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BRIEF COMMUNICATIONS

Factors affecting isolation and propagation of bovine coronavirus in human rectal tumor-18 cell line

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Bovine coronavirus (BCV) is an important cause of calf enterocolitis[4] and respiratory disease.[12] It is the second major cause of viral diarrhea in calves, with rotavirus being the first.[5] At the Wisconsin Animal Health Laboratory during 1993-1994, BCV was detected in 93 cases of calf scours out of 1,058 bovine fecal samples examined by direct electron microscopy. Electron microscopy is used commonly for the diagnosis of enteric viruses, including BCV.[11] The advantages of electron microscopy are that diagnosis can be made rapidly and multiple pathogens, a common feature in enteritis, can be detected simultaneously. Using electron microscopy, more than a dozen novel enteric viruses have been described in the last 2 decades. However, electron microscopy has some limitations; approximately 1 million viral particles should be present to detect a virus by electron microscopy. Thus, it lacks sensitivity[8] and can lead to false-negative results. In addition, some viruses, especially coronaviruses, can be confused morphologically with nonviral particles such as intestinal brush border epithelium and with other morphologically similar viruses, leading to false-positive results.[1]

Virus isolation is not commonly used for the diagnosis of BCV.[4] However, 1 advantage is that the virus propagated in cell culture can be used for further antigenic and genomic characterization. To improve BCV isolation from clinical samples, factors affecting its isolation and propagation in human rectal tumor- 18 (HRT- 18) cell line were investigated. In a previous study,[10] HRT-18 was found to be a suitable cell line for BCV isolation. In this study, BCV propagated in vitro showed a change in hemagglutination pattern from that of the BCV from the original clinical samples. It is not known if this change correlates with changes in antigenicity and immunogenicity of the virus.

HRT-18[a] and human colon tumor-8 (HCT-8) cells are derived from adenocarcinomas of human rectum and colon, respectively.[16] The 51 samples included in this study were provided by the Wisconsin Animal Health Laboratory (WAHL), Madison (n = 27), the California Veterinary Diagnostic Laboratory (CVDL), San Bernardino (n = 6), and another diagnostic laboratory (referred to as VDL for confidentiality) (n = 18). Samples were obtained as 20% fecal suspensions in phosphate-buffered saline (PBS) (pH 7.2), as ultracentrifuged pellets (approximately 100,000 x g), or as pieces of spiral colon. All samples were positive only for BCV by either direct electron microscopic examination or fluorescent antibody test.

For trial 1, fecal suspensions were treated with dihydrostreptomycin (1 mg/ml), penicillin (300 units/ml), and fungizone (7.5 µg/ml) for 30 minutes at 37 C. Samples were then inoculated onto HRT- 18 cells grown in 12-well plates.[b] For trial 2, approximately 1 ml of fecal suspension was clarified by centrifugation at 1,000 x g for 10 minutes, diluted 1:10 in minimum essential medium (MEM), and passed through a 0.45-µm filter.[c] Before the samples were filtered, the membranes were wetted with MEM to avoid loss of the virus through viral absorption and adsorption.

Before adsorption of the virus, HRT-18 monolayers were washed once with calcium- and magnesium-free PBS (CMF-PBS) containing trypsin (5 µg/ml). After the wash was discarded, the virus was adsorbed for 1 hour at 37 C. Samples were rocked twice during adsorption and then MEM with L-glutamine, gentamicin, penicillin, dihydrostreptomycin, fungizone, trypsin (5 µg/ml), and pancreatin (5 µg/ml) was added. Flasks were observed daily for 3-5 days. When cytopathic effects, such as rounding and syncytia formation, became evident, the cells were harvested and then frozen and thawed 3 times.

To study the effect of centrifugation of samples on the isolation of BCV, HRT- 18 cells were plated in 12-well tissue culture dishes.[d] After the monolayers became confluent, the wells were washed with CMF-PBS containing trypsin (5 µg/ml). Approximately 300 µl of sample dilution (1: 10) was added, and the plates were centrifuged at 2,000 x g for 1 hour at 20 C (centrifuged group). After centrifugation, MEM containing trypsin (5 µg/ml) and pancreatin (5 µl/ml) was added. To study the effect of centrifugation of samples on BCV isolation, an identical set of plates was inoculated with the samples but not centrifuged (stationary group). After incubation of the plates for 3-5 days, the cells were examined by a direct fluorescent antibody test for BCV antigen.

