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Sensitivity comparison for detection of respiratory bovine coronaviruses in nasal samples from feedlot cattle by ELISA and isolation with the G clone of HRT-18 cells

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Abstract. A monoclonal antibody-based capture enzyme-linked immunosorbent assay (ELISA) was developed to detect respiratory bovine coronavirus (RBCV) antigens in nasal swabs collected from cattle showing signs of respiratory tract disease following shipping. These samples had been previously tested for RBCV by inoculation of G clone cultures of human rectal tumor cells (HRT-18G) and for bovine herpes virus 1, parainfluenza virus 3, bovine adenovirus, bovine respiratory syncytial virus, and bovine viral diarrhea virus on other specifically permissive cell cultures. RBCV has not previously been recognized as an important etiological factor in the bovine respiratory disease complex of feedlot cattle. Thirty of 100 samples tested positive for RBCV antigen by capture ELISA in contrast to 38 of 100 samples that yielded RBCV isolates in G clone cells. Samples yielding other bovine respiratory viruses in the absence of RBCV were negative in the capture ELISA, which was based on the use of a single monoclonal antibody that recognizes one RBCV epitope on the S glycoprotein with the broadest reactivity with different strains of RBCV tested. Some RBCV strains may not be detected by this ELISA, which may account for the higher percentage of RBCV-infected cattle detected by RBCV isolation. However, the ELISA was simple to perform, sensitive, and specific and was more rapid than virus isolation. This assay will be useful for processing large numbers of field samples in future epidemiologic and diagnostic studies of RBCV infections of cattle.

Bovine respiratory disease is the single most important syndrome affecting 6–8-month old beef cattle after entry into feedlots in North America.[7] Bacteria, mycoplasma, and viruses such as bovine herpes virus 1, parainfluenza virus 3 (PI-3), bovine respiratory syncytial virus, and bovine viral diarrhea virus in conjunction with environmental stress factors have been implicated as causes of respiratory tract infections of cattle.[1,7] In one investigation conducted in the USA to assess prevailing viral respiratory infections in cattle, a high percentage of cattle arriving at feedlots yielded isolates of an ''emerging'' respiratory bovine coronavirus (RBCV).[11] These viruses were recovered from nasal swab samples collected from cattle that experienced acute respiratory distress following shipping.

The frequent isolation of RBCV from the respiratory tract of cattle requires further investigation and justifies the development of more rapid and sensitive tests, such as the enzyme-linked immunosorbent assay (ELISA), that can improve diagnosis of RBCV. Other investigators have provided serologic evidence for respiratory coronavirus infection in adult cattle.[2,3] The RBCVs have not been isolated previously from feedlot or other adult cattle by employing conventional cell culture techniques, yet these viruses may be a contributing factor in respiratory tract disease in cattle. The ELISA can facilitate processing of large numbers of samples, compared with current cell culture methods. The objective of this study was to develop a capture ELISA for the detection of RBCV antigen in nasal swab samples from cattle with respiratory tract disease and to compare the results with findings achieved through virus isolation techniques.

Material and methods

Cell lines. The G clone of human rectal tumor cells (HRT-18G) comprises a cell line cloned and selected by medium modulation from the parent HRT-18 cells previously established.[5,8,13] This cell clone was maintained in Dulbecco's modified Eagle's medium (DMEM)[a] supplemented with 5% (v/v) fetal bovine serum (FBS), 4.5 g/liter glucose, and 25 mM HEPES.

Viruses. The prototype RBCV OK-0514 was isolated from the nasal swab of a feedlot steer with clinical respiratory tract disease subsequent to shipping from Oklahoma to a feedlot in Kansas.[11] The virus was propagated in G clone cells and characterized as previously described.[7] Forty-eight-hour postinfection cell cultures developed abundant cytopathic effects (CPE) and then were frozen at −60 C, thawed 3 times, and sonicated twice for 15 sec at a power setting of 4 using a Cell Disrupter 200.[b] The lysate was centrifuged at 2,000 × *g* for 20 min, and the resulting supernatant was

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stored at −60 C. The virus infection titer was 4×10^8 plaque-forming units (PFU)/ml. Cell lysates of normal cell cultures were also prepared.

