

## Isolation of Bovine Coronavirus from Feces and Nasal Swabs of Calves with Diarrhea

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**ABSTRACT.** Fecal and nasal samples were collected from 180 calves with diarrhea and 36 clinically normal co-habitants, and tested for virus using HRT-18 cell cultures derived from human rectal adenocarcinoma. A cytopathic virus was isolated from 5 fecal and 56 nasal samples obtained from diarrheic calves. All calves in which the virus was isolated from diarrheic feces were positive for virus isolation from nasal swabs. The virus was also isolated from the nasal swabs of 10 clinically normal calves that were co-habitants with diarrheic calves. Because they were morphologically similar to coronavirus, agglutinated mouse erythrocytes and serologically identical with the Nebraska calf diarrhea coronavirus, new isolates were identified as bovine coronavirus. The demonstration of viral antigens in nasal epithelial cells by a direct immunofluorescence was in close agreement with the virus isolation in HRT-18 cell cultures. This is the first report on the isolation of bovine coronavirus from newborn calves with diarrhea in Japan. The evidence that the virus was frequently isolated from nasal swabs is of great interest for understanding the pathogenesis of bovine coronavirus infection.—**KEY WORDS:** bovine coronavirus, calf, diarrhea, virus isolation.

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Bovine coronavirus (BCV) is known as a primary cause of diarrhea in newborn calves in North America and Europe [2, 7, 13, 16]. In addition, some recent reports have suggested that BCV possesses a tissue tropism to the upper respiratory tract as well as the small intestine of calves [15, 18, 19]. Although some isolates of BCV were shown to replicate in organ cultures and in cell cultures [2, 7, 11, 15], the primary isolation of BCV in cell cultures is still difficult, and therefore, there have been only a few investigations on antigenic comparison of BCV strains. In Japan, Takahashi *et al.* [22] reported that BCV was an agent causing epizootic diarrhea of adult cows. However, there is no report on the detection and isolation of BCV from newborn calves with diarrhea in Japan.

The purpose of this report was to describe the isolation of BCV from feces and nasal swabs of newborn calves using a human rectal adenocarcinoma (HRT-18) cell line, and antigenic comparison of the isolates with the Nebraska calf diarrhea coronavirus (NCDC). In addition, direct immunofluorescence (IF) of nasal epithelial cells was compared with virus isolation from nasal swabs with HRT-18 cells for diagnosis of BCV infection in the upper respiratory tract of calves.

### MATERIALS AND METHODS

*Clinical specimens:* Samples of feces and nasal swabs were obtained from 180 diarrheic calves aged 3 to 30 days on the onset of diarrhea at 4 beef farms in Hokkaido, Japan. Samples were also collected from 36 clinically normal calves that were kept in the same barns with diarrheic calves. Feces were diluted 1:10 in 0.01 M phosphate buffered saline (PBS; pH 7.4) and clarified by low-speed centrifugation at 3,000 g for 10 min, and the supernatants were used for virus isolation. Nasal secretions were collected with cotton-tipped swabs, extracted with 2 ml of Eagle's minimum essential medium (EMEM) supplemented with 0.15% sodium bicarbonate and 50 µg/ml of gentamicin, and clarified by low-speed centrifugation. The supernatants were used for virus isolation, and the precipitates, which contain nasal epithelial cells, were used for direct IF to examine BCV. Fecal samples were also examined for bovine rotavirus as previously described [24].

*Cell cultures:* HRT-18 cells that are derived from a human rectal adenocarcinoma [23] were kindly supplied by Dr. H. Kida (Faculty of Veterinary Medicine, Hokkaido University, Sapporo), and used for virus isolation and neutralization test. The growth medium was EMEM supplemented with 10% calf serum, 10% tryptose phosphate broth

(Difco), 0.15% sodium bicarbonate and 50 µg/ml of gentamicin. The maintenance medium was EMEM supplemented with 0.11% bovine serum albumin and the same concentrations of sodium bicarbonate and gentamicin as the growth medium.

*Reference virus:* The NCDC strain of BCV passaged in HRT-18 cells was kindly supplied by Dr. Y. Murakami (Department of Exotic Disease, National Institute of Animal Health, Kodaira, Tokyo). This strain was originally isolated by Mebus *et al.* in U.S.A. [16].

