Detection of bovine viral diarrhea virus, using degenerate oligonucleotide primers and the polymerase chain reaction

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SUMMARY

A technique for detection of bovine viral diarrhea virus (BVDV) from circulating blood leukocytes, using the polymerase chain reaction, is described. The published nucleotide sequences of 2 strains of BVDV and that of hog cholera virus were aligned and the information was used to design oligonucleotides coding for 2 regions of amino acid homology. The oligonucleotides were a mixed population including all possible codons for the conserved amino acids. These degenerate oligonucleotides were used in the polymerase chain reaction to detect viral RNA in cells infected in vitro, or in circulating blood leukocytes from infected animals. Virus was detected in over 60 samples from diverse isolates. The detection of BVDV by the polymerase chain reaction is a rapid, sensitive, and specific technique, which represents an improvement over existing technology.

Bovine viral diarrhea virus (BVDV) is a pathogen that causes a variety of disease syndromes resulting in economic losses in domestic cattle herds. Transmission may be vertical or horizontal, and may result in acute or persistent infections. Infection in utero may result in abortion, teratogenesis, or the birth of persistently infected, immunotolerant animals.

Persistent infection is usually established by noncytopathic biotypes of BVDV by infection of fetuses in utero before 110 to 120 days of gestation. Mutation of noncytopathic BVDV to a cytopathic biotype, or superinfection with an antigenically related cytopathic biotype in persistently infected animals, leads to the development of the usually fatal mucosal disease. Persistently infected animals are often “poor doers” and are the main source of infectious virus in a herd. Calves that are offspring of persistently infected animals are also persistently infected and immunotolerant to strains of BVDV that are antigenically similar to the persistently infecting strain.

Horizontally transmitted virus may result in diarrhea in young calves and the virus has been suggested to be immunosuppressive, having an auxiliary role in the establishment of opportunistic infections by other pathogens.

The control of BVDV-related problems in a herd requires the identification and elimination of persistently infected cattle. These animals shed BVDV, although they may have low to undetectable serum antibody titers against the virus. Animals with normal antibody titers against BVDV may also be persistently infected, because persistently infected animals superinfected with a serologically distinct strain of BVDV may mount a normal response against the superinfecting virus.

Current methods of detecting BVDV in clinical samples rely on serial passage in cell culture and subsequent detection of BVDV antigens. In infected cells by immunofluorescence or immunoperoxidase staining. This technique is labor-intensive, and noncytopathic variants may require passage 2 or 3 times in cell culture before detectable amounts of viral antigen are produced. In contrast, sensitive techniques for the detection of BVDV RNA would be quicker and would be able to identify infectious virus, as well as virus that has been rendered inactive. Detection of BVDV by hybridization of nucleic acid probes to viral RNA has been reported. However, the variation in nucleic acid sequence between isolates suggests that a probe prepared from the cDNA of 1 strain of BVDV may not detect genomic RNA from all BVDV strains.

The recent development of the polymerase chain reaction (PCR) for the amplification of nucleic acid, allows for the amplification of 1 copy of the viral genome in a sample. The amplified product can then be analyzed by use of agarose gel electrophoresis and possible hybridization to viral-specific probes. The ability of the PCR to detect all isolates of a particular virus depends on the design of oligonucleotide primers representing regions of the viral genome whose nucleotide sequence is conserved among all strains of the virus. The variability of the BVDV genome has made it difficult to design primers that will bind to any but those virus strains most closely related to the NADL strain, which was the only strain from which sequence information had been published. However, because sequence information is now available from the NADL and Osloss strains of BVDV and from the closely related hog cholera virus, the purpose of the study reported here was to overcome this problem by identifying highly conserved amino acid sequences, and designing “degenerate” oligonucleotide primers that include all possible codons for the conserved amino acids. These primers should recognize all BVDV strains. We have used these primers to identify several reference strains and field isolates, using the PCR.
Materials and Methods

Cell culture and virus strains—The cytopathic NADL strain,¹ WCVM field isolates,² Angel isolate of BVDV,³ cytopathic Oregon strain,⁴ and noncytopathic Draper⁵ and NY-1 strains⁶ were obtained as supernatants of infected cell cultures. Field isolates that included both cytopathic and noncytopathic strains, were also obtained as infected cell cultures or supernatants.⁷

Bovine tracheal (EBTR) cells⁸ were grown in Dulbecco modified eagle medium (DMEM) + 10% horse serum.⁹ These cells were determined to be free of noncytopathic BVDV contamination by indirect fluorescence antibody (IFA) staining with BVDV-specific antibodies, and were routinely tested subsequently by PCR and shown to be uninfected. Cells were infected with 100 µl of infected cell supernatant and grown for 48 to 96 hours at 37 C and 5% CO₂.