Clinical specimens. Nasal swabs from 50 cattle of ages 6–8 mo delivered to a Kansas feedlot from Oklahoma, Texas, and Arkansas and from 50 cattle of the same age delivered to an Arizona feedlot from California were used in this study. One swab was used to scrape the nasal mucosa of each test animal and was then placed in 1 ml transport medium consisting of minimal essential medium (MEM) buffered with 25 mM HEPES, transported on dry ice, and stored at −60 C until processing. For processing, the swabs were discarded, and the transport medium was diluted 1:2 with MEM and centrifuged at $2,000 \times g$ for 30 min. The resulting supernatants were filtered through sterile 0.45-μm filters and stored at −60 C. For virus isolation, 0.1 ml of sample was added to confluent G clone monolayers in 24-well plates.[c] Plates were incubated at 37 C and microscopically examined for CPE over a period of 7 days. Two to 4 wells on each plate were not inoculated to serve as normal cell controls.

Antibodies. The monoclonal antibody (MAb) MAb13[4] was used in these studies. This MAb was prepared against the cell-culture-adapted enteric bovine coronavirus (EBCV) strain L9 in the 72nd cell culture passage and had the broadest reactivity with different strains of RBCV tested (data not shown). It reacts with the S glycoprotein. Polyclonal antiserum 1745 used in this study was obtained from naturally infected cattle and has a neutralization index of 4.5 against EBCV-L9.[9,10]

Quantitation of RBCV antigen by hemagglutination test. The hemagglutination (HA) test was performed in 96-well U-bottom culture dishes[d] according to a previously described procedure.[12] Test samples were diluted 2-fold in sterile Dulbecco's phosphate-buffered saline (DPBS) containing 0.5% (v/v) bovine serum albumin (BSA). An equal volume of 0.5% (v/v) washed rat red blood cells in DPBS containing 0.5% (v/v) BSA was added to each well. After gentle agitation, the plates were incubated for 1 hr at 4 C. The HA titer was expressed as the reciprocal of the highest virus dilution inducing complete HA.

One-step replication curve of prototype RBCV OK-0514 in G clone cells. Confluent monolayers of G clone cells in 25-cm^2 flasks were washed with DPBS and infected with 0.25 ml of inocula containing 256 HA units of RBCV OK-0514-4. Cells were incubated at 4 C for 60 min to synchronize infection and then washed with DPBS, and DMEM without serum was added. Flasks were further incubated at 37 C. The medium from one cell culture was collected at intervals of 0, 2, 5, 8, 10, 12, 20, 24, 30, 36, 44, 52, and 60 hr postinfection and stored at −20 C until assayed by ELISA and the HA test.

Detection of RBCV antigens using capture ELISA. MAb13 was diluted to 2.5 μg protein/ml in 0.1 M carbonate buffer, pH 9.6, and the MAb dilution was added to each well of 96-well flat-bottom Immulon 2 plates. All solutions of the ELISA were added at 100 μl/well. The plates were stored overnight at 4 C. The wells were then treated with blocking buffer (NET; 0.15 M NaCl, 1 mM ethylenediaminetetroacetic acid, 0.01 M Tris, pH 7.4) containing 1% (w/v) BSA. After 2 hr, plates were washed 6 times with washing NET buffer containing 0.05% Tween 20 and tapped to remove excess buffer. Culture supernatant or clinical samples were diluted 1:2 in NET buffer containing 1% (w/v) BSA and 0.05% Tween 20, and each sample was added to 3 wells. Plates were incubated at room temperature for 30 min and washed as above. Thereafter, anti-bovine coronavirus serum 1745 diluted 1:100 was added to each well for incubation at room temperature for 30 min. After washing, goat anti-bovine IgG (H + L) horseradish peroxidase conjugate[e] diluted 1:1,000 was added. Plates were incubated at room temperature for 30 min and then washed as above. Substrate solution containing H_2O_2 and the chromogen tetramethylbenzidine[f] was added. After 10 min, 0.1 M H_2SO_4 was used to stop the reaction. Optical density (OD) was determined at 492 nm with a Dynatech MR 5000 ELISA reader.[c]

