Bovine Epizootic Diarrhea Resembling Winter Dysentery Caused by Bovine Coronavirus

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Introduction

The etiologic factors of bovine diarrhea are complex. Many viruses associated with this disease have been reported.

Epizootic diarrhea in cattle resembling commonly called winter dysentery[4] has been described in Australia,[10] Canada,[8] England,[17] France,[5,17] Israel[12] and United States.[12,16] We also observed outbreaks of the disease in ten or more areas in Japan during the winter of 1976 and 1977. More than 3000 adult cattle in 12 prefectures in the northern, central and western parts of Japan developed profuse diarrhea, and the milk production in these animals declined by 20–25% during the disease and recovered to only 80% of the previous production levels at 3 or 4 weeks after the disease. The purpose of the present report is to give a serological evidence for the etiological role of bovine coronavirus in the epizootic diarrhea of adult cattle, with supportive electron-microscopical and cell culture studies.

Detection of virus particles in diarrheal feces

Diarrheal feces were collected from an affected cow and prepared for demonstration of virus particles by electron microscopy. The feces were homogenized to make a 5% suspension in PBS. The suspension was clarified by centrifugation at 1,500×g for 20 min and at 20,000×g for 30 min. The resulting supernatant fluid was centrifuged at 100,000×g for 2 hr and the pellet obtained was suspended in 1/50 the original volume of PBS. The suspension was then centrifuged at 1,500×g for 10 min. Immunoelectron microscopic examination of the supernatant fluid by the phosphotungstic negative staining technique revealed numerous coronavirus-like agents, from 60 to 120 mm in diameter (Plate 1).[20]

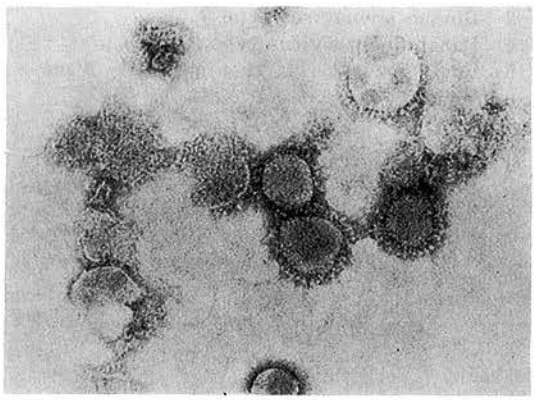

Plate 1. Electron micrograph of bovine coronavirus-like particles obtained from diarrheal fecal extract
Negative stain ×79,000

Serological evidence

Serological tests with various viruses including bovine coronavirus were carried out on paired sera subjected to diagnostic purpose which were sampled from various areas during the outbreak. A relatively high rate (59.0% : 59/100) of adult cattle clinically diagnosed as having the disease showed a significant rise in antibody titer for bovine coronavirus,[19] whereas only small fractions of those animals showed serological evidences

for recent infection with bovine rotavirus, bovine adenovirus type 7, parainfluenza virus type 3 and bovine viral diarrhea-mucosal disease virus (Table 1).[20] In further serological tests on paired sera, 153 cows or 81.8% of the total of 187 cows tested exhibited a significant titer rise for bovine coronavirus (Table 2) (Inaba et al., unpublished data).

Table 1. Seroconversion in paired serum of cattle suffering from epizootic diarrhea

Prefecture	BCV[1]	BRV[2]	BAV-7[3]	PIV-3[4]	BVD-MDV[5]
Shizuoka	38/65*	3/65	5/65	7/65	3/65
Chiba	11/21	8/21	4/21	0/21	2/21
Tochigi	4/5	0/5	0/5	0/5	0/5
Shimane	6/9	0/9	0/9	0/9	0/9
Total	59/100	11/100	9/100	7/100	5/100

* Number of seroconverted cows/number of cows tested
1 Bovine coronavirus
2 Bovine rotavirus
3 Bovine adenovirus type 7
4 Parainfluenza virus type 3
5 Bovine viral diarrhea-mucosal disease virus

Table 2. HI test with BCV on paired sera sampled for diagnosis from various areas during the outbreak

