Immunoperoxidase Procedures for Rapid Detection of Bovine Viral Diarrhea-Mucosal Disease Virus Antigen
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ABSTRACT. Bovine viral diarrhea-mucosal disease (BVD-MD) virus antigen was detected in bovine fetal muscular cells from sera of 42 cattle persistently infected with noncytopathic BVD-MD virus by the indirect immunoperoxidase procedure (IIP) but not from sera of 100 apparently healthy cattle. These findings agreed with the results obtained by the interference method. Furthermore, BVD-MD virus antigens were detectable in the smears of buffy coats by the IIP procedure and in the formalin-fixed tissue sections by the avidin-biotin-peroxidase complex procedure. These procedures are therefore expected to be useful for rapid detection of BVD-MD virus antigen.—KEY WORDS: bovine viral diarrhea, immunoperoxidase, persistent infection.


Bovine viral diarrhea-mucosal disease (BVD-MD) virus has long been recognized in Japan in association with bovine abortion, congenital anomalies and mucosal diseases [6, 10, 11]. Recently, it has become evident that many cattle are persistently infected with noncytopathic (NCP) BVD-MD virus caused by transplacental fetal infection [9, 12] and that this persistent infection is related to many diseases with transient fever, to respiratory disease with or without diarrhea and to immunosuppressive and mucosal diseases [1]. The serological types isolated from persistently infected cattle in Japan are reported to be the Nose group and the KS86-1 group [11]. These NCP viruses have been commonly detected by the interference method [4]. Recently indirect immunoperoxidase (IIP) procedures have been successfully applied to the diagnosis of several viral infections [3, 5, 8]. IIP procedures, in common with the fluorescent antibody (FA) procedure, provide a simple, rapid means for detecting viral antigens in tissues. In addition, the sensitivity of these techniques may be superior to that of the FA procedure [5], and avidin-biotin-peroxidase complex (ABC) procedures provide a more permanent record and preserve histological details [7]. The present report describes the detection of BVD-MD virus antigen in serum and buffy coat by the IIP procedures for the rapid diagnosis of cattle persistently infected with the BVD-MD virus, and the immunohistochemical staining to demonstrate the BVD-MD virus antigen by the ABC procedure.

BVD-MD virus isolation from serum specimens: Serum specimens were collected from 1987 through 1982, from 42 cattle that were persistently infected with the NCP BVD-MD virus for more than two weeks, and stored at -80°C. NCP BVD virus strains isolated from these sera were cloned 4 times by the terminal dilution method and examined antigenically by neutralization test [12]. In this study, 38 Nose and 4 KS86-1 serogroup strains respectively, were used. As negative controls, serum specimens having no BVD-MD virus were collected from 100 healthy cattle. Bovine fetal muscular (BFM) cell cultures prepared by an ordinary tissue-culture method were used for virus isolation within 20 passages [12]. Antiserum to identify the isolated virus as BVD-MD virus was prepared from cattle [12]. The neutralization titers of the antiserum to the Nose and KS86-1 strains were 1:128 and 1:64, respectively, and antiserum diluted to 1:16 with growth medium was employed for the test. For simultaneously isolating and identifying the virus, 50 μl of each specimen was added to 3 wells with 50 μl of growth medium and to 3 wells with 50 μl of the antiserum on a 96-well microplate, and incubated for 1 hr at 37°C for neutralization to occur. Then 100 μl of BFM cell suspension (2 × 10⁵ cells/ml) was added to all wells. The cells were incubated at 37°C in 5% CO₂ for 3 days, and examined by the IIP and interference methods [12] to detect the BVD-MD virus.

The IIP procedure was performed according to the method of Guy et al. [5]. The BFM cells were washed twice with phosphate-buffered saline (PBS) and fixed in cold 20% buffered acetone (200 ml of bovine serum albumin, 200 ml of acetone and 800 ml of PBS) at 4°C for 10 min and dried at 37°C overnight. These cells were washed briefly with 0.15 M NaCl solution once and reacted with 50 μl of mouse anti-BVD-MD/monoclonal antibody (JCU Tropical Biotechnology Inc., Cat. No. JCU/BVD/CF10) diluted to 1:1000 with PBS, at 25°C for 1 hr in a humidity box. The cells were washed 4 times with a washing solution (0.05% of Tween 20 with PBS) and reacted with 50 μl of horseradish peroxidase conjugated rabbit anti-mouse IgG (Rockland Inc., Code No. 310-4304) diluted to 1:1000 with PBS, at 25°C for 1 hr in the humidity box. The cells were then again washed 4 times with the washing solution and reacted with the substrate, chromogen consisting of hydrogen peroxide and amionoethyl carbazole, for 15 to 30 min at 25°C. These cells were then washed with distilled water, air-dried and examined under a microscope. If reddish-brown staining of cell cytoplasm was observed in the 3 wells with the specimen and growth medium and not in the 3 wells with the specimen and antiserum, viral isolation from the specimen was judged to be positive. If no staining was observed in either one, the result was considered to be negative. If both wells were stained, this was judged to be due to nonspecific reactions.

