifuged at 100,000 g for 2 h and the resultant

pellets were suspended in one-hundredth of the original volume of PBS. The suspension was clarified by centrifugation at 20,000 *g* for 20 min and layered on top of a 5–60% (*w/w*) sucrose linear gradient to be centrifuged again using a SW 40 Ti rotor (Beckman, California, U.S.A.) at 100,000 *g* for 2 h. The gradient was then fractionated by a density gradient fractionator (ISCO, Nebraska, U.S.A.). Each of the fractions obtained was assayed for infectivity and hemagglutinin activity.

#### *Electron microscopic observation*

A drop of the peak fraction with a density of 1.182 was placed on a carbon collodion-coated grid and stained with 2% phosphotungstic acid adjusted to pH 6.7 with 1 *N* NaOH. The specimen was examined by a JEM-100CX electron microscope (Jeol Ltd., Tokyo, Japan).

#### *Immunization*

Antigen for hyperimmunizing guinea-pigs was prepared as follows. Infective culture fluids were concentrated by ultra-centrifugation. The resulting suspensions were mixed with a CsCl solution to a density of 1.25 and centrifuged at 100,000 *g* for 20 h. Fractions showing the highest hemagglutinin titer were collected. Four guinea-pigs were injected intramuscularly with 1 ml of a mixture of equal parts of antigen and complete Freund's adjuvant. They were bled at 2-week intervals after three injections.

#### *Neutralization (NT) test*

Each serum dilution was mixed with an equal volume of virus dilution containing 200 TCID<sub>50</sub>, and incubated at 37°C for 1 h. Each mixture was then assayed for infectivity by using two tubes per serum dilution. The tests were read after incubation at 37°C for 7 days. The antibody titer was expressed as the reciprocal of the highest serum dilution that showed no cytopathic changes in at least one of the two tubes.

#### *Hemagglutination (HA) and hemagglutination inhibition (HI) tests*

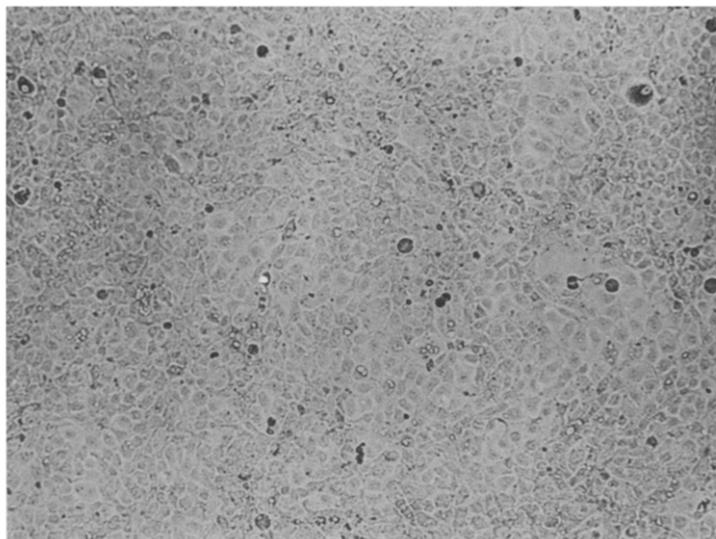
The HA and HI tests were carried out by the methods described by Sato et al. (1977b).

### RESULTS

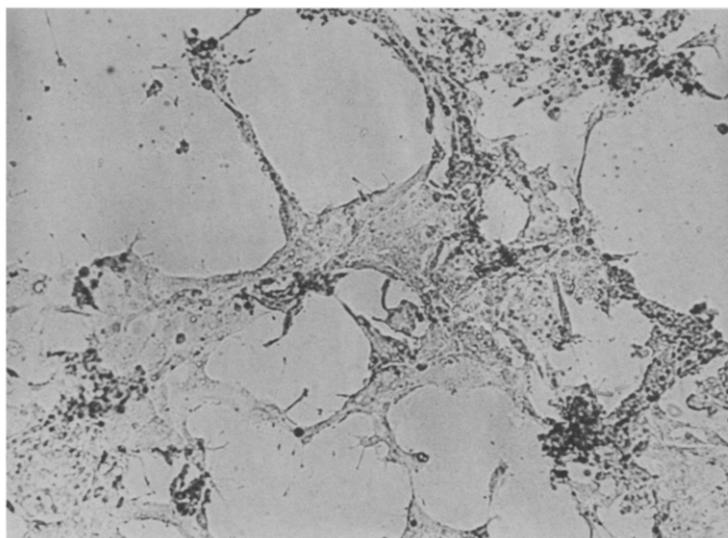
#### *Behavior of the isolate in BEK-1 cell culture*

