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Microtiter Virus Isolation and Enzyme Immunoassays for Detection of Bovine Viral Diarrhea Virus in Cattle Serum

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Cattle immunotolerant to and persistently infected (PI) with bovine viral diarrhea (BVD) virus (BVDV) constitute the mechanism by which BVDV persists in cattle herds. Two procedures for using serum to detect PI cattle were developed and evaluated. BVDV was found to remain viable for 7 days in serum samples stored at room temperature. The tests use cell culture virus isolation (VI) in 96-well microtiter plates, followed by immunostaining of cell monolayers with monoclonal antibodies. One technique, the immunoperoxidase monolayer assay (IPMA), forms a red intracellular precipitate while the other, the monolayer enzyme-linked immunosorbent assay (M-ELISA) produces a yellow color in solution. The optimal incubation period for microtiter VI was determined to be 4 days. Optimal IPMA staining was obtained by fixing cell monolayers with 20 to 30% acetone, whereas a simple dry-rehydrate-dry cycle provided optimal M-ELISA staining. The M-ELISA and IPMA had the same sensitivities and specificities, but the M-ELISA was a more rapid procedure and use of a spectrophotometer for reading samples allowed for greater objectivity. When compared to standard VI with routine samples submitted for the diagnosis of BVD, M-ELISA and IPMA had a relative sensitivity of 85% and a relatively specificity of 100%. When only samples from cattle suspected of being PI were considered, these two parameters were 100% for both IPMA and M-ELISA. The two procedures, especially the M-ELISA, are suitable for whole-herd testing to identify PI cattle. The appeal of these tests is derived from the convenience of using serum as a diagnostic sample and the ability to rapidly screen large numbers of samples at low cost.

Bovine viral diarrhea (BVD) is one of the most economically important infectious diseases of cattle in the United States and worldwide. The causative agent, BVD virus (BVDV), is a member of the genus *Pestivirus* in the *Flaviviridae* family (12). On the basis of growth characteristics in cell cultures, naturally occurring BVDV strains are divided into cytopathic (CP) and noncytopathic (NCP) biotypes; CP strains cause visual cytopathic effects, while NCP strains grow in cells without visual cytopathic effects (8).

BVDV causes a variety of clinical syndromes in cattle, including diarrhea, reproductive failure, congenital defects, respiratory disease, mucosal disease, and hemorrhagic syndrome resulting from thrombocytopenia (2, 24). A significant consequence of BVDV infection, from the epidemiologic standpoint, is the birth of calves persistently infected (PI) with BVDV as a result of transplacental infection of the fetus from pregnant cows that become infected with NCP strains during the first trimester of gestation (19). PI animals shed large amounts of BVDV into the environment throughout their lives and, thus, represent a mechanism by which the virus persists and spreads among cattle populations. The detection and elimination of PI cattle, along with vaccination, are necessary for control of BVD in cattle herds.

The standard method of detection of BVDV-infected cattle is virus isolation (VI) in cell cultures followed by fluorescent-antibody (FA) staining with BVDV-specific antibody. FA staining is required to identify NCP strains, which constitute about 90% of BVDV field isolates (9). Standard virus isolation is laborious and not practically applicable to large numbers of samples. In recent years, following an apparent recrudescence of BVD in the United States and a subsequent increased awareness of its importance among veterinarians and cattle producers, diagnostic laboratories are experiencing increased demands for whole-herd screening to detect and eliminate PI cattle. PI animals secrete large amounts of BVDV in their sera (usually 10^3 to 10^5 cell culture infective doses per ml) (5, 20). Due to the ease of collection, shipping, and handling in the laboratory, serum is a very convenient diagnostic sample for identifying PI animals. Measurement of serum antibodies to BVDV, although easier than VI, is not applicable to the identification of BVDV-infected animals for two reasons. First, PI animals, whose detection and elimination are the goal of whole-herd testing, are, by definition, immunotolerant to BVDV and may not develop anti-BVDV antibodies (19). Second, in the general cattle population the prevalence of BVDV antibody-positive animals is high, typically about 70% (22).