*Virus isolation:* The confluent monolayers of HRT-18 cell cultures grown in roller tubes were washed 3 times with Earle's balanced salt solution (EBSS). Four tubes were inoculated with 0.1 ml of fecal or nasal samples. After adsorption for 60 min at 37°C, the cultures were washed once with EBSS and received 0.5 ml of the maintenance medium. The cultures were incubated in a roller drum for 4 to 5 days at 37°C, and examined for cytopathic effect (CPE). After incubation, the cells of one tube of each sample were scraped off and examined for BCV by direct IF. Cells of remaining tubes were harvested by being frozen and thawed once, and subsequent passages were carried out in the same manner with 0.1 ml of cell suspensions. Three to 7 passages were done if neither CPE nor fluorescent cells were detected. After 3 passages, 14 isolates (4 derived from feces and 10 from nasal swabs) were cloned 3 times in HRT-18 cells by limiting dilution and used for electron microscopy (EM), hemagglutination (HA) test and neutralization test.

*Preparation of antiserum:* The NCDC strain and the 2 cloned isolates (216XF and 119WN strains isolated from a feces and a nasal swab, respectively) were purified by sucrose density ultracentrifugation as described previously [3]. Hyperimmune sera were prepared by inoculating guinea pigs with the purified viruses. The sera were heated for 30 min at 56°C and used in the neutralization test.

*IF:* Nasal epithelial cells from nasal swabs and HRT-18 cells from the inoculated cultures were washed twice with PBS, smeared onto the 10-well multitest slides and examined for BCV by direct IF [18, 19]. The hyperimmune serum against the NCDC of BCV was fractionated and conjugated with fluorescein isothiocyanate (FITC) according to the procedures of Kawamura [12].

*EM:* The supernatants of HRT-18 cell cultures infected with the cloned isolates were concentrated by centrifugation at 57,000 g for 2 hr. The pellets

were resuspended in PBS and centrifuged at 78,000 g for 2 hr through 20% (w/w) sucrose cushion. The pellets were resuspended in PBS in 1/1,000 of original volumes, stained with 1% uranyl acetate, placed on carbon-coated collodion grids, and examined with an electron microscope (JEM-1200EX; Nippon Denshi Co.).

*Virus titration and neutralization test:* Virus titration and neutralization test were performed in HRT-18 cell cultures grown in 96 well microplates. For the titration, serial 10-fold dilutions of viruses were made with the maintenance medium. Four wells of HRT-18 cells were inoculated with 0.1 ml of each dilution, incubated for 7 days at 37°C and examined for CPE. Infective titers were expressed as median tissue culture infective doses (TCID<sub>50</sub>)/ml. For the neutralization test, serial 2-fold dilutions of serum were mixed with an equal volume of virus suspensions containing 200 TCID<sub>50</sub>/0.1 ml and incubated for 60 min at 37°C. Four wells of HRT-18 cells were inoculated with 0.1 ml of each virus-serum mixture and incubated for 7 days at 37°C. Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that inhibited 50% of CPE.

*HA test:* HA test was carried out by the microtiter method. Supernatants of HRT-18 cell cultures infected with the NCDC and the isolates were tested for HA using human (O), cattle, sheep, horse, mouse, guinea pig and rabbit erythrocytes. Serial 2-fold dilutions of infected fluids were prepared in 0.025 ml of 0.01 M PBS (pH 7.4) supplemented with 0.2% bovine serum albumin and mixed with 0.025 ml of 1% erythrocyte suspensions in the same buffer. The mixtures were then incubated for 2 hr at either 4, 20 or 37°C, and the tests were read at the end of the incubation period.

## RESULTS

*Virus isolation:* Cytopathic BCV was isolated from 5 (3%) fecal samples and 56 (31%) nasal swabs of 180 diarrheic calves (Table 1). The virus was also isolated from the nasal swabs, but not from feces, of 10 (28%) of 36 clinically normal calves which were co-habitants with diarrheic calves. All calves, even those in which BCV was isolated from nasal swabs, showed no clinical signs of respiratory disease. In 5 calves which were positive for BCV isolation from diarrheic feces, BCV was isolated simultaneously from nasal swabs. Further 3 of the 5 calves de-

Table 1. Isolation of BCV from feces and nasal swabs of 180 diarrheic and 36 normal calves in HRT-18 cell cultures

Calf	BCV isolation from			
	Feces		Nasal swabs	
	+	-	+	-
Diarrheic (n=180)	5	175	56	124
Normal (n=36)	0	36	10	26

veloped dehydration and died 3 to 6 days after the onset of diarrhea. Two of them were also positive for bovine rotavirus in diarrheic feces.