Cytopathic changes were first observed 2–3 days after inoculation. They were characterized initially by granulation and syncytial formation of BEK-1

cells. These changes were followed by cellular degeneration and sloughing of the cells from the glass wall (Figs. 1 and 2).



**Fig. 1.** Normal BEK-1 cell culture 7 days after seeding ( $\times 100$ ).



**Fig. 2.** Cytopathic changes of BEK-1 cells 72 h after inoculation with the Kakegawa isolate ( $\times 100$ ).

*Characterization of the isolate*

The influence of IUDR upon the experimental results is illustrated in Fig. 3. The Kakegawa isolate multiplied in the presence of IUDR, as did the American strain, and it was therefore assumed to be an RNA type virus. BEV was not affected by IUDR, but BHV-1 was prevented from growing in the presence of IUDR, as expected.

The Kakegawa isolate and the American strain were inactivated completely within 5 min treatment with ether and chloroform (Table I).

Both viruses decreased slightly in titer by 0.8 and 0.7 logs, respectively, when placed at pH 3.0, but were scarcely affected at pH 5.0 and 7.0 (Table II).

The Kakegawa isolate passed a membrane filter of 200 nm pore size, but was retained by one of 100 nm pore size. The American strain, however, passed a membrane filter of 100 nm pore size (Table II).

Although the American strain was completely inactivated by heating at 70°C, the Kakegawa isolate retained infectivity at this temperature. However

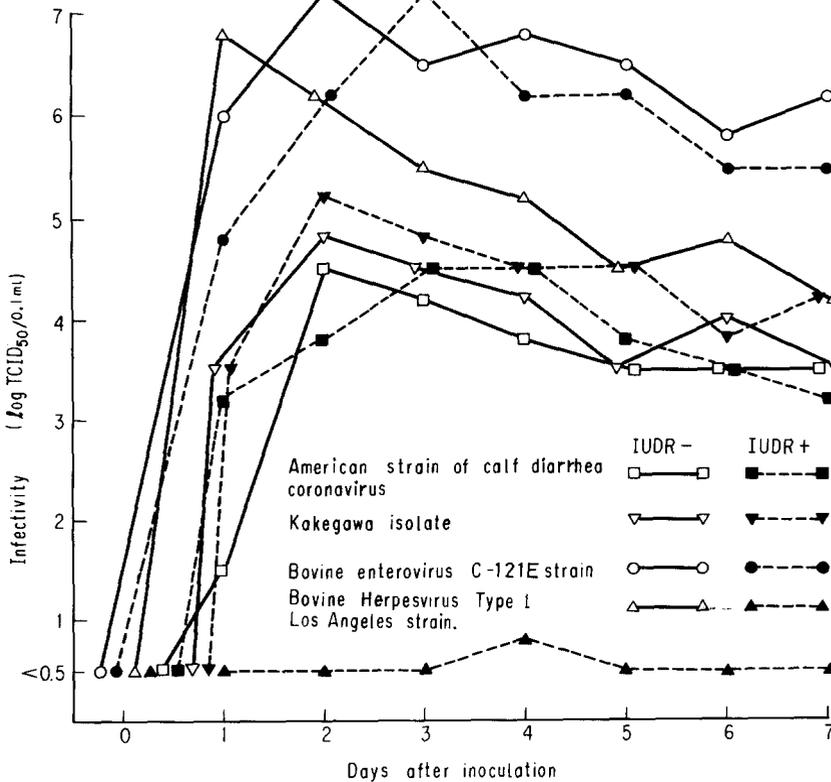


Fig. 3. Influence of 5-iodo-2'-deoxyuridine.