Demonstration of BVD-MD viral antigen on leucocytes: Blood samples were obtained from 3 calves persistently infected with NCP BVD-MD virus isolated from the serum by the interference method. Seven blood samples of normal calves having no BVD-MD virus served as negative controls. These blood samples were centrifuged and fractionated in Ficoll-Conray solution to obtain
lymphocytes and granular leukocytes. These preparations were washed 2 times with PBS and smeared on glass slides, air-dried and examined by the IIP procedure and indirect fluorescent antibody (IFA) methods [7]. Before IIP staining, preparations were treated with 0.5% hydrogen peroxide in methanol at 25°C for 20 min to remove endogenous peroxidase from cells such as granular leukocytes and monocytes.

Demonstration of BVD-MD viral antigen by immunohistological staining: Mesenteric lymph nodes of a 12-month-old persistently infected cow having acute mucosal disease and of a cow having no BVD-MD virus were fixed in 10% buffered neutral formalin. Sections of these tissues were routinely processed and stained by the ABC procedure with mouse anti-BVD-MD monoclonal antibody and an ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.). The ABC procedure was performed according to the manufacturer's instructions.

As a result of virus isolation by the IIP procedure, all 42 serum specimens from the persistently infected cattle showed positive results and all 100 serum samples from the healthy cattle showed negative results. These results agreed with those obtained by the interference method. Thirteen samples of CP BVD-MD virus derived from mucosal disease were positive by the IIP procedure, but not by the interference method. There was no sample showing a nonspecific reaction (Fig. 1). Antigen-positive lymphocytes were detected in theuffy coats of 3 persistently infected cattle by the IIP procedure but not in those of 7 normal cattle tested (Fig. 2). These results

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Fig. 1. Indirect immunoperoxidase staining of NCP BVD-MD virus antigen in bovine fetal muscular cells (3 days after inoculation). a: The KS86-1 strain. × 200. b: Uninfected cells. × 200.

Fig. 2. a: Immunohistological staining of BVD-MD virus antigen in mesenteric lymph node from calf suffering from BVD-MD by ABC procedure. × 300. BVD-MD virus antigen-specific staining is seen in most lymphocytes. b: Indirect immunoperoxidase staining of BVD-MD virus antigen in the smear of lymphocytes from persistently infected calf. × 300.
agreed with those obtained by the IFA method. Viral antigen in mesenteric lymph nodes was detected by the ABC procedure in the cow with mucosal disease but not in the normal cow. BVD-MD virus antigen-positive cells were scattered in the cortex and medulla in lymph nodes. The cortex contained slightly more antigen-positive cells. Most of the antigen-positive cells were lymphocytes and a few were macrophages (Fig. 2).

The IIP procedure can be done on microplates and it is easy to examine many samples. Furthermore, reddish-brown staining in BFM cells can be detected by the naked eye and stored for a long time. BVD-MD virus isolation from serum specimens by the interference method has required 8 days after inoculating the samples. But by the IIP procedure both isolation and identification of the virus were possible in 4 days. As a result of this procedure for testing samples from persistently infected cattle, both CP and NCP viruses were detectable regardless of the Nose and KS86-1 serogroups. For detecting the antigen in lymphocytes and formalin-fixed tissue sections, counter staining is available and identification of stained cells is possible. The probability of virus detection from auffy coat by the IIP procedure makes it possible to identify the virus from persistently infected cattle and from cattle with mucosal disease in one day. It is difficult to isolate the BVD-MD virus from the sera of persistently infected newborn calves due to the influence of maternal antibody. Occasionally the virus is isolated from leukocytes, even if it was not isolated from the serum [2]. Thus the detection of BVD-MD viral antigen fromuffy coat by the IIP procedure is considered to be available for the diagnosis of persistently infected newborn calves as well. Furthermore, since the antigen was detectable by applying the ABC procedure to formalin-fixed lymph nodes, it is considered that old formalin-fixed samples can be followed-up.

REFERENCES