BCV antigens were demonstrated by IF in HRT-18 cells inoculated with the 71 samples at the first or second passage. The number of fluorescent cells increased after further serial passages. After the second or third passage, CPE, that is characterized by the appearance of granular cells and the formation of cell clumps, occurred in the cultures inoculated with the samples positive for IF antigens. CPE appeared 48 to 72 hr after inoculation, and the granular cells were detached from the surface of tubes or plates and floated in the medium. Eventually, many "craters" were formed on the cell monolayers, which appears like the moon surface. Virus titers of the cloned viruses reached  $10^{5.7}$  to  $10^{7.5}$  TCID<sub>50</sub>/ml at the 6th passage.

**EM test:** Viral particles characteristic of coronavirus were seen in the culture fluids of HRT-18 cells infected with the cloned isolates. The particles were approximately 80 to 140 nm in diameter and had club-shaped projections of 15 nm in length (Fig. 1).

**HA test:** Mouse erythrocytes were agglutinated by the supernatants of HRT-18 cell cultures infected with the 14 cloned isolates and the NCDC strain. HA titers ranged from 16 to 64 and no difference was observed in HA titers among reaction temperatures at 4, 20 and 37°C. No erythrocytes of other animals were agglutinated by the infected culture fluids.

**Cross neutralization test:** The 14 cloned isolates and the NCDC strain were compared antigenically by the cross neutralization test using antisera to the NCDC strain, and the 216XF and the 119WN strains of the isolates. As shown in Table 2, the NCDC strain and the isolates were neutralized equally with each antiserum. This indicates that they are serologically identical one another.

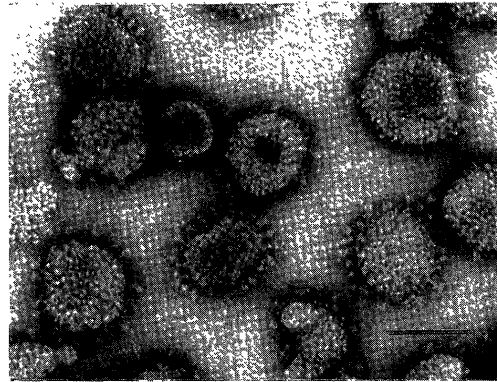


Fig. 1. Electron micrograph of the 216XF isolate (passage 6 on HRT-18 cells). Negative stain. Bar, 100 nm.

Table 2. Serological characterization of newly isolated BCV by neutralization test

Virus	Antiserum		
	NCDC	119WN	216XF
NCDC (Control)	46300 <sup>a)</sup>	2900	5800
119WN	23200	2900	5200
216XF	20600	2900	8200
339XF	23200	2600	5800
45XF	32800	4100	5800
48XF	23200	4100	8200
207XN	23200	2600	5200
224XN	46300	2600	5800
406XN	23200	2900	5200
14XN	23200	4100	5800
107XN	26000	5200	5800
315XN	20600	5800	8200
408WN	26000	5800	8200
238WN	23200	5800	8200
339XN	32800	4100	8200

N and F indicate the sources of virus isolation, nasal swabs and feces, respectively.

a) Neutralizing antibody titers (reciprocal of the serum dilution which inhibited 50% of CPE).

**Detection of BCV in nasal epithelial cells by direct IF:** BCV antigens were detected from nasal epithelial cells in 49 (27%) of 180 diarrheic calves and in 12 (33%) of 36 normal calves by direct IF. Figure 2 shows specific fluorescence observed in nasal epithelial cells stained with FITC-conjugated anti-BCV (NCDC strain) antibody. Fluorescing cells in the BCV positive samples ranged from less than 1% to 20% of total cells. The results of direct IF of nasal epithelial cells were in close agreement with those of virus isolation from nasal swabs in HRT-18 cell cultures. Agreement rate of the results obtained from both tests was 87.5% (Table 3).

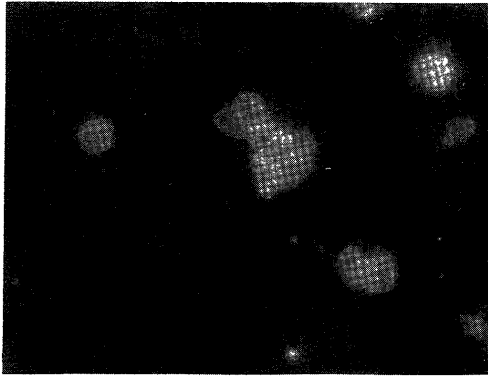


Fig. 2. Infected nasal epithelial cells from a diarrheic calf. The cells were stained with FITC-conjugated anti-BCV (NCDC) serum. Fluorescence is seen in the cytoplasm of infected cells.  $\times 280$ .