TABLE I

## Influence of ether and chloroform

Virus	Control	Infectivity after treatment at room temperature for 5 min	
		Ether	Chloroform
American strain	5.5 <sup>1</sup>	≪ 0.5	≪ 0.5
Kakegawa isolate	5.5	≪ 0.5	≪ 0.5

<sup>1</sup> Log TCID<sub>50</sub>/0.1 ml.

TABLE II

## Influence of acid pH and filtration

Influence of acid pH			Filtration		
pH	American strain	Kakegawa isolate	Pore size (nm)	American strain	Kakegawa isolate
PBS (7.2)	4.2 <sup>1</sup>	4.8	Before	5.8	4.8
7.0	4.2	4.5	200	4.5	4.8
5.0	3.8	4.8	100	1.8	< 0.5
3.0	3.5	4.0	50	< 0.5	< 0.5

<sup>1</sup> Log TCID<sub>50</sub>/0.1 ml.

TABLE III

## Influence of heating for 1 h

Virus	Before heating	Temperature (°C)			
		50	60	70	80
American strain	5.2 <sup>1</sup>	2.5	0.5	< 0.5	< 0.5
Kakegawa isolate	5.2	1.8	0.5	— <sup>2</sup>	< 0.5

<sup>1</sup> Log TCID<sub>50</sub>/0.1 ml.<sup>2</sup> Not calculated; CPE appeared in two of three tubes inoculated with undiluted materials.

when heated at 50°C, both viruses showed a gradual loss of infectivity, over a period of 3 h, and it is presumed therefore that both viruses are moderately sensitive to heat (Tables III and IV).

Both viruses were stabilized with 1 M MgCl<sub>2</sub> (Table V), and were sensitive to high concentrations of trypsin (Table VI).

Buoyant density determination of the Kakegawa isolate was performed in a sucrose linear density gradient. The peak of infectivity and hemagglutinin

TABLE IV

Influence of heating at 50°C

Virus	Before heating	Incubation time (min)					
		5	10	15	30	60	180
American strain	5.5 <sup>1</sup>	5.2	4.6	4.5	4.6	3.2	≤ 0.5
Kakegawa isolate	5.5	5.2	5.5	5.8	4.5	3.5	0.8

<sup>1</sup> Log TCID<sub>50</sub>/0.1 ml.

