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Development of an Antigen Spot Test for Detection of Coronavirus in Bovine Fecal Samples

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We have developed a rapid and sensitive microimmunodot blot assay, the antigen spot test (AST), for the detection of bovine coronavirus (BCV) antigen from neonatal calf fecal samples. The AST procedure can be completed in 3.5 h, whereas the previously reported immunodot blot assays require 10 to 12 h. Ninety-six samples can be tested per membrane, and 10 membranes (960 samples) may be processed by a single technologist in 1 working day. The effects of detergents, oxidizing chemicals, chaotropic agents, and enzyme substrates in improving the sensitivity and signal-to-noise ratio of the AST were studied. Finally, the sensitivity and specificity of AST for the detection of BCV antigen were compared to those of a sandwich enzyme-linked immunosorbant assay (ELISA) and a hemagglutination assay (HA). Of 347 field samples tested by all three methods, 94.2% were positive by AST, 91.4% were positive by ELISA, and 86.7% were positive by HA. The sensitivity of the AST was determined to be 100% compared to the results of the ELISA reference method. The specificity of the AST was 67%, which reflects a lower limit of detection of 10^4 viral particles per ml in a 10% fecal suspension.

Bovine coronavirus (BCV), an enveloped, positive-strand RNA virus, is an important cause of diarrhea in calves of up to 4 weeks of age (13, 22), and it is frequently detected in the feces of adult cattle with winter dysentery (1, 2, 18–20, 22). Neonatal diarrhea caused by BCV is responsible for heavy economic losses in dairy and beef cattle (11). BCV is second only to rotavirus as a cause of enteric infection in calves between 1 and 16 weeks of age (8, 9, 12, 14, 16, 17).

Virus isolation in cell culture is considered to be the "gold standard" for the detection of most viruses in clinical samples. This technique, however, is rarely used for the diagnosis of BCV infection because no cell culture system which can reliably propagate BCV to a high titer from clinical samples has been identified (4, 5, 21). Monoclonal antibody (MAb) technology has facilitated the development of sensitive and specific tests for the detection of many microbial and viral antigens in clinical specimens. Several immunological techniques which incorporate the use of MAbs have been described, including enzyme-linked immunosorbent assays (ELISAs) (4, 5, 21, 24), direct- and indirect fluorescent-antibody tests (9, 20, 23), immunohistochemical staining of fixed tissues (6, 26), and immunoblot assays (10). The advantages of using dot blot immunoassays for the detection of viral antigens directly from tissues, swabs, washings, and body secretions are (i) culture of virus in cells, eggs, or laboratory animals is not required; (ii) large numbers of specimens can be handled simultaneously; and (iii) immunoassays are sensitive and specific and can be performed rapidly.

In this study, a rapid blot assay for the detection of BCV spike protein and nucleoprotein developed in this laboratory is described. The assay has increased sensitivity compared to the previously described sandwich ELISA (21). The effects of treatment of the antigen blots with detergent, chaotropic agents (which change the structure of biomolecules, such as protein conformation), and oxidizing solutions and the use of two detection substrates were studied to optimize the sensitivity of the immunoblot technique.

MATERIALS AND METHODS

Specimens. Fecal samples were obtained from 347 neonatal, diarrheic calves submitted to the Kansas State University Veterinary Diagnostic Laboratory, Manhattan. Fecal samples from naive, BCV-free calves obtained immediately after birth and kept in an isolation facility were used as negative controls. All fecal samples were processed before testing by vortexing 0.5 g of feces in 5 ml of 0.01 M phosphate-buffered saline (PBS; pH 7.0) and then centrifuging at 1,800 × g for 5 min at room temperature. The supernatants, which were used for testing, were transferred to a sterile vial, and were stored at −70°C. Stock cultures (10^8 PFU/ml) of two BCV isolates, California-1 (CA-1) and Wisconsin-1 (WI-1-SK), were used as positive controls for all tests.

