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Detection of bovine viral diarrhea virus in serum from cattle by dot blot hybridization assay

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ABSTRACT

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A dot blot hybridization assay was developed for use as a rapid screening test to detect bovine viral diarrhea virus (BVDV) in serum from infected cattle. A 1.1 kilobase cDNA, prepared from the BVDV genome, was molecularly cloned and used in this study. Insert cDNA was removed from the pUC9 plasmid vector by Pst-I restriction endonuclease digestion and purified from plasmid DNA by agarose electrophoresis and electroelution. The hybridization probe was prepared by nick translation in the presence of gamma dCTP32P and labelled to a specific activity of 2 × 108 cpm/μg of DNA. Specificity was determined by dot blot hybridization of infected cell culture supernate from nine different BVDV strains. The probe hybridized equally with all strains of BVDV tested, which included four cytopathic and five noncytopathic strains of BVDV. Serum was collected from veal calves with respiratory tract disease, unthriftiness, anorexia, and/or poor conditions. Serum samples were treated with nonidet P40 detergent and denatured with formaldehyde and heat prior to application on 1.2 micron nylon membrane filters using a vacuum dot blot apparatus. Hybridization was done under relatively stringent conditions (50% formamide at 42°C). A total of 141 serum samples from different calves were tested and of these samples, 55 (39%) were positive by dot blot hybridization for BVDV RNA. Eight calves (33%) out of 24, tested 3 to 4 weeks later, remained positive for BVDV RNA.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) remains widespread and is a major contributor of disease in cattle (Reggiardo, 1979; Duffel and Harkness, 1985; Harkness, 1987). Transplacental infection is a common sequel in persistently-infected cattle or after BVDV infections in susceptible, pregnant heifers and cows (Orban et al., 1983; Liess et al., 1984). The virus is fetopathic, primarily early in pregnancy; however, fetal infections may result in persistent immunotolerant infections in postnatal life (Duffel and Harkness, 1985; Harkness, 1987). Such animals may appear to be healthy but some die pre-
maturely, often after chronic illness, and all have the potential of developing mucosal disease (Orban et al., 1983; Liess et al., 1984; Duffel and Harkness, 1985). Evidence indicates that mucosal disease is precipitated by superinfection of persistently-infected, seronegative animals with a different strain of BVDV and thus represents the final outcome of in utero infection (Lies et al., 1984). Another significant repercussion of in utero infection is that persistently-infected cattle may be the primary source of BVDV in nature (Harkness, 1987). Novel approaches for identifying persistently-infected animals are required since methods currently employed are inefficient, expensive and of questionable accuracy (Coria et al., 1984; Howard et al., 1985).

Since the virus is endemic in the U.S.A., attempts to maintain a herd free of infection to the virus likely would invite disastrous economic losses (Harkness, 1987). The aim of rational control measures should be to break the cycle of transmission by identifying (and removing) persistently-infected animals and by preventing transplacental infections (Duffel and Harkness, 1985; Harkness, 1987). Another important objective must be prevention of BVDV infection of stressed animals (Harkness, 1987). Effective BVDV control, therefore, would require screening of herds for persistently-infected cattle and use of effective vaccines.

Measures for the control and prevention of BVDV are based on the detection and identification of persistently-infected individuals within a herd. At present, virus isolation is the only method available to detect persistently-infected animals (Duffel and Harkness, 1985; Harkness, 1987; Bezek et al., 1988). A rapid, specific, and sensitive detection assay would facilitate herd screening for BVDV infection. The purpose of this study was to evaluate the use of a dot blot hybridization assay to detect BVDV in serum from infected cattle.

MATERIALS AND METHODS

Cell cultures and virus

BVDV strains used in this study included five cytopathic strains (72, Auburn, NADL, Singer, Oregon C24V) and five non-cytopathic strains (2724, 7443, New York-1, TGAN, and NEB). The origin of strains 72 and 2724 has been described (Potgieter et al., 1984); the other BVDV strains were obtained from Dr. S.R. Bolin, National Animal Disease Center, Ames, IA. Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum that had been treated with beta-propiolactone (0.05% final concentration) to remove adventitious viruses was used for cell growth (Brock et al., 1988). Primary bovine turbinate (BTU) cell cultures, passaged 5 to 12 times and negative for BVDV by indirect immunofluorescence and dot blot hybridization assay, were used for propagating BVDV.
**Virus isolation and indirect fluorescent antibody assay**

