

Short communication. First report in Cuba of bovine coronavirus detection in a winter dysentery outbreak

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Abstract

Bovine coronavirus (BCoV) infection causes epidemics of acute diarrhea in calves and winter dysentery (WD) in adult cattle. The disease in adult cattle can cause a decrease in milk production resulting in serious economic losses. In Cuba, BCoV infections have not been previously reported. During 2004, many outbreaks of enteric disease have occurred in adult cattle from thirteen dairy farms in the central and western part of the island. The clinical features of the outbreaks resembled those of WD, such as decrease in milk production and, 24 hours later, diarrhea, sometimes bloody, that lasted until the animals recovered in about 7-15 days. Laboratory confirmation of BCoV infection was provided by hemagglutination test (HAT) in 24 samples from four dairy farms from three provinces; RT-PCR assays confirmed the presence of BCoV in three of these samples. Cell culture isolation in secondary calf kidney cells was obtained from four pools of fecal diarrheic samples from each dairy farm. It was remarkable that the disease was also observed during summer. Studies of molecular characterization of the viral strain are in progress.

Additional key words: BCoV infection, diagnosis, diarrhea, hemagglutination test, RT-PCR assays.

Resumen

Nota corta. Primer reporte en Cuba de presencia de coronavirus bovino en un brote de disentería invernal

Las infecciones por coronavirus bovino (BCoV) causan epidemias de diarrea aguda en terneros y disentería invernal (WD) en el ganado bovino adulto. En este último, el descenso de la producción de leche da lugar a graves pérdidas económicas. En Cuba, donde no se habían descrito previamente infecciones por BCoV, se presentaron durante el año 2004 brotes de enfermedad entérica en ganado adulto en trece vaquerías del oeste de la isla. Las características clínicas eran las de WD: se iniciaba con un descenso en la producción de leche, 24 horas después aparecían diarreas, sanguinolentas en la mayoría de los casos, que duraban entre 7 y 15 días, y una final recuperación. Se confirmó la presencia de BCoV mediante la prueba de hemaglutinación (HA) en 24 muestras de heces de cuatro vaquerías ubicadas en las tres provincias afectadas, así como mediante ensayos de RT-PCR en tres de las muestras positivas por HA. El virus fue aislado en cultivos secundarios de riñón de ternero de cuatro mezclas de heces de cada vaquería. Es de destacar que la enfermedad ocurrió también en el verano. Se están realizando estudios de caracterización molecular del virus.

Palabras clave adicionales: diagnóstico, diarrea, ensayos de RT-PCR, infección por BCoV.

Bovine coronavirus (BCoV) infection causes epidemics of acute diarrhea in calves and adult cattle (Saif, 2002). In the latter, the disease is called winter dysentery (WD), because the highest incidence occurs during the winter months, with high morbidity (50- 100%), low mortality (1-2%) and a severe decrease in milk

production that can result in serious economic losses (Mc Arthur, 1997). WD has been reported in Europe, Japan, Canada, the United States and more recently in Brazil (Brandão *et al.*, 2002). To prevent the disease, vaccines have been developed, but their efficacy is questionable (Takamura *et al.*, 2002).

In Cuba, BCoV had not been reported previously, although surveys had been carried out to clarify the etiology of calf diarrhea (Frías, 1983). During 2004,

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many outbreaks of enteric disease occurred in adult cattle from nine dairy farms from the province of Matanzas and four dairy farms from the province of Havana (both in the western part of the island); the disease was disseminated to another 19 dairy farms of the central and eastern provinces. The clinical features of the outbreaks resembled those of WD: the dairy cows started with a dramatic decrease in milk production and, 24 hours later, diarrhea that could be bloody and lasted until the animals recovered in about 7-15 days. Although BCoV causes acute diarrhea in calves (Cho *et al.*, 2001), calves did not seem to be involved in the first outbreaks in Cuba although they became ill when they stayed with their dams.

The objective of this work was to confirm BCoV infection in Cuban cows by hemagglutination test (HAT), RT-PCR and cell culture isolation.

Twenty-eight fecal samples were collected from adult cows with clinical signs of WD in four dairy farms epizootiologically connected by the vehicles used to collect the milk: three from the province of Havana (a farm called *Sureste Típico Habana* located in the Southeast of the province, another at Guayabal and a third one from the *Centro Nacional de Sanidad Agropecuaria*, CENSA, located in San José de Las Lajas) at the beginning of the scouring outbreaks (November 2003-February 2004) and the fourth from a dairy farm of Cienfuegos province, situated in the central part of the island, infected later (August, 2004) (Table 1). Ten additional samples taken from healthy cows in the dairy farm of CENSA before the beginning of the outbreak were used as controls. Fecal suspensions were prepared according to Brandão *et al.* (2002). Briefly,

Table 1. Reciprocal of hemagglutination titer of the feces of animals with clinical signs of winter dysentery in seven samples from one and three dairy farms, respectively, from the Cienfuegos and Havana provinces (these localised at Guayabal, Southeast Havana and CENSA, San José de Las Lajas)

Sample No.	Herd			
	Cienfuegos	Guayabal	Southeast Havana	CENSA
1	16	128	16	32
2	128	128	16	32
3	32	128	32	128
4	32	128	8	16
5	8	16	16	16
6	68	16	8	32
7	8	16	128	64

fecal samples were diluted in 0.01M PBS, 0.1% BSA pH 7.2 (PBS-BSA) to a 1:4 final dilution, centrifuged (12,000g, 30 min, 4°C) and the supernatant was used to detect BCoV by HAT in 96-well U-bottom plates with 25 µl of serial two-fold dilution of the samples and 0.4% hamster red cells in PBS-BSA to each well. After 2 h at room temperature, end point titers were read. The samples with HAT titers > 4 were tested in hemagglutination inhibition test (HI) with 25 µl of serial two fold dilution of samples and kaolin-treated anti-BCoV convalescent bovine serum with 8 HI units to each dilution. The serum was collected from a cow which had recovered from the infection, with blood extraction 21 days after the recovery. The serum was treated with kaolin (Gibco) to remove non-specific inhibitors of hemagglutination frequently found in animal sera, and reacted specifically with the reference strain. After one hour at 37°C, 25 µl of 0.4% HRC in PBS-BSA were added and the plates incubated for 2 h at room temperature. End point titers were read. Samples were considered positive if at least a 4-fold fall in titer was observed.