Plaque assay. The assay was performed as previously reported[6] using 6-well flat-bottom tissue culture plates[g] with G clone confluent monolayers. Cells were maintained in DMEM with 4.5 g/liter of glucose buffered with 44 mM $NaHCO_3$ and supplemented with 5% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Inocula were serially diluted in 10-fold steps. Cell monolayers were inoculated with 0.5 ml of the appropriate dilutions adsorbed for 1 hr at 37 C, overlayed with 0.5% agarose in DMEM, and incubated at 37 C for 2–4 days until CPE was observed. Cells were then stained with neutral red in phosphate-buffered saline (PBS) (0.5 g/liter) and fixed with 4% formalin in PBS for plaque quantification. Noninoculated cell cultures were used as negative controls.

Results

Comparison of the capture ELISA and HA assay in the measurement of free virus in a 1-step replication curve. The first rise of RBCV antigen released into the medium was detected at 12 hrs postinfection with both HA test and ELISA even though signs of CPE were observed in the monolayers of infected cells at 10 hrs. The highest amount of RBCV antigen in the medium was recorded at 44 hrs postinfection, and results were similar for the HA test and the ELISA. The kinetics of HA and antigen production during the 1-step replication curve for prototype virus RBCV OK-0514-4 in G clone cells are shown in Fig. 1.

Correlation analyses were performed with Spearman's rank order coefficient, and results indicate a strong correlation between these tests in measuring RBCV antigens released from infected G clone cells into the medium ($r = 0.951$).

Comparison of results from capture ELISA and plaque assay. Five different preparations of the RBCV OK-0514 strain were previously quantitated by plaque assays, which gave virus titers of $10–10^8$ PFU/ml (Fig. 2). These samples were also evaluated by capture ELISA. Comparison of results from plaque assays and capture ELISA indicated that an increase in PFU/ml paralelled increasing OD values. The correlation between the 2 assays was 0.975 (Fig. 2).

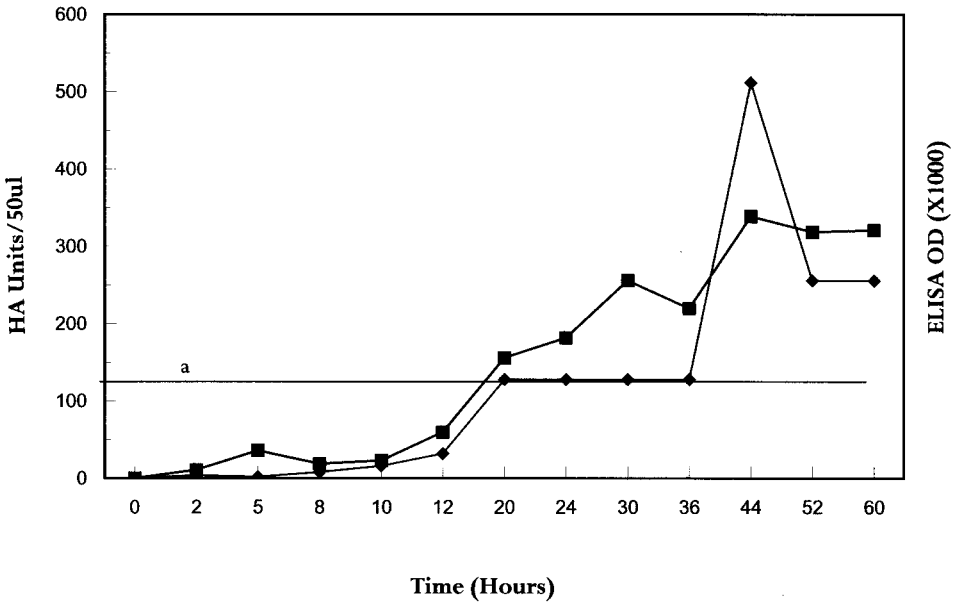

Figure 1. One-step replication curve of prototype RBCV OK-0514-4 in HRT-18G cells. ■=antigen released into the medium and assayed by capture ELISA; ♦=antigen released into the medium and assayed by HA; a=cutoff for antigen detection by capture ELISA defined as the mean of the CPE-negative samples plus 3 SD (OD = 0.103).

