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Development and Applications of a Bovine Coronavirus Antigen Detection Enzyme-Linked Immunosorbent Assay†

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We developed a monoclonal antibody-based, antigen capture sandwich enzyme-linked immunosorbent assay (ELISA) for bovine coronavirus. We compared the ELISA with electron microscopy and the hemagglutination test and found a close correlation between them. The sensitivity of the ELISA was 10^4 bovine coronavirus particles per ml of 10% fecal suspension. Compared with electron microscopy, bovine coronavirus ELISA had 96% specificity.

Bovine coronavirus (BCV) is an economically significant cause of calf scours and winter dysentery of adult cattle (9) and may cause respiratory disease in calves (6, 7). BCV is second only to rotavirus as a frequent cause of calf scours (1). Currently, no enzyme-linked immunosorbent assay (ELISA) is commercially available for BCV detection in feces. Although electron microscopy is commonly used to detect enteric viruses, it lacks sensitivity. Therefore, we have developed an economical, rapid, sensitive, and specific antigen capture ELISA for BCV.

(This work was conducted by Shanon Lynn Schoenthaler to fulfill the requirements for the Bachelor of Science honors program in the College of Arts and Sciences, Kansas State University, Manhattan.)

Feces were obtained from three different sources: diagnostic specimens, mostly from calves up to 3 months old with signs of diarrhea, from Kansas and Nebraska submitted to Kansas State University (KSU) Veterinary Diagnostic Laboratory, Manhattan ($n = 323$); three experimentally infected newborn calves ($n = 158$); and a KSU herd of 24 adult dairy cattle ($n = 117$) sampled weekly over a 4-month period during 1997. These cows were randomly selected and had no signs of diarrhea. All the samples were stored at −70°C.

For experimental infection, two different isolates of BCV were administered soon after birth. Calves A and B were given BCV isolate CA-1 and calf C was given BCV isolate WI-1-SK. Calf A failed to exhibit signs of BCV infection because of inadvertent administration of BCV antibodies in the calf milk replacer (Farmhand, Kansas City, Mo.). The presence of antibodies (1:2,000) in calf milk replacer was determined by an indirect fluorescent-antibody test. Fecal samples were collected from calves B and C several times daily for the next 5 days. Fecal samples from all sources were prepared as either a 10% (wt/vol) suspension of solid or semisolid feces in 0.01 M phosphate-buffered saline (PBS) or as a 20% (vol/vol) suspension of liquid feces in 0.01 M PBS. All samples were then centrifuged (1,500 × *g*); supernatant was saved and stored at −80°C.

Standardization of the BCV ELISA was achieved by applying monoclonal antibody (Z3A5) and purified BCV stock (WI-1-SK) in a checkerboard pattern to Immulon 1 96-well microtiter ELISA plates (Dynatech Technologies, Chantilly Va.). In the rows, dilutions of semipurified Z3A5 monoclonal antibody were applied (10 to 1,000 ng/well), increasing in concentration from the bottom to the top rows. In the columns, concentrated and purified BCV stock dilutions (10^8 PFU/ml) were added, increasing in concentration from right to left. Standardization of the secondary antibody and horseradish peroxidase (HRPO) conjugate was achieved in a simlar way. The goal was to achieve the largest difference in absorbance between the known negative and positive BCV fecal samples (Fig. 1).

The details of Z3A5 production have been described before (13). Z3A5 has neutralizing activity against BCV and specificity for the spike subunit of BCV. It reacted with 90 BCV isolates collected from eight states (California, Kansas, Minnesota, Nebraska, North Dakota, Oklahoma, Wisconsin, and Wyoming) and stored at −84°C (13), and it has an immunoglobulin G1 isotype.

For semipurification and concentration of Z3A5, ammonium sulfate (50% saturation) precipitation was carried out (5) and Z3A5 was suspended at a concentration of 1 mg/ml. For BCV ELISA, Z3A5 was diluted (1:4,000) in 0.05 M carbonate coating buffer (pH = 9.6). Diluted Z3A5 (50 μl, 250 ng/ml) was then added to each well of an Immulon 1 flat-bottom microtiter plate, which was incubated overnight at 4°C. After incubation, the plate was washed five times with a wash solution of 0.01 M PBS and 0.05% Tween 20 (Sigma Chemical, St. Louis, Mo.), desiccated, and stored at 4°C. A 0.4% blocking solution (casein enzymatic hydrolysate [CEH]) and 0.01 M PBS were mixed just before use, filtered (0.45-μm pore size), and added to each well (100 μl). The plate was incubated for 30 min at 37°C and washed. Fecal sample (50 μl) was added, and the plate was incubated at 37°C for 25 min, followed by washes. A polyclonal porcine anti-BCV (1:500 dilution, from National Veterinary Services Laboratory, Ames, Iowa) was made by using 0.4% CEH–PBS as a diluent. Fifty microliters was added to each well, and the plate was incubated at 37°C for 25 min, followed by washes. Next, a 1:16,000 dilution (50 μl) of goat anti-porcine immunoglobulin G (heavy plus light chain, affinity

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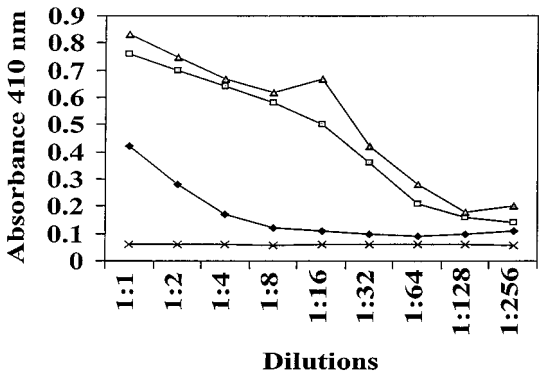

