Application of Immunohistochemistry and In Situ Hybridization for Detection of Bovine Coronavirus in Paraffin-Embedded, Formalin-Fixed Intestines†

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Bovine coronavirus (BCV) causes severe diarrhea in calves and winter dysentery in cattle (2). When fecal samples are submitted to laboratories, BCV is diagnosed by electron microscopy (EM) (3) or antigen capture enzyme-linked immunosorbent assay (4). When unfixed intestines are submitted, a fluorescent-antibody test (FAT) is often used. When only formalin-fixed intestines are submitted, diagnosis of BCV infection is difficult, as lesions of BCV infection are nonspecific. A monoclonal antibody (MAb) (Z3A5) developed for this study was found to be specific for detection of BCV in paraffin-embedded, formalin-fixed intestines.

An isolate of BCV (WI-1-SK) (7) from the United States was used to immunize mice for hybridoma production. BALB/c mice were immunized with sucrose gradient-purified, heat-inactivated (55°C, 30 min) BCV mixed with Ribi adjuvant system (Ribi, Hamilton, Mont.). Mice were immunized five times at 3-week intervals with 100 µg of BCV. Spleen cells (~2 × 10⁶ cells/spleen) were mixed with 0.5 × 10⁶ to 1 × 10⁶ myeloma cells (Ag8), fused with polyethylene glycol 6600, resuspended in hypoxanthine-aminopterin-thymidine medium, plated, and incubated (37°C, 3 to 4 weeks). Supernatants from wells with clones were screened by an indirect fluorescent-antibody test (IFAT). About 50 µl of hybridoma cell culture fluid was added to BCV-infected cells, followed by sheep anti-mouse immunoglobulin G (IgG) labeled with fluorescein isothiocyanate.

MAbs against BCV were isotyped with a mouse typer kit (Bio-Rad, Hercules, Calif.). All MAbs had κ light chains. Of 18 MAbs, 12 were IgG1 (including Z3A5), 3 were IgG2a, 1 was IgG2b, 1 was IgG3, and 1 was IgA.

Biological activity of the MAbs was evaluated by a hemagglutination inhibition test (11), a plaque reduction assay (6), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) followed by Western blot assay. None of the MAbs showed hemagglutination inhibition activity. Nine MAbs (including Z3A5) showed neutralizing activity against BCV. All of the MAbs recognized a BCV protein with a molecular weight of 86 kDa, a spike protein subunit (8). By an IFAT, MAb Z3A5 reacted with all 90 BCV isolates collected from 8 states. This MAb was selected for further evaluation.

Sections from formalin-fixed intestines (n = 61) were examined by immunohistochemistry. Sections (4 µm thick) were heat fixed (55°C, 30 min) and deparaffinized in three changes of Hemo-de (Fisher, St. Louis, Mo.) for 20 min. The sections were rehydrated and treated with 0.01% trypsin (10 min, room temperature). To quench endogenous peroxidase, 3% H₂O₂ was applied to the tissues (5 min, room temperature). A protein blocker (PBA; Lipshaw, Pittsburgh, Pa.) was applied to the sections (10 min, room temperature). Diluted Z3A5 (1:300) was applied to the tissues and incubated (2 h, 37°C). After a 10-min wash in phosphate-buffered saline–TWEEN 20, slides were incubated with a biotinylated anti-mouse IgG (Vector, Burlingame, Calif.) and washed with phosphate-buffered saline–TWEEN 20, and avidin-biotin complex (ABC; Vector) was applied for 30 min at room temperature. Aminoethyl carbazole was applied (30 min) and the sections were washed, counterstained with hematoxylin (30 sec), and mounted with 50% glycerol gelatin. Of the 18 MAbs, three (including Z3A5) detected BCV antigen in intestinal sections. BCV-infected cells were distributed in the epithelial linings of the crypts (Fig. 1) and enterocytes of the villi and rarely as single enlarged cells between infected crypts in the lamina propriae of the intestines. Inclusions were present in the cytoplasm of infected cells. Exfoliated and degenerated cells were seen many times in the lumens of the infected crypts of the colon (Fig. 1). MAb Z3A5 reacted with only antigenic group II of the mammalian coronaviruses, which includes BCV and elk coronavirus (5). The MAb did not react with transmissible gastroenteritis virus, feline infectious peritonitis virus, infectious bovine rhinotracheitis virus, bovine virus diarrhea virus, parainfluenza virus-3, or bovine respiratory syncytial virus on the IFAT or immunoperoxidase test.

When immunohistochemistry and FAT results were compared in 19 cases, there was 52% correlation. The discrepancy between FAT with a polyclonal anti-BCV antibody (National Veterinary Services Laboratory, Ames, Iowa) and immunohistochemistry with Z3A5 may have been due to the segmental and/or focal distribution of BCV antigen and because different parts of the intestines from the same animal were submitted for FAT and immunohistochemical examination.

When EM and immunohistochemistry results were compared in 13 cases, we observed 62% correlation. The discrepancy between the two tests may have been due to detection of
free virus in the feces by EM versus tissue-associated virus by immunohistochemistry. After the acute phase of BCV diarrhea, the enterocytes are lost and EM may give false-negative results. However, immunohistochemistry may still give positive results due to the virus present in the crypts.

A full-length nucleoprotein cDNA (3B6) with more than 95% homology with the Mebus nucleoprotein gene sequence was used as a probe for in situ hybridization (ISH) (1). Specificity of the 3B6 probe was established by Northern blot hybridization (10). Probe 3B6 specifically hybridized with BCV RNAs, and no signal was observed with total RNA extracted from uninfected HRT-18 cells. BCV nucleoprotein cDNA was isolated by restriction digestion and electrophoresed in 1% low-melting-temperature agarose gel, and the nucleoprotein band was excised from the gel. The 3B6 probe was labeled by random prime labeling.

To validate the specificity of Z3A5, we performed ISH on the same tissue blocks (n = 60). Thirty-two sections were negative by both immunohistochemistry and ISH. In 22 sections, both immunohistochemistry and ISH were positive. In one section, ISH was negative while immunohistochemistry was positive. In five cases, immunohistochemistry was negative but ISH gave weakly positive signals. On the basis of this study, we found that immunohistochemistry with Z3A5 had 82% sensitivity, 97% specificity, and 90% accuracy for the diagnosis of BCV infections.

Immunohistochemistry with MAb Z3A5 was found useful for establishing a confirmed diagnosis of BCV-associated enteritis when only formalin-fixed intestines were available. Compared to FAT, immunohistochemistry provides better cellular detail and histologic architecture so that smaller numbers of BCV-infected cells and lesions may be studied in the same section and with more confidence. Collection and archiving of frozen tissues for FAT is difficult, but we were able to easily perform retrospective studies (1994 to 1996) using immunohistochemistry. We conclude that MAb Z3A5 is an effective reagent for BCV diagnosis and for study of the pathogenesis of BCV infections.

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REFERENCES

FIG. 1. Immunohistochemical staining of a bovine colon sample positive for BCV (primary MAb, Z3A5). BCV antigen (red staining) is present in the cytoplasms of infected crypt epithelia (arrowheads) and in the sloughed necrotic cells in crypt lumens. Chromogen, AEC; counterstain, hematoxylin. Magnification, ×450.