Antibodies. MAbs Z3A5, to the BCV spike protein (26), and 8F2 (6), to the BCV nucleoprotein, were prepared and tested in this laboratory as described previously (6, 26). Both MAbs were of the immunoglobulin G1 (IgG1) isotype. The secondary antibody used was horse anti-mouse IgG–horseradish peroxidase (HRPO) conjugate (Vector Laboratories, Burlingame, Calif.) supplied at a protein concentration of 1 mg/ml. The antigen-capture antibody for the sandwich ELISA was MAb Z3A5. Primary antibody for the ELISA was polyclonal porcine anti-BCV serum (National Veterinary Services Laboratory, Ames, Iowa), and the secondary antibody was goat anti-pig IgG–HRPO (heavy and light chains; ICN Biomedicals, Aurora, Ohio).

Antigen spot test (AST). A bio-dot microfiltration unit (Bio-Rad Laboratories, Richmond, Calif.) was used to bind BCV antigen-containing samples onto nitrocellulose membranes (Gelman Sciences, Ann Arbor, Mich.) for testing by immunoassay techniques in a manner similar to that for a 96-well ELISA plate format. The apparatus connects to a vacuum with a three-way valve which allows adjustment for passive filtration or for rapid vacuum-assisted washes. Prior to assembly of the membrane sandwich, the nitrocellulose and filter paper were prewetted in deionized, distilled water. All reagents were prewarmed for 15 min in a 37°C water bath before use, and all procedures were carried out at room temperature with gentle agitation (40 to 50 rpm) on an orbital shaker. Clinical samples (50 µl) were applied to the wells and were allowed to adsorb to the membrane. Excess fluid was drawn through the membrane by vacuum. Each well was vacuum rinsed with 100 µl of 0.01 M PBS (pH 7.0), and then the nitrocellulose membrane was removed from the bio-dot unit and was allowed to dry for 15 min. The membranes were soaked for 30 min in PBS containing $3 \times 10^{-5}\%$ H_2O_2 and were then transferred to a blocking solution of 10% adult horse serum (Sigma, St. Louis, Mo.) in 0.025 M Tris-free base (pH 7.4) in 0.8% NaCl (TBS). The samples were probed for 30 min with a combination of MAbs (MAbs Z3A5 and 8F2 diluted 1:100 in PBS). The membranes were washed five times for 5 min each time in TBS, and were then covered with horse anti-mouse-IgG–HRPO-conjugated secondary antibody diluted 1:1,000 in TBS for 5 min. After five 5-min washes in TBS, the membranes were flooded with peroxidase substrate. Metallo-

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3,3′-diamino-benzinine (metallo-DAB; Pierce Chemical, Rockford, Ill.), which was prepared according to the manufacturer's instructions, was used as the chromogen. The samples were examined visually, and a dark brown color indicated the presence of BCV antigen.

Optimization of AST sensitivity. To study the effects of detergents, chaotropic agents, and oxidizing solutions on the detection of BCV antigen by AST, the following chemicals were used to treat the nitrocellulose blots after application of clinical samples: (i) urea (Bio-Rad Laboratories) at concentrations of 4 and 8 M, (ii) sodium dodecyl sulfate (SDS; Bio-Rad Laboratories) at five concentrations of between 0.05 and 1.0%, (iii) guanidine hydrochloride (GuHCl; Amresco, Solon, Ohio) at concentrations of 3 and 6 M, and (iv) H_2O_2 at six concentrations of between 3×10^{-5} and 6×10^{-3}%. To study the effects of incubation time on the detection of BCV antigen by AST, the nitrocellulose membranes were treated with the solutions for eight incubation times of between 1 and 60 min at room temperature before they were probed with the primary antibody. Two different substrates were used for detection of the bound secondary antibody signal on separate blots. Metallo-DAB, as described above, was compared to 4-chloro-1-naphthol (Pierce Chemical), which was used at a concentration of 1 g/ml in TBS, for chromogen development.

