Application of PCR for Specific Identification of Epizootic Hemorrhagic Disease Virus Serotype 2

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What is This?
Epizootic hemorrhagic disease virus (EHDV), an arthropod-borne double-stranded (ds) RNA virus, is a member of the genus *Orbivirus* in the family Reoviridae. EHDV infects domestic, captive, and free-ranging ruminants; the whitetailed deer (*Odocoileus virginianus*) is the species most seriously affected with the disease. The white-in-the-family Reoviridae. The white-in-the family Reoviridae.

In a previous study, experimental EHDV infection in calves resulted in transient infection that could be detected by virus isolation and serological tests. However, few studies have shown that the virus can also cause bluetongue (BT)-like disease in cattle. There are 10 serotypes of EHDV worldwide, but only serotypes 1 and 2 are enzootic in the United States. Recent epidemiological studies of the disease indicated that EHDV-2 was more prevalent than EHDV-1. Even in the absence of the disease, there is restriction on the international movement of livestock and/or their germplasm from countries suspected to harbor the disease to EHDV-free countries unless the animals are certified free of EHDV infection by serology or virus isolation. Such a restriction could lead to economic losses for EHDV-endemic countries that rely on the sale of livestock and their germplasms for foreign exchange.

EHDV isolation procedures presently lack adequate sensitivity. Identification of EHDV field isolates includes direct isolation of the virus in baby hamster kidney (BHK-21) cells or initial inoculation of embryonating chicken eggs (ECE), followed by subsequent passages on cell culture for serotyping. Serum neutralization and plaque inhibition tests are commonly used for EHDV serotype-specific identification. However, serumology is complicated by cross-reactions within EHDV serogroups and among non-EHDV orbiviruses.

The surge of new techniques in cell immunology and molecular biology has made possible the development of improved diagnostic tests. A previous report described a competitive enzyme-linked immunosorbent assay (cELISA) protocol for detection of antibodies to EHDV. However, the cELISA technique required collection of blood samples from animals that have been infected for at least 2 weeks to detect EHDV antibody. cDNA probes were also developed for detection of EHDV serogroup-specific and serotype-specific sequences to serve as efficient alternatives for serotyping. However, these cDNA probes have not proven satisfactory for the detection of quantities less than a few nanograms of EHDV RNA with the dot-blots hybridization technique. This low sensitivity of the cDNA probes limits their application for direct detection of virus nucleic acid sequences present at very low amounts in biological specimens. A polymerase chain reaction (PCR)-based assay for direct detection of EHDV serogroup in clinical samples from experimentally and naturally infected ruminants was recently developed. In this report, we describe a rapid, sensitive, and specific assay for specific identification of EHDV serotype 2 in cell culture and clinical samples using PCR technology.

The 2 prototype serotypes of EHDV present in the United States (EHDV-1 and EHDV-2), EHDV field isolates (14 samples of EHDV-2, 5 samples of EHDV-1), and the 5 BT virus (BTV) prototypes and field isolates were propagated on confluent monolayers of BHK-21 cells. The infectious material was harvested and centrifuged at 1,500 x g for 30 minutes, and the cell pellet was used for the dsRNA extraction.

The EHDV and BTV dsRNA was extracted from the infected cells as previously described. The pellet from the infected cells was resuspended in a buffer containing 0.1 M NaAc and 10% sodium dodecyl sulfate (SDS) and extracted twice with phenol and water-saturated ether. Total nucleic acid was ethanol precipitated. Viral dsRNA was purified by differential lithium chloride precipitation, resuspended in 100 µl of double-distilled water, and quantified using a spectrophotometer at 260 nm wavelength.

Heparinized blood samples were collected from clinically normal animals. In addition, blood, lung, and spleen samples were collected from EHDV-2-infected deer. Because of unavailability of clinical samples from EHDV-1-infected animals, a 4-month-old calf was experimentally infected with EHDV-1 as described previously. Processing of the clinical samples for viral nucleic acid extraction was as previously described. Two hundred fifty microliters of processed blood or spleen was digested with SDS and proteinase K. The sample was phenol extracted twice. Total nucleic acid was ethanol precipitated and resuspended in 20 µl of double-distilled water. Five microliters of the resuspended nucleic acid was used in the PCR assay.

A pair of primers were designed from the sequence of genome segment 2 of EHDV-2 (Alberta strain) (unpublished data) and used in these EHDV-2 PCR assays. Primers 1 and 2 (P1 and P2) were selected for the synthesis of specific EHDV-2 PCR product. P1 included bases 901-914 of the positive sense strand of genome segment 2; P2 included bases 1936-1952 of the complementary strand: 5'-TCTCCGCTGTCTATACC-3'.