Complete monolayers of BTU cell cultures (25 cm$^2$) were inoculated with 0.5 to 1.0 ml of test serum for 1 h. The inoculum was removed after 1 h, replaced with medium, and incubated at 37°C in a 5.0% CO$_2$ incubator for 5 days. Cell cultures were examined daily for development of cytopathic effect. Cells were removed by scraping and a drop of cell suspension was placed onto a glass slide and fixed in acetone for 10 min. BVDV antigen was detected by indirect fluorescence assay using an anti-BVDV antiserum (NADL and New York-1 strain) prepared in a gnotobiotic calf. The indirect fluorescence assay also was done to determine serum antibody titers to BVDV using BTU cell cultures infected with the NADL strain of BVDV. A 1.0 ml aliquot of the cell suspension from the first passage was inoculated into BTU cell cultures for a second passage as described above and tested by indirect fluorescence assay for BVDV antigen.

**Complementary DNA**

cDNA was obtained from purified BVDV (strain 72) genomic RNA and cloned into the pUC9 plasmid vector (Brock et al., 1988). A 1.1 kilobase (kb) cDNA clone was identified from the cDNA clones and used to prepare hybridization probes (Brock et al., 1988). The insert cDNA fragment was removed from the pUC9 plasmid by restriction endonuclease digestion with $Pst$-I and purified from plasmid DNA by agarose electrophoresis and electroelution (Maniatis et al., 1982).

**Preparation of cDNA probes**

Purified cloned cDNA sequences were labelled with dCT$^{32}$P to a specific activity of 1–4 × 10$^8$ cpm/g by nick translation as described by Rigby et al. (1977). Labelled DNA was separated from unincorporated nucleotides by gel filtration through Sephadex G-50 (Maniatis et al., 1982). Prior to hybridization, probe was heated for 5 min at 100°C and chilled on ice.

**Samples**

Whole blood was collected from veal calves with respiratory tract disease, unthriftness, anorexia, and/or poor condition. Serum was removed from clotted blood samples following centrifugation at 1000 g for 30 min. Serum samples (0.45 ml) were immediately denatured as described below and applied to membrane filters. The remaining portion was frozen at –80°C for further use. Negative control samples from uninfected BTU cell culture supernate and positive control samples from BVDV-infected cell cultures (strain 72, 10$^5$ CCID$_{50}$/ml) also were denatured and applied to each membrane filter to be hybridized.
Hybridization assay

Hybridization blots were prepared, hybridized, and washed following hybridization as described by Maniatis et al. (1982). Hybridization of cDNA probes with BVDV RNA was done by the dot blot method as described by Shockley et al. (1987). Samples tested included various strains of cell culture-grown BVDV, uninfected cell culture supernatant fluids, and serum. Specimens were clarified by centrifugation at 2000 g for 10 min. Samples were denatured by adding nonidet P-40 to a concentration of 0.5% and the mixture was incubated on ice for 10 min (White and Bancroft, 1982). Then an equal volume of 40% formaldehyde and 60% 20× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) was added and the mixture was heated at 60°C for 15 min. A 100 μl volume of the treated sample was applied to a 1.2 micron nylon membrane filter (Biodyne membranes, ICN Biomedicals, Irvine, CA) using a dot blot apparatus. The nylon membrane filters were air-dried and baked at 80°C for 90 min before they were placed into plastic bags with prehybridization solution at 42°C for 4–6 h with gentle agitation. The cDNA probes (10^7 cpml/ml) were denatured by heating at 100°C for 5 min followed by rapid cooling in ice and added to the hybridization solution (50% formamide, 5× SSC, 0.1% SDS and 1000 μl/ml denatured salmon sperm DNA). Hybridization was allowed to proceed at 42°C for 16–24 h. The hybridized filters were washed (2× SSC and 0.1% SDS, four times for 5 min at 22°C and then 0.1× SSC and 0.1% SDS, twice for 15 min at 50°C) and dried at room temperature on filter paper before their placement into radiographic film cassettes containing radiographic film and an intensifying screen for 24–72 h.

RESULTS

The cDNA hybridization probe reacted equally with infected cell culture supernates of all cytopathic strains (72, Auburn, NADL, Singer, Oregon C24V) and non-cytopathic strains (2724, 7443, New York-l, TGAN, and NEB) tested. Previously, as little as 10–20 pg of purified BVDV RNA resulted in detectable hybridization signals with the hybridization probe used in this study (Brock et al., 1988).