FIG. 1. Dose relationship between absorbance and sample dilutions. Absorbances for a weakly BCV positive sample (◆), a tissue-culture propagated BCV sample (□), an EM-positive BCV sample (△), and a negative fecal control (×) are shown.

purified, HRPO conjugated; ICN Biomedicals, Aurora, Oh.) was made by using 0.01 M PBS as a diluent and added to each well, and the plate was incubated at 37°C for 25 min and washed. Lastly, 50 μl of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] peroxidase substrate (ABTS; Kirkegaard & Perry Laboratories, Gaithersburg, Md.), warmed for 1 h at room temperature, was added to each well, and the plate was incubated for 15 to 30 min at 37°C. Absorbance was read with an Anthos Labtec 2001 plate reader (Labtec, Salzburg, Austria) at 405 nm. Blocking solution, secondary antibody, and HRPO conjugate were made fresh for each assay and were prewarmed at 37°C for 15 min before addition to the plate.

Types 1, 2, and 4 Immulon microtiter plates were tried to determine the best type of plastic on which to bind the anti-BCV Z3A5. Both Immulon 2 and 4 produced higher background reactions in negative control wells; therefore, Immunlon 1, which showed no background reaction, was chosen for the BCV ELISA. The optimal concentration of Z3A5 used in the ELISA was determined to be 250 ng/ml by checkerboard titration. A positive absorbance reading was determined to be equal to or more than two times the absorbance of the average fecal negative controls in the same assay. On average, the absorbance of a negative control well was 0.075, whereas the average absorbance of a positive well was about 0.25. Positive absorbance values ranged from borderline positive at 0.150 to highly positive at 0.6. A few samples from experimentally infected calves had an absorbance reading of 1.3.

The 117 fecal samples collected and tested for BCV from adult cows in the KSU dairy herd gave 64 positive and 53 negative findings. Adult cows shed very low levels of BCV but did not exhibit clinical signs. For the experimentally BCV-infected calves, 101 positive and 57 negative results were obtained. Of these, 97 random samples were also tested by a hemagglutination (HA) test, and an 81% correlation between ELISA and HA results was observed (Table 1). This relatively low correlation between HA assay and ELISA was due to the lower sensitivity of the HA assay for BCV in fecal samples. A total of 323 diagnostic fecal samples was submitted to our laboratory for BCV ELISA. Seventy-four were positive and 249 were negative. A total of 216 of these fecal samples were also tested for BCV by electron microscopy (EM), and a 92% correlation was observed (Table 1). The remaining specimens tested by ELISA were not tested by EM or by HA assay.

The BCV ELISA extended both sensitivity and specificity in detection of the virus. The high level of sensitivity of the ELISA allowed detection of the virus in fecal samples containing as few as 10^4 particles, whereas the sensitivity of EM allows detection of virus only in samples containing 10^5 particles or more (Fig. 1). A dose relationship was observed between absorbance reading and serial dilution of BCV positive samples: the presence of a greater number of virus particles produced a higher absorbance reading (Fig. 1).

TABLE 1. Correlation between detection of BCV by ELISA and detection by EM and HA assay

Assay and result	No. of samples with ELISA result		% Correlation
	Positive	Negative	
EM			
Positive	40	10	92
Negative	7	159	
HA			
Positive	40	8	81
Negative	10	39	

We have developed a diagnostic test for BCV that combines the advantages of sensitivity and specificity yet minimizes the disadvantages of previous attempts to develop a BCV ELISA: long protocols, nonspecific reactions, and limited numbers of samples studied (2–4, 8, 10–12). There was a 92% correlation between ELISA and EM. One possible reason for this disparity was that BCV ELISA was more sensitive than EM. A second possibility is that certain nonviral particles, e.g., brush border or antigenically different coronavirus-like particles (such as Breda virus), present in feces could be mistaken for virus particles in EM (6, 8). Because no "gold standard" test for BCV exists, the greater specificity and sensitivity of our ELISA make it more attractive for BCV detection than EM. However, when compared with EM, BCV ELISA had a specificity of 96%. We further validated our ELISA with samples from experimentally BCV-infected calves. It was easy to test a large number of specimens with BCV ELISA. The fecal specimens tested in the BCV ELISA ranged from low positives (dairy cow herd) to high positives (diagnostic submissions and fecal specimens from other species—porcine, equine, and canine). No nonspecific reactions were detected, and all the specimens collected before experimental BCV infection and specimens from other species with diarrhea were negative.

Finally, our ELISA has a distinct advantage by producing rapid results. Previous BCV ELISAs have ranged in total incubation times (from blocking until reading the plate for absorbance) from overnight (6) to 18+ h (12), 6 h (8), or around 3 h (3, 10) after coating. For the same steps in our procedure, total incubation time is 130 min and total protocol time, including washing of the plate and preparation of reagents, is around 3.5 h for an average batch of 25 specimens submitted to the diagnostic laboratory. BCV-ELISA has been found to be useful for screening clinical samples from animals exhibiting diarrhea or pneumoenteritis. Furthermore, it can be helpful for tracking the cause of diarrhea in herds so that suspect animals can be isolated in the event that BCV is detected. The sensitivity, reproducibility, and specificity of this BCV ELISA make it a good assay for large volumes of fecal samples.

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