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EHDV PCR using primers P1 and P2 would result in a 1,051-bp product. For synthesis of a probe complementary to the predicted amplified viral sequences generated by P1 and P2, oligonucleotide primers (P3 and P4) were designed from the same sequences. P3 and P4 were internal to the annealing sites of P1 and P2. P3 consisted of bases 1214-1233 of the positive strand: 5'-GTTGCGAACAAGCTACGTGG-3'. P4 was designed from the complementary strand between bases 1770 and 1786: 5'-CCGGTTAAGCATAGAGT-3'. PCR amplification using P3 and P4 would result in a 572-bp PCR product internal to the annealing sites of P1 and P2. All primers were synthesized on a DNA synthesizer and purified using oligo-pak oligonucleotide purification columns as per the manufacturer’s instructions. The amplification product produced by P3 and P4 was purified using DNA binding beads according to the manufacturer’s instructions and used as a probe for chemiluminescent hybridization.

The PCR protocol used in this study was basically as previously described except that the primers were used at a concentration of 10 µmole and 5.0 U/µl of the Taq polymerase was used per reaction. The thermal cycling profiles were as follows: a 2-minute incubation at 95 C followed by 40 cycles of 95 C for 1 minute, 55 C for 30 seconds, and 72 C for 45 seconds, and a final incubation at 62 C for 10 minutes. Thermal profiles were performed on a thermal cycler. Following amplification, 20 µl from each PCR reaction containing amplified product were loaded onto 1.0% SeaKern agarose and electrophoresed. The gels were stained with ethidium bromide, and the specific 1,051-bp PCR products were visualized under UV light. Southern blot hybridization was performed basically as previously described. Agarose gels from PCR reaction were denatured, neutralized, and transferred to nylon membranes under vacuum at 50 mm Hg. Southern blotted nucleic acid was UV cross-linked to the nylon membrane. Chemiluminescent hybridization was performed using a kit according to the manufacturer’s instructions. The probe prepared by PCR was labeled with peroxidase in the presence of glutaraldehyde. Southern blots were prehybridized with hybridization buffer containing 5% blocking agent and 0.85 M NaCl at 42 C for 1 hour. The labeled probe was added to the hybridization buffer, and the membranes were hybridized at 42 C for at least 12 hours. After posthybridization washing, detection reagents were applied to the membranes for 1 minute. The membranes were then sealed in plastic wrap and exposed to X-ray film for 1-60 minutes with an intensifying screen.
Figure 3. Specificity of PCR for RNA from EHDV-2, EHDV-1, BTV, or other extracts. Amplification product was not detected from a high concentration of 1.0 ng of EHDV-1 RNA, BTV RNA from all US BTV prototypes, or from total nucleic acid extracts from BHK-21 cells. Lane MW: molecular weight marker; lane 1: 1 pg EHDV-2; lane 2: EHDV-1 prototype; lanes 3-7: EHDV-1 field isolates; lanes 8-12: BTV prototype serotypes 2, 10, 11, 13, 17; lane 13: BHK-21 total nucleic acid extract.

The described PCR-based assay afforded a simple, rapid, sensitive, and specific detection of EHDV prototype serotype 2 and EHDV-2 field isolates used in this study. The specific 1,051-bp PCR product was visualized on ethidium-bromide-stained gel from ≥ 100 fg of EHDV-2 RNA (Fig. 1A). Southern blot with chemiluminescent hybridization detected as little as 1.0 fg of the EHDV RNA target (Fig. 1B).

Using 1.0 pg of EHDV-2 RNA target with chemiluminescent hybridization, the 1,051-bp specific PCR product was detected in the 14 EHDV-2 field isolates (Fig. 2).

The 1.0 ng RNA from EHDV-1 prototype or field isolates, the US BTV prototype serotypes 2, 10, 11, 13, and 17, and the total nucleic acid extracts from uninfected BHK-21 cells failed to demonstrate PCR products (Fig. 3). With chemiluminescent hybridization, the specific PCR product was detected directly from unfractionated lysed blood, lung, and spleen samples of infected animals. Clinical samples from uninfected animals failed to produce positive hybridization signals (Fig. 4).