This report describes the development and application of microtiter techniques for screening large numbers of serum samples for BVDV. VI was performed in microtiter plates and one of two techniques was used to detect the isolated virus (generally NCP BVDV). One technique, the immunoperoxidase monolayer assay (IPMA), produces a red intracellular precipitate which is detected by the naked eye or by light microscopy, while the second technique, a monolayer enzyme-linked immunosorbent assay (M-ELISA) produces a yellow color in solution. The intensity of the M-ELISA color is quantified with a spectrophotometer.

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MATERIALS AND METHODS

Cells and viruses. Bovine turbinate (BT) cells (National Veterinary Services Laboratories, Ames, Iowa) and Madin-Darby bovine kidney (MDBK) cells (ATCC CCL 22) were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% BVDV-free fetal bovine serum (FBS) and antibiotics (50 μg of gentamicin per ml, 200 IU of penicillin per ml, and 200 μg of streptomycin per ml). These two cell lines were chosen because they are widely used in diagnostic laboratories for BVDV isolation. Low-passage primary bovine testicle (B.test) cells were grown in minimal essential medium with Earle's salts supplemented with 10% FBS, 200 IU of penicillin per ml, 200 μg of streptomycin per ml, and 2.5 μg of amphotericin B per ml. The B.test cells were used exclusively in one of our laboratories (that of E. J. Dubovi) for testing sera by the IPMA method. The data presented concerning development of test procedures involve only BT and MDBK cells, while the data on the application of the M-ELISA and IPMA involve BT and B.test cells, respectively. Several known BVDV were used, including the NCP strains NY-1 and Draper and the CP strains Singer, NADL, and the type 2 125 strain (National Veterinary Services Laboratory).

MAbs, field samples, and VI. Because most field isolates of BVDV are NCP, immunological identification of virus isolates is required following virus isolation in cell cultures. Two monoclonal antibodies (MAbs), MAbs 15c5 and 20.10.6, previously shown to be reactive with all available BVDV strains (7), were used. Field samples consisted of bovine sera, buffy coat cell suspensions, and 10% homogenates of fresh tissues (lung, spleen, lymph nodes, intestine, and fetal organs) submitted for the diagnosis of BVD.

For standard VI, samples were inoculated (150 μl/well) into 24-well cell culture plates seeded the previous day with BT cells at 120,000 cells/well. Inoculated cultures were subcultured after 5 to 7 days of incubation by trypsinization. After incubating the second passage for another 5 to 7 days, the presence of BVDV was detected by trypsinizing the cells and spotting them onto Teflon-coated slides (Cell-line Associates, Newfield, N.J.); this was followed by FA staining with a 1:1,000 dilution of MAbs 15c5 and 20.10.6 as described previously (7).

IPMA. The IPMA was a modification of a previously described method (20). Briefly, BT and/or MDBK cells seeded into 96-well cell culture microtiter plates (Costar Corp., Cambridge, Mass.) at 15,000 cells/well were infected in suspension with 25 μl of test sample (serum, buffy coat cells, or tissue homogenates) per well. After incubating for 4 days at 37°C in 5% CO_2, the medium was removed and the plates were dried at 37°C for 1 h before fixing. The dried cell monolayers were fixed with 20% acetone in phosphate-buffered saline (PBS; 100 μl/well) for 10 min and were dried again at room temperature overnight. The monolayers were then reacted with a 1:1,000 dilution of a mixture of MAbs 15c5 and 20.10.6 in binding buffer (PBS plus 0.5 M NaCl and 0.01% Tween 20) for 1 h at 37°C. Following four washes with PBS containing 0.05% Tween 20, the plates were reacted with a 1:500 dilution of biotinylated rabbit anti-mouse immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) in binding buffer supplemented with 60% chicken serum for 1 h at 37°C. After another wash, a 1:500 dilution of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, Calif.) was added to the plates for 1 h at 37°C. After a final wash, substrate consisting of 280 μg of 3-amino-9-ethylcarbazole (Sigma) per ml and 0.01% hydrogen peroxide in 50 mM acetate buffer (pH 5.0) was added, and the mixture was incubated in the dark. A positive reaction was the appearance of a red intracellular precipitate after 25 to 30 min of incubation at room temperature. The samples were tested in duplicate, with one negative and two positive control samples included on each plate. Test samples were considered positive if they produced in at least one well distinct red cytoplasmic staining in at least a cluster of infected cells (a plaque). Samples were scored negative if they yielded a uniform colorless translucence.