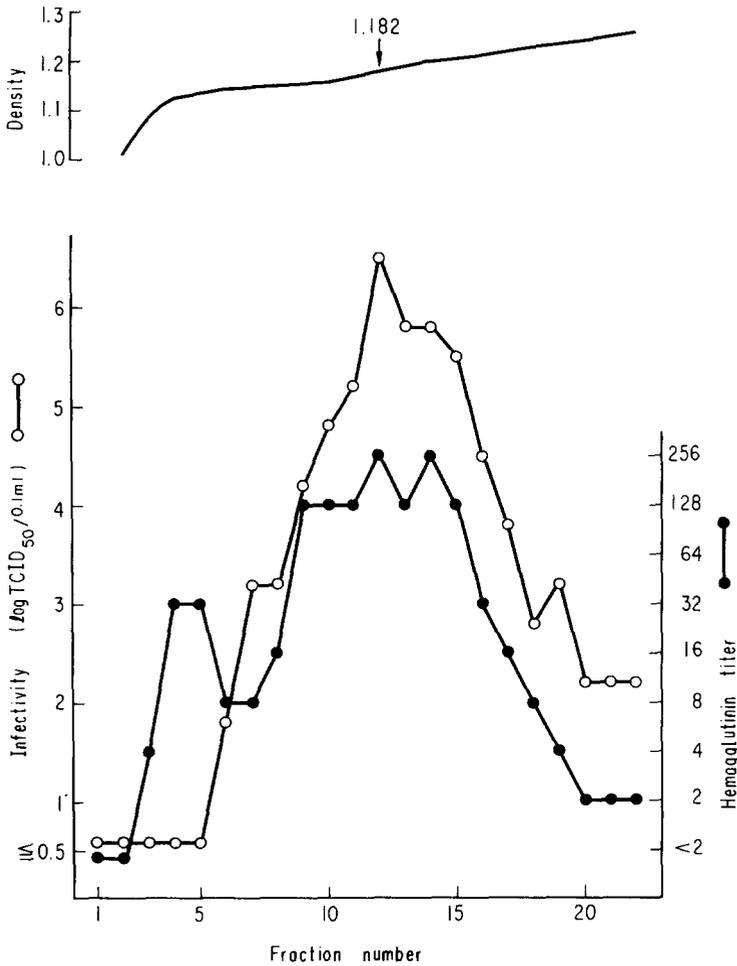


Fig. 4. Sucrose linear density gradient centrifugation of Kakegawa isolate.

TABLE V

Cationic stabilization (at 50°C for 1 h)

Virus	Before heating	Heating in		
		1 M MgCl <sub>2</sub>	MM <sup>1</sup>	H <sub>2</sub> O
American strain	4.2 <sup>2</sup>	3.8	2.2	< 0.5
Kakegawa isolate	4.8	4.0	1.5	1.5

<sup>1</sup> Maintenance medium.<sup>2</sup> Log TCID<sub>50</sub>/0.1 ml.

TABLE VI

Influence of trypsin treatment (at 37°C for 1 h)

Virus	Control	Concentration of trypsin (%)				
		1	0.5	0.25	0.125	0.0625
American strain	4.2 <sup>1</sup>	2.2	2.2	3.2	3.8	4.2
Kakegawa isolate	3.8	2.5	2.5	3.2	3.8	3.5

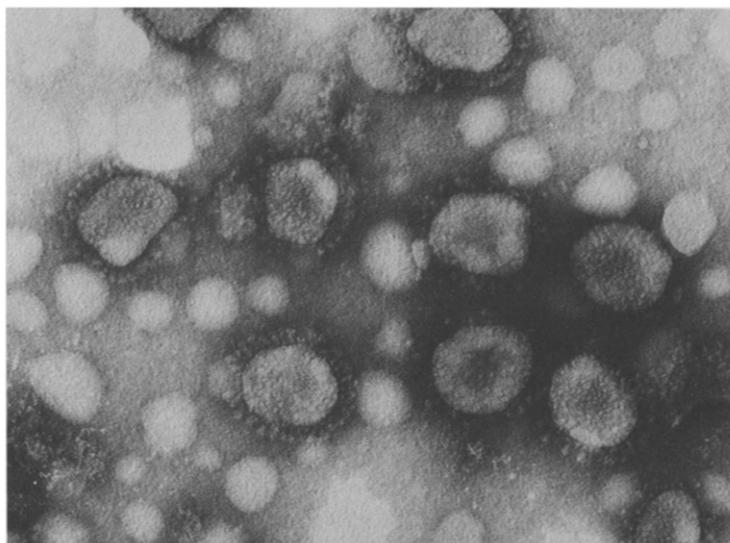
<sup>1</sup> Log TCID<sub>50</sub>/0.1 ml.

Fig. 5. Particles of the Kakegawa isolate negatively stained with PTA. Widely spaced petal-shaped projections are observed ( $\times 100,000$ ).

activity was located in fraction 12 with a buoyant density of 1.182 (Fig. 4).

Electron microscopy of this material revealed numerous spherical virions of 120 nm average diameter. They were pleomorphic and had widely spaced petal-shaped surface projections about 20 nm in length (Fig. 5).

TABLE VII

## Cross neutralization and hemagglutination inhibition tests

Immunized by	Guinea-pig number	Neutralizing titer		Hemagglutination inhibiting titer	
		Kakegawa isolate	American strain	Kakegawa isolate	American strain
Kakegawa isolate	1	5,120	5,120	320	640
	2	5,120	5,120	640	640
	3	10,240	10,240	640	640
	4	5,120	10,240	640	320
American strain	1	5,120	10,240	1,280	1,280
	2	2,560	5,120	640	640
	3	2,560	5,120	640	640
	4	5,120	10,240	1,280	320

*Immunological properties of the isolate*

The results of cross NT and HI tests with guinea-pig hyperimmune sera are shown in Table VII. These indicate that there is a close antigenic relationship between the Kakegawa isolate and the American strain.