To study the effect of rolling, HRT- 18 cells were grown in 16- x 125-mm tissue culture tubes.[b] Each sample was inoculated in an identical manner into 2 tubes. One set of tubes was kept stationary, and the other set was rotated at 2-3 rpm. After 6 days, presence of BCV antigen was detected by a fluorescent antibody test.

The hemagglutination test was performed in V-bottom plates.[e] Two-fold dilutions of samples (25 µl) were prepared in PBS (0.01 M) containing bovine serum albumin (0.1%).

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Table 1. Effect on isolation of bovine coronavirus of centrifugation of samples onto HRT-18 cell culture.

Sample no.*	Hemagglutination†		Virus isolation‡	
	Stationary	Centrifuged	Stationary	Centrifuged
WI-1, SK original	32	<2	+ve	+ve
WI-1, TC-1	128	1,024	NA	NA
WI-2, TC-1, TC-2	<2	<2	FR	FR
WI-4, TC-1, TC-2	<2	<2	−ve	−ve
WI-9, original	16	64	+ve	+ve
WI-9, TC-1, TC-2	16	16	NA	NA
WI-10, original	<2	<2	F	F
WI-10, TC-1, TC-2	<2	<2	F	F
WI-11, original	<2	<2	F	F
WI-11, TC-1, TC-2	<2	<2	F	F
WI-17, TC-1, TC-2	<2	<2	F	F
WI-18, original	16	<2	+ve	−ve
WI-18, TC-1	512	<2	NA	NA
WI-18, TC-2	32	<2	NA	NA

* Original = first inoculation of a clinical sample on HRT-18 cell line; TC-1, TC-2 = tissue culture passage no.

† Hemagglutination titer per 25 μl of culture supernatant with mouse erythrocytes.

‡ +ve = virus isolated after preparation of sample by antibiotic treatment; −ve = virus could not be isolated either by antibiotic treatment or filtration of the samples; NA = not applicable; F = isolated after filtration of the sample; FR = isolated only after filtering of the sample and rolling the cell culture tubes.

After addition of 25 μl of PBS-hemagglutination buffer and mouse (1%) or chicken (0.5%) erythrocytes, the hemagglutination plates were incubated at 4 C for 1 hour. Hemagglutination titers were expressed as the reciprocal of the highest dilutions of the samples showing complete hemagglutination.

To check the presence of BCV antigen, HRT-18 cells were scraped, washed once with PBS (pH 7.2), spotted on clean glass slides, and air dried at room temperature. After fixation with cold acetone for 20 minutes, the anti-BCV fluorescein isothiocyanate (FITC)-labeled conjugate[f] with Evan's blue as a counterstain was added onto the cells. After incubation at 37 C for 30 minutes, the unbound FITC conjugate was washed off. Characteristic apple-green, granular cytoplasmic fluorescence was observed in BCV-positive cells, and negative cells were brick red.

In trial 1, samples were treated with antibiotics and fungizone. Of the 18 samples (WI-1-18), many inoculated wells were lost due to bacterial contamination or cytotoxicity. Only 3 BCV isolates (WI-1.SK, WI-9, WI-18) could be propagated (see Table 1). However, in trial 2, the fecal samples (WI-1-27) were filtered before inoculation on HRT-18 cell line. Of the 27 samples (fecal suspensions) testing positive for BCV by direct electron microscopy at the WAHL, BCV was isolated from 9 in HRT-18, for an isolation rate of 33%. Of the 6 samples (spiral colon pieces about 3 cm in length) testing positive by a direct fluorescent antibody test at the CVDL, BCV was isolated from all 6 in HRT-18 cell culture. Of the 18 samples (fecal samples) testing positive by direct electron microscopy at the VDL, BCV could not be isolated.

To further evaluate the effect of centrifugation of samples on BCV isolation, we performed a hemagglutination test on culture supernatants from 8 samples. Centrifugation of the samples onto cells did not improve the isolation of BCV in HRT-18 cell line. To evaluate the effect(s) of rolling on the isolation of BCV in HRT- 18, 7 clinical samples were examined; 3 of these samples were positive by both stationary and rolling cell culture and 4 were negative (Table 2). The hemagglutination titers were similar. Thus, on the basis of these results, rolling did not improve isolation of BCV in HRT-18.

Table 2. Effect on isolation of bovine coronavirus of rolling of samples on HRT-18 cell culture.

Sample no.	Hemagglutination*		Virus isolation	
	Stationary	Rolling	Stationary	Rolling
WI-2	ND	ND	+	+
WI-3	<2	<2	−	−
WI-4	<2	<2	−	−
WI-5	<2	ND	−	−
WI-6	<2	<2	−	−
WI-9	≥4,096	≥4,096	+	+
WI-18	≥4,096	≥4,096	+	+

* Hemagglutination titer with mouse erythrocytes. ND = not done.