Blood sample collection—Blood samples were collected from a BVDV-negative herd at the Animal Diseases Research Institute (ADRI) Lethbridge, Alberta, from a herd in High Prairie, Alberta, and from several other herds in Alberta and Saskatchewan, and were submitted in EDTA vacuum tubes.¹⁰ One bull from the High Prairie herd was suspected of being BVDV-infected. Noncytopathic virus was isolated on 2 occasions (October 1989 and May 1990) by the Alberta Animal Health laboratory. A sample was submitted to the diagnostic virology laboratory for virus isolation, and to our laboratory for PCR analysis. This herd had a history of abnormally high numbers of abortions, stillbirths, congenital malformations, and diarrhea in both adult animals and calves. Bovine viral diarrhea virus was isolated from 3 aborted or stillborn fetuses from this group.¹²

Virus isolation and IFA—Blood samples were subjected to centrifugation at 1,000 x g for 5 minutes and theuffy coat was aspirated into transport media containing a balanced salt solution, gentamicin, and antifungal agents. The cell suspension was added to freshly seeded EBTR cells and grown in DMEM + 10% horse serum for 7 days, at which time the cells were harvested onto slides and tested by IFA using a monoclonal antibody against BVDV.¹¹ Cytoassays were performed by resuspending cells from infected cultures in 1 ml of phosphate-buffered saline solution (PBS) and seeding 30 µl of cell suspension on poly-D-lysine coated, 12-well microscope slides. The cells were freeze-thawed to promote cell disruption, blocked by use of 50 µl of 5% skim milk, and incubated with pooled monoclonal antibodies against BVDV.¹² Virus was detected by use of a biotinylated secondary antibody, and an avidin peroxidase conjugate with 3,3'-diaminobenzidine tetrahydrochloride as a chromogenic agent. Monoclonal antibodies to parainfluenza-3 virus were used to detect nonspecific binding. All assays were performed in duplicate.

RNA extraction—Infected EBTR cells were harvested from flasks by scraping, or buffy coat was aspirated from vacuum tubes containing blood in EDTA following centrifugation at 1,000 x g.¹³ The cell pellet was resuspended in 1 ml of PBS and transferred to a 1.5-ml tube and washed twice with cold PBS. Total cellular RNA was extracted,¹⁴ and resuspended in 50 µl of diethylpyrocarbonate-treated water.¹⁵

Synthesis of cDNA—Five microliters of RNA was heated to 65 C, cooled on ice, and transferred to a 0.5-ml tube containing 200 µM deoxynucleotide triphosphates,¹⁶ 50 pmol of DNA hexamers, 2.5 µg of RNA's,¹⁷ 2.5 µg of avian myeloblastosis virus reverse transcriptase,¹⁸ and 1 x PCR buffer, in a total volume of 20 µl. The mixture was incubated at 22 C for 10 minutes and 43 C for 120 minutes, then heated to 95 C for 5 minutes and quickly cooled.

Polymerase chain reaction—To the cDNA reaction was added 100 pmol of each primer, 2.5 µg of Taq 1 DNA polymerase,¹⁹ 1 x PCR buffer (50 mM KCl, 10 mM tris, 2.0 mM MgCl₂, 0.01% gelatin, pH 8.8), to a total volume of 100 µl. Annealing temperatures for the PCR were determined on the basis of the length of the primers and amplification cycles were as described (Fig 1). A 10 µl aliquot of the reaction product was analyzed by electrophoresis in 1% GTG agarose/1% agarose.²⁰ The plasmid pBVD-p80²¹ containing the p80 coding region from the NADL strain of BVDV cloned in pGEM²² was included as a positive control.