## DISCUSSION

Isolation of bovine coronaviruses in tissue culture is difficult (Woode et al., 1978) and only the American strain develops cytopathic changes in fetal bovine kidney cells (Mebus et al., 1973) and BEK-1 cells (Inaba et al., 1976). Other isolates propagated in cell culture (Bridger et al., 1978b) and organ culture (Stott et al., 1977; Bridger et al., 1978a), are non-cytopathic.

In the present study, the Kakegawa isolate was readily propagated in BEK-1 cells. Cytopathic changes appeared within 2–3 days after virus inoculation and resembled those obtained in BEK-1 cells infected with the American strain of coronavirus (Inaba et al., 1976). It is presumed, therefore, that BEK-1 cells are preferable to primary bovine kidney cells for the propagation of this virus.

Because multiplication of the virus was not affected by the action of IUDR, this indicates that its nucleic acid is RNA. The presence of an envelope, containing structural lipids in the virus can be presumed from its sensitivity to ether and chloroform, and this suggestion was supported by electron-microscopic observation. These basic properties are in common with those of the American strain of calf diarrhea coronavirus (Sharpee et al., 1976) and other members of the coronaviridae (McIntosh, 1974).

The isolated virus was almost completely inactivated by heating at 50°C for 3 h and was considered to be moderately sensitive to heat. It also showed

increased stability against heat inactivation in the presence of 1 M MgCl<sub>2</sub>. These findings are in agreement with those previously reported by other workers (Sharpee et al., 1976; Sato et al., 1977a). In contrast it should be noted that the thermostability of bluecomb coronavirus of turkeys is decreased in the presence of 1 M MgCl<sub>2</sub> (Deshmukh and Pomeroy, 1974), and that no effect of MgCl<sub>2</sub> occurs on the thermostability of human coronavirus 229-E (Hierholzer, 1976).

Sato et al. (1977a) reported that the American strain decreased slightly in infectivity at pH 3.0, while the same strain was found to be resistant to acid by Sharpee et al. (1976). Although the virus isolated by the present authors revealed a slight loss of infectivity at pH 3.0, it was considered to be acid stable.

It was also affected by a high concentration of trypsin to a similar degree as the American strain, although Sato et al. (1977a) reported that the American strain was inactivated completely when treated with a concentration of 5% or higher of trypsin. These conflicting results may be due to differences in viral titers and/or the type of trypsin used.

The buoyant density of the virus was estimated at 1.182 in sucrose gradient which is in agreement with the previous results obtained with the American strain (Sharpee et al., 1976) and some other coronaviruses (McIntosh, 1974; Pensaert and Callebaut, 1978).

There was an antigenic similarity in the NT and HI tests between the Kakegawa isolate obtained from a cow and the American strain of calf diarrhea coronavirus. This suggests that bovine coronavirus may be of a single serotype which is capable of causing diarrhea in a wide age-range of cattle, regardless of the age of the animal.

Further detailed studies will be necessary to compare the pathogenicity of the Kakegawa isolate with that of calf diarrhea coronaviruses, and its antigenicity with that of coronaviruses from cattle and other species.

## REFERENCES

- Acres, S.D., Laing, C.J., Saunders, J.R. and Radostits, O.M., 1975. Acute undifferentiated neonatal diarrhea in beef calves. I. Occurrence and distribution of infectious agents. *Can. J. Comp. Med.*, 39: 116-132.
- Bridger, J.C., Caul, E.O. and Egglestone, S.I., 1978a. Replication of an enteric bovine coronavirus in intestinal organ cultures. *Arch. Virol.*, 57: 43-51.
- Bridger, J.C., Woode, G.N. and Meyling, A., 1978b. Isolation of coronaviruses from neonatal calf diarrhoea in Great Britain and Denmark. *Vet. Microbiol.*, 3: 101-113.
- Deshmukh, D.R. and Pomeroy, B.S., 1974. Physicochemical characterization of a bluecomb coronavirus of turkeys. *Am. J. Vet. Res.*, 35: 1549-1552.
- Doughri, A.M., Storz, J., Hajer, I. and Fernando, H.S., 1976. Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. *Exp. Mol. Pathol.*, 25: 355-370.
- Durham, P.J.K., Stevenson, B.J. and Fargharson, B.C., 1979. Rotavirus and coronavirus associated diarrhoea in domestic animals. *N.Z. Vet. J.*, 27: 30-32.

