Comparative Evaluation of the Fluorescent Antibody Test and Microtiter Immunoperoxidase Assay for Detection of Bovine Viral Diarrhea Virus from Bull Semen

Ahmad Afshar, Gilles C. Dulac, Claude Dubuc and Thomas H. Howard

ABSTRACT

An indirect immunoperoxidase staining technique (IP) is described for the detection of bovine viral diarrhea virus (BVDV) in bovine semen. The performance of the IP was compared to the reference immunofluorescent staining test in its ability to detect BVDV in 23 coded field semen samples. The IP assay which can be applied with ease to a large number of samples and does not require expensive fluorescence microscope equipment, appears to be an alternative method for BVDV detection. The IP assay can be strongly recommended for certification of BVDV-free bovine semen for artificial insemination and trading purposes and for laboratories which are not equipped for performing the immunofluorescent test.

RÉSUMÉ

Une technique de coloration indirecte par immunoperoxydase (IP) a été évaluée afin de démontrer la présence du virus de la diarrhée virale bovine (BVD) dans la semence de taureaux. Une comparaison entre cette méthode et la technique usuelle de détection du virus a été réalisée sur 23 échantillons de semence. La technique IP s'est averée une alternative valable pour la détection du virus compte tenu du grand nombre d'échantillons pouvant être traités et ce, à moindre coût. Cette technique pourrait être avantageusement utilisée dans des laboratoires qui ne sont pas équipés pour les tests d'immunofluorescence ainsi que pour certifier l'absence du virus de la semence pour fin d'insémination artificielle ou de vente à l'étranger. *(Traduit par Dr Pascal Dubreuil)*

Bovine viral diarrhea virus (BVDV) is the causative agent of two distinct clinical entities affecting cattle, viral diarrhea and the mucosal disease complex (1-3). The BVDV is a ubiquitous pathogen, to which a high percentage of cattle have serum antibodies (4,5). An important epizootiological feature of noncytopathic (NCP) BVDV and other pestiviruses is their ability to establish persistent infection in immunologically immature fetuses (6,7).

Such persistently infected calves may remain seronegative, have a persistent viremia and shed large amounts of virus in all body secretions and excretions, including semen, over prolonged periods. These chronically infected animals transmit BVDV infection to susceptible cattle with which they come in contact (1). The semen produced by persistently infected bulls often contains virus and may transmit infection by either natural service or artificial insemination (AI) (8). Bulls may acquire BVDV infection from infected herdmates with subsequent seminal shedding of the virus. However, in such cases the excretion of BVDV in the semen is transient and the amount of virus is lower than that in persistently infected bulls (9-12). Detection and elimination of persistently infected bulls are essential components of BVD control programs, especially in AI centers (8). Virological examination of serum and semen samples provides a reliable means to establish the BVD status of a bull. Until recently, the immunofluorescent (IF) test was the most common technique used for detection of NCP strains of BVDV (8,13). The method was also used to identify cytopathic strains. In recent years, with the advent of enzyme immunoassays and the use of microtiter techniques, a semiautomated immunoperoxidase (IP) test for BVDV isolation (14-17) has replaced the IF technique and the method is used for the routine monitoring of bulls in AI centers (Lucas MH, Drew TW, personal communication). Since 1989, we have adapted an indirect IP test and we have successfully applied it in the detection of persistently infected animals. In the present communication, the adaptation and comparison of IP and IF tests for detection of BVDV in bull seminal samples are described.

Samples of extended semen from 23 semen collections from five Canadian and seven American bulls (Table I) were coded and tested blindly in this study. The samples had been stored and were transported in liquid nitrogen. For the indirect IF test, bovine kidney (BK) cells, between the second to the fifth passage, were grown in 25 cm^2 Falcon flasks and on glass coverslips in Leighton tubes, in Earle's minimum essential medium (MEM) supplemented with 10% fetal

Agriculture Canada, Animal Diseases Research Institute, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9 (Afshar, Dulac, Dubuc) and American Breeders Service, P.O. Box 459, DeForest, Wisconsin 53532 (Howard).

Submitted June 7, 1990.