Cloning and sequencing of reaction products—The amplified cDNA was eluted from the agarose gel,²³ digested by Xba I and Sac I,²⁴ and ligated into the phagemid vector pEMBL19,²⁵ Single-stranded DNA was prepared,²⁶ and sequenced.²⁷ Sequence data were analyzed by use of the SEQWCE software package,²⁸ and sequences were aligned by use of CLUSTAL²⁹

Synthesis of oligonucleotides—Oligonucleotides for the PCR, sequencing, and random primed cDNA synthesis were synthesized in our laboratory.³⁰

Results

Design of oligonucleotides—To identify conserved regions of amino acid sequence, the amino acid sequences of the NADL and Ossloss strains of BVDV and the hog

* Beckman TJ-6 refrigerated centrifuge, Beckman Canada, Mississauga, Ontario.
* Bio-Rad Laboratories (Canada) Ltd, Mississauga, Ontario.
* Sigma Chemical Co, St Louis, Mo.
* Pharmacia (Canada) Ltd, Baie d'Urfe, Quebec.
* Promega Corp, Madison, Wis.
* Ampliqon, Cetus Corp, Emeryville, Calif.
* The PCR reactions were done in a thermocycler, Perkin-Elmer/Cetus Corp, Norwalk, Conn.
* Nu-Sieve GTG and SeaKem agarose, FMC BioProducts, Rockland, Me.
* From Dr. Marc Collet, Molecular Vaccines Inc, Gaithersburg, Md.
* Sequenced using Sequenase (US Biochemicals Ltd, Cleveland, Ohio) according to the manufacturer's directions.
* Delney Software Ltd, Vancouver, BC.
* Oligonucleotides were synthesized by use of model 381A DNA synthesizer, Applied Biosystems, Foster City, Calif.

cholera virus were aligned by use of the CLUSTAL software package. From this alignment, highly conserved regions within the gene for the p80 protein of BVDV were identified. Oligonucleotides were designed on the basis of the following criteria: the pair of oligonucleotides amplified a region of between 100 and 600 nucleotides (33 to 200 amino acids); each oligonucleotide corresponded to a region of 6 amino acids conserved between Osloss and NADL strains of BVDV and the hog cholera virus; and the 6 amino acids selected had the minimal degree of codon degeneracy while satisfying the first 2 criteria. Two oligonucleotide primers were designed that satisfied all 3 criteria and amplified a region of 562 bp. Each oligonucleotide contained 17 bases, specifying codons for 5 conserved amino acids and the first 2 bases of the codon for a sixth conserved amino acid, plus an additional 10 bases coding for recognition sites for either Sac I or Xba I restriction enzymes. These restriction sites facilitated the rapid cloning and sequencing of viral isolates (Fig 2).

Determination of PCR sensitivity and specificity.—To determine the sensitivity and specificity of the PCR for detection of BVDV, we first amplified DNA from the pbVD-p80 plasmid in concentrations ranging from 1 ng to 0.1 pg/50-μl reaction, along with RNA from cells infected with NADL strain, and cRNA produced by in vitro transcription of the p80 gene cloned into plasmid pbVD-p80 (data not shown). The presence of BVDV cDNA in the 562 bp band was confirmed by hybridization to a probe prepared from the cloned p80 sequence.

The ability of the PCR to amplify different strains of BVDV was determined by amplifying RNA from EBTR cells infected with either reference strains of BVDV, or with field isolates collected in Saskatchewan, Alberta, Ontario, or the United States. Plasmid DNA from pbVD-p80 was used as a positive control. Negative controls included 1 for the CDNA synthesis from which RNA was omitted, and 1 for the PCR in which the cDNA synthesis step was omitted. The PCR detected virus in all infected cell cultures (Fig 3). The presence of virus in the isolates from Cornell University was confirmed by virus isolation and by use of a fluorescent antibody test. Virus was detected in all infected cell cultures by the PCR, in 9 of 10 cultures by cytoassay, and in 6 of 10 by cell culture followed by IFA (Table 1). In total, 5 reference strains, 25 Cornell isolates, 10 WCVM isolates, and 10 isolates from the Ontario Animal Health Laboratory have been tested, and virus has been amplified in every case. Uninfected EBTR cells from which RNA was extracted consistently tested negative for BVDV (data not shown).