Initial hemagglutination titers of clinical samples can be taken as a correlate of the amount of BCV in the clinical samples. Samples from which we could successfully isolate

Table 3. Correlation of virus isolation (VI) results with hemagglutination (HA) titers of original clinical samples.

Sample no.	HA titer*		VI
	Mouse rbc (1%)	Chicken rbc (0.5%)	
WI-1.SK	≥256	not done	+
WI-2	2,048	2	+
WI-3	32	8	−
WI-4	<2	4	−
WI-5	32	−ve	−
WI-6	8	32	−
WI-7	256	256	−
WI-8	16	32	−
WI-9	2,048	32	+
WI-10	1,024	2	+
WI-11	≥4,096	256	+
WI-12	64	128	−
WI-13	8	4	−
WI-14	4	2	−
WI-15	1,024	128	−
WI-16	<2	<2	−
WI-17	4,096	64	+
WI-18	256	16	+
WI-19	8	16	−
WI-20	128	128	+
WI-21	8	32	−
WI-22	32	128	−
WI-23	≥4,096	2,048	+
WI-24	2	4	−
WI-25	4,096	32	−
WI-26	16	16	−
WI-27	128	128	−

* After propagation in HRT-18 cells, none of the samples showed any hemagglutination activity with chicken erythrocytes.

BCV had hemagglutination titers of at least 1:128 or more with mouse erythrocytes (Table 3). With chicken erythrocytes, the same samples showed comparatively lower hemagglutination activity. BCV could not be isolated from many samples with nearly equal hemagglutination titers for both mouse and chicken erythrocytes (Table 3). The reason for this isolation failure is not known.

There are only a few reports on the success of various cell lines (Vero, Madin-Darby bovine kidney, porcine kidney-15)[7,9] and organ cultures (intestinal,[3] tracheal[15]) for the isolation of BCV. HRT-18 has been used for isolation of calf coronavirus and winter dysentery coronavirus. However, primary isolation of BCV in cell culture is difficult[4] especially under a routine diagnostic virology setting.

Handling of samples during transportation affected the viability and integrity of the virus particles. All samples from the WAHL were maintained frozen at -70 C, and 9 of 27 samples yielded BCV. Samples (pieces of spiral colon) from the CVDL were mailed to our laboratory on dry ice (- 40 C) and were received frozen. All of these samples were positive for BCV in our laboratory. Fecal samples from the VDL were mailed over cold packs (-0 C) and had a putrid smell after thawing. None of these samples yielded virus in cell cultures, possibly because the virus was degraded during transit. Thus, samples for BCV isolation should be shipped overnight and held on dry ice (-40 C) while in transit.

Bacteria in clinical samples can affect virus isolation. Treatment with antibiotics or antifungal agents was not sufficient to prevent bacterial contamination, and the inoculated cell culture became contaminated during incubation at 37 C. However, none of the samples became contaminated after 0.45 µm filtration, which resulted in a higher success rate of BCV isolation (Table 1).

Proteolytic enzymes (trypsin and/or pancreatin) added to the culture medium enhance the isolation of BCV in HRT-18.[14] Even for subsequent propagation of isolated BCV, the presence of trypsin and pancreatin is essential. Age of the HRT-18 cells after plating had an effect on BCV propagation and formation of cytopathic effect(s). The best time to use HRT-18 cell cultures is 24 hours after the monolayers become confluent; cytopathic changes due to BCV were more distinct at this time.

Effect of physical conditions, such as centrifugation of samples onto cells and rolling, on BCV isolation in HRT-18 cells was studied. On the basis of our experiments (Tables 1, 2), centrifugation of the samples onto the cell culture and rolling did not improve the isolation rate of BCV in HRT-18.

