J Vet Diagn Invest 7:301-304 (1995)

Evaluation of shell vial cell culture technique for the detection of bovine coronavirus

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Abstract. The effect of blind passage and centrifugation on the isolation of bovine coronavirus in human rectal tumor cells cultured in shell vials was investigated. A total of 68 fecal samples known to be positive for bovine coronavirus by transmission electron microscopic (TEM) examination were used. The samples were centrifuged onto human rectal tumor cell monolayers and incubated in the presence of trypsin. The growth of bovine coronavirus in infected cells was demonstrated by fluorescent antibody staining, and the extracellular virus was detected and confirmed by hemagglutination and hemagglutination-inhibition tests, respectively. Of the 68 TEM-positive samples, 51 (75%), 58 (85%), and 61 (90%) grew in shell vial cell cultures at first, second, and third passages, respectively. Of the 51 cultures positive on first passage, 19 were examined by TEM; 18 of these were positive for bovine coronavirus. The shell vial technique was also compared with direct detection of bovine coronavirus by staining cryostat sections of infected tissues in a direct fluorescent antibody assay. The results of direct fluorescent antibody assay were available for 54 of the 68 samples, of which 53 (98%) and 43 (80%) were positive by shell vial technique and direct fluorescent antibody assay, respectively. For identification of bovine coronavirus, shell vials using human rectal tumor cells in the presence of trypsin is more sensitive than direct fluorescent antibody assay but is relatively less sensitive than transmission electron microscopy.

Bovine coronavirus (BCV) is 1 of the agents[23] that causes diarrhea in calves of up to 4 weeks of age[13] and has frequently been detected in the feces of adult cattle with winter dysentery.[1,20,21,23] Neonatal diarrhea caused by BCV is responsible for heavy economic losses in both dairy and beef cattle.[10] In addition, BCV can cause respiratory tract infection in calves between 2 and 16 weeks of age.[9,12,16,19,22]

At present, transmission electron microscopic (TEM) examination of negatively stained preparations of intestinal contents and direct fluorescent antibody (DFA) test on cryostat sections of intestinal tissue[13] are the 2 most widely used diagnostic tests for the detection of BCV in diarrheic animals. However, because of the requirement for skilled technicians and sophisticated and costly equipment, most laboratories are not equipped with TEM facilities, especially in developing countries. Furthermore, TEM is not always reliable because more than 10^6 viruses per gram of specimen are needed for the detection of viruses by TEM.[4] The DFA test is simple and rapid but can only be done on carcasses and not on living animals.

Virus isolation in cell cultures is considered to be a standard method for the detection of most viruses but is rarely used for the detection of BCV because the virus grows poorly in cell cultures. At present, no cell culture system is available in which BCV can grow to a significantly high titer during primary virus isolation. Human rectal adenocarcinoma (HRT-18) cells[27] are highly susceptible to BCV,[5,14,29] especially when trypsin is added to the culture medium.[5,6]

Centrifugation-enhanced shell vial technique (SVT) followed by fluorescent antibody test (FAT) on infected cell cultures is rapid and sensitive for the detection of certain human[2,17,15] and animal[18,25] viruses. The purpose of this study was to evaluate SVT for the detection of BCV from clinical specimens using the HRT-18 cell line and to compare the results with those of direct detection of virus by TEM. We also compared the sensitivity of SVT with that of DFA test on frozen tissue sections for the diagnosis of BCV infection.

Materials and methods

Cells and medium. The HRT-18 cells were grown in RPMI 1640 media with L-glutamine, sodium bicarbonate, and 25 mM HEPESa supplemented with 15% fetal bovine serum (FBS).[b] The inoculated monolayers were maintained in Dulbecco's modified Eagle's medium[a] containing 5 µg/ml trypsin. Shell vials containing 12-mm round coverslips were seeded with 1.5 x 10^5 cells suspended in 1 ml of growth medium and incubated at 37 C in a humidified atmosphere containing 5% CO_2. Cultures were confluent within 2-3 days.

Specimens. A total of 68 specimens of intestinal contents or fecal material from calves with gastroenteritis were included in this study. TEM examination of all of these specimens revealed BCV or BCV-like particles but no other virus.

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Received for publication August 5, 1994.

A 10% suspension of the samples was prepared in Hanks' balanced salt solution (HBSS) using a homogenizer.[c] The homogenates were clarified by low-speed centrifugation at 2,200 x gat 4 C for 1 hr, and the supernatants were sterilized by passage through 0.45-µm membrane filters. To avoid virus loss from adsorption of virus to membrane filters, the filters were coated by passage of 0.5 ml of HBSS containing 10% FBS. The filtrates were stored at -70 C until inoculation.