Identification of RBCV in clinical specimens using capture ELISA. Nasal swab samples from the 100 feedlot cattle were tested for the presence of RBCV antigen by capture ELISA, and the results were compared with results achieved by virus isolation on G clone cells.[11] Samples with OD greater than the mean of the samples negative for virus isolation plus 3 SD were defined as positive (OD = 0.103) (Fig. 3). The capture ELISA detected RBCV antigen in 28 of 50 samples from the Kansas feedlot cattle, and 32 of 50 samples harbored the viral agent only multiplying in G clone cells. The capture ELISA detected RBCV in 2 of 50 samples from the Arizona feedlot cattle compared with 6 of 50 samples that were positive by virus isolation. Comparison of results from virus isolation and capture ELISA agreed in 29 positive samples and 61 negative samples (Table 1). Virus isolation did not detect RBCV in 1 ELISA-positive sample, and ELISA failed to detect RBCV antigen in 9 samples that were positive in G clone cultures: 5 from the Kansas feedlot cattle and 4 from the Arizona feedlot cattle. The sensitivity and specificity of virus isolation compared with ELISA were 96.0% and 98.4%, respectively. The sensitivity and specificity of ELISA compared with virus isolation were 76.5% and 95.4%, respectively.

Although the sensitivity of the capture ELISA was lower than that of virus isolation, the specificities were similar. Other respiratory viruses, including PI-3, were isolated from the nasal swabs of 7 animals (data not shown). These samples were all negative by capture ELISA, further substantiating the specificity of the assay.

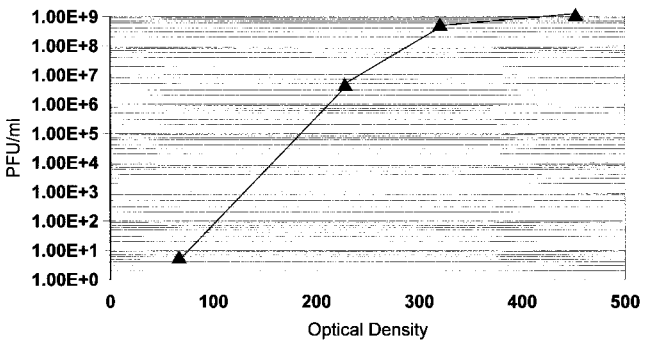

Figure 2. Correlation between numbers of plaque-forming units induced by RBCV OK-0514 and absorbance values (×1000) in capture ELISA.

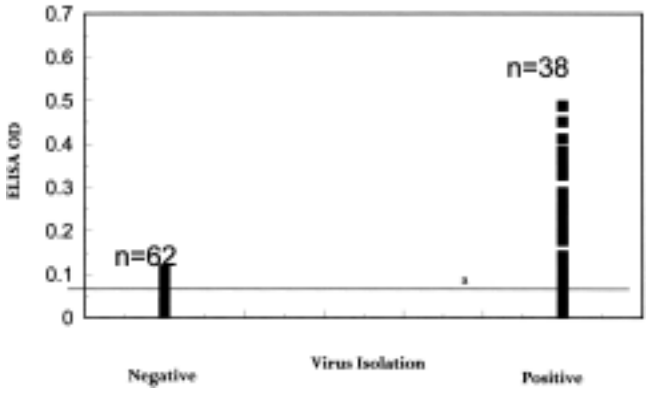

Figure 3. Capture ELISA detection of RBCV antigen from nasal samples collected from 100 6–8-month-old beef cattle. The horizontal continuous line indicates the cutoff value for antigen defined as the mean of the CPE-negative samples plus 3 SD (OD = 0.103).