Amplification of BVDV from blood samples.—Of the 145 samples collected from the herd in High Prairie, Alberta, 30 have been analyzed by the PCR. Virus was detected in 4 of these by PCR. Sixteen of these samples also have been tested for serum antibody neutralization titers, and viral isolation by cell culture. Virus was isolated from 3 of the

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* Dubovi E, Department of Pathology, Cornell University, Ithaca, NY: Personal communication, 1990.*
be attributed to the poor state of the sample on arrival
passaged cells 5 times prior to testing, whereas the WCVM
at the WCVM, or to the fact that the Alberta laboratory
was identified after agarose gel electrophoresis. Negative controls for the
variety of BVDV reference and field isolates as shown. Synthesis of CDNA
laboratory passaged cells only once. Some of the animals
Hinc\s used as a size marker.

4 samples that were positive by the PCR (Table 2). Of the
4 positive animals, 1 (calf) has since died having been
“unthrifty” since birth and having had congenital eye de-
fects. Another cow diagnosed as positive by PCR had a
history of abortions, and this year’s calf died soon after
birth. Although virus was isolated from 1 bull (Table 2)
on 2 occasions by the Alberta laboratory, and by the PCR,
virus was not isolated by the WCVM laboratory. This could
be attributed to the poor state of the sample on arrival
at the WCVM, or to the fact that the Alberta laboratory
passed cells 5 times prior to testing, whereas the WCVM
laboratory passaged cells only once. Some of the animals
that tested positive by the PCR did have antibody titers
against BVDV (Table 2).

The samples collected from the BVDV-free herd from
ADRI Lethbridge were also analyzed by the PCR. These
animals are routinely tested for serum antibody titers to

Table 1—Detection of bovine viral diarrhea virus by three
techniques

<table>
<thead>
<tr>
<th>Virus strains</th>
<th>Type</th>
<th>Cytoassay</th>
<th>IFA</th>
<th>PCR</th>
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<tr>
<td>NADL</td>
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<td>+</td>
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<td>+</td>
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<td>V607</td>
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IFA = indirect fluorescence antibody; PCR = polymerase
chain reaction; CP = cytopathic; NCP = noncytopathic; ND
= not determined. + = positive; − = negative or no titer.

Table 2—Detection of BVDV by IFA and PCR in blood

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Antibody titer</th>
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<tr>
<td>16t</td>
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</table>

* Virus was isolated from this bull twice at the
Animal Health Laboratory in Alberta, but not at the
Western College of Veterinary Medicine Diagnostic
Virology Laboratory. All virus isolated from blood
samples was noncytopathic where confirmed by vi-
rus isolation. † Yearling heifers.
− = no detectable titer, + = moderate titer, and
++ = high titer.

BVDV to ensure that the herd has not been exposed to
BVDV. Viral nucleic acid was not detected in any of the
14 samples from this herd (data not shown).

Cloning and sequencing of PCR products—The ampli-
fied products from several of the WCVM isolates were cloned
as described. After digestion with the restriction enzymes
Sac I and Xba I, several restriction length polymorphisms
were identified in certain isolates. The NADL strain of
BVDV contains no internal Sac I or
Xba I sites in this
region. The nucleotide sequence was determined for 3 iso-
lates and compared with the published sequences for NADL
and Osloss strains (Fig 4). The predicted amino acid se-
quences are identical to the published BVDV sequences.
However, the nucleotide sequences vary by as much as
20% from the published sequences.

Discussion

Bovine viral diarrhea virus is difficult to detect for sev-
eral reasons. The virus, particularly the noncytopathic
biotype, grows slowly in cell culture and many require
been suspected that the nucleic acid sequence would be highly variable, which is a common feature of RNA viruses. Recent work has suggested that this may be the case, and indeed, our sequence data (Fig 4) tend to confirm this idea. This suggests that detection by hybridization to nucleic acid-based probes prepared from single cDNA clones may fail to detect some isolates, unless hybridization is carried out under conditions of low stringency. Recent work in which cDNA and oligonucleotide probes were used to detect paired isolates from mucosal disease cases and our findings (data not shown) support this idea.