M-ELISA. The plates for the M-ELISA were prepared as described above for IPMA. The M-ELISA was performed on cell monolayers following a modification of established indirect ELISA procedures (10) as described previously (28). Briefly, fixed and dried cell monolayers were rehydrated for 10 min with PBS and were then reacted successively with a 1:1,000 dilution of a mixture of MAbs 15c5 and 20.10.6, a 1:2,000 dilution of peroxidase-conjugated sheep anti-mouse immunoglobulin G (Sigma), and a 3,3′,5,5′-tetramethylbenzidine–hydrogen peroxide chromogen–substrate mixture. The samples were tested in duplicate, with one negative and two positive control samples included on each plate. Test samples were considered positive if they yielded a mean optical density (OD) at least equal to twice the mean OD for the negative control wells.

Optimization of test conditions. (i) Determination of optimal fixation conditions. Fixation of a cell monolayer before immunostaining is necessary to ensure the monolayer's integrity throughout the staining procedure while preserving antigen reactivity. To determine optimal fixation conditions, each pair of four pairs of 96-well cell culture plates was seeded with freshly trypsinized BT or MDBK cells, respectively, at a density of 15,000 cells/well. Six of the eight rows in each plate were infected with NCP BVDV NY-1 at a multiplicity of infection of 4 cell culture infective doses per cell (25 μl/well), while two rows were mock inoculated with virus diluent. After 4 days of incubation at 37°C in 5% CO_2, the 12 columns of each plate were fixed as described above with either PBS (pH 7.6) or various dilutions of acetone (2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, or 90%) in PBS. A set of four plates (two with BT cells and two with MDBK cells) was dried for 3 h at 37°C, while a second set of four plates was dried overnight at room temperature. IPMA and M-ELISA were then performed with two plates from each set immediately following the drying period.

(ii) Determination of optimal incubation period. Six 96-well plates were seeded with BT cells (top half of each plate) and MDBK cells (bottom half of each plate). Duplicate wells of each plate and each cell line were then inoculated with 25 μl of either normal FBS and normal cow serum (negative controls), BVDV NY-1 (positive control), or diagnostic samples (10 serum samples, 7 buffy coat samples, and 4 tissue homogenates) from which BVDV had been isolated by standard VI. One plate was fixed every day starting from 2 days postinoculation (dpi) to 7 dpi. M-ELISA was performed after all the plates were fixed at 7 dpi. The binding ratio (BR) for each sample was computed by dividing the mean OD for the sample by the mean OD for the negative controls for each cell line. A sample was scored positive for BVDV if it yielded a BR of ≥2.0.

Comparison of IPMA and M-ELISA versus VI. To compare the sensitivities of IPMA and M-ELISA, parallel titrations of 32 BVDVs were performed by the two procedures. The viruses included the five laboratory strains listed above and 27 isolates obtained from field samples by standard VI. Serial 10-fold dilutions of each virus were added to quadruplicate wells containing BT cells seeded in two series of 96-well plates. Two columns of wells mock inoculated with virus diluent (DMEM plus 5% FBS) served as a negative control. After a 4-day incubation period at 37°C in 5% CO_2, the plates were fixed and stained either by M-ELISA or by IPMA. All inoculated wells were scored as positive (virus growth) or negative (no virus growth). The endpoint titer for each virus was determined by following the method of Reed and Muench (26). To compare statistically the endpoint titers determined by M-ELISA and IPMA, the mean numbers of positive wells for each of 30 viruses for which endpoints were obtained by both methods were compared by the Student *t* test for independent samples (30). Furthermore, 225 field serum samples were tested by the two methods for the presence of BVDV. Finally, VI was compared with IPMA and M-ELISA by simultaneously testing 324 diagnostic specimens (225 serum specimens, 54 buffy coat specimens, and 45 tissue homogenates) by the three methods.

Stability of BVDV in serum. The underlying premise of this study is that serum is a valid sample for the isolation of BVDV in these test systems. It was thus important to determine the stability of BVDV in field serum samples. Three serum samples that were from calves known to be PI and that were submitted by a veterinarian were divided into four aliquots, and the aliquots were stored as follows: (i) at −70°C for 7 days and then heat inactivated at 56°C for 1 h, (ii) at laboratory room temperature (23 ± 2°C) for 7 days, (iii) at 4°C for 7 days, and (iv) at −20°C for 7 days. After 7 days the sera were titrated for BVDV by M-ELISA, with endpoints determined by the method of Reed and Muench (26).

Application of IPMA and M-ELISA in testing of herds for PI cattle. The M-ELISA was used to test 5,649 serum samples at the Oklahoma Animal Disease Diagnostic Laboratory during a 2-year period (June 1994 to July 1996). These included 4,338 samples from 89 cattle herds and 1,311 samples submitted for various serologic tests. The IPMA has been used at the Diagnostic Laboratory, Cornell University, for 4 years to test a total of 36,522 serum samples. Where possible, a second serum sample was obtained from positive animals after an interval of at least 3 weeks to confirm the presistent infection status as opposed to the presence of a possible acute infection at the time of initial testing.

RESULTS

IPMA and M-ELISA. BVDV-infected cell monolayers could be stained specifically by both methods. The staining conditions described did not produce any remarkable background staining by either procedure. IPMA-stained infected cell monolayers produced intense bright red intracellular staining that could be read for several weeks by the unaided eye or, in the case of weakly positive samples, under an inverted microscope. M-ELISA-stained cell monolayers produced a bright yellow solution, but OD measurement needed to be performed immediately following staining.

Optimal fixation conditions. Drying of the plates for 1 h followed by rehydration and fixation for 10 min and then drying for at least 3 h at 37°C or overnight at room temperature preserved the cell monolayer throughout the staining procedure. Figure 1 presents the effects of various fixation conditions on the M-ELISA reactivities of BVDV-infected cells with MAbs. The reactivity patterns of the two MAbs were identical for both BT and MDBK cell lines (data not shown). For the M-ELISA, PBS without acetone was selected as the fixative, since it systematically yielded the highest ODs without increased background staining (Fig. 1). For the IPMA, visual blinded scoring of the intracytoplasmic color established that

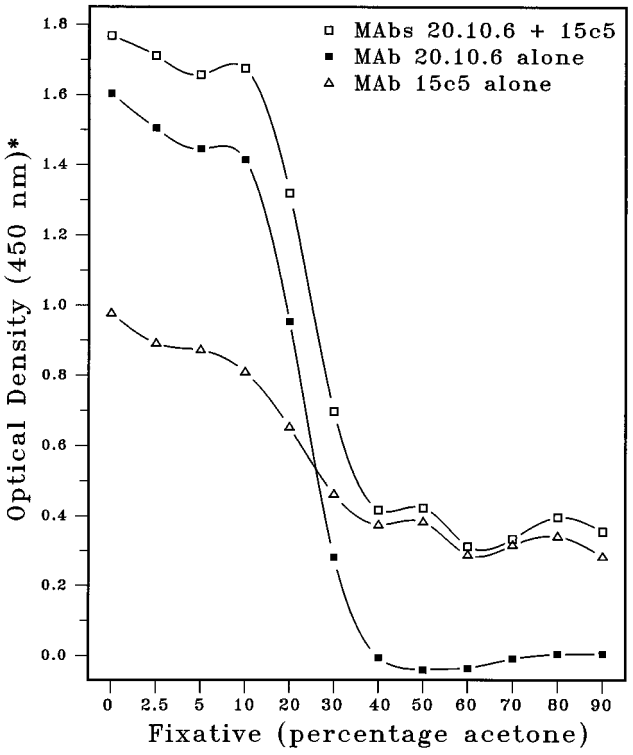

FIG. 1. BVDV-infected BT cell monolayers fixed in PBS containing various concentrations of acetone (0 to 90%) were stained for BVDV by M-ELISA. Because all infected wells contained the same amount of antigen prior to fixation, the ODs are a reflection of reactivity under each fixation condition. *, corrected OD = mean OD for infected wells − mean OD for control (uninfected) wells.

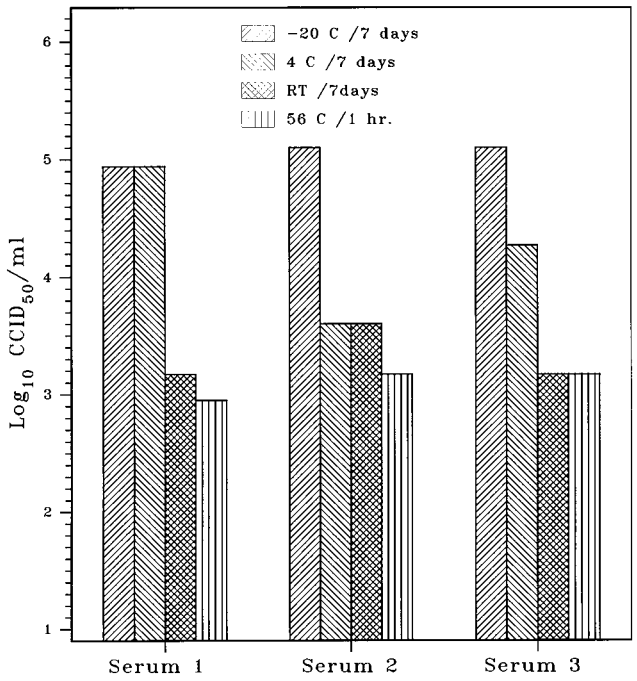

FIG. 2. Stability of BVDV in three serum samples under various storage conditions. The sera were treated as described on the figure (RT, room temperature) and were then titrated for the presence of live BVDV by microtiter VI followed by M-ELISA staining.

the best staining was obtained with 20 and 30% acetone for BT and MDBK cells, respectively.

Optimal incubation period. Since the IPMA and M-ELISA do not detect virus released into solution, it is important to fix cell monolayers at a time when the antigen concentration is highest within the cells. Table 1 presents the M-ELISA reactivities of cells inoculated with 21 field samples and fixed between 2 and 7 dpi. The field samples were selected to include a broad range of positive samples (very weak to strongly positive) as determined by VI. Fixation on 4 dpi gave the highest BR as well as the highest number of positive samples. BT cells proved to be more sensitive than MDBK cells because more positive samples were detected earlier and with a generally higher mean BR (Table 1).

Comparison of M-ELISA and IPMA versus VI. When 32 viruses were titrated by IPMA and M-ELISA, endpoint titers could be determined for all 32 viruses by M-ELISA, but the titers for 2 viruses that were highly cytopathic could not be determined by IPMA because of total cell monolayer destruction. Although there was individual variation in the titers determined for each virus by the two methods, the correlation coefficient was very high (0.96). The total number of positive wells for the 30 viruses that could be titrated by both methods was identical, and there was no significant difference in the titers ($P = 1.000$). This method of comparing titers by using the mean numbers of positive wells in a titration series is valid because the titer is directly proportional to the total number of positive wells in the method of Reed and Muench (26). When only 225 field serum samples were tested for BVDV by IPMA and M-ELISA, both tests gave identical results: 4 positive and 221 negative samples.

VI was more sensitive than IPMA and M-ELISA when 324 diagnostic specimens (from animals with acute infections plus animals suspected of being PI) were tested by the three methods. VI detected 34 samples as positive, while IPMA and M-ELISA each detected 29 positive samples. All IPMA- or M-ELISA-positive samples were also VI positive. By using VI as the "gold standard," the relative sensitivity of IPMA and M-ELISA was 85%, while the specificity was 100%. When only the subpopulation of 225 field serum samples was considered, the sensitivity of IPMA and M-ELISA relative to that of VI was 100%. In all subsequent work (data not shown), all samples from PI cattle diagnosed to be positive by VI systematically tested positive by IPMA or M-ELISA.

Stability of BVDV in serum. The stability of BVDV in serum under various storage conditions is indicated by the endpoint BVDV titers presented in Fig. 2. The virus showed good stability under all conditions studied, with heat inactivation at 56°C for 1 h and room temperature storage for 7 days being relatively deleterious. Even under these conditions, all three

TABLE 1. Determination of optimal incubation period

dpi	BT cells		MDBK cells	
	No. positive (n = 21)	Mean BR (range)[a]	No. positive (n = 21)	Mean BR (range)
2	9	2.8 (2.0–4.7)	7	3.8 (2.2–7.6)
3	15	5.9 (4.2–8.7)	14	5.4 (2.1–8.1)
4	15	6.3 (3.5–9.7)	15	5.6 (2.8–9.0)
5	15	5.7 (2.5–8.6)	14	4.4 (2.3–7.3)
6	14	4.8 (2.2–9.2)	13	5.0 (2.8–7.1)
7	15	7.8 (4.3–10.2)	15	5.0 (2.3–6.3)

[a] The given ranges are for positive samples. The range of BR values for negative samples was 0.7 to 1.8.

serum samples remained positive when they were tested undiluted (the normal testing condition).

Application of M-ELISA and IPMA to testing of herds. Application of M-ELISA and IPMA to the screening of large numbers of serum samples for BVDV in two laboratories yielded BVDV viremia rates of 1.86% (105 of 5,649 samples) and 0.925% (336 of 36,522 samples) in Oklahoma and New York, respectively. Of the 441 viremic cattle, only about 16% (about 70) were confirmed to be PI by follow-up testing. Difficulties in confirming persistent infection status arose from cattle producers' reluctance to spend more money on animals that they had already decided to cull after receiving the first test result.

DISCUSSION

This study has described two simple techniques, IPMA and M-ELISA, for the detection of BVDV in cell monolayers following virus isolation in microtiter plates. The IPMA produces a precipitate in the cell monolayer, while the M-ELISA results in the formation of a colored product in solution. The two techniques have identical sensitivities and specificities, but the M-ELISA has four advantages over the IPMA: (i) the readings are more objective, (ii) the procedure is faster by 1 h, (iii) M-ELISA works equally well for both CP and NCP viruses (IPMA requires the presence of cells to be visualized), and (iv) there is the possibility of automating readings by programming software to automatically compute ODs and express them as positive or negative, taking into account the background staining of the negative control. The convenience of using serum for virus isolation and the low cost (about $2.20 per sample) renders these techniques ideal tools for screening large herds and eliminating PI cattle.

Although the IPMA and M-ELISA were less sensitive than VI when all diagnostic samples (samples from animals with acute infections plus samples from animals suspected of being PI) were considered, they had the same sensitivity as VI when only samples from animals suspected of being PI were considered. Indeed, all samples (buffy coat cells, tissues, and serum samples) from animals that were confirmed to be PI by VI were systematically positive by both methods. Furthermore, all samples that were VI positive but IPMA and M-ELISA negative were tissue homogenates or buffy coat cells from clinically sick animals that most likely were not PI. These microtiter procedures were designed for PI animals, which are known to secrete large amounts of BVDV in their sera. They are therefore recommended for use in whole-herd screening for persistent infection status when serum is used as the test sample. They may not be completely adequate for diagnostic testing for acute BVDV infections, in which virus is secreted in relatively small amounts over a short time period.

Several antigen-capture ELISAs have been described for the direct detection of BVDV antigens in blood and tissues (11, 13, 21, 29). While these tests have a sensitivity rate comparable to that of IPMA (16) and can be completed in 1 day, they cannot be used with cell-free specimens like serum and thus require time-consuming tissue processing steps such as buffy coat separation or preparation of tissue homogenates. This shortcoming, together with the relatively higher cost, make the antigen-capture kit tests less attractive to laboratories that have established cell culture systems, in which the expense of cell culture ceases to be a limiting factor. Antigen-capture ELISAs, however, remain a good option for testing for PI status in laboratories without adequate cell culture facilities. Some workers (16) have reported problems with the IPMA when toxic or contaminated samples are used. In our experience, such problems occur only rarely with properly handled serum samples tested by IPMA and M-ELISA. The use of an appropriate cell line is also important. In our studies, the BT cells were more sensitive to BVDV (Table 1), while they were more resistant to the toxic effects of inocula than MDBK cells (data not shown).

In recent years, several PCR methods for the diagnosis of BVD have also been described (1, 3, 6, 14–16, 27). These methods are highly sensitive, rapid, and applicable for all types of BVDV-containing specimens. However, their high cost and relative technical sophistication make them unsuitable for large-scale testing. We believe that the M-ELISA is the test of choice for routine laboratory testing of whole herds to screen out PI cattle. Its appeal to diagnostic laboratories with adequate cell culture facilities derives from the ease of collection and handling of the test specimen (serum), the low cost of testing, objective reading, and the possibility of performing large-scale testing. It takes one technician two 8-h working days to test 540 specimens. One potential disadvantage of using serum is that the presence of antibody could interfere with virus isolation. We have avoided this potential problem by using serum only from animals that are at least 3 months old. Because of the large amount of BVDV antigen produced by PI cattle, we suggest that all maternally derived antibody is rapidly cleared from the calf's circulation. This suggestion is supported by other workers who reported a rapid decline in colostral antibody titers of eight PI calves from whose sera virus could be readily isolated after 8 weeks of age, even in the presence of low neutralizing antibody titers (17). In another study with 11 PI calves that had received colostrum at birth, while no antibody could be detected at 8 weeks of age, BVDV was isolated from all calves (23).

The high stability of BVDV in serum is well established. Indeed, BVDV is one of the most frequent viral contaminants of commercial fetal bovine serum (4, 18). In this study, the fact that BVDV remains viable in serum following heat inactivation at 56°C for 1 h or storage at room temperature for 7 days means that under normal conditions of sample submission to a diagnostic laboratory, a delay of up to 1 week between specimen collection and testing would not compromise virus isolation in serum samples. Our results with field samples confirm and extend those of other workers (25).

The IPMA and M-ELISA procedures have been applied to large-scale screening for BVDV in the United States. Our results indicate that the rate of BVDV viremia may be 1.86% among cattle in certain herds. A similar viremia rate (1.9%) has been reported in another study in the United States (5). However, a true rate of persistent infection could not be determined for all the animals in our study because of our inability to obtain a second sample. Indeed, BVDV viremia at a single point in time could result from either an acute or a persistent infection; a second positive sample from the same animal after a 3-week interval confirms the persistent infection status.

The MAbs used in this study were selected because of their broad reactivities with BVDV strains. Measurement of their separate M-ELISA reactivities with infected cells fixed under various conditions revealed two distinct reactivity patterns: MAb 15c5 remained reactive at acetone concentrations up to 90%, while the reactivity of MAb 20.10.6 was completely extinguished at acetone concentrations of $\geq$40%. This observation was surprising in view of the fact that MAb 20.10.6 remains reactive when FA staining of cells fixed onto glass slides with 100% acetone is used. The difference might be due to the nature of the solid support. While glass is inert to acetone, the plastic in microtiter plates is soluble in acetone. MAb 20.10.6

might bind to a conformational epitope that is denatured when acetone reacts with the plastic. Traditional belief holds that acetone (or other organic solvents such as methanol) is required for good cell staining. It was therefore surprising to find that unfixed infected cell monolayers gave a higher M-ELISA signal than acetone-fixed monolayers. While acetone might be required for cells on glass surfaces, our results indicate that a simple dry-rehydrate-dry cycle is enough to fix cells onto plastic surfaces for immunostaining.

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