Prefecture	No. of cattle showing 4-fold or greater antibody rise/ No. of cattle tested	Rate of significant rise in antibody
Hokkaido	33/39	77.8
Fukushima	24/36	66.7
Toyama	22/30	73.3
Ishikawa	10/10	100
Nagano	6/6	100
Yamaguchi	14/14	100
Saga	10/10	100
Oita	34/42	81.0
Total	153/187	81.8

Isolation of the virus

Attempts were made to isolate the virus from the feces of the cow Kakegawa No. 16, a stabled dairy cattle, showing a typical epizootic diarrhea characterized by a brief attack of severe diarrhea and remarkable reduction in milk production.

Primary bovine kidney (BK) cell cultures were prepared in 100×11 mm tubes. Each of the BK cell cultures was inoculated with 0.1 m*l* volume of the purified material obtained from feces as described above. After virus adsorption at 37°C for 2 hr the cell cultures were washed three times with Earle's solution, fed with 0.5 m*l* of maintenance medium per each and incubated at 37°C in a roller drum. The cultures were checked for virus growth at each passage level by the direct immunofluorescence technique with antiserum to bovine coronavirus[18] supplied by courtesy of Dr. C. A. Mebus (Nebraska, U.S.A.).

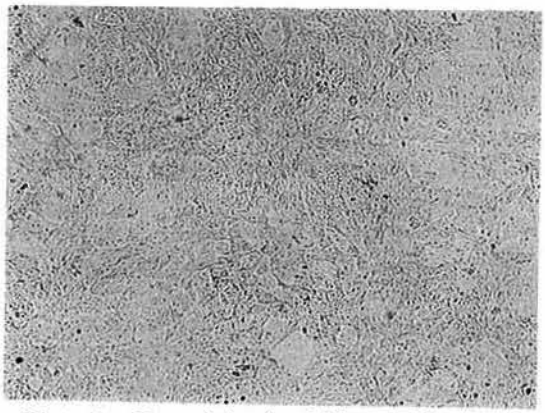

Plate 2. Normal bovine kidney cell culture 7 days after seeding ×90

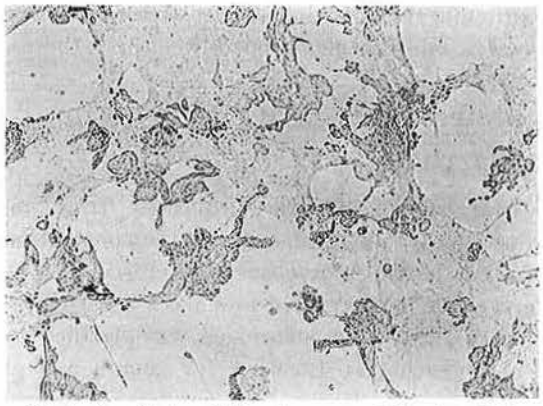

Plate 3. Cytopathic changes of bovine kidney cell cultures 3 days after inoculation with the Kakegawa isolate ×90

Table 3. 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

A few fluorescent cells were observed in the cultures at the second passage level and many distinct fluorescent cells were easily observed at the further passage levels. The CPE was recognized at the 8th passage of culture. The CPE appeared 3 to 4 days after the inoculation and was characterized by syncytium formation and granulation of the cells (Plate 2, 3).[1,20] As the passages increased, syncytia developed more quickly and were more distinct. The CPE was neutralized with the rabbit antiserum to bovine coronavirus which was used for immunofluorescence staining. Further identification of specific antigen of bovine coronavirus[19] in infected cells was confirmed by the electron microscopic examination with the purified viral preparations obtained by sucrose gradient centrifugation. The negatively stained viral particles were similar to those detected in diarrheal feces from an affected cow (Plate 4). The isolate was designated as strain "Kakegawa". Neutralization and hemagglutination-inhibition tests showed a close serological relationship between the Kakegawa strain and the American strain of calf diarrheal coronavirus (Table 3).[1]