ELISA for BCV antigen detection. Complete details of the ELISA procedure have been described previously (21). For the BCV ELISA, MAb Z3A5 was diluted 1:4,000 in 0.05 M carbonate coating buffer (pH 9.6), and then 50 μl was added to each well of an Immulon 1 (Dynex Technologies, Chantilly, Va.) flat-bottom microtiter plate. The plates were incubated overnight at 4°C and were then washed five times with PBS containing 0.05% Tween 20 (Sigma), desiccated, and then stored at 4°C until they were used for testing. All steps in the ELISA were performed in a humidified chamber at 37°C, and all reagents were prewarmed in a 37°C water bath for 15 min before use. A blocking solution of 0.4% casein enzymatic hydrolysate in PBS was clarified with a 0.45-μm-pore-size filter and was then added in 100-μl aliquots to each well. The plates were incubated for 30 min and were washed five times with PBS-Tween. Fecal suspensions and positive and negative controls (50 μl) were added to their respective wells, and then the plates were incubated for 25 min followed by five washes with PBS-Tween. Primary polyclonal porcine anti-BCV antibody was prepared at a 1:500 dilution in the blocking solution. Fifty microliters was added to each well, and the plate was incubated for 25 min, followed by five washes with PBS-Tween. Secondary goat anti-porcine IgG–HRPO-conjugated antibody was diluted 1:16,000 in PBS, and then 50 μl was added to each well. After incubation for 25 min, the plates were washed five times with PBS-Tween. Color was developed by adding 50 μl of 2,2′-azino-di-(3-ethylbenzthia-zoline sulfonate) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) HRPO substrate to each well and then incubating the plates for 15 to 30 min. The absorbance at 405 nm was read on an Anthos Labtec 2001 plate reader (Labtec, Salzburg, Austria). The average optical density for three negative samples was doubled to determine the cutoff optical density. Positive samples had absorbence values greater than the cutoff value, and negative samples had absorbance values less than the cutoff value.

HA for detection of BCV. Hemagglutination assay (HA) testing for BCV was conducted by the microtiter method as described previously (23). Briefly, 25 μl of diluent (0.2% bovine serum albumin in PBS) was added to each well of a V-bottom, polystyrene microtiter plate 1 (Dynex Technologies, Chantilly, Va.). Control and test specimens in 25-μl volumes were added to their respective wells in duplicate, and then serial twofold dilutions were made across 12 wells. Fresh erythrocytes from BALB/c mice were diluted to 1% in bovine serum albumin-PBS diluent, and 25 μl was added to each well. The plates were incubated for 90 min at 4°C. The presence of BCV antigen was indicated by the dispersal of erythrocytes in a latticework across the bottom of the well. The formation of a tight button of erythrocytes at the bottom of a well indicated the absence of BCV, and the test result was read as negative.

Calculation of assay specificity and sensitivity. The following formulas were used to calculate specificity and sensitivity (25): sensitivity = $[TP/(TP + FN)] \times 100$, where TP is the number of samples with a true-positive result as determined by the reference assay and FN is the number of samples with a false-negative result, and specificity = $[TN/(TN + FP)] \times 100$, where TN is the number of samples with a true-negative result and FP is the number of samples with a false-positive result.

RESULTS

Optimization of AST sensitivity. Optimization of the AST by treatment of the antigen dot blots prior to antibody probing was aided by incubation for 30 min in 3×10^{-5}% H_2O_2. Although the sensitivity of AST was enhanced by incubating the antigen blots with either 6 M GuHCl or 0.05% SDS, nonspecific staining of the immunoblots occurred. The results were similar when the antigen dot blots were incubated with PBS alone. Nonspecific staining was significantly less when the antigen blots were treated only with the 3×10^{-5}% H_2O_2 solution, yet sensitivity remained high. This was the antigen blot treatment selected for use in the final AST protocol. Substrate choice was also shown to affect the sensitivity of antigen de-

TABLE 1. Agreement of results of AST with those of ELISA and HA for detection of BCV in calf feces[a]

Result by the following assay:			No. (%) of positive specimens
AST	ELISA	HA	
+	+	+	301 (86.7)
−	−	−	20 (5.8)
+	+	−	16 (4.6)
+	−	−	10 (2.9)

[a] A total of 347 samples were tested.

tection. Chromogen development by DAB was shown to be superior to that by 4CN; thus, DAB was used as the substrate for the AST protocol.

Comparison of AST, ELISA, and HA for detection of BCV. Of 347 field samples tested by all three methods, 94.2% were positive by AST, 91.4% were positive by ELISA, and 86.7% were positive by HA. The results of the three testing protocols were compared. A total of 301 samples (86.7%) which tested positive by HA were also positive by AST and ELISA. Twenty samples (5.8%) were negative by all three assays (Table 1); thus, the three assays displayed 92.5% agreement of results. The ELISA detected an additional 16 positive samples which were also found to be positive by AST. Additionally, AST detected 10 positive samples which tested negative by both ELISA and HA. These 10 samples were collected from newborn calves that were fed colostrum but that were challenged with BCV at 2 days of age.