- Hierholzer, J.C., 1976. Purification and biophysical properties of human coronavirus 229E. *Virology*, 75: 155—165.
- Horner, G.W., Hunter, R. and Kirkbride, C.A., 1976. A coronavirus-like agent present in faeces of cows with diarrhoea. *N.Z. Vet. J.*, 23: 98.
- 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.
- Kurogi, H., Inaba, Y., Takahashi, E., Sato, K. and Omori, T., 1976. Separation and properties of enterovirus and reovirus recovered from a fecal sample of calf with diarrhea. *Natl. Inst. Anim. Health Q. (Jpn)*, 16: 49—58.
- McIntosh, K., 1974. Coronaviruses: A comparative review. *Curr. Top. Microbiol. Immunol.*, 63: 85—129.
- Madin, S.H., York, C.J. and Mckercher, D.G., 1956. Isolation of the infectious bovine rhinotracheitis virus. *Science*, 124: 721—722.
- Mebus, C.A., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J., 1973. Neonatal calf diarrhea: Propagation, attenuation, and characteristics of a coronavirus-like agent. *Am. J. Vet. Res.*, 34: 145—150.
- Morin, M., Larivière, S. and Lallier, R., 1976. Pathological and microbiological observations made on spontaneous cases of acute neonatal calf diarrhea. *Can. J. Comp. Med.*, 40: 228—240.
- Pensaert, M. and Callebaut, P., 1978. The coronaviruses: Clinical and structural aspects with some practical implications. *Ann. Med. Vet.*, 122: 301—322.
- Sato, K., Inaba, Y., Kurogi, H., Takahashi, E., Ito, Y., Goto, Y., Omori, T. and Matumoto, M., 1977a. Physico-chemical properties of calf diarrhea coronavirus. *Vet. Microbiol.*, 2: 73—81.
- Sato, K., Inaba, Y., Kurogi, H., Takahashi, E., Satoda, K., Omori, T. and Matumoto, M., 1977b. Hemagglutination by calf diarrhea coronavirus. *Vet. Microbiol.*, 2: 83—87.
- 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.
- Stott, E.J., Thomas, L.H., Bridger, J.C. and Jebbett, N.J., 1977. Replication of a bovine coronavirus in organ cultures of foetal trachea. *Vet. Microbiol.*, 1: 65—69.
- Takahashi, E., Inaba, Y., Sato, K., Ito, Y., Kurogi, H., Akashi, H., Satoda, K. and Omori, T., 1980. Epizootic diarrhoea of adult cattle associated with a coronavirus-like agent. *Vet. Microbiol.*, 5: 151—154.
- Wellems, G., Antoine, H., Botton, Y. and Van Opdenbosch, E., 1977a. La fréquence du virus Corona dans les troubles digestifs du jeune veau en Belgique. *Ann. Med. Vet.*, 121: 411—420.
- Wellems, G., Oyaert, W., Muylle, E., Thoonen, H. and Van Opdenbosch, E., 1977b. Isolatie van een corona-virus bij kalveren. *Vlaams Diergeneesk. Tijdschr.*, 46: 249—255.
- Woode, G.N., Bridger, J.C. and Meyling, A., 1978. Significance of bovine coronavirus infection. *Vet. Rec.*, 102: 15—16.
- Zygraich, N., Georges, A.M. and Vascoboinic, E., 1975. Étiologie des diarrhées néonatales du veau. Résultats d'une enquête sérologique relative aux virus reo-like et corona dans la population bovine belge. *Ann. Med. Vet.*, 119: 105—113.