TABLE I. Correlation between the results obtained by indirect immunofluorescent (IF) and immunoperoxidase (IP) tests in the detection of bovine viral diarrhea virus in 23 coded bull semen samples

Code no.	Bull no.	Semen identification no./ Ejaculation no.	Results IF	Results IP
1	1	ADRI[a] x -5969/1	-	-
2		" x -5969/2	-	-
3		" x -5969/3	-	-
4		" x -5969/4	-	-
5		" x -5969/5	-	-
6		" x -5969/6	-	-
7	2	" x -6810/1	+	+
8	3	" x -6845/1	+	+
9	4	" Vagabond, ET	+	+
10	5	" Musk Mat	+	+
11	6	ABS[b] 29B3716/1	+	+
12		" 29B3716/2	+	+
13		" 29B3716/3	+	+
14		" 29B3716/4	+	+
15	7	" 29J2841/1	+	+
16		" 29J2841/2	+	+
17		" 29J2841/3	+	+
18		" 29J2841/4	+	+
19	8	" 29H3853/1	-	-
20	9	" 29H4111/1	-	-
21	10	" 29H3570/1	-	-
22	11	" 29H5040/1	-	-
23	12	" 29H4750/1	-	-

[a]ADRI = Animal Diseases Research Institute, Nepean's Accession
[b]ABS = American Breeders Service's Accession

bovine serum (FBS) (Gibco, Grand Island, New York) and antibiotics. The cells were monitored routinely for BVDV and the FBS was pretested for absence of anti-BVDV antibody and BVDV and was treated at 0-4° C with 1 Megarad of gamma rays.

After washing the monolayers with 0.01 M phosphate buffered saline, pH 7.6 (PBS), two Falcon flasks were each inoculated with 0.5 mL of a 1:5 dilution of semen samples in MEM. After an adsorption period of 60 min at 37°C, the inoculum was decanted, cell monolayers were washed with warm MEM and the cultures were maintained in MEM containing 2% FBS. The cells were observed daily for characteristic cytopathic effect (CPE). After incubation for five days the cultures were frozen and thawed and the fluids were passaged once in Leighton tubes, each receiving 200 μL of inoculum. The monolayers grown on coverslips were washed in PBS (pH 7.4), followed by a quick rinse in distilled water (DW) before being fixed with absolute acetone for 10 min at 4°C. The indirect IF staining method described previously (18) was used except for the primary antibody which was raised in cattle against several strains of BVDV. The primary antiserum was used at 1:50 dilution in PBS Tris/HCl buffer. The fluorescein conjugate was rabbit antibovine IgG (heavy and light chain) (Lot 29009; Cappel, Cooper Biomedicals, West Chester, Pennsylvania) and used at 1:40 dilution. Each antibody incubation period was 30 min and the coverslips were mounted in glycerinated Tris HCl buffer medium and the slides were examined by experienced microscopists, using an epifluorescence microscope, for fluorescent staining.

The IP technique was a modification of the previously described assays (14,15,18) and used serially passaged bovine turbinate (BT) cells, originally obtained from Mr. T. Drew (Central Veterinary Laboratory, Weybridge, England). Ten μL of the harvest of the first passage of semen sample in BK cells were dispensed in duplicate wells of tissue culture microplates (Falcon - 96 wells). Each well received 100 μL (approx. 300,000 cells/mL) of freshly trypsinized BT cells in MEM supplemented with 5% FBS or horse serum (Flow Laboratories, Mississauga, Ontario). After brief and gentle shaking the plates were incubated at 37° C for 96 h in a humid atmosphere of 5% CO_2 and 95% air after which the overlay medium was discarded by gentle inversion. The monolayers were rinsed gently with warm PBS, drained and fixed for 10 min at room temperature (RT) with 20% acetone in PBS containing 0.02% bovine serum albumin. The fixative was discarded and the monolayers were dried under a bench lamp (30° C) for 3 h. The staining method was as described previously (18) except that the primary antibody was that used in indirect IF at 1:50 dilution in PBS containing 0.05% Tween 20 and 2.95% NaCl (PBSTN).

The peroxidase conjugate was rabbit antibovine IgG (heavy and light chain) (Cappel) used at 1:500 dilution in PBST with 2% normal rabbit serum. Each antibody incubation was 15 min at RT, with a three-cycle wash in PBST. The bound peroxidase activity was detected using 3-amino-9-ethylcarbazole (AEC) substrate, essentially as described previously (18). In a glass tube 8 mg of AEC were dissolved in 1.0 mL of N,N-dimethylformamide and added slowly to 19 mL of 0.05 M sodium acetate buffer, pH 5.0, containing 66 μL of 3% H_2O_2. Specific brown staining developed within 10 min, whereupon the substrate was replaced with PBS and then with tap water. The plates were dried and readings were done with an inverted light microscope.

The comparative results of IF and IP testing on 23 extended seminal samples, collected from 12 bulls, are presented in Table I. Twelve samples were positive and 11 were negative for BVDV by both tests. None of the positive samples induced any CPE in cell cultures. For both IF and IP tests known quality control samples (positive and negative) were included to monitor the test performance. When samples were examined in duplicate by the IP test, in microtiter plates, no discrepancy was observed between the duplicate results. Postulating that the reference test (IF) had a specificity and sensitivity of 100%, the performance of IP in detecting BVDV was in complete agreement with the IF assay. The indirect IF and IP procedures follow two major principles; amplification of BVDV, if present, in the cell cultures and its detection by an immunostaining technique. Therefore, the agreement between the two tests is not surprising. Nevertheless, the IP assay has several advantages over the IF test. It can be applied with ease to a large number of samples, is less expensive, and it does not require fluorescent microscope equipment and expertise in reading. In addition, once the enzyme chromogen is developed in the cell monolayer, it provides a permanent record.

