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 104 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 (106 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 similar 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 [CEHI]) 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% CEHI–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...
purified, HRPO conjugated; ICN Biomedicals, Aurora, Oh.)
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, Immulon
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
titrations. A positive absorbance reading was determined to be
equal to or more than two times the absorbance of the average
fetal 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 in-
fected 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 ob-
tained. 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 contain-
ing 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 ab-
sorbance reading and serial dilution of BCV positive samples;
the presence of a greater number of virus particles produced a
higher absorbance reading (Fig. 1).

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 ex-
perimentally 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 spec-
imens 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 in-
cubation times (from blocking until reading the plate for ab-
sorbance) 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, in-
cluding 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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![Graph](image-url)

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