Table 3. Comparison of direct immunofluorescence in nasal epithelial cells and virus isolation from nasal swabs with HRT-18 cell cultures for detection of BCV

	Virus isolation	
	Positive	Negative
Direct immunofluorescence		
Positive	50 (23.1%)	11 (5.1%)
Negative	16 (7.4%)	139 (64.4%)

#### DISCUSSION

Because coronaviruses are difficult to be adapted to growth in cell cultures, there have been a few reports dealing with the isolation of BCV in cell cultures and the antigenic comparison among BCV strains [9, 18]. Recently, it has been reported that HRT-18 cells derived from human rectal adenocarcinoma are susceptible to bovine, canine, human and turkey enteric coronaviruses [1, 6, 14]. In this study, calves with diarrhea and their co-habitants were examined for BCV using HRT-18 cell cultures, and many cytopathic viruses were successfully isolated from diarrheic feces and nasal swabs. They agglutinated mouse erythrocytes and were serologically identical to the NCDC strain of BCV [20, 21], indicating that the isolates are BCV. These results suggest that HRT-18 cells are considerably susceptible to BCV and suitable for the virus isolation as previously described [1, 14].

In Japan, the etiological importance of BCV in newborn calf diarrhea remains obscure, because the

virus has only been isolated from adult cows affected with diarrhea [22], but not from newborn calves. The results of the present study appear to suggest that BCV is one of etiological agents causing newborn-calf diarrhea in Japan as well as in North America and Europe.

It is of great interest that BCV was isolated from many nasal swabs of not only diarrheic but also clinically normal calves that were co-habitants with diarrheic calves. Reynolds *et al.* [18] have reported that there were no differences in serological and biological properties between the enteric and respiratory isolates of BCV. Our studies demonstrated that BCV isolates recovered from diarrheic feces and nasal swabs were serologically indistinguishable from each other, therefore, they may be identical. The observation that BCV was recovered from the nasal swabs of all calves in which the virus was isolated from diarrheic feces appears to support the above conclusion.

Although the pathogenic significance of BCV isolated in the present study should be confirmed in the further studies, it is reasonable to consider that diarrhea investigated was probably associated, at least in part, with the infection of the isolated virus. The reasons for the low frequency of virus isolation from diarrheic feces are unknown, but the negative results in the virus isolation may not always indicate the absence of the virus in fecal samples. It is likely that the fecal samples that were negative for virus isolation still contain BCV. The efforts with the application of other methods, such as a immune EM and enzyme-linked immunosorbent assay, may prove the presence of BCV in the diarrheic feces that were negative for virus isolation [4, 5, 8, 10, 13, 17].

The evidence that BCV was isolated from the nasal swabs of many diarrheic and clinically normal calves indicates that the virus has a tissue tropism to the upper respiratory tract as well as the intestinal tract. This agrees with the previous reports describing that the upper respiratory epithelial cells are highly sensitive to the infection with BCV [10, 15, 18, 19]. However, the pathogenic importance of BCV in the respiratory diseases is not defined. McNulty *et al.* [15] have reported that BCV isolated from the respiratory tissues induced mild clinical signs of the respiratory diseases in experimentally inoculated calves. By contrast, some other workers have failed to produce the experimental respiratory diseases in inoculated calves [10, 18, 19]. In this

study, no clinical signs of the respiratory diseases were observed in any calves, even in which BCV was recovered from nasal swabs. Further studies are expected to elucidate the association of BCV with bovine respiratory diseases.

The evidence that the frequency of BCV isolation from nasal swabs was considerably high suggests that intestinal infection of calves with BCV may occur by the transmission via aerosol-nasal route as well as fecal-oral route [19]. For the prevention of calves from diarrhea associated with BCV infection, it is important to investigate the mechanisms of the virus transmission and compare intestinal pathogenicity among BCV strains.

The detection of viral antigens in nasal epithelial cells by the direct IF is as sensitive as the virus isolation. The direct IF method in the detection of BCV is recommended for practical examination, because of its simplicity.

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