To determine the minimum detection level of BVDV in serum, serial tenfold dilutions of BVDV infected cell culture supernatant (10^5 CCID50/ml) were made in BVDV negative, fetal bovine serum. Virus was detected by dot blot hybridization assay to minimum level at a 10^-4 dilution in the serum. A total of 141 serum samples from different calves were tested and 55 (39%) of these samples were positive by dot blot hybridization for BVDV RNA. An autoradiograph of a hybridized dot blot membrane filter containing 22 test samples is shown in Fig. 1. Virus isolation was attempted on the first 30 samples collected in addition to dot blot hybridization assay. BVDV was present in 8 of the 30 samples determined positive by dot blot hybridization assay.
Fig. 1. Dot blot hybridization assay of serum samples from calves. Radiographic film was exposed for 24 h following hybridization as described in Materials and Methods. Column C, row 7 and 8 are positive and negative wells, respectively. Note the different intensities of hybridization signals, arrows indicate samples considered to be positive. All other samples are considered negative.

TABLE 1

| Sample | Isolation | Antibody titer$^2$ |
|--------|-----------|-------------------|
| 1      | -         | 1:160             |
| 2      | -         | 1:80              |
| 3      | +         | 1:40              |
| 4      | -         | < 1:40            |
| 5      | -         | < 1:40            |
| 6      | +         | < 1:40            |
| 7      | -         | < 1:40            |
| 8      | -         | 1:160             |

$^1$Samples positive by hybridization assay of the first 30 samples collected.
$^2$Virus isolation and antibody titers were done as described in Materials and Methods.
Virus was isolated from three of the 30 samples. One of the serum samples from which virus was isolated was negative for BVDV by dot blot hybridization assay. Serum antibody levels of the eight dot blot hybridization positive samples ranged from $< 1:40$ to $1:160$ (Table 1). Serum from eight calves (33%) out of 24, tested 3 to 4 weeks later, remained positive for BVDV RNA. Results from the dot blot hybridization assay were available within 36 to 48 h.

DISCUSSION

The sensitivity and specificity of hybridization probes for the presence of viruses is equal or greater than other laboratory methods such as immunoassays, virus isolation, and electron microscopy (Richman et al., 1984; Teramoto et al., 1984). The sensitivity of hybridization of probes with target sequences is dependent on the methods of labelling, hybridization conditions, detection system, and hybridization system (Richman et al., 1984). From the results of this study, it is concluded that a dot blot hybridization assay can detect BVDV in serum from naturally-infected cattle and may be applicable as a diagnostic assay. However, the hybridization results do not correlate well with standard diagnostic methods such as virus isolation since virus was isolated from only two out of eight dot blot positive samples (Table 1).

When detection of virus passaged in BTU cell cultures by hybridization is compared with virus isolation, the results highly correlate. However, dissimilar results were obtained from individual animals samples. Comparing hybridization results of virus-laden serum with virus isolation, virus was detected in a $10^5$ dilution of cell culture supernate ($10^6$ CCID$_{50}$) in fetal bovine serum. In this study, BVDV was isolated from only 25% (2/8) of samples positive by hybridization assay.

The presence of anti-BVDV serum antibody in some samples may account for the difficulty in the isolation of virus from serum (Table 1). Following the acute phase of postnatal BVDV infections, virus can not be isolated from serum. Virus may remain in serum in antibody complexes following the development of antibody. Therefore, samples taken 7 to 10 days after infection may be negative for virus by isolation in cell culture but positive by hybridization. The preferred sample for isolation of BVDV from infected animals is white blood cells (buffy coat) from heparinized whole blood instead of serum (Bezek et al., 1988). Also, virus isolation may not have correlated with hybridization results due to insufficient detection of some antigenically-heterologous field strains of BVDV by the anti-BVDV antibody used in the indirect fluorescence antibody assay (Castrucci et al., 1975; Coria et al., 1984). Also it may be possible that antibody present in FBS used to supplement cell culture interfered with the isolation of BVDV.