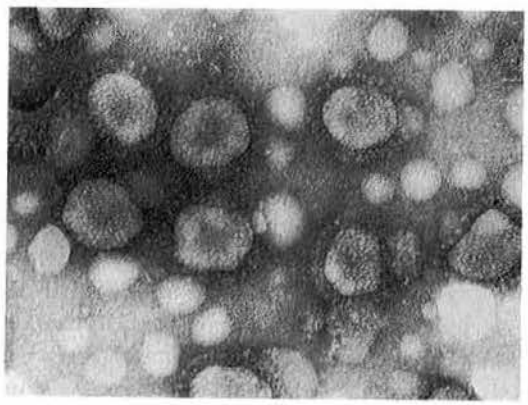

Plate 4. Particles of the Kakegawa isolate negatively stained with phosphotungstic acid
Widely spaced petal-shaped peplomers are observed ×79,000

Properties of the virus

The Kakegawa strain isolated from a cow showing epizootic diarrhea was grown in BEK–1 cells and examined for biochemical and biophysical properties. The Kakegawa strain was able to be replicated in the presence or absence of 5-iodo-2′-deoxyuridine, indicating that its viral nucleic acid was RNA.[1] It was highly sensitive to ether and chloroform, and moderately sensitive to trypsin and heat.[1] It was, however, readily stabilized by treatment with cation at 50°C for 1 h.[1] Its infectivity was slightly reduced at pH 3.0.[1] The virus passed through a membrane filter of 200 nm pore size, but not through that of 100 nm pore size.[1] 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 (Fig. 1)[1] which is in agreement with the previous results obtained with the American strain of calf coronavirus[15,18] and some other coronaviruses.[13]

The Kakegawa strain was easily propagated

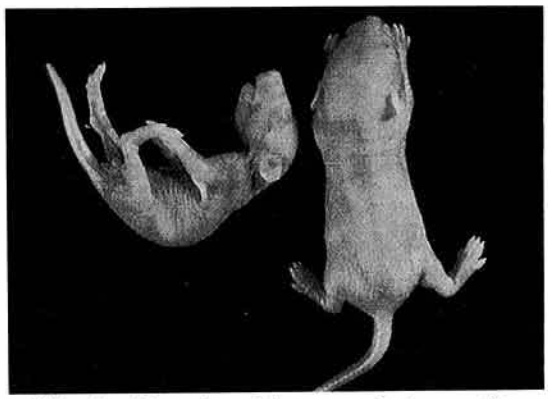

Plate 5. Four-day-old mouse 3 days after intracerebral inoculation with the 3rd mouse brain passaged Kakegawa strain (left) and an uninoculated 4-day-old mouse (right)

in suckling mice. Infected animals died with nervous symptoms, and serial passage was readily accomplished by intracerebral inoculation with brain emulsions (Plate 5).[2] The 3rd passage viral material from infected mice evoked the same disease in suckling mice, rats and hamsters inoculated by the intracerebral or by the subcutaneous route. Viruses recovered from mice, rats and hamsters could be clearly differentiated from mouse hepatitis virus by the neutralization test (Table 4).[2]

Table 4. Neutralization of the viruses recovered from the brains of affected animals with the anti-Kakegawa strain of bovine coronavirus and the anti-mouse hepatitis virus (MHV) strain 2 rabbit immune sera

Virus	Neutralizing antibody titer	
	Anti-Kakegawa strain	Anti-MHV strain 2
Mouse i. c.[a]	2048	16
Mouse s. c.[a]	1024	16
Rat i. c.	1024	16
Hamster i. c.	1024	16

[a] Viruses recovered from the brains of affected mice by the intracerebral (i.c.) or the subcutaneous (s.c.) inoculation with the 3rd passage viral matrials in suckling mice.

Discussion and conclusion

Coronavirus has been known to be associated with diarrhea in calves in the United States,[19] Great Britain and Denmark.[3] Horner et al.,[9] and Durham et al.[6] and Espinasse et al.[7] reported the observations of the virus in feces of cows with diarrhea in New Zealand and France. However, the etiological role of coronavirus in outbreaks of diarrhea among adult cattle remained unknown.