The specific PCR-based assay using primers derived from genome segment 2 of EHDV-2, which codes for VP2, reproducibly and specifically detected EHDV-2 in all samples tested at the stringency conditions described in this study. Viral dsRNA extracted from EHDV prototype serotypes 1 and 2, EHDV-1 and EHDV-2 field isolates, and BTV prototype serotypes 2, 10, 11, 13, and 17 were used in this study. Based on the total molecular mass of the EHDV genome as 11.44 x 10^6 Daltons, the sensitivity of the PCR assay was 100 fg of EHDV-2 viral RNA (equivalent to 6 x 10^7 viral particles) with ethidium-bromide-stained agarose gels. Chemiluminescent hybridization increased the sensitivity of the PCR assay by 100 times, and specific signals were detected from 1 fg of viral RNA (equivalent to 60 viral particles).

Figure 4. Detection of the 1,051-bp specific EHDV-2 PCR product by Southern blot with chemiluminescent hybridization from clinical samples. Lane MW: molecular weight marker; lane 1: spleen homogenates from a deer with clinical hemorrhagic disease; lane 2: lung homogenate from naturally infected deer; lanes 3-7: unfractionate lysed blood cells from EHDV-2-infected deer; lane 8: unfractionated lysed blood from EHDV-1 infected calf; lanes 9,10: unfractionate lysed blood cells from uninfected animals.
The specificity studies indicated that the specific 1,051-bp PCR product was not amplified from a relatively high concentration of 1.0 ng of RNA from EHDV-1 prototype or field isolates, from the US BTV prototype serotypes 2, 10, 11, 13, and 17, from total nucleic acid extracts from BHK-21 cell controls, or from clinical samples from uninfected animals under the same stringency conditions described in this study. This finding agrees with that of a previous report, which indicated that genome segment 2 of EHDV-2 has a variable nucleotide sequence compared with that of EHDV-1. Additional research is necessary to confirm that assumption with the remaining serotypes of the EHDV serogroup. Temperature and time for denaturation, primer annealing and extension, enzyme and MgCl₂ concentration, and number of cycles of the 3 temperatures per time segments were very important for maintaining sensitivity and specificity of the PCR reaction.

Virus isolation is a lengthy, cumbersome procedure, and serology is complicated by cross-reactions between non-EHDV orbiviruses and within EHDV serogroups. These observations render PCR amplification and the hybridization techniques superior diagnostic alternatives to serotyping. The PCR assay is simpler than other molecular biological techniques, many of which are lengthy and cumbersome. The PCR-based assay for detection of EHDV-2 dsRNA in susceptible ruminants would greatly facilitate clinical disease investigation and molecular studies of the virus and would promote intercontinental transfer of livestock and associated germplasm. Because of the time required for isolation of EHDV, it would be highly advantageous to make the diagnosis of EHDV-2 infection directly from an infected clinical sample without initial isolation of the virus in a susceptible cell line or ECE. The PCR assay with chemiluminescent hybridization can efficiently reduce the time required to identify EHDV-2, especially during an outbreak of the disease in susceptible ruminants. Using the described PCR assay, a positive hybridization signal can be obtained from clinical samples from EHDV-2 infected animals in 3 days, whereas conventional virus isolation and identification procedures take 3-4 weeks. Conventional methods of serotyping are more costly and require a longer time for interpretation of the results than does PCR technology.

The PCR product can be visualized on an ethidium-bromide-stained agarose gel for tentative diagnosis of EHDV-2 infection. However, chemiluminescent hybridization remains important to increase the sensitivity of the PCR-based assay and to confirm the specificity of the amplified product. In the present study, the use of nonradioactive chemiluminescent hybridization removes the hazardous and cumbersome radioactive laboratory procedures of working with ³²P or ³⁵S. This EHDV-2 PCR-based assay can be used as an efficient alternative to EHDV-2 serotyping, thereby reducing the cost of maintaining reference sera and high-quality cell culture supplies. The first report of the successful use of PCR for detection of EHDV serogroup³ and the development of a PCR-based assay for specific identification of EHDV-2 would provide the basis for future diagnostic techniques. Similar studies on molecular diagnosis of BTV were conducted in different laboratories. The PCR amplification technology should start the revolution of orbivirus diagnosis on a practical scale within this decade.

The described EHDV-2 PCR assay using primers derived from genome segment 2 of EHDV-2 could provide a rapid, sensitive, and specific diagnostic method for serotype-specific identification of EHDV-2 field isolates in cell culture and a variety of clinical samples.

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a. Arthropod-Borne Animal Disease Research Laboratory, Laramie, WY.
b. National Veterinary Services Laboratories, USDA, APHIS, Ames, IA.
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m. Vacugene XL Vacuum Blotting System, Pharmacia LKB Biotechnology, Piscataway, NJ.
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