Table 1. Comparison of virus isolation and capture ELISA for detection of RBCV in nasal swab samples from naturally infected cattle.

Virus isolation	Capture ELISA		Correlation (%)
	Positive	Negative	
Positive	29	9	72
Negative	1	61	

Discussion

Virus isolation with the G clone of HRT-18 cells is the most sensitive method available to detect RBCV in naturally infected cattle. These RBCV isolates replicated only in G clone cells,[11] but this procedure is not practical for routine investigations when numerous samples need to be rapidly processed. Unlike virus isolation, the capture ELISA requires minimal preparation of samples prior to testing. The capture ELISA can be used to study RBCV infections under conditions in most veterinary diagnostic laboratories because it does not require specialized cell cultures and has the advantage of being inexpensive for the simultaneous processing of large numbers of samples. The results of this study confirm the validity of the capture ELISA in detecting coronavirus antigen released into the medium by infected G clone cells as well as in detecting RBCV in nasal swab samples from cattle that have experienced respiratory disease following shipping. The ELISA is specific and reproducible, and the results correlated well with those of virus isolation for detecting RBCV in the majority of virus isolates. Statistical analysis based on Spearman's rank order coefficient showed a strong correlation between these 2 RBCV detection tests ($r = 0.723$). The correlation analysis between plaque assay and capture ELISA for detecting RBCV antigen in 5 samples was performed using Spearman's rank order coefficient. The results indicated a high correlation between the ELISA results and the number of plaques ($r = 0.975$). These findings suggest that our capture ELISA is reliable, practical, economical, and less time consuming.

A large number of samples from cattle delivered to the Kansas feedlot, especially from Oklahoma, were RBCV positive as indicated by virus isolation and by capture ELISA. The ELISA failed to detect RBCV in 5 samples from the Kansas feedlot cattle that yielded RBCV by virus isolation.[11] We did not isolate other viruses from the cattle of the Kansas feedlot. In cattle of the Arizona feedlot, ELISA failed to detect RBCV antigen in 4 samples that yielded RBCV by virus isolation. Viruses other than RBCV were isolated from 7 cattle of the Arizona feedlot, but the samples from all these cattle were negative by capture ELISA, which substantiates the specificity of our ELISA. These viruses were isolated from cattle that did not shed RBCV, indicating that individual cattle did not have multiple viral infections (data not shown). It is unknown at this time if the 4 Arizona animals identified as positive by virus isolation but negative by ELISA were all shedding the same RBCV. Future studies of the RBCV isolated from these cattle will address this issue. Previous investigations have documented that antigenic variabilities may occur among strains of coronaviruses.[4,14] Amino acid substitutions in the antigenic region of the S glycoprotein might be associated with a modification in tropism and virulence of these viruses.[14] Although MAb13 recognized the broadest range of RBCV isolates in our studies, which included strains from Oklahoma, Texas, Louisiana, Mississippi, Arizona, and Arkansas (data not shown), it did not recognize 9 samples that previously yielded RBCV isolates in G clone cells. The differences in the reactivities of MAbs are possibly attributed to epitopic variation among bovine coronavirus strains,[4,14] which may be responsible for the failure of MAb13 to detect some of the RBCV strains using our capture ELISA. The capture ELISA described here differentiated infected from uninfected cattle. A combination of MAbs against different epitopes of the S glycoprotein or hyperimmune antiserum may improve the sensitivity of future tests. Nevertheless, a capture ELISA was devised to detect coronavirus antigen in cattle infected with RBCV. The test has practical applications for future studies of incidence and prevalence of RBCV infections and for diagnosing causes of respiratory tract infections of cattle.

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Sources and manufacturers

a. Sigma Chemical Co., St. Louis, MO.
b. Branson Ultrasonics, Danbury, CT.
c. Dynatech Laboratories, Sullyfield, VA.
d. Costar, Cambridge, MA.
e. Jackson Immune Research, Avondale, PA.
f. Kirkegaard & Perry Laboratories, Gaithersburg, MD.
g. Becton-Dickinson, Lincoln Park, NJ.

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