Infection of monolayers in shell vials. Upon confluency of the monolayer, the growth medium was removed and the specimens were inoculated into duplicate shell vials (0.3 ml/vial). The inocula were adsorbed onto the monolayers by centrifugation of vials at 700 x g for 60 min at 30 C. Inocula were then removed, monolayers were washed twice with HBSS, and 1 ml of maintenance medium was added to each vial. Vials were then inoculated at 37 C in 5% CO^2 for 3 days. The cultures were monitored daily for the appearance of cytopathic effects (cpe). Each specimen was blind passaged 3 times. All inoculated cultures, irrespective of the appearance of cpe, were examined by FAT and hemagglutination (HA) and hemagglutination-inhibition (HI) tests. In addition, 19 inoculated cultures from first cell culture passage were examined by TEM.

Fluorescent antibody test on infected monolayers. Immunofluorescent staining of infected monolayers was carried out as described previously.[11,25] The culture medium was removed, and the monolayers were washed twice with phosphate-buffered saline (PBS, pH 7.2) and fixed in cold (-20 C) acetone for 10 min. The acetone was then removed, and the monolayers were washed again. After air drying, the monolayers were covered with a working dilution (1:40) of fluorescein-conjugated anti-BCV IgG.[d] After incubation at 37 C for 30 min, the conjugate was removed, and the monolayer was washed in PBS (pH 8.5), counterstained with 0.05% Evan's blue for 1 min, and then rinsed in distilled water. Coverslips from the vials were then removed with a needle and forceps and were allowed to air dry before they were mounted on glass slides with the cell side down. The slides were then examined with a fluorescence microscope for the presence of cytoplasmic fluorescence.

Hemagglutination test. Culture fluids from infected cell cultures were examined by the HA test. Serial 2-fold dilutions of culture fluids (0.05 ml) in PBS (pH 7.2) were mixed with 0.05 ml of a 1% (v/v) suspension of washed rat red blood cells (RRBC) in PBS containing 0.2% bovine serum albumin. The plates were incubated at 4 C for 90 min, and the HA titer was calculated as the highest dilution showing complete hemagglutination.

Hemagglutination-inhibition test. The HI test was performed using anti-BCV antiserum to confirm the identity of the hemagglutinating virus in culture fluids. Serial 2-fold dilutions, starting with 1:10 dilution of antiserum, were prepared and mixed with equal volume (0.05 ml) of culture fluids, which were adjusted to have 8 HA units. The mixtures were incubated at 37 C for 1 hr, followed by the addition of 0.05 ml of a 1% suspension of RRBC to each mixture, and the plates were incubated at 4 C for 90 min. The presence of the virus was confirmed when hemagglutination was inhibited.

Transmission electron microscopy. Fecal samples were processed for TEM examination as described previously[8] for the detection of BCV from naturally infected animals. In addition, 19 fluid samples from shell vial cultures were also examined by TEM to confirm the presence of BCV. The culture fluids were clarified by centrifugation at 2,500 x g for 30 min. The supematants were then centrifuged at 30,000 x g for 120 min, and the pellet was resuspended in water. After staining with phosphotungstate (pH 6.4), the suspension was nebulized on a 200-mesh collodion-coated grid and examined using a TEM at a magnification of 180,000 x .

Direct fluorescent antibody test on cryostat sections of tissues. Direct detection of BCV in cryostat sections of small intestine and colon was performed as described previously.[17] Flash frozen tissue samples were mounted on a specimen disk and sectioned at 4-6 µm with a cryostat (-18 to -25 C). The tissue sections were placed on glass slides, air dried, and fixed in acetone. After quick rinses in distilled water and PBS, the slides were dried, and fluorescein-conjugated anti-BCV IgG was applied. After incubation at 37 C for 30 min, the conjugate was washed off with PBS (pH 8.5) and the slides were counterstained with Evan's blue. The cover slips were then applied, and slides were examined under a fluorescence microscope.

Results

Sixty-eight fecal specimens known to be positive for coronavirus by TEM examination were included in this study. All of these specimens were inoculated in HRT-18 cells by the shell vial technique for a total of 3 passages. A tissue-culture-adapted Nebraska strain of BCV was used as a positive control. Cytopathic virus was detected in 36, 59, and 61 specimens at first, second, and third passages, respectively (Table 1). The cpe were characterized by rounding and detachment of cells.

Although only 36 samples produced cpe on first passage, culture fluids from 51 samples were positive by FAT (Table 1). Also, culture fluids from 54 infected cultures agglutinated RRBCs, and fluids from 52 of these cultures were inhibited by anti-BCV antibody. On examination of 19 culture fluids by TEM, 18 showed the presence of coronavirus. Uninfected cultures did not show cpe and were negative by FAT and the HA test.

At second passage, 59 specimens were positive for cpe and HA and 58 were positive by FAT. On third passage, 61 samples showed cpe and all of them were positive by FAT and the HA and HI tests. No TEM examination was done on second- and third-passage culture fluids. Specimens negative by SVT were tested by indirect fluorescent antibody test for the presence of morphologically similar bredavirus but were negative (data not shown).