The convalescent bovine anti-BCoV serum was selected from 10 recovered cows and tested by HI using as antigen the suspension of a virulent BCoV Kakegawa strain (from the Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil) following the protocol described above.

The material for virus isolation corresponded to diarrheic and bloody stool samples from the same four farms, diluted with tissue culture medium Dulbecco MEM (DMEM, Sigma) and antibiotics (300 UI ml⁻¹ penicillin; 300 µg ml⁻¹ streptomycin sulphate; 7.5 µg ml⁻¹ Fungizone (Sigma) to produce a 10% suspension. Suspensions were clarified and filtered following the standard method for transmissible gastroenteritis virus (OIE, 2000). The samples were inoculated in secondary newborn calf kidney cell culture monolayers in 25 cm² culture flasks, previously washed with 0.01 M PBS and treated with DMEM plus 10 µg ml⁻¹ trypsin and 20 mM Hepes (Sigma) (Paton *et al.*, 1997). After incubation at 37°C for 2 h, the cell sheets were washed twice and overlaid with DMEM.

Twenty-four out of 28 samples from sick animals were positive to HAT with a HA titer between 16-128 (Table 1). The hemagglutination was specifically inhibited by the antiserum. No hemagglutination was observed in samples from the healthy animals.

The diagnosis was confirmed in three fecal samples by a RT-PCR assay carried out with primers and conditions as described by Tsunemitsu *et al.* (1999) to amplify

a 407-bp fragment of BCoV nucleoprotein gene (positions 92 to 498 of *N* gene Mebus strain). Briefly, total RNA was extracted from fecal suspensions by Tripure Isolation Reagent (Boehringer Mannheim) and c-DNA synthesis was carried out in a 20 μ l reaction with 5 μ l of RNA, 50 pmol of anti sense primer, 10 mM dNTPs (Promega), 1 μ l of RNAsin (Promega) and 12.5 units of AMV reverse transcriptase (Promega) in 1X RT buffer. PCR reactions were performed in a 50 μ l volume with 5 μ l of cDNA, 10 mM dNTPs, 50 pmol of sense and anti sense primers, 0.5 μ l (2.5 units) of Taq DNA polymerase (Promega) in a thermocycler MS Research. Then, 12 μ l of PCR product were loaded on a 2% ethidium bromide stained agarose gel for electrophoresis. Figure 1 shows that the expected fragment of 407 bp was observed in the three samples, as well as in the Kakegawa strain; no bands were observed in the negative control.

Bovine coronavirus was isolated from feces of all animals tested from the four places described above. In all cases, the cytopathic effect appeared from 96 hours post-inoculation, and was characterized by syncytial presentation and cell degeneration. The RT-PCR assay was used to identify the isolated virus (manuscript in preparation).

It can be concluded that BCoV was involved in the outbreaks of enteric disease described here, and the

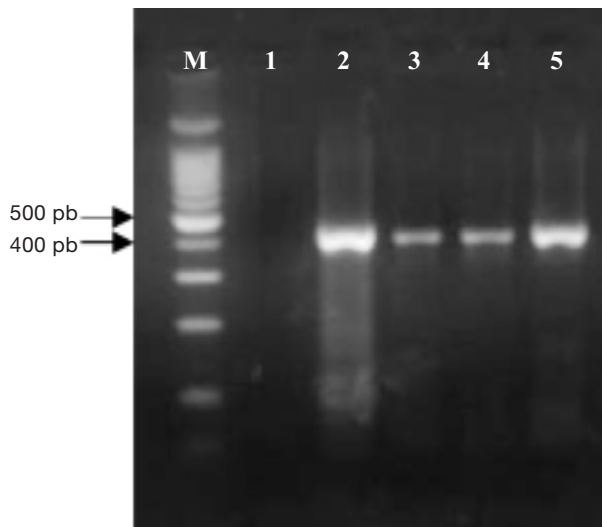


Figure 1. Ethidium bromide-stained 2% agarose gel electrophoresis of the RT-PCR products obtained from RNA extracted from three positive samples from dairy farms of Havana province by viral isolation and hemagglutination test. M: 100-bp marker (Promega), 1: negative control (H_2O), 2: Kakegawa strain, 3: sample VB 73/04 from Guayabal, 4: sample VB 97/04 from CENSA, 5: sample VB 773/03 from Southeast Havana.

clinical signs resembled winter dysentery. Moreover, as the outbreaks occurred in regions with temperatures ranging from 15°C to 32°C in winter and summer, unlike the expected pattern of WD, it can be suggested that BCoV causing dysentery in adult bovines in Cuba may lack the seasonal trend and may occur during the whole year, increasing the impact of the disease in dairy farms. This is in agreement with previous reports of enteric disease caused by BCoV in higher tropical temperatures (Martínez *et al.*, 2002). The large number of cows that were affected in a very short period of time, suggests that the virulence and transmissibility of the virus in Cuba were high. Molecular studies are being conducted in order to characterize the BCoV strains involved in the outbreak and the source of the infection.

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