In the present report a coronavirus was isolated from the feces of a cow with diarrhea and this virus was found to be morphologically, physico-chemically and antigenically similar to the coronavirus isolated from a neonatal calf with diarrhea in the United States.

A serological evidence for the etiological significance of bovine coronavirus was provided by examination of animals which were clinically diagnosed as having the disease. A majority of animals showed significant rises in antibody titers to bovine coronavirus, whereas only a small minority showed serological evidences of recent infection with bovine rotavirus, bovine adenovirus type 7, parainfluenza virus type 3, or bovine viral diarrhea-mucosal disease virus. These serological data, together with the facts that numerous coronavirus-like particles were detected in diarrheal fecal samples from affected cows and that a coronavirus was isolated from the diarrheal fecal sample, strongly suggest that bovine coronavirus is the etiological agent of the epizootic diarrhea of adult cattle, although further investigations are needed to prove the mode of etiological role of the virus.

On the other hand, there was an antigenic similarity in the neutralization and hemagglutination-inhibition tests between the Kakegawa strain obtained from a cow and the American strain of calf diarrhea coronavirus. This suggests that bovine coronavirus may be a single serotype which is capable of causing diarrhea in a wide age-range of cattle, regardless of the age of the animal.

Similar outbreaks which are now presumed to have been caused by bovine coronavirus

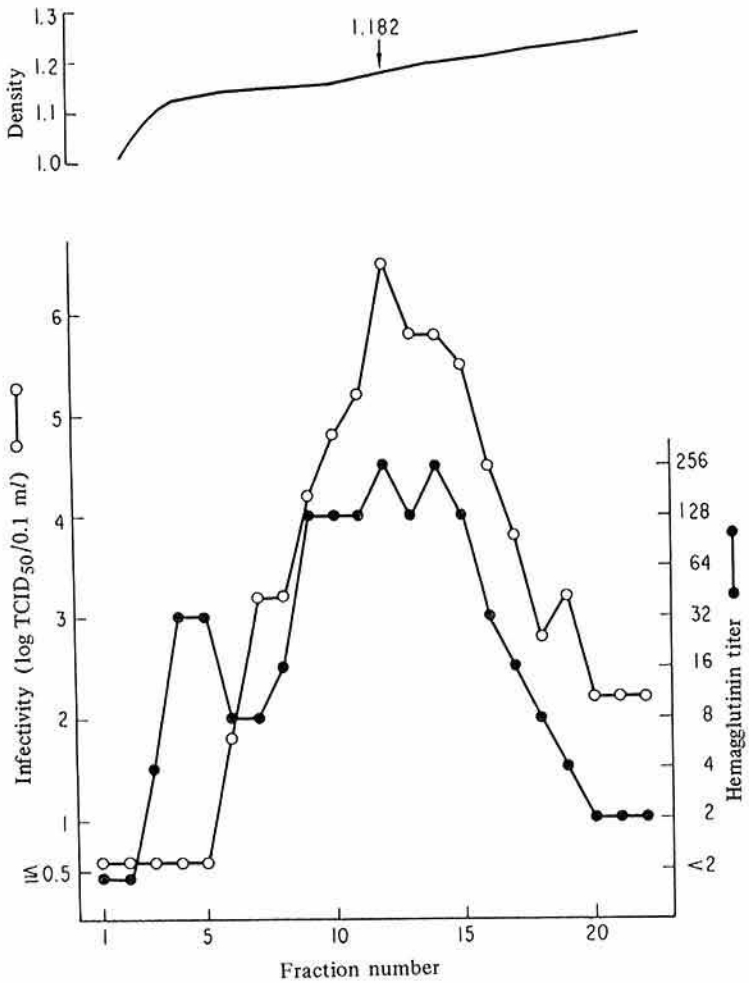

Fig. 1. Sucrose linear density gradient centrifugation of Kakegawa isolate

were recorded in 1951–52, 1956–57, 1963–64 and 1967–68, in Japan (Inaba et al., unpublished data). Those outbreaks resembled the 1976–77 outbreak in epidemiological and clinical features, suggesting the same etiology as that of the 1976–77 outbreak.

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