The HA titer of culture fluids ranged from 1:2 to 1:256. Of the 54 samples showing positive HA at first passage, 27 had titers of ≥ 1:64 and 25 had titers be-

Table 1. Effect of passage on the detection of bovine coronavirus in shell vial cell cultures.*

Passage no.	No. samples showing cpe†		No. samples confirmed positive‡			
	2 dpi	3 dpi	FAT	HA	HI	TEM
1	7	29	51	54	52	18§
2	24	35	58	59	ND	ND
3	33	28	61	61	61	ND

* Sixty-eight samples (intestinal contents or feces) showing bovine coronavirus (BCV) and BCV-like particles by electron microscopy were used in this study.

† cpe = cytopathic effect; dpi = days postinfection.

‡ FAT = fluorescent antibody test; HA = hemagglutination test; HI = hemagglutination-inhibition test; TEM = transmission electron microscopy. ND = not done.

§ Of the 19 culture fluids tested by TEM, 18 were positive for coronavirus.

tween 18 and 1:32 (data not shown). The number of culture fluids showing HA titers of ≥ 1:64 increased to 35 at second passage, whereas titers of the remaining 23 samples ranged between 1:8 and 1:32. No significant increase was observed in the HA titer at third passage; however, infected cell cultures developed earlier cpe and showed a marked increase in the number of infected cells by FAT. The HA titer of culture fluids from cultures inoculated with the tissue-culture-adapted Nebraska strain of BCV remained within the range of 1:64 to 1:256 at all three passages.

Cryostat sections of small intestines from 54 of the 68 animals from which these samples were obtained were examined by the DFA test. Of these 54 specimens, 42 (78%) were positive for BCV by both the SVT and DFA test, 11 (20%) were positive only by the SVT, and 1 specimen was positive only by the DFA test (Table 2). Overall, 53 (98%) specimens were detected by SVT as compared with 43 (80%) detected by the DFA test.

Table 2. Comparison between direct fluorescent antibody test (DFAT) and shell vial technique (SVT) for the detection of bovine coronavirus (BCV) in 54 positive specimens.

DFAT*	SVT	No. specimens positive for BCV
+	+	42 (78%)
−	+	11 (20%)
+	−	1 (2%)

* Direct fluorescent antibody staining on cryostat sections of intestinal tissue.

Discussion

A simple and reliable technique is needed for the detection of BCV in clinical specimens. Currently, TEM examination of intestinal contents and DFA staining of cryostat sections of intestinal tissue are the most widely used techniques for the diagnosis of BCV infection.[17] Although DFA staining of intestinal tissue is a rapid and simple technique as compared with virus isolation and TEM,[19] DFA staining of fecal or rectal smears is not as reliable because of the presence of fewer cells in fecal smears and because of nonspecific fluorescence.

Isolation of BCV in cell culture systems is not routinely used because the virus is difficult to grow in cell cultures, especially on primary isolation. The shell vial technique is sensitive for the diagnosis of several human viruses and is used routinely in many human diagnostic laboratories. In the present study, SVT detected as many as 61 of 68 (90%) TEM-positive samples by third cell culture passage. The 7 samples positive by TEM and negative by SVT did not react with bredavirus antiserum. If the samples did contain true BCV particles, they did not grow in shell vials because they were nonviable or because they were associated with immune complexes.

Of the 61 specimens in which BCV was detected by SVT, 83%, 95%, and 100% were detected at first, second, and third passages, respectively. If TEM results are considered 100% accurate, SVT detected virus in 75%, 85%, and 90% of the specimens at first, second, and third passages, respectively. A high rate of virus replication in shell vials in this study may have been due to the activation of viral infectivity by exogenous trypsin,[24] enhanced uptake of the virus particles by the cells as a result of the centrifugal field, or enhanced cell fusion activity as a result of both of these factors. Enhanced and earlier cpe, characterized by rounding of cells, appeared after each passage. Over time, these cells fused with neighboring cells, resulting in the formation of holes in the monolayers. Later, these cells detached and floated in the medium. Our observations are similar to those of other workers,[26,28] who have reported an increase in the number of infected cells following blind cell culture passages.

The FAT was an effective and reliable method for detecting BCV in HRT-18 cells used in the SVT. Fluorescence in infected cells was observed within 3 days postinfection in this study; in earlier studies fluorescent cells were seen 3-7 days after infection of primary calf kidney and BEK-1 cells with the British[3] and the Nebraska[11] strains of BCV, respectively. The usefulness of HA and HI tests for confirming the presence of BCV was also shown in this study.

In a comparison of the SVT and the DFA test results, SVT detected 98% of the positive specimens and the DFA test detected 80%, indicating that virus isolation in shell vials was more sensitive than direct detection of BCV by the DFA test but was relatively less sensitive than TEM. Electron microscopy, therefore, remains an effective method for BCV diagnosis. An advantage of

TEM is that it can detect not only BCV but also any other virus present in the sample. However, TEM is limited in that a relatively small number of samples can be examined in a given day.

Sources and manufacturers

a. Celox, Hopkins, MN.
b. Hyclone, Logan, UT.
c. Tekmar, London, England.
d. National Veterinary Services Laboratory, Ames, IA.

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