To study the effect of cell culture passage of BCV in HRT-18 on hemagglutination activity of BCV, fecal samples positive for BCV and cell-culture-propagated BCV were tested using mouse and chicken erythrocytes. After propagation in HRT-18 cells, the ability of BCV to agglutinate chicken erythrocytes was abolished (Table 3), which probably indicates that the epitopes that interact with chicken erythrocytes were altered. Also, after 2 or 3 passages in HRT-18, the hemagglutination activity with mouse erythrocytes started decreasing. Presence of trypsin (5 µg/ml) in the culture medium has been reported to abolish the ability of the BCV to lyse mouse erythrocytes.[14] Also, the BCV particles produced in trypsin-treated HRT-18 culture have uniformly shorter surface projections and exclude phosphotungstic acid stain, 2 characteristics not found in BCV particles produced in cell culture medium without trypsin.[14]

In a previous study,[10] several cell lines were compared for primary isolation and propagation of BCV, and HRT-18 was found to be the most permissive. Other cell lines, such as human lung carcinoma (A549), bovine embryonic kidney, bovine turbinate, mink lung, primary calf testicle cell culture, and Madin-Darby bovine kidney, were not suitable.[10] Recently, primary spiral colon cells were not conducive to BCV replication and propagation (S. Kapil and J. Nietfeld, unpublished observations). HCT-8 cells were less suitable than HRT-18 cells.[10] Different clones of HRT-18 cells may differ in their susceptibility and, thus, suitability for isolation of BCV from clinical samples.[6]

All samples that were isolated successfully after 1 passage in HRT-18 cells had a hemagglutination titer of at least 128 units per 25 µl of the 20% fecal suspension. Thus, usefulness of virus isolation for rapid diagnosis on the basis of 1 passage is limited by the lack of sensitivity of the virus isolation system. Although repeated passages improved the isolation rate, they also increase the turnaround time.[10] Combined cell culture propagation and reverse-transcriptase polymerase chain reaction techniques may help to improve the sensitivity and turnaround time for diagnosis. The pH of cell culture media affects the isolation of BCV and treatments with dactinomycin (0.05 µg/ml), DEAE-dextran, and hypertonic medium increase the production of BCV, its cytopathology, or both.[7] Temperature of incubation affected the propagation of BCV. In this study, BCV isolates at passage 4 did not grow at room temperature (approximately 25 C). However, the same BCV isolates were easy to propagate at 37 C. A large number of clinically normal calves excrete BCV in feces. Thus, positive isolation of BCV should be correlated with histopathology and clinical history to make a proper diagnosis. In addition, lack of a positive isolation does not rule out BCV as the cause of diarrhea in an individual calf.

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Evaluation of two antigen-capture ELISAs using polyclonal or monoclonal antibodies for the detection of bovine coronavirus

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Bovine coronavirus (BCV) is recognized as a common causative agent of neonatal calf diarrhea.[21] It has also been incriminated by several researchers from around the world as a causative agent for winter dysentery (WD), an acute diarrhea1 disease of adult cattle.[1,8,17,20,22,25,29-31] Review articles concerning BCV[4] and the immunology of coronaviruses have recently been published. Currently, the diagnosis of BCV infection is usually made by identifying the virus in feces.[4] Because isolation of BCV in cell culture is difficult, this method is rarely employed as a diagnostic test; electron microscopy (EM) is often used for the identification of BCV in feces.[2,10] Although the intact virion of BCV is fairly characteristic in appearance, it is not uncommon for the identifying surface projections of the virus to be lost during sample preparation or storage, making it more difficult to properly identify virus particles by EM. Immunoelectron microscopy (IEM), utilizing specific antibodies against BCV, is used to increase the sensitivity and specificity of EM.[4,10] Enzyme-linked immunosorbent assays (ELISAs) have been described for the detection of BCV antigen in feces, but these assays have lacked sensitivity when compared with similar assays for other enteric pathogens.[6,7,10,15,23] The use of monoclonal antibodies rather than polyclonal antibodies has increased the sensitivity and specificity of BCV ELISAS.[7,23] ELISAs offer an advantage over EM and IEM of being able to rapidly evaluate large numbers of samples. This advantage is important for epidemiologists conducting large surveys and diagnosticians who must examine many submissions.

In this report, we describe the development and evaluation of 2 ELISAs for detection of BCV antigen in feces. In 1, polyclonal antibodies were used for antigen capture (PACELISA); the other was identical except for the use of monoclonal antibodies (MAbs) (MACELISA). We also present new information related to evaluation of the 2 assays for detection of both calf and WD BCV strains and the sensitivity and specificity obtained with the MACELISA.