On the basis of our findings, degenerate oligonucleotides, representing highly conserved amino acid sequences, are capable of acting as primers for the polymerase chain reaction and amplifying BVDV RNA in infected cell cultures or in circulating blood leukocytes from persistently infected animals (Fig 3; Table 2). This approach has been previously described as having been successful for cDNA cloning of rare mRNA transcripts. The speed and sensitivity of the PCR makes it a useful method for screening large numbers of samples in a relatively short time. These, plus the ability to detect diverse isolates, are critical considerations for assaying the extent of infection in large herds.

Bovine viral diarrhea virus cDNA was detected in all cases where the presence of virus could be confirmed by virus isolation and IFA, and in several cases where virus was not detected by isolation (Table 1). In these latter cases, the virus may have been inactivated by serum neutralizing antibodies or by physical means. Current methods of detection depend on the presence of infectious BVDV for detection. By contrast, the PCR will detect viral nucleic acid even though the virus may have been inactivated. The ability of the PCR to amplify strains containing considerable genetic diversity, combined with the ease with which reaction products may be cloned, suggests that the PCR may be a useful method for studying the evolution and pathogenesis of BVDV.

The PCR has been used to amplify BVDV in other reports. In the first case, a limited number of isolates were tested and the technique was not applied to blood from field samples. The second report describes viral isolation of BVDV directly from serum from a variety of isolates with extreme sensitivity. We believe that our study supports the idea that the PCR can be used to amplify a wide range of BVDV isolates, and can be used successfully on blood samples submitted for routine BVDV detection.

Of the 5 animals in the herd from High Prairie, Alberta, that were BVDV-positive by the PCR, 3 were seronegative, 1 had a low antibody titer, and 1 was seropositive with a moderate-to-high antibody titer against BVDV. This supports other work that has shown that persistently infected animals can mount an immune response against a superinfecting, antigenically distinct strain. Therefore, in a case where BVDV is thought to be a problem within a herd, it may be necessary to screen the entire herd for persistently infected animals, not only seronegative animals. To do this by virus isolation would be prohibitively expensive and time-consuming. By contrast, the initial screening of 30 suspect or high-risk animals by the PCR technique took < 1 week from time of receipt of the samples.

Animals persistently infected with BVDV are thought to shed virus continuously and in most bodily secretions. This is thought to be the mechanism by which persistently infected animals transmit the virus to other members of the herd. However, virus cannot be isolated from immunocompetent acutely infected animals by current techniques by 2 weeks after infection. To date, it has not been shown at what point after an acute infection that virus can no longer be detected by the PCR. We are currently addressing this question by use of blood samples collected over a 3-week period from experimentally infected animals. Until these data are available, it is impossible to state with certainty that the animals from the Alberta herd in which BVDV was detected were persistently infected. This is particularly true of the animals that were seropositive. In most of the BVDV-positive cases, it was impossible to get a second sample for virus isolation and PCR. One animal died within 24 hours of the first sample being collected, and several others were shipped for sale soon after sampling.

The sequence data confirm previous ideas that there is considerable variability in the BVDV genome (Fig 4). In a region where the amino acid sequence is identical in 2 reference strains and 3 field isolates of BVDV, the nucleic acid sequence differs by up to 20%. A probe derived from any of these strains would hybridize to all other strains only if the hybridization and washing temperatures were decreased by 15 to 20 degrees from the optimal temperature calculated for perfect homology between the probe and target sequences. This has been confirmed by work in our laboratory and by other groups. In regions where amino acid sequence is not so highly conserved, the amount of genomic variation would be expected to increase. We believe these data validate the use of PCR for detection of BVDV, and further, validate the use of degenerate oligonucleotides as primers in the PCR.

The PCR is proving to be an invaluable technique for the detection and study of a wide range of viruses. The ability to amplify small amounts of viral nucleic-acid from samples ranging from blood and tissue to foodstuffs and preserved material has made the PCR invaluable for a variety of forensic, pathologic, and biological studies. We believe that the application of this technique to the detection and analysis of BVDV will advance the understanding of BVDV biology and the management of BVDV-related conditions in cattle.

References