The disparity between the hybridization results and virus isolation would
suggest the hybridization probe is reacting nonspecifically. However, several factors support the specificity of the hybridization probe for BVDV. The hybridization probe consisted only of insert DNA removed from plasmid DNA by restriction endonuclease digestion and electroelution to prevent nonspecific reactions by plasmid DNA. Control pUC9 plasmid DNA did not hybridize with the BVDV hybridization probe and probe prepared from pUC9 plasmid DNA alone did not hybridize with samples identified as positive with the BVDV hybridization probe. The hybridization probe has previously been used against several bovine RNA and DNA viruses (bovine respiratory syncytial virus, bluetongue virus, bovine coronavirus, and bovine herpesvirus I) without any cross-hybridization (Brock et al., 1988). The possibility that the probe reacts nonspecifically with a factor in bovine serum samples is not probable. Hybridization results of serum samples taken from a different population of 575 steers and bulls identified only five animals that were positive by hybridization using identical assay conditions (unpublished data). Assuming comparable results, if random nonspecific reactions occur, more positive animals would have been expected. Also in this study, initial samples collected from some animals were positive and following the second sampling 3 to 4 weeks later, the samples taken from the same animals were negative. One animal was negative from the first sample taken and positive on the second sample by hybridization.

Another important factor in the evaluation of the assay results was the definition of the positive endpoint of the dot blot hybridization assay. In this study, samples that had an equal intensity of hybridization signal compared with the positive control dots of BVDV-infected cell cultures were considered positive for BVDV RNA by hybridization (Fig. 1). The variability in the hybridization signals was likely due to the varying amounts of BVDV RNA present in the samples. Hybridization of some samples resulted in low level signals below the intensity of the controls and were considered negative although virus may have been detectable by virus isolation (Fig. 1). Although the hybridization probe can detect a minimum quantity of 10 to 20 pg of BVDV RNA (Brock et al., 1988), the evaluation of hybridization results from clinical samples at this low threshold level is difficult and impractical as a diagnostic assay. The use of polymerase chain reaction technology to amplify the template in the samples may improve definition between positive and negative signals when a minimum amount of BVDV RNA is present.

It was thought that serum was the best sample for testing for BVDV infection. Serum samples would be easier to obtain from a field situation than white blood cells from buffy coats. Control and prevention of BVDV infection centers around the ability to detect and remove persistently-infected animals which may have levels of $10^2$ to $10^5$ CCID$_{50}$/ml of serum (Harkness, 1987). White blood cells may remain infected with BVDV following the clearance of virus from the serum in acutely-infected animals (Malmquist,
1968). Therefore, the detection of BVDV in buffy coat cells would not differentiate acute and persistent infections of BVDV. Although the presence of virus in serum does not indicate a persistent infection, a continual presence of virus in the serum of persistently-infected animals would be expected.

The high percentage (40%) of calves infected with BVDV demonstrates that BVDV is very prevalent especially in stressful environments. The calf sample population did not represent a normal population in this study, therefore the prevalence of BVDV infection may actually be lower in a given herd. The eight out of 24 calves that remained positive by hybridization 3 to 4 weeks after initial testing were not characterized as persistently-infected animals which indicates that acute BVDV infections may not be resolved following the development of antibody. It is probable that the animals identified as positive by hybridization represented postnatal infections and were not persistently infected since virus was not isolated from serial samples and most animals had anti-BVDV serum antibody. Reoccurring, postnatal infections with antigenically-heterologous BVDV strains may be common in mixed and stressed populations of animals such as veal calves.

The results from this study are not considered conclusive. The disagreement between virus isolation and hybridization requires further investigation. However, the results are important in demonstrating the difficulty encountered in comparing two assays; the ability to detect infectivity of BVDV and the detection of BVDV nucleic acids. Due to the potential problems that may be encountered in attempting to isolate BVDV it is difficult to ignore the potential detection of BVDV by hybridization assay. However, BVDV hybridization assay results must be examined closely along with virus isolation until further controlled studies can be done to support results obtained by hybridization assay.

Although current theories on the pathogenesis of mucosal disease, acute BVDV infections, and persistent infections from in utero infection are accepted by many researchers, many aspects of the complex pathogenesis of BVDV infection remain unknown (Orban et al., 1983; Liess et al., 1984; Duffel and Harkness, 1985; Harkness, 1987; Horzinek and Van Berlo, 1987). The development of new technologies such as hybridization probes to detect viral genomes may provide new or different information than is currently accepted on the pathogenesis of BVDV infection. Hybridization probes are very sensitive and specific for detecting BVDV RNA, and although correlating results with standard tests such as virus isolation is difficult, there are important diagnostic applications of these methods.

CONCLUSION

Dot blot hybridization can be used to detect BVDV in serum from cattle naturally-infected with BVDV. The hybridization assay is extremely sensitive
and specific for detection of BVDV RNA. However, the results of the hybridization assay in detecting natural infections did not correlate with virus isolation as a diagnostic assay.

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