Nine strains of BCV adapted to cell culture in human rectal tumor (HRT-18) cells as previously described[3] were used to evaluate the ELISAs. Of the 9 strains evaluated, 2 (Mebus, DB2) were isolated from diarrheic calves and 7 (DBA, SD, BE, BM, AW, TS, CN) were isolated from adult cows clinically affected with WD.[3,14,28]

Sixty reference fecal samples were collected from gnotobiotic calves or field cases of neonatal calf scours for which the BCV infection status was determined by EM, IEM, or immunofluorescence. These samples were diluted 1:25 in phosphate-buffered saline (PBS) and centrifuged (850 x g,

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Corrigendum

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In the article "Experimental rayless goldenrod (*Isocoma pluriflora*) toxicosis in goats" by Bryan L. Stegelmeier et al., the published mean body weight and the means and statistics of serum biochemistries were carried out on groups of 4 animals, not 3, as described in the Material and Methods section. The additional animal in each group was part of an auxiliary physiologic study and though the animals were dosed and treated the same, they were not necropsied and were not included in the histologic study. To correct this oversight, the corrected weight and chemistry table (shaded cells indicate corrected numbers) are listed below. The differences are minimal and do not alter the conclusions. In addition, reference 7 has been deleted.

Material and Methods: "Fifteen, yearling, female Spanish goats weighing **29.4 ± 3.4 kg** (mean ± standard deviation) were randomly divided into 5 groups with 3 animals per group."

References: Reference 7 should be deleted

Corrected Table 1. Selected mean serum biochemical data from groups of 3 goats dosed with rayless goldenrod (*Isocoma pluriflora*) to obtain benzofuran ketone doses of 0, 10, 20, 40, and 60 mg/kg body weight for 7 days.*

Serum test (reference range†)	Dose	Serum result (mean ± standard deviation)			
		Day 0	Day 3	Day 6	Day 7
Creatinine kinase (< 350 U/l)	0	226 ± 93	107 ± 6	73 ± 16[a]	66 ± 30[a]
	10	226 ± 160	118 ± 8	206 ± 184[a]	495 ± 623[ab]
	20	967 ± 1233	306 ± 276	240 ± 113[a]	497 ± 277[ab]
	40	125 ± 18	117 ± 24	6,699 ± 5,329[b]	16,270 ± 11,054[b]
	60	202 ± 93	202 ± 124	2,987 ± 3,701[a]	10,433 ± 4,326[ab]
Cardiac troponin-I (<0.40 U/l‡)	0	<0.02 ± 0.0	<0.02 ± 0.0	<0.02 ± 0.0	<0.02 ± 0.0
	10	<0.02 ± 0.0	<0.02 ± 0.0	<0.02 ± 0.0	<0.02 ± 0.0
	20	<0.02 ± 0.0	0.17 ± 0.26	0.05 ± 0.03	<0.02 ± 0.0
	40	<0.02 ± 0.0	<0.02 ± 0.0	1.98 ± 3.39	1.79 ± 2.97
	60	<0.02 ± 0.0	<0.02 ± 0.0	1.38 ± 2.31	0.13 ± 0.18
Aspartate aminotransferase (<125 U/l)	0	96 ± 7	91 ± 6	83 ± 2[a]	72 ± 3[a]
	10	147 ± 69	104 ± 11	89 ± 8[a]	97 ± 13[a]
	20	164 ± 82	284 ± 248	293 ± 252[ab]	376 ± 256[a]
	40	112 ± 17	102 ± 12	991 ± 184[c]	3,277 ± 1,556[b]
	60	96 ± 13	115 ± 31	819 ± 571[bc]	2,095 ± 1,333[b]
Alanine aminotransferase (<55 U/l)	0	39 ± 3	37 ± 3	38 ± 0[a]	43 ± 18[a]
	10	44 ± 1	42 ± 3	39 ± 2[a]	37 ± 1[a]
	20	41 ± 9	57 ± 34	63 ± 38[ab]	61 ± 25[a]
	40	46 ± 2	44 ± 4	134 ± 24[a]	333 ± 127[b]
	60	40 ± 7	44 ± 5	118 ± 84[ab]	267 ± 176[b]
Lactate dehydrogenase (<1,560 U/l)	0	1,061 ± 145	1,075 ± 62	875 ± 213[a]	573 ± 115[a]
	10	1,334 ± 668	1,050 ± 223	942 ± 265[a]	709 ± 182[a]
	20	1,650 ± 1,546	2,617 ± 2,685	1,185 ± 449[a]	753 ± 447[a]
	40	1,054 ± 201	1,162 ± 130	5,996 ± 2,491[b]	9,891 ± 3,210[b]
	60	1,026 ± 287	1,277 ± 348	3,623 ± 2,924[ab]	7,011 ± 5,205[a]

*Different means (<0.05) between groups are indicated with superscript letters.
†Estimates of normal range were determined as 2 standard deviations from mean values of control goats and pretreatment samples. These ranges are probably laboratory and assay specific.
‡Cardiac troponin-I concentrations below detection limits are reported as <0.02 ng/ml.