Sensitivity and specificity. Because no established gold standard for the detection of BCV in clinical specimens exists, the sensitivities and specificities of the three tests were computed in three ways: each assay was used as the reference method against which the other two tests were compared (Table 2).

DISCUSSION

One advantage of the immunodot blot technique is that the synthetic membranes used to bind the proteins also allow their retention of antigenicity and accessibility to antibody. Direct coating of polystyrene or polyvinyl chloride ELISA plates with the protein of interest can result in the deformation of the antigenic epitope against which the MAbs react. To avoid this potential problem, a sandwich ELISA is often designed such that the microtiter plates are required to be coated with capture antibodies that serve to anchor the protein of interest and the antigen retains its native conformation. Another improvement of the AST described here over the sandwich ELISA is

TABLE 2. Sensitivities and specificities of the various assays compared to results of three reference methods

Reference method	Assay	Sensitivity (%)	Specificity (%)
AST	AST	100	100
	ELISA	96.9	100
	HA	92.1	100
ELISA	AST	100	67
	ELISA	100	100
	HA	95	100
HA	AST	100	43.5
	ELISA	100	65.2
	HA	100	100

[a] A total of 347 samples were tested. Sensitivity and specificity were calculated as described in Materials and Methods.

that fewer antibody reagents are necessary for performance of the test. Nitrocellulose can bind greater quantities of protein per surface area than polystyrene or polyvinyl chloride plates, and it binds protein quantitatively up to concentrations of 80 $\mu g/cm^3$ (15). Laboratories which routinely perform HA must maintain mouse colonies as sources of the fresh erythrocytes that are required for this assay. Many laboratories are discouraged from performing tests which necessitate the killing of animals. Time factors for the completion of the three tests are comparable; however, storage space for supplies and equipment is considerably less for the AST than for either ELISA or HA.

Attempts to improve the sensitivities of immunoassays usually focus on optimization of detection by varying the levels of antibody, by using antibody which is more specific to the antigen, and by using different enzyme conjugate-substrate systems including signal amplification schemes. Treatment of antigen with detergents, chaotropic chemicals, and ozidizing or reducing agents is another approach which may be considered for increasing the sensitivity of an immunoassay because these treatments have the potential to make reactive epitopes more accessible to the antibodies in the detection system (3). The sensitivity of our AST was increased by using 6 M GuHCl or 0.05% SDS. This was expected because both chemicals dissociate the capsid proteins of the bound BCV, making the nucleoprotein antigen more accessible to antinucleoprotein MAb 8F2, one of the primary antibodies used in the AST. However, nonspecific staining also occurred, negating any beneficial effect that these treatments had on increasing the sensitivity. The optimal protocol for AST used treatment of the antigen dot blots with $3 \times 10^{-5}\%$ H_2O_2 for 30 min prior to probing with antibody. The favorable effect of H_2O_2 on increasing the sensitivity of detection may be explained by its effect on destroying endogenous peroxidase-containing cellular components, such as intestinal epithelium, that are shed in the feces and that remained in the fecal supernatants tested rather than by a reaction of H_2O_2 with the antigen epitopes.

The improved AST which was developed was compared to a sandwich ELISA protocol that has been reported on previously (21) and to a standard HA for BCV (23). Viruses such as the influenza virus and some enteroviruses and coronaviruses have an associated hemagglutinin protein which causes aggregation of erythrocytes from some mammalian species. This phenomenon is nonspecific because it is not the result of immunoserological interactions; thus, HA detects the presence of hemagglutinating viruses generally. The potential for obtaining a positive HA result from non-BCV-infected bovine fecal samples exists; therefore, this test is not truly specific. Although the sensitivity of HA (Table 2) is shown to be greater than 92%, the inability of HA to detect very low titers of BCV is an additional concern. Both AST and ELISA are capable of detecting as few as 500 BCV particles in a 50-μl sample. AST was found to be rapid, sensitive, and specific for the detection of BCV in bovine feces.

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