We have adapted and applied the IP test in a microtiter system for detecting BVDV in naturally infected bull seminal samples which were submitted in the context of semen exportation. The IP test is recommended as an alternative to the standard IF for certification of BVDV-free bovine semen for AI and trading purposes. The method is a complement to the detection of BVDV in serum with the IP test.

ACKNOWLEDGMENTS

We thank Dr. Ron Schultz and Dr. Doug Hare for providing some of the samples and Paul Chatillon, Marcel Ranger and Michelle Fraser for their technical assistance.

REFERENCES

1. **DUFFELL SJ, HARKNESS JW.** Bovine virus diarrhea-mucosal disease infection in cattle. Vet Rec 1985; 117: 240-245.
2. **BAKER JC.** Bovine viral diarrhea virus. A review. J Am Vet Med Assoc 1987; 190: 1449-1458.
3. **RADOSTITS OM, LITTLEJOHNS IR.** New concepts in the pathogenesis, diagnosis and control of diseases caused by the bovine viral diarrhea virus. Can Vet J 1988; 29: 513-528.
4. **KAHRS RF, ATKINSON G, BAKER JA, CARMICHAEL L, COGGINS L, GILLESPIE J, LANGER P, MARSHALL V, ROBSON D, SHEFFY B.** Serological studies on the incidence of bovine viral diarrhea, infectious bovine rhinotracheitis, bovine myxovirus parainfluenza-3, and *Leptospira pomona* in New York State. Cornell Vet 1964; 54: 360-369.
5. **HARKNESS JW, SANDS JJ, RICHARDS MS.** Serological studies of mucosal disease virus in England and Wales. Res Vet Sci 1978; 24: 98-103.
6. **ROEDER PL, HARKNESS JW.** BVD virus infection: prospects for control. Vet Rec 1986; 118: 143-147.
7. **BROWNLIE J, CLARKE MC, HOWARD CJ, POCOCK DH.** Pathogenesis and epidemiology of bovine virus diarrhea virus infection of cattle. Ann Rech Vét 1987; 18: 157-166.
8. **AFSHAR A, EAGLESOME MD.** Viruses associated with bovine semen. Vet Bull 1990; 60: 93-109.
9. **WHITMORE HL, GUSTAFSSON BK, HAVARESHTI P, DUCHATEAU AB, MATHER EC.** Inoculation of bulls with bovine diarrhea virus: excretion of virus in semen and effects on semen quality. Theriogenology 1978; 9: 153-163.
10. **BARLOW RM, NETTLETON PF, GARDINER AC, GREIG A, CAMPBELL JR, BONN JM.** Persistent bovine virus diarrhea virus infection in a bull. Vet Rec 1986; 118: 321-324.
11. **MEYLING A, JENSEN AM.** Transmission of bovine virus diarrhea virus (BVDV) by artificial insemination (AI) with semen from a persistently infected bull. Vet Microbiol 1988; 17: 97-105.
12. **PATON DJ, GOODEY R, BROCKMAN S, WOOD L.** Evaluation of the quality and virological status of semen from bulls acutely infected with BVDV. Vet Rec 1989; 124: 63.
13. **ROBERTS PC, ETCHISON JR, BOND CW.** A rapid, quantitative assay for titration of bovine virus diarrhea-mucosal disease virus. Vet Microbiol 1988; 18: 209-217.
14. **MEYLING A.** Detection of BVD virus in viraemic cattle by an indirect immunoperoxidase technique. Curr Top Vet Med 1984; 29: 37-46.
15. **HOWARD CJ, BROWNLIE J, THOMAS LH.** Prevalence of bovine virus diarrhea virus viraemia in cattle in UK. Vet Rec 1986; 119: 628-629.
16. **KATZ JB, LUDEMANN L, PEMBERTON J, SCHMERR MJ.** Detection of bovine virus diarrhea virus in cell culture using an immunoperoxidase technique. Vet Microbiol 1987; 13: 153-157.
17. **SMITH GH, COLLINS JK, CARMAN J, MINOCHA HC.** Detection of cytopathic and noncytopathic bovine viral diarrhea virus in cell culture with an immunoperoxidase test. J Virol Methods 1988; 19: 319-324.
18. **AFSHAR A, DULAC GC, BOUFFARD A.** Application of peroxidase labelled antibody assays for detection of porcine IgG antibodies to hog cholera and bovine viral diarrhea viruses. J Virol